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City University of New York

PH.D. 1983

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ENZYMATIC AUTOMODULATION OF CHEMICAL PRESYNAPTIC NEUROTRANSMISSION

by

NATHAN MOSKOWITZ

**A dissertation submitted to the Graduate Faculty
in Biomedical Sciences in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
The City University of New York.**

1983

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1983

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Enzymatic Automodulation of Chemical Presynaptic Neurotransmission

by

Nathan Moskowitz

Adviser: Professor Saul Puszkin

A variety of enzymes in brain synaptic and coated vesicles and presynaptic plasma membrane were characterized. It was found that synaptic and coated vesicles and synaptic plasma membrane had endogenous calcium-dependent phospholipase A_2 (PLA₂). In all three membrane preparations, calmodulin and prostaglandin $F_{2\alpha}$ (PGF_{2 α}) stimulated whereas ATP inhibited both synaptic vesicle and synaptic plasma membrane PLA₂ activities, whereas it stimulated coated vesicle PLA₂ activity. Cyclic-AMP by itself inhibited coated vesicle and synaptic plasma membrane PLA₂ activities, whereas it stimulated synaptic vesicle activity. Furthermore, conditions which were conducive to stimulation of a cAMP dependent protein kinase in synaptic vesicles also stimulated synaptic vesicle PLA₂ activity. The modulation of PLA₂ activities in the three membrane preparations studied were correlated with function via light scattering, light microscopic and electron microscopic techniques. Conditions in general which stimulated endogenous membrane PLA₂ activity induced vesicle aggre-

gation and membrane distortion, conditions conducive to both exocytosis and endocytosis. Based on enzymatic and protein cross-linking studies it was found that a variety of purified soluble PLAs₂ were modulated by calmodulin, PGF_{2α}, cAMP and cGMP. On the basis of this data a scheme for PLA₂ polymerization pathways was postulated. Synaptic and coated vesicles also were found to have endogenous calcium/calmodulin-dependent protein kinase, [Ca²⁺-Mg²⁺]-ATPase and PGF synthetase activities. Synaptic vesicles in addition were found to have endogenous cAMP-dependent protein kinase activity. This thesis presents a unified theory of presynaptic neurotransmission which invokes a series of parallel and interconnecting enzymatic cascades which operate simultaneously and synergistically to modulate presynaptic chemical neurotransmission.

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CONTENTS

ACKNOWLEDGMENTS.....	iv
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	xv
LIST OF ILLUSTRATIONS.....	xvii
A. INTRODUCTION.....	1
1. The role of calcium in neurotransmitter release, exocytosis and membrane fusion.....	4
a. Calcium involvement in neurotransmitter release.....	5
b. Calcium involvement in exocytosis.....	8
c. Calcium involvement in membrane fusion.....	9
2. The vesicle hypothesis.....	10
3. The origin of synaptic vesicles.....	17
4. Protein and lipid characteristics of synaptic vesicles.....	19
5. Morphological structures in the axon terminal closely associated with synaptic vesicle fusion.....	19
a. The presynaptic densities and grid.....	19
b. Synaptopores.....	22
c. The presynaptic axon terminal calcium channel.....	23
6. Pertinent physical chemical properties of calcium.....	27
7. Theories on the mechanisms of the direct action of calcium in exocytosis and fusion.....	29
8. Coated pits/vesicles.....	38
a. The discovery of coated vesicles and their distribution.....	40
b. Morphological structure of coated vesicles.....	41

c.	Coated vesicle lipid composition.....	43
d.	Coated vesicle protein composition.....	43
e.	Function of coated vesicles in neurons.....	49
	(i) Recycling of synaptic vesicles.....	50
	(ii) Molecular filter functions.....	53
	(iii) Exocytosis.....	55
	(iv) Synaptogenesis.....	55
f.	Non-enzymatic theories on the mechanism of formation of coated pits and in the transformation of coated pits into vesicles.....	55
g.	Calcium dependence of endocytosis.....	58
9.	Calmodulin.....	59
a.	Discovery of calmodulin and the enzymes which it activates.....	59
b.	Physical-chemical properties.....	60
c.	Calcium binding and calcium loops.....	62
d.	Model of calmodulin enzyme activation.....	64
e.	Tissue distribution.....	65
f.	Subcellular distribution.....	65
g.	Antagonists.....	67
h.	Involvement in exocytosis and endocytosis.....	67
10.	Phospholipase A ₂	69
a.	Effects of exogenous phospholipases on membrane structure and function.....	70
b.	Effects of lysolecithin on membrane structure and function.....	78
c.	Theories on the mechanism of lysolecithin action.....	79

d.	Endogenous brain phospholipases.....	80
	(i) PLA ₂ , PLA ₁ and lysolecithin.....	80
	(ii) Phospholipase C and diacylglycerol.....	82
	(iii) Phospholipase D.....	83
e.	Endogenous phospholipases in non-brain tissues.....	83
	(i) PLA ₂ and lysolecithin.....	83
	(ii) PLA ₁	86
	(iii) PLC.....	86
f.	Involvement of phospholipases in endocytosis.....	90
g.	Other possible functions of PLA ₂	91
	(i) Metabolism.....	91
	(ii) Regulation of membrane proteins, receptors and channels.....	91
	(iii) Chemotaxis.....	93
	(iv) Prostaglandin production.....	94
h.	Modulators of PLA ₂ activity.....	94
	(i) Neurotransmitters, hormones, peptides and physical stimuli.....	95
	(ii) Methyltransferase.....	96
	(iii) Anesthetics.....	98
	(iv) Antibiotics.....	98
	(v) Cyclic AMP.....	99
	(vi) Lipomodulin.....	99
	(vii) Modulation of synaptic vesicle, coated vesicle and synaptic plasma membrane PLA ₂	100
i.	Purified soluble Phospholipases A ₂	103
	(i) Pancreatic PLA ₂	103

(ii)	Naja naja PLA ₂	106
(iii)	Crotalus adamanteus PLA ₂	107
(iv)	Crotalus atrox PLA ₂	108
(v)	Bee venom PLA ₂	109
j.	Theories on the mechanism of Ca ²⁺ activation of PLA ₂	109
k.	Sequence homology of phospholipases A ₂	111
11.	Prostaglandins.....	113
a.	Discovery.....	113
b.	Chemistry.....	115
c.	Biosynthesis.....	116
d.	Location and synthesis in the brain.....	120
e.	Metabolism.....	123
(i)	General.....	123
(ii)	Brain.....	124
f.	The association between nerve stimulation and PG release.....	127
g.	Effects of PGE on neurotransmission.....	128
(i)	Inhibition.....	128
(ii)	Theories on the mechanism of PGE presynaptic inhibition.....	130
(iii)	Direct effect of PGE on neurons.....	131
(iv)	PGE and fever, pain and schizophrenia.....	131
h.	Effects of PGF _{2α} on neurotransmission.....	132
(i)	Stimulation.....	132
(ii)	Inhibition.....	133
i.	Prostaglandins and cyclic nucleotides.....	134

(i)	PGE and cAMP.....	134
(ii)	cAMP and PGE biosynthesis.....	135
(iii)	PGF _{2α} and cGMP.....	136
j.	Function of arachidonic acid and PGs in membranal events.....	136
(i)	Secretion.....	136
(ii)	Cell aggregation.....	137
(iii)	Chemotaxis.....	137
k.	Concluding statement.....	138
12.	Cyclic nucleotide and Ca ²⁺ /Calmodulin dependent protein kinases.....	139
a.	History of cAMP and protein kinases in the brain.....	139
b.	Concentration and location of cAMP in brain.....	140
c.	Generation and degradation of cAMP.....	141
(i)	Adenylate cyclase.....	141
(ii)	Phosphodiesterase.....	142
d.	cAMP dependent protein kinase and substrates.....	143
e.	cAMP involvement in neurotransmitter release.....	145
f.	cAMP and calcium mobilization.....	146
g.	cAMP involvement in general brain functions.....	146
h.	Calcium/Calmodulin dependent protein kinases.....	147
i.	Calcium/calmodulin and cAMP interrelationship.....	148
13.	Other proteins, enzymes and nucleotides possibly involved in mediating exocytosis and endocytosis.....	150
a.	Actomyosin.....	150
b.	Microtubules.....	151
c.	Glycoproteins.....	152

d.	Adenine nucleotides.....	152
e.	Transglutaminase.....	153
14.	Control of axon terminal calcium concentration.....	154
a.	Brain $[Ca^{2+}-Mg^{2+}]-ATPase$	155
b.	Mechanism of $[Ca^{2+}-Mg^{2+}]-ATPase$ action.....	156
c.	PLA ₂ stimulation of $[Ca^{2+}-Mg^{2+}]-ATPase$	157
d.	Calmodulin stimulation of $[Ca^{2+}-Mg^{2+}]-ATPase$	157
B.	MATERIALS AND METHODS.....	160
1.	Materials.....	160
2.	Methods.....	160
a.	Preparation of synaptic, coated, and decoated vesicles.....	160
b.	Decoated vesicle preparation.....	163
c.	Synaptic plasma membrane preparation.....	163
d.	Calmodulin preparation.....	164
e.	Calmodulin association with synaptic and coated vesicles.....	167
	(i) Radioimmunoassay (RIA).....	167
	(ii) Calcium dependence of calmodulin binding to synaptic and coated vesicles.....	168
	(iii) Specificity of vesicle binding sites for calmodulin.....	169
	(iv) Identification of synaptic and coated vesicle calmodulin binding proteins.....	170
f.	Phospholipase A ₂ assay.....	171
g.	Synaptic and coated vesicle and synaptic plasma	

membrane (SPM) PLA ₂ characteristics.....	171
(i) Ca ²⁺ dependence.....	171
(ii) pH dependence.....	171
(iii) Time dependence.....	171
(iv) Substrate concentration dependence.....	172
(v) Effects of modulators on membranal PLA ₂ activities.....	171
h. Vesicle metabolism of arachidonic acid.....	172
i. Functional biophysical and morphological correlation of endogenous membranal PLA ₂	173
(i) Synaptic vesicle light-scattering experiments...	173
(ii) Coated vesicles.....	174
(iii) Phase contrast microscopy.....	176
(iv) Electron microscopy.....	176
j. Purified phospholipases A ₂	177
(i) Calmodulin affinity chromatography.....	177
(ii) Dimethylsuberimidate (DMS) cross-linking.....	177
(iii) PLA ₂ assay.....	178
(iv) Naja naja PLA ₂	178
(a) PLA ₂ assay.....	178
(b) DMS-crosslinking.....	179
k. Effect of prostaglandins on purified PLA ₂ function.....	179
l. Phosphorylation studies.....	180
(i) Effect of antibodies.....	181
(ii) Gel electrophoresis, autoradiography and microdensitometry.....	181
m. [Ca ²⁺ -Mg ²⁺]-ATPase.....	181

n.	Calcium uptake determinations.....	182
o.	Miscellaneous procedures.....	182
C.	RESULTS.....	183
1.	Membrane preparations.....	183
a.	Synaptic vesicles.....	183
b.	Coated vesicles.....	183
c.	Synaptic plasma membrane.....	190
2.	Calmodulin purification.....	202
3.	Calmodulin association with brain synaptic and coated vesicles.....	207
4.	Membranal PLA ₂ activity.....	227
a.	Synaptic vesicles	227
b.	Synaptic plasma membrane.....	258
c.	Coated vesicles.....	265
5.	Synaptic and coated vesicle prostaglandin synthesis.....	292
6.	Calmodulin association with purified phospholipases A ₂	295
a.	PLA ₂ activity.....	298
b.	DMS-crosslinking.....	303
7.	Interaction of calmodulin, PGF _{2α} , PGE ₂ , cAMP, and cGMP with Naja naja phospholipase A ₂	313
8.	Synaptic and coated vesicle protein kinase activities.....	328
9.	Synaptic and coated vesicle [Ca ²⁺ -Mg ²⁺]-ATPase activities...	341
D.	DISCUSSION.....	346
1.	Calmodulin association with brain synaptic and coated vesicles.....	346

2.	Membrane PLA ₂	348
3.	Synaptic and coated vesicle prostaglandin and Thromboxane synthetase.....	360
4.	Purified phospholipases A ₂	346
5.	Speculations on the evolutionary importance of PLA ₂	372
6.	Synaptic and coated vesicle protein kinases.....	373
7.	Synaptic and coated vesicle [Ca ²⁺ -Mg ²⁺]-ATPase.....	379
8.	Speculations on the diverse effects of calcium on neurotransmitter release.....	380
9.	Unified theory of presynaptic chemical neurotransmission....	380
	a. SV-SPM fusion.....	381
	b. Coated pit/vesicle retrieval of synaptic vesicles.....	401
10.	Vesicle Mobility.....	413
E.	REFLECTIONS ON THE PAST AND THOUGHTS ON THE FUTURE.....	415
F.	REFERENCES.....	418

LIST OF TABLES

INTRODUCTION

Table 1	Comparison of pH and calcium requirements of phospholipases in different tissues and cellular compartments.....	88
Table 2	PLA ₂ stimulators and inhibitors mentioned in past literature with pertinent references.....	101

RESULTS

Table 1	Affinity values of specific [¹²⁵ I]-calmodulin binding to synaptic and coated vesicles.....	224
Table 2	Effects of different modulators on synaptic vesicle PLA ₂ V _{max}	236
Table 3	A comparison of the effects of several conditions on synaptic vesicle PLA ₂ activity light scattering profile and morphology as judged by light and electron microscopy...	257
Table 4	Effectors of synaptic plasma membrane PLA ₂ kinetic parameters.....	263
Table 5	The effects of a variety of modulators on synaptic plasma membrane PLA ₂ activity.....	266
Table 6	Correlation of the effects of a variety of modulators on synaptic plasma membrane PLA ₂ activity and morphology as judged by electron microscopy.....	271
Table 7	The effects of a variety of modulators on coated vesicle PLA ₂ kinetic parameters.....	277

Table 8	The relative change in coated vesicle PLA ₂ activity induced by various conditions and the kinetic parameters affected.....	278
Table 9	The effects of various conditions on coated vesicle PLA ₂ V _{max} and k values.....	279
Table 10	A comparison of the effects of a variety of modulators on coated vesicle PLA ₂ activity, light scattering profile and morphology as determined by light microscopy....	291
Table 11	Kinetic parameters of PLA ₂ s from four different sources in the presence or absence of calmodulin.....	304
Table 12	Kinetic parameters of pancreatic PLA ₂ species in the presence or absence of calmodulin.....	305

DISCUSSION

Table 1	A comparison of the different characteristics of synaptic vesicle, coated vesicle, and synaptic plasma membrane PLA ₂ activities.....	351
Table 2	Comparison of the effects of various modulators on synaptic vesicle, coated vesicle and synaptic plasma membrane PLA ₂ activities.....	359

LIST OF ILLUSTRATIONS

INTRODUCTION

- Figure 1. The general chemical structure of a phosphoglyceride.....71
- Figure 2. Structures of arachidonic acid and some representative
eicosanoid metabolites and their trivial names.....117
- Figure 3. Some important metabolic reactions of prostaglandins....125
- Figure 4. Proposed mechanism of sarcoplasmic-reticulum
[Ca²⁺-Mg²⁺]-ATPase action.....158

RESULTS

- Figure 1. Electron micrograph of synaptic vesicles.....184
- Figure 2. Polypeptide composition of synaptic vesicles
as determined by 5-15% SDS PAGE.....186
- Figure 3. Elution profile of synaptic vesicle preparation by
controlled pore glass bead chromatography.....188
- Figure 4. Electronmicrograph of coated vesicles obtained
after pore glass-bead chromatography.....191
- Figure 5. Polypeptide composition of coated vesicles obtained
prior to pore glass-bead chromatography; decoated
vesicles prepared by extraction procedure (a); and
coated vesicles obtained after pore-glass-bead
chromatography.....193
- Figure 6. Electronmicrograph of decoated vesicles prepared by
extraction procedure (a).....195
- Figure 7. Polypeptide composition of coated vesicles obtained
prior to pore glass-bead chromatography; decoated

	vesicles prepared by extraction procedure (b); and the protein extract of coated vesicles obtained by decoating extraction procedure (b).....	197
Figure 8A.	Electronmicrograph of rat brain synaptic plasma membranes.....	199
Figure 8B.	Polypeptide composition of rat synaptic plasma membranes as determined by 5-15% SDS PAGE.....	199
Figure 9.	Elution profile of ¹²⁵ I-calmodulin on a chloraminopropyl phenothiazine (CAPP) affinity column.....	203
Figure 10.	Polypeptide composition of the calmodulin preparation before and after it was passed through the CAPP affinity column.....	205
Figure 11.	Sucrose gradient profile of synaptic vesicles.....	208
Figure 12.	Sucrose gradient profile of coated vesicles.....	210
Figure 13.	Competition radioimmunoassay curves for chicken gizzard calmodulin synaptic and coated vesicles.....	212
Figure 14.	Competition of unlabelled calmodulin and troponin-C with radio-labelled calmodulin in synaptic (A) and coated (B) vesicles.....	214
Figure 15.	Scatchard analysis of specific [¹²⁵ I]-calmodulin binding to synaptic and coated vesicles.....	216
Figure 16.	Elution profile of solubilized synaptic vesicle proteins on a Sepharose-4B-CNBR-calmodulin affinity column.	219
Figure 17.	Calmodulin affinity chromatography elution profiles of Triton X-100-treated coated vesicle extract (A), purified clathrin (B), and purified 30,000 M _r protein	

	doublet (C).....	221
Figure 18.	Synaptic vesicle PLA ₂ activity plotted as a function of Ca ²⁺ concentration.....	228
Figure 19.	Characteristics of synaptic vesicle PLA ₂ activity.....	230
Figure 20.	Lineweaver-Burk transformations of synaptic vesicle PLA ₂ substrate concentration curves in the presence of CaCl ₂ , CaCl ₂ and PGE ₂ , or CaCl ₂ and PGF _{2α}	233
Figure 21A.	The effect of Ca ²⁺ followed by PLA ₂ Viperra russelli or lysolecithin on synaptic vesicle light scattering.....	237
Figure 21B.	The effect of the sequential addition of Ca ²⁺ and calmodulin on synaptic vesicle light scattering.....	237
Figure 21C.	The effect of the sequential addition of Ca ²⁺ and calmodulin after preincubation with PGF _{2α} on synaptic vesicle light scattering.....	237
Figure 21D.	The effect of sequential addition of CaCl ₂ , MgCl ₂ , cAMP and ATP on synaptic vesicle light scattering.....	237
Figure 22.	The percent increase of light scattering as a function of PLA ₂ or lysolecithin concentration.....	239
Figure 23.	Synaptic vesicle light scattering as a function of calmodulin concentration.....	242
Figure 24.	Synaptic vesicle light scattering as a function of PGF _{2α} concentration.....	244
Figure 25.	Light microscopy of synaptic vesicles untreated (A), treated with PLA ₂ (Viperra russelli) (B); lysolecithin (egg yolk) (C); PGF _{2α} (D); cAMP, ATP, MgCl ₂ and CaCl ₂ (E).....	248

Figure 26.	Electron microscopy of synaptic vesicles untreated (A); treated with CaCl_2 and PLA_2 (<i>Vipera russelli</i>) (B); CaCl_2 and CaM (C); CaCl_2 and $\text{PGF}_{1\alpha}$ (D); CaCl_2 , Mg^{2+} , ATP and cAMP (E).....	252
Figure 27.	Synaptic plasma membrane PLA_2 activity plotted as a function of calcium concentration.....	259
Figure 28.	Characteristics of synaptic plasma membrane PLA_2	261
Figure 29.	Electron microscopy of synaptic vesicles and synaptic plasma membrane untreated (A); treated with Ca^{2+} and PLA_2 (<i>Naja naja</i>) (B); Ca^{2+} and CaM (C); Ca^{2+} and $\text{PGF}_{1\alpha}$ (D); pretreated with parabromophenacylbromide prior to incubation with $\text{PGF}_{1\alpha}$ (E).....	267
Figure 30.	Coated vesicle PLA_2 activity as a function of calcium concentration.....	273
Figure 31.	The pH (A) and time (B) dependencies of coated vesicles PLA_2 activity.....	275
Figure 32.	Light scattering profiles of decoated vesicles treated with calcium and PLA_2 (<i>Vipera Russellii</i>) or lysolecithin (egg yolk).....	281
Figure 33.	Light microscopy of decoated vesicles untreated (A) or treated with lysolecithin (B).....	283
Figure 34.	Light scattering profiles of decoated vesicles incubated with CaCl_2 and calmodulin (A), $\text{PGF}_{1\alpha}$ (B) or PGE_2 (C)	286
Figure 35.	Light microscopy of decoated vesicles treated with PGE_2	289
Figure 36.	Radiometric scan of thin layer chromatogram of	

	synaptic (A) and coated vesicle (B) arachidonic acid metabolites.....	293
Figure 37.	Phase contrast microscopy of synaptic vesicles treated with indomethacin and then by PLA ₂ (<i>Vipera russelli</i>).....	296
Figure 38.	Calmodulin affinity chromatography elution profiles of phospholipases A ₂ from CDT venom (A), CA venom (B), Bee venom (C), <i>Naja naja</i> venom (D), and porcine pancreas (E).....	299
Figure 39	Polypeptide composition of DMS-crosslinked phospholipases A ₂ obtained from CA venom (A), CDT venom (B), bee venom (C), Pancreatic _T (D), Pancreatic _C (E), and Pancreatic _P (F) as determined by 5-15% SDS gel analysis.....	308
Figure 40.	<i>Naja naja</i> PLA ₂ activity as a function of calmodulin concentration.....	314
Figure 41.	<i>Naja naja</i> PLA ₂ activity as a function of PGE ₂ or PGF _{2α} concentration in the presence of CaCl ₂ , or CaCl ₂ and calmodulin.....	316
Figure 42.	<i>Naja naja</i> venom PLA ₂ as a function of cAMP (A) or cGMP (B) concentration in the absence or presence of calmodulin.....	319
Figure 43.	Polypeptide composition of DMS-cross linked <i>Naja naja</i> PLA ₂ in the presence of CaCl ₂ (Lane 1), EGTA (Lane 2), PGF _{2α} and CaCl ₂ (Lane 3) and PGF _{2α} and EGTA (Lane 4)....	321
Figure 44.	Polypeptide composition of <i>Naja naja</i> PLA ₂ DMS crosslinked in the presence of Ca ²⁺ and calmodulin	

	(Lane 1), Ca^{2+} , CaM and $\text{PGF}_{1\alpha}$ (Lane 2). Ca^{2+} , CaM and PGE_1 (Lane 3), Ca^{2+} , CaM, and cAMP (Lane 4), Ca^{2+} , CaM and cGMP (Lane 5). Ca^{2+} , CaM, cGMP, cAMP, PGE_1 and $\text{PGF}_{1\alpha}$ (Lane 6).....	326
Figure 45.	Light microscopy of synaptic vesicles pretreated with PGE_1 prior to treatment with PLA_2 (Viperra Russell)....	329
Figure 46.	Electron microscopy of synaptic vesicles and presynaptic plasma membrane pretreated with: (A) PGE_1 ; (B) PGE_1 ; (C) PGA_2 or (D) PGB_2 prior to incubation with <i>Naja naja</i> PLA_2	331
Figure 47.	Autoradiogram of synaptic vesicle proteins incubated in the presence of $[\gamma^{32}\text{P}]\text{-ATP}$	335
Figure 48.	Percent $[\gamma^{32}\text{P}]$ incorporation into the major calmodulin-mediated synaptic vesicle phosphorylated protein bands of M_r 55K and 53K as a function of anti-30K IgG concentration.....	337
Figure 49.	Autoradiogram of coated vesicle proteins incubated in the presence of $[\gamma^{32}\text{P}]\text{-ATP}$	339
Figure 50.	Synaptic vesicle $[\text{Ca}^{2+}+\text{Mg}^{2+}]\text{-ATPase}$ activity.....	342
Figure 51.	Coated vesicle $[\text{Ca}^{2+}+\text{Mg}^{2+}]\text{-ATPase}$ activity.....	344

DISCUSSION

Figure 1.	Proposed metabolic pathway of arachidonic acid in synaptic vesicles.....	361
Figure 2.	Proposed metabolic pathway of arachidonic acid in coated vesicles.....	361
Figure 3.	Hypothetical model of the possible <u>in vivo</u> calcium-	

	calmodulin pancreatic PLA ₂ interactions.....	367
Figure 4.	Proposed scheme of PLA ₂ branched polymerization pathways.....	370
Figure 5.	Scheme of events postulated to be involved in synaptic vesicle-synaptic plasma membrane fusion and subsequent neurotransmitter release from axon terminals.....	389
Figure 6.	Ca ²⁺ -cAMP synarchy in modulation of synaptic vesicle protein kinase activity.....	393
Figure 7.	Ca ²⁺ -cAMP synarchy in modulation of synaptic vesicle PLA ₂	395
Figure 8.	Scheme of events hypothesized to be involved in coated pit and coated vesicle formation.....	406
Figure 9.	Model of the possible functional significance of synaptic and coated vesicle protein kinase activities...	408

"The basic mechanisms of the modulation of enzyme activity are a marvel of biological engineering that greatly contribute to the high efficiency of the present forms of life."

Alberto Sols, 1979 (1)

A. INTRODUCTION

Chemical neurotransmission is the predominant form of communication from one neuron to the next neuron in central and peripheral synapses (2). The presynaptic neuron transmits information by releasing neurotransmitters which cross the synaptic cleft and then interact with the postsynaptic membrane inducing its excitation or inhibition via electrochemical membrane alterations (3). The typical neuron has between 1,000 to 10,000 synapses and receives information from approximately 1,000 other neurons (4). It is therefore evident that the modulation of transmitter release at one synapse can directly affect the transmission of information to more than one thousand other neurons each of which subsequently can affect more than one thousand other neurons ad infinitum. The modulation of presynaptic neurotransmission could therefore affect local brain functions such as nervous system information processing, learning, memory and behavior (5, 6).

A modulator as defined in 1967 by Florey (7) is any compound of cellular and non-synaptic origin that can affect the excitability of nerve cells. In 1977, Burnstock (8) redefined a modulator as a substance that acts on a presynaptic site of another neuron or on the nerve terminals from which it is derived, inhibiting or accelerating the release of neurotransmitter.

Evidence for presynaptic modulation came from Eccles in 1961 (9) who demonstrated that monosynaptic excitation of motor neurons was depressed by conditioning presynaptic volleys and occurred in the absence of any post-synaptic membrane alteration. Further evidence for presynaptic modulation came from the demonstration that repetitive

stimulation of presynaptic terminals could produce facilitation, depression or potentiation of neurotransmission (10).

Automodulation of presynaptic neurotransmission is classically interpreted as a mechanism whereby a neurotransmitter somehow inhibits its own release by interacting with a presynaptic receptor (11). Thus if the concentration of a particular neurotransmitter is increased at the synapse, it would automodulate, i.e. decrease its own release.

In this work, I attempted to focus on non-transmitter induced automodulation of chemical neurotransmission. Automodulation is defined here as those presynaptic membranal and cytoplasmic elements -- protein, nucleotide or lipid in nature -- which upon stimulation are excited and/or released within the axon terminal and consequently act to either enhance or depress the release of neurotransmitter.

Specifically, I have chosen to study some of the enzymatic properties of brain synaptic and coated vesicles and the presynaptic axonal membrane. Synaptic vesicles store neurotransmitter and are thought to release their contents by fusing with presynaptic plasma membrane. Coated pits/vesicles retrieve the synaptic vesicles from the presynaptic membrane. The modulation of coated vesicle function in turn could affect the neurotransmission process by regulating the number of existing synaptic vesicles.

In the course of this study, I have found that synaptic vesicles and coated vesicles have the endogenous enzymes phospholipase A₂, prostaglandin synthetase, [Ca²⁺-Mg²⁺]-ATPase, and protein kinases dependent on Ca²⁺/Calmodulin and cAMP. The end products of certain enzymes were found to modulate a variety of axon terminal membrane-bound enzymes. Therefore, the history, description, discovery and functions

of phospholipase A₂ prostaglandin synthetase, [Ca²⁺-Mg²⁺]-ATPase, Calcium/Calmodulin and cAMP dependent protein kinases, calmodulin and prostaglandins within the context of neuronal function are carefully outlined and presented.

Because of the importance of calcium as the key initiator of neurotransmitter release, and the absolute cofactor and activator of many of the enzymes studied, a detailed description of its physical and chemical properties and purported roles in neurotransmitter release, endocytosis and membrane fusion will be emphasized.

It is the ultimate goal of this work to present a unifying hypothesis on the mechanism of calcium mediated neurotransmission and neuromodulation. In essence, the hypothesis invokes the direct role of calcium on membranes as well as the ions ability to synergistically and simultaneously activate a host of enzymatic cascades dependent and regulating one another whose products can either amplify or depress synaptic vesicle exocytosis and synaptic vesicle regeneration via the coated pit/vesicle system.

The idea that enzymes function in neurotransmitter release has been criticized and looked upon with a great deal of skepticism because of the extreme rapidity of the neurosecretory process despite the fact that the efficiency of enzymatic multicyclic cascades with respect to its rate amplification and flexibility has been recognized in many metabolic systems (11).

The details and historical background for the formulation of the concept that enzymatic cascades may be involved in neurotransmitter release are presented in this work.

1. The role of calcium in neurotransmitter release, exocytosis and membrane fusion.

"From the neurobiological point of view, calcium is currently regarded as one of the key factors in the operation of nerve cells, not only through its mediation of synaptic transmission but also, and in a deeper sense, in the maintenance of long-term neuronal viability."

R. R. Llinas, 1977 (12)

"Although exocytosis is in some respects, a delightfully simple process, our present knowledge of the mechanism involved is limited. The exocytosis-vesiculation sequence is clearly a complex cellular activity that offers abundant scope for experimental inquiry."

W. W. Douglas, 1974 (13)

Membrane fusion is an essential event in a variety of cell functions such as cell fusion, uptake of extracellular material (endocytosis) and release of cell products (exocytosis). Elucidation of the molecular mechanism of membrane fusion has been hampered by the lack of suitable systems for studying this process. Studies with intact cells have not yielded data which lead to ready interpretation concerning subcellular processes. On the other hand, studies with artificial membranes may be only tangentially related to the fusion of biological membranes."

M. Gratzl and G. Dahl, 1978 (14)

a. Calcium involvement in neurotransmitter release.

In the 19th and early 20th centuries it was being debated whether neurotransmission was chemical or electrical in origin. DuBois-Reymond postulated in 1848 that synaptic neurotransmission was electrical in nature (15). In 1904, Elliott (16) demonstrated that adrenaline, an extract of the adrenal gland, mimicked the action of sympathetic neurons and therefore suggested that adrenaline was secreted from nerve terminals and might act as a chemical transmitter. In 1921, Loewi demonstrated that sympathetic nerves exerted their effects by releasing a chemical substance (17). It was Henry Dale in 1937 (18) who established the principle of chemical neurotransmission, i.e., that the passage of an impulse from one neuron to another involves release from an active neuron of a chemical that has the specific property of stimulating or inhibiting the next neuron.

In the late 1800's and early 1900's while it was being debated whether the nerve impulse was chemical or electrical in nature, information was gathering that the nerve impulse whatever its origin was dependent on calcium. This was first implicated by Locke in 1894 (19) who showed that a muscle if bathed in a calcium deficient medium could not respond to nerve stimulation after 1 or 2 hours even though the muscle would contract upon direct stimulation. In 1912, Hagan and Ormond (20) observed that the ability of the vagus nerve to inhibit the amphibian heart beat was calcium dependent. When calcium was removed from the bathing medium, vagus nerve inhibition was depressed. Upon calcium restoration to the medium, the nerve inhibition was completely resumed. They therefore suggested that calcium was necessary in the nerve apparatus itself for conveyance of nerve impulses to the heart

muscle. Feng in 1936 (21) demonstrated in the frog neuromuscular junction that there existed an antagonism between calcium and curare in eliciting a muscular contraction and therefore maintained that calcium facilitated muscular contraction by acting directly on the synapse and not on the muscle.

The technological achievement of measuring acetylcholine output from nerves led to further advances in elucidating the role of calcium in transmitter release. The first investigators to make use of this technique were Harvey and MacIntosh in 1940 (22) who employed the isolated perfused cat cervical sympathetic ganglion to demonstrate that if preganglionic nerve endings were stimulated either by an electric current or by potassium injection, acetylcholine was not released in the absence of calcium. They maintained that calcium deprivation had a direct effect on neurotransmitter release and did not lead to alteration of nerve conduction.

In 1944, Kuffler demonstrated that during calcium deprivation, there was a decrease in the endplate potential at the frog neuromuscular junction despite the fact that the nerve could still conduct impulses (23). He concluded that the major effect of calcium deprivation was due to a decrease in transmitter release. Fatt and Katz and Del Castillo and Stark in 1952 confirmed these findings with intracellular recordings and extended the findings to the mammalian neuromuscular junction, (24, 25). Hutter and Kostial in 1955, (26) showed that acetylcholine release elicited by electrical stimulation was increased by calcium and that the presence of sodium ions was not required for acetylcholine release. The effects of calcium were definitively ascribed to its capacity for increasing the probability of releasing acetylcholine quanta during

depolarization by Katz and Miledi in 1967 who demonstrated that the action of calcium was on neurotransmitter release and was not mediated by axon terminal impulse conduction alteration (27, 28, 29). Electrophoretic application of calcium to the presynaptic axon terminal increased quantal components of the end-plate potential even when the action potential of the nerve terminal diminished in size. The fact that calcium increased transmitter release only when immediately preceded by a depolarizing pulse suggested that calcium was involved in an essential step between membrane depolarization and acetylcholine release that was not related to the effects of the electrical properties of the axon terminal. Furthermore, when mammalian preparations were bathed in calcium free medium in the presence of calcium chelators, the spontaneous release of acetylcholine as detected by the frequency of miniature end plate potentials was greatly reduced or abolished (30, 31, 32, 33). It has also been demonstrated that magnesium antagonizes the effect of calcium at acetylcholine peripheral synapses (34, 35).

The action of calcium on acetylcholine release from mammalian central synapses is similar to its actions on peripheral axon terminals. The spontaneous and electrically evoked release of acetylcholine was reduced by calcium deprivation and increased upon doubling the calcium concentration (36).

At the same time that the role of calcium was being elucidated at cholinergic synapses the effect of calcium on promoting neurotransmitter release from adrenergic peripheral and central synapses was being established as well. In 1962, Hukovic and Muscholl (37) demonstrated that decreasing the calcium concentration of the bathing medium in isolated rabbit hearts decreased stimulation induced norepinephrine

output. Removal of calcium from the perfusion fluid of the isolated spleen abolished stimulation induced noradrenaline release (38, 39). It was demonstrated in 1967 that norepinephrine output varied directly with external calcium concentration (40). Magnesium antagonized calcium's effect on evoked release whereas barium and strontium substituted for calcium in sustaining the release process (38, 40). Blaustein et. al. found that norepinephrine release from central brain synaptosomes also was entirely calcium dependent (41).

A similar dependence on calcium for neurotransmitter release was found at other chemical synapses (42, 43). In 1968, it was shown that L-glutamate release from the insect neuromuscular junction was increased and depressed, respectively, by calcium and magnesium (42). The release of gamma-butyric acid (GABA) from mammalian brain slices was also inhibited by calcium deprivation (44).

b. Calcium involvement in exocytosis.

Adrenal medulla which has a common ectodermal embryological origin with the nervous system is often employed as a model for neurosecretion. Calcium has been demonstrated to be essential for the exocytotic release of catecholamines from the adrenal medulla. In the isolated cat adrenal gland and in bovine adrenal slices calcium deprived perfusion solutions were shown to depress or abolish acetylcholine induced secretion of catecholamines (45-49). It was only in the presence of calcium that the adrenal medulla secreted catecholamines in response to a variety of stimuli (50-53). In the absence of calcium, spontaneous catecholamine release was very low (53, 54).

Subsequently the role of calcium in releasing secretory substances

has been demonstrated in a wide variety of cell types and tissues. Calcium was demonstrated to be involved in the release of vasopressin and oxytocin from the neurohypophysis (56-62). The release of the anterior pituitary hormones; thyrotropin, leutinizing hormone, adrenocorticotrophin and prolactin are also all calcium dependent (63). Calcium was also demonstrated to stimulate insulin secretion from the pancreas (64-67). Various stimuli such as glucose, potassium, ouabain and glucagon, enhanced pancreatic insulin secretion in a calcium dependent manner (64-67). As in the other secretory systems mentioned, magnesium not only did not substitute for calcium, it inhibited insulin secretion when added to calcium containing perfusion solutions (64-69).

Calcium in addition has been implicated in the release of water, protein and electrolyte secretions from the salivary gland (70), HCl, pepsin and gastrin secretions from the gastric mucosa (71-77), protein secretion from leukocytes (78), and histamine release from a variety of tissues (79-87). Calcium is also necessary in antigen and thrombi induced release of 5-HT from blood platelet granules (88-93).

c. Calcium involvement in membrane fusion.

Calcium induces fusion of isolated secretory vesicles from a variety of cell types (94-101). Douglas demonstrated that in mast cells, an external stimulus initiated fusion between secretory vesicle membranes and the cell membrane followed by fusion of other vesicles to the original vesicle (102). This phenomenon was observed in a variety of cell types (95-100). According to Douglas, intervesicular fusion and exocytosis are parts of the same process (102). When vesicles were isolated from β -pancreatic cells they fused in the presence of calcium

(103). In isolated liver secretory vesicles Dahl and Gratzl demonstrated that out of the various cations examined — Mg^{2+} , Sr^{2+} , Ba^{2+} and Ca^{2+} — only calcium could induce fusion of vesicles (103). Mg^{2+} antagonized calcium's effect and decreased the amount of vesicle fusion.

In summary, experiments performed over the past 90 years have established that calcium is the universal requirement for neurotransmitter release at most if not all peripheral and central chemical synapses. This cation is also absolutely necessary for the release of secretory material from cell types, and for the fusion of discrete membranal components. In most of these phenomena, it has been established that Mg^{2+} antagonizes the action of calcium.

2. The vesicle hypothesis

After the elucidation of the chemical nature of neurotransmission, after and during the establishment of calcium's role in releasing neurotransmitters and hormones, and facilitating membrane fusion, Del Castillo and Katz in 1954 (104) enunciated the quantal theory of synaptic transmission. Shortly afterwards, small vesicles were observed using electron microscopy in axon terminals by De Robertis and Bennett in 1955 (105). Del Castillo and Katz in 1956 (106) suggested that these synaptic vesicles were morphological counterparts of the quanta and they hypothesized that the synaptic vesicles could quantitatively release their contents into the synaptic cleft by exocytosis. The demonstration by Whittaker, Michaelson and Kirkland in 1964 (107) that synaptic vesicles contained the neurotransmitter acetylcholine provided physiological, morphological and biochemical observations, which were

unified into a theory of transmitter storage and release that was henceforth called "the vesicle hypothesis." Since the formulation of this theory, the quantal nature of transmitter release has been established in a variety of vertebrate (108, 109) and invertebrate (110, 111) synapses, and synaptic vesicles have been found in many types of chemical synapses (112).

Supporting the vesicle hypothesis, electrophysiological studies estimated that the number of molecules released in the quantal packet was found to be the same order of magnitude as that determined to be inside the synaptic vesicles. It was estimated at the neuromuscular junction that the quantal packet contained 1000-6000 molecules of neurotransmitter (113, 114). Synaptic vesicles isolated from brain nerve endings were found to contain 1000-2000 molecules of acetylcholine (115). Similarly, vesicles isolated from the bovine superior cervical ganglion were estimated to contain 1600 molecules of acetylcholine (116).

Nevertheless, despite the inherent logic of the "vesicle hypothesis," morphological studies in the past have failed to confirm it. When thin sections of stimulated glutaraldehyde fixed nerve endings were analyzed by electron microscopy, synaptic vesicles could not be seen to fuse with the presynaptic membrane (117). Additionally, the lack of precise parallelism between altered transmitter levels and vesicle abundance in stimulated neurons implied that under certain experimental conditions, the most recently synthesized transmitter seemed to be rapidly used (118). Other phenomena such as the presence of synaptic vesicles at synapses where transmission occurred by electrotonic coupling (119), and the finding that enzymatic destruction

of cytoplasmic acetylcholine blocked neurotransmission (120) were not in line with the scheme that synaptic vesicles serve as neurotransmitter storage areas. Therefore, it was alternatively postulated that transmitters are released from the cytosol through voltage dependent pore openings, rather than from synaptic vesicles (118).

The development of new techniques over the past several years, however, has provided striking morphological evidence that synaptic vesicles do indeed fuse with the pre-synaptic plasma membrane upon stimulation. Some of these techniques include rapid freezing, freeze-fracture and immunoflorescent studies.

Heuser (121) by quick freezing frog neuromuscular junctions captured synaptic vesicles in the process of exocytosis. After applying 4-aminopyridine to neuromuscular junctions to dramatically increase the amount of secretion following an impulse he observed that the earliest events in the unfixed freeze-etched secretion faces were small depressions along the active zones. Shortly afterwards, widened pores developed in these locations, and synaptic vesicles were shown to open after the triggering of secretion.. Heuser et al. (122) also showed that the number of exocytotic vesicles caught by quick freezing increased proportionally, indicating that one vesicle undergoes exocytosis for each quantum that is discharged. Also statistical analysis performed by this group on the spatial distribution of synaptic vesicle discharge sites along the active zones marking secretory regions of the nerves showed that individual vesicles fuse with the plasma membrane independently of one another.

Similarly Ciccarelli et al. (123) have used the freeze fracture technique to observe vesicle exocytosis in the frog neuromuscular

junction. They intensified transmitter discharge with black widow spider venom which they maintained produces an effect similar to depolarization by excess potassium. They concluded from their work, as did Heuser et al., that synaptic vesicles on fusion collapse completely into the membrane and that fusion of the vesicle and the release of quantum of neurotransmitter occur almost simultaneously.

Von Wedel et al. (124) utilized immunofluorescent techniques to show that synaptic vesicles were fusing with the plasma membrane. Antiserum was raised against purified cholinergic synaptic vesicles from the electric organ of the marine electric ray. When this antiserum was added to the bathing medium of intact frog neuromuscular junctions prior to stimulation, binding of antibody was undetectable. However, when intact preparations were stimulated to release their vesicular contents by the addition of LaCl_3 , antibody binding to the intact nerve terminals became striking. These findings suggested that synaptic vesicle antigens were accessible to the outer surface of nerve terminals only in the excited state, but not in the resting state.

There are other studies that point to the validity of the "vesicle hypothesis." When nerve terminals were repeatedly stimulated to the point of fatigue, ultrastructural changes in wet fixed tissues generally showed a loss of synaptic vesicles (125-127) and a corresponding increase in the plasma membrane of the terminal (125-126). It has also been shown that the sum of the areas of cytoplasmic membranes and the external plasma membrane stayed constant upon stimulation (125,128). These results could only be expected under experimental conditions where incorporation of the vesicle membrane into the outer membrane due to exocytosis exceeded the rate of subsequent retrieval of membrane

segments. It was shown that cooling of the tissue during activation inhibited vesicle retrieval more than vesicle fusion, thereby causing a prominent enlargement of the presynaptic membrane surface (127, 129). At the frog neuromuscular junction, there was no loss of vesicles upon stimulation at 0.2 Hz continued for a long time, whereas stimulation at higher frequencies led to a rapid decrease in synaptic vesicle number (130). Similarly in the Torpedo electric organ, a sustained stimulation at 5 Hz led to a massive depletion of synaptic vesicles, whereas sustained low frequency stimulation did not induce a loss in synaptic vesicle number (131).

The application of lanthanum chloride to frog neuromuscular junctions has been shown to lead to a 10,000 fold increase in the frequency of quantal release in unfixed tissues. If the same procedure was performed and the tissue was fixed, no evidence for depletion could be detected (132). Black widow spider venom which is known to cause a burst of quantal release also caused a marked depletion of synaptic vesicles (133).

Pysh and Wiley (128) analyzed the ultrastructure of presynaptic terminals after short periods of acetylcholine secretion in the cat superior ganglion in vivo. Rapid stimulation of the ganglia produced not only an absolute decrease in the number of synaptic vesicles but also an enlargement of the presynaptic terminal membrane.

Heuser and Reese (134) demonstrated by quick freezing nerves at various times of stimulation that vesicles not only collapsed after exocytosis but that also the components of their membrane mixed intimately with the plasma membrane. Three or more unusually large intramembranous particles characterized the membrane of each fractured

synaptic vesicle. After vesicles fused with the plasma membrane and collapsed into it, these particles were seen diffusing away from the active zone. The fusion of the synaptic vesicles and the plasma membrane began at a minute spot where the vesicle first touched the plasma membrane, and membrane fusion proceeded to completion in less than a millisecond. Thereafter, the vesicle opening enlarged progressively until the vesicle had collapsed flat into the presynaptic membrane. After vesicles fused with the plasma membrane and collapsed into it, the particles diffused away from the active zone. After long periods of stimulation these particles appeared to distribute uniformly over the plasma membrane.

If the vesicle hypothesis is correct, then the following steps which are hypothesized to occur in the general fusion of membranes (135) most likely also occur upon fusing of the synaptic vesicle with the plasma membrane. Small portions of the two independent membranes become closely apposed to eliminate all intervening layers of water. At the region of apposition the membranes become destabilized and their molecular components mix with each other. In the destabilized region membranal fission occurs to create continuity between the outer portions of the membrane. Once vesicle apposition has been achieved, the remaining steps proceed rapidly and spontaneously, and well defined intermediate stages have rarely been observed. Palade (136) distinguished the above mentioned three successive steps of exocytosis and referred to them as apposition, fusion and fission.

When the presynaptic axonal membrane is split by freeze fracture, the intramembrane components are revealed (137-139). On the PF (P Face), the outer face of the inner lamella of the plasma membrane, the

active zones appear as gentle transverse ridges bounded on each side by a double row of large intramembrane particles. The areas of membrane between the active zones appear relatively uniform and contain both small and large intramembrane particles distributed randomly over the face. On the EF (E face) the active zones are marked by gentle furrows bordered on some places by double rows of pits or large or small particles. Upon repeated stimulation of a nerve, numerous circular or ellipsoidal dimples appear on the PF of the presynaptic membrane and numerous circular protuberances with central craters appear on the EF. Similar circular deformations on the PF of the cell membrane have also been observed in the Tetrahymena membrane stimulated to discharge mucocysts (140, 141) in nerve terminals (142), in the neurohypophysis (143), in stimulated parotid acinar cells (144), in pancreatic β cells (145, 146) and in the adrenal medulla (147). It is thus well established in these secretory systems that the dimples or protuberances represent fractured connections between the plasmalemma and membranes of secretory vesicles. Similar findings in these other secretory systems buttress the interpretation that the dimples and protuberances seen on freeze fractured nerve terminals mark connections between the plasma membrane and the membrane of synaptic vesicles (148-152). The results of freeze fracture therefore provided definitive proof that synaptic vesicles fuse with the plasma membrane upon nerve stimulation and that fusion of the synaptic vesicle and the release of a quantum of neurotransmitter are closely linked in time (134).

Recently, spontaneous release of cholinergic transmitters from minced mousebrain was shown to occur independently of calcium and had a ratio of false to true transmitter similar to that of the cytoplasm,

whereas the evoked transmitter release required calcium and had a ratio of false to true transmitter similar to the vesicle fraction (153). These studies demonstrated that spontaneous release of cholinergic transmitter occurred from the cytoplasm independently of calcium, whereas the evoked release of cholinergic transmitter occurred from synaptic vesicles.

Because "the vesicle hypothesis" has appeared to withstand the test of time, this work uses this hypothesis as its basic starting point and intends to build upon it.

3. The origin of synaptic vesicles

If synaptic vesicles fuse at a constant rate with the plasma membrane, new vesicles must be formed by some mechanism. The regeneration of synaptic vesicles by the coated pit/vesicle system will be described and discussed later on. At this point it is of interest to discuss the original genesis of synaptic vesicles within the neuron.

There are two major theories which describe the origin of synaptic vesicles. The first theory (154) maintains that synaptic vesicle components are synthesized in the perikaryon and are then transported down the axon by microtubules and filaments. This theory is supported by electron microscopic observations delineating the presence of similar vesicles in the cell body and axon terminals in a variety of neuronal cell types (155, 156). The site of origin within the perikaryon has been speculated to be either the Golgi (157) or the Endoplasmic Reticulum (158). Vesicles have also been hypothesized to arise from a network of tubules located away from the Golgi which represent a form of agranular reticulum (154, 155).

Morphological and autoradiographic studies indicate that synaptic vesicles are translocated "in tact" from perikaryon to axon terminal (159). Holtzman, however, argues against the theory that synaptic vesicles originate in the perikaryon because upon observation of normal vertebrate axons in central and peripheral synapses there are very few synaptic vesicles seen along the axon (154). Although Dahlstrom (160) has shown that if axons are ligated, proximal to their ligation there is an accumulation of vesicles, this may not be sufficient evidence according to Holtzman (154), since vesicles can also originate locally at interruptions from larger sacs or tubules (161,162). This type of origin has been noted along axons and terminals of normal neurons (150).

The second major theory of synaptic vesicles genesis propagated by Holtzman maintains that the vesicles originate locally in the axon terminal from axonal sacs and tubules (156). The sacs and tubules are histochemically defined as agranular reticulum (156). It has been proposed from serial reconstructions (158) that this reticulum is continuous along the length of the axon and may stretch as a single structural entity from the cell body to the axon terminal. The relationship between the axonal reticulum and the perikaryon are not clearly defined. According to Teichberg and Holtzman, the axonal reticulum branches into networks of tubules in axon terminal and these branches are the chief source of axonal synaptic vesicles (156). It has also been hypothesized that the smooth endoplasmic reticulum is continuous from the perikaryon and that newly synthesized proteins and phospholipids are transferred to the smooth endoplasmic reticulum and are rapidly transported down the reticulum and form synaptic vesicles within the axon terminal (152).

4. Protein and lipid characteristics of synaptic vesicles

Proteins with the following M_r 's have been reported in synaptic vesicles: 128K, 96K, 64K, 58K, 53K, 48K, 41.5K, 37.5K, 32.5K, 29.6K, 24.5K, 21.1K, 18.2K, 13.8K and 12K (163). Moreover, Matthew et al. reported that a protein with M_r 65K is specific to synaptic vesicles (164). In addition, glycoproteins in synaptic vesicles with the following M_r 's have been reported: 120K, 68K and 45K (163).

Deutsch and Kelly purified synaptic vesicles from the electric organ of the marine ray *Narcine brasiliensis* and analyzed the lipid and fatty acid content of their membranes (165). The major lipids (mole %) were phosphatidylcholine (32.3%), phosphatidylethanolamine (20.5%), phosphatidylserine (6.1%), sphingomyeline (3.0%) and cholesterol (33.3%). This lipid profile was significantly different from the parent electric organ. The vesicles were enriched with docosahexenoic acid (22:6). The phospholipid:protein ratio in synaptic vesicles is 5.3:1 by weight (166). The vesicles did not contain any unusual phospholipids. Lysophosphatidylethanolamine was a minor component. The synaptic vesicle preparation of Carlson et al. (166) did not exhibit vesicle-vesicle aggregation or fusion in the presence of calcium. They admit however that during the course of their extensive purification, some protein component which might be relevant to fusion could have been lost.

5. Morphological structures in the axon terminal closely associated with synaptic vesicle fusion.

a. The presynaptic densities and grid.

Presynaptic densities are found in peripheral nervous tissue and

the presynaptic grid is found in the central nervous system. Both structures have been implicated in somehow guiding synaptic vesicles to the specific site of exocytosis.

It has been found in the frog neuromuscular junction that the presynaptic densities occur as thin transverse bands directly above the postsynaptic fold (167). Synaptic vesicles collect in rows on both sides of the dense bands and it is there that exocytosis is found to occur with freeze fracture techniques (168). The evidence thus suggests that presynaptic densities mark the active zones at synapses where exocytosis occurs. If their function is to align the synaptic vesicles at the presynaptic membrane in preparation for exocytosis, then these structures should be reliable indicators of synaptic activity. Govind and Meiss (168) found a good correlation between the surface area of dense bars and the mean quantal output at muscle fibers. Govind and Chiang (169) compared low and high output terminals arising from a single motor axon to the crustacean accessory flexor muscle. Thin section electron microscopy showed that even though the size of the synapses was similar in two types of neuromuscular terminals, the high output terminals had a significantly greater number and total surface area of presynaptic dense bands than their low output counterparts. This seemed to support the hypothesis that presynaptic densities may determine transmitter output at crustacean neuromuscular junctions. Since the densities are also found at sites not differentiated into synapses it was suggested that they represent an early stage of synaptogenesis. Govind et al. (170) examined the three dimensional ultrastructure of presynaptic densities by serial section electron microscopy at the neuromuscular synapse of the lobster. They found that the cross-

sectional profile of the density resembled that of an asymmetric hour glass. The bar has a height of 55-60 nm and varies in length from 75-600 nm. In its dimensions it resembles the dense projections found in the CNS. In all cases, the densities are intimately in contact with synaptic vesicles on both sides.

Akert (1971) found in the central nervous system that the presynaptic grid is on the cytoplasmic side of the presynaptic membrane and consists of an assembly of dense projections which are arranged in a triangular lattice and interconnected by filamentous cross-bridges. The holes of the grid have roughly the dimensions required to accommodate synaptic vesicles. Thus Akert maintains that the grid provides the most economical geometry of placing transmitter storage vesicles as closely as possible to the expected release sites. This grid is consistently found in chemical synapses of the spinal cord, cerebellar cortices, basal ganglia and midbrain tectum of avian and mammalian species. This seems to indicate that the grid is an exclusive feature of the CNS where it seems to be coextensive with the active zone. Akert tried to correlate morphological changes within the grid with different states of synaptic activity. There was a high ratio of unoccupied to occupied grids. He looked at the characteristics of synaptic membranes in animals anesthetized with sodium pentobarbital compared to unanesthetized animals. Freeze fracture replicas of membrane faces in the region of the grid revealed striking changes that accompanied the shift of synaptic activity from the anesthetized state compared to the unanesthetized state. There was a higher frequency of grid occupation with synaptic vesicles in the unanesthetized state compared to the anesthetized state. These observations were consistent with the

decrease in mean quantum content of the presynaptic transmitter under the influence of pentobarbital in spinal motor neurons.

Thus it appears that the presynaptic densities in peripheral synapses and the presynaptic grid which in essence consists of presynaptic densities interconnected by filamentous cross bridges in the central nervous system serve to align synaptic vesicles with the presynaptic membrane to undergo exocytosis. Why is it that this alignment is achieved in peripheral synapses with dense projections, whereas in central synapses a grid is necessary? No one has yet commented on this. Perhaps there is a greater need of segregation of different synaptic vesicles, or greater need for the regulation of neurotransmitter exocytosis by synaptic vesicles in the central nervous system.

The other question which arises is: why should the synaptic vesicle need any alignment at all? Perhaps the answer can be deduced from an analogous secretory system in *Tetrahymena Pyriformis*. In this organism, Satir et al. (172) found that there were precise morphological markers for exocytosis of mucocyst discharged from granules. Only when secretory vesicles aligned themselves with rosettes present in the plasma membrane (freeze fracture study) did exocytosis occur. Perhaps in the nervous system dense projections and grids ensure the exact alignment of secretory sites on the synaptic vesicle with the secretory sites of the presynaptic membrane.

b. Synaptopores.

Freeze cleavage of presynaptic membranes in the vertebrate central nervous system revealed specialized structures which have been called synaptopores (148), presynaptic membrane modulations (173) or vesicle

attachment sites (173). These structures are protuberances extending towards the presynaptic cytoplasm and have been shown to be loci of direct contact with synaptic vesicles and exocytotic release (148, 173). It has been postulated that these sites must have specific properties to permit vesicle-plasma membrane interaction. Invariably, images of synaptic vesicle fusion are lined up between these zones. Rarely has a vesicle opening been observed as far as $500\text{\AA}$ away from these structures. Thus synaptic vesicle exocytosis is very strictly localized to the active zone. In the discussion, I will elaborate the idea that the active zone, the presynaptic densities and synaptopores align the vesicle such that the necessary enzymes, phospholipid, and proteins in the synaptic vesicle and presynaptic membrane are optimally arranged and aligned to facilitate rapid exocytosis.

c. The presynaptic axon terminal calcium channel.

It has been estimated by Llinas et al. that as soon as the electron motive force for calcium is increased by membrane depolarization, calcium enters the axon terminal and within 200 μsec triggers neurotransmitter release (174). This time period assumes that the site of calcium entry and the site of transmitter release are very close to each other since the distance which calcium can move during this time is very limited (174).

Parsegian (175) calculated that the diffusion coefficient (D) of calcium determined by Hodgkins and Keynes (176) in the squid giant axon is $6 \times 10^{-7} \text{ cm}^2/\text{sec}$ which is one order of magnitude lower than the ion's diffusion coefficient in water. Thus Parsegian calculated that in 200 μsec calcium can diffuse only $1500\text{\AA}$ from its site of entry at the presynaptic membrane. This calculation is based on the Stokes-Einstein

equation that states that the mean square displacement of calcium is represented by the equation " $\sqrt{x^2} = 2DT$ " where x = distance and t = time. Therefore, the time available for diffusion of calcium is less than 200 μ sec. Because calcium may move only a few hundred $\text{\AA}$ into the axon terminal during the synaptic delay, only synaptic vesicles in close proximity to the calcium entry sites can undergo exocytosis. The fact that calcium must exert its effects at its site of entry was confirmed by experiments in which calcium was injected into a cell and visualized by the glow of aequorin, demonstrating that calcium did not move within the cell but remained near the tip of the micropipette and then disappeared into the cytosol (177).

Experiments with aequorin have also revealed that following depolarization calcium ions enter the axon terminal at a rate that roughly parallels the rise of sodium conductance and subsequently the rise of potassium conductance (178). It appeared that the early phase of calcium entry resulted from calcium entering through a channel that was pharmacologically distinct from the sodium and potassium channels. This special channel has been called the calcium channel. Evidence points to the existence of special voltage sensitive calcium channels distributed throughout the length of the axon terminal through which calcium can enter and initiate neurotransmitter release. Like the sodium channel, the calcium channel opens transiently in response to a maintained depolarization. However, whereas the sodium channel closes or is inactivated within a few msec, inactivation of calcium channels takes several seconds. Although calcium also enters the axon terminal through sodium channels and contributes to secretion-activation, its contribution to the process is far less significant. Even after

pharmacological blocking of voltage dependent sodium and potassium channels in the squid presynaptic terminal, transmitter was still released by depolarization of the terminal (179). The fact that synaptic transmission is accompanied by an inward calcium current was suggested by the discovery of tetrodotoxin-insensitive presynaptic spikes (180).

It was shown that the temporal sequences between depolarization and transmitter release could be divided into two parts (12). The first component is related to the time required for opening the calcium channels and the second component relates to the time after the channel opening to the time of transmitter release. The brevity of delay, Llinas maintains, indicates that the site of calcium entry is located near the release site.

The density of calcium channels is much higher at the axon terminal than anywhere else along the axon (12). It has also been demonstrated that the ratio of calcium to sodium channels is much higher at the axon terminal (12).

It has been proposed by Dreyer et al. (138), Heuser and Reese and Peper et al. (139, 181) that the intramembranous particles observed in freeze fractures of active zones in the frog neuromuscular junction are the morphological representations of calcium channels. Heuser estimated that if those particles are calcium channels then the calcium channel density is approximately $150/\mu\text{m}^2$. Assuming that there is a total calcium ion density of 1.14×10^3 ions/ mm^2 /spike (180), there would be an average calcium ion flow of 8 ions per impulse per channel, assuming all calcium ions open during the action potential.

If it is assumed that calcium enters the nerve and is uniformly

distributed throughout the $200\text{\AA}$ layer of cytoplasm closest to the pre-synaptic membrane, in the squid stellate ganglion the internal calcium concentration at the release site was calculated to reach about 10^{-5}M (174). Kelly et al. (182) speculated that the estimated value would be higher if (a) the calcium diffusion constant were lower than $6 \times 10^{-7}\text{cm}^2/\text{sec}^{-1}$ in the nerve terminal; (b) calcium entered only at the active zones; or (c) if the internal calcium were not uniformly distributed but preferentially associated with negatively charged membrane surfaces. Because of these unknown parameters Kelly et al. speculated that the internal calcium concentration in the region of the exocytotic site during the peak of transmitter release may reach between 10^{-6} - 10^{-3}M .

Llinas et al. derived a theoretical model of the calcium gate suggesting that the calcium channel is formed of five monomers each having one gating charge and capable of assuming in a noncooperative manner a molecular structure which permits calcium flow through the channel (174). At rest, each monomer is positive at the inner surface and negative on the outer surface. A membranal depolarization would induce a change in the electric field across the membrane, redistributing the charge across each monomer, producing a conformational change such that calcium is allowed to move in without obstruction. The synaptic delay in exocytosis, Llinas speculated, is probably related to the time dependence of the calcium current.

Depolarizing the presynaptic membrane potential in *Aplysia* enhances transmitter release by activation of a steady state calcium current, and decreases the outward potassium current indicating that the amount of calcium coming into the presynaptic terminal with each action potential is not fixed but can be modulated by membrane voltage and chemical

neurotransmitters (183). This suggests that calcium current modulation may be a mechanism for the presynaptic regulation of transmitter release.

6. Pertinent physical chemical properties of calcium

It is apparent that calcium is involved in the release of a variety of neurotransmitters, hormones and granule components. Because of its importance in these processes as well as being important in the regulation of cell function in general and its ability to act as a messenger that coordinates a multiplicity of intraneuronal reactions involving membranous and enzymatic events which will be the topic of this thesis, it is at this point relevant to discuss certain physical and chemical properties of calcium. Awareness of these properties is necessary to obtain a complete understanding of calcium dependent exocytosis and endocytosis.

Calcium is maintained at an intracellular concentration of 10^{-8} - 10^{-7} M (184). In squid axon, the free calcium concentration is estimated to lie between 20×10^{-9} - 50×10^{-9} M (184). This intracellular level is much lower than any other intracellular ion. Sodium is maintained at a level of 12-20mM. Potassium is maintained at 150 mM and Mg^{2+} at 2.8mM (185).

The ionic radius of calcium is $0.94 \text{ \AA}$. The ionic radii of Mg^{2+} , Na^{+} , and K^{+} are respectively, 0.65, 0.98 and $1.33 \text{ \AA}$. (186). When calcium is bound it is primarily dehydrated and the coordination requirements are provided essentially by the ligand (187). Carafoli (188) suggested that calcium was chosen as a major intracellular regulator and messenger as opposed to Mg^{2+} because Mg^{2+} is small and

therefore limits the possibility of it being controlled since it is difficult to build cavities small enough to provide a selective fit for it. A Mg^{2+} selective cavity would require only six oxygen coordinating atoms around the central Mg^{2+} ion. This, Carafoli maintains, is improbable in view of the mutual repulsion of the atoms on the coordination sphere. With eight coordinating atoms, the cavity would become too large to provide an optimal fit.

Other than the size of the cation, other factors which are involved in metal complexation with ligands are the charge and polarizing power of the ion. The polarizing power of an ion is directly proportional to its radius. Mg^{2+} has a smaller radius than Ca^{2+} and thus a higher polarizing power. Mg^{2+} therefore exerts more attraction than calcium on the polarizable atoms of the coordination sphere. Therefore donor atoms, e.g., oxygen, nitrogen and sulfur, would assume a regularly spaced structure determined by the strong attraction exerted by Mg^{2+} . The relatively weaker polarizing power of Ca^{2+} would allow the donor atoms to maintain irregular spacings which would be determined by their mutual repulsion. The optimal coordination number of Ca^{2+} varies from 6-8. Because of the high polarizing power of Mg^{2+} its coordination bonds are always of similar length and angle, thereby maintaining a regular octahedral geometry around the cation, whereas those of calcium may vary widely, allowing irregular geometries of the coordination site. Calcium is therefore far more versatile than Mg^{2+} and can adapt to a constellation of irregular binding sites.

Another shortcoming of Mg^{2+} is its tendency to retain part of the hydration water when forming complexes. Mg^{2+} can interact only with coordination centers of irregular binding sites that are compatible with

its requirement for fixed bond length and angle preserving the octahedral geometry through partial hydration.

It is interesting at this point to recall that Mg^{2+} antagonizes the effect of Ca^{2+} in mediating neurotransmitter release, exocytosis, and membrane fusion. As will be discussed later, Mg^{2+} can indeed compete with Ca^{2+} for binding sites on certain proteins, e.g., calmodulin and phospholipase A_2 . Thus understanding of the release process may be obtained by physical-chemical analysis of ion-protein interactions.

The two cations that interact most closely at the membrane level are calcium and sodium. It is possible that calcium and sodium originally represented equivalent evolutionary choices (189). The ionic radius of the two ions are nearly the same and may be important for the close interplay between these two ions. Similarly, the coordination bonds of Na^+ may vary greatly in length and angle allowing irregular geometries of the coordination site. This is probably why calcium enters the axon terminal via the sodium channel during the initial phase of membrane depolarization (188).

Kretsinger (190) has proposed that calcium functions as a second messenger because in the evolutionary sequence it was first extruded from cells to prevent calcium phosphate precipitation. Cells then exploited the outward and inward calcium gradients to transmit information.

7. Theories on the mechanisms of the direct action of calcium in exocytosis and fusion.

This section will deal with the various theories generated

throughout the years concerning the direct nonenzymatic actions of calcium on fusion. Other mechanisms involving contractile proteins, enzymes and interactions with calmodulin will be detailed in later sections of this introduction.

The arrival of the action potential at the nerve terminals causes a large increase in the rate of transmitter release. Within a few milliseconds several hundred packets of transmitter are liberated to produce a post-synaptic potential. The increased release rate is dependent on external calcium concentration. In the absence of extracellular calcium, nerve impulses fail to cause an increase in the probability of release (180).

The increase in neurotransmitter release when a second nerve impulse invades the nerve terminals after the first was termed facilitation by Eccles et al. in 1941 (191). It has been demonstrated that this facilitation is dependent on calcium ions (192) and is probably due to residual calcium ions left at the site of transmitter release. The increase in transmitter release following tetanic stimulation is also affected by the presence of calcium ions in the extracellular medium (193). Thus all phenomena of transmitter release which are dependent on the arrival of a nerve impulse at the axon terminal are dependent on calcium.

Jenkinson (194) in 1957 showed that the relationship between calcium concentration and evoked transmitter release was nonlinear. Quantitative analysis at the frog neuromuscular junction by Dodge and Rahamimoff in 1967 (195) showed that a high powered sigmoidal relationship existed between calcium concentration and transmitter release. They assumed that there is a cooperative action of four

calcium ions in the release of a quantum of transmitter by the nerve impulse and that there exists a calcium receptor site. They proposed a model which involved a stoichiometric reaction between four divalent cations and a membrane component that had four independent binding sites. To be effective for release, they maintained, all four sites must be occupied by calcium. Similar quantitative dependence of neurosecretion on external calcium has been observed at the mammalian neuromuscular junction (196), at the squid giant synapses (197) and noradrenaline secretion at the guinea pig vas deferens (198). The sigmoidal relationship has been interpreted to mean that several calcium ions have to cooperate in the release of a quantum of neurotransmitter. Whittaker and Sheridan (199) proposed the existence of intracellular tubular connections from synaptic vesicles to the external membrane. They maintained that calcium in some way enlarged the lumen of these tubules to facilitate exocytosis.

Katz in 1969 (200) proposed a mechanism for calcium invoked exocytosis. He thought that vesicles moved within the axon terminal in brownian motion, and as a result were constantly colliding with the presynaptic membrane. However, Katz hypothesized that vesicles can only fuse and release neurotransmitter if they strike a reactive site on the membrane that can initiate the exocytotic process. He hypothesized that calcium increased the number of reactive sites in the presynaptic membrane and therefore allowed many more collisions to be effective.

Bass and Moore (201) also proposed that vesicles containing Acetylcholine (ACh) undergo brownian motion in the nerve terminals, and that ACh is released whenever a vesicle touches the inner face of the plasma membrane of the nerve terminal membrane. They maintained that

the frequency of contact is limited by the energy barrier that must be overcome before the vesicle can touch the plasma membrane. The barrier, they think, is composed of electrostatic repulsion between positive fixed charges on the vesicles and a relative positive potential at the face of the plasma membrane as well as a layer of water molecules held to the vesicle by the surface charge. A variation of this hypothesis by Van der Kloot and Kita suggests that the vesicle and the inner face of the membrane are assumed to have fixed negative charges responsible for the energy barrier (202). Their data indicated that only vesicles with sufficient thermal energy to overcome a barrier could reach the release site on the inner face of the plasma membrane. The barrier, they maintained, is the rate limiting step in quantal release. The role of calcium, they speculated, is to bind ionic groups near release sites reducing the surface charge and accelerating release.

The hypothesis that vesicles undergo brownian motion has been proven incorrect. Axoplasm is highly viscous. The cytoplasm is viewed as a two phase system in which local areas of low viscosity are within the interstices of a fibrous actin gel meshwork (203). Crick in 1950 (204) demonstrated that the axoplasm is elastic in the living cell. Therefore, if synaptic vesicles are embedded in this matrix, they cannot possibly have any brownian motion.

The prime advocate for the direct role of calcium in membrane fusion is Papahadjopoulos. Calcium which induces charge neutralization, he maintains, is not sufficient in itself to induce fusion of membranes (205). The key event for fusion of lipid bilayer membranes is the isothermic induction by Ca^{2+} of a phase transition from fluid to crystalline state viz. a condensed acyl chain packing. During the

addition of calcium, a transient unstable state in the membrane is produced making it susceptible to fusion. This state is created by the asymmetric distribution of calcium across the phospholipid bilayer membrane, the structural discontinuities between different phospholipid domains and the transient local release of heat liberated by the crystallization of the phospholipid acyl chain. The presence of calcium induces a phase transition or phase separation inducing either a partial or complete crystallization of the outer monolayer of the vesicle membrane. The molecular condensation would create structural defects with exposure of the hydrocarbon-water contact points. Papahadjopoulos deals with purified synthetic phospholipid vesicles and has amassed a good deal of elegant proof to verify this theory (206). In the absence of calcium, the membranes in the form of closed vesicles are stable at temperatures above and below the gel to fluid lipid transition temperature of the lipids in the membrane and therefore do not fuse. Aggregation and fusion of vesicles prepared from pure acidic lipids such as phosphatidylserine can be induced simply by the addition of calcium at temperatures at which the lipids are fluid. The calcium concentration needed is 10^{-2} - 10^{-4} M. Vesicles, however, composed of neutral phospholipids do not fuse under these conditions. It also appears that the effects on acidic lipids is calcium specific. Mg^{2+} causes rapid vesicle aggregation but fusion does not occur.

Papahadjopoulos emphasizes that based on his experiments the finding that aggregation of vesicles can be seen without fusion means that when two membranes are close to each other and apposed, it does not necessarily lead to fusion even if the membranes are fluid. Fusion therefore requires a calcium induced lateral phase separation which

results in formation of a rigid crystalline domain of acidic lipids within a mixture of lipid membrane. The role of calcium in the fusion process consists of facilitating apposition of two membranes by enhancing electrostatic interactions between them, and would also facilitate fusion of membranes by inducing destabilization via the formation of domains of crystalline acidic phospholipids. The factors which are important in thinking about fusion of biological membranes, according to Papahadjopoulos, are the contents of and distribution of phospholipids in the membrane and the concentration of calcium in the vicinity of the membrane. It is of interest, therefore, that phosphatidylserine is present almost exclusively in the inner cytoplasmic half of the bilayer while the outer layer consists predominantly of neutral phospholipids, e.g. phosphatidylcholine and sphingomyelin in erythrocytes (207). Papahadjopoulos predicts that the absence of phosphatidylserine on the outer side of the membrane would make it resistant to fusion, whereas the inner surface of the membrane at the proper calcium concentration would be susceptible to fusion. He proposed that ordinarily membrane fusion does not occur under normal conditions because of steric hindrances which frustrate the close apposition of membranes and the low calcium availability in the vicinity of the membranes.

Ekerdt and Papahadjopoulos (208) have demonstrated that membranes achieve a close contact that allows the formation of a specific 1:2 Ca^{2+} /phosphatidylserine complex which is stable and anhydrous, and serves as the trigger for membrane fusion.

Using synthetic phospholipids as a model for biological fusion has been criticized based on the fact that calcium induces permeability and

lysis of synthetic vesicles, not fusion (209).

It has been proposed that both calcium and magnesium neutralize the net surface charge of secretory granules from the submaxillary gland, suggesting that calcium reduces the electrostatic surface charge, thereby controlling the fusion process (210). This theory does not explain the opposing action of Mg^{2+} .

It has also been hypothesized that calcium acts to neutralize opposing electrostatic forces between the adrenal chromaffin granule and the cell membrane (13, 50, 102) to induce fusion and exocytosis. This has been criticized because the theory tends to minimize the importance of the direct participation of metabolic energy in the secretory process which has been clearly demonstrated in a number of secretory systems.

Hauser et al. in 1975 (211) demonstrated that divalent cations interact with phospholipid polar groups to cause water exclusion, making the phospholipid-divalent cation complex appear to be more hydrophobic. Breisblatt and Ohki (212) therefore maintained that the two essential factors for membrane fusion to occur are instability of the membrane and hydrophobicity of the membrane surface. Calcium, although stabilizing the membrane and in turn decreasing the membrane fluidity, makes acidic lipids more hydrophobic.

Bilinski et al. (213), looking at secretion from paramecium cells, have demonstrated that calcium as well as other cations precipitates phosphate ions and produces protein decondensation leading to discharge of secretory vesicle material.

Poste and Allison (214) implicated calcium, ATP and ATPase activity to explain secretion in a wide variety of cells. They maintained that fusion is initiated by the displacement of calcium and ATP from the

membrane. Calcium and ATP stabilize membrane. Woodin and Wieneke (215) found that fusion of lysosomes with the plasma membrane was inhibited by high concentrations of ATP and calcium. They maintained that an ATPase functions to sequester calcium and thereby initiates calcium removal and fusion. This was strengthened by the finding that ATPase inhibitors prevent the release of many types of secretory granules by inhibiting fusion of the granule membrane with the plasma membrane. Loewenstein (216) and Politoff et al. (217) maintained that the function of the fused membrane is dependent on the continuous displacement of Ca^{2+} by $[\text{Ca}^{2+} - \text{Mg}^{2+}]$ -ATPase.

Poste and Allison's (214) proposal for membrane fusion requires the displacement of Ca^{2+} from the membrane to provide ionic sites for molecular interaction with other membranes. The following is their outline for fusion: 1) Contact and close apposition of the membranes so that separation is less than $10 \text{ \AA}$; 2) the displacement of Ca^{2+} and ATP from the membranes; 3) the establishment of stable inter-membrane linkages; 4) the recovery of the newly fused membranes to a normal state. According to them the removal of Ca^{2+} and ATP from the membrane increases the molecular freedom of the membrane macromolecules, by producing conformational changes in the molecules and increasing the number of potential interaction sites, providing an increased number of free cationic and anionic sites. Membrane stabilization would entail the reversal of destabilization and involve the replacement of Ca^{2+} and ATP to their original binding sites with the membrane. This theory, however, seems untenable in the light of Papahadjopoulos' studies showing that calcium induces apposition and fusion of vesicles (205, 206).

It has been hypothesized that calcium acts as an ionic bridge linking anionic groups on the granule to membrane surfaces (218, 219). Matthews (219) and Cooke et al. (220) proposed that calcium diminishes energetic barriers between secretory particles and the cell membrane.

Kosower and Werman (221) postulated that calcium induces transmitter release by forming calcium-dithiolate formation, thereby inducing a shorter sulphur-sulphur distance and subsequent contraction of the local region of membrane. The cooperative actions of several calcium ions could lead to contractions of two Ca^{2+} -dithiolates producing a corresponding expansion of the membrane at the area of attachment of the vesicles, permitting release of acetylcholine content. An oxidizing agent such as glutathione disulphide can oxidize the membrane SH groups with subsequent disulphide formation and induce even greater contraction of the membrane. They proposed that calcium independent release of neurotransmitter may be due to a small amount of free glutathione reductase in the cell.

Evidence has accumulated in recent years that calcium by itself cannot induce neurotransmitter release. Thus, although calcium may play a direct role in the fusion of membranes by one or all of the various mechanisms theorized in this section, calcium must exert other than merely direct effects on the membrane. This might include interaction with calmodulin and a host of enzymes. This of course will be the topic of the following sections.

8. Coated pits/vesicles.

"One of the continuing intellectual challenges in the field of coated vesicle research is extrapolation from morphological experimental data obtained from observations at single points in time to a concept of vesicle behaviour as continuum."

C.D. Ockleford and E.A. Munn, 1980 (222)

"Although the endocytotic process resulting in the pinching off of coated vesicles is now well documented, very little is known of the cellular regulation of this process."

P.K. Nandi, P.K. Van Jaarsveld, R.E. Lippold
and H. Edelhoch, 1981 (223)

"The significance of endocytosis in general is not established. It may assist in the delivery of the ligand to the cytoplasmic matrix or in the initiation of some signal."

S.C. Silverstein, R. M. Steinman and Z.A. Cohen, 1977 (224)

Until this point, I have been dealing with neurotransmitter release from axon terminals which occurs by exocytosis. Exocytosis is defined as the release of secretory products from cells within membrane limited vesicles or granules (225). After the synaptic vesicle fuses and mixes with the presynaptic membrane it must somehow be retrieved. The retrieval of synaptic vesicles occurs by endocytosis.

Endocytosis is defined as the uptake of extracellular material within membrane limited vacuoles or microvesicles (223). There are three types of endocytosis. The first type is phagocytosis which is

defined as the ingestion of particles with a diameter greater than $1\mu\text{m}$. Pinocytosis is a term that was introduced in 1931 by Lewis (226) to encompass macropinocytosis and micropinocytosis; the two other types of endocytosis. Macropinocytosis involves the uptake of fluid into large vacuoles ranging in size from 300-1000 nm in diameter which are visible by light microscopy (225). Micropinocytosis involves the formation of microvesicles that have an approximate diameter of 70 nm and can only be visualized by electron microscopy (225).

The retrieval of synaptic vesicles occurs by the coated pit/vesicle endocytotic pathway. This type of endocytosis falls under the heading of micropinocytosis. More specifically, it has been called adsorptive pinocytosis or receptor mediated endocytosis (227). These definitions focus on the fact that this type of endocytosis is primarily concerned with the selective uptake of membrane areas rather than particles or fluid.

Coated pits in axon terminals are invaginated areas of the presynaptic plasma membrane which by electron microscopy appear to have a fuzzy coat (228). Because of this morphology, they are called "coated pits." The coated pits selectively retrieve synaptic vesicle membrane, pinch off and become coated vesicles, i.e., enclosed membrane bilayers surrounded by what appears morphologically as a coat (228). The coated vesicles in axon terminals thus participate in the regeneration of synaptic vesicles.

This section describes the discovery and description of the coated pit/vesicle system in animal cells and particularly in neurons as well as the details of their putative functions. General nonenzymatic theories involving the mechanism of transformation of coated pits to

coated vesicles will be presented. Enzymatic theories concerning the mechanism of coated pit/vesicle function will be discussed in future sections.

a. The discovery of coated vesicles and their distribution.

Coated vesicles were first discovered by Bessis and Gorius in 1957 (229) and further described by Gray in 1961 (238). Coated vesicles are distributed ubiquitously in a variety of cell types including muscle cells (230, 231), kidney proximal tubule cells (232), epithelial cells (233), odontoblasts (234), chondrocytes (235), endothelial cells, brain arterioles, capillaries and venules (236), lymphocytes (237) and many other cell types.

Gray was the first to report the presence of coated vesicles in neurons in 1961 (238). He described them as 60-80 nm spheroidal complex vesicles in mossy fiber endings of the rat brain cerebellar cortex which were surrounded by shells consisting of closely packed 15-20 nm vesicular bodies. Palay in 1963 (239) reported that coated vesicles were restricted to the neuronal perikaryon. Andres in 1964 (240), however, described plasma membrane invaginations in the axon terminal which were coated on the cytoplasmic surface (coated pits) and appeared to give rise to coated vesicles by budding inward and pinching off. He speculated that after coated vesicles were formed they fused with intracellular membranous cisternae. The cisternae were coated in portions of their surface and eventually gave rise to uncoated synaptic vesicles.

Roth and Porter in 1964, described the coating of vesicles as bristles (241). Other terms over the years used to describe this morphology include: spikes, spines, hair-like projections, stria and fuzzy coats (222).

Friend and Farquhar in 1967 (242) suggested that coated vesicles in the rat vas deferens occurred in two different sizes, each having discrete functions. The small vesicles were less than 75 nm in diameter and performed Golgi associated or lysosomal functions. The larger vesicles were greater than 100 nm in diameter and performed endocytotic functions. This morphological distinction has not been found in other tissues.

Douglas et al. reported the presence of coated pits and vesicles in the rat hypophyses (243). Bunt reported the presence of coated vesicles in the neurosecretory axon terminals of the Crustacean Sinus gland (244).

The difficulty in the resolution of the 3-dimensional organization of the vesicle coat led Gray in 1972 (245) to postulate that coated vesicles in thin sections represented fixation artifacts arising from the denaturation of either microtubules or the cytonet. This concept was not supported by studies done on coated vesicles isolated from unfixed brain tissue. It also became evident by Markham rotations performed by Woods et al. in 1978 that the coat is a highly regular structure unlike aggregates of denatured protein (246).

b. Morphological structure of coated vesicles.

Palay in 1963 (239) observed coated vesicles in the purkinje cells of the rat cerebellum and suggested that the vesicle projections represented the vertical walls of a honeycomb which made up the limiting membrane of the vesicle. Bowers in 1964 (247) observed the coating in the aphid pericardial cells and was the first to suggest that the honeycomb probably consisted of pentagons and hexagons covering the spherical surface.

Kanaseki and Kadota isolated coated vesicles from the nerve endings of pig brain in 1971 (228). Using electron microscopy (EM) they found that the coat consisted of a spherical polygonal network. The hairlike projections of the coat they found were actually the superimposed visual images of regular hexagons and pentagons of the coat basket, each with sides 24 nm in length.

Crowther et al. in 1976 (248) similarly showed the EM ultrastructure of coated vesicles. Tilted specimens of coated vesicles showed that their coats are polyhedral lattices consisting of 25 hexagons and a variable number of pentagons.

The most elegant photographs of the structure of the coated pit/vesicle system is that obtained by Heuser's rapid freezing technique (249). The photographs display with clarity the coats that appear underneath fibroblast membranes at the start of receptor mediated endocytosis. The coats appear to be 7 nm strands arranged in 30 nm polygons, most of which are hexagons but some of which are 5 and 7 sided rings. The proportion of pentagons in each network increased as the coated area of the plasma membrane puckered up to its most sharply curved condition as a pinched off coated vesicle. Since the coated pit/vesicle system is found in most cells, and clathrin, the major coat constituent is highly conserved, it is likely that these photographs are representative of the system present in neurons as well as other cells.

Estimates of the length of the projections on coated vesicles in different cell types vary between 15-25 nm, although in any one coated vesicle the projections are fairly uniform in size (250). The distance between each projection is approximately 20-25 nm and the width of each projection is approximately 5 nm. Where the plane of the section is

tilted, as was demonstrated by Ockelford in 1976 (251), the projections have been inferred to represent a polygonal network. A comparison of coated vesicles from different tissues (246) revealed no significant differences between the structure and chemical properties in neurons and in other cells.

c. Coated vesicle lipid composition.

In 1969, Schjeide et al. (252) studied the lipid composition of oocyte coated vesicles. They were 48% lipid. The lipid was predominantly phospholipid, of which the most abundant species was phosphatidylcholine. The rest of the lipid composition consisted of 10-15% sterol, 3% sterol esters, 7-10% triglyceride and 1.5% non-esterified fatty acid. The fatty acids present were oleic, palmitic, stearic, linoleic and arachidonic acids. Pearse in 1975 studied brain coated vesicle lipid composition (253) and found that there was a 3:1 protein/lipid ratio and that the lipid composition of brain coated vesicles was very similar to coated vesicles isolated from other tissues, indicating that the basic biochemical composition of coated vesicles was relatively conserved. Brain coated vesicles, as determined by Pearse, contained 42-43% Phosphatidylcholine, 30-33% Phosphatidylethanolamine, 10-11% Phosphatidylinositide, 4% Phosphatidylserine, and 8-12% sphingomyelin. The cholesterol levels were very low, which may indicate, according to Pearse, that coated vesicles selectively internalize areas of cholesterol deficient plasma membrane.

d. Coated vesicle protein composition.

An analysis by Pearse of brain coated vesicles revealed that clathrin, a 180,000 M_r protein, is the predominant protein of the coated

vesicle (254). This protein was shown to be on the cytoplasmic surface of the vesicle and to be the major constituent of the coat. When coated vesicles were treated with trypsin or pronase, naked vesicles were observed by electron microscopy, and SDS-gel analysis revealed the absence of clathrin (253). Pearse decided to name the protein by the Greek term "Clathrin" meaning lattice.

Coated vesicles isolated from chicken oocytes, brain, human placenta and a variety of other tissues all have clathrin (246, 255, 256). Clathrin thus appears to be conserved across tissues and species. Amino acid analysis and one dimension peptide analysis of brain and lymphoma clathrin peptide fragments indicated that clathrin is indeed conserved (254). Clathrin has an isoelectric point of 5.8, and because it does not stain with periodic shiff reagent (257) probably does not have any carbohydrates associated with it. Pearse suggested that the absence of carbohydrates makes clathrin capable of reversible association with membranes (254).

In brain, clathrin represents approximately 0.1% of the total protein. Because coated vesicles are found in axon terminals (121) and the source of most polypeptides is the neuronal cell body, clathrin must be axonally transported down to the axon terminal. The fast component of axonal transport is the primary source of membrane elements (258), whereas the slow component of axon transport is the source of cytoskeletal elements for the axon terminal such as tubulin, actin and neurofilaments (259). Garner and Lasek have demonstrated that clathrin is transported down the axon with the cytoskeletal components via the slow transport system (260).

Clathrin itself is capable of two ordered states: filamentous

structures and baskets (261). Schook et al. demonstrated in 1979 that these two ordered structures are dependent on pH. At or below pH 6.5, baskets spontaneously formed from the filamentous structures (261).

Woodward and Roth (262) maintained that Mg^{2+} is important in modulating the polymerization-depolymerization equilibrium at pH 8.0. Basket assembly at physiological pH was facilitated in the presence of Mg^{2+} , suggesting that Mg^{2+} may stabilize the coat lattice. Keen et al. (263) maintained that this effect is specific for Mg^{2+} , because in the presence of other cations, e.g., calcium, zinc or manganese, filaments rather than baskets are formed.

Nandi et al., on the other hand, demonstrated that calcium and Mn^{2+} strongly enhanced the rate of basket polymerization (223). They also found that Clathrin polymerized at pH values below 6.8 with the absorption of one proton/mole clathrin (264). Nandi et al. (223) speculated that the binding of other positively charged substances could also initiate polymerization. Calcium and Mn^{2+} not only accelerated the rate of clathrin polymerization at pH values where clathrin spontaneously polymerized, but also induced polymerization at physiological pH values and above. They demonstrated that polymerization could be reversed by chelating calcium. In addition, they found that a positively charged basic protein lysozyme stimulated the rate of basket polymerization. It would thus appear that the binding of positive charges, be it Ca^{2+} , Mg^{2+} , H^+ , Mn^{2+} , or positively charged proteins, enhances the rate of basket polymerization. Physiologically, any one of multiple factors may be responsible for clathrin-clathrin interaction to form a polygonal lattice around coated pits and coated vesicles.

Ungewickell and Branton separated clathrin from most of its associated polypeptides, but failed to remove polypeptides of M_r 33-36K. By virtue of this property, they named this protein doublet clathrin light chains (265).

Since this observation, it has been demonstrated that the basic assembly unit of the polygonal lattice surrounding the coated vesicles is a molecular complex of 630,000 daltons, composed of three clathrin chains, each having a M_r of 180K and approximately three proteins of M_r 33-36K (265). These subunits are organized as a triskelion and are composed of three legs of equal size joined at one end, with each leg bending at the midpoint (265). The triskelions can be dissociated from the coated vesicles by manipulations that include change in pH, ionic strength and low concentrations of Urea. Under appropriate conditions the triskelions can assemble in vitro into baskets whose structural features are very similar to the lattice that surrounds the coated vesicle (253, 262, 263).

Unanue et al. (266) have demonstrated that the triskelions bind with a very high affinity to protease sensitive sites on the coated vesicles after the vesicles are depleted of their endogenous clathrin. The binding, they showed, is high-affinity and saturable, and requires the presence of a pronase sensitive polypeptide on the vesicles. They do not know whether each triskelion has one or three binding sites. When they used elastase which selectively cleaved a 110 K protein, the binding was considerably reduced. Therefore, they speculated that the 110 K M_r protein participates in anchoring the triskelion to the membrane.

Keen et al., in 1979, suggested that a 110 K M_r protein derived

from coated vesicles is a basket assembly factor. Unanue et al. speculated that these two proteins are probably one and the same. There is a flaw in this logic however. Keen et al. (263) suggested that a 110K M_r protein is a factor necessary for clathrin-clathrin interactions to form a basket in the absence of membrane. Unanue et al. demonstrated that a 110K protein was necessary to anchor clathrin to the membrane. The properties and mechanisms, however, of clathrin-clathrin and clathrin-membrane interactions are different processes. Therefore each group of workers has suggested that a 110K protein is necessary for two distinct coated pit/vesicle phenomena. There is as yet no indication that these two proteins are identical. In fact, it would seem that these proteins must be different.

Crowther and Pearse have outlined a model for the packing of triskelions into larger assemblies (267). They proposed that because of the three dimensional nature of polygonal structure of coats and the larger array of structures seen in freeze-fractured cells, the vertex of the triskelion must be at the vertex of the polygonal coat.

Because the trimer is capable of forming pentagons, hexagons and heptagons (249), Crowther and Pearse postulated that there must be a triskelion at every vertex in a completed structure. Although a hexagon could be formed from three triskelions placed at alternate vertices, this arrangement would not generalize to allow for the formation of pentagons and heptagons. If there is a triskelion at each vertex, a pentagon and heptagon can be formed from a hexagon by removing or adding a triskelion. Such assemblies would be accommodated with only small distortions of the molecule.

Crowther and Pearse speculated that in a cell, clathrin triskelions

probably add onto the already existing areas of the hexagonal-clathrin lattice of the coated pit. In vivo, 0.5 mg clathrin/ml of cytoplasm is in the coat membranes. They speculated that the cycle of coated vesicle function and the control of clathrin assembly and dissociation must involve other factors. The basic basket structure is formed from one clathrin triskelion at each polyhedral coordinated vertex extending along two neighboring polyhedral edges. The polyhedral edge therefore consists of the proximal parts of two legs and the distal parts of two other legs. Crowther and Pearse proposed that packing triskelions involves a small clockwise rotation of the triskelion relative to the underlying polyhedral lattice, so that the legs of the triskelion do not point directly at the neighboring legs but slightly to one side, and that each leg has an anticlockwise kink. They also maintained that the clathrin triskelion is a highly extended fibrous molecule with its nearest and distant neighbors. They compared the packing of clathrin molecules to the packing of tropomyosin which makes contact between itself and other muscle proteins in fibrillar aggregates. Clathrin uses similar extended fibrous contacts. This specificity implies that clathrin must be a very stiff structure, containing a variable joint at the triskelion vertex. Such a molecular design, Crowther and Pearse maintain, allows the formation of shells with a wide variety of curvatures while maintaining construction specificity and conferring the mechanical strength and flexibility that is needed when a vesicle is being pinched off from the membrane.

Schmid et al. (268) have obtained a proteolytic domain of clathrin having the molecular weight of 110,000 that has the capacity to polymerize into cages in the absence of the 30K M_r proteins. This is a

capacity that intact clathrin molecules lack. They proposed that the light chains are not always present or active throughout the clathrin coated vesicle cycle of assembly and disassembly. Therefore they speculated that these light chains provide a possible point at which the clathrin coated vesicle cycle may be regulated.

Immunocytochemical results by Cheng et al. (269) of the localization of clathrin in the rodent cerebellum at both the light and electron microscope level have revealed that coated vesicles are highly concentrated in presynaptic terminals. Within the terminals, the coat of coated vesicles was heavily labelled with antisera, in addition to labelling a pool of protein throughout the cytosol of the axon terminal. They speculated that there is a pool of nonlattice clathrin in the cytoplasm which is available for transformation into the lattice upon demand. This pool is primarily localized in the presynaptic terminal and is not distributed throughout the neuron.

e. Function of coated vesicles in neurons.

Palay, in 1963 (239), was the first to speculate on the function of coated vesicles in neurons. Because coated vesicles were associated with different cellular membranes, he postulated that they were participating in the circulation of membrane components between the surface membrane and the internal membranal compartments. After Roth and Porter in 1964 (241) reported that coated vesicles in mosquito oocytes functioned in the uptake of yolk protein, it was suggested that coated vesicles in the brain by analogy micropinocytosed extracellular fluid and solutes (240). This was based on the observation of coated pits on the plasma membrane of a variety of neurons, as well as coated vesicles located close to the coated pits within the cytoplasm (270,

271). These views of coated pits and vesicles were consistent with the function of this organelle in either exocytosis or endocytosis. The introduction of dyed extracellular tracers clarified that coated vesicles function primarily in endocytosis as opposed to exocytosis (272). This section will explore all the possible functions of coated vesicles.

1. Recycling of synaptic vesicles. Bittner and Kennedy (273) suggested that it is unlikely that synaptic vesicles are replaced by de novo synthesis and assembly of their components. In studying the opener stretcher neuron of the crayfish they estimated that at an impulse rate of 20/second, the contents of approximately 4.5×10^9 synaptic vesicles were discharged per hour. They estimated that for mammalian neurons this number would be lower by a factor of ten. They concluded that the incorporation of vesicle membrane into the presynaptic membrane must be balanced by retrieval and reformation of synaptic vesicles. From studies conducted in the past decade it seems that the coated pit/vesicle system is responsible for the selective retrieval of the synaptic vesicles.

The idea that coated vesicles are involved in synaptic vesicle recycling first came from Heuser and Miledi in 1971 (132). Upon treatment of frog neuromuscular junctions with lanthanum, they observed by EM that the number of coated vesicles increased with the duration of lanthanum treatment. After 12 hours of treatment there was a predominance of coated vesicles in the terminals. Synaptic vesicles were absent. Tracer techniques have served to confirm the hypothesis that coated vesicles are involved in synaptic vesicle recycling. Heuser and Reese (125, 134), after applying tetanic stimulation to frog

sartorius junctions, noticed that the number of synaptic vesicles decreased and that coated vesicles increased in a reversible fashion. When Horse Radish Peroxidase (HRP) was present in the bathing medium, the tracer first appeared in coated vesicles which budded off the membrane, then appeared in cisternae, after which it appeared in the synaptic vesicles. Heuser and Reese have shown, however, in 1977 (134) that synaptic vesicles were formed directly from the coated vesicle after the loss of its coat. The synaptic vesicles did not bud off cisternae. The cisternae originally observed represented an artifact of excessive stimulation. It was also observed that the total membrane area remained constant during recycling, and that HRP which was incorporated into the synaptic vesicles was removed from the nerve terminal during a second period of stimulation. Similarly, Turner and Harris (274) have shown in the cerebral cortex that, after one hour of HRP application subarachidally, presynaptic terminals showed labelling of synaptic and coated vesicles with HRP. Jones et al. (275) introduced HRP directly into the cerebral cortex of rats, and after 60 minutes the rats were killed, their brains perfused and fixed. Quantitative analysis revealed an early rise in the rate of labelling of both synaptic and coated vesicles, after which synaptic vesicle labelling rose slowly to a plateau. Fried and Blaustein (276) showed that even in isolated brain synaptosomes HRP was incorporated into synaptic and coated vesicles. Stimulus dependent incorporation of HRP into synaptic vesicles was reported in the photoreceptors of various animals (277, 278). Other extracellular tracers such as ferritin and thorium dioxide were observed to be taken up within coated vesicles by neurons and neurosecretory cells.

More proof that coated vesicles are involved in synaptic vesicle recycling came out of Heuser and Reese's laboratory with their freeze-fracture studies on the frog neuromuscular junction (279). In images of freeze-fractured terminals, there could be seen in association with the synaptic vesicles a set of particles larger than the particles that could be seen at the release sites. According to Heuser and Reese, each synaptic vesicle contained 2-3 of these particles. During stimulation, these particles were incorporated into the presynaptic membrane in the region of the active zones and then appeared to move laterally away from the release sites in a direction roughly parallel to the synaptic cleft. At the sites of endocytosis, within the coated vesicle opening, these particles formed clusters or mounds. Since it is likely that the intramembranous particles are aggregates of intrinsic membrane proteins, some of which span the lipid bilayer, it was suggested that clathrin, the major coat constituent associated with the cytoplasmic face of the plasma membrane, interacts with the synaptic vesicle particles which extend into the cytoplasm. The idea of intramembranous particles being internalized by coated membrane sites was supported by freeze-cleaving studies of neurosecretory endings in the neurohypophysis (138). Pits were seen in the plasma membrane of stimulated cells which corresponded in size to the coated invaginations seen in thin sections. These pits contained a higher density of particles than the surrounding areas of membrane. In this system, however, retrieval by coated pits occurs at the site of exocytosis, not at a site adjacent to the active zone.

The rapid freezing technique developed by Heuser and Reese led to the improved visualization of the recycling process (134). After stimulating discharge of neurotransmitter with the concomitant collapse

of the synaptic vesicle into the presynaptic membrane, it could be seen in the succeeding seconds that equivalent amounts of membrane were being retrieved from the presynaptic membrane by two different forms of endocytosis which were different with respect to time and specificity. One form involved the formation of large vesicles during the first one or two seconds and was nonspecific. The other was highly selective and retrieved from the membrane large intramembranous particles that looked like those present in synaptic vesicle membranes. Together, these two forms of endocytosis compensated for expansion of the presynaptic membrane into new generations of synaptic vesicles.

The coated pit/vesicle system is found in almost all eukaryotic cells (280). In all cells other than neurons, it is postulated to take part in the recycling of membrane. Further support for this idea came from Goodrum and Tanaka (281). They analyzed synaptic vesicle, coated vesicle and presynaptic membranes by polyacrylamide slab gel electrophoresis and found that all these membranes had a very similar polypeptide composition.

11. Molecular filter functions. Coated pits in general act as molecular filters, selecting some proteins and excluding others (282), e.g., Low Density Lipoprotein (LDL) specifically binds to the LDL receptor and is then taken up by coated pits/vesicles (283). Maxfield et al., using fluoresceine labelled alpha two macroglobulin and rhodamine labelled derivatives of insulin and epidermal growth factor, demonstrated that all these ligands are internalized by the coated pit/vesicle system in fibroblasts (284). On the basis of their data, they postulated that the coated pit/vesicle may represent a specific organelle that is adapted for the binding and the rapid internalization

of a variety of macromolecules that exert regulatory functions on the cell.

Willingham et al. (285) also demonstrated in fibroblasts that macroglobulin binds to specific receptors and is taken up by this system. Coated vesicles in the human placenta have been demonstrated to selectively endocytose ferritin, transferrin and immunoglobulin G (286a).

Ligands taken up by the coated pit/vesicle bind to glycoprotein receptors. Ligands however that bind to ganglioside receptors, e.g. cholera toxin, are specifically taken up by noncoated invaginations (286b).

It has been suggested by Wild in 1973 (287) that the coat of coated vesicles functions to protect the vesicle and its contents from lysosomal attack. In the axon terminal, however, where there are no lysosomes, the coat probably does not function in this capacity.

In general, it is agreed that specific ligands and their receptors are internalized by coated pits/vesicles. The presynaptic axonal membrane has presynaptic neurotransmitter receptors. Although it has been demonstrated that synaptic vesicle membrane is selectively retrieved by coated pits/vesicles, it is also quite conceivable that coated pits, in addition, selectively endocytose neurotransmitter receptors along with their neurotransmitters. This may be a mechanism for receptor turnover and/or neurotransmitter uptake. Neither of these functions to date have been established or investigated. It is conceivable that the uptake of neurotransmitter, the regulation of presynaptic membranal receptor turnover, and the regeneration of synaptic vesicle membrane may all be tightly coupled in the coated

pit/vesicle system.

iii. Exocytosis. There is some evidence indicating that coated vesicles function in exocytosis in certain non-neuronal cell types. In rat hepatocytes coated vesicles containing very low density lipoprotein fuse with the plasma membrane (288). The release of casein granule containing coated vesicles has been demonstrated in the epithelial cells of the rat mammary gland (289). In the epithelial cells of the vas deferens, some lysosomal enzymes are excreted from the cell via coated vessels (290). Although in neurons it is established that coated vesicles act in endocytosis, the possibility that coated vesicles may function in a limited sense in exocytosis should be kept in mind.

iv. Synaptogenesis. There is evidence that during embryogenesis of the nervous system, coated vesicles convey to the synaptic region components of the postsynaptic membranal specialization. Fusion of coated vesicles with the postsynaptic membrane at developing synapses in the chick cervical spinal cord was noted by Stelzmer et al. (291). Similar observations were made by Rees et al. (292).

f. Non-enzymatic theories on the mechanism of formation of coated pits and in the transformation of coated pits into vesicles.

Roth and Porter suggested in 1964 that the free ends of the coated pit lattice may repel each other and might therefore generate the process of infolding of the plasma membrane with subsequent formation of a vesicle (241).

Kanaseki and Kadota in 1969 (228) suggested that the transformation of coated pit hexagons into pentagons is the driving force for pinching off the coated pit to form the coated vesicle. The precise mechanism whereby the hexagons are transformed into pentagons was not mentioned by

Kanaseki and Kadota. However, Ockleford and Munn (293) have suggested that the transformation probably involves the ejection of clathrin molecules. Because clathrin molecules in the original coated pit lattice are in all probability equivalent, the transformation of any polygon may be triggered by the binding of a ligand to a receptor. If a random transformation would occur, this would then give a random formation of different polygonal structures. When the concentration of ligand is high, the receptors would all rapidly saturate, producing a large proportion of pentagons and a smaller radius of curvature in the lattice, and therefore smaller vesicles. However, if this were the case, they maintain it would be very inefficient. Their theory therefore states that when specific receptors are exposed at the external surface of the phospholipid bilayer, either a portion of the receptor molecule or a portion of the clathrin molecule to which it may become connected spans the bilayer of the membrane, or alternatively these two molecules may be linked together through a third molecule which spans the phospholipid layer. The linking of clathrin to the receptor might be initiated through binding of a ligand to the receptor. The binding of the molecule to the receptor would induce a change in the linker which would permit binding of clathrin which would in turn be altered so that it became suitable for polymerization. The growth of the coated pit lattice would induce curvature of the membrane until the vesicle separated from the membrane and the curved lattice formed a coated vesicle. What would initially give rise to lattice formation of membranes would result from clathrin cross-linking and multivalent ligands attaching to more than one receptor.

Ockleford and Munn bring as evidence for their theory the following

phenomena: a) Despite the fact that coated vesicles occur in nearly all cells, they are most common in tissues which function in the selective uptake of protein; b) Within the tissues where coated vesicles are common, there is a high rate of protein uptake, e.g. intestinal epithelial cells between the bases of the microvilli. Their model requires that receptors and the clathrin lattice become co-extensive, which they propose occurs via linkage molecules binding receptors to clathrin through the membrane. They state that the linker may be an arm of clathrin, a separate molecule, or even a portion of the receptor.

Buttressing Ockleford and Munn's hypothesis is the data of Anderson et al. in 1969 (294) demonstrating that there are some mutant fibroblasts in which receptors are mislocated. Binding of the ligand to the receptor occurs, but the receptor cannot be incorporated into coated pits. Goldstein et al. (295) maintained that the LDL receptor has at least two active sites. One of these sites is necessary for the receptor to become incorporated into the pit, and the other for the binding of the ligand (LDL). Separate mutations can occur in the gene encoding the LDL receptor, so that either the binding activity or the internalization activity is destroyed.

Nagura and Asai in 1976 (296) indicated that coated vesicles in macrophages do not require metabolic energy. However, Roth et al. in 1976 indicated that, in certain circumstances, metabolic energy is required. Palade and Fletcher (297) have found that anoxia interrupts coated vesicle formation and makes coat material aggregate into small empty baskets in pancreatic acinar cells. Similarly, Heuser (249) observed that after adding metabolic inhibitors to frog nerves, coated

coated vesicle formation was perturbed and many empty baskets appeared.

It thus appears that although clathrin-clathrin polymerization may occur in vitro, the interaction of clathrin with the membrane in vivo probably requires O_2 and metabolic energy, i.e. ATP. The possible involvement of enzymes utilizing ATP in coated pit/vesicle function will be described in a later section.

g. Calcium dependence of endocytosis.

Endocytosis in general is considered to be a calcium dependent process. Allison and Davies presented evidence that calcium plays a very important role in endocytosis (298). Prusch in 1980 (299) demonstrated, using the amoeba proteus as a model for endocytosis, that the addition of the calcium ionophore A23187 resulted in the influx of calcium and the subsequent endocytosis of sucrose from the medium. This provided further evidence that calcium is crucial for endocytosis.

More specifically, coated vesicle mediated endocytosis has been postulated to be calcium dependent by Douglas in 1974 (102). His experiments indicated that calcium ions were essential for the formation of coated vesicles. Ceccarelli and Hurlbut in 1980 noticed that the Black Widow spider venom, which rapidly depletes synaptic vesicles (300), is highly dependent on the presence of calcium. They therefore suggested that the profound depletion of synaptic vesicles observed in the absence of calcium occurs because calcium is required for endocytosis at the frog neuromuscular junction. Calcium in addition stabilizes the clathrin polymerized structure of coated pits and vesicles (218).

9. Calmodulin.

"The pivotal role of Ca^{2+} in cellular functions has been appreciated for over 30 years; only within the last few years has it been known that most of the calcium effects are mediated through a homologous class of calcium binding proteins, of which calmodulin appears to be the most ubiquitous and versatile".

Wai Yiu Cheung, 1982 (301)

Calmodulin is the major calcium binding protein in eukaryotic cells by which calcium exerts many of its physiological effects (301). Because neurotransmitter release is entirely dependent on the presence of calcium, the physical-chemical properties of calmodulin, its mode and mechanism of action, its location and distribution in the brain would be of fundamental significance in understanding what possible effects calmodulin might play in neurotransmitter release.

a. Discovery of calmodulin and the enzymes which it activates.

Calmodulin (CaM) was originally discovered by Cheung in 1967 as an activator of cyclic nucleotide phosphodiesterase (302). Kakiuchi et al. in 1970 (303) demonstrated that the activator of brain phosphodiesterase was dependent on the presence of calcium. Teo and Wang in 1973 demonstrated that a phosphodiesterase activator was a calcium binding protein (304). Wolff and Siegel in 1972 (305) purified a calcium binding protein from the brain which they called calcium binding protein and which they later demonstrated activated phosphodiesterase in a calcium dependent manner (306). Vanaman et al. (307) and Kuo and Coffee (308) were analyzing a brain and adrenal medulla calcium binding protein

which had many similarities with Troponin C. Because of this property, they named it Troponin C-like-protein. Watterson et al. in 1976 (309) sequenced the protein and renamed it calcium modulator protein to emphasize its modulatory role. The name "calmodulin" was subsequently derived from this name.

After its initial characterization as a protein calcium receptor, and activator of cyclic nucleotide phosphodiesterase, calmodulin was shown to activate a variety of enzymes previously shown to be calcium dependent. Among the brain enzymes now known to be calmodulin activated are nucleotide phosphodiesterase, adenylate cyclase, cGMP protein kinase, synaptic plasma membrane Ca^{2+} - Mg^{2+} ATPase, Phosphoprotein phosphatase, Tryptophan 5' monooxygenase and N-Methyltransferase (310-316). Additional calmodulin activated enzymes present in non-neuronal tissues and yet to be established as being activated in brain are Guanylate cyclase, myosin light chain kinase, phosphorylase kinase, glycogen synthetase kinase, NAD kinase, phospholipase A_2 , succinate dehydrogenase, and O-Methyltransferase (317-321). The list of calmodulin dependent enzymes is continually growing.

b. Physical-chemical properties.

Calmodulin has a molecular weight of 16,700 (322) and is a heat resistant (not stable) protein with a half-life of 2 minutes at 100°C (323). Calmodulin has been sequenced from bovine brain (322), rat uterus (324), rat testis (325), and rabbit skeletal muscle (326). Amino acid analysis revealed that the protein has 148 amino acids and that it is highly conserved. In all the known sequences there are only 7 amino acid substitutions which have been produced from point mutations.

Calmodulin has an N-terminal acetylated alanine and a carboxyl

terminal lysine. It is characterized by the absence of tryptophan and cysteine, a high threonine:serine ratio (12:4), 27 glutamate and 23 aspartate residues, and a single histidine and trimethyllysine (322). Very few proteins have this latter unique posttranslational modification. Studies have demonstrated, however, that this amino acid residue does not play a significant role in calmodulin enzyme activation (327). Because of its high content of acidic amino acids, its isoelectric point is 3.9 (328).

Because of calmodulin's high ratio of phenylalanine (8 residues) to tyrosine (2 residues), calmodulin displays a distinctive ultraviolet absorption pattern with 5 peaks at 253, 259, 265, 269 and 277 nm and a shoulder at 282 nm — a spectrum characteristic of the fine structure of phenylalanine (328). Cheung observed that the lack of cysteine and hydroxyproline might allow calmodulin to assume a steric structure with a great deal of flexibility allowing it to interact with a variety of receptor proteins.

Carboxylation of calmodulin's sole histidine residue (329), modification of both tyrosine residues (330-332), and modification of the six arginine residues do not affect its ability to activate enzymes. Furthermore, a calmodulin derivative obtained by successive modifications of the tyrosine, histidine, and arginine residues does not alter calmodulin activity either (333). Modification, however, of some of the methionine, lysine, and carboxyl residues (322, 334) has led to varying degrees of inactivation.

Modification of two lysine residues led to a 50% decrease in activity (329). The remaining lysines are modified slowly without accompanying loss of calmodulin activity. Walsh and Stevens therefore

suggested that one or two lysine residues situated on the surface of the activated calmodulin molecule may be important in the activation of enzymes.

c. Calcium binding and calcium loops.

Calmodulin is now recognized to be one of a group of homologous calcium binding proteins. On the basis of the crystal structure of parvalbumin, Kretsinger and Barry proposed putative calcium binding domains in the family of homologous binding proteins (335). According to their model, the structure of calcium binding domains consists of helical regions connected by a loop of 12 amino acids with appropriately positioned calcium binding residues. This structure was coined the "EF hand." Calmodulin is capable of binding 4 mol calcium/molecule because it has 4 calcium binding domains. Each calcium binding loop consists of 12 amino acids with 6 oxygen ligands which are not exactly alike. Calcium binding is maximal throughout the pH range from 6-7.5, but decreases precipitously from pH 5-3.5 probably due to titration of the ionized carboxyl groups at the four binding sites (301).

The calcium binding loops have been shown to consistently contain sequences favorable for the formation of 8 turns (336). Amino acid analysis has revealed that domain 1, beginning at the NH₂ terminal portion of the molecule, is highly homologous with domain 3, and that domain 2 is highly homologous with domain 4. Approximately 70% of the amino acids in domains 1 and 3, and in domains 2 and 4, are homologous, indicating that calmodulin may have arisen by gene duplication (322).

It is hypothesized that Troponin C, a homologous calcium binding protein, evolved from calmodulin at the time that the vertebrates appeared (337). The sequence of bovine brain calmodulin has been

aligned with bovine heart Troponin C by the introduction of a three residue gap at position 80 in calmodulin. About 74-76 residues (approximately 50%) are identical between Troponin C and calmodulin. In addition, 40 residues are functionally and genetically conserved (332). Furthermore, it has 11 fewer amino acids than Troponin C, and has a distinct structural conformation. Troponin C contains 3 classes of metal binding sites whereas calmodulin contains only four sites and all bind calcium (338). Troponin C and calmodulin are generally poor substitutes for each other in regulating phosphodiesterase or muscle actomyosin ATPase activities (339).

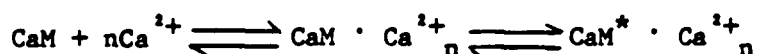
Equilibrium dialysis experiments have revealed that all the 4 metal binding sites in calmodulin are calcium specific and equivalent. Scatchard plot analysis of calcium binding to calmodulin indicates a single slope with a K_d of $2.4\mu\text{M}$ (338). There is great controversy as to the effects of Mg^{2+} and salts on the binding of calcium to calmodulin. Wolff et al. (340) maintain that Mg^{2+} can bind to the metal binding site. They observed that 1mM Mg^{2+} decreased the affinity of all sites for calcium and that in the presence of Mg^{2+} , calmodulin only binds 3 moles of calcium/mole. When 10mM Tris buffer was used, millimolar concentrations of Mg^{2+} affected the circular dichroic spectrum of calmodulin. Dedman et al., however, did not observe competition between Ca^{2+} and Mg^{2+} for calmodulin binding (339). Klee et al. maintained that there is a positive cooperativity in calcium binding to calmodulin (341).

Terbium, a trivalent lanthanide like calcium, possesses a similar ionic radius, coordination number, and preference for binding to charged or uncharged oxygen groups (342). Terbium can replace calcium in many enzymes (343) and Wallace et al. demonstrated (344) that Tb^{3+}

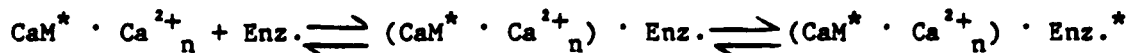
effectively substituted for calcium in many attributes of calmodulin function, including activation of phosphodiesterase. The sequence of Tb^{3+} occupation of calmodulin binding sites in calmodulin is: a) domain 1 or 2; b) domain 3 or 4; c) domain 2 or 1, depending on which wasn't filled initially. They did not differentiate whether domains 3 and 4 were filled randomly or sequentially.

d. Model of calmodulin enzyme activation.

Kinetic studies reveal that calmodulin may affect the enzymes' substrate K_m and/or V_{max} (341). The calcium dependent interaction of calmodulin with the enzymes under its control has been hypothesized to occur in two steps (341): First, calmodulin binds to calcium according to the following equation:



When calcium binds to calmodulin it undergoes a conformational change. At that point it is in its activated form (denoted by an asterisk). Calmodulin then interacts with the enzyme which is inactive or partially active. The enzyme undergoes a conformational change according to the following equation.



It is therefore proposed that calmodulin induces a conformational change in the enzyme which is its most active form (indicated by asterisk).

Addition of calcium leads to changes in the conformational

properties of calmodulin including resistance to proteolysis. Ca^{2+} induces changes in the ultraviolet absorption, fluorescence emission, circular dichroism, optical rotary dispersion and other physical properties (341) and leads to a 5-10% increase in alpha helix (341).

It is known that calmodulin can exist in one of several conformations depending on the number of calcium ions bound/molecule. According to Klee et al. (341), 16 such conformational states exist, and each may be recognized by a different protein or groups of proteins. The fact that the structure of calmodulin is almost perfectly conserved throughout evolution suggests to them that each of the four calcium sites and other important structural features are used in protein function and that different classes of effectors could interact with calmodulin at different degrees of calcium occupancy. Calmodulin could, therefore, translate a quantitative difference in calcium concentration into qualitatively different cellular responses. For example, CaM-Ca_2^{2+} may activate adenylate cyclase whereas, CaM-Ca_1^{2+} may activate phosphodiesterase and myosin light chain kinase.

e. Tissue distribution.

Thus far, calmodulin has been found in a variety of tissues and is considered ubiquitous. Brain and testes have a particularly high concentration of CaM, having several hundred mg CaM/kgm tissue (345). It is estimated that the brain has a 10^{-5} M calmodulin concentration. Most of the protein is found in the grey matter of the brain (346). The high concentration of calmodulin in brain may imply that it plays an important role there.

f. Subcellular distribution.

It was found that calmodulin is partly soluble and partly

particulate in most mammalian tissues, including brain, kidney, liver, platelets, erythrocytes, adrenal medulla and smooth, cardiac and skeletal muscles (347-351).

Calmodulin was localized in rat cerebellum by immunoelectron microscopy and found to be located in the dendrites, membranes of the smooth endoplasmic reticulum (SER), and in mitochondria (352). At the synaptic junctions, calmodulin was observed attached to the inner surface of the postsynaptic membrane, whereas very little was observed in the presynaptic membrane or in synaptic vesicles. Lin et al. (352) suggested that calmodulin is synthesized in the ribosomes and discharged into the cytosol and subsequently becomes associated with a variety of intracellular organelles. The location of calmodulin at the synaptic junction, they maintain, indicates that it may play a role in the mediation of calcium effects there. They performed radioimmunoassay on brain particle fractions and demonstrated that calmodulin concentration ($\mu\text{g}/\text{mg}$) in cerebellar homogenate was 9.5, in the nuclei it was 6.7, in mitochondria 4.4, in microsomes 19.3, in plasma membranes 11.0, in polyribosomes 3.6, in synaptic plasma membranes 10.6, and in postsynaptic densities it was 30.8. These data suggested that calmodulin represented a major protein component of post-synaptic densities.

Harper et al. (353) localized calmodulin in liver, skeletal muscle and adrenal slices. In the liver, calmodulin was located in the cytoplasm, nucleus and plasma membrane. Much of the cytoplasmic calmodulin was localized within glycogen particles. In the skeletal muscle it was also associated with glycogen particles. In adrenal cortex cells, it was located in the cytoplasm and nucleus. They suggested that

calmodulin regulates hormone effects on nuclear processes.

g. Antagonists.

Weiss and Levin (354) revealed that the binding of phenothiazines to CaM was calcium specific and interaction with these drugs prevented stimulation of phosphodiesterase activity, as well as a variety of other enzymes. Calmodulin binds to two moles of phenothiazine in the presence of calcium. Troponin on the other hand exhibits a much poorer affinity for these agents (354).

Van Eldik and Watterson (355) have described the antigenic site on calmodulin that associates with a series of antibodies. This site was shown to be restricted to an 18 amino acid region in the COOH terminal portion of the protein. Whether these antibodies prevent the stimulation of enzymes and phenothiazine binding by calmodulin remains to be established.

h. Involvement in exocytosis and endocytosis.

It has been suggested that the key role for calcium in triggering exocytosis may be mediated by calmodulin. Trifluoperazine inhibits exocytosis in sea urchin eggs (356), inhibits the release of catecholamines from the adrenal medulla (357) and also inhibits glucose stimulated insulin secretion (358). Calmodulin has also been demonstrated to be involved in the release of neurotransmitter from synaptic vesicles in the brain (359).

Salisbury et al. (360) demonstrated that recruitment of clathrin coats to the membrane opposite ligand receptor clusters is sensitive to stelazine, while subsequent coated vesicle formation and migration is unaffected. This suggests that CaM is involved in the regulation of clathrin recruitment to cell surface receptors bound for internalization

by the coated pit/vesicle system.

There are therefore indications in the literature that calmodulin mediates the effects of calcium in exocytosis and endocytosis. The question is, how? It is evident that the only way thus far known that calmodulin may affect cellular phenomena is via enzymatic activation. Thus, if calmodulin is involved in calcium mediated exocytosis and endocytosis, it is only logical to assume that exocytosis and endocytosis are mediated by Ca^{2+} /Calmodulin dependent enzymes, at least in part. What enzymes are activated in axon terminal synaptic vesicle exocytosis and coated pit/vesicle endocytosis? How does this activation lead to the precise membranal events involved?

It is important initially to determine whether calmodulin is associated with synaptic and coated vesicles. Calmodulin content of synaptic and coated vesicles was determined in this work by radioimmunoassay. Kinetic parameters of calmodulin binding -- K_d and B_{max} -- were determined by radiolabelled calmodulin binding studies. Calmodulin binding proteins were identified by using calmodulin affinity chromatography, enzymatic and immunological techniques. Preliminary results also suggested that PLA_2 and Ca^{2+} /Calmodulin, as well as cAMP dependent protein kinases, are intimately involved in synaptic vesicle exocytosis and coated pit/vesicle endocytosis. The following sections go into detail on the description and possible importance of these enzymes in brain function.

10. Phospholipase A₂.

"In view of the widespread occurrence of the phenomenon of phosphoglyceride turnover in nature, it is not amazing that phospholipase A and lysophospholipase have been reported to be widely distributed (361). As holds in general for the metabolism of phosphoglycerides, very little is known about the factors that regulate the activity of the enzymes involved in the catabolism of these membrane constituents. Undoubtedly this is due largely to the fact that many intracellular phospholipases A occur in membrane bound form, and little progress has been made in obtaining such enzymes in purified form. On the other hand, it is conceivable that disrupting the membrane structure to get the enzyme out will introduce enough variation in the environment and conformation of the enzyme to change considerably the effects of factors that possibly control the activity of the enzyme in its natural membrane-bound state. Studies with purified and membrane-bound phospholipases will be needed to fully comprehend such regulatory aspects."

H. Van den Bosch and A.M.H.P. Van den Besselaar, 1978 (362)

Phospholipase A₂ (PLA₂) catalyzes the fatty ester hydrolysis of 1, 2-sn phosphoglycerides in the two position resulting in the production of lysophosphoglyceride and fatty acid (363). The enzyme is ubiquitously distributed and almost invariably displays an absolute dependence on calcium ions (364). The dependence of exocytosis and endocytosis in nerve axon terminals on calcium invokes the possibility that endogenous brain membranal PLA₂, if present, may somehow be

involved in the initiation and/or facilitation of these two processes. To critically analyze this possibility it is necessary to review the literature and see whether and how the addition of exogenous PLA₂ to various brain preparations affects neurotransmitter release and coated pit/vesicle formation. In addition, all the extant data on the involvement of endogenous PLA₂ in cell secretion, neurotransmitter release and endocytosis will be presented.

Other phospholipases include: a) Phospholipase A₁ (PLA₁) which catalyzes the hydrolysis of the fatty acid at the 1 position of 1, 2-sn phosphoglycerides; b) Phospholipase B (PLB) which sequentially catalyzes the fatty acids at the 1 and 2 position; c) Phospholipase C (PLC) which catalyzes cleavage of the bond between phosphoric acid and glycerol; and d) Phospholipase D (PLD) which removes the polar head group leaving phosphatidic acid and free choline, ethanolamine, etc.

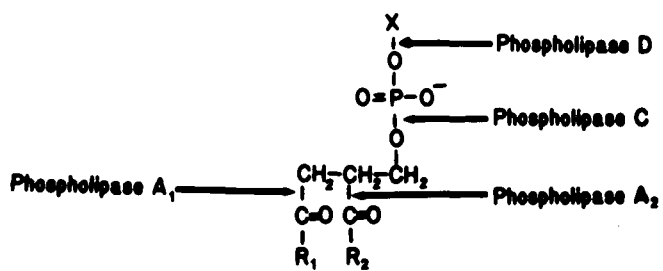
A diagram of the phosphoglyceride substrates for the various phospholipases and their site of action are indicated in Figure 1.

We are primarily interested in PLA₂. The other phospholipases will be mentioned peripherally for the purpose of placing PLA₂ within the context of other phospholipases. Also, because PLA₁, PLC and PLD are found in the brain, and because PLC has been implicated in secretory events in non-brain tissue, these phospholipases are of potential interest to the general discussion of neurotransmission and secretion.

a. Effects of exogenous phospholipases on membrane structure and function.

In 1938, Feldberg and Kallaway (365) demonstrated that cobra venom which contains PLA₂ induced histamine release from the guinea pig lung. They suggested that a lytic substance such as lysophosphatidyl-

Figure 1. The general chemical structure of a phosphoglyceride. Arrows indicate the site of action of Phospholipases A₁, A₂, C and D.



X=

phosphatidylethanolamine $-\text{CH}_2-\text{CH}_2-\text{NH}_2$

phosphatidylcholine $-\text{CH}_2-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$

phosphatidylserine $-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COO}^-$

phosphatidylinositol $-\text{C}_6\text{H}_8\text{O}_6$

R_1-R_2 fatty acids

choline (lysolecithin), which is one of the end products of PLA₂-phosphatidylcholine hydrolysis, was formed and acted as a histamine releasing agent. Branganca and Quastel in 1952 (366) demonstrated that acetylcholine was released from brain homogenate by treatment with cobra venom.

Kelly et al. (367) studied the biochemical properties of β -bungarotoxin, a presynaptic neurotoxin from the venom of the snake *Bungarus multicinctus* and the physiological changes it produced in neuromuscular transmission. They showed that this toxin is a calcium dependent PLA₂ which causes initial enhancement of spontaneous evoked and delayed release of neurotransmitter. They suggested that bungarotoxin binds to the presynaptic plasma membrane and in the presence of calcium perturbs the membrane's structure. This gives rise to an enhanced rate of transmitter release by either inactivating the rate of calcium removal or by increasing the probability of synaptic vesicle fusion with the presynaptic membrane via increasing the concentration of lysophospholipid and/or fatty acids. After the initial enhancement of neurotransmitter release, the toxin causes failure of neurotransmission. Kelly et al. maintained that the decline and eventual failure of transmitter release is not due to the depletion of synaptic vesicles, to depolarization of the nerve terminal or to uncoupling of mitochondria, because all these phenomena enhance rather than diminish transmitter release. They maintained that prolonged exposure to toxin resulted in the inactivation of PLA₂. This is buttressed by the fact that prolonged or brief treatment of synthetic phospholipid vesicles with PLA stabilizes or destabilizes vesicles, respectively (365).

Chang et al. (369), employing phrenic nerve diaphragm preparations

to study the effect of β Bungarotoxin, confirmed the findings of Kelly et al. that the toxin initially increased the miniature end plate potentials, then progressively decreased the output until there was a complete inhibition of ACh release. Sen and Cooper confirmed that bungarotoxin acts as a PLA₂, liberating fatty acids from synaptic membrane phospholipids in a calcium dependent manner (370). Similarly, Chen and Lee (371) demonstrated by electron microscopy that the injection of bungarotoxin produced a depletion of synaptic vesicles. In the mammalian brain synapse it was demonstrated that the toxin resulted in the efflux of GABA, norepinephrine, serotonin (372) and 2-deoxyglucose (373).

Sen et al. (374) using synaptosome preparations showed that choline acetyltransferase activity was not affected by β -Bungarotoxin. The toxin reduced choline uptake and increased transmitter release. They maintained that in synaptosomes the initial response to the toxin is due to an increase in transmitter release, and that the subsequent inhibition of transmitter release is due to the toxin's inhibition of choline transport. Because transport is inhibited, ACh concentration is reduced inside the terminal. Mitochondria were not affected in this study.

Wagner et al. (375), on the other hand, demonstrated that the extent of calcium uptake into mammalian brain mitochondria was noncompetitively inhibited by β -bungarotoxin, not because calcium uptake sites were blocked but because ATP synthesis was inhibited by the toxin. This toxin therefore de-energized the mitochondria by destroying the proton motive force.

Napias and Heilbrunn (376) demonstrated that β -bungarotoxin specifically attacks axon terminals because synaptosomal phosphatidyl-

choline embedded in a membrane containing a low amount of cholesterol is a highly specific substrate for the toxin. Mitochondria, on the other hand, are a poor substrate for the toxin. They suggested that mitochondria are susceptible to the toxin's attack because they are more fragile than synaptosomes. Oberg and Kelly showed along the same lines that β -Bungarotoxin binds more to brain membranes than to liver or red cell membranes (377).

It was demonstrated in rat brain cortex slices that ACh uptake and storage were impaired in the presence of purified PLA₂ (378, 379). A progressive breakdown of pre and post synaptic membranes was observed, and, in addition, synaptic vesicles appeared to form clusters with a concomitant decrease in their total number. According to Heilbrunn, cytoplasmic ACh was probably released from these slices as a consequence of membranal rupture. Additionally, cortical synaptic vesicles were found to be sensitive to exogenous PLA₂, losing most of their ACh content at the early stages of PLA₂ attack (380). Morphologically, the vesicles were found to swell and become diffuse. Their granularity increased and the membranes lost contrast. At a later stage, the vesicles appeared as debris with a fibrous appearance. The vesicle debris never fused again. In contrast membranes reorganized themselves into larger rings or sacs. Heilbrunn, therefore, concluded that phospholipids susceptible to PLA₂ are essential to the structure of synaptosomal membranes and cholinergic synaptic vesicles (381) and that these findings "keep open the possibility of an enzymic release of ACh at the synaptic junction either by the formation of pores in the vesicle membrane or by fusion of the synaptic vesicle with the presynaptic plasma membrane."

The application of PLA₂ to the rat motor cortex caused nerve terminals and mitochondria to become distended. The membrane of the tissue appeared ruptured in small pieces (382). Quantitative analysis revealed that the number of synaptic vesicles/unit surface area decreased, while ruptures of the plasma membrane increased with increasing PLA₂ concentration. Cedergren et al. suggested that the effect of PLA₂ is due to mechanical weakening of the membrane. It was also confirmed that PLA₂ treatment of rat motor cortex led to ruptures of synaptosomal membranes (378). In addition, they demonstrated that ACh was released and its uptake was inhibited.

Boyne (383) demonstrated that low levels of PLA₂ induced efflux of ACh and ATP from synaptic vesicles isolated from the *Narcine Brasilenis* electric organ. Boyne also showed that lysolecithin (300ng/ml) increased release of ACh and ATP from these vesicles.

Rotman (384) demonstrated that the uptake of norepinephrine and serotonin by rat brain synaptosomes was reduced as a result of pretreatment with PLA₂ or PLC. This was not due to inhibition of Na⁺-K⁺-ATPase but was due to hydrolysis of neuronal membrane phospholipids, predominantly phosphatidylcholine, which apparently is important in the uptake of neurotransmitter.

Wang (385) and Armstrong et al. (386) demonstrated that PLA₂ destroyed the sodium influx in axon membranes without affecting the potassium current, which, they maintain, indicated that there was extensive disruption of the membrane structure. Heilbrunn et al. in 1970 (387) showed that PLA and PLC decreased the uptake of ACh from brain slices. Electronmicroscopic analysis of PLA₂ treated slices suggested that a decrease, but not a complete loss, of synaptic vesicles

was induced. Similarly, PLA and PLC inhibited histidine uptake by brain slices (388).

PLA₂ from bee venom preferentially hydrolyzed synaptic membrane phosphatidylserine and inhibited opiate binding. This inhibition was reversed by the addition of phosphatidylserine (389). Similarly, *Vipera russelli* PLA₂ inhibited opiate binding to brain membranes (390).

Taniguchi and Tonomura in 1970 (391) have shown that treatment of ox brain-microsomes with PLC led to inactivation of the (Na⁺-K⁺)-ATPase. Eldefrawi et al. (392) found that nicotinic receptors from torpedo electroplax were susceptible to PLC treatment. Albuquerque and Thesleff (393) found that PLC significantly increased the ionic permeability of the muscle membrane and reduced the resting membrane potential of innervated fibers. The action potential generating mechanism in fast and slow muscle was completely and irreversibly blocked by PLC. They suggested that the apparent action of PLC on the action potential generation mechanism suggests that polar heads of phospholipid molecules serve the function of ion carriers in the muscle membrane.

Zwall et al. (394) demonstrated that *Naja naja* and bee venom PLA₂s hydrolyzed phospholipids in intact RBC's. However, pancreatic and *Crotalus adamanteus* PLA₂ did not have any effect. Chap et al. (395) showed that PLC did not produce lysis or phospholipid hydrolysis in intact platelets, however, *Naja naja* venom PLA₂ in the absence of albumin induced extensive aggregation of platelets followed by lysis.

Banjo et al. (396) found that PLA₂ perturbation in the human erythrocyte membrane resulted in a decrease in the uptake of D-glucose. These effects were reversed by removal of the phospholipid

byproducts with defatted bovine serum albumin, suggesting that the phospholipid byproducts somehow reversibly inhibited the interaction of D-glucose with its receptor. Their findings indicated that marked changes in the phospholipid hydrophobic environment may alter the conformation of the functional membrane proteins that results in a decrease of their activity. They suggested that this might be due to destruction of vital phospholipids that accompanied such treatment and/or to the disturbance of the interaction between lipid and protein components of the membrane. Their results suggested that PLA₂ affects the structures of the phospholipids proximal to or bound to membrane proteins. This in turn leads to modification of their glucose uptake activities.

Lowy et al. exposed *Drosophila* larva and Hela cell membranes to bee venom PLA₂ and demonstrated that PLA₂ induced a concentration dependent swelling of the membranes by creating holes that permitted leakage of metabolites through the membrane (397).

Zwaal et al. in 1973 (398) demonstrated that both crude sea snake venom (*Enhydriana Schistosa*) and purified *N. naja* venom, both of which contain PLA₂, produced phospholipid breakdown in human erythrocytes without causing lysis. These venoms decreased the stability of the cell membrane as manifested by an increase in osmotic fragility.

b. Effects of lysolecithin on membrane structure and function.

The mechanism whereby PLA₂ may lead to its fusogenic effects and membranal alterations is by the release of lysolecithin. It is important to be familiar with some of the mechanistic theories of lysolecithin action and the various membranal alterations it induces.

Burdzy et al. in 1964 (399) noticed that macrophages fused upon

treatment with lysolecithin. Because lysolecithin is one of the main lipids in adrenal chromaffin granules, Blashko suggested that its formation may precede secretion of catecholamines (400).

Poole et al. (401) demonstrated that lysolecithin induced cell fusion leading to multinucleated syncytia and heterokaryons. They speculated that in the living cell lysolecithin may be produced at localized sites so that the overall integrity of the membrane is not threatened in vivo. They also suggested that in vivo transient changes in the level of lysolecithin may occur, and that the degradation and regeneration of lysolecithin provides a potential controlling system whereby membranes may be changed to allow fusion.

Cullis and Hope (402) also demonstrated that lysolecithin induced erythrocyte fusion. Lysolecithin caused lysis of RBCs and artificial phospholipid membranes. Lysolecithin also decreased the electrical resistance for the latter membrane (403), which indicated that an increase in membrane permeability occurred.

Lysolecithin is used as a tool to produce cell hybrids (404). It has been demonstrated that treatment of myoblasts with lysolecithin abolished their ability to fuse (405). The inhibition of fusion in this system has not been elucidated.

c. Theories on the mechanism of lysolecithin action.

Lucy (406) suggested that for fusion to occur membranes must have a high proportion of their phospholipid molecules in the micellar configuration. Lysolecithin, they speculated, constitutes one of the simplest agents that could facilitate membrane fusion. As a consequence of its wedge shape, the incorporation of lysolecithin into a bilayer would produce a transition from a bimolecular leaflet to a transiently

oriented molecules (407). When lysolecithin is dispersed in water it forms globular micelles (408), and the effectiveness with which lysolecithin micelizes membrane lipids is probably responsible for the lytic properties of the molecule. Lysolecithin probably also interacts with membrane proteins. Treatment of myelin with lysolecithin yielded a complex protein-lipid-lysolecithin micelle (409). NMR studies of erythrocyte membrane codispersed with lysolecithin also indicated that lysolecithin forms an additive complex with membrane material (410). These studies showed that treatment of plasma membranes with lysolecithin resulted in changes in membrane protein structure.

In summary, exogenous PLA_2 or lysolecithin leads to: 1) enhancement of transmitter release; 2) synaptic vesicle and synaptic plasma membrane lysis and rupture; 3) inhibition of neurotransmitter uptake; 4) uncoupling of mitochondria; 5) inhibition of ligand binding to membranes; 6) inhibition of sodium influx into the axon terminal; and 7) membranal fusion.

Presumably if the very systems that were treated with exogenous material had endogenous PLA_2 , it can be speculated that a controlled, less intense manifestation of exogenous in vitro PLA_2 application may happen in vivo. It is now crucial to review the literature on endogenous brain Phospholipase activity.

d. Endogenous brain phospholipases

1. PLA_2 , PLA_1 , and lysolecithin. In 1962, Gallai-Hatchard et al. demonstrated that brain extracts had endogenous PLA_2 activity (411). In 1963 it was demonstrated that respiring rat cerebrum slices had endogenous PLA_2 activity (412). Webster and Cooper in 1968 demonstrated PLA_1 and PLA_2 activities existed in brain homogenates at pH 7.4 (413).

Neuronal and glial PLA₁ have pH optima of 7.2 and 5.4, respectively (414). Neuronal and glial PLA₂ have pH optima of 5.4 and 8.0, respectively. PLA₂ is eight fold higher in neurons compared to glia (414).

Bazan in 1971 (415) carried out the first investigation of the subcellular localization of brain PLA. He studied rat brain cerebral cortex and hypothalamus at pH 7.2 and found that microsomes have predominantly PLA₁ whereas mitochondria have predominantly PLA₂ activity (416). Synaptosomes were demonstrated to contain a calcium independent PLA₂ activity at all pH values (416). The synaptosomal localization of this enzyme activity was further investigated, and two peaks of PLA₂ activity having pH optima of 4.8 and 8.4 were obtained. The PLA₂ that had the highest activity at pH 4.8 was localized in synaptic vesicles. The enzyme with the pH optimum of 8.4 was located in the intraterminal mitochondria.

Woelk et al. (417) showed that PLA₁ activity in synaptosomes was located primarily in microsomes and synaptosomal membranes, whereas PLA₂ was located primarily in synaptic vesicles and mitochondria. They suggested that PLA₂ functions in the renewal and turnover of neuronal and glial plasma membranes.

Woelk et al. (418) also found that PLA₂ activity was two to three times higher in neurons than in glial cells. Neuronal PLA₂ released arachidonic acid more readily than linolenic acid from both phosphatidylethanolamine (PE) and phosphatidylcholine (PC).

Low levels of lysophosphatidylcholine have been found in synaptic vesicles of rat brains (419), serotonin granules from blood platelets (420) and synaptic vesicles from splenic nerve (421). Although the

absence of lysophospholipid generation during stimulation may not support its role in neurotransmitter release, it is possible that there is a small pool of lysolecithin undergoing rapid turnover which is either removed by breakdown or reacylation after discharge of vesicle contents. Maintaining low levels of lysolecithin would avoid the toxic effects associated with these agents (422).

Baker et al. (423) analyzed synaptic vesicles from the electric organ of *Torpedo marmorata* and from guinea pig cerebral cortex. The authors demonstrated that there was not a significant turnover of lysolecithin in response to neurotransmitter release by their method of detection. They therefore concluded that PLA_2 is not involved in neurotransmitter release.

PLA_1 has been purified from the brain (424) and has been found to be located in neurons and glia (425). Subcellularly it has been detected in microsomes and synaptosomes (414,417,425). Its pH optimum ranges from 4.0-7.2 depending on the study. It is heat labile and is not calcium dependent.

Woelk purified a PLA_1 from the brain with a M_r of 75K (426). Doherty and Rowe (427) have demonstrated a cytoplasmic brain PLA_1 that hydrolyses phosphatidylethanolamine with a pH optimum of 9.5 and is stimulated by calcium.

11. Phospholipase C and diacylglycerol. Electrical stimulation of synaptosomes was correlated with calcium dependent increased labelling of phosphatidic acid by ^{32}P in synaptic vesicles (428). Hawthorne and Bleasdale speculated that synaptic transmission is accompanied by a change in phospholipid metabolism which may involve phosphatidylinositol (PI) and phosphatidic acid such as occurs in secretion of enzymes by

acinar pancreatic cells and secretion of catecholamines by chromaffin cells. The following hypothesis was put forward to relate phosphatide metabolism and transmitter release. Electrical stimulation depolarizes the presynaptic plasma membrane leading to entry of calcium and apposition of the synaptic vesicles to the membrane. Plasma membrane phosphatidate phosphatase converts phosphatidate of the vesicle membrane to diacylglyceride promoting fusion of the membranes and transmitter release. Resealing of the vesicles after repolarization of the plasma membrane would involve resynthesis of phosphatidic acid by diglyceride kinase. Hence they invoke the enzymes phosphatide phosphatase and diacylglyceride kinase in the regulation of transmitter release. Diacylglyceride, a potent fusogen according to these authors, mediates the release process. Recently, a PLC which is capable of releasing diacylglycerol, has been found in the brain. Its location and function in the brain awaits elucidation.

iii. Phospholipase D. Taki and Kanfer (429) purified phospholipase D from rat brain and characterized some of its properties. The Ph optimum is 6.0. Although the presence of Ca^{2+} is not absolutely necessary for its function, it does stimulate its relative activity.

e. Endogenous phospholipases in non-brain tissues.

1. PLA₂ and lysolecithin. Francioli in 1933 (430) noted that there was PLA in the bovine adrenal gland. Winkler et al. in 1967 found that it was in the adrenal medulla (431).

Compound 48/80, an extract from the eelworm of the swine *Ascaris lumbricoides* and from the jellyfish, *Cyanea capitata*, liberated histamine from mast cells (432). Hugberg and Uvnas in 1957 (433) tested

many enzymes on their potential to liberate serotonin from mast cells. Only PLA was capable of doing so. This suggested to them that 48/80 might activate a PLA in mast cells. They demonstrated that lysolecithin was capable of disrupting mast cells, however only at high concentrations (200-5000 $\mu\text{g/ml.}$). Compared to 48/80, it was a weak disrupting agent. The disruptive action of 48/80 was inhibited by the same compound that inhibited PLA. This was supported in 1979 by Hirata et al. who showed that when PLA_2 was inhibited in mast cells, the amount of histamine release was reduced (434).

Rubin et al. (435) speculated that nonlysosomal PLA_2 required calcium for activity and the enzyme is of potential importance in secretion since calcium is ubiquitous and critical in stimulus secretion coupling. They demonstrated (436) that ACTH in adrenocortical cells caused a calcium dependent activation of PLA_2 which brought about an increased turnover of arachidonyl phosphatidylinositol which was linked in the ACTH induced activation of steroid release. Using neutrophils as a model for the investigation of secretion, Rubin et al. (435) postulated that calcium dependent PLA_2 mediated increase in the turnover of arachidonyl PI may be a general mechanism for altering membrane function during secretory activity. Pancreas, small intestine, testis, spleen, lung, liver, kidney, heart and skeletal muscle of the rat have PLA_2 activity (437). All have approximately similar levels of PLA_2 except for the pancreas and small intestine which have the first lowest and second lowest activities, respectively (437,438). In the liver, PLA_2 is located in mitochondria, lysosomes, plasma membranes and microsomes (416).

Stoffel and Greten (439) and Smith and Winkler (440) have postu-

lated that the ability of primary lysosomes to fuse with digestive vacuoles and with the plasma membrane may be due to lysosomal PLA₂ or PLA₁.

Lapetina et al. found in platelets a PLA₂ that specifically degrades phosphatidic acid (441).

In 1970, a calcium dependent PLA₂ with optimal activity at pH 7-8 was found in rat liver lysosomal membranes (442). Rahman et al. (442) suggested that this membranal PLA₂ may be involved in the binding of lysosomes to membranes and subsequent fusion. Ahkong et al. (443) have shown that an increase in temperature leads to an increase of fusion of hen erythrocytes. They postulated that the increased temperature perhaps facilitates activity of membrane bound PLA₂ or other lipases.

PLA₂ seems to be a ubiquitous enzyme present in many eukaryotic organisms and organelles. In addition, PLA₂ has been detected in the membranes of some prokaryocytes.

Ono and Shoshici in 1968 (444) demonstrated PLA₂ activity in the membrane fraction of growing cells of *Mycobacterium phlei* with pH optima at 5 and 4. The activity was stimulated either by Mg²⁺ or Ca²⁺. PLA of bacterial origin has been reported in far fewer cases than in animals and fungi. The results of these authors were the first indication of a close association of PLA with the bacterial membrane. They postulated that PLA₂ functions in the turnover of phospholipids within the growing bacterial cell.

Nurminen and Shuomalainen in 1970 (445) found PLA in the cell envelopes of bakers yeast predominantly in the plasma membrane. They suggested that PLA functions in controlling the permeability of the plasma membrane, and also participates in transport processes by changing the permeability properties of the membrane.

ii. PLA₁. PLA₁ has been detected in liver lysosomal membrane plasma membrane and microsomes (416). Cytoplasmic PLA₁ in liver and adrenal medulla lysosomes was inhibited by calcium (417). PLA₁ has also been detected in pancreas and intestinal mucosa (418).

iii. PLC. PLC has been implicated in releasing arachidonic acid from membranes as well as being involved in secretory phenomena (446). The involvement of PLC in both these phenomena is based on the assumption that PLC is Ca²⁺ dependent. Whether or not PLC is Ca²⁺ dependent is controversial. Billah et al. demonstrated that platelet membrane PLC is Ca²⁺ dependent and specifically hydrolyzes phosphatidylinositol (PI) to diacylglycerol which can be either degraded by 1,2 diacylglycerol lipase or phosphorylated by 1,2 diacylglycerol kinase to phosphatidic acid (447). Phosphatidic acid (PA) may then be hydrolyzed by a Ca²⁺ dependent PA specific PLA₂ (448). Rittenhouse-Simmons, however, disagrees with Billah and maintains that Ca²⁺ does not activate platelet PLC. They speculate that PLC is stimulated by a physiological receptor or stimulus (449).

Allan and Michell (450) speculated that probably only a very small area of membrane is involved in the initiation of a fusion event. Therefore, proteins which facilitate fusion may be minor membrane components that are concentrated close to the actual point of fusion. Removal of polar head groups by PLC could then play a part in decreasing intramembrane charge repulsion at a very localized point at which fusion is initiated. They speculated that diacylglycerol, because of its hydrophobicity, may itself increase the likelihood of membrane fusion. There is evidence that during exocrine pancreatic ACh secretion diacylglycerol is considerably elevated.

The most direct evidence that PLC functions in fusion comes from the observation that PLC treatment rendered RBC membranes fusible (451). Similar findings were made by Mitchell et al. (452). PLC is an endogenous Ca^{2+} activated enzyme of the red cell membrane which specifically converts polyphosphoinositides to 1, 2-diacylglycerols and inositol di and tri phosphates. Although this enzyme only removes the head groups from approximately 1% of charged lipid, the molecules involved have the bulkiest and most highly charged head groups of any ubiquitous phospholipid and therefore seem to be those likely to effectively prevent membrane fusion. Vesiculation in human RBC has been correlated with accumulation of 1, 2, diacylglycerol (452-455).

There is evidence that diacylglycerol can move rapidly between the two lipid leaflets of a membrane. Its unilateral generation in one leaflet could give rise to increased membrane curvature (455). Thus diacylglycerol accumulation may initiate the production of localized membrane curvature, an essential prelude to the membrane fusion event, thereby causing vesiculation.

It was found that PLA_2 in platelets is largely membrane associated, whereas PLC is primarily cytosolic (456). PI is a poor substrate for PLA_2 , whereas it is the preferential substrate for PLC. PLA_2 acts primarily on phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). According to Billah et al., both require calcium (456).

Table 1 summarizes the location and properties of some phospholipases A_1 , A_2 , C and D.

It is evident that PLA_2 as well as several other phospholipases are in fact endogenous to brain tissue and have been invoked in secretion in various tissues. The fact that certain brain phospholipases A_2 were

Table 1

Comparison of pH and calcium requirements of phospholipases in different tissues and cellular compartments.
 0 = not necessary, (+) = stimulates, (-) = inhibits.

Enzyme	Tissue or cell type	Subcellular Location	pH Optimum	Ca ²⁺	Miscellaneous	Reference(s)
PLA ₂	Brain	homogenate (rat)	5.5	0	cAMP (+)	413
	Brain	homogenate (human)	5.0	0		413, 457, 458
	Brain	glia	8.0	?		414
	Brain	neurons	5.4	?		414
	Brain	mitochondria	8.4	0		413
	Brain	synaptosomes	4.8, 8.4	0	cAMP (-)	416, 460
	Brain	synaptic vesicles	4.8	0		416
	Brain	SV's, SPM	3.5, 6.5	?		419
	Liver	homogenate	?	?	cAMP (0)	459
	Liver	mitochondria	9.5	+		416
	Liver	lysosomes int.	4.0	-		416
	Liver	lysosome-membrane	7-8	+		442
	Liver	plasma membrane	9.0	+		416
	Liver	microsomes	8.0	+		416
	Lung	Fat cell homog.	?	?		459
	Mast cell	plasma membrane	?	?		433

Table 1 (continued)

Enzyme	Tissue or cell type	Subcellular Location	pH Optimum	Ca ²⁺	Reference(s)
PLA ₁	Brain	Homogenate (rat)	4-4.2	0	424, 426, 458
	Brain	homogenate (human)	4.6	0	458
	Brain	neurons	7.2	?	414
	Brain	glia	5.4	?	414
	Brain	synaptosomes	6.4	?	416
	Brain	microsomes	6.8	0	413
	Brain	cytoplasm	9.5	+	426
	Liver	homogenate	4.0	0	424
	Liver	lysosomes	4.0	-	416
	Liver	plasma membrane	9.0	+	416
	Liver	microsomes	9.0	+	416
	Adrenal medulla	lysosomes	4.2, 6.5	?	400
	Pancreas	homogenate	9.0, 4.5	+	461, 437, 438
	Intest. mucosa	homogenate	7.0	?	437
PLC	Platelets	membranes	9.0	0/+	449, 446
	Brain	homogenate	?	+	479, 480
PLD	Brain	homogenate	6.0	+	429

shown to have acidic pH optima and not to be dependent on calcium indicated that the PLA₂ looked at in these systems was either entirely lysosomal or contaminated with lysosomal PLA₂ that was not calcium dependent. Investigation therefore of a brain synaptosomal calcium dependent PLA₂ would be of great importance in understanding neurotransmission.

I have therefore chosen to characterize brain cortex synaptic vesicle and presynaptic plasma membrane PLA₂ activities in terms of calcium, time and substrate concentration dependence. The kinetic parameters, K_m and V_{max} were determined.

f. Involvement of phospholipases in endocytosis.

It was suggested in 1965 that lysolecithin may be involved in the pinching off of plasma membrane (462), and in the fusion of phagocytic vacuoles with lysosomes of granulocytes.

Singer et al. (463) in 1972 viewed endocytosis as a "bilayer couple" based on the fact that different lipids are present in the two bilayers of the membrane and that the two halves can independently be affected by a particular perturbation. He viewed local membrane invagination as a local relative increase of the area occupied by the inner layer compared to the outer half layer. The clustering of membrane proteins, he hypothesized, could induce a local distribution of lipids or might activate a local PLA₂ which would in turn lead to relative localized area changes that would be required for endocytosis.

Rat nerve terminals exposed to β -bungarotoxin exhibited a slight decrease in the number of synaptic vesicles as well as an increase in the number of plasma membrane invaginations (464). Kelly and Brown demonstrated that β -bungarotoxin led to an increase in the number of

coated vesicles in axon terminals (465). The treatment of frog nerve terminals with β -bungarotoxin by Heuser and Reese led to an enormous increase in coated pits (134).

The internalization of EGF which is specifically taken up by the coated pit/vesicle system in fibroblasts was shown to be inhibited by quinacrine and p-bromophenacylbromide, two PLA_2 inhibitors, as well as by chlorpromazine, a PLA_2 and calmodulin inhibitor (466).

In addition, PLC has been implicated in endocytosis because application of PLC to macrophages has been demonstrated to increase the number of coated pits (467).

There are, therefore, some indications from the literature that exogenous PLA_2 is capable of inducing coated vesicle formation and that there may be endogenous PLA_2 in fibroblast coated pits. To date, there is no information on endogenous brain coated vesicle PLA_2 . Investigation of this enzyme in coated vesicles if present might lead to a better understanding of the molecular mechanisms of endocytosis. Therefore, I decided to determine the kinetic parameters and calcium, pH, time and substrate dependencies of brain coated vesicles.

g. Other possible functions of PLA_2 .

i. Metabolism. PLA_2 was shown to be involved in the metabolism of brain mitochondrial and microsomal membranes (425).

ii. Regulation of membrane proteins, receptors and channels. In general, lipids can modulate enzymatic activity by affecting ligand-binding properties of the membrane. The lipid annulus of receptors, the lipids that surround the receptors, modulate the receptors' three-dimensional structure and hence their ligand affinity for the receptor (468). In addition, the physical state of the lipid domain in which the

receptor is situated regulates the receptor's lateral mobility and in turn the interaction of the ligand-receptor complex with effector molecules such as adenylate cyclase (468). Phospholipases can modify ligand receptor interactions and their ligand coupling to receptors by altering the lipid annulus of the receptor's domain. When Limbird and Lefkowitz (469) treated erythrocyte membrane with PLA, PLC and PLD, there was a decline of adrenergic agonist binding to the membrane. PLA₁, PLA₂, and PLC, but not PLD, treatment inhibited hormone binding to bovine corpus luteum, thereby demonstrating that lipids surrounding receptors have a vital role in ligand-receptor interactions (468). Similar modification of receptor binding by phospholipase application could be observed with the insulin receptor. PLA₂ treatment also decreased dopamine receptor binding and inhibited GABA binding to the rat cerebellum. GABA binding was enhanced, however, by PLC treatment (468). Important roles for phospholipase in ligand-receptor interactions have also been suggested for ACTH, LH, HCG, adrenergic and cholinergic receptors, all of which exhibited a decrease in ligand binding after phospholipase treatment (468).

The activities of $\text{Na}^+ - \text{K}^+$ ATPase and adenylate cyclase were decreased by PLA₂ treatment (468,471). Thus PLA₂ may affect ion transport by altering protein lipid interactions around proteinaceous ion channels (471). Banjo et al. (396) showed that PLA₂ treatment of erythrocyte membranes inhibited D-glucose uptake. They therefore speculated that marked changes in the phospholipid hydrophobic environment may alter the conformation of functional membrane proteins which then resulted in a decrease of their activity. The structure of the phospholipids proximal or hydrophobically bound to membrane

structures were affected by PLA₂ and hence modified D-glucose uptake activity.

iii. Chemotaxis. Hirata et al. (472) demonstrated a close association between chemotaxis in leukocytes and the degradation of phospholipids by PLA₂. After chemotaxis was stimulated, an accumulation of lysolecithin occurred. Lysolecithin has been reported to translocate rapidly across membranes (473) and may, Hirata suggests, regulate the dynamic aspects of chemotaxis. They demonstrated that lysolecithin regulated guanylate cyclase whose product cGMP was closely related to chemotaxis. They further speculated that there are probably localized areas in the membrane where phospholipid methyltransferase, PLA₂, and chemotactic receptors are clustered.

iv. Prostaglandin production. Flower and Blackwell in 1976 (474) summarized the following available evidence of phospholipase A₂ involvement in prostaglandin formation: (a) In 1968, it was demonstrated that phospholipids can act as stores of precursor for prostaglandins (475, 476); (b) In 1966, it was demonstrated that perfusion of guinea pig lungs or frog intestine with PLA₂ led to a rapid release of large quantities of prostaglandins (477, 478); (c) Flower and Blackwell demonstrated that spleen slices incorporated [1 - ¹⁴C]-arachidonic acid chiefly into the 2 position of lecithin; (d) During cellular damage caused by homogenization, mechanical stimulation or anaphylactic shock there was a loss of esterified labelled prostaglandin substrate from the phospholipid fraction, as well as an increase in the free substrate levels and in the biosynthesis of prostaglandins.

Flower and Blackwell maintained that PLA₂ hydrolysis products are the source of prostaglandin mobilization. They also concluded that

normal cells that synthesize prostaglandins must first undergo stimulation. They speculated that stimuli effective in PG release share the common property of activating PLA₂ which then releases fatty acids.

Vogt (364) maintained that with the exception of the lysosomal species, all PLA₂'s are absolutely dependent on Ca²⁺ ions. The PLA₂'s of plasma membranes and microsomes, the membranes most likely involved in prostaglandin synthesis, are Ca²⁺ dependent. Thus, according to Vogt, the sequence of PLA₂ stimulation followed by prostaglandin synthesis is tenable because it is well established that Ca²⁺ ions are stimulatory for PG synthesis.

In the past few years, however, it has been recognized that in tissues such as platelets and fibroblasts, arachidonic acid which is metabolized to prostaglandins may be released by PLA₂ or by the sequential action of PLC and diglyceride lipase (446,447). The relative contribution of these two pathways in platelets is controversial. Whether the second pathway exists at all in the brain and to what extent it contributes to the readily releasable pool of arachidonic acid is unknown at present. It should be noted that both PLC and diacylglycerol lipase have been detected in brain (479,480). Whichever pathway is predominant, it is certain that PLA₂ does release arachidonic acid. The production of prostaglandins and their role in the nervous system is the topic of the following section.

h. Modulators of PLA₂ activity.

Calcium is required as an absolute cofactor in PLA₂ hydrolysis in many membranes. Mg²⁺ inhibits platelet PLA₂ (481). A variety of compounds have been implicated in the literature as modulators of PLA₂ activity. Very often PLA₂ stimulation is implied by the subsequent

release of prostaglandins. It is unknown whether many of these compounds have direct or indirect effects. Because it has become evident in recent years that arachidonic acid may be released by enzymes other than PLA₂, it is possible that some of these putative stimulators may represent stimulators of PLC or diglyceride lipase. Nevertheless, with all the shortcomings it should be kept in mind that all the compounds to be discussed have been implicated either directly or indirectly in PLA₂ modulation.

(1) Neurotransmitters, hormones, peptides and physical stimuli. Catecholamines have been found to stimulate PLA₂ in brain, fat tissue, spleen and stomach (482-486). 5-hydroxytryptamine stimulated PLA₂ in brain, fat tissue, and stomach (482,484). Acetylcholine stimulated PLA₂ in skin, intestine, adrenals and fat tissue (482, 487, 488).

The hormones TSH, ACTH, LH and glucagon stimulated PLA₂ in the thyroid, adrenal, uterus, liver and stomach (489-492,486). The peptides bradykinin and angiotensin stimulated PLA₂ in lungs and spleen, respectively (493, 494). Collagen and thrombi stimulated platelet PLA₂ (495, 496). Nerve stimulation of brain, stomach, spleen, kidney and fat tissue led to PLA₂ stimulation in these tissues (485, 496-500). Mechanical stimulation of the spleen (501) and distension in the lung, uterus and stomach (497, 502, 503) also stimulated PLA₂. Piracetam (2-oxopyrrolidine-1-acetamide) stimulated neuronal and synaptosomal PLA₂ (504).

Cells in culture can be stimulated to produce PGs by serum, thrombin, bradykinin, angiotensin, arginine vasopressin, cAMP, histamine, serotonin, and mechanical stimulation (506).

Hong and Deykin (507) provided evidence that PLA_2 hydrolysis of PI in mouse cells subsequently led to PG synthesis. These results provided direct evidence for the involvement of PLA_2 in prostaglandin synthesis. They showed that bradykinin and thrombin led to the release of lysoPI. They maintained that within the limits of detection, PLC and diglyceride lipase were not activated and were not responsible for the release of arachidonic acid. Thus bradykinin and thrombin acted by directly stimulating PLA_2 .

Woelk et al. in 1978 (425) found that norepinephrine injected into the lateral ventricle of rabbit brain stimulated hydrolysis by PLA_2 . So did electric shock and a variety of convulsive drugs. These results suggested a connection between fatty acid formation, electrical activity, and release of transmitter substances to these authors. They also suggested that PLA_2 may be connected with the molecular changes taking place in membranes during synaptic transmission. However, they were cautious in this interpretation because the same changes that occurred in neurons also occurred in glia cells.

Kunze (460) demonstrated that guinea pig brain synaptosomes had PLA_1 and PLA_2 activities and that they were both stimulated by norepinephrine, acetylcholine and 5-HT.

(ii) Methyltransferase. Hirata and Axelrod (508) proposed that catecholamines bind to β -adrenergic receptors and stimulate Methyltransferase I and phospholipid Methyltransferase II which sequentially increase the methylation of PE to phosphatidyl-N-Monomethyl ethanolamine and then to PC.

Methyltransferase I uses PE as substrate, requires Mg^{2+} , and has a high affinity for the methyl donor S-Adenosyl-L-Methionine (SAM).

Methyltransferase II methylates phosphatidyl-monomethylethanolamine to PC and has a low affinity for SAM. The asymmetric arrangement of the two enzymes across the membrane makes possible the stepwise methylation of PE located on the cytoplasmic side and facilitates the rapid transmembrane transfer of the final product, PC, to the external surface of the membrane.

As the phospholipids are methylated, they flip flop and increase the fluidity of the membrane and hence increase the mobility of adrenergic receptors to interact with adenylate cyclase and guanyl nucleotide factor. Axelrod maintains that phospholipid methylation is also closely coupled to PLA_2 activation. Phospholipid methylation increases calcium influx and the subsequent release of histamine from mast cells presumably by stimulating PLA_2 . The increase in membranal fluidity brought about by phospholipid methylation would affect the membrane microenvironment which would in turn open calcium channels and thereby activate PLA_2 . Methylated phospholipids are translocated in the membrane and thus could bring the substrate phospholipid in juxtaposition to PLA_2 . Because mepacrine, a PLA_2 inhibitor, blocked histamine release from mast cells, Axelrod postulated that arachidonic acid or its metabolites participate in the release of histamine as a result of PLA_2 activation. Supporting the hypothesis of indirect methyltransferase stimulation of PLA_2 is the fact that inhibition of methylation in certain cell types blocks calcium influx, the release of arachidonic acid, and the formation of lysolecithin. Thus ligand activation of membranal methyltransferase could indirectly stimulate membranal PLA_2 by increasing the fluidity of the membrane and hence bringing the phospholipid substrate closer to PLA_2 as well as open calcium channels

leading to the influx of calcium and the subsequent stimulation of PLA₂.

Direct ligand activation of PLA₂ without intermediate methyltransferase involvement has been demonstrated in the macrophage. A surface membrane protein was shown to be bifunctional in that it was both a receptor for the F_C portion of IgG and a PLA₂ (509).

(iii) Anesthetics. It has been demonstrated that the local anesthetics, dibucaine, tetracaine benzocaine and propranolol, inhibit stimulus activation of PLA₂ in platelets (510). Local anesthetics inhibit thrombin and collagen induced platelet aggregation and secretion of contents from storage granules (511). Local anesthetics also inhibited snake venom seminal vesicle, pancreatic mitochondrial and microsomal PLA₂ (512-514).

Local anesthetics have been known to antagonize the binding of calcium to phospholipids (515). Scherphof et al. postulated that local anesthetics are positively charged and hence displace Ca²⁺ from membranes. Therefore, activation of PLA₂ is inhibited because the formation of an enzyme-Ca²⁺-substrate complex is inhibited. According to Scherphof, PLA₂ inhibition leads to a decrease in Na⁺ and K⁺ membrane permeability, thereby inducing anesthesia.

Other theories on the mechanism of local anesthetic inhibition of PLA₂ include the possibilities that the anesthetic binds to either (a) the phospholipid substrate and modifies its physicochemical properties and its ability to be hydrolyzed or to (b) PLA₂, displacing calcium from the enzyme itself and thereby inhibiting it (515). It has been demonstrated that purified phospholipids bind to local anesthetics. Anesthetics have also been demonstrated to bind directly to PLA₂.

(iv) Antibiotics. Sugatini et al. (516) demonstrated that the

antibiotics tetracycline, macrolides, chloramphenicol and carbenicillin inhibited the activity of *Crotalus adamanteus* PLA₂. These antibiotics also had variable inhibitory effects on PLC and PLB.

(v) Cyclic AMP. Cyclic AMP has been reported to either stimulate or inhibit PLA₂ depending on the tissue looked at and the individual investigator. Rittenhouse-Simmons and Deykin (517) found that cAMP inhibited platelet PLA₂. They speculated that cAMP may directly inhibit PLA₂ by binding to the enzyme or to the substrate. Alternatively, cAMP, they speculated, could stimulate an inhibitory phosphorylation of PLA₂ or of a protein which as a result would in some way restrict the effective interaction of enzyme and substrate. They also thought it is also possible that cAMP may promote the active storage of calcium.

Kunze demonstrated that cAMP inhibited PLA₂ in rat brain synaptosomes (460). In rat liver microsomes cAMP neither stimulated nor inhibited PLA₂.

Van den Bosch and Van den Besselar demonstrated that cAMP stimulated PLA₂ activity 2-4 fold in fat cell homogenates (362). The authors suggested that cAMP mediated hormonal regulated lipolysis in fat cell homogenates by stimulating PLA₂. Increased cAMP has been implicated in the stimulation of PLA₂ by TSH in thyroid (489).

Similarly, Imri (459) found that cAMP activated PLA₂ in lung tissue.

(vi) Lipomodulin. Hirata et al. (518) demonstrated that glucocorticoids induce the synthesis of the PLA₂ inhibitory protein, lipomodulin, in rabbit neutrophils. This synthesis was blocked by inhibitors of RNA and protein synthesis. The induction was mediated by cytoplasmic glucocorticoid receptors. They suggested that the presence of PLA₂

inhibitory proteins might provide a control mechanism for the activation of PLA₂. They maintained that the anti-inflammatory capacity of glucocorticoids may reside in the capacity of cells to synthesize lipomodulin. Lipomodulin has a M_r of 40,000 and is possibly a glycoprotein. The existence of lipomodulin has also been reported in rat lung (35).

Lipomodulin itself apparently can be modulated by cellular regulating mechanisms. In vitro cAMP dependent protein kinase or in vivo calcium dependent protein kinase phosphorylation of lipomodulin caused a decrease in its capacity to inhibit PLA₂.

It is thus apparent that PLA₂, by mechanisms to date not entirely established, is modulated, i.e. stimulated and/or inhibited by a variety of compounds. Some of the modulators that were mentioned are summarized in Table 2.

Van den Bosch and Van den Besselaar in contemplating how PLA₂ could be modulated by so many different compounds speculated that the different modulators may affect access of the substrate to PLA₂ (362).

(vii) Modulation of synaptic vesicle, coated vesicle and synaptic plasma membrane PLA₂. In view of the presence in axon terminals of certain key modulators of neurotransmission — PGE₂, PGF_{2α}, cAMP, ATP (to be discussed later) and calmodulin — their effects on modulating synaptic and coated vesicle and presynaptic membrane PLA₂ were investigated. In an attempt to correlate the effects of these compounds on the various membranal PLA₂'s with function in neurotransmission, the techniques of nephelometry, phase contrast microscopy and electron microscopy were used to evaluate the effects of the above mentioned compounds on synaptic vesicle-vesicle interaction, decoated vesicle-vesicle interaction and synaptic plasma membrane integrity.

Table 2

Variety of PLA₂ stimulators and inhibitors mentioned in the literature are presented with pertinent references.

PLA₂ modulators

Stimulators

Neurotransmitters: Norepinephrine, 5-Hydroxytryptamine, Acetylcholine, Histamine, Serotonin (425, 506, 460, 482-488)

Hormones: TSH, ACTH, Glucagon, Pentagastrin, Vasopressin (486, 489-492)

Peptides: Bradykinin, Angiotensin (493, 494, 506)

Proteins: Collagen, thrombin (495, 496, 506)

Physical stimuli: Nerve stimulation, Mechanical vibration, Distension (425, 485, 496-503)

Modulator proteins: Calmodulin (319)

Enzymes: Methyltransferase (516)

Nucleotides: cAMP (459, 489)

Pharmacological agents: Piracetam (504)

Prostaglandins: PGF_{1α} (505)

Ions: Ca²⁺, Ba²⁺, Sr²⁺ (538, 550, 551)

Table 2 (cont'd)

Inhibitors

Anesthetics: Dibucaine, Tetracaine, Benzocaine, Propranolol,
Nupercaine (510-514)

Antimalarial: Mepacrine (520)

Antipsychotic: Chlorpromazine (521-524)

Halo-ketone: p-Bromophenacylbromide (530-532)

Tetracycline antibiotics: Macrolide, Chloramphenicol, Carbenicillin
(512)

Lipids: Lysolecithin, Long chain fatty acids (543, 549)

Modulator proteins: Lipomodulin (519)

Nucleotides: cAMP (460, 517)

Ions: Mg^{2+} (481)

1. Purified soluble Phospholipases A₂.

In order to gain insight into the mechanism of PLA₂ hydrolysis, its substrates, modulators and physical-chemical properties, it is desirable to study the purified enzyme. Phospholipase A₂ has been purified from a variety of sources where it is soluble. In contrast, few membrane bound Phospholipases A₂ have been purified. As Van den Bosch and Van den Besselar have noted (362), even if membrane-bound phospholipase A₂ were purified, it is not certain that the membrane extracted PLA₂ would behave in vitro as it does in the in vivo membrane. I have therefore attempted to understand PLA₂ actions by looking at both membranal and purified soluble phospholipases A₂. Some of the properties and mechanisms of the purified phospholipases that I have studied for this thesis are discussed in this section.

(1) Pancreatic PLA₂. Bokay, in 1877, first suggested that PLA existed in the pancreas (526). Belfanti in 1932 (527) and Gronchi in 1936 (528) provided evidence for the existence of PLA in pancreas. Magee et al. obtained a highly purified PLA₂ from the pancreas in 1963 (461).

De Haas et al. (529) purified PLA₂ from pig pancreas and found that it has a M_r of 13,800 and that it is crosslinked by 7 disulphide bonds. It occurs primarily as a zymogen in the pancreas and is activated by trypsin which specifically splits an arginine-alanine linkage in the N terminal region of the polypeptide chain giving rise to an active PLA₂ and a heptapeptide. Pancreatic PLA₂ has a digestive function in the small intestine and is transported to the juice as the harmless zymogen protein.

The zymogen consists of a single polypeptide chain 130 amino acids

crosslinked by 7 disulphide bridges. Pieterse et al. and Volwerk et al. in 1974 (530, 531) demonstrated that several features of the active site of the enzyme are also present in the zymogen. Calcium binds in a molar ratio of 1:1 to both proteins inducing similar conformational changes. In addition, histamine 53 reacted at the same rate with parabromophenacylbromide producing a nonactivatable modified zymogen (530-531). Although the zymogen can attack lecithin in its monomeric form, it is probably inert because it cannot attack organized lipid-water interfaces due to the absence of a recognition site for the interface (532). Pieterse et al. maintain (532) that the interaction with the interface in the activated enzyme is controlled by a single amino acid residue with a pK of 8.1. When trypsin hydrolyses the arginine-alanine bond, a limited conformational change takes place in which the newly formed protonated alpha amino group ($\alpha\text{-NH}_3^+$) of the N terminal alanine forms a salt bridge (an ion pair) with a buried carboxylate. Volwerk et al. speculated that the formation of the salt bridge triggers the induction of the recognition site at surfaces with no direct effect on the active site. Within their conceptual framework, the enzymatic activity of a lipolytic enzyme is largely determined by the physicochemical properties of the interface. Small differences in the chemical structure of the substrate are known to produce a different water-lipid interface, interacting in a different way with the recognition site. They maintain that metabolic activity can induce slight alterations in lipid structure and therefore could exert a powerful control of membrane-linked enzymes.

The conformational change induced by calcium in PLA₂ is due to a partial shielding of a tyrosine residue from the aqueous solvent.

Substitution by barium and strontium resulted in a similar but different PLA₂ conformational change. Volwerk et al. suggested that the PLA₂ calcium binding site contains one or more carboxylates in addition to histidine. The conformational change induced by calcium induces stabilization of the structure, i.e. there is a diminished susceptibility to denaturation, peptide bond cleavage by proteolytic enzymes or reduction of disulfide bridges.

The introduction of parabromophenacylbromide (pBP), a haloketone, to pancreatic PLA₂ severely distorted binding of Ca²⁺ to the enzyme (532). Volwerk et al. concluded that histidine 53 is the active center of the enzyme. The data support the fact that His 53 is preserved in the primary structure of all vertebrate Phospholipases A₂ (531).

Volwerk et al. (531) demonstrated that pancreatic PLA₂ as well as its zymogen are completely inactivated upon reaction with p-bromophenacylbromide. P-bromophenacylbromide alkylates the imidazole side chain of histidine-53 in pancreatic PLA₂. This reagent, which does not possess a well defined group specificity or a structural resemblance to the substrate, is capable of producing selective modification of an essential residue on the enzyme. It is not simply a histidine reagent. It does not react with free histidine. Volwerk et al. suggested that there exists a special complementarity between the modification site in the protein and pBP which favors covalent bond formation. They suggested that the reaction between pBP and Histidine 53 is in fact catalyzed by the enzyme and that pBP is directed at the active site.

Pancreatic PLA₂ seems to be the only PLA₂ which requires proteolysis for its activation. The only exception I have come across

in the literature is a PLA₂ existing in the erythrocytes which is activated by trypsin (533).

The mammalian pancreas was found to contain PLA₂ isoenzymes (534). The enzyme and isoenzymes were characterized by few substitutions. From the porcine pancreas approximately 5% can be isolated as an isoenzyme. Both are similar with respect to binding calcium and substrate requirement. Amino acid analysis revealed that the isoenzyme contained one histidine and methionine residue less than the major occurring enzyme. Multiple pancreatic isoenzymes have been isolated by several groups (535, 536).

Pancreatic PLA₂ only behaves as active monomers (537). X-ray crystallographic studies revealed that the pancreatic enzyme is highly alpha helical with a flat disc shape. A long central stretch of alpha helix contains the active histidine site.

(ii) Naja naja PLA₂. *Naja naja* PLA₂ is calcium dependent (538). Salach et al. demonstrated that there are 11 isoenzymes of *Naja naja* PLA₂ with isoelectric points ranging from pH 4.0 to 5.66 and M_rs ranging from 8500 to 20,000 (538). Incidentally, the term isoenzyme was proposed by Markert and Moller (540) to describe different proteins with similar enzymatic activities and occurring in the same source but different in some of their physical chemical properties.

Experiments using dimethylsuberimidate (DMS) as a cross-linking reagent demonstrated that this PLA₂ dimerized at 0.5 mg/ml and that even larger aggregates occurred at enzymatic concentrations of 2.5 mg/ml (539, 540, 541).

Roberts et al. (542) speculated, based on their experimental observations, that two phospholipid molecules are required for the

activity of *Naja naja* PLA₂. One is necessary to sequester the enzyme to the interface, and the other is necessary for subsequent catalysis. The lipid substrate, they maintained, is essential for enzyme aggregation. They proposed that the enzyme first binds calcium and then binds one phospholipid. This leads to a conformational change in the enzyme leading to its dimerization. A second phospholipid is then bound to the dimer at a functional site and then catalysis occurs.

It was found that long chain fatty acids (palmitate, oleate and linoleate) inhibited *Naja naja* PLA₂ (543). It was hypothesized that this inhibition was due to fatty acid interaction with substrate micelles.

(iii) *Crotalus adamanteus* PLA₂. *Crotalus adamanteus* exists as alpha and beta isoenzymes (544). Both are dimeric proteins with M_r s 30,000 composed of identical monomeric subunits with approximate M_r s of 15,000. The monomeric subunits differ by their replacement of either an aspartic acid or glutamic acid residue. The dimers disassociate either at low pH or in 8M urea. Only the dimer is catalytically active (544,545).

The unique requirement of dimeric structure could be an indication of structural and possibly mechanistic differences between the C. adamanteus (C.A.) enzyme and other monomeric phospholipases A₂. The active dimeric form of the C.A. enzyme has been demonstrated by Shen et al. (545) to be in equilibrium with enzymatically inactive subunits. They found that the dimer was the sole active species and that dimerization was independent of calcium. The requirement of the dimeric form of the enzyme is of interest in that other phospholipases A₂ are active as monomers.

Wells (546) demonstrated that two tryptophans are essential for the activity of C.A. PLA₂. Loss of activity occurred when these two residues were modified, leaving histidine completely untouched. He also reported that two lysines are important to the catalytic activity of the enzyme. Since the protein contains two identical subunits, it was assumed that each subunit contains one of each of these residues. He suggested that the peculiar site of tryptophan in the active site might arise due to subunit interactions upon dimerization. He also suggested that calcium activates *Crotalus adamanteus* PLA₂ by inducing a conformational change in the enzyme allowing the substrate to bind to the enzyme. Calcium binding to the enzyme leads to deprotonation of the lysine and the burial of its NH₂ group. This buried NH₂ group, he suggested, may be a nucleophile promoting hydrolysis of the ester bond. Alternatively he suggested that it is possible that the role of lysine is to control the conformation of the active site without being directly involved in catalysis.

(iv) *Crotalus atrox* PLA₂. PLA₂ from *Crotalus atrox* is also a dimer of apparently identical subunits and is activated by calcium (547). The M_r of the monomer has been reported to be 14,700. Both *C. adamanteus* and *C. atrox* exist as dimers at neutral pH and dissociate into monomers at low pH. *C. Atrox*, however, has a much lower alpha helix content (9%) than *C. Adamanteus*, which contains 70% helix (547).

Keith et al. (548) reported that *C. Atrox* remains a dimer at concentrations as low as 10^{-11} M and is probably a dimer during catalysis.

Both *Crotalus Adamanteus* and *C. Atrox* are subject to strong product-lysolecithin inhibition which is relieved by serum albumin (549).

(v) Bee venom PLA₂. Bee venom PLA₂ has a M_r of 14,000 and is calcium dependent. Its amino acid sequence was elucidated in 1970 by Shipolini et al. (550). Its amino acid sequence is completely different from all the other PLA₂'s thus far sequenced with the exception of 8 residues that have been conserved. It is inactivated by parabromophenacylbromide. Information as to whether it exists as an active monomer or dimer is lacking at this point.

j. Theories on the mechanism of Ca²⁺ activation of PLA₂.

Verheij et al. (551) postulated that the role of calcium may be to bind the negative charges of the substrate phosphate. This function may be fulfilled by barium ions as well. They suggested that water attacks the substrate carbonyl atom, and then the imidazole ring histidine picks up a proton from the water, thereby facilitating the reaction. The proton is then donated to the alkoxy oxygen of aspartic acid. Comparison of the primary structure of all vertebrate PLA₂'s shows an absolute conservation of His 48, Asp 49, Asp 99, and Tyr 52. They therefore suggested that an Asp-His couple functions in catalysis.

Wells (546) suggested that calcium might facilitate the deprotonation of a nucleophile which plays an important role during catalysis. Dijkstra et al. (551, 552) suggested that the active His 48 site forms a hydrogen bond with the carboxyl group of Asp 99. A water molecule liganded to a calcium ion in the PLA₂ active site area is expected to act as a nucleophile for attacking the carbonyl atom of the substrate. Yang and King (553) modified Hemachatus PLA₂ with pBP and found that the enzyme lost its activity and toxicity. Only one His residue-47 was modified. In pancreatic PLA₂ His 55 was the only modified residue. Calcium exerted a pronounced protection on the

inactivation process by pBP. Wells et al. suggested that His 48 may act as a charge relaying group and upon calcium binding the charge release resulting from deprotonation could induce a tryptophan perturbation.

Dijkstra et al. (552) proposed a mechanism for PLA₂ hydrolysis in 1979. In the region of the active site His 48 forms a hydrogen bond with the carboxyl group of Asp 99. A water molecule which is hydrogen bonded to both the His 48 imidazole and calcium acts as a nucleophile attacking the carbonyl atom of the substrate. The calcium ion is liganded with four amino acid residues near the active site (Tyr 28, Gly 30, Gly 32 and Asp 49) and the other water molecule. The resulting tetrahedral intermediate is stabilized by calcium. Subsequently, the ester bond is broken as the other proton of the water molecule is donated to the ester bond.

Dijkstra et al. showed that three backbone carbonyl oxygens, Tyr 28, Gly 30, Gly 32 and a carboxyl of Asp 49, form a calcium coordination site. Keith et al. (548) refer to this region as the calcium binding loop. The central role for this region in the function of the enzyme is reflected in the fact that several sequence positions in the loop in all PLA₂'s studied to date have been conserved.

The crystal structure of bovine pancreatic PLA₂ revealed that Asp 49 utilizes both oxygens of the carboxylate to coordinate calcium. Keith et al. demonstrated that the architecture of the C. Atrox dimer provides a basis for the cooperative interactions between protomers in response to the binding of substrate and calcium (548). They suggested that the interprotomer contacts of the calcium loops (Asp 49 of one protomer and Lys 64 in the opposite protomer) and the binding of substrate and calcium to one protomer may affect the conformation of the dimer.

k. Sequence homology of phospholipases A₂.

The complete amino acid sequences have been reported for PLA₂ from porcine and equine pancreas (554-556), honey bee venom (550), *Naja naja atra* venoms (557,558), the Australian tiger snake venom (559), the gabbin adder (560), *C. Adamanteus* and *C. Atrox* venoms (561,562,563). All of the phospholipases with the exception of Bee Venom exhibit strong sequence homology. Sequence analysis revealed that there is a high conservation of disulphide bond placement and several functionally important sequences (562). The isoenzymes of *C. Adamanteus* are chromatographically and electrophoretically distinct (564). However, they are indistinguishable based on their molecular weight and amino acid composition. The complete amino acid analysis of *C. Adamanteus* alpha was determined by Henrikson et al. (561). The enzyme monomer has 122 amino acids and has 7 inter-chain disulphide bonds. Henrikson et al. grouped PLA₂'s into 2 distinct groups. Group I consists of PLA₂ from elapid and pancreatic sources. Group II contains enzymes from *Crotalus* and *Viper* venoms. The differences between groups I and II are based on sequence homology, unique disulphide bonds, and the existence of characteristic structural elements. The most significant difference between the two groups is the existence of a COOH terminal extension of 6 or 7 amino acid residues in group II enzymes. Both groups I and II contain a high degree of conservation of certain domains which may be important in catalysis. Both contain a common highly conserved structural matrix maintained by disulphide bonds. The Group II enzymes are not inhibited by p-bromophenacylbromide. The presence of His 47 and Asp 48 are invariant in all phospholipases A₂ including the Honey Bee Venom.

There is also a correlation between the sequence analysis of the PLA₂'s and the taxonomy of the venomous reptiles' fang structures and a variety of phyletic characteristics. Pancreatic venom and salivary glands share a common embryological origin and contain acinar cells which secrete a number of hydrolytic enzymes. Heinrichson et al. proposed that pancreatic tissue evolved from a common ancestral tissue. Bee venom, with the exception of the sequence (Cys-Cys-Arg-Thr-His-Asp-Met-Cys), is entirely different from all other PLA₂'s and is put into a different class.

The enzymes found in all snake venoms characterized to date have approximately 120 amino acids and 7 disulphide bonds. Despite apparent structural similarity they display a great deal of variability in terms of neurotoxicity, myotoxicity and anticoagulant activity (564). It is also interesting to note that *Naja naja* PLA₂ acts optimally at pH 8.9 whereas pancreatic PLA₂ acts optimally at pH 6.5 despite extensive similarities in their amino acid sequences (364).

I have chosen to use purified PLA₂'s from the following sources to examine whether calmodulin exerts a direct effect on the enzyme: *Crotalus Adamanteus*, *Crotalus Durissus Terrificus*, Bee Venom and *Naja naja* venoms. The following three correlative experiments were undertaken for this analysis: 1) The effect of calmodulin on these enzymes' V_{max} and K_m was determined, 2) The ability of these enzymes to bind to a calmodulin affinity column was examined and 3) It was determined whether these enzymes could be cross-linked in a calcium dependent manner to calmodulin with Dimethylsuberimide (DMS), a protein crosslinking reagent.

I have chosen to examine *Naja naja* PLA₂ activity to elucidate

whether the same compounds that were examined for their effects on synaptic and coated vesicle and synaptic plasma membrane PLA₂ activities (PGF_{2α}, PGE₂, cAMP and ATP) have a direct effect on the PLA₂ as opposed to an effect on an enzyme or protein in the membrane that could indirectly affect PLA₂. This enzyme was chosen because of its strong sequence homology with the mammalian PLA₂. The dose dependent effects of these modulators were studied as well as their ability to effect PLA₂ polymerization as determined by DMS crosslinking experiments.

11. Prostaglandins.

"Few would question that one of the most exciting and important developments in biochemistry and physiology in recent years has been the emergence of prostaglandins as a new class of molecules mediating and modulating hormonal, neurohormonal, or other stimuli on most mammalian tissues."

L. S. Wolfe, 1978 (565)

After discussing the potential of brain PLA₂ involvement in neuronal events, it is only natural that a discussion of its major endproducts, arachidonic acid, and its metabolites, the eicosanoids, should be discussed. The history of prostaglandins and thromboxanes will be presented along with evidence of their location and function in the brain and their involvement in membranal events.

a. Discovery

The first historical mention of what can retrospectively be attributed to prostaglandins (PGs) was made in 1905 by Japelli and Scarfa

(566) who reported that an injection of dog and bull prostate extract led to an increase in canine blood pressure. In 1930 Kurzrok and Lieb (567) observed that seminal fluid increased the contractility of smooth muscle strips. Von Euler (568) extended these observations shortly afterwards and observed that seminal fluid from many other species contained factors that reduced blood pressure and contracted smooth muscle. Von Euler speculated that in the above mentioned extracts the active material involved was present in the lipid soluble acid fraction of the semen and originated from the prostate gland. Thus he coined the term "prostaglandins". It is now known, however, that it is the seminal vesicle fraction from semen that contains the highest concentration of prostaglandins. The development of chromatographic, isotopic and mass spectrometric techniques led to the isolation and characterization of these lipid soluble PGs by Bergstrom and Sjovall in 1955 (569). In 1960, Bergstrom et al. reported the isolation in crystalline form of $\text{PGF}_{1\alpha}$, $\text{PGF}_{1\beta}$, and PGE_1 , and elucidated their structures (570).

Bioassays utilizing pure prostaglandins have shown that both PGE_2 and $\text{PGF}_{2\alpha}$ enhance uterine contractility (571). PGE_2 and $\text{PGF}_{2\alpha}$ are also potent vasoactive agents which either cause vasodilation or vasoconstriction depending on the animal species (572). In general PGFs are vasoconstrictive, whereas PGEs dilate all vessels except in the nasal mucosa, where they are vasoconstrictive. PGE_2 also dilates bronchial smooth muscle and relaxes the circular muscle of the gastrointestinal tract. $\text{PGF}_{2\alpha}$ has opposing effects in both these systems.

In 1975, the structure of Thromboxane A_2 (573), a bicyclic unstable

oxane derivative of arachidonic acid, which was previously referred to as aorta contracting substance (574), was elucidated. The name Thromboxane denotes that it is an oxane compound that was originally discovered in platelets (thrombo). It is a vasoconstrictor and a platelet aggregator.

In 1976, another prostaglandin, prostaglandin I_2 (PGI_2) or prostacyclin, was discovered and its structure elucidated (575-577). It is a vasodilator and a platelet antiaggregator acting in direct opposition to Thromboxane A_2 . The location and function of these arachidonic acid derivatives in the brain are discussed later on in this section.

b. Chemistry.

Prostaglandins are a family of oxygenated and unsaturated carbon fatty acids that contain a cyclopentane ring. They are derived from polyunsaturated fatty acids by most vertebrate and invertebrate tissues. PG classification is based on the structural differences in the cyclopentane ring (578). PGEs are β hydroxyketones. PCFs are 1, 3-diols. PGA, PGB and PBC are derived from PGE and are β unsaturated ketones. The PGs are further classified by the number of carbon-carbon double bonds in their side chains. The subscripts 1, 2 and 3 refer to the number of double bonds. They are further subdivided into stereoisomers, and the subscripts Alpha and Beta refer to cis and trans isomers, respectively.

Thromboxane A_2 is a nonstable bicyclic oxane derivative of arachidonic acid which has a half life of 30 seconds and then stabilizes to the hemiacetal Thromboxane B_2 or PHD (8-1-hydroxy-3-oxopropyl-9-12L-dihydroxy-5-heptadecadienoic acid (577). Prostaglandin I_2 is 6,9, α

epoxy-11- α -15-dihydroxyprosta-5, 13-dienoic acid (576, 577). See Figure 2 for the structures of some representative eicosanoids.

c. Biosynthesis.

The primary eicosanoids are formed by the bioconversion of unesterified 8, 11, 14-eicosatrienoic acid (homo- γ -linolenic acid) to PGE₁ and PGF_{1 α} , 5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid) to PGE₂, PGF_{2 α} , TxA₂ and PGI₂ and 5, 8, 11, 14, 17-eicosapentaenoic acid to PGE₃ and PGF_{3 α} . In most mammalian tissues, the essential fatty acid, arachidonic acid, is quantitatively the most important precursor for endogenous prostaglandin and thromboxane synthesis (578).

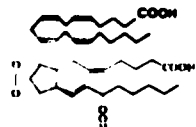
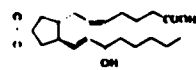
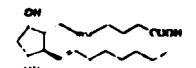
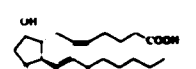
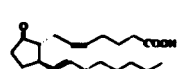
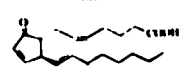
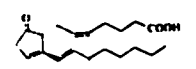
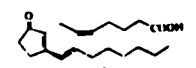
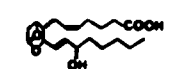
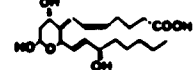
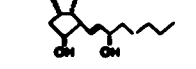
The transformation of unsaturated fatty acids to PGs is catalyzed by a group of membrane bound enzymes collectively called PG synthetase. Because there is little free unsaturated fatty acid within the cells, these precursors are stored in ester linkage of phospholipids, cholesterol esters, and triglycerides in association with the cell membrane. Present theory is that PGs are synthesized by splitting a fatty acid from these storage forms by PLA₂, PLC followed by diglycerol lipase, cholesterol lipase or triglyceride lipase. The liberated fatty acids are then metabolized to PGs by the PG synthetase system (579).

The release of fatty acids by acylhydrolases into a specific membranal pool available to the prostaglandin synthetase multienzyme complex is therefore the rate limiting step in endogenous PG synthesis.

A detailed study of the transformation of arachidonic acid to PGs has been detailed by Samuelsson (580). The initial reaction is the stereoselective elimination of the 13 hydrogen from the precursor

Figure 2. Structures of arachidonic acid and some representative eicosanoid metabolites and their trivial names.

Structures of arachidonic acid and some of its metabolites

Structure	Trivial and abbreviated name
	Arachidonic Acid
	Prostaglandin G ₂ (PGG ₂)
	Prostaglandin H ₂ (PGH ₂)
	Prostaglandin F _{2m} (PGF _{2m})
	Prostaglandin D ₂ (PGD ₂)
	Prostaglandin E ₂ (PGE ₂)
	Prostaglandin A ₂ (PGA ₂)
	Prostaglandin C ₂ (PGC ₂)
	Prostaglandin B ₂ (PGB ₂)
	Thromboxane A ₂ (TXA ₂)
	Thromboxane B ₂ (TXB ₂)
	Prostaglandin I ₂ (PGI ₂) or Prostacyclin

polyunsaturated acid followed by isomerization of the 11 double bond into the 12 double bond position and stereospecific hydroperoxidation at the 11 α position. The 11 α hydroperoxide then attacks the 9 α position of the 8 double bond with concomitant conrotatory cyclization between C-11 and C-12, isomerization of the 12 double bond into the 13 double bond position and another stereospecific hydroperoxidation at the 15 position. This reaction requires oxygen and is catalyzed by cyclooxygenase, a dioxygenase. PGG₂, 15-hydroperoxyprostaglandin endoperoxide, the resultant product, is reduced to PGH₂, 15-hydroxyprostaglandin endoperoxide. PGH₂ acts as a substrate for several reactions. It can be converted to PGE₂ by an endoperoxide isomerase, and to PGF_{2 α} by an endoperoxide reductase (581). The PGE prostaglandins can be converted by dehydration, possibly enzymatic into PGA (Ps) and then by isomerases into PGC and then PBG (582). The β hydroxyketone function of PGE is not stable to acidic or basic conditions. With the exception of pH range 5-8 dehydration of PGE occurs giving rise to PGA, which then rearranges to PGB under basic conditions (583). PGA, PGB, and PGC are not present or formed in all species and tissues. There is evidence that prostaglandin endoperoxide isomerase and reductase are separate enzymes (579). Therefore, different tissues could vary in their capacity to form PGEs and PGFs.

PGH₂ can also be converted to PGD₂ by PGD isomerase (584), to PGI₂ by prostaglandin endoperoxide I isomerase (585) which in the presence of H₂O is converted to its more stable derivative 6-keto-PGF_{1 α} . PGH₂ can also be converted to TxA₂ (573) by prostaglandin endoperoxide Thromboxane A isomerase. TxA₂ in the presence of H₂O is rapidly converted to TxB₂, its more stable form.

Arachidonic acid can also be acted upon by another enzyme, lipoxygenase, to form various non-endoperoxide hydroxyderivatives (HETEs) (586). This enzyme and its derivatives will not be the topic of our discussion.

The multi-enzyme complex prostaglandin synthetase is a heme protein (587). It is probably the iron in the heme that is the active site of the enzyme and is critically involved in fatty acid oxidation (588). Peterson et al. suggested that arachidonic acid interacts with Fe^{2+} at two sites, one site to bind the carboxyl group and a second site where oxygen bound to Fe^{2+} can interact within the fatty acid double bond.

The enzyme has a M_r of 124 K and is composed of two subunits. It has a high content of hydrophobic amino acids, proline and histidine (589). In 1971, it was discovered that aspirin probably exerts its anti-inflammatory properties by inhibition of PG synthetase (590). Aspirin acetylates the active site of the enzyme (591). Indomethacin, another PG synthetase inhibitor, however, does not covalently modify the enzyme (592).

d. Location and synthesis in the brain

In 1964, Samuelsson identified $\text{PGF}_{2\alpha}$ in the ox brain (593). Some later studies indicated that $\text{PGF}_{2\alpha}$ is the predominant PG in the brains of cat, dog, rabbit, mouse and chicken (594-597). PGE_2 and $\text{PGF}_{2\alpha}$ are usually present in the brain in concentrations of nanogram/gm weight, e.g. $\text{PGF}_{2\alpha}$ concentration ranges from 10-300 ngm/gm wet weight.

Cat brain cerebral cortex, cerebellum, caudate nucleus and hypothalamus were found capable of endogenous PG synthesis. In each case more $\text{PGF}_{2\alpha}$ compared to PGE_2 was synthesized (565).

Biosynthesis of PGs from arachidonic acid in brain homogenate and

in cerebral cortex slices was linear for 15 and 60 minutes, respectively (598, 599).

With regard to the subcellular distribution of PGEs and PGFs, it was found that homogenates of the rat cerebral cortex contain PGE₁, PGE₂, PGF_{1α} and PGF_{2α} which are concentrated mainly in the light microsomal and mitochondrial fractions (600). Only the microsomal fractions exhibited ability to synthesize PGE₁ and PGF_{1α}, from bis-homo-linolenic acid. After fractionation of the crude mitochondrial fraction, PGEs and PGFs were found mainly in the cholinergic and noncholinergic nerve endings. Kataoka et al. (600) therefore concluded that the nerve endings are storage sites of PGs, whereas the microsomes are sites of synthesis. They concluded that PGs released from cortex and spinal cord upon electrical stimulation were derived from synaptic vesicles in cholinergic and noncholinergic nerve endings.

There have been reports that TxA₂ is the major arachidonic acid derivative of the brain (601-602). Wolfe et al. (599) demonstrated that TxB₂ was formed from endogenous precursors during short incubations of guinea pig and rat cortex. The amount of TxB₂ was 5-6 times higher than the formation of PGE₂ or PGF_{2α}. Noradrenaline was found to stimulate TxB₂ formation.

The recent re-examination of the subcellular distribution of PG synthetase enzymes revealed that the highest activity was located in microsomes (603). In the adult brain PGE isomerase has low activity. The major enzymes were thromboxane synthetase and the endoperoxide reductase which forms PGF_{2α}. Because the activities of prostaglandin endoperoxide and isomerase differ considerably in different brain

regions, it was suggested that this may indicate different functional roles of PG types in relationship to specific neuronal pathways (565).

The enzyme PGE-9-keto-alpha reductase which converts PGE_2 to $\text{PGF}_{2\alpha}$ has been recently described in the brain of various animals and in the spinal cord of the frog (604). It has been suggested that the preferential if not exclusive formation of $\text{PGF}_{2\alpha}$ (565) upon CNS stimulation may reflect a change in the direction of PG biosynthesis at the endoperoxide step and/or the direct conversion of PGE_2 into $\text{PGF}_{2\alpha}$ and may be important in the termination of PG effects.

On the other hand, Bishai and Cocceani found that the enzyme is absent in the brain of sheep and cats (605). Feline brain lacks the capacity to produce $\text{PGF}_{2\alpha}$ or PGD_2 . The feline brain does however have PGE_2 isomerase, and PGI and TxA_2 synthetase.

Studies by Abdel-Halim et al. demonstrated that rodent brain in vitro synthesized more PGD_2 than any other PG (606). An endoperoxide D isomerase has high activity in the cytosol fraction of brain and spinal cord with the highest activity in the thalamus and hypothalamus (607). Because it was 40 times higher in neuroblastoma cells compared to glial cells and stimulated adenylate cyclase, a significant role for PGD_2 in neuronal function was postulated. This was buttressed by the findings that intravenous injections of PGD_2 induced a sedative effect (608). However, it has been criticized that in the cat and human brain there is very low synthesis of PGD_2 (609). The only cells where PGD_2 has been found in humans is platelets and mast cells (610).

Thus there is conflicting data as to whether $\text{PGF}_{2\alpha}$, TxA_2 , or PGD_2 is the predominant PG of the brain.

PGI₂ is the principal PG formed by cerebral blood vessels (611).

It relaxes artery strips and is a potent vasodilator of the cerebral vasular bed (611-614). Incidentally, all primary PGs and PGI₂ are formed in cerebral blood vessels. PGE and PGF are both constrictors of cerebral vessels, although PGE has variable effects (615,616). TxA₂ is not formed in cerebral vessels (609).

Because there is a higher concentration of PGs in the grey matter of brain compared to white matter, it is assumed that this reflects the possibility that PGs are localized more in neurons than in glia (617). Indirect immunofluorescent techniques used to determine the localization of prostaglandin forming cyclooxygenase in the cerebellar cortex of the pig, rat mouse, cow, rabbit and sheep revealed that cyclooxygenase antigenicity was detected in the cell bodies of glial cells in the Purkinje cell layer of cerebellar cortex, in the small arterioles throughout the cerebellar cortex, and the endothelial cells of large arteries (618). Cyclooxygenase activity was present in the neuronal cell bodies of granule, basket, stellate and Purkinje cells.

e. Metabolism

(1) General. The initial step in PG metabolism is the oxidation of the hydroxyl group at the C-15 position of PGs and TxB₂ by a specific NADH-dependent 15-hydroxyprostaglandin dehydrogenase present in the cytoplasm. The highest activities of this enzyme occur in the lung, spleen and kidney cortex. It is also present with much less activity in many other tissues. It has been suggested that PGA and PGB may be true circulating hormones because they can escape inactivation by prostaglandin dehydrogenase. But this is not conclusive. Dehydrogenation is followed by reduction of the double bond 13 by a specific reductase to form di-hidro compounds. The activity of this

reductase differs in different tissues and species.

β -oxidation occurs in mitochondria to form C-18 (dinor-) and C-16 (tetranor-) prostaglandins. The liver is the principal site of these transformations which may occur before or after dehydrogenation and reduction. Prostaglandin E_2 or prostaglandin $F_{1\alpha}$ forms the same metabolites as PGE_2 or $PGF_{1\alpha}$, respectively, after the second β -oxidation step because of isomerization of the double bond to the α , β -position before oxidative chain shortening.

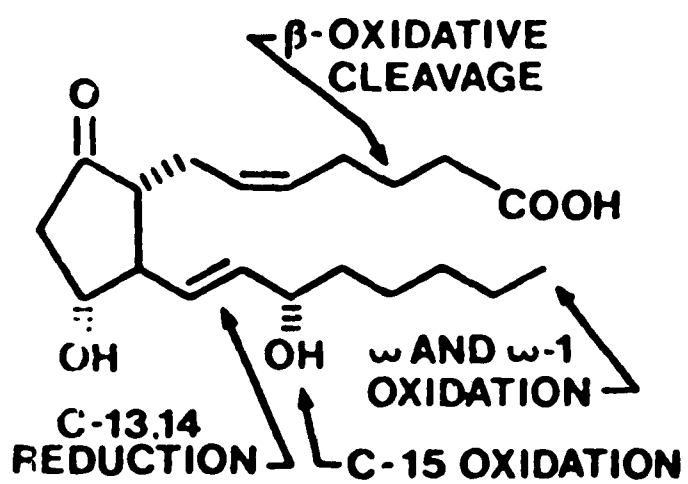
Microsomal ω -oxidation occurs principally in the liver and kidney and transforms PGE and PGF metabolites into dicarboxylic acids.

The sites of PGE that are acted upon by various metabolic enzymes are demonstrated in Figure 3. See Wolfe (565) for a review on PG metabolism.

(11) Brain. Conflicting reports exist as to the existence of PG-degrading enzymes in the brain. Pig brain has been reported to contain less 15 keto-dehydrogenase than other tissues but appears to contain Δ^1 -reductase (619). Siggins et al. (620) reported little or no degradation activity in all regions of the brain except cerebellar cortex. On the other hand, incubated homogenates of cerebral cortex and cerebellum are almost devoid of catabolic enzymes (621). The data indicate that in general there are very low levels of degradative enzymes in the brain and that PGs are probably released into the venous circulation by active transport across the blood-CSF and blood-brain barriers (622).

The basic vertebrate cell membrane, e.g. the RBC, is impermeable to $PGEs$, $PGFs$, TxB_2 and PGI_2 (623) and thus the passage of PGE and PGF through cells probably occurs through tight junctional epithelia or

Figure 3. Some important metabolic reactions of prostaglandins. Arrows indicate sites of metabolic action. The diagram is taken from L.S. Wolfe, 1975 (565).



active transport processes.

f. The association between nerve stimulation and PG release.

If PGs are present in neurons and somehow function in the transmission process, then it would be expected that nerve stimulation would be correlated with PG synthesis and release. This is indeed the case. The literature is presented in this section.

Stimulation of sympathetic nerves is associated with release of PGE_2 and $\text{PGF}_{1\alpha}$. The rate of PG release is related to stimulus frequency and decreases to spontaneous levels upon cessation of stimulation (624,625). The accelerated synthesis is thought to arise from stimulation of acylhydrolases that liberate free fatty acid from precursor membrane lipids.

In 1953, Hokin and Hokin reported a phospholipid effect following cholinergic stimulation of exocrine and endocrine glands and central synapses in which the turnover of phosphatidylinositol and phosphatidic acid was greatly increased (626). This effect has recently been shown to be coupled to arachidonic acid release and the synthesis of PGs (627).

Ramwell and Shaw demonstrated that afferent stimulation of peripheral nerves stimulated a marked increase in PG content of cerebral cortex superfusate (624). The electrical stimulation of somatosensory cortex or reticular formation also increased PG levels. Upon nerve stimulation in the peripheral nervous system, PGE seems to be the predominant PG released. In the central nervous system, it is $\text{PGF}_{1\alpha}$ which is the predominant PG released (628,629).

Many psychotropic drugs inhibit PG synthesis, including a variety of antidepressants (e.g., imipramine and Pargyline), antipsychotics

(e.g., chlorpromazine and haloperidol), antianxiety drugs (e.g. diazepam) and others, e.g. cannabinoids (630).

Upon nerve stimulation, are PGs released from the postsynaptic or the presynaptic cell?

The following evidence is summarized by Westfall (631) in favor of PGE being released post junctionally: 1) PG release can be evoked by many different forms of stimulation other than nerve stimulation, including neurohormones, mechanical stimulation and hypoxia; 2) The administration of alpha adrenergic antagonists prevents the release of PGs induced by norepinephrine, epinephrine, or nerve stimulation; 3) The administration of epinephrine can still stimulate the release of PGs in the denervated spleen. The following provides evidence that PGs are released presynaptically from adrenergic neurons themselves: 1) PGs have been found in pure nerve tissue such as the vagus and splenic nerve trunk; 2) blocking PG synthesis still results in a marked increase in norepinephrine secretion from the isolated field stimulated vas deferens in which muscular contraction was prevented by use of low calcium concentrations in the medium; 3) Propanolol, a norepinephrine antagonist, did not inhibit the norepinephrine induced release of PGs in the rabbit heart.

In addition, it was suggested that PGs can be released from the presynaptic neuron during vesicle fusion and rupturing of the presynaptic membrane (631). This is supported by the findings of Gilbert et al. (632) that synaptosomes spontaneously release PGs. It is therefore likely that PGs are produced in both pre- and post-synaptic sites.

g. Effects of PGE on neurotransmission

(1) Inhibition. Hedqvist hypothesized that endogenous PG formed

and released from post synaptic membranes during stimulation inhibits the release of norepinephrine from presynaptic sympathetic neuromuscular terminals in the cat spleen, guinea pig vas deferens and rabbit heart. This inhibition represents a short negative feedback loop (633-637). He speculated that the effect of PG constitutes a general principal functioning in most sympathetically innervated nerves.

There is a variety of evidence which seems to confirm this theory. It was found that eicosatetraynoic acid, a PG synthetase inhibitor, led to augmented release of noradrenaline from rabbit heart preparations. Inhibition of local PG formation in cat mesenteric artery and kidney and guinea pig vas deferens was also associated with increased neurotransmitter release upon nerve excitation (638). Johnson et al. demonstrated that in the innervated guinea pig vas deferens, PGE₂ reduces stimulated release of norepinephrine and dopamine from presynaptic terminals (639). Bergstrom et al. (640) showed that PGE₂ reduces stimulated release of norepinephrine and dopamine from rat cerebral cortex and neostriatum in vitro.

The effect of PGE on cholinergic neurotransmission is not very clear. Cerebral ventricular perfusion with PGE₁ reduced the release of ACh into the ventriculo-cisternal perfusate of the cat brain (641).

The Hedqvist hypothesis however has been criticized. The major difficulties of the hypothesis stated by Wolfe (642) are: 1) the species dependent nature of the effect; 2) the sensitivity to the frequency of stimulation; 3) the lack of generality to all adrenergically innervated tissues. Whereas PGE₂ inhibited noradrenaline overflow in rat cortex slices, it did not in the rabbit cortex or caudate nucleus, nor did it release dopamine from the rat striatum. In addi-

tion, it was found that PGE₂ enhanced noradrenaline release from central synapses, and although it has been reported that PGEs are released from neuromuscular junctions, they do not appear to have any neuromodulatory role there; 4) the inconsistency of synthesis inhibitor effects; and 5) the paucity of evidence that PGE neuromodulation occurs in vivo.

Despite these criticisms it is clear, however, that PGE in general leads to inhibiting CNS effects. Horton in 1964 demonstrated that PGE₂ caused sedation and a stuporous state in cats (643). Although the mechanism of this effect is unknown, it is possible that the site of action is on the brain stem column pathways passing through that area.

PGE also potentiates barbiturate sleeping time and prevents convulsions induced by electrical stimulation (572). In addition, penicillin induced generalized epilepsy in the cat was inhibited by PGE₂ (644).

(11) Theories on the mechanism of PGE presynaptic inhibition.

There are indications that PGE does not interfere with norepinephrine synthesis, or facilitate its metabolism or uptake, since PGE₂ did not alter the removal of norepinephrine infused into the cat spleen (645).

Hedqvist hypothesized in 1970 (646) that PGE₂ may inhibit sympathetic neurotransmission in the cat spleen by preventing calcium from gaining entry into sympathetic nerve terminals where it promotes secretion of noradrenaline. He based this on his findings that increasing the concentration of calcium antagonized the effects of PGE₂ on the system.

Stjarne found that the inhibitory effect of PGE₂ on sympathetic neurotransmitter release depended on the presence of calcium. He suggested that PGE₂ mediates its effect by promoting efflux of

intraaxonal calcium as opposed to antagonizing calcium influx (647).

Other speculations on the sedative and inhibitory action induced by PGE have been attributed to an increased turnover of 5-HT (648), stimulation of adenylate cyclase (649), or an alteration in the cerebral circulation (650).

Because PGE₂ is more active than PGI₂ and 6-keto-PGF_{1α} in inhibiting sympathetic neurotransmission in the field stimulated guinea pig vas deferens, it was concluded that the inhibitory capacity of PGE resides in the C-9 carbonyl group of the PGE₂ molecule (651).

(iii) Direct effect of PGE on neurons. When PGs are applied microiontophoretically, they have varying effects. In brain stem neurons it was reported that PGE₂ and PGF_{2α} have predominantly excitatory effects. Neurons in the cerebral cortex and cuneate nucleus were unresponsive (652-654). Bloom's group found excitation by PGE₂ primarily in the neurons of cerebellar cortex hippocampus and brain stem (655).

Using microelectrophoretic experiments, Hoffer et al. demonstrated that PGE₁ and PGE₂ antagonized the reduction in the discharge rate of cerebellar Purkinje cells produced by norepinephrine (656).

Kaplan et al. demonstrated that PGE₁ and PGF_{2α} stimulate brain stem cardio regulatory centers (657, 658). The major effect of PGs on the spinal cord reflexes is also primarily on the excitation of alpha motor neurons through a transmitter modification role (659).

(iv) PGE and fever, pain and schizophrenia. The following observations are in favor of PGE involvement in fever generation as discussed by Wolfe (642): 1) endogenous pyrogen increases cerebral PG synthesis, which results in increased PG levels in CSF; 2)

intraventricular injection of PGE₂ into the preoptic anterior hypothalamus elicits febrile response; 3) Nonsteroidal anti-inflammatory antipyretic drugs decrease the CSF elevated PGE₂ levels, accompanied by a fall in body temperature.

Recently, there has been some controversy over this matter. Microiontophoresis of PGE₂ into hypothalamic neurons showed little correlation between neuronal stimulation and inhibition, and thermogenesis (660). This issue is yet to be resolved.

PGE can sensitize the afferent nerve endings and increase the pain producing activity of bradykinin (572).

Consistent with the hypothesis that PGE exerts a CNS effect, in recent years it has been suggested that schizophrenia may be related to a prostaglandin deficiency (661). In studying platelets in patients with depression, mania and schizophrenia, it was found that schizophrenics had a complete inhibition of PGE₂ synthesis (662). Alternatively, it has been postulated that catalepsy develops in cats upon injection of PGE₂ into their ventricles (663). When fever and PGs are brought down catalepsy disappears. They also speculated that febrile episodes are genuine symptoms of schizophrenia.

h. Effects of PGF_{2α} on neurotransmission

(1) Stimulation. There is growing evidence that PGF_{2α} in general has a facilitory action on neurotransmission. Brody and Kadowitz in 1974 demonstrated that PGF_{2α} increased responses of the perfused hind paw of the dog to norepinephrine (625). PGF_{2α} has also been reported to enhance peripheral adrenergic stimulation in the following end organs: dog muscle, spleen, tibial artery, uterine artery, pulmonary, lumbar artery and veins, saphenous veins, hind limb superficial vein, and the

rat mesenteric artery and kidney.

Brody and Kadowitz presented evidence that $\text{PGF}_{2\alpha}$ causes significant potentiation of response to nerve stimulation by facilitating transmitter release rather than having an effect on the adrenergic neuroeffector or on the muscle itself. Their data are consistent with the hypothesis that $\text{PGF}_{2\alpha}$ may act as a modulator of sympathetic neurotransmission.

Consistent with the hypothesis that $\text{PGF}_{2\alpha}$ potentiates neurotransmission, it was demonstrated that $\text{PGF}_{2\alpha}$ but not PGE_2 induced convulsions and the appearance of epileptiform spike activity in several patients receiving therapeutic doses of PGs (664). In addition, Rosenkranz found that PGEs inhibited, whereas PGFs stimulated, the incidence of electroshock induced tonic convulsions when injected into the ventricles of mice (665). It is interesting to note that PGE_2 but not $\text{PGF}_{2\alpha}$ can decrease the electrically induced release of dopamine from rat striatal slices (666).

Present evidence also suggests that $\text{PGF}_{2\alpha}$ can facilitate the release of catecholamines from the adrenal medulla (625).

(ii) Inhibition. $\text{PGF}_{2\alpha}$ has been demonstrated to have inhibiting effects on neurotransmissions. $\text{PGF}_{1\alpha}$ and $\text{PGF}_{2\alpha}$ and PGE_1 and PGE_2 given intravenously had a central depressant effect on the rat (667). Puddubio and Kleinrok speculated that this is somehow caused by the indirect action of PGs on the concentration and turnover of various neurotransmitters (667).

Furthermore, synaptic transmission throughout the cuneate nucleus was depressed by $\text{PGF}_{2\alpha}$ after it was applied to the surface of the medulla oblongata (668). Cocceani postulated that a post synaptic site

is the most likely target of this action. PGE₁, on the other hand, had no effect in this system. Microiontophoresis of PGF_{2α} or PGE₂ usually leads to neuronal excitation.

1. Prostaglandins and cyclic nucleotides

(1) PGE and cAMP. PGE has been shown to interact with adenylate cyclase in a variety of tissues (491). Samuelsson (506) speculated that the reason for PGE's wide spectrum of effects may be attributed to this function. The ability of TSH to stimulate the release of thyroid hormones depends on cAMP. Like TSH, PGE stimulates adenylate cyclase in the thyroid tissue. It has been suggested that PGs are messengers between hormones and cAMP. The evidence for this is that antagonists of PGs inhibit the stimulating effect of TSH and PGE₂ on adenylate cyclase. PGE₁ also increased cAMP in platelets and pituitary glands (506).

Partington et al. (669) demonstrated that PGE is necessary for the expression of the alpha adrenergic receptor mediated activation of cAMP in brain tissue (669).

Cyclic-AMP also induces neuronal differentiation and growth. PGE does this presumably by activating adenylate cyclase (669). This is supported by the fact that both PGs and cAMP are present in the immature brain (668).

It is hypothesized that PGs induce hyperanalgesia by lowering the threshold of C fiber polymodal nociceptors (670). Ferreira speculated that hyperanalgesia is a result of a cAMP/Ca²⁺ dependent process.

The idea that PGE activates adenylate cyclase is supported by the finding that specific binding of PGE to a variety of cells was correlated with an accumulation of cAMP (671) or with the activation of adenylate cyclase (672). PGE₁ and PGE₂ stimulate adenylate cyclase in

normal liver membranes (673) and normal kidney cells (674). It has been documented by several workers that $\text{PGF}_{2\alpha}$ is incapable of stimulating adenylate cyclase or increasing cAMP levels.

There are PGE receptors in adipocytes, stomach, thyroid gland, adrenal gland, corpus luteum, fibroblasts, liver, neuroblastoma cells, platelets, thymocytes and uterus (675-678). The above studies support the fact that receptors are involved in PG stimulation of adenylate cyclase.

There is also saturable, reversible and specific high-affinity binding of PGE_2 to rat brain membranes that are maximally distributed in the hypothalamus and posterior pituitary. The receptors are subcellularly in the synaptosomal fraction. In the peripheral nervous system, receptors for PGs have been described as being specific, high affinity and coupled to adenylate cyclase (679). Malet et al. (680) proposed that binding of PGE to brain membrane may be prerequisite for stimulation of adenylate cyclase.

There is also evidence that PGE acts by preventing norepinephrine activation of adenylate cyclase. Thus norepinephrine and PGE may influence Purkinje cells via reciprocal opposing interaction with adenylate cyclase (655).

(11) cAMP and PGE biosynthesis. The stimulation by cAMP of PG synthesis has been demonstrated in cultured mammalian cells, graffian follicles, thyroid cells, adrenal cortex and adipocytes (506,681,683,684,687). Cyclic AMP activated triglyceride lipase in thyroid which liberates arachidonic acid (680). In contrast cAMP inhibited PG formation in platelets (682). The ability of PGEs to stimulate adenylate cyclase, and that of cAMP in turn to stimulate PGE

synthesis has been postulated to represent a positive feedback system.

(iii) PGF_{1α} and cGMP. It has been demonstrated that PGF_{1α} raised the level of cGMP in the rat uterus (683). The same phenomenon has been demonstrated in liver and other cells (684,686). The Yin-Yang hypothesis (688) proposes that cAMP and cGMP are the opposing arms of a bidirectional intracellular control system. There is evidence for this hypothesis in the contraction of vascular muscle and myocardium, the release of lysosomal enzymes, leukocyte chemotaxis, and other functions. PGE/cAMP relax, whereas PGF_{1α}/cGMP enhance the above phenomena. An extension of this hypothesis holds that adrenergic ligands mediate effects via PGE stimulation of adenylate cyclase and hence increase cAMP levels, whereas cholinergic ligands activate guanylate cyclase through PGF_{1α} and hence increase cGMP levels.

This hypothesis to date is very speculative and much further work must be done before it can be completely accepted.

j. Function of arachidonic acid and PGs in membranal events

(i) Secretion. Recently a model of exocytosis has been developed for the chromaffin granule system. When activated by calcium, synexin, a protein with a M_r of 47 K, polymerizes to form small rods which self-associate and bind to chromaffin granules and plasma membrane. It is suggested that granules attached to the membrane are exposed to high extracellular chloride concentrations. This ion is then driven into granules as a counter ion to protons being pumped out by granule membrane Mg²⁺-ATPase. Consequently the osmotic strength inside the granule increases and eventually ruptures releasing its contents (689-692).

Creutz (693) presented evidence that in the presence of small

amounts of unsaturated fatty acids, the regions of contact between granules by synexin lead to breakage and fusion of the granules and the formation of larger vesicles. Arachidonic acid was demonstrably the most effective fatty acid in inducing fusion. PGE_2 did not lead to fusion. Thus they provided evidence that one of the products of PLA_2 hydrolysis -- arachidonic acid -- facilitates fusion directly without being converted to PGs.

Addition of arachidonic acid to pancreas stimulated enzyme secretion. Also stimulation of enzyme secretion in pancreas leads to the formation of PGE_2 . Marshall et al. (694) provided evidence that breakdown of PI in animal tissue which consists largely of 1-stearoyl-2-arachidonyl sn-glycero-3-phosphoinositol leads to the formation of PGE_2 which is directly involved in stimulus secretion coupling. Exogenous PGEs stimulated secretion. PGE_1 , PGE_2 , PGI_2 , and $\text{PGF}_{2\alpha}$ all stimulated amylase secretion.

(ii) Cell aggregation. PGE_1 and $\text{PGF}_{2\alpha}$ were shown to inhibit peptide induced aggregation of polymorphonucleocytes (695). TxA_2 caused release of 5-HT and irreversible platelet aggregation (696). There is evidence that Leukotriene B_4 , a lipoxygenase derivative of arachidonic acid, is a potent aggregator of polymorphonucleocytes (697).

(iii) Chemotaxis. Polymorphonuclear (PMN) cells in the process of phagocytosing produce PGs. PGE_1 and TxB_2 are chemotactic for other PMNs, whereas PGE_2 and $\text{PGF}_{2\alpha}$ have little or no chemotactic effects (698, 699).

Arachidonic acid is metabolized by lipoxygenase to produce mono, di and tri hydroxyeicosatetraenoic acids (HETEs). MonoHETEs are chemotactic (700). The mechanism of the induction of chemotaxis by

HETEs is unknown, however, it has been demonstrated that neutrophils incorporate exogenous 5-HETE and 12-HETE into phosphoglycerides and triglycerides. The alteration in the membranal phospholipid fatty acid composition may be responsible for the chemotactic effect (701). Ford-Hutchinson demonstrated that the chemotactic and aggregating activity released from polymorphonuclear cells are due primarily to 5, 12-diHETE (702).

k. Concluding statement

In conclusion, PGE_2 and $\text{PGF}_{2\alpha}$ have been reported to both stimulate and inhibit neurotransmission and secretion. At times, these PGs have been reported to have absolutely no effect on neurotransmission. Apparently the actions of PGs are complicated because they probably have effects on the pre and post synaptic cell and can either stimulate or inhibit adenylate or guanylate cyclases. In addition, PGs are not the only variable in the system and it is therefore difficult to define a precise role for them. In order to elucidate what exactly PGs do and how they regulate neurotransmission, it would probably be important to devise experimental conditions where there is a clear separation of pre and post synaptic effects and in which it is possible to isolate a specific enzymatic or nonenzymatic membranal effect of the PG under study.

I have therefore chosen to isolate the presynaptic organelles, synaptic and coated vesicles and to determine if they are capable of prostaglandin synthesis and to attempt identification of the prostaglandins produced. As I mentioned in the previous section, I studied the effects of PGE_2 and $\text{PGF}_{2\alpha}$ on presynaptic membranal PLA_2 to elucidate their possible functions in presynaptic neurotransmission.

12. Cyclic Nucleotide and Ca^{2+} /Calmodulin dependent protein kinases

"The cyclic phosphorylation and dephosphorylation of serine and threonine hydroxyl groups in cellular proteins is now recognized as a ubiquitous regulatory mechanism in living organisms, modulating a whole range of cellular activities."

M. Williams and R. Rodnight, 1977 (703)

The covalent modification of protein substrates in the brain via phosphorylation by cAMP and Ca^{2+} /Calmodulin dependent protein kinases has been implicated in playing a role in both pre and post synaptic events (704). The possible involvement of protein phosphorylation in neurotransmitter release and secretion are reviewed here. There is no mention in the literature of the existence or function of protein phosphorylation occurring in endocytosis or in coated pits/vesicles. This will be presented later in the results and discussion sections. Cyclic AMP and the enzymes adenylate cyclase and phosphodiesterase which generate and catabolize cAMP, respectively, will be mentioned with respect to their location and function in brain. Although the history, chemistry and function of calmodulin has been discussed in a previous section, it is important at this point to discuss the unique interrelationships between Ca^{2+} , calmodulin and cAMP since they are present in brain and may all function synergistically in releasing neurotransmitter.

a. History of cAMP and protein kinases in the brain.

The first mention of cAMP with respect to brain function was made

in 1957 when Heald speculated that the increased phosphorylation of endogenous membrane proteins induced by a brief electrical depolarization of respiring guinea pig cortex sections might be attributed to cAMP (705).

In 1963, Sutherland and his group demonstrated that norepinephrine stimulated cAMP formation in nervous tissue homogenates (706). From 1967-1969, it was found that when intact cell preparations of brain slices were exposed to depolarizing stimuli and certain biogenic amines, these preparations manifested a large increase in cAMP (707-709).

The first definitive evidence for the role of cAMP in brain came in 1969 when Miyamoto, Kuo and Greengard described the properties of a soluble cAMP dependent protein kinase from rabbit brain which catalyzed the phosphorylation of exogenous histones (710). Kuo and Greengard in 1970 (711) subsequently formulated the concept that the intracellular actions of cAMP are mediated through enzymatic phosphorylation or dephosphorylation of enzyme substrates.

It was demonstrated in the early 1970's that cAMP was involved in synaptic processes. This was evident by the finding that synaptic membrane fractions were good substrates for cAMP dependent phosphorylation (712,713).

b. Concentration and location of cAMP in brain.

The concentration of cAMP in vertebrate brain is approximately ten times greater than that found in nonneuronal tissues (714). In the brain, the concentration ranges from 1-2 pmol/mg wet weight. It would therefore be logical to assume that cAMP plays an important role in the brain. Using histochemical techniques to localize cAMP in the brain it was revealed that most bound cAMP in the cerebellum and brainstem occurs

in neuronal cell bodies and dendrites rather than in glia and vascular elements. Virtually no immunofluorescence was seen in the white matter. This method, it was emphasized, cannot detect soluble cAMP (715).

c. Generation and degradation of cAMP.

(1) Adenylate cyclase. Cyclic AMP is formed by the activation of membrane bound adenylate cyclase from adenosine 5' triphosphate (ATP). In addition to ATP, adenylate cyclase requires the presence of millimolar concentrations of Mg^{2+} or Mn^{2+} (716). Lead, mercury and copper inhibit this enzyme (717, 718), whereas millimolar concentrations of fluoride, micromolar concentrations of calcium, and calmodulin stimulate it (704). It was found that brain contains a calmodulin and noncalmodulin sensitive adenylate cyclase that could be separated from each other by calmodulin affinity chromatography (719).

Adenylate cyclase was localized initially in post-synaptic membranes (720) and later in the presynaptic plasma membrane (721). Adenylate cyclases are coupled to a host of neurotransmitter receptors in a variety of membranes such that binding of the neurotransmitter to the receptor leads to stimulation of adenylate cyclase and subsequently the formation of cAMP. The neurotransmitter receptors that are associated with either central or peripheral neuronal adenylate cyclase include dopamine, norepinephrine, serotonin, histamine and Substance P receptors (704).

Greengard postulated that after neurotransmitters are released into the synaptic cleft, binding to their respective receptors, which are coupled to adenylate cyclase on the postsynaptic membrane, leads to stimulation and formation of cAMP. Cyclic AMP then stimulates a protein

kinase which subsequently phosphorylates membranal proteins. The latter phenomenon Greengard speculated is responsible for inducing permeability changes in the membrane leading to influx of ions. According to Greengard, this is the mechanism whereby neurotransmitters communicate with the postsynaptic cell (720). Because adenylate cyclase (721) as well as a variety of presynaptic receptors are present in the presynaptic plasma membrane, it is possible that a similar scheme may take place in the presynaptic membrane upon binding of neurotransmitter to presynaptic receptors.

As has been mentioned in the previous section, PGs interact with adenylate cyclases in the brain. The current concepts are that there is a specific PGE receptor linked to adenylate cyclase and that binding of PGE would stimulate it with subsequent increase of cAMP formation. There is also evidence that PGE can compete with norepinephrine and dopamine for binding to receptors linked to adenylate cyclase. The action of PGE therefore would be to prevent certain neurotransmitters from binding to their receptors leading to a resultant inhibition of neurotransmission. As has been mentioned, PGE blocks the slow inhibitory post synaptic potential induced by electrical stimulation.

(11) Phosphodiesterase. Cyclic nucleotide phosphodiesterase converts cAMP to adenosine 5' monophosphate (AMP) and thus serves to terminate reactions initiated by cyclic nucleotides (722). The enzyme is highly concentrated in the brain and is inhibited by methylxanthines, i.e., caffeine, theophylline and theobromine. Inhibition of phosphodiesterase (PDE) augments and prolongs cAMP dependent reactions. PDE was the first enzyme demonstrated to be activated by calmodulin.

Regional PDE distribution studies demonstrated that cerebral

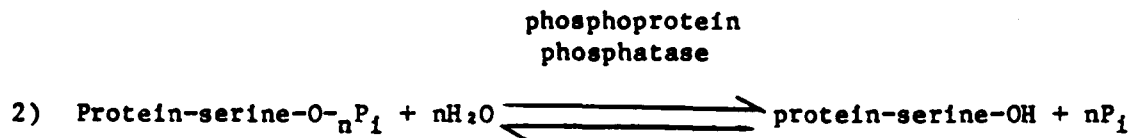
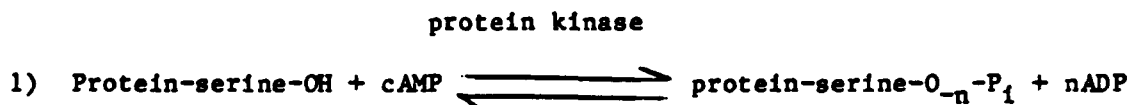
cortex, striatum and limbic forebrain have relatively high levels of PDE, whereas cerebellum and brainstem have lower activities (723, 724). With regard to its subcellular distribution it has been demonstrated that it is highly concentrated in synaptic membranes, synaptic vesicles and microsomes (725-727).

Because hydrolysis by PDE is the only enzymatic mechanism for inactivating cAMP, its regulation is complex. It is not a single enzyme but a class of enzymes with different properties that can be separated (728). It is also possible that cAMP can be actively or passively extruded through the plasma membrane into the extracellular medium (729).

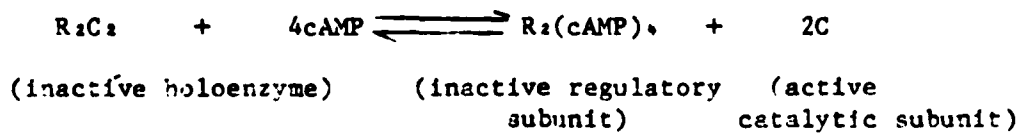
d. cAMP dependent protein kinase and substrates

It now appears that most of the physiological and biochemical actions of cAMP in various tissues are mediated by regulation of the levels of substrate protein phosphorylation through the action of cAMP dependent protein kinases and phosphatases (720). Cyclic AMP dependent protein kinase requires Mg^{2+} or Mn^{2+} and is inhibited by Ca^{2+} , ADP and adenosine.

The reactions involved in enzyme catalyzed phosphorylation and dephosphorylation of proteins are shown in equations 1 and 2 (730) below:



The amino acid to which the phosphoryl group is transferred is usually a serine or threonine. Transfer to histidine and lysine residues has also been documented (730). cAMP dependent protein kinases are composed of two dissimilar subunits termed regulatory and catalytic (731). The postulated mechanism of activation is shown in the equation below:



Binding of cAMP to the regulatory subunit of the inactive holoenzyme results in the dissociation of the tetramer into a regulatory subunit dimer containing 4 mol of bound cAMP. The free catalytic subunits are now capable of expressing its phosphotransferase activity transferring the gamma phosphate of ATP to protein residues. Phosphoprotein phosphatase hydrolyzes the phosphate from the phosphoresidues.

cAMP dependent protein kinases are highly concentrated in synaptic membrane, microsomal and synaptic vesicle containing fractions (704). There is also a limited amount of soluble cAMP dependent protein kinase. Phosphoprotein phosphatase is also present in synaptic membrane fractions, although a more significant amount is found in cytosol (704).

Synaptic membrane, synaptic vesicle and microsomal fractions all exhibit a high rate of incorporation of phosphate into phosphoprotein that is increased by cAMP (704). High resolution separation of synaptic membrane proteins phosphorylated by endogenous cAMP dependent protein kinases in brain has revealed that two proteins in particular undergo phosphorylation. One of these proteins is a doublet designated protein I, of approximate M_r 80K, which appears to be unique to the nervous

system. The other is protein II, approximate M_r 60K, and is present in the membrane and cytoplasm of a variety of tissues. The phosphorylation of protein I is extremely rapid, occurring in less than 5 seconds (704).

e. cAMP involvement in neurotransmitter release

In 1967, Breckenridge postulated that cAMP may be involved in neurotransmitter release at the neuromuscular junction based on the observation that theophylline enhanced neuromuscular transmission (732). Cyclic AMP and PDE inhibitors have subsequently been shown to increase the spontaneous release of catecholamines from the adrenal medulla and from central and peripheral sympathetic terminals (733-736). In the rat phrenic nerve, cAMP increased both the spontaneous and stimulated release of ACh (737). Cyclic AMP can also initiate activity in unstimulated motor axons in the cat soleus nerve and induce stimulus bound repetitive activity in stimulated axons (738). In the cat spleen and in rat striated slices, cAMP stimulated the facilitated but not spontaneous release of neurotransmitter (739, 740).

Cyclic AMP additionally has been observed to increase insulin release from the pancreas (741) and polypeptide release from the pituitary (704).

There have been objections to the concept that cAMP is physiologically involved in transmitter release because of the temporal lag observed between increase in cAMP content and release of material. However, Williams and Rodnight (742) state that while there may be temporal objections to the relationship between increase in cAMP and increased transmitter release, it is possible that sufficient cAMP is produced to have a physiological effect although it escapes detection in whole assays. Nathanson feels that conceptually the involvement of

adenylate cyclase and protein kinases in initiation of transmitter release is unlikely in view of the speed of events involved (704). He does think, however, that cAMP may modulate neurotransmission as either part of a feedback mechanism or in relation to long-term modulation of transmitter release.

f. cAMP and calcium mobilization

Although most of the effects of cAMP are mediated by protein phosphorylation, there is the possible exception of cAMP involvement in calcium mobilization.

In 1974, Borle (743) demonstrated that cAMP could evoke an instantaneous release of calcium from liver, kidney and heart mitochondria. Cyclic AMP could also mobilize intracellular calcium in the fetal mouse brain (703). Purpura and Shofer suggested that this particular function of cAMP may explain the facilitory effects of cyclic nucleotides in neocortex transmission (744). Kandel et al. also invoked calcium release as a mechanism for cAMP facilitation of serotonergic neurotransmission in the *Aplysia Californica* (745).

Apparently cAMP dependent protein phosphorylation is not involved in cAMP induced calcium release from mitochondria. The mechanism whereby cAMP mobilizes calcium without affecting protein phosphorylation remains elusive.

g. cAMP involvement in general brain functions

According to Williams and Rodnight (742), "associative processes occurring in the brain during learning result in the repetitive activation of new neuronal pathways with the progressive development of persistent changes in the properties of their component synapses." Greengard and Kuo in 1970 suggested that the phosphorylation state of

proteins may be related to short term memory (746). He proposed that alterations in the phosphorylated state of proteins in the postsynaptic cell could lead to changes in its properties and interneuronal connectivity. Supporting this hypothesis it was found that short term behavioral experiences in mice and rats resulted in changes in synaptic protein phosphorylation. Additionally, intracerebral or intraventricular injection of cAMP resulted in the general arousal of behavior and an increase in motor activity and convulsions (747).

Other possible roles of cAMP in brain function include the regulation of neuronal growth and movement, and in the trophic effects of nerve and muscle, the induction of neuronal differentiation in embryonic cells and the stimulation of nerve regeneration (704). All these effects are presumed to be the result of protein phosphorylation.

h. Calcium/Calmodulin dependent protein kinases

It has been demonstrated that calcium ions regulate the endogenous phosphorylation of specific proteins found in synaptic plasma membrane, synaptic vesicles, cortical brain slices, synaptosomes and synaptosomal lysates (748-754), and in neuronal cytosol (755).

Krueger et al. demonstrated that agents which increased calcium transport into synaptosomes stimulated ^{32}P incorporation into synaptosomal proteins (752). DeLorenzo demonstrated that calcium ions caused a marked increase in the level of endogenous phosphorylation of proteins from synaptosomal fractions and speculated that the effects of calcium on neurotransmitter release may be modulated by the phosphorylation of specific synaptosomal proteins (750). It was also demonstrated that calcium dependent phosphorylation of brain synaptic vesicles and synaptosomal soluble proteins is in fact Calcium/Calmodulin dependent (750, 755).

There is some evidence that Ca^{2+} /CaM dependent protein kinase activity is involved in the release of neurotransmitter and in secretion in general. DeLorenzo demonstrated that neurotransmitter release from synaptic vesicles was correlated with endogenous Ca^{2+} /Calmodulin dependent protein phosphorylation. Cyclic AMP and calcium, independently triggered secretion of insulin from the β -pancreatic cell (741). Concomitant with stimulation there was phosphorylation of certain proteins. Calmodulin was shown to be involved in calcium dependent phosphorylation in that trifluoperazine inhibited insulin release as well as protein phosphorylation. Schubert et al. (741) postulated that Calcium/Calmodulin and cAMP dependent protein kinases accelerate stimulus secretion coupling in β -pancreatic cells. Similarly, both Ca^{2+} /Calmodulin and cAMP dependent phosphorylation were found in the adrenal granule membrane (756). Calcium/Calmodulin dependent phosphorylation has also been invoked in stimulating secretion from mast cells and platelets (757,758).

1. Calcium/calmodulin and cAMP interrelationship

It has been noted that because calmodulin serves as an intracellular calcium receptor, the hypothesis that calcium has a second messenger role analogous to cAMP may be supported (335). The mechanism of action of Ca^{2+} and cAMP are similar in that each binds to a specific protein receptor. Calcium binds to calmodulin whereas cAMP binds to cAMP dependent protein kinases. Binding of calcium to calmodulin induces a conformational change in the protein so that it can interact with a variety of enzymes and stimulate their activities. In a similar sense cAMP binding to the regulatory components of protein kinase produces a conformational change resulting in the release of

catalytic activity (731).

Both Ca^{2+} /Calmodulin and cAMP stimulate protein phosphorylation. A major difference between these two systems with regard to phosphorylation, however, is that mammalian cells contain only one catalytic subunit of cAMP dependent protein kinase that phosphorylates a variety of proteins, whereas many different protein kinases with restricted substrate specificities are activated by calmodulin (759).

Cyclic AMP and calcium/calmodulin protein kinase activities are closely linked and may often be interchangeable in different tissues. Cyclic AMP protein kinases may often phosphorylate calmodulin dependent enzymes, e.g. phosphorylase kinase and myosin light chain kinase. In addition, calcium and cAMP often phosphorylate the same protein substrates, although at distinct sites due to the high specificity of the kinase, e.g. protein I and tyrosine hydroxylase in the brain (759).

As has just been noted, both calcium and cAMP have been implicated in secretion. Rasmussen (835) speculated that because calcium alone cannot induce neurotransmitter release, cAMP must participate in the process.

The cellular levels of calcium and cAMP are reciprocally regulated. As has been noted, cAMP releases calcium from intracellular storage sites (743), thus increasing intracellular calcium. Calcium can directly counteract this cAMP effect by stimulating $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase activity, leading to calcium reuptake into intracellular storage areas or extrusion out of the cell through the plasma membrane. Ca^{2+} /Calmodulin regulates the cellular level of cAMP by activating both adenylate cyclase and phosphodiesterase (301). The precise coordination of calmodulin activation of these two opposing enzymes may be mediated

by the number of calcium ions bound to calmodulin (341).

A major difference between cAMP and Ca^{2+} /Calmodulin mediated regulation is that protein phosphorylation by cAMP dependent protein kinase is the sole mediator of cAMP. Calcium/Calmodulin, however, regulates many enzymes other than protein kinases (731).

In this thesis, an attempt was made to characterize and compare Calcium/Calmodulin and cyclic AMP dependent protein kinase activities in synaptic and coated vesicles by phosphorylation experiments and immunological techniques.

13. Other proteins, enzymes and nucleotides possibly involved in mediating exocytosis and endocytosis.

a. Actomyosin.

Because calcium ions initiate contraction in skeletal muscle, it has been proposed that similar contractile filaments regulate the intracellular movement of secretory granules. Pumplin and McClure in 1974 (760) showed that cytochalasin B, an actin disrupter, inhibited the release of ACh from the sympathetic ganglion. This compound also inhibited secretion from mast cells, lymphocytes and the adrenal gland (761). The role of actin in neurotransmitter exocytosis is buttressed by the finding that there is actin in torpedo synaptic vesicles (762) and actin and myosin in nerve endings (763).

Puszkín and Berl in 1968 postulated that calcium stimulated actomyosin was responsible for calcium stimulated release of neurotransmitter by moving the synaptic vesicle to the presynaptic plasma membrane leading to neurotransmitter exocytosis (764). This hypothesis has been criticized, however, on the grounds that the fastest

actomyosin system could translocate synaptic vesicles only 0.7nm within 100 seconds (182).

There is evidence that supports the idea that actomyosin ATPase and therefore the contractile apparatus in many smooth muscle and nonmuscle cells is controlled by phosphorylation and dephosphorylation of the regulatory light chains of myosin by myosin light chain kinase. Phosphorylation of myosin results in the activation of actomyosin ATPase and dephosphorylation is accompanied by enzymatic inactivation. Thus in various smooth muscle cells phosphorylation of myosin may be the main mechanism by which the effect of calcium is mediated. Both myosin light chain kinase activity and actomyosin ATPase activities have been shown to be stimulated by calmodulin in the chicken gizzard (301).

Recently calmodulin stimulated myosin light chain kinase has been implicated in platelet secretion (765). The fact that calmodulin and actomyosin are present in axon terminals leaves open the possible involvement of actomyosin in neurosecretion.

Schook et al. demonstrated that Clathrin of coated vesicles has affinity for actin (261). It is therefore possible that actomyosin may be involved in some fundamental way in coated pit/vesicle mediated endocytosis.

b. Microtubules

Evidence for the involvement of microtubules in exocytosis is based on the effects of colchicine, an antimitotic that disrupts the mitotic spindle and other microtubular systems. This compound was shown to inhibit secretion of a variety of granules. The use of colchicine, however, as a marker for microtubular involvement should be made with caution since it binds to membranes as well and inhibits a number of

transport mechanisms (766).

Zisapel et al. have shown that tubulin is an integral protein of synaptic vesicle membranes, and Babitch has shown that it is an integral component of synaptic plasma membrane (767,768). Both groups have suggested that tubulin in these membranes may be involved in the secretory process. Burke and DeLorenzo demonstrated that Ca^{2+} /Calmodulin stimulated a synaptic terminal tubulin kinase system leading to phosphorylation of synaptic membrane tubulin. They suggested that this process might in some way mediate exocytosis of neurotransmitter (769). Microtubules are also associated with coated pits (770) and therefore may be involved in endocytosis.

Wang et al. demonstrated that μM concentrations of calcium lead to slight inhibition of microtubule assembly, whereas in the presence of calmodulin, polymerization of microtubules is completely inhibited (771). Tubulin has been demonstrated to bind to calmodulin with low affinity (772).

c. Glycoproteins

Transmitter release has been demonstrated to be inhibited after presynaptic injection of neuraminidase into the giant synapse (773), which suggests that specific glycoproteins are of major importance in the fusion process. In addition, ganglioside GM3 has been detected in the secretory vesicle of the adrenal medulla (774) and implicated in fusion. It has been speculated that glycoproteins might create a certain microenvironment conducive for fusion to occur.

d. Adenine nucleotides

Adenine nucleotides have been shown to reduce the release of ACh and noradrenaline from different nerve terminals (775). Adenosine

completely blocks the release of ACh induced by postganglionic stimulation. It is postulated that adenosine may prevent the spread of impulse at the nerve terminal varicosities. This, however, is unlikely since ACh release induced by high frequency stimulation is not affected by adenine nucleotides. Alternatively it may prevent the entry of calcium into the nerve terminals. This too is unlikely because its inhibitory actions were not prevented by calcium excess (775). It has been postulated that ATP may be involved in a negative feedback mechanism whereby under conditions of repetitive stimulation sufficient ATP is released from the nerve terminal to depress the release of transmitter by subsequent stimuli (775). I determined that the effect of ATP on synaptic membranal PLA₂ activity to see whether ATP affects presynaptic neurotransmission via interaction with membranal PLA₂.

e. Transglutaminase.

Transglutaminase catalyzes the calcium dependent formation of ϵ (γ -glutamyl)-lysine bonds between protein molecules and also catalyzes the coupling of amines and diamines to the gamma carboxyl of glutamine. All transglutaminases are calcium dependent and inhibited by aliphatic amines (776). Davies et al. (777) found a correlation between the ability of compounds to inhibit fibroblast transglutaminase and their ability to inhibit internalization of α_2 macroglobulin. Therefore, they concluded that protein cross-linking is essential for coated pit endocytosis. They proposed a model for the mechanism of coated pit to vesicle transformation in which the formation of small reversible aggregates in coated pits may promote local calcium influx and local activation of transglutaminase. This would subsequently lead to cross-linking proteins in the complex. The cross-linked proteins might be

receptors coupled to each other, to intrinsic membrane proteins, or to cytoskeletal proteins. Supporting this hypothesis is the finding that insulin and thrombin are cross-linked to their receptors during receptor mediated endocytosis. The net effect of covalent crosslinking receptor proteins, Davies et al. maintain, would make the aggregation process irreversible and would result in the progressive accumulation of ligand receptors in the coated pit. Ultimately, a coated pit would pinch off and become internalized.

It has also been demonstrated that transglutaminase inhibitors inhibit EGF and β adrenergic receptor internalization (778).

14. Control of axon terminal calcium concentration

"During the last decade, the study of calcium transport and calcium regulation of cellular functions has expanded explosively and calcium is now regarded as one of the key factors in the regulation of a variety of cellular events in a variety of cells."

A. Scarpa, 1978 (779)

I have presented the literature discussing how calcium either by direct non-enzymatic mechanisms or by indirect enzymatically controlled mechanisms may control exocytosis and endocytosis. By controlling the level of calcium, these two processes may be regulated. A brain membranous enzyme that is capable of such control is $[Ca^{2+}-Mg^{2+}]-ATPase$. Its function in secretion, its mechanism of action and its location in the brain will be presented. Other enzymes possibly involved in controlling calcium axonal concentration will also be mentioned.

Calcium may be buffered in the brain by a variety of ways. Mitochondria is a strong neuronal calcium buffer. It has been observed that the number of brain mitochondria calcium carrier sites are higher than that in liver, heart or kidney mitochondria (780).

Mitochondrial inhibitors augment the release of transmitter from nerve. The mitochondrial content of the motor nerve terminals is approximately 6.59% (781). Mitochondria play a role in transmitter release by participating in the regulation of the intracellular Ca^{2+} . The observation that calcium uptake by mitochondria is a fast process starting to operate in less than a msec after exposure substantiates this hypothesis (782).

Calcium ions may be extruded against the existing concentration gradient either by counter transport with sodium ions or driven out by the $[\text{Na}^+-\text{K}^+]\text{-ATPase}$ or by a direct ATP dependent transport system (783).

a. Brain $[\text{Ca}^{2+}\text{-Mg}^{2+}]\text{-ATPase}$.

$[\text{Ca}^{2+}\text{-Mg}^{2+}]\text{-ATPase}$ is present in axon terminal smooth endoplasmic reticulum (783) and synaptic plasma membrane (784,785) and is capable of controlling cytoplasmic calcium concentration.

Michaelson et al. (786) and Breer et al. (787) demonstrated that synaptic vesicles isolated from the Torpedo electric organ possess $[\text{Ca}^{2+}\text{-Mg}^{2+}]\text{-ATPase}$ activity and postulated that the vesicles were involved in calcium ion sequestration.

Blitz et al. demonstrated that brain coated vesicles manifested $[\text{Ca}^{2+}\text{-Mg}^{2+}]\text{-ATPase}$ activity and that the vesicle may be involved in regulating axon terminal calcium concentration (788). It was also shown that both synaptic and coated vesicles isolated from bovine neurohypophyses contain $[\text{Ca}^{2+}\text{-Mg}^{2+}]\text{-ATPase}$ activity (789).

Adrenal chromaffin granules are also capable of sequestering calcium. The content of calcium in these granules is higher than that found in mitochondria (790).

b. Mechanism of $[Ca^{2+}-Mg^{2+}]$ -ATPase action.

A model for the mechanism of action of sarcoplasmic reticulum $[Ca^{2+}-Mg^{2+}]$ -ATPase has been proposed (791). Refer to Figure 4.

The substrate of $[Ca^{2+}-Mg^{2+}]$ -ATPase is the $Mg\cdot ATP$ complex. The binding of calcium to a high affinity site of the enzyme located on the outer surface of the membrane leads to the initiation of ATP hydrolysis by the transfer of the terminal phosphate of ATP to an aspartyl residue of the enzyme forming an acyl phosphate (Fig. 4, 2-3). Calcium is then translocated through the membrane and released in the vesicular lumen prior to hydrolysis of the phosphoenzyme (Fig. 4,5). Mg^{2+} accelerates the phosphoenzyme hydrolysis (Fig. 4, 6). Two calcium ions are translocated for each molecule of ATP consumed. The release of P_i from the enzyme (Fig. 4, 7) leads to conversion of the activated enzyme back to its native conformation in the presence of ATP (Fig. 4, 8).

This sequence includes two distinct functional states of the enzyme, E and *E. The E form Ca^{2+} binding site faces the outer surface of the vesicle and binds Ca^{2+} with high affinity. The *E form Ca^{2+} binding site faces the inner surface of the vesicle and binds Ca^{2+} with low affinity.

The proteins of the sarcoplasmic reticulum with M_r s 100K, 60K and 80K constitute 50% of the total protein. The 100K protein was deduced to be the ATPase since ^{32}P from γ - ^{32}P ATP was incorporated exclusively into this component. The 56K protein is a high affinity calcium binding protein (792).

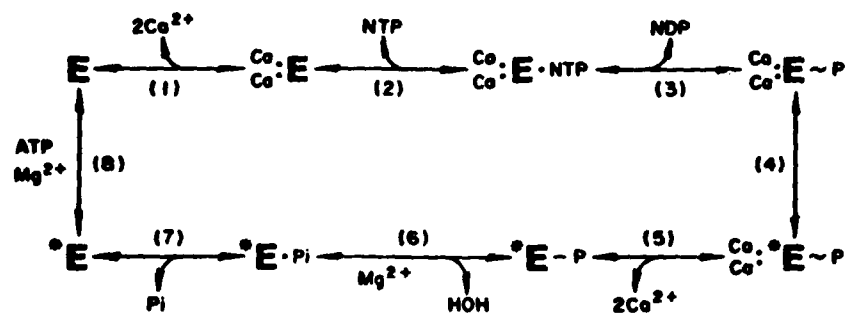
c. PLA₂ stimulation of [Ca²⁺-Mg²⁺]-ATPase.

It was demonstrated that PLA₂ can stimulate [Ca²⁺-Mg²⁺]-ATPase in a manner similar to calmodulin and that the stimulation may be the result of PLA₂ perturbation of membrane organization or by PLA₂'s direct hydrolytic effect on the ATPase (793).

d. Calmodulin stimulation of [Ca²⁺-Mg²⁺]-ATPase.

Calmodulin has been reported to activate [Ca²⁺-Mg²⁺]-ATPase in the red blood cell, renal plasma membrane, pancreatic islet cell membrane and synaptic plasma cell membrane (794-798). Because both synaptic and coated vesicles have been reported to contain [Ca²⁺-Mg²⁺]-ATPase activity, it was of interest to find out for this work if calmodulin can stimulate [Ca²⁺-Mg²⁺]-ATPase and calcium uptake activities in synaptic and coated vesicles.

Figure 4. Proposed mechanism of sarcoplasmic-reticulum $[Ca^{2+}-Mg^{2+}]$ -ATPase action. Numbers are referred to in the text. The diagram is from de Meis and Vianna, 1979 (791).



B. MATERIALS AND METHODS

1. Materials.

[γ - ^{32} P]-ATP (specific activity, 8-10mCi/ μ mol), [Ca^{2+}] (specific activity 91.2 μ Ci/mg), and arachidonic acid (specific activity 55.8 mCi/mmol) were purchased from New England Nuclear, Massachusetts.

[^{125} I]-calmodulin was a generous gift from D.M. Watterson of The Rockefeller University, New York. Sodium-dodecylsulfate and polyacrylamide were from Bio-Rad Lab., Richmond, California.

α -stearol-8-[^{14} C]-arachidonyl-L-phosphatidylcholine (specific activity 54 mCi/mmol) was purchased from Amersham, Arlington Heights, Illinois. Parabromophenacylbromide was from Aldrich, Milwaukee, Wisc. Phospholipase A₂ obtained from porcine pancreas, bee, *Naja naja*, *Crotalus adamanteus* and *Crotalus durissus terrificus* venoms, lysolecithin (egg yolk), arachidonic acid, PGA₂, PGB₂, PGE₂, PGF_{2 α} , cAMP, ATP, indomethacin, and quinacrine dihydrochloride were from Sigma, St. Louis Mo. Thromboxane A₂ was a generous gift supplied by B. Wexler, Cornell Medical Center, N.Y. Silica-G chromatogram sheets (100 μ m thick) were from Fisher Scientific Co., Pittsburgh, Penn.

All other reagents were of analytical Grade I. Fresh chicken gizzards and bovine brains were delivered on ice from a local slaughterhouse.

2. Methods.

a. Preparation of synaptic, coated, and decoated vesicles.

All vesicle types were prepared from the grey matter of bovine brain which was removed by means of suction. All isolation steps took place at 4°C.

Synaptic vesicles were prepared by adapting the technique described by DeLorenzo and Freedman (799). Approximately 150 gms of grey matter was homogenized in 600 ml of 10% sucrose (w/v) in a Waring blender with 2 x 5 second bursts. The homogenate was centrifuged at 2,000 x g for 10 minutes to pellet debris and nuclear waste material. The supernatant was centrifuged at 10,000 x g for 20 minutes; the resultant pellet containing synaptosomes was resuspended and washed in an equal volume of 10% sucrose, and centrifuged at 10,000 x g for 20 minutes. This washed pellet was subjected to osmotic shock by resuspending it in 5.5 times its volume in distilled water, using 10 strokes with a Teflon glass homogenizer. Reagents were then immediately added in one tenth the volume giving a solution containing 160 mM KCl, 5 mM NaCl, and 0.1 M MES buffer to prevent osmotic shock of the synaptic vesicles liberated from the synaptosomes. The preparation was centrifuged at 20,000 x g for 25 minutes resulting in pelleting of unshocked synaptosomes and cellular debris. The supernatant containing synaptic and coated vesicles and soluble proteins was centrifuged at 55,000 x g for 60 minutes, leading to sedimentation of coated vesicles. The resulting soluble proteins and synaptic vesicles were treated with 1 mM MgCl₂ and centrifuged at 135,000 x g for 45 minutes. The final synaptic vesicle pellet was resuspended in 0.1 M MES, pH 6.5 (Buffer A).

To further assess the purity of the synaptic vesicles, approximately 2-3 mg of synaptic vesicles (800) was resuspended in 2 ml of 10 mM MES, pH 6.5, containing 0.2 M sucrose and 0.19 M NaCl (Buffer G.P.B.) (800). This was loaded onto a controlled pore glass column (1.5 x 90cm) and chromatographed at a rate of 14 ml/hr with Buffer G.P.B. Optical density at 280 nm was determined for every 2 ml fraction. The

polypeptide composition of the vesicles obtained was analyzed by SDS gel electrophoresis using a 5-15% gradient for the separating gel and was run at 6.5 mAmps.

Coated vesicles were prepared by adapting the method of Keen et al. (801). Approximately 300 gm of grey matter was homogenized in 600 ml of Buffer A in a Waring blender with 3 x 10 seconds bursts. The homogenate was centrifuged at 34,000 x g for 15 minutes, leading to pelleting of debris and nuclear material. The supernatant was then centrifuged for 60 minutes at 80,000 x g, resulting in crude coated vesicle pellets. Coated vesicles were purified by layering the resuspended crude coated vesicle fraction on discontinuous sucrose gradients containing the following volumes of sucrose concentrations (w/v): 4.5 ml 5%, 9 ml 10%, 9 ml 40%, 4.5 ml 50% and 4.5 ml 60% sucrose. The gradients were centrifuged at 65,000 x g in an SW-27 rotor for one hour. The material at the interface between the 10-40% sucrose layers was diluted to 3 times its volume with Buffer A and centrifuged at 80,000 x g for 60 minutes. The resulting pellet was a relatively pure coated vesicle preparation. To obtain highly purified coated vesicles, this pellet was resuspended in Buffer A, and the sucrose gradient procedure repeated. The resultant coated vesicle pellet was resuspended in Buffer A. These vesicles were used for all the studies with the exception of the coated vesicle phospholipase A₂ assays. For this assay coated vesicles were purified further as described previously (800) by resuspending them in 0.1 M MES Buffer, pH 6.5, containing 0.2 M sucrose and 0.15 M NaCl. A 2 ml aliquot of the vesicle suspension was loaded onto a controlled pore glass column 3000 (1.5 x 90 cm) and chromatographed at a rate of 14 ml/hr. Protein concentration was monitored by optical density

measurements at 280 nm for every 2 ml fraction. Three protein peaks were obtained. The first contained large contaminating membranes, the second peak contained a mixture of membranes and coated vesicles, and the third peak contained highly purified coated vesicles. The third peak was concentrated by centrifugation at 80,000 x g for 45 minutes. The pellet was resuspended in Buffer A and dialyzed against 2 liters Buffer A for 16 hrs.

b. Decoated Vesicle Preparation.

Decoated vesicles were prepared by denuding coated vesicles that were prepared as described above.

Coated vesicles (10-12 mg protein) were suspended in either (a) 0.5 M Tris-HCl Buffer, pH 7.0, or (b) 5 mM TES, pH 8.4, for 30 minutes and centrifuged at 80,000 x g for 30 minutes. The decoated vesicles prepared by procedure (a) were used for calmodulin radioimmunoassays. Vesicles prepared by procedure (b) were used for PLA₂ and protein kinase studies. Under either of these extraction procedures, coat proteins disassemble from the coat and remain in the supernatant (801). The resultant pellets in both cases were then resuspended in Buffer A.

c. Synaptic plasma membrane preparation.

These membranes were prepared by a variation of the method of Jones and Matus (802). Approximately 200 gms of bovine brain was homogenized in 600 ml of 10% sucrose (w/w). The homogenate was centrifuged at 800 x g for 20 minutes and the supernatant removed and centrifuged at 9,000 x g for another 20 minutes. This pellet was washed once with 10% sucrose to yield a crude mitochondrial fraction which was resuspended in a hypotonic buffer of 5 mM Tris-HCl, pH 8.1, and incubated at 0°C for 30 minutes followed by six strokes in a hand-operated glass Teflon

homogenizer. The lysate was made up to 34% sucrose (w/w) by the addition of the appropriate volume of 48% sucrose (w/w). The upper phase of 28.5% sucrose (w/w) was overlaid above the sample phase and a small volume of 10% sucrose (w/w) was overlaid onto this upper phase to give a gradient of a total volume of 38 ml. These density gradients were centrifuged at 100,000 x g for 110 minutes in an SW-27 swinging bucket rotor. The enriched synaptic plasma membrane fraction was recovered from the interface (28.5% and 34%), diluted 1:3 in 0.1 M MES buffer pH 6.5, and centrifuged at 60,000 x g for 60 minutes. The final pellet was resuspended in 0.1 M MES pH 6.5.

In all enzymatic studies performed, it was determined whether there was calmodulin activation. Because these membrane preparations may contain endogenous calmodulin, in order to see a calmodulin effect on membrane enzymes it was at first necessary to deplete the membranes of endogenous calmodulin. Synaptic and coated vesicles and SPM were depleted of calmodulin by suspending them in 0.1 M MES buffer, 2mM EGTA, 2mM EDTA, pH 6.5 for 15 minutes, followed by centrifugation at 150,000 x g for 30 minutes. This step was repeated once. The final pellets were resuspended in Buffer A and dialyzed against the same buffer for 16 hours.

d. Calmodulin preparation.

Calmodulin was prepared from chicken gizzards that were delivered fresh on ice from a local slaughterhouse by a modification of a procedure described by Jamieson and Vanaman (803). Gizzards were thawed at room temperature for two hours and homogenized in 2 volumes (2 ml/gm) of buffer 1 (0.05 M tris buffer, 1mM EGTA, 1mM β -mercaptoethanol, pH 8.0), with 3 x 30 second bursts in a Waring blender. The homogenate was

centrifuged at 10,825 x g for 30 minutes. The supernatant was poured through two layers of cheesecloth and saved. The pellets were resuspended in one volume of Buffer 1, after which the homogenization and centrifugation procedure was repeated. The supernatant was poured through cheesecloth and then combined with the previous supernatant. The combined supernatants were then made 60% saturated with ammonium sulfate. The solution was then stirred for 30 minutes and then centrifuged for 60 minutes at 10,825 x g. Ammonium sulfate leads to the precipitation of many proteins except calmodulin. The supernatant was then brought to pH 4.0 with 50% H₂SO₄, the isoelectric point of calmodulin, followed by stirring for 60 minutes. The solution was centrifuged for 60 minutes at 10,825 x g. The pellet was resuspended in a small volume of distilled water and brought to pH 7.5 with 1M Trizma base which led to the solubilization of calmodulin. The resuspended material was dialyzed for two hours at 4°C against 4 liters of H₂O and then overnight against 2 liters of Buffer 2 (0.01 Tris-OHCl Buffer, 1mM EGTA, 1mM EGTA, 1mM β-mercaptoethanol, 0.2 M NaCl, pH 8.0). The material was then centrifuged at 73,400 x g for 60 minutes. The supernatant obtained was applied to a DEAE Sephadex A-50 column (4.0 x 20 cm) equilibrated with Buffer 2. Two column volumes of Buffer 2 were passed through the column to elute proteins not bound to the ion-exchange column. Calmodulin (an acidic protein with an overall negative charge) was eluted along with other acidic proteins with Buffer 3 (Buffer 2 containing 0.7 M NaCl). The column flow was adjusted to 2 ml/min. The eluate was monitored for protein content at 280 nm. The peak was pooled, dialyzed overnight against 4 liters H₂O, lyophilized and resuspended in Buffer 4 (0.01 Tris-HCl Buffer, 1 mM MgCl₂, 1mM-β-

mercaptoethanol, 2mM anhydrous CaCl_2 , pH 8.0). The protein eluted from the DEAE column has many contaminating proteins. The essential element in obtaining pure calmodulin entails passing this eluate through a 2-chloro-10-(3-aminopropyl) phenothiazine (CAPP) Sepharose-4B conjugated column (803). The basis of the separation involves the calcium specific binding of calmodulin into this phenothiazine. CAPP is a derivative of chlorpromazine with a free amine group enabling CNBR coupling of the phenothiazine to Sepharose-4B.

The CAPP-Sepharose-4B column was prepared as described (803). A solution of 61 mg of CAPP dissolved in 20 ml of 5 mM HCl was added dropwise to a 30 gm suspension of cyanogen bromide activated sepharose 4B resin with rapid stirring. Because phenothiazines are poorly soluble at the pH utilized for coupling amine groups to cyanogen bromide activated resins, the phenothiazines were dissolved in a diluted acid solution. Coupling was performed overnight at 4°C with gentle stirring. Because phenothiazines are light sensitive, coupling was carried out in a beaker wrapped with aluminum foil. Following coupling, the resin was filtered and washed on a sintered glass funnel with 1 liter of cold de-ionized H_2O , followed by 1 resin volume of 2 M ethanolamine-HCl (pH 8.0). The resin was then resuspended in equal volume of the same buffer and incubated at 4°C with gentle stirring for two hours to block any remaining activated groups. The resultant CAPP-Sepharose-4B conjugate was washed on a sintered glass funnel with 1 liter of cold distilled H_2O , followed by 500 ml of 1 mM HCl, 2mM β -mercaptoethanol to ensure removal of any free phenothiazine present in precipitated form in the matrix. The resin was washed with 5 volumes of 100 mM sodium acetate pH 5.5, 1 mM β -mercaptoethanol, and 1 mM sodium

azide, in which it was suspended as a 2:1 (buffer:resin) slurry and stored at 4°C in a light tight container.

The crude calmodulin sample was applied to the CAPP Sepharose column and allowed to flow on by gravity and then incubated at 0 flow for 30 minutes. The column was run at room temperature and eluted by pumping at 36 ml/hr collecting 10 minutes fractions. Buffer 4 was passed through the column for 4 hours, followed by passing through Buffer 5 (Buffer 4 and 0.2 M NaCl) for 3 hours. These conditions eluted proteins that did not bind to phenothiazine in a calcium specific manner. Calmodulin was eluted by washing with Buffer 6 (0.01 M Tris-HCl Buffer, 1mM MgCl₂, 1mM β-mercaptoethanol, 4mM EGTA, 0.4 M NaCl, pH 8) for three hours. Nonspecific proteins were eluted by washing with Buffer 7 (Buffer 6 and 8 M Urea) for two hours. The column was re-equilibrated by washing for four hours with Buffer 4. The fractions were monitored for calmodulin at an optical density of 235 nm. The protein peak obtained with Buffer 6 was pooled and dialyzed overnight against 4 liters H₂O, lyophilized, and resuspended in a small volume of H₂O. This method of calmodulin purification allows the purification of calmodulin without any contaminants and fully retaining its enzymatic activating capabilities (803).

e. Calmodulin association with synaptic and coated vesicles:

(i) Radioimmunoassay (RIA). Radioimmunoassay for calmodulin in all vesicles was performed using chicken gizzard calmodulin [¹²⁵I]-calmodulin, and calmodulin antibodies prepared and raised by Dr. Watterson and his group at Rockefeller University, New York, as described (804). Their materials, buffers and facilities were used for this quantitation. Competition radioimmunoassay was performed in RIA

buffer (0.1M NaCl, 1mM EDTA, .02M Tris-HCl, pH 7.6), containing 0.2% (w/v) bovine serum albumin. Reaction mixtures containing a limited dilution of anticalmodulin sera (that amount predetermined by direct radioimmunoassay to give 50% precipitation of the ^{125} I counts) and varying concentrations of competing antigens (coated, decoated, or synaptic vesicles) and unlabelled chicken gizzard calmodulin were incubated at 4°C overnight. [^{125} I]-calmodulin (1 ng; approximately 50,000 cpm) was then added and the solution was incubated at 4°C overnight. Goat antirabbit serum was then added and the mixture was incubated at 4°C for an additional 8-24 hours. The mixture was washed with RIA Buffer and centrifuged at 900 x g for 10 minutes, washed again, and radioactivity in the resultant pellets was determined in a Packard gamma counter.

The quantity of calmodulin in all the vesicle fractions was determined by comparing the amount of vesicle and unlabelled calmodulin protein needed to competitively inhibit 50% of radiolabelled calmodulin binding. This method is reliable and accurate (804).

(11) Calcium dependence of calmodulin binding to synaptic and coated vesicles. Approximately 4-6 mg of vesicles were incubated for 1½-2 hours at 25°C with 17-20 µg ^{125} I-calmodulin in the presence of 10^{-6} M CaCl_2 , or 10^{-3} M EGTA and brought to a final volume of 3 ml with Buffer A. The samples were layered on discontinuous sucrose gradients. Each layer had a volume of 5 ml and consisted of the following sucrose concentrations (w/v): 10%, 20%, 30%, 40%, 50% and 60% for coated vesicles, and 20%, 30%, 40%, 50%, 55%, 60% and 70% for synaptic vesicles. Samples pretreated with EGTA were layered on sucrose gradients containing 10^{-6} M EGTA. Gradients were centrifuged at 80,000

x g for 90 minutes. Aliquots of 1.5 ml were withdrawn sequentially, and optical density at 280 nm and radioactivity were recorded. The sedimentation of vesicles allowed the separation of vesicles from free calmodulin which remained in the top layer. This procedure provided information on the calcium dependence of calmodulin binding to the vesicles and the sedimentation pattern of vesicles in sucrose.

(iii) Specificity of vesicle binding sites for calmodulin. To determine whether sites on the vesicles were specific for calmodulin, a series of 0.4 mg aliquots of vesicles were incubated for two hours at 25°C with increasing amounts of unlabelled calmodulin or troponin C (0-10,000). Troponin C was prepared as described (805) and was a generous gift from J. Maimon. Radiolabelled calmodulin (10 ng) was then added to all the samples and incubated for an additional two hours. Ability of troponin C to compete for binding sites provided insight into the specificity of the interaction. Samples were then centrifuged at 150,000 x g for 30 minutes at 4°C. This procedure allowed the separation of bound calmodulin (pelleted) from free (supernatant). Radioactivity in pellets was determined using a gamma counter. The amount of radiolabelled calmodulin binding that was achieved in the presence of the highest concentration of unlabelled calmodulin was determined as the amount of nonspecific binding.

Affinity of calmodulin binding to synaptic and coated vesicles (0.5 gm) was determined by incubating a range of 0.1-22.0 μg ^{125}I -calmodulin for two hours at 25°C in the presence of 1mM CaCl_2 in a final volume of 1 ml Buffer C.B. (0.1 M MES, 1mM β -mercaptoethanol, pH 6.5). Subsequent steps were identical to those steps described above. Specific binding was determined by subtracting nonspecific binding (determined as

described above) from total binding. Data were analyzed by Scatchard analysis. Calmodulin binding under these conditions reach equilibrium (preliminary results) and good separation of bound from free calmodulins was obtained.

(iv) Identification of synaptic and coated vesicle calmodulin binding proteins: Calmodulin binding proteins were identified by incubating extracts of vesicles on a Sepharose-4B-CNBR-calmodulin affinity column prepared as described (806). Essentially activated cyanogen bromide coupled sepharose-4B was mixed in a 40 ml slurry of Buffer Aff. (0.05 M triethanolamine-HCl, 1mM CaCl₂, pH 8.5). Calmodulin (10 mg) was added dropwise to the slurry and was then stirred continuously at 4°C for 8 hours. Afterwards the gel was washed with 40 ml of Buffer Aff., resuspended in 20 ml of 1M ethanolamine-HCl, pH 8.0, and then stirred for two hours at room temperature. The gel was then washed exhaustively with 40 mM Tris-HCl, 1 mM CaCl₂, pH 8.0, and stored as a 1:1 slurry in the same buffer.

Vesicles were treated with 2.5% Triton X-100 and 2.5 M NaCl for 30 minutes and centrifuged for 10 minutes at 20,000 x g. The supernatant was then applied to the the column (1.5 x 10 cm). Proteins that did not bind in a Ca²⁺ dependent manner were eluted with 10 mM Tris buffer, 1 mM MgCl₂, 2 mM CaCl₂, 1 mM β-mercaptoethanol, pH 8.0. Proteins binding in a Ca²⁺-dependent manner were eluted with 10 mM Tris Buffer, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 10 mM EGTA, pH 8.0. Eluates were dialyzed against 10 liters of de-ionized H₂O lyophilized and analyzed by 5-15% SDS polyacrylamide gel electrophoresis (SDS-PAGE).

f. Phospholipase A₂ Assay.

All reaction mixtures were incubated with 100 µg of synaptic vesicles, coated vesicles or synaptic plasma membrane protein, using β ¹⁴C-arachidonic acid-phosphatidylcholine as substrate (S.A. = 54 mCi/mmol). The substrate chosen had arachidonic acid in the two position, because it was necessary to determine whether vesicle PLA₂ was capable of releasing arachidonic acid, the precursor of prostaglandins. The study of arachidonic acid metabolism to prostaglandins by these vesicles will be described later. The incubations took place in a final volume of 200 µl with 100 mM Tris-HCl Buffer. Variations in terms of Ca²⁺ and substrate concentration, incubation time and pH are detailed below.

g. Synaptic and coated vesicle and synaptic plasma membrane (SPM) PLA₂ characteristics.

(i) Ca²⁺ dependence. Reaction mixtures were incubated with 1.85-2.8 nmol [¹⁴C]-phosphatidylcholine in the presence of 5 mM EGTA and increasing Ca²⁺ concentrations (10⁻⁷ - 10⁻¹ M).

(ii) pH dependence. A series of reaction mixtures were incubated with 1.85 nmol [¹⁴C]-phosphatidylcholine in buffers with increasing pH values ranging from 4.0-12.0. Synaptic vesicles, coated vesicles, and SPM were incubated for 60, 10 and 30 minutes, respectively.

(iii) Time dependence. A series of reaction mixtures were incubated with 1.85 nmol [¹⁴C]-phosphatidylcholine, at pH 9.0, 9.0, and 7.0 for synaptic vesicles, coated vesicles and SPM, respectively. The Ca²⁺ concentration in the reaction mixtures was 2 x 10⁻³, 1 x 10⁻³ and 1.0 x 10⁻⁷ M for synaptic vesicles, coated vesicles, and SPM, respectively. Reaction mixtures were stopped at time intervals ranging from 5-120 min.

(iv) Substrate concentration dependence. A series of reaction mixtures were incubated with increasing concentrations of [^{14}C]-phosphatidylcholine ranging from 0.5-6.0 nmol at optimal time, pH, and Ca^{2+} concentrations for all the membrane preparations.

(v) Effects of modulators on membranal PLA₂ activities. A series of reaction mixtures were incubated with increasing substrate concentrations (0.2-4.0 nmol) at the optimal Ca^{2+} and pH concentrations and optimal time values. Different series of reaction mixtures were incubated in the presence of either 4.0 nmol PGE₂, 4.0 nmol PGF_{2 α} , 1 μM calmodulin, 1 mM cAMP, 1 mM cAMP and 1.6 mM ATP. The latter two conditions were incubated in the presence of 2 mM MgCl₂ for the synaptic vesicle assay. All the reactions were stopped by the addition of 600 μl ethanol, and were then dried down under nitrogen, resuspended in a small volume of chloroform:ethanol:water (63:27:4), layered on Silica G thin layer chromatogram sheets (100 μm thick) and developed in the same solvent used for suspension (807). The spots corresponding to phosphatidylcholine and arachidonic acid were scraped off and counted in a liquid scintillation counter to determine the amount of arachidonic acid released.

h. Vesicle metabolism of arachidonic acid.

Vesicle membrane was incubated with 1- ^{14}C -arachidonic acid (2 μg , S.A. = 55.8 mCi/mmol) for 20 minutes at 37°C in the presence of 2 mM CaCl₂ (319). The reaction was stopped by acidification to pH 3.0 with 0.1 M HCl. The suspension was extracted three times with 2 ml ethyl acetate. The organic phase was pooled and dried under N₂. Products were separated by thin-layer chromatography, using a solvent system containing chloroform:methanol:acetic acid:water (90:8:10:8). The

radioactive peaks were localized with authentic prostaglandin standards and a comparison of R_f values was performed.

1. Functional biophysical and morphological correlation of endogeneous membranal PLA_2 .

(i) Synaptic vesicle light-scattering experiments. A Behring laser nephelometer model 7601 (La Jolla, Calif.) was used to measure light scattering of synaptic vesicles. Light scattering was recorded every 5-10 seconds by Data Processing module, model 100 (La Jolla, Calif.) in nephelometer units that were directly proportional to the intensity of scattered light detected by a photodiode-generated signal. Baseline light scattering of synaptic vesicles was recorded prior to incubations. Various compounds were incubated with synaptic vesicles (30 $\mu\text{g/ml}$) at 25°C in 1 min. sequences to observe their effects on light scattering (total volume: 300-600 μl). The following 4 series of compound additions were performed on synaptic vesicles.

1. 2 mM CaCl_2 and 20 μg PLA_2 (*Vipera russelli*) were added sequentially to the synaptic vesicle suspension. This species of PLA_2 was chosen because its pH optimum (6.5) was close to that of the synaptic vesicle suspension buffer.
2. 2 mM Ca^{2+} and 25 μg lysolecithin were added sequentially to the synaptic vesicle suspension. This sequence was performed to see if the lysolecithin light-scattering profile was comparable to that induced by exogenous PLA_2 .
3. 2 mM CaCl_2 and 1.0 μM calmodulin were added sequentially to the synaptic vesicle suspension to see if the light-scattering profile produced by calmodulin was due to interaction with endogenous synaptic vesicle PLA_2 . The sequence was repeated after

preincubation for 60 minutes with quinacrine or parabromophenacylbromide (0.8-80 mM). To see the effect of PGE₂ and PGF_{2α} on calmodulin-dependent light-scattering profile, the above sequence was repeated after synaptic vesicles were preincubated for 10 min with 4.0 nmol of PGE₂ or 4.0 nmol PGF_{2α}.

4. 2 mM CaCl₂, 10 mM MgCl₂, 1 mM cAMP and 2mM ATP were added sequentially to the synaptic vesicle suspension. The sequence was repeated after preincubation with parabromophenacylbromide (.08-80 mM).

Additionally, the same compounds mentioned above were added in a dose-dependent manner to see the effect on light scattering with the following concentration ranges. PLA₂ (10⁻⁵ - 25.0 μg), lysolecithin (10⁻⁵ - 25.0 μg), calmodulin (10⁻⁵ - 7.0 μg), PGF_{2α} (10⁻⁶ - 10 μg).

The purpose of the light scattering experiments was to correlate endogenous PLA₂ activation or inhibition by key neuromodulators with vesicle fusion, the process whereby neurotransmitters are released into the synaptic cleft. This model, of course, has its limitations. It measures synaptic vesicle-vesicle interactions in an in vitro environment as opposed to the in vivo situation of synaptic vesicle fusion with the presynaptic plasma membrane.

(11) Coated vesicle light scattering. Because it was difficult to devise experiments that would functionally measure increased or decreased endocytotic function of coated vesicles, vesicle aggregation — a phenomenon that is measurable and related to endocytosis — was studied. The pinching off of a coated pit (endocytosis) and vesicle fusion (exocytosis) are analogous in that both involve membranal distortions and fissions at one point in the process. Before two

membranes fuse, fission must occur in the complementary outer halves of the bilayers to allow intermixing of the membranes and subsequent fusion. Similarly for coated pit endocytosis to occur, membranal fission occurs. However, in this process, fission occurs in both sides of the coated pit membrane in both inner and outer leaflets. When the two ends of the coated pit membrane join together to become a vesicle, the two ends intermix with one another, i.e., they fuse. Since aggregation and fusion are membranal events, nephelometry and light microscopy were performed on decoated vesicles (vesicles whose coats were removed so that all that remained was the vesicle) to assess the effects of a variety of modulators on coated vesicle function. Because the coat of coated vesicles might be subject to reversible assembly and disassembly under certain nephelometry conditions, it would be difficult to ascertain if light scattering changes were due to vesicle interactions or clathrin polymerization/depolymerization if performed with coated vesicles. Nephelometry with coated vesicles was therefore not performed. Various compounds were incubated with decoated vesicles (10 µg/ml) at 25°C in 1-5 minute sequences to observe their effects on light scattering. The total volume was 300-600 µl. The vesicles were suspended in 100 mM Tris-HCl buffer, pH 9.0.

The following series of sequential compound additions were performed:

- 1) 1mM CaCl₂ and 20µg PLA₂ (Naja naja venom). This PLA₂ was chosen because optimal coated vesicle PLA₂ activity occurred at pH 9.0.
- 2) 1mM CaCl₂ and 20µg lysolecithin.
- 3) 1mM CaCl₂ followed by 1µM calmodulin, or 4.0 nmol PGE₂ or PGF_{2α}.

These conditions were repeated after preincubation with 1.0×10^{-5}

M parabromophenacylbromide.

- 4) 1 mM CaCl_2 followed by 1mM cAMP or 1mM ATP. These conditions were also repeated after preincubation with 1×10^{-8} M parabromophenacylbromide.

(iii) Phase contrast microscopy. In order to interpret the light scattering profile observed in both synaptic and decoated vesicles, conditions similar to those conducted for the light scattering experiments were performed on the vesicles (0.75mg/ml) in a total volume of 500 μ l and observed under a Zeiss phase contrast microscope. The exact details appear in the figure legends in the Results section.

To observe the morphological effects of prostaglandin synthesis inhibition on synaptic vesicle interaction, synaptic vesicles (0.75 mg/ml) suspended in 200 μ l 0.1 M MES buffer, pH 6.5, were treated with 1.0×10^{-5} M indomethacin for 20 minutes prior to treatment with 20 μ g PLA₂ (*Viperra russelli*) at 25°C, and observed under a Zeiss phase contrast microscope.

(iv) Electron microscopy. To further assess the morphological effects of a variety of modulators on synaptic vesicles, several treatments of synaptic vesicles were performed and observed by electron microscopy. Synaptic vesicles (100 μ g) in a total volume of 200 μ l were either: a) untreated, or treated with b) 1 mM CaCl_2 and 25 μ g PLA₂ (*Viperra russelli*), c) 2 mM CaCl_2 and 1 μ M calmodulin, d) 2 mM CaCl_2 and 4.0 nmol $\text{PGF}_{2\alpha}$, or e) 2 mM CaCl_2 , 10 mM MgCl_2 , 1 mM ATP and/or 1 mM cAMP. All the vesicles were treated for 30 minutes at 25°C and suspended in 0.1 M MES pH 6.5. The reactions were stopped with the addition of 400 μ l 3% gluteraldehyde phosphate buffer, centrifuged at 2,000 x g for 10 minutes and observed under a JEOL 100 B electron microscope.

To assess the effects of a variety of modulators on possible synaptic vesicle-SPM interactions, synaptic vesicles (100 μ g) and synaptic plasma membrane (100 μ g) suspended in 0.1 M MES, pH 7.0, were mixed in a 1:1 protein ratio in a total volume of 400 μ l and were: a) untreated, or treated with b) 1 mM CaCl_2 and 10 μ g PLA_2 (Naja naja venom), c) 1 mM CaCl_2 and 1 μ M calmodulin, d) 1 mM CaCl_2 and 4.0 nmol $\text{PGF}_{2\alpha}$ or PGE_2 , e) 1 mM CaCl_2 and 1 mM ATP and/or 1 mM cAMP, or f) preincubated with 1.0×10^{-6} M parabromophenacylbromide for 30 minutes prior to incubation conditions c and d. Vesicles were incubated for 30 minutes at 25°C and stopped with 400 μ l 3% gluteraldehyde phosphate buffer, centrifuged at 2,000 x g for 10 minutes and observed under a JEOL 100 B electron microscope.

j. Purified phospholipases A_2 .

(i) Calmodulin affinity chromatography. A variety of five different phospholipases A_2 (0.50-1.0 mg) were incubated on the calmodulin affinity column (1.5 x 5 cm) for 30-60 minutes. Enzymes not binding to calmodulin were eluted with 0.1M Tris buffer, 1 mM MgCl_2 , and 1 mM CaCl_2 . Enzymes binding to calmodulin were eluted with 0.1M Tris buffer, 1 mM MgCl_2 , and 10 mM EGTA. The pH of the buffers was adjusted to the optimum of the various enzymes pH viz. pH 8.0, 8.5, 8.9, 8.9, 8.9 for PLA_2 's from porcine pancreas, bee, naja, naja, Crotalus Adamanteus and Crotalus Durissus Terrificus venoms, respectively. The eluates were lyophilized and resuspended in Tris-HCl buffer and analyzed by 5-15% SDS gel analysis.

(ii) Dimethylsuberimidate (DMS) cross-linking. DMS is an alkylimido ester which reacts rapidly and specifically with the unprotonated ϵ -Amino group of lysine or NH_2 terminal alpha amino groups

of proteins and peptides (808). This reagent has been used in various systems to join proteins or protein subunits within a complex in close proximity. Proteins capable of being cross-linked with DMS exist as complexes at most within 11 Å from each other in solution (808). The 5 phospholipases A₂ mentioned above (20 µg) were incubated with 0.42 µg/ml DMS (dissolved in 20 mM Triethanolamine, pH 8.5) for 30 minutes in a total volume of 60 µl 1 mM imidazole buffer, pH 6.5. Four different conditions of cross-linking were employed. The enzyme was cross-linked in the presence of: 1) 1 mM CaCl₂; 2) 1 mM EGTA; 3) 1 mM CaCl₂ and 7.0 µg calmodulin; or 4) 1 mM EGTA and 7.0 µg calmodulin. The reaction was stopped with 14 µl phenol red solution (10% SDS and 1 mM β-mercaptoethanol). Protein composition and polymerization were analyzed by 5-15% SDS PAGE and visualized with Coomassie Blue. Molecular weights of the protein aggregates were determined by comparison to standards.

(iii) PLA₂ assay. Reaction mixtures (1.0 pgm PLA₂) were incubated with increasing 1-¹⁴C-β-arachidonyl-phosphatidylcholine concentrations (0.2-2.8 nmol) in the presence of 1mM CaCl₂ with or without 7.0 µg calmodulin in a final volume of 100 µl for 10 minutes. The reactions were stopped and processed as described for the membranal PLA₂ assay.

(iv) Naja naja PLA₂. This PLA₂ was chosen for extensive analysis due to its amino sequence homology to mammalian PLA₂.

(a) PLA₂ assay. Reaction mixtures containing 1 pgm PLA₂, 1 mM CaCl₂ and 1.8 nmol 1-[¹⁴C]-β-arachidonyl-phosphatidylcholine were incubated with the following conditions: 1) increasing concentrations (10⁻⁹ - 10⁻⁵ M) calmodulin, PGE₂, PGF_{2α}, cAMP or cGMP; 2) calmodulin (10⁻⁵ M) and increasing concentrations of PGE₂, PGF_{2α}, cAMP or cGMP (10⁻⁹ - 10⁻⁵). These studies were undertaken to analyze the direct

effects of these modulators on PLA₂ activity, and their potential to act either synergistically or antagonistically on the same enzyme. The reactions were stopped and processed as described for membranal PLA₂ assays.

(b) DMS-crosslinking. DMS-crosslinking was performed as described above. Naja naja PLA₂ (20 µg) was incubated with: a) PGE₂ (10µg), b) PGF_{2α} (10µg), c) cAMP (10^{-5} M) or d) cGMP (10^{-5}). For each of these 4 conditions the following 4 subconditions were performed: incubation in the presence of: 1) 1 mM CaCl₂, 2) 1 mM EGTA, 3) 1 mM CaCl₂ and 7.0 µg calmodulin, or 4) 1 mM EGTA and 7.0 µg calmodulin.

In addition, to examine the synergistic effects of these modulators on PLA₂, PGF_{2α} (10µg), PGE₂ (10µg), cAMP (10^{-5} M) and cGMP (10^{-5} M) were simultaneously incubated under the 4 incubation subconditions mentioned above. The reactions were stopped with 14 µl phenol red solution (10% SDS and 1 mM β-mercaptoethanol). Protein composition was analyzed by 5-15% SDS PAGE. The relative ratios of protein complexing were determined by densitometry of a positive film of the gel at 500 nm. The background darkness of the film was taken as baseline values of these determinations.

k. Effect of prostaglandins on purified PLA₂ function.

To verify whether prostaglandins could affect purified PLA₂ function, I wanted to see whether a variety of prostaglandins would alter the morphological effect of PLA₂ on synaptic vesicle and SPM morphology, which is an expression of the membranal hydrolytic function of PLA₂. Synaptic vesicles (0.75 µg/ml) in a total volume of 200 µl suspended in 0.1 M MES, pH 6.5, were pretreated with 10^{-6} - 10^{-5} M parabromophenacylbromide (used as a comparison control) or 4.0 nmol

PGA₂, PGB₂, or PGE₂ for 20 minutes prior to treatment with 25 µg PLA₂ (*Viperra russelli*). The suspension was observed 30 minutes later under a Zeiss phase contrast microscope.

In addition, synaptic vesicles and SPM (100 µg of each) mixed in a 1:1 protein ratio in a total volume of 400 µl, suspended in 0.1 M MES buffer, pH 7.0, were pretreated with 4.0 nmol PGE₂, PGA₂ or PGB₂ for 30 minutes prior to incubation with 10 µg PLA₂ (*Naja naja* venom) at 25°C. The reaction was stopped with 800 µl 3% gluteraldehyde phosphate buffer. The mixtures were centrifuged at 2,000 x g for 10 minutes. The resultant pellets were observed under a JEOL 100 B electron microscope.

1. Phosphorylation studies.

The standard phosphorylation assay involved incubation of 100 µg synaptic or coated vesicle protein in a total volume of 100 µl 40 mM Tris-HCl buffer at 30°C. For determination of Ca²⁺/Calmodulin dependence, the vesicles were suspended in 80 mM KCl, 2.5 mM NaCl, 10 mM MgCl₂ and a) EGTA, b) 1 mM CaCl₂ with or without 1.0 µM calmodulin or with c) 1 mM CaCl₂, 1 µM calmodulin and 1.0 x 10⁻⁶ M trifluoperazine in Tris buffer, pH 7.0. For determination of cAMP dependence, vesicles were suspended in Tris-HCl buffer, pH 7.4, with or without 10 mM MgCl₂ and 10 x 10⁻⁵ M cAMP.

For immunogen protein and antibody preparation, clathrin and affinity-purified anti-clathrin antibodies were prepared and raised as described (261, 809) and were a generous gift from Elaine Hua. Affinity-purified IgG antibodies to the 30,000 M_r protein doublet (30K protein) from coated vesicles were prepared as described (810) and were a generous gift from Jonathan Maimon. Immunological reactivity of the antibodies was tested by the Ouchterlony double-gel-diffusion technique (811).

(i) Effect of antibodies. Anti-clathrin (1.0 μ g) or anti-30K protein IgG (0.5-1 μ g) was preincubated with vesicles for 1 hour at 25°C prior to initiation of the assay in those conditions where either $MgCl_2$ and cAMP, or Ca^{2+} and calmodulin were present. The phosphorylation reaction was initiated after five minutes preincubation with [$\gamma^{32}P$]-ATP by addition of either synaptic or coated vesicle proteins. After five minutes of incubation, the reaction was terminated by addition of 50 μ l SDS stop solution (10% SDS, 7% β -mercaptoethanol, 15% glycerol and 0.1% bromophenol blue). Mixtures were then placed in boiling water for three minutes.

(ii) Gel electrophoresis, autoradiography and microdensitometry. Aliquots (65 μ l) of solubilized proteins were resolved by 5-15% SDS-PAGE. Gels were fixed and stained with 0.1% Coomassie blue, 50% methanol, 7% acetic acid before being dried under heat and reduced pressure by a Bio-Rad slab-gel drier. Autoradiography was carried out for 2-5 hours with Kodak NS-5T film. Incorporation of phosphate into specific protein bands was estimated by microdensitometry of autoradiographs with a Gilford spectrophotometer, model 240, at 615 nm. Protein phosphorylation was quantified from the densitometric scans by integration of areas under the peaks corresponding to specific protein bands using an electronic planimeter, model 1250, Numonics Corporation. Background darkness of the autoradiographs was taken as baseline values for these determinations.

m. [Ca^{2+} - Mg^{2+}]-ATPase.

Calmodulin depleted synaptic and coated vesicles (0.5 mg total protein) were suspended in 40 mM Tris HCl-buffer, 0.2mM Ca^{2+} , 0.1 mM ouabain, pH 7.0, in the presence or absence of 1 μ g calmodulin. Samples

were preincubated for 30 minutes at 37°C prior to addition of 0.4 mM ATP. ATP hydrolysis was allowed to proceed for 45 minutes at 37°C and samples were withdrawn at various time intervals. The reactions were stopped with Trichloroacetic acid. ATPase activity was determined by the release of γ ^{32} P from ATP using a procedure described previously (811). Briefly, molybdate was added to form a phosphomolybdate complex, as well as citrate to complex any free molybdate (812). The phosphomolybdate complex was extracted with butanol. Aliquots of the butanol phase were counted in a scintillation counter to determine the amount of phosphate released. The radiometric procedure allowed the accurate determination of P_i released in the pmol range (811).

n. Calcium uptake determinations.

Calmodulin depleted synaptic and coated vesicles were incubated with 2mM $MgCl_2$, 3.2mM ATP, and 2mM $CaCl_2$ ($^{45}Ca^{2+}$, S.A = 91.2 mCi/mg) in the presence or absence of calmodulin and brought to a final volume of 100 μ l with buffer C.U. (0.13 M KCl, 20 mM imidazole buffer, pH 7.0) (788). Incubation proceeded for 10 minutes at 25°C. The reaction was quenched with 0.4 ml 0.37M sucrose, 20mM imidazole buffer, pH 7.0. Vesicles with sequestered calcium were separated from free calcium by passing the vesicles through a Sephadex G-50 column (15 x 1.5 cm). Vesicles with sequestered calcium were eluted with buffer C.U. The eluate was monitored for protein content spectrophotometrically at O.D. 280nm and for radioactivity by scintillation counting.

o. Miscellaneous procedures.

Protein concentrations were determined by the method of Lowry et al. (813) using bovine serum albumin as standard.

All sodium-dodecyl-sulfate-PAGE (5-15%) was performed as described by Laemmli (814).

C. RESULTS

1. Membrane preparations

a. Synaptic vesicles

Electron microscopy of the synaptic vesicle preparation after depletion of their endogenous calmodulin revealed that the preparation was enriched with synaptic vesicles which measured approximately 400 Å in diameter (Fig. 1). Most of the synaptic vesicles in the preparation appeared to be intact. There also appeared to be a small number of open vesicles and a minor degree of small membrane fragments. The latter structures could have arisen from synaptic vesicles or from minor membranal contaminations. The polypeptide composition of synaptic vesicles as determined by 5-15% SDS PAGE appears in Fig 2.

The elution profile of the synaptic vesicle preparation on a controlled pore glass bead column is illustrated in Figure 3. It is apparent that the vesicles separated into two peaks. Sodium dodecyl sulfate PAGE analysis revealed that the polypeptide composition of the synaptic vesicles prior to chromatography and the vesicles obtained in both peaks were virtually identical. A small amount of a protein with M_r of approximately 180K seemed to be more concentrated in the second peak. Electron microscopy revealed that the vesicles were separated by virtue of their state of aggregation with no observable differences in degree of membrane contamination. The first peak was slightly more aggregated than the second peak.

b. Coated vesicles

Electron microscopy revealed that coated vesicles were homogenous with few membrane contaminants (Figure 4). The diameters of coated

Figure 1. Electron micrograph of synaptic vesicles. Magnification x 120,000.



Figure 2. Polypeptide composition of synaptic vesicles as determined by
5-15% SDS PAGE.

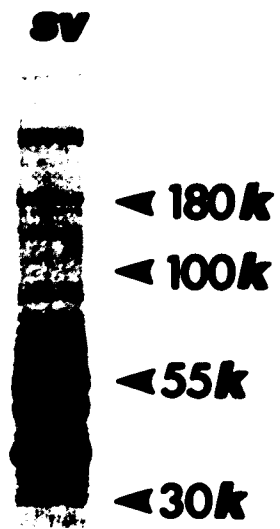
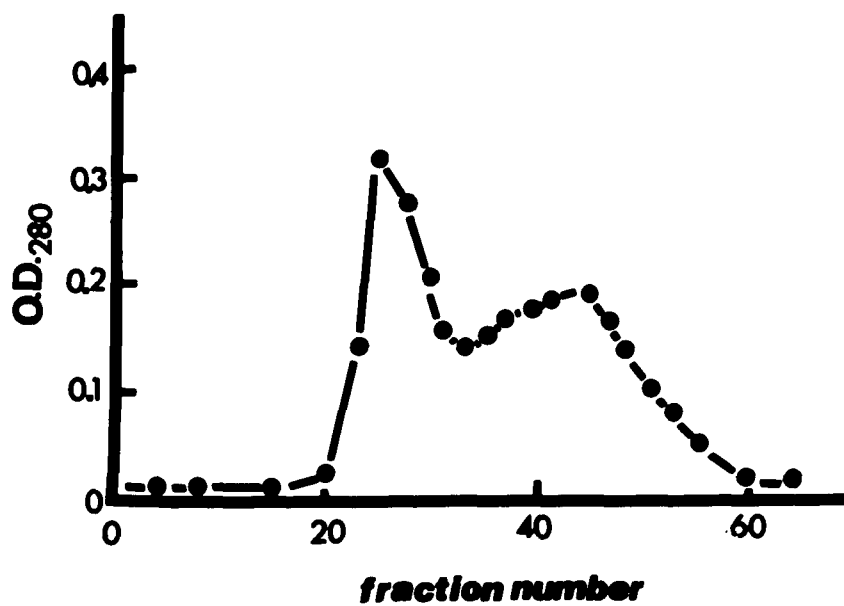


Figure 3. Elution profile of synaptic vesicle preparation by controlled pore glass bead chromatography. The synaptic vesicles were suspended in 2ml 10 mM MES, pH 6.5, containing 0.2M sucrose and 0.19 M NaCl, and chromatographed at a rate of 14ml/hr with the suspension buffer. Optical density at 280nm was determined for every 2ml fraction.



vesicles measured between 500-700 $\text{\AA}$. The morphological appearance of coated vesicles was identical before and after pore glass chromatography. SDS PAGE analysis (5-15%) revealed that coated vesicles obtained after pore glass bead chromatography had fewer protein bands (Fig. 5). All enzymatic assays with the exception of the PLA assay was performed on coated vesicles obtained prior to pore glass bead chromatography. The coated vesicle PLA assay was performed on coated vesicles obtained after this chromatographic procedure.

Electron microscopy of de-coated vesicles revealed that the coated vesicles were successfully decoated (Figure 6). The clathrin baskets typically observed on coated vesicles were not present. The morphological appearance of decoated vesicles prepared by extraction procedure (a) - 0.5M Tris-HCl buffer, 1mM 2-mercaptoethanol, pH 7.0 for 30 minutes or by extraction procedure (b) - 5mM TES pH 8.4 for 30 minutes, were virtually identical. The decoated vesicles had diameters of approximately 400 $\text{\AA}$ (Figure 6). There appeared to be a certain amount of fragmented and open vesicles and debris. SDS analysis (5-15%) revealed that decoated vesicles prepared by extraction procedure (a) removed most of the clathrin, M_r 180K (Figure 5). SDS-PAGE revealed that extraction by procedure (b) removed clathrin as well as most of an accompanying 30K polypeptide doublet (Figure 7).

Decoated vesicles prepared by procedure (a) were used for calmodulin binding and RIA experiments. Decoated vesicles prepared by procedure (b) were used for immunological-phosphorylation experiments.

c. Synaptic plasma membrane

Electron microscopy of the synaptic plasma membrane preparation after removal of endogenous calmodulin revealed that the preparation

Figure 4. Electronmicrograph of coated vesicles obtained after pore glass-bead chromatography. Magnification. x 120,000.



Figure 5. Polypeptide composition of: 1) coated vesicles obtained prior to pore glass-bead chromatography; 2) decoated vesicles prepared by extraction procedure (a); and 3) coated vesicles obtained after pore-glass-bead chromatography. The protein composition was determined by 5-15% PAGE.

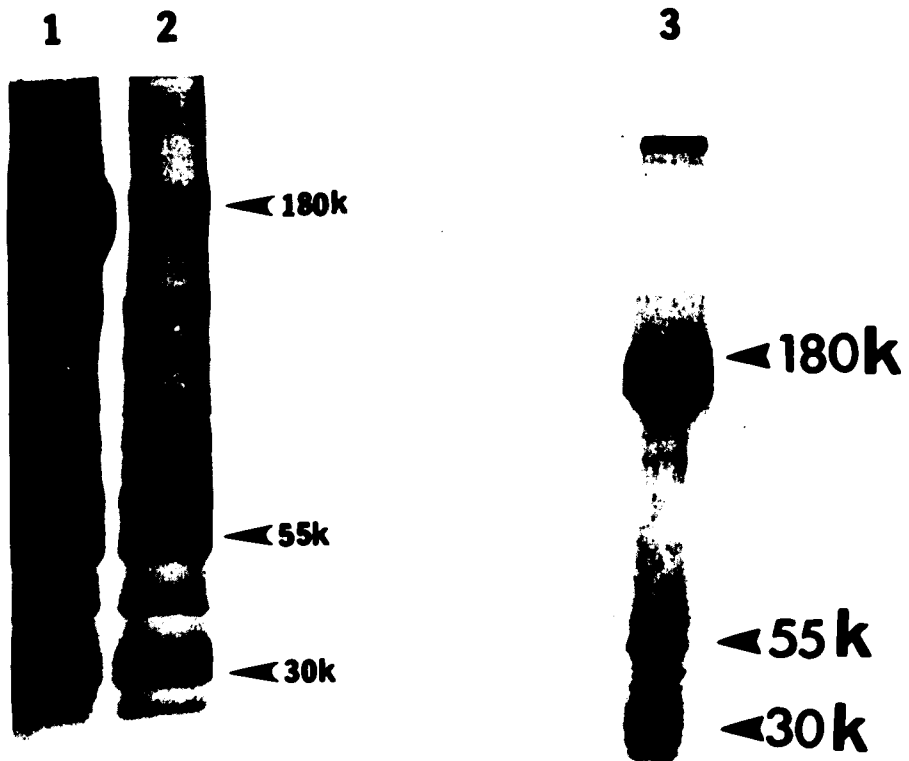


Figure 6. Electronmicrograph of decoated vesicles prepared by extraction procedure (a). Magnification x 120,000.



Figure 7. Polypeptide composition of: A) coated vesicles obtained prior to pore glass-bead chromatography; B) decoated vesicles prepared by extraction procedure (b); and C) the protein extract of coated vesicles obtained by decoating extraction procedure (b). Protein composition was determined by 5-15% PAGE.

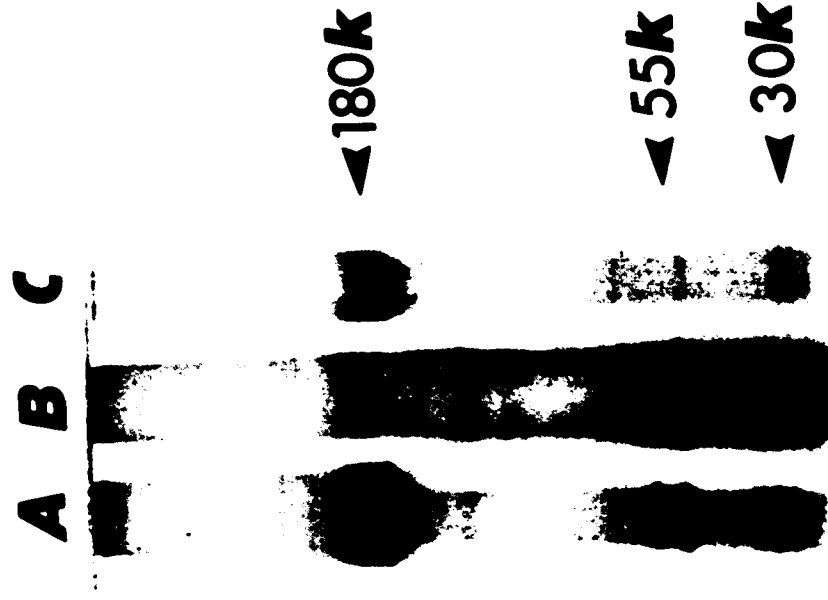
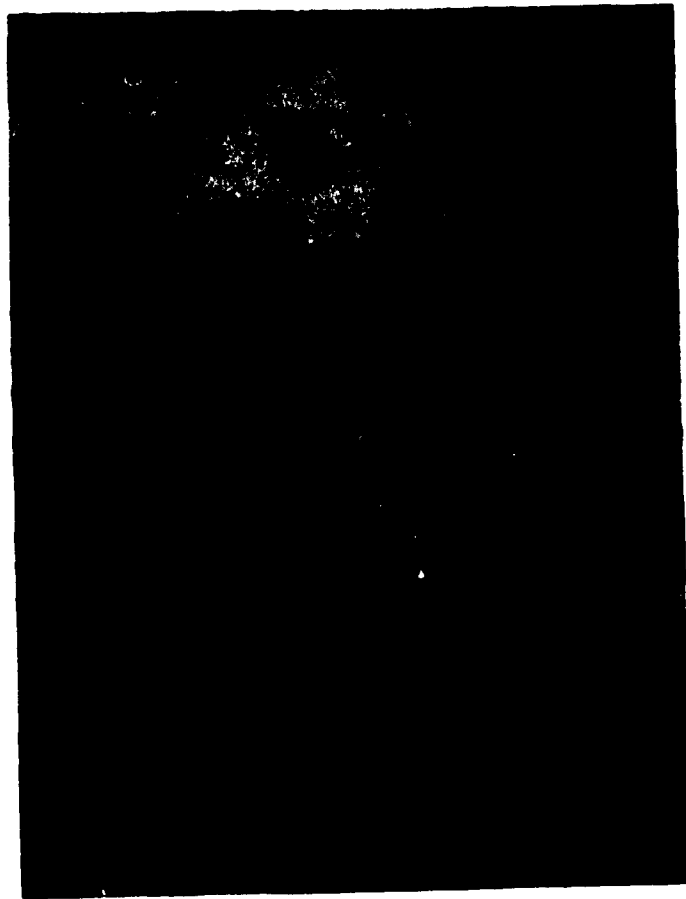


Figure 8. A) Electronmicrograph of rat brain synaptic plasma membranes. Magnification x 120,000. B) Polypeptide composition of rat brain synaptic plasma membranes as determined by 5-15% SDS PAGE.



A

SPM



< 175k

< 55k
< 40k

B

consisted mostly of elongated and closed membranes (Figure 8A). There appeared to be a variable number of synaptosomes, synaptic vesicles and debris. The polypeptide composition appears in Fig. 8B.

The following observations implied that the three different membrane preparations — synaptic and coated vesicles and synaptic plasma membranes — were relatively pure and not significantly cross-contaminated: 1) Synaptic and coated vesicles had different sedimentation profiles in sucrose (to be described in detail later in this section); 2) Enzymatic characteristics of all three preparations were different; specifically: (a) the calcium, pH and time dependencies of endogenous PLA₂ were different in all three preparations and (b) endogenous protein kinases in synaptic and coated vesicles had different conditions necessary for their stimulation and different membrane protein substrates (to be described in detail later on in this section).

It therefore appeared that each membrane fraction was suitable for characterization of its properties. The possibility exists that there may be minimal cross-contamination of the three different membranes as well as minimal contamination from membranes of other types.

2. Calmodulin purification

Figure 9 demonstrates the elution profile obtained by passing radio-labelled calmodulin through the CAPP affinity column. The bulk of calmodulin eluted in the presence of EGTA. The polypeptide composition of the crude calmodulin preparation prior to loading on the CAPP affinity column and that obtained in the EGTA eluate is shown in Figure 10. It is apparent that the EGTA buffer eluted calmodulin (M_r 17,000) free of contaminating proteins. The purification procedure employed therefore yielded highly purified calmodulin.

Figure 9. Elution profile of ¹²⁵I-calmodulin on a chloraminopropyl phenothiazine (CAPP) affinity column. After the calmodulin was loaded on the column and incubated for 30 minutes, it was sequentially eluted with 10 ml Calcium, 20 ml EGTA and 10 ml Urea buffers.

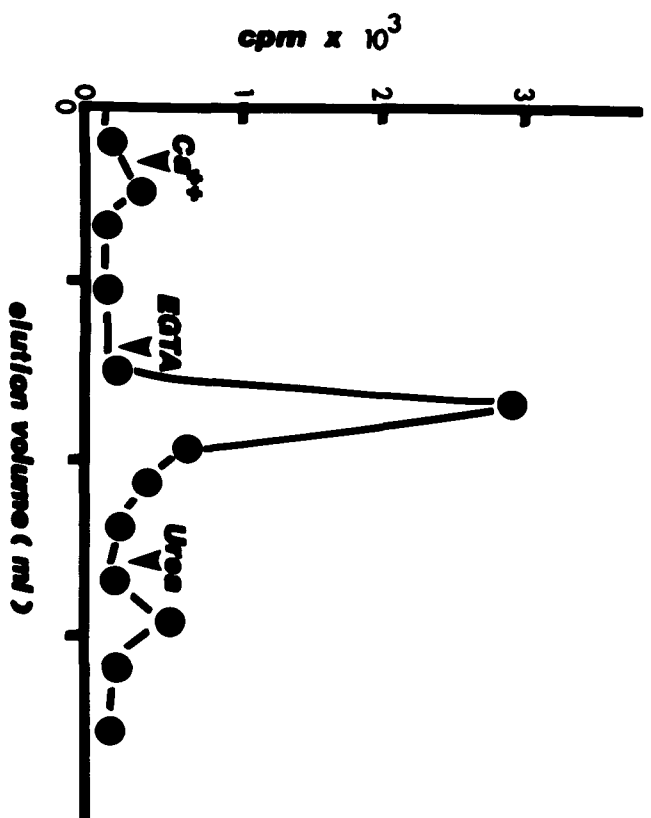


Figure 10. Polypeptide composition of the calmodulin preparation before (1) and after (2) it was passed through the CAPP affinity column. Lane 2 represents the polypeptide composition of the CAPP affinity column-EGTA eluate. Protein composition was determined by 5-15% SDS PAGE.

1 2



3. Calmodulin association with brain synaptic and coated vesicles

The sedimentation profiles of synaptic and coated vesicles in discontinuous sucrose gradients is illustrated in Figures 11 and 12, respectively. Synaptic and coated vesicles equilibrated in the 60% and 30-40% sucrose concentration regions, respectively. The fact that both vesicle types sedimented in one major region with different sedimentation patterns implied that the vesicles were not substantially cross-contaminated with each other and that they were relatively homogeneous.

Because a fraction of the synaptic vesicles were partly aggregated, it is likely that the entire vesicle population cosedimented in the discontinuous sucrose gradient at the 60% sucrose concentration region.

The sedimentation of ^{125}I -calmodulin with both synaptic and coated vesicles (Figures 11 and 12) only in the presence of Ca^{2+} indicated that calmodulin binding to the vesicles was Ca^{2+} dependent. Calmodulin radioimmunoassay competition curves for chicken gizzard calmodulin, and synaptic and coated vesicles are displayed in Figure 13. As determined by this procedure, synaptic, coated and decoated vesicles contain 0.98, 1.05 and 1.10 μg immunoreactive calmodulin/mg protein, respectively. When synaptic coated and decoated vesicles were treated to remove their endogenous calmodulin, calmodulin content decreased below the limit of detection.

The effects of increasing concentrations of troponin-C and unlabelled calmodulin on the binding of [^{125}I]-calmodulin to synaptic and coated vesicles is illustrated in Figures 14A and 14B, respectively. Troponin-C, a homologous Ca^{2+} -binding protein found in striated muscle, inhibited 12% and 2% of [^{125}I]-calmodulin binding at the maximum concentrations used in synaptic and coated vesicles, respectively. Unlabelled

Figure 11. Sucrose gradient profile of synaptic vesicles. Calmodulin-depleted synaptic vesicles (6.0 mg total protein) were incubated with 25 μ g [^{125} I]-calmodulin (total cpm=100,262) in the presence of 10^{-6} M CaCl_2 or 10^{-3} M EGTA and centrifuged at 80,000 x g. Aliquots of 1.5 ml were withdrawn and the values measured are plotted as a function of optical density at 280 nm (Δ) and cpm x 10^3 . (O)=cpm in the presence of 10^{-6} M Ca^{2+} , $\bullet$ =cpm in the presence of 10^{-3} M EGTA.

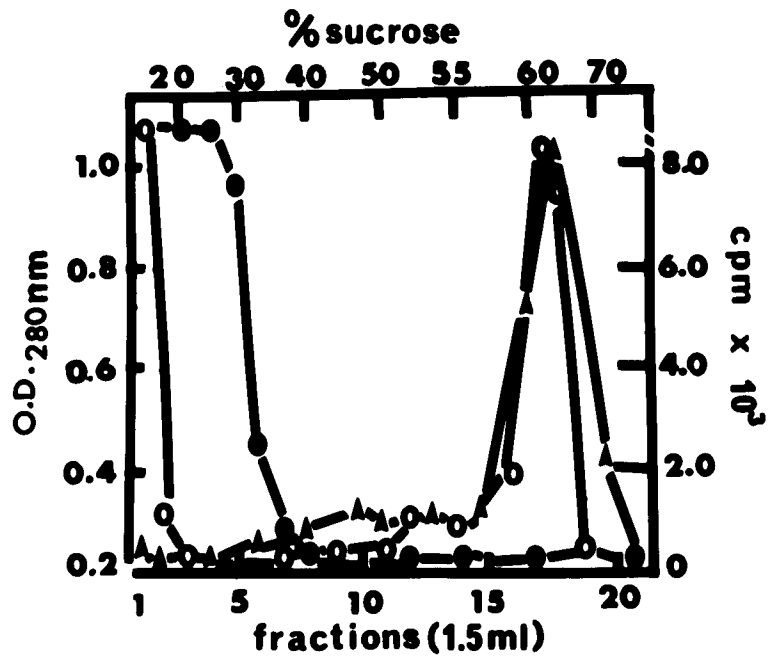


Figure 12. Sucrose gradient profile of coated vesicles. Calmodulin coated vesicles (4.5 mg total protein) were incubated with [^{125} I]-calmodulin and treated as described in legend for Fig. 11. (Δ)=optical density 280 nm; ($\bigcirc$)=cpm in the presence of 10^{-8} M Ca^{2+} ; ($\bullet$)=cpm in the presence of 10^{-3} EGTA.

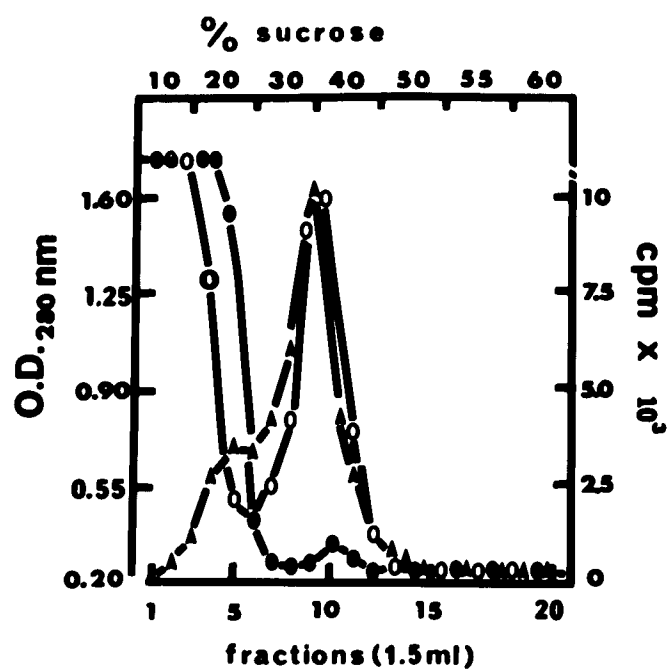


Figure 13. Competition radioimmunoassay curves for chicken gizzard calmodulin (■) synaptic (●) and coated vesicles (▲). Reaction mixtures containing a limiting dilution of anti-calmodulin sera and varying concentrations of unlabelled calmodulin, synaptic or coated vesicles were incubated at 4°C for 8 hours. ¹²⁵I-calmodulin was added and the solution incubated at 4°C for an additional 8 hours. Goat anti-rabbit serum was then added and the mixtures incubated at 4°C for an additional 8-24 hours. The mixtures were washed and then centrifuged at 900 x g for 10 minutes. This procedure was repeated once. Radioactivity in the pellets was determined. Calmodulin concentration in synaptic and coated vesicles was quantitated by comparing the amounts of vesicle protein and unlabelled calmodulin protein that inhibited 50% of ¹²⁵I-calmodulin antibody binding.

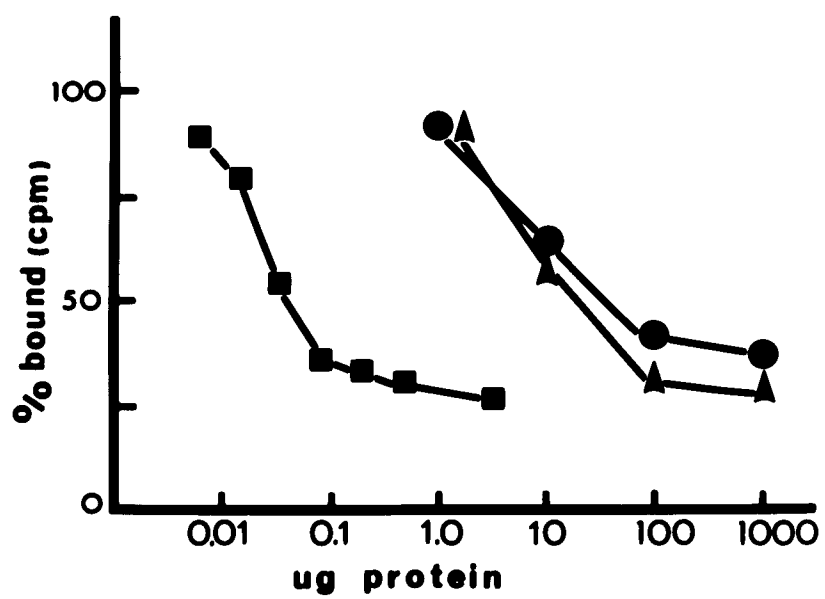
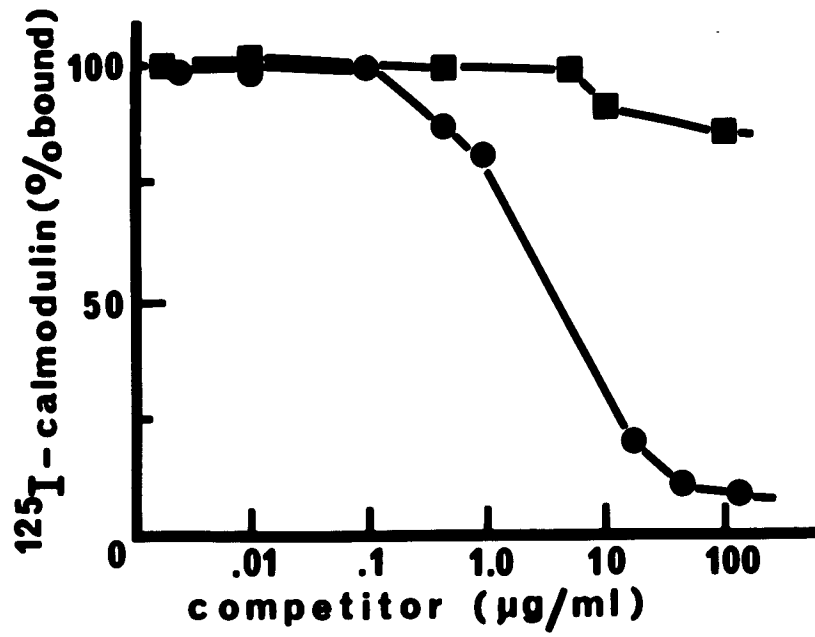


Figure 14. Competition of unlabelled calmodulin and troponin-C with radio-labelled calmodulin in synaptic (A) and coated (B) vesicles. A series of 0.4 mg total protein of synaptic or coated vesicles were incubated with a range of 0-100 μ g of troponin-C or unlabelled calmodulin for two hours at 25°C. Radio-labelled calmodulin (10 ng) was added and the suspension incubated for an additional two hours in the presence of Ca^{2+} . The samples were centrifuged at 150,000 x g for 30 minutes and radioactivity in the pellet measured. [^{125} I]-calmodulin (% bound) is plotted as a function of competing concentrations of calmodulin (●) and troponin-C (■).

A



B

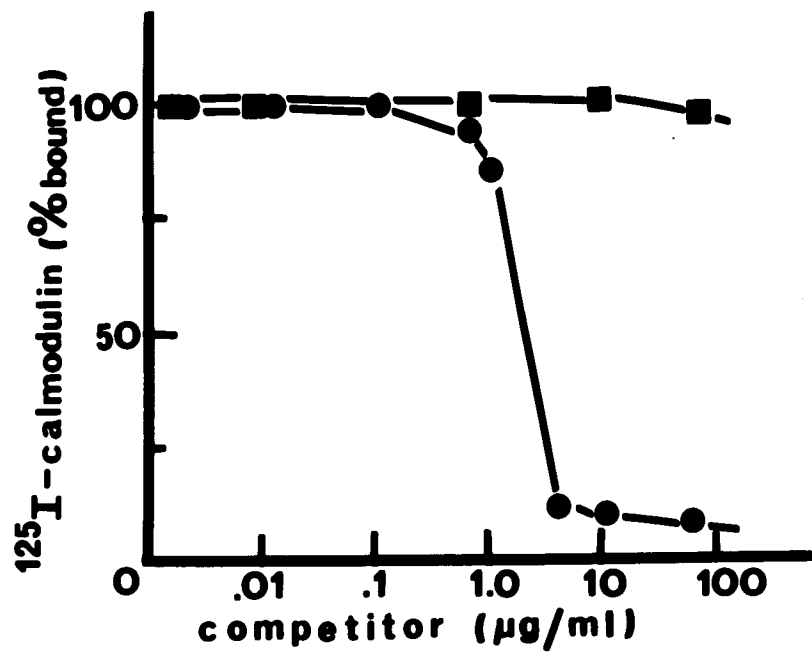
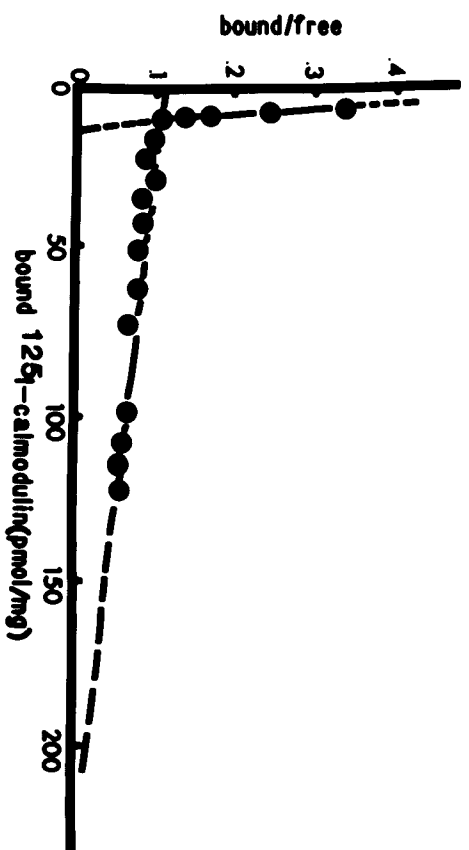
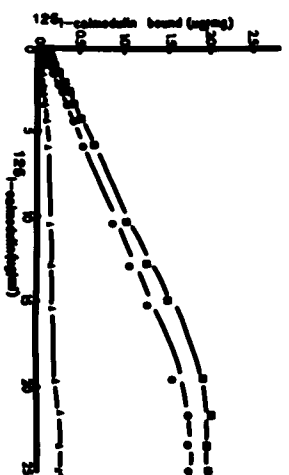


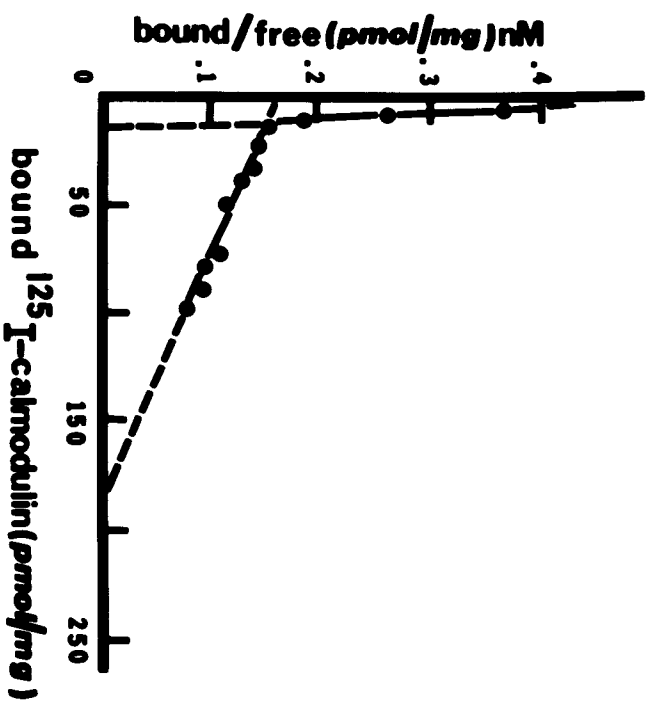
Figure 15. Scatchard analysis of specific [^{125} I]-calmodulin binding to synaptic (A1) and coated (B1) vesicles. Data points fit two straight lines indicating more than one binding site. Dashed lines are extrapolations derived from the solid lines constructed between the experimental data points. Nonspecific binding ($\blacktriangle$) was subtracted from the total binding ($\blacksquare$) to determine specific binding ($\bullet$) (Figures A2 and B2).



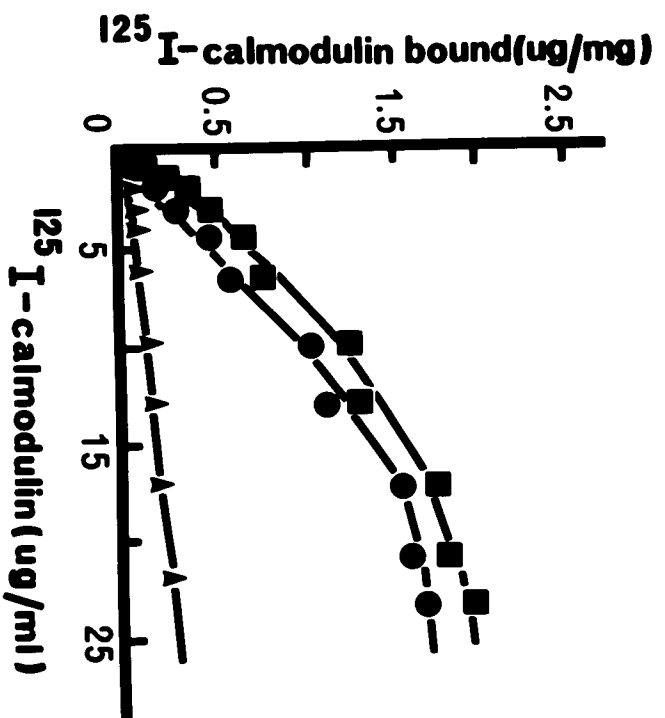
A1



A2



B1



B2

Figure 16. Elution profile of solubilized synaptic vesicle proteins on a Sepharose-4B-CNER-calmodulin affinity column. Optical density at 280 nm is plotted as a function of elution volume (ml). Elution peak is accompanied by its corresponding polypeptide composition determined by 5-15% SDS-PAGE. Twenty-five ml of the calcium buffer and 15 ml of the EGTA buffer were used for protein elutions.

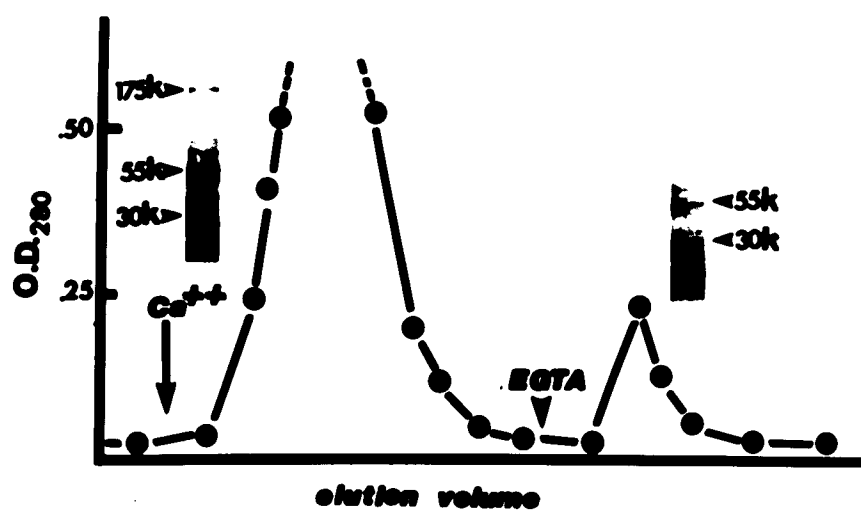
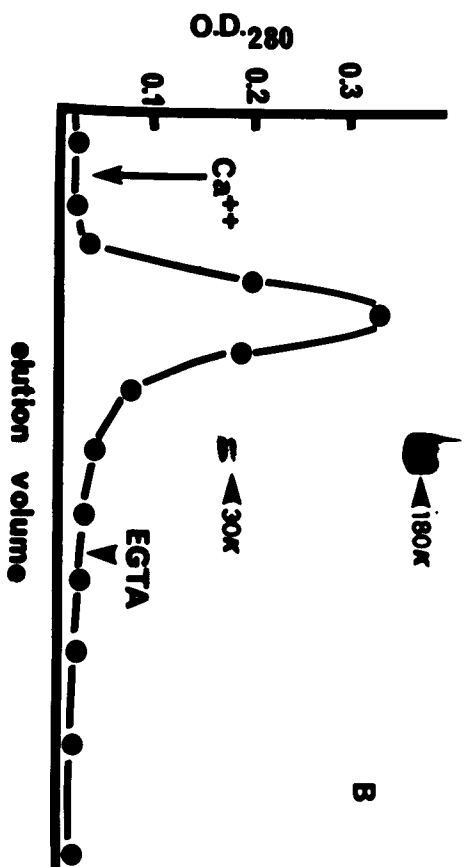
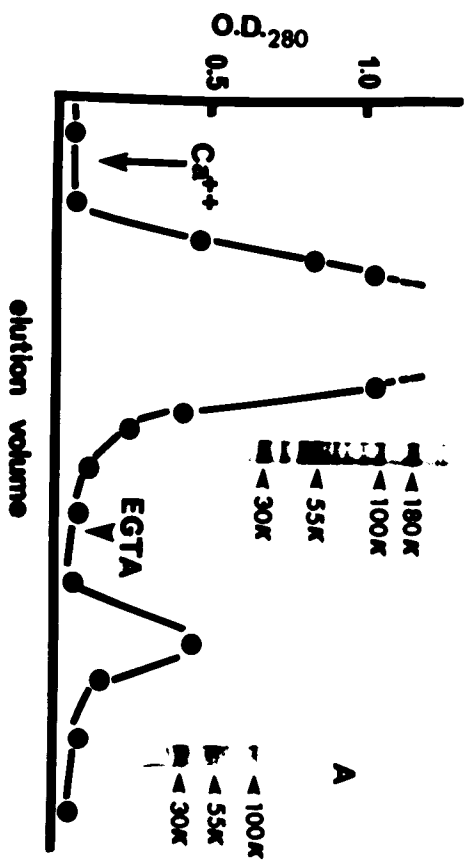


Figure 17. Elution profiles of Triton X-100-treated coated vesicle extract (A), purified clathrin (B), and purified 30,000 M_r protein doublet (C). Polypeptide composition as determined by 5-15% SDS-polyacrylamide gel electrophoresis accompanies each elution peak. The eluate was collected in 1-ml fractions; 25 ml of the Ca^{2+} buffer and 15 ml of the EGTA buffer were used for protein elutions. Optical density at 280nm is plotted as a function of elution volume.



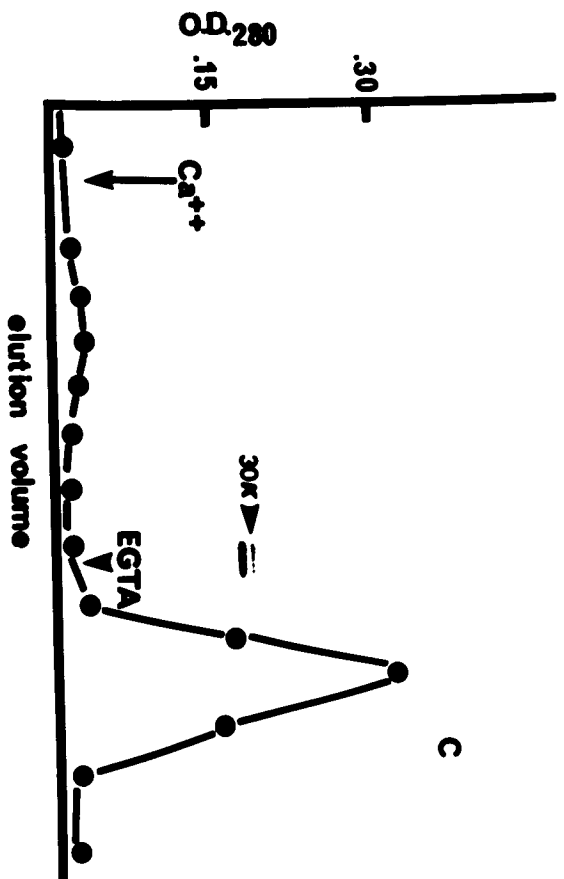


Table 1

Affinity values of specific [^{125} I]-calmodulin binding to synaptic and coated vesicles.

Vesicle type	Site I		Site II		$B_{\max}$ total pmol/mg
	K_d nM	$B_{\max}$ pmol/mg	K_d nM	$B_{\max}$ pmol/mg	
Synaptic	1.0 (± 0.15)	4.1 (± 0.6)	177 (± 12.0)	202 (± 15.0)	206
Coated	3.9 (± 0.6)	16.3 (± 2.4)	102 (± 15.0)	151 (± 23.0)	167

K_d and $B_{\max}$ values were obtained from Scatchard analysis of specific [^{125} I]-calmodulin binding to synaptic and coated vesicles shown in Figures 15A1 and 15B1, respectively.

calmodulin blocked 89% and 88% of [^{125} I]-calmodulin binding at the highest concentrations used in synaptic and coated vesicles, respectively. The concentration of unlabelled calmodulin which inhibited 50% of [^{125} I]-calmodulin binding (IC_{50}) was 400 and 300 nM for synaptic and coated vesicles, respectively. The relatively ineffective competition of troponin-C for radio-labeled binding sites implied that the sites were specific for calmodulin.

A Scatchard plot of specific [^{125} I]-calmodulin binding to synaptic and coated vesicles is illustrated in Figures 15A1 and 15B1, respectively. Levels of non-specific binding determined from the aforementioned competition studies were subtracted from the total binding to calculate specific binding. Scatchard plots for both synaptic and coated vesicles displayed curves with downward slopes not corresponding to straight lines, suggesting more than one binding site. Affinity values obtained, based on the assumption of two existing binding sites, are presented in Table I. The K_d s for both sites are similar for the two types of vesicles. The B_{max} values obtained for each site and the B_{max} total for both vesicle types also are comparable. There appeared to be one high-affinity, low-binding-capacity site, and one low affinity, high binding-capacity site.

The elution profile of synaptic and coated vesicle proteins by calmodulin affinity chromatography is shown in Figures 16 and 17A, respectively. As determined by optical density measurements at 280nm, most of the synaptic and coated vesicle proteins were eluted by calcium. The protein composition of the calcium eluates as detected by SDS gel analysis appear next to the calcium elution peaks in Figures 16 and 17A. The polypeptide composition of the EGTA protein eluates

appears to the right of the EGTA elution peak in Figures 16 and 17A, respectively. The major synaptic vesicle polypeptides that bound to the calmodulin affinity column in a calcium dependent manner were protein doublets of approximate M_r s 55K and 30K. The major coated vesicle polypeptides that bound to the column in a calcium dependent manner had M_r s of approximately 100K, 55K and 30K. The proteins that eluted with EGTA appeared to have the same approximate M_r s as some of the proteins that were eluted by calcium. The proteins in the two different eluates could represent different comigrating proteins with similar M_r s that separated from each other by calmodulin affinity chromatography. Alternatively, the proteins in both eluates may represent the same proteins. It is possible that only a fraction of the proteins bound to the calmodulin affinity column, either because the protein binding capacity of the column was exceeded or because only a fraction of the proteins bound with high enough affinity to the column.

The fact that multiple proteins bound to the calmodulin affinity column in both synaptic and coated vesicles implied that the downward slope of the Scatchard plot was indicative of the vesicles having multiple calmodulin binding sites.

To verify whether clathrin of coated vesicles itself was capable of binding to calmodulin, the protein was passed through the calmodulin-affinity column. The elution profile of clathrin is shown in Fig 17B. Clathrin and the 30,000 M_r doublet that consistently co-purifies with it did not bind to calmodulin, and both were eluted in the buffer containing Ca^{2+} . Figure 17C illustrates the elution profile of the purified 30,000 M_r protein doublet. The protein was eluted entirely with the EGTA buffer. The 30,000 M_r doublet protein did not bind to calmodulin

when it was co-purified with clathrin; however, it did bind after it was purified by itself. This may be due to the fact that the 30,000 M_r doublet protein represents a minor portion of the purified clathrin preparation. Alternatively calmodulin may compete with clathrin for binding to the 30K doublet.

The possible identities of the calmodulin binding proteins will be presented in the discussion.

4. Membranal PLA₂ activity

a. Synaptic vesicle PLA₂

Synaptic vesicle PLA₂ activity as a function of Ca^{2+} concentration is demonstrated in Figure 18. It is apparent that there is a very low baseline activity in the absence of Ca^{2+} . Activity increased with increasing Ca^{2+} concentration reaching maximal activity at 10 μM Ca^{2+} and began to decline beyond 5mM $CaCl_2$. Dependence of PLA₂ on pH, incubation time and substrate concentration is shown in Figure 19. The pH curve of the enzyme is broad, with a pH optimum of 9.0 (Fig. 19A). Maximum activity manifested after 60 minutes of incubation (Fig. 19B) and 2.8 nmol of substrate (Fig. 19C). Transformation of the linear portion of the substrate concentration curve (Fig. 19C) into a double reciprocal Lineweaver-Burk plot is displayed in Figure 20. The K_m was 60 μM and the V_{max} was 2.0 nmol/mg/h.

Effects of various modulators on synaptic vesicle PLA₂ activity: No modulator significantly altered the apparent K_m . Effects were manifested as an inhibition or stimulation of V_{max} . Figure 20 graphically compares the effects of PGE₂ and PGF_{2 α} on the Lineweaver-Burk plot. In the presence of PGF_{2 α} and PGE₂, the V_{max} was 16.0 and 0.6 nmol/mg/hr,

Figure 18. Synaptic vesicle PLA₂ activity is plotted as a function of Ca²⁺ concentration. Reaction mixtures were incubated with 1.85-2.8 nmol [¹⁴C]- α -arachidonyl phosphatidylcholine in the presence of 5mM EGTA and increasing calcium concentration.

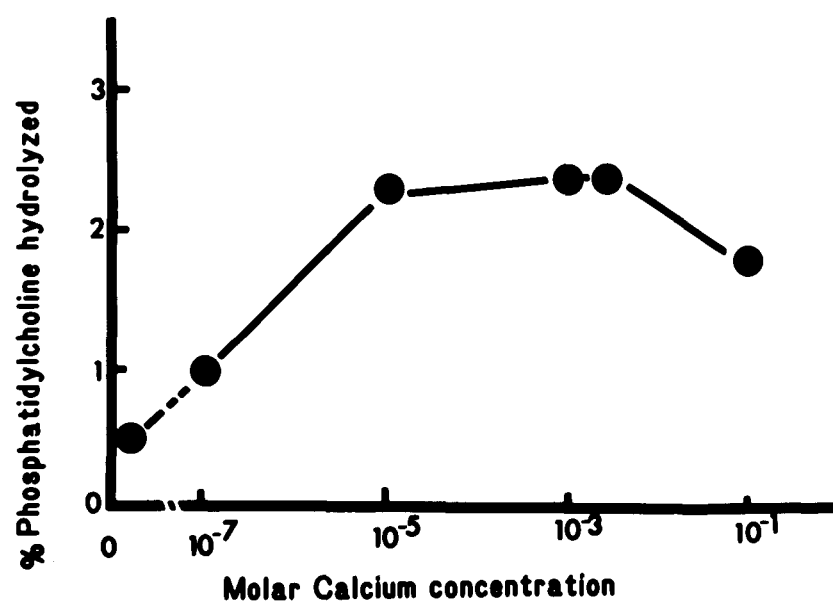
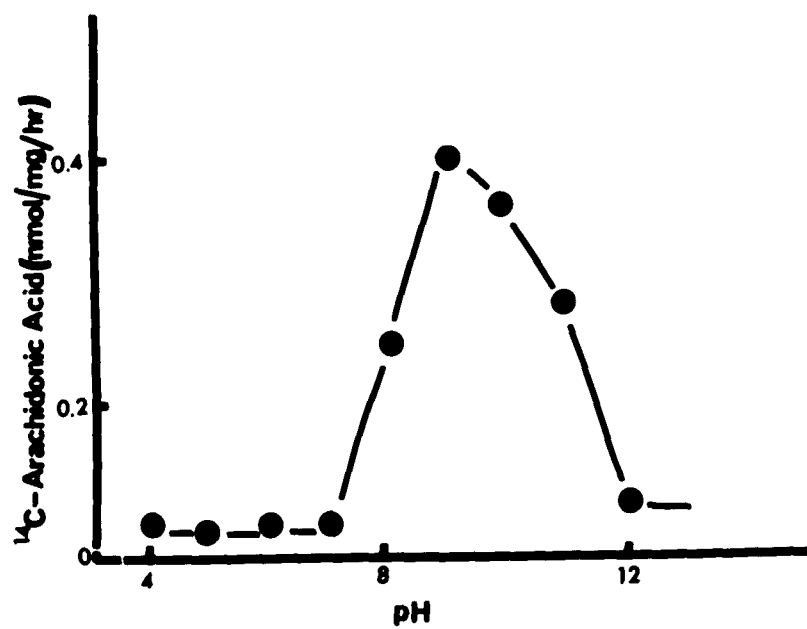
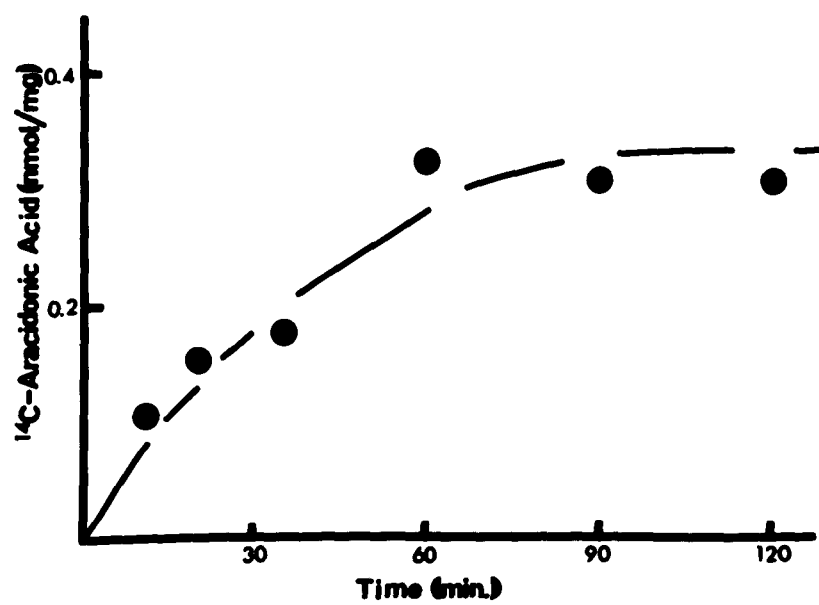


Figure 19. Characteristics of synaptic vesicle FLA₂: The pH (A), time (B) and substrate concentration (C) ranges were determined in the presence of 2 mM CaCl₂.

A



B



C

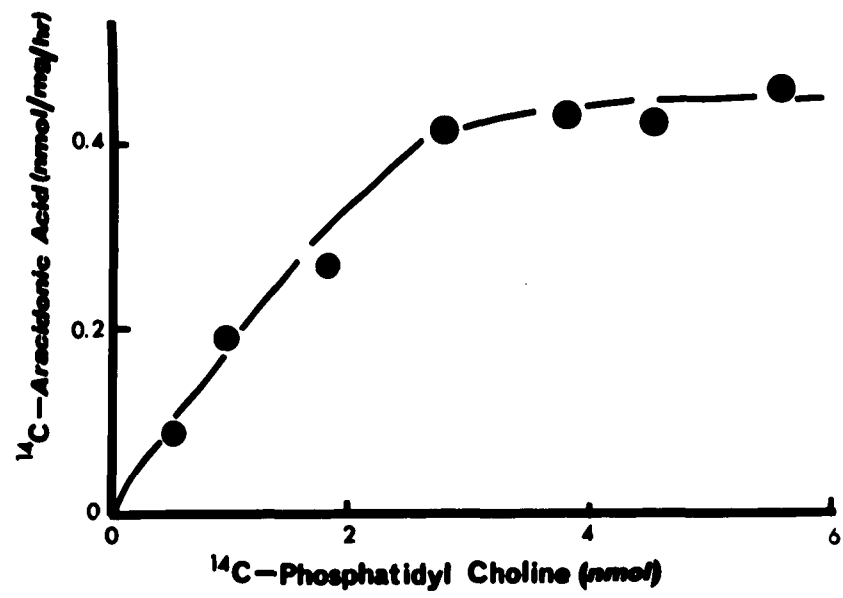
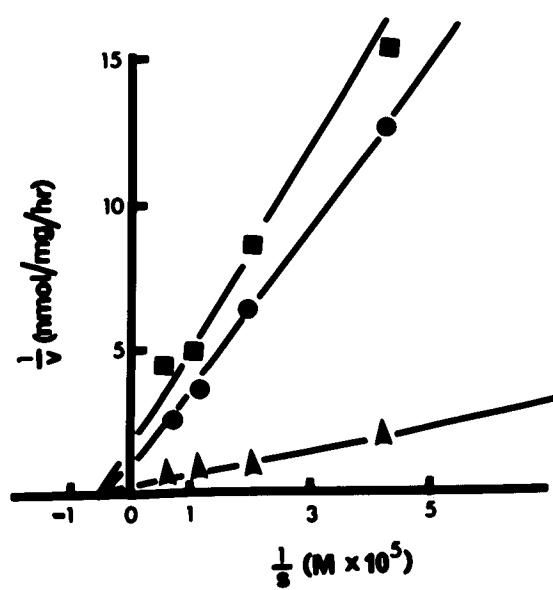


Figure 20. Lineweaver-Burk transformations of synaptic vesicle PLA₂ substrate concentration curves in the presence of CaCl₂ (●), CaCl₂ and PGE₂ (■), or CaCl₂ and PGF_{2α} (▲). ($r = -0.99$).



respectively. Thus $\text{PGF}_{2\alpha}$ stimulated and PGE_2 inhibited PLA_2 activity by 700% and 70%, respectively. Table 2 illustrates the effects of other conditions on the enzymatic $V_{\max}$ as determined from Lineweaver-Burk plots ($r=0.97-0.99$). Cyclic-AMP stimulated the $V_{\max}$ by only 50%; however, cAMP and ATP together stimulated the $V_{\max}$ 200%. Prostaglandin E_2 not only inhibited baseline PLA_2 activity but also inhibited calmodulin-mediated PLA_2 stimulation. To correlate the modulation of synaptic vesicle PLA_2 activity with function, synaptic vesicle aggregation was assayed with a Behring Laser nephelometer which measured the amount of light scattered by synaptic vesicles. When synaptic vesicles were incubated with 2mM CaCl_2 , only a 40% increase in light scattering was observed (Figure 21). Exogenous PLA_2 induced a rapid increase in light scattering within 10 seconds after its addition (Figure 21A). As will be detailed later, this profile was indicative of synaptic vesicle aggregation and sedimentation. The entire process of aggregation and sedimentation occurred within 60 seconds (Figure 21A). A light scattering profile similar to that obtained with PLA_2 was obtained by adding lysolecithin (Figure 21A). This suggested that exogenous PLA_2 hydrolyzed synaptic vesicle phosphatidylcholine, leading to production of lysolecithin which induced the subsequent light scattering profile. Both PLA_2 (*Vipera russelli*) and lysolecithin induced a dose dependent increase of synaptic vesicle light scattering (Figure 22).

Conditions modulating PLA_2 activity were tested for their potential for altering the synaptic vesicle light scattering profile. Calmodulin and Prostaglandin $\text{F}_{2\alpha}$ both increased synaptic vesicle light scattering in a dose dependent manner (Figures 23 and 24). Calmodulin had no significant effect on light scattering below a concentration of

Table 2

Effects of different modulators on synaptic vesicle PLA₂ V_{max}.

Modulator	V _{max} nmol arachidonic acid /mg/hr	% change/stimulation (+), inhibition (-)
cAMP (1 mM)	3.0	50 (+)
ATP (1mM)	1.0	50 (-)
cAMP (1mM) and ATP (1 mM)	6.0	200 (+)
Calmodulin (1μM)	9.0	300 (+)
PGE ₂ (4.0 nmol) and Calmodulin (1μM)	1.0	50 (-)

The V_{max}s were determined from Lineweaver-Burk transformations of substrate concentration curves (r=0.97-0.99).

Figure 21A. The effect of Ca^{2+} followed by PLA_2 *Vipera russelli* (●) or lysolecithin (■) on synaptic vesicle light scattering.

Figure 21 B. The effect of the sequential addition of Ca^{2+} and calmodulin (CaM) (●) on synaptic vesicle light scattering. The effect of preincubation of synaptic vesicles with PGE_2 = (■), with parabromophenacylbromide (PB) = (X), or with quinacrine (Qn) = (▲) are illustrated.

21C. The effect of the sequential addition of Ca^{2+} and calmodulin after preincubation with $\text{PGF}_{2\alpha}$ (●) on synaptic vesicle light scattering. Preincubation of synaptic vesicles with parabromophenacylbromide (PB) prior to addition of $\text{PGF}_{2\alpha}$ = (■), is illustrated.

21D. The effect of sequential addition of CaCl_2 , MgCl_2 , cAMP and ATP (●) on synaptic vesicle light scattering. The effect of the same sequence after preincubation with parabromophenacylbromide (PB) = (■). Light scattering in nephelometer units are plotted as a function of time.

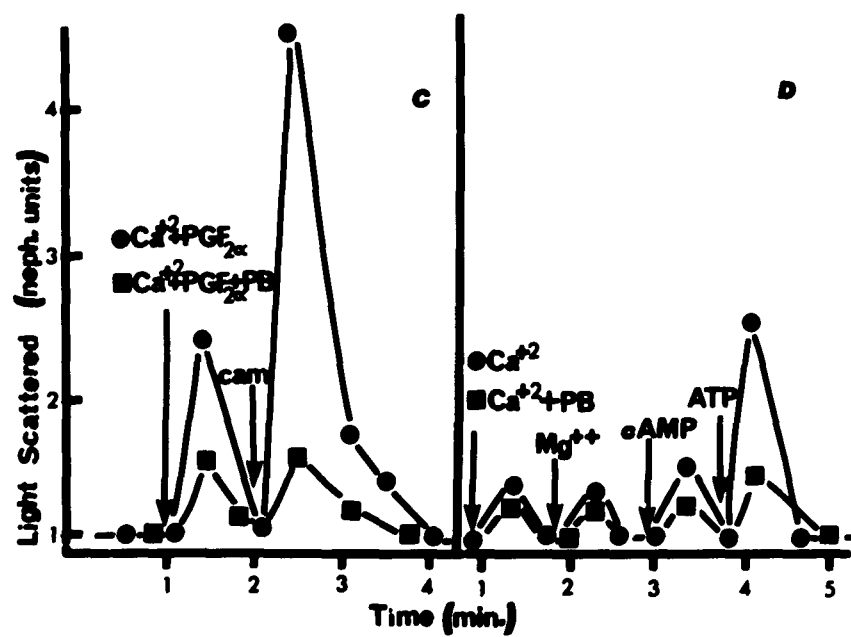
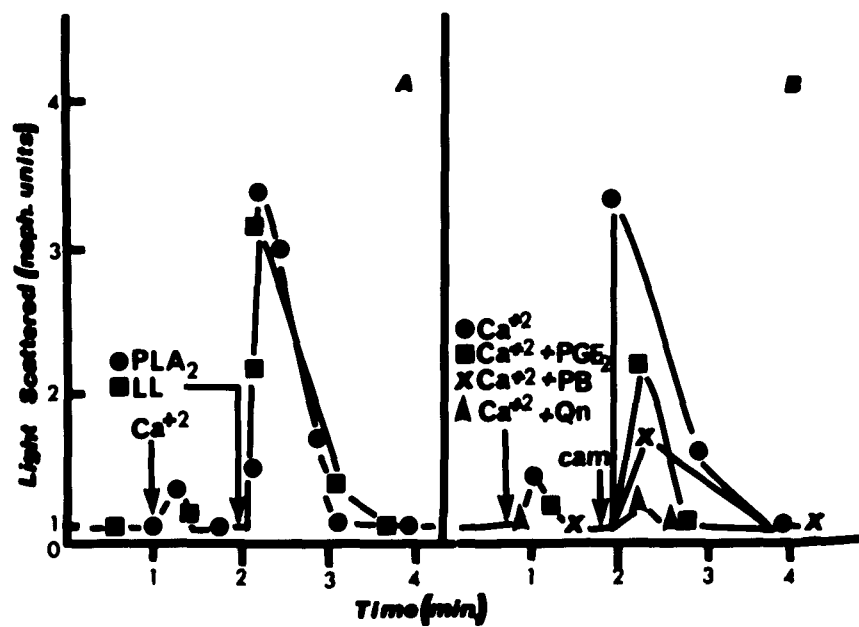
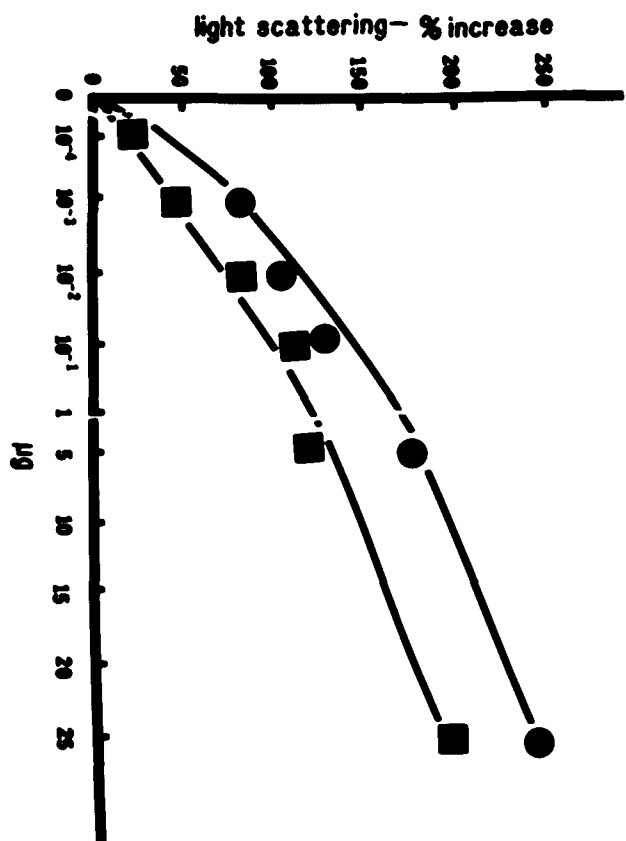


Figure 22. Synaptic vesicles (30 μ g/ml) were incubated at pH 6.5 with increasing concentrations of PLA₂ (●) or lysolecithin (■) in the presence of 2mM CaCl₂ in a total volume of 300 μ l. Plotted is the percent increase of light scattering as a function of PLA₂ or lysolecithin concentration. The points on the graph represent the peak of synaptic vesicle light scattering induced by PLA₂ or lysolecithin within 10-15 seconds of its addition to the synaptic vesicle suspension.



1.0×10^{-2} $\mu\text{g}/300\mu\text{l}$. Increasing concentrations of calmodulin beyond this concentration led to further increases in light scattering, reaching a maximal effect at a concentration of $5.0 \mu\text{g CaM}/300\mu\text{l}$ (Figure 23). $\text{PGF}_{2\alpha}$ led to a dose dependent increase in light scattering, reaching its maximal effect at 3.0×10^{-3} $\mu\text{g}/100\mu\text{l}$ (Figure 24). The light scattering profile of synaptic vesicles induced by $1\mu\text{M}$ calmodulin is displayed in Fig 21C. Calmodulin increased light scattering within 10 seconds of its introduction. Baseline light scattering returned within 2 minutes. The profile was similar to that induced by exogenous PLA_2 .

Prostaglandin E_2 (4.0 nmol) which inhibited the calmodulin-induced stimulation of PLA_2 activity, inhibited calmodulin-mediated light-scattering increase (Fig. 21B). Prostaglandin E_2 alone produced no observable effects. In the presence of Ca^{2+} , $\text{PGF}_{2\alpha}$ (4.0 nmol) stimulated an increase in synaptic vesicle light scattering (Fig. 21C) within 10 seconds as well as potentiating calmodulin-mediated light scattering increase (Fig. 21C).

Inhibition of these effects by preincubation with parabromophenacylbromide demonstrated they were due at least in part to interaction of $\text{PGF}_{2\alpha}$ with synaptic vesicle PLA_2 . Effects of the synaptic vesicle light-scattering profile by sequential addition of Ca^{2+} , Mg^{2+} , and 1mM ATP are illustrated in Figure 21D. Calcium and Mg^{2+} produced small effects. Introduction of cAMP induced a 50% increase in light scattering; addition of ATP induced a 150% increase. The latter effect was equivalent to that attained by $\text{PGF}_{2\alpha}$. Reduction of these light-scattering effects upon preincubation with parabromophenacylbromide demonstrated that these effects were due in part to interaction with synaptic vesicle PLA_2 .

Figure 23. Synaptic vesicles (30 μ g/ml) were incubated with increasing concentrations of calmodulin in the presence of 2mM CaCl₂, pH 6.5 in a total volume of 300 μ l. The percent increase of synaptic vesicle light scattering is plotted as a function of calmodulin concentration. The points on the graph represent the maximal increase of light scattering induced by calmodulin within 10-15 seconds of its addition to the synaptic vesicle suspension.

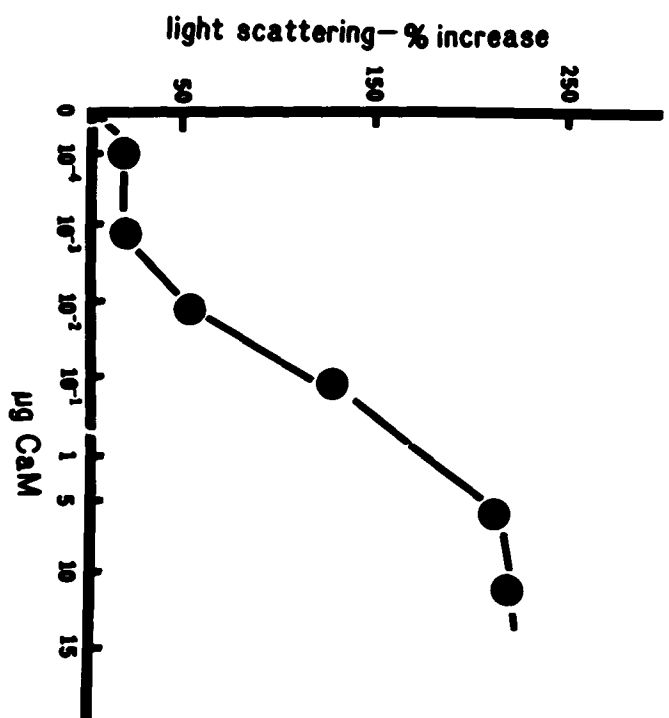
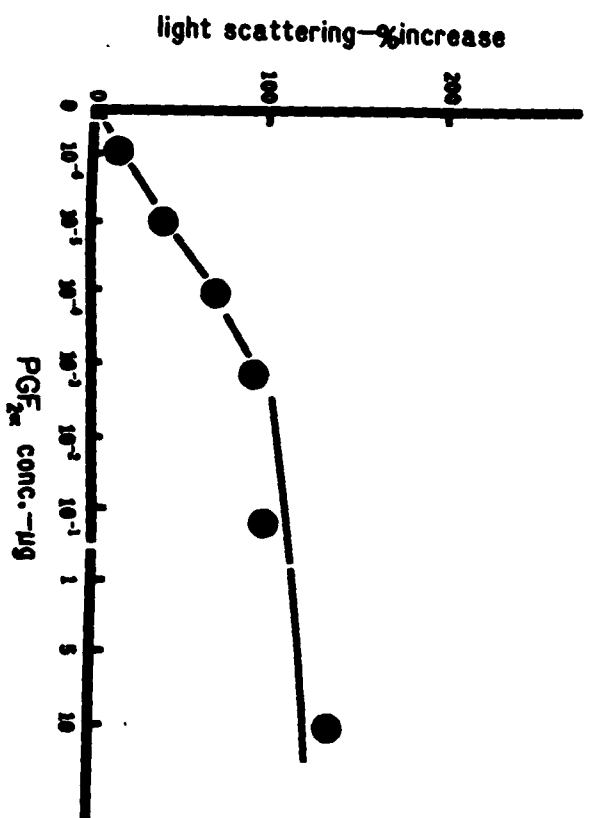


Figure 24. Synaptic vesicles (30 μ g/ml) were incubated with increasing concentrations of PGF_{2 α} in the presence of 2mM CaCl₂, pH 6.5 in a total volume of 300 μ l. The percent increase of synaptic vesicle light scattering is plotted as a function of PGF_{2 α} concentration. The points on the graph represent the maximal increase of light scattering induced by PGF_{2 α} within 10-15 seconds of its addition to the synaptic vesicle suspension.



All the light scattering experiments were performed at 25°C at pH 6.5. When these experiments were performed at pH 9.0 the results were similar to the ones obtained under the above conditions. However, at pH 9.0 parabromophenacylbromide induced a 24.3% (± 8.4) greater inhibition of light scattering than at pH 6.5. This was in line with the observation that this compound exerts its maximum inhibitory effect on PLA₂ at basic pH values.

Although maximal PLA₂ activity as determined by the enzymatic assay was achieved at pH 9.0, it is emphasized that this assay measured the amount of exogenously added phosphatidylcholine capable of being hydrolyzed by endogenous synaptic vesicle PLA₂. The high pH may be necessary for the maximum exposure of synaptic vesicle membrane PLA₂ to come into contact with and subsequently hydrolyze exogenous substrate. The nephelometer experiments, however, represent a functional manifestation of PLA₂ hydrolysis of endogenous synaptic vesicle phosphatidylcholine. At pH 6.5 there is probably adequate interaction between the substrate and the enzyme.

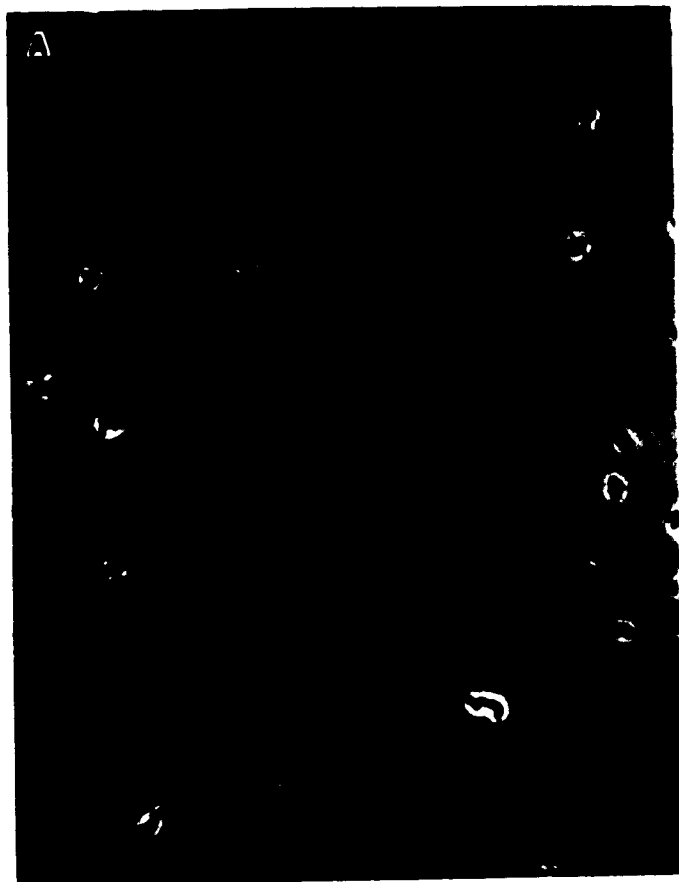
In order to interpret the significance of the light scattering profiles induced by the various modulators, phase contrast and electron microscopy were employed. Untreated synaptic vesicles (0.75mg/ml) appear to contain a small amount of clumped and aggregated vesicles observable by phase contrast microscopy (Figure 25A). In the presence of calcium and exogenous PLA₂ (*Vipera russelli*) or lysolecithin (egg yolk), a large increase in the size of the vesicle aggregates occurred (Figures 25B and 25C). When the vesicles were incubated with only 2mM calcium or with 2mM calcium and 4.0nmol PGE₂ or 1mM cAMP and 10mM Mg²⁺, the morphological appearance of the vesicles was identical to that of

untreated vesicles. Incubation of the synaptic vesicles with 2mM calcium and 4.0nmol $\text{PGF}_{2\alpha}$ induced a significant increase in the size of the vesicle aggregates (Figure 25D). This effect was similar to that induced by PLA_2 or lysolecithin (Figures 25B and 25C). Incubation of synaptic vesicles with 1mM Ca^{2+} , 10mM Mg^{2+} , 1mM cAMP and 1mM ATP also induced moderate increase in the number and size of synaptic vesicle aggregates (Figure 25E).

Electron microscopy of synaptic vesicles prior to any experimental treatment appears in Figure 26A. Synaptic vesicles appear to be relatively intact. Incubation of synaptic vesicles with 1mM calcium and 25 μg exogenous PLA_2 (*Viperra russelli*) induced vesicle aggregation, lysis and possible fusion of lysed membranal fragments as judged by electron microscopy (Figure 26B). Incubation of the synaptic vesicles with either 2mM calcium or 2mM calcium and 4.0nmol PGE_2 , or 1mM cAMP and 10mM Mg^{2+} , or 1mM ATP and 10mM Mg^{2+} did not lead to any morphological changes in any way different from that observed in untreated vesicles. Incubation of the synaptic vesicles with 1 μM calmodulin or 4.0nmol $\text{PGF}_{2\alpha}$ in the presence of 2mM calcium led to morphological membranal changes very similar to that induced by exogenous PLA_2 (Figures 26C and 26D). Cyclic-AMP, ATP and Mg^{2+} induced slight membranal perturbations (Figure 26E).

In all the electron micrographs, either the most prevalent morphological entities observed or that morphological entity which appeared with greater frequency throughout the pellet compared to the untreated samples are presented. In all cases, with the exception of incubation with exogenous PLA_2 , vesicles of normal and intact morphology were also observed. Exogenous PLA_2 however induced alterations in all the

Figure 25. Light microscopy of synaptic vesicles (0.75mg/ml) in a total volume of 200 μ l, untreated (A); treated with 25.0 μ g PLA₂ (*Viperra russelli*) (B); 20 μ g lysolecithin (egg yolk) (C); 4.0 nmol PGF_{2 α} (D); 1mM cAMP, 1mM ATP, 10mM MgCl₂ and 2mM CaCl₂ (E). Magnification x 4,330.



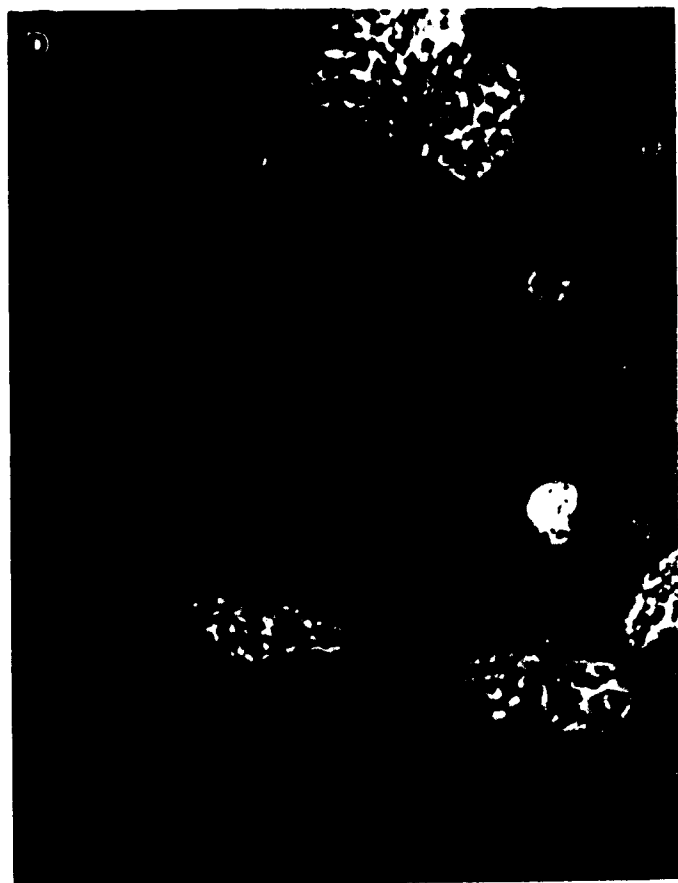
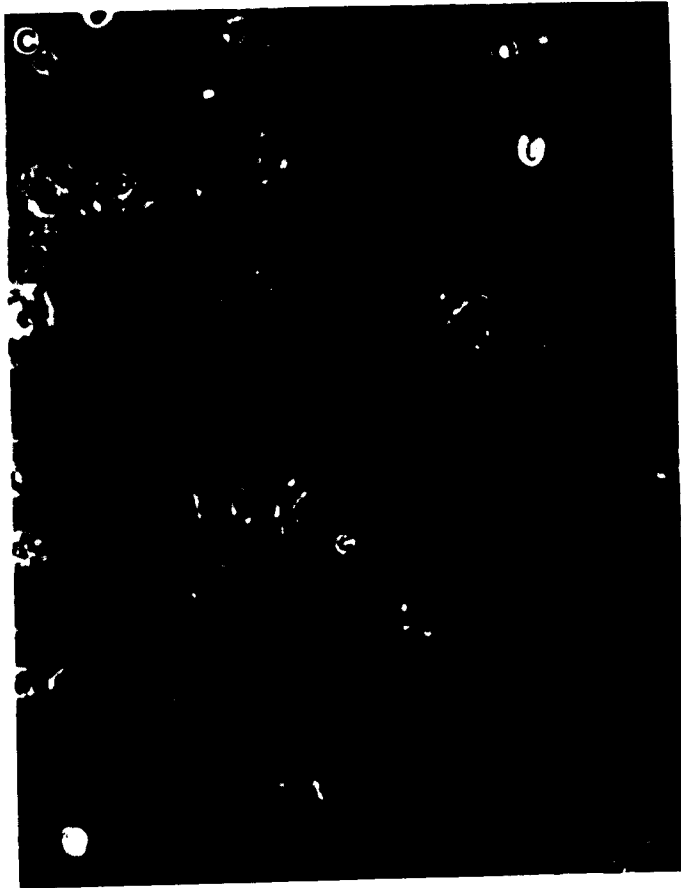
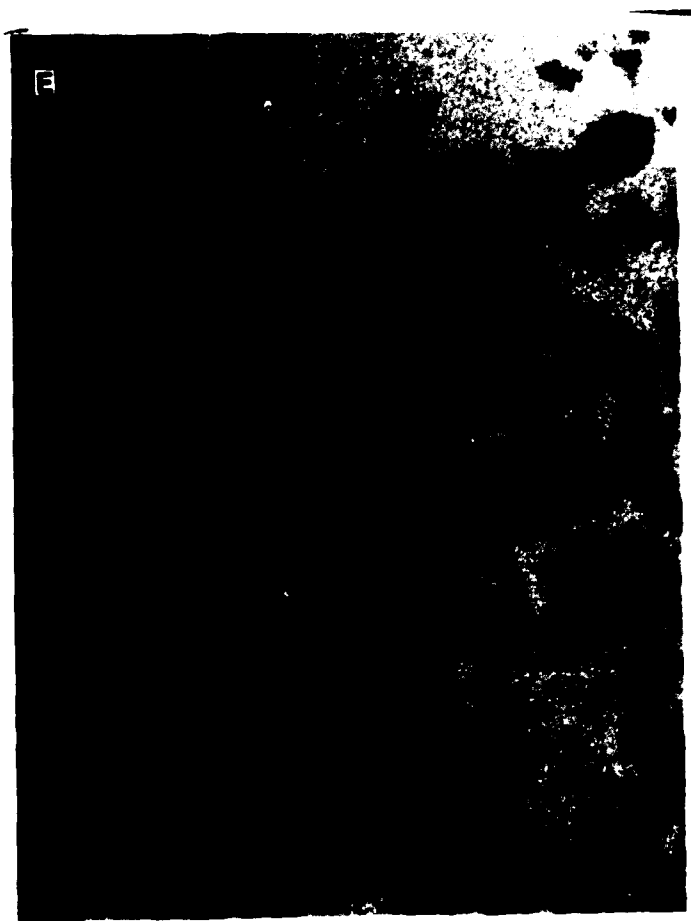




Figure 26. Electron microscopy of synaptic vesicles (100 μ g) in a total volume of 200 μ l untreated (A); treated with 1mM CaCl₂ and 25 μ g PLA₂ (Viperra russelli) (B); 2mM CaCl₂ and 1 μ M CaM (C); 2mM CaCl₂ and 4.0nmol PGF_{2 α} (D); 2mM CaCl₂, 10mM Mg²⁺, 1mM ATP and 1mM cAMP (E). All the vesicles were treated for 30 minutes under the above conditions and stopped with 400 μ l 3% gluteraldehyde buffer, centrifuged at 2,000 x g for 10 minutes and observed under a JEOL 100 B electron microscope. Magnification x 120,000.







synaptic vesicles viewed by electron microscopy.

Table 3 summarizes the effects of the modulators tested on synaptic vesicle PLA₂ activity, light scattering profiles and morphological changes detected by light and electron microscopy.

In general, those modulators which inhibited PLA₂ activity — PGE₂ and ATP — did not induce any significant changes in the light scattering profile of synaptic vesicles or in their morphological structure. Calmodulin, which stimulated PLA₂ activity, increased light scattering of the synaptic vesicles and induced observable membranal alterations on the electron microscopic level but not on the light microscopic level. Apparently, calmodulin may have induced a small scale aggregation of synaptic vesicles which was not visible on the light microscopic level. However, observation by electron microscopy did detect the aggregation induced by calmodulin. PGF_{2α}, which induced the greatest stimulation of PLA₂ activity of all the modulators tested, also induced the greatest change in morphology as detected by both light and electron microscopic observations. However, it did not increase light scattering to the same extent as calmodulin did, which stimulated PLA₂ activity less than PGF_{2α} did. cAMP and ATP induced a light scattering profile similar to that induced by PGF_{2α} even though these compounds led to a 150% increase in PLA₂ activity and led to only minor structural changes in synaptic vesicles morphology. The PLA₂ effects of cAMP and ATP in relationship to protein phosphorylation will be presented later on in the discussion.

It can therefore be concluded that light scattering of synaptic vesicles is a rough indication of synaptic vesicle interactions which can be characterized as aggregation, lysis and possibly fusion.

Table 3

A comparison of the effects of several conditions on synaptic vesicle PLA₂ activity, light scattering profile and morphology as judged by light and electron microscopy.

Condition	% stimulation (+)/ inhibition (-)		Δ Morphology	
	PLA ₂ activity	light scattering	Light Microscopy	Electron Microscopy
Ca ²⁺	control	40 (+)	0	0
Calmodulin	350 (+)	250 (+)	0	+++
PGF _{2α}	700 (+)	150 (+)	+++++	+++++
PGE ₂	50 (-)	40 (+)	0	0
cAMP	50 (+)	90 (+)	0	0
ATP	50 (-)	30 (+)	0	0
cAMP + ATP	150 (+)	150 (+)	++	++

The effects of the conditions on PLA₂ activity are presented as the percent change of synaptic vesicle PLA₂ V_{max} from baseline conditions in the presence of 2mM CaCl₂. The change in light scattering is presented as the percent change induced at the peak of light scattering relative to baseline conditions in the absence of calcium. The morphological changes observed by light and electron microscopy are increased aggregation and/or lysis of synaptic vesicles relative to untreated conditions. The number of pluses (+) is correlated with the intensity of the morphological change. (0) indicates that no significant morphological alterations were observed.

It appears from Table 3 that endogenous synaptic vesicle PLA₂ upon stimulation may lead to synaptic vesicle-vesicle aggregation and fusion. The fact that quinacrine and parabromophenacylbromide inhibited the effects of these compounds supports the hypothesis that endogenous synaptic vesicle PLA₂ is involved in synaptic vesicle interactions.

b. Synaptic plasma membrane PLA₂ activity

The calcium concentration, pH and time dependencies of synaptic plasma membrane PLA₂ are illustrated in Figures 27 and 28. PLA₂ activity was maximally active at 10^{-7} - 10^{-8} M calcium concentration. Beyond this calcium concentration PLA₂ activity dropped drastically reaching almost negligible values at 10^{-1} M CaCl₂ (Figure 27). The pH curve is rather sharp, displaying maximal activity at pH 7.0 (Figure 28A). The enzymatic activity still remained linear after 80 minutes of incubation (Figure 28B).

The enzyme had a K_m of 62 μ M and a V_{max} of 98.0 nmol arachidonic acid/mg/hr at the optimal incubation conditions viz. incubated in the presence of 10^{-7} - 10^{-8} M CaCl₂, pH 7.0 for 30 minutes.

Table 4 lists the effects of several modulators on the enzymatic parameters of PLA₂ activity under optimal conditions. Calmodulin decreased both the K_m and V_{max} of the enzyme, decreasing the K_m more significantly, resulting in a 638% increase in the substrate turnover rate. PGE₂ by itself led to a small (8%) decrease in the substrate turnover rate. However, PGE₂ almost completely inhibited the stimulating effect of calmodulin by inhibiting calmodulin's capacity to significantly decrease the K_m leading to an overall 46% stimulation of the substrate turnover rate.

Table 5 lists the effects of some other modulators on one point

Figure 27. Synaptic plasma membrane PLA₂ activity plotted as a function of calcium concentration. Reaction mixtures were incubated with 5mM EGTA and increasing concentrations of Ca²⁺.

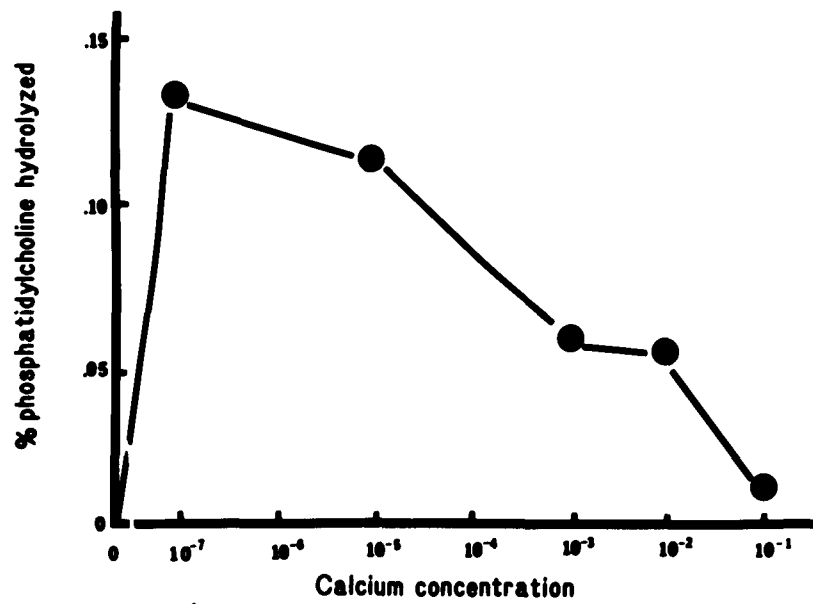


Figure 28. Characteristics of SPM PLA₂: The pH (A) and time dependencies (B) were determined in the presence of 10^{-7} M - 10^{-8} M Ca^{2+} .

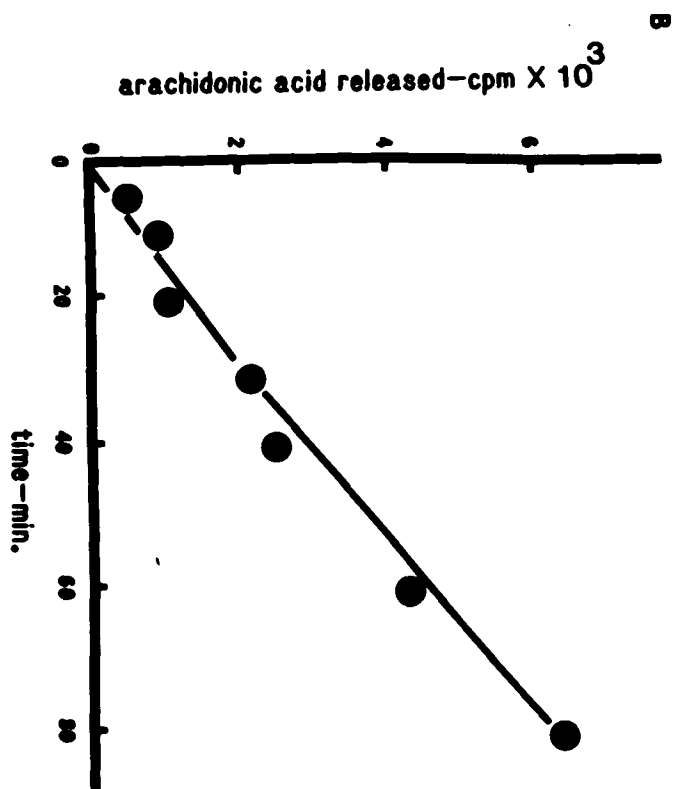
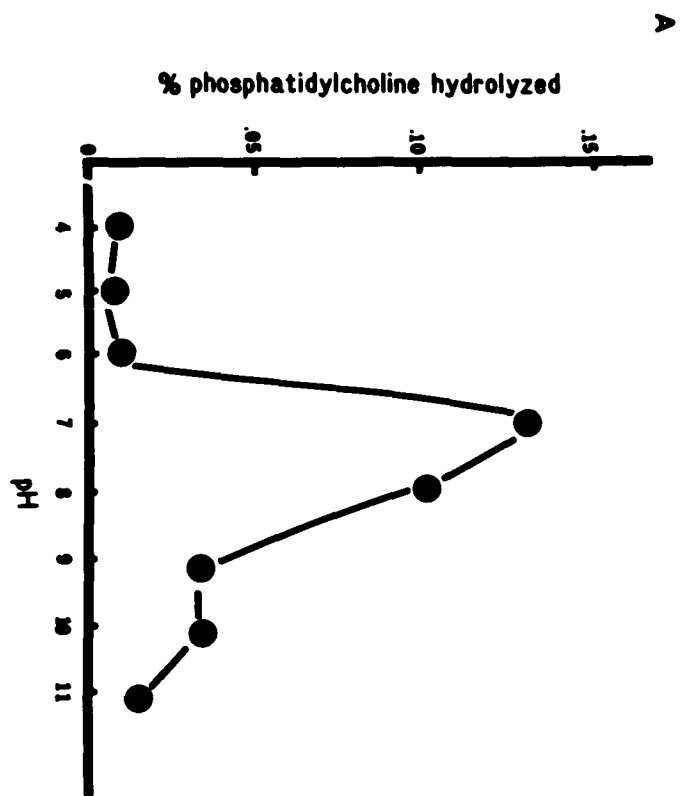


Table 4

Effectors of synaptic plasma membrane PLA₂ kinetic parameters.

Effectors (Concentration)	Apparent K_m (μM)	Relative V_{max} (nmol arachidonic acid/mg/hr)	Substrate turnover k (mg-1/min-1)	% Change k , stimulation (+) inhibition (-)
Ca^{2+} (0.1-1.0 μM)	62.0	98.0	0.13	control
Calmodulin (1 μM)	1.4	16.0	0.96	638 (+)
PGE ₂ (4.0nmol)	80.0	117.0	0.12	7.7 (-)
Calmodulin (1 μM) + PGE ₂ (4.0 nmol)	40.0	88.0	0.19	46.0 (+)

Presynaptic plasma membrane (100 μg) was incubated for 30 minutes at 37°C in the presence of 0.1-1.0 μM $CaCl_2$ with [^{14}C]- β -arachidonyl-phosphatidylcholine (0.2-2.8 nmol). Kinetic parameters were determined from Lineweaver-Burk transformations of substrate concentration curves ($r=0.96-0.99$).

determinations of synaptic plasma membrane phospholipase A₂ activity.

To correlate endogenous synaptic plasma membrane PLA₂ with function, electron microscopy was employed. Synaptic plasma membrane and synaptic vesicles were mixed in a ratio of 1:1 (100 μ g/100 μ g) in a total volume of 400 μ l in 0.1M MES buffer pH 7.0. The effects of several compounds on membrane morphology were then observed by electron microscopy. Synaptic vesicles and synaptic plasma membrane were mixed in an attempt to stimulate in vivo physiological conditions of synaptic plasma membrane-synaptic vesicle interactions. An electron micrograph of the untreated brain membranal mixture prior to any treatment appears in Figure 29A. All the components of the synaptic plasma membrane can be seen viz. the elongated opened and closed membranes, synaptosomes and debris. In addition, synaptic vesicles can also be observed. Although synaptic vesicles and synaptic plasma membrane were mixed in equal protein ratios, it appears that the synaptic membrane preparation is the more prominent morphological structure. The synaptic membranes may have a higher lipid:protein ratio than the synaptic vesicles and therefore appear more abundant.

Addition of 10 μ g exogenous PLA₂ (*Naja naja*) led to a complete alteration in the morphology (Figure 29B). Very few membranes were left intact. Hydrolysis of membranes occurred and there also appeared aggregated areas of membranal fragments and vesicles possibly undergoing refusion.

Incubation of this preparation with 1mM calcium or with 1mM calcium and 4.0 nmol, PGE₂, 1mM cAMP, 1mM ATP, or 1mM cAMP and 1mM ATP did not in any way alter the morphological structure. Incubation with 1mM calcium and 1 μ M calmodulin or with 1mM calcium and 4.0nmol PGF_{2 α} led to

varying degrees of membranal lysis which appeared with greater frequency throughout the pellet as compared to the untreated preparation (Figures 29C and 29D). The morphological changes induced were not as severe as those induced by exogenous Phospholipase A₂. Many more membranes and synaptosomes were left intact. The small effect of CaM and PGF_{2α}, however, was inhibited if the preparation was preincubated with 10⁻⁵ M parabromophenacylbromide (Figure 29E) prior to treatment with these modulators. This implied that the morphological changes induced by calmodulin and PGF_{2α} were at least in part attributed to endogenous synaptic membranal PLA₂. Table 6 summarizes the effects of a variety of modulators on endogenous synaptic membranal PLA₂ activity and morphological structure. Modulators which inhibited SPM PLA₂ — PGE₂, ATP and cAMP — did not lead to any apparent morphological change as determined by electron microscopy. PGF_{2α} and calmodulin, which both stimulated PLA₂, did lead to increased membranal lysis. These data, along with the fact that parabromophenacylbromide prevented the morphological changes induced by these modulators which stimulated endogenous PLA₂, implied that synaptic plasma membrane PLA₂ functions in membranal lysis. Lysis of membranes is the prerequisite for the induction of fusion. Thus it is possible that SPM PLA₂ partakes in this physiological process in this regard.

c. Coated vesicle PLA₂

The calcium concentration, pH, and time dependencies of coated vesicle PLA₂ are illustrated in Figures 30 and 31. Coated vesicle PLA₂ displayed a sharp peak of activity at 10⁻⁵ M calcium. Between calcium concentrations of 10⁻⁷ to 10⁻⁵ M and over 10⁻³ M Ca²⁺, PLA₂ activity was approximately one third of its peak activity (Figure 30).

Table 5

The effects of a variety of modulators on synaptic plasma membrane PLA₂ activity.

Modulator (Concentration)	% stimulation (+)/inhibition (-) PC hydrolyzed
PGF _{2α} (4.0 nmol)	70 + (±28.6)
cAMP (1mM)	23 - (±7.1)
ATP (1mM)	36 - (±7.0)
cAMP (1mM and 1 mM ATP)	70 - (±3.6)

Synaptic plasma membrane (100 µg) was incubated for 30 minutes at 37°C in the presence of 0.1-1.0µM CaCl₂ with 0.46 nmol ¹⁴C-β-arachidon-ylphosphatidylcholine. Percent stimulation and inhibition represent values relative to reactions incubated in the absence of modulators. SPM PLA₂ hydrolyzed 0.24 nmol of exogenous phosphatidylcholine (1.85 nmol) in the absence of modulators.

Figure 29. Electron microscopy of synaptic vesicles and synaptic plasma membrane suspended in 0.1M MES, pH 7.0 mixed in a 1:1 protein ratio in a total volume of 400 μ l, untreated (A); treated with 1mM Ca^{2+} and 20 μ g PLA_2 (*Naja naja*) (B); 1mM Ca^{2+} and 1 μ M CaM (C); 1mM Ca^{2+} and 4.0 nmol $\text{PGF}_{2\alpha}$ (D); pretreated with 10^{-5} M parabromophenacylbromide for 30 minutes prior to incubation with 1.0 nmol $\text{PGF}_{2\alpha}$ (E).

The preparation was incubated for 30 minutes and stopped with 400 μ l 3% gluteraldehyde phosphate buffer, centrifuged at 2,000 x g for 10 minutes and observed under a JEOL 100 B electron microscope. Magnification x 120,000.



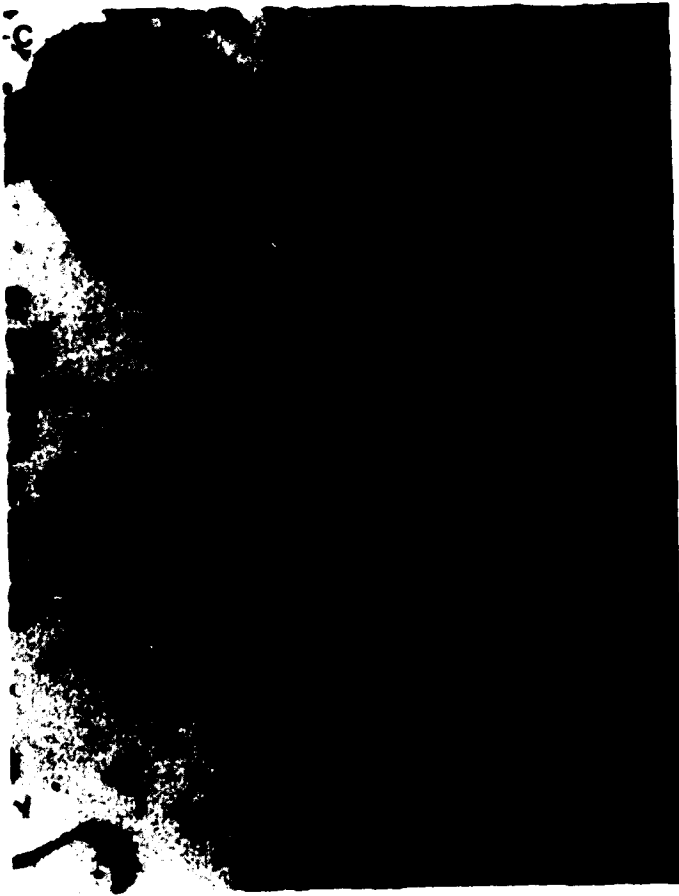




Table 6.

Correlation of the effects of a variety of modulators on synaptic plasma membrane PLA₂ activity and morphology as judged by electron microscopy. (+) = increased lysis relative to control conditions. (0) = no change in morphology relative to control conditions.

Modulators	% Stimulation (+)/Inhibition (-) SPM PLA ₂ activity	Δ Morphology (electron microscopy)
Calmodulin	638 (+)	+
PGF ₂ α	70 (+)	+
PGE ₂	8 (-)	0
cAMP	23 (-)	0
ATP	36 (-)	0

The enzyme had a pH optimum of 9.0 (Figure 31A). Enzymatic activity was linear for the first 5 minutes, after which it declined slowly over the next 75 minutes (Figure 31B). When coated vesicles were incubated with ^{14}C -8-arachidonic acid-phosphatidylcholine under optimal conditions viz. incubated with 1mM Ca^{2+} , pH 9.0 for 5 minutes, it was found that the K_m was $3.5 \times 10^{-6}\text{M}$ and the V_{max} was $6.86\text{ nmol a.a./mg/hr.}$ Various compounds which are present in nerve endings and could therefore possibly influence coated pit/vesicle function were analyzed for their effects on coated vesicle PLA_2 activity. The results are presented in Table 7. Calmodulin, PGE_2 , and $\text{PGF}_{2\alpha}$ stimulated substrate turnover by 131%, 31%, and 168%, respectively. PGE_2 and calmodulin potentiated each other and stimulated PLA_2 activity by 238%. Both cAMP and ATP inhibited PLA_2 activity by 37.5% and 97.0%, respectively. Table 8 summarizes the % change in PLA_2 activity induced by the different modulators studied and lists the kinetic parameters affected.

Assuming that clathrin constitutes 70% of coated vesicle protein concentration, the appropriate corrections for the V_{max} and the first order rate constants for the vesicle (without the coat) have been made in Table 9. Under control conditions, coated vesicle PLA_2 in the presence of 1mM CaCl_2 hydrolyzes $5.3\% \text{ PC}/10^{-1}\text{ second.}$

To correlate coated vesicle PLA_2 with function, the coated vesicles were de-coated as mentioned in the materials and methods section, and nephelometry and phase contrast microscopy were employed to assess vesicle interactions. Figure 32 illustrates the light scattering profile induced by PLA_2 (*Vipera russelli*) and lysolecithin. Addition of calcium to the vesicle suspension in concentrations ranging from 10^{-7} - 10^{-3} M at pH 6.5-9 did not lead to any increase in light scattering.

Figure 30. Coated vesicle PLA₂ activity is plotted as a function of calcium concentration. Coated vesicles were incubated with EGTA and increasing amounts of calcium in the presence of 1.85 nmol [¹⁴C]-phosphatidylcholine and incubated for 5 minutes at 37°C at pH 9.0

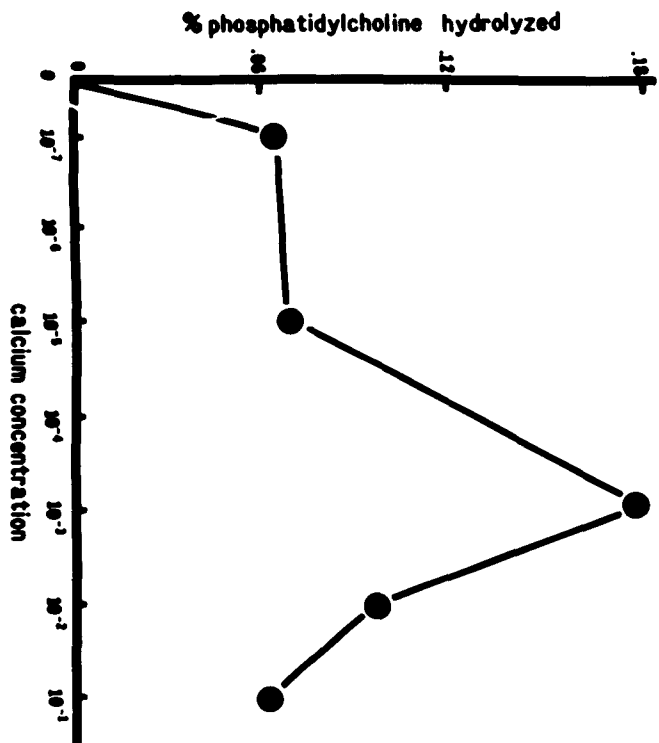


Figure 31. The pH (A) and time (B) dependencies of coated vesicles PLA₂ in the presence of 1mM Ca²⁺ incubated under the same condition as in Figure 30.

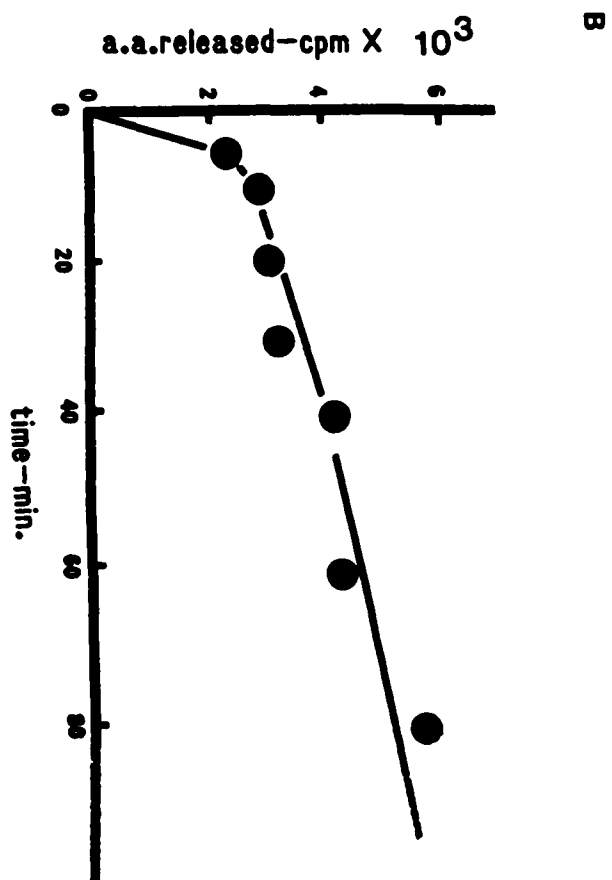
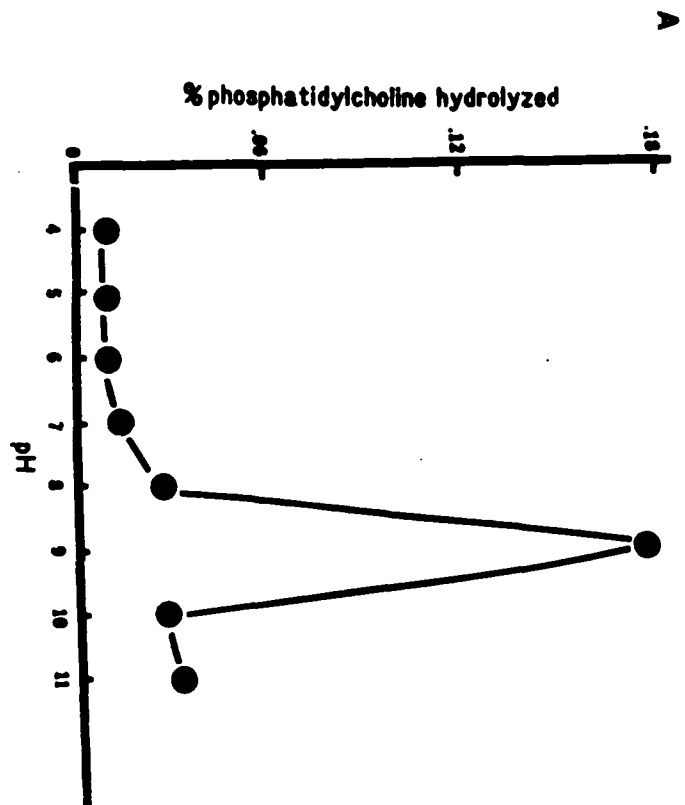


Table 7

The effects of a variety of conditions on coated vesicle PLA₂ kinetic parameters.

Condition (Concentration)	r^2	K_m (μ M)	V_{max} (nmol a.a. /mg/hr)	k (mg^{-1}/min^{-1})
1) Control (1mM)	.99	3.5	6.86	.160
2) CaM (1 μ M)	.98	2.2	10.00	.370
3) PGE ₂ (4.0 nmol)	.97	5.6	14.40	.210
4) PGE ₂ +CaM (4.0 nmol)	.95	2.9	18.80	.540
5) PGF _{2α} (4.0 nmol)	.96	3.6	18.40	.730
6) CAMP (1mM)	.95	7.1	8.03	.100
7) ATP (1mM)	.99	5.0	0.30	.005

Coated vesicles (100 μ g) were incubated for 5 minutes at 37°C in the presence of 10⁻³ M CaCl₂ with [¹⁴C]-arachidonylphosphatidylcholine (0.2-2.8 nmol). Kinetic parameters were determined from Lineweaver-Burk transformations of the substrate concentration curves.

Table 8

The relative change in coated vesicle PLA₂ activity induced by various conditions and the kinetic parameters affected.

Condition	% stimulation (+)/ inhibition (-) PLA ₂ activity	Change in Kinetic Parameters	
		K _m	V _{max}
CaM	131 (+)	-	+
PGE ₂	31 (+)	+	+
PGE ₂ + CaM	238 (+)	-	+
PGF _{1α}	168 (+)	0	+
cAMP	37.5 (-)	+	-
ATP	97.0 (-)	+	-

The values shown are derived from Table 7. (-) = decreased, (+) = increased, and (0) = no change.

Table 9

The effects of various conditions on coated vesicle PLA₂ V_{max} and k values. Values have been corrected based on the assumption that clathrin constitutes 70% of the protein concentration in coated vesicles.

Condition	V _{max1}	V _{max2}	k ₁ (mg ⁻¹ /min ⁻¹)	k ₂ (mg ⁻¹ /.1 min ⁻¹)
Control	6.86	22.6	.160	.053
CaM	10.0	33.0	.370	.100
PGE ₂	14.4	43.2	.210	.070
PGE ₂ + CaM	18.8	63.0	.540	.180
PGF _{2α}	18.4	61.0	.430	.140
cAMP	6.86	22.6	.090	.030
ATP	0.30	0.99	.005	.002

V_{max1} and k₁ are the original values derived from Table 7. V_{max2} and k₂ are the corrected values representing coated vesicle PLA₂ activity/decoated vesicle protein concentration.

Addition of 0.1 μg PLA_2 at pH 6.5 in the presence of 1mM Ca^{2+} led to a 200% increase in light scattering within 10 seconds (Figure 32). Light scattering decreased rapidly within the next 30 seconds at which point it increased slightly by 25% and reached baseline values again 60 seconds thereafter (Figure 32). Addition of lysolecithin led to a 100% increase in light scattering within 10 seconds which returned to baseline values within the next 10-15 seconds and then 40 seconds later led to a 100% increase in light scattering which then declined to baseline values 20 seconds thereafter. Figure 33A reveals that in the absence of any reagents, vesicle aggregates were not observable. Phase contrast microscopy revealed that upon addition of lysolecithin very large visible aggregates became observable (Figure 33B). Addition of calcium at any concentration did not induce visible aggregation. Thus it would appear that exogenous PLA_2 may have hydrolyzed phosphoglycerides leading to production of lysolecithin which induced aggregation of vesicles. The fact that both PLA_2 and lysolecithin induced biphasic increases in light scattering may imply that the vesicles were associating and dissociating sequentially. Alternatively, different populations of decoated vesicles may have been aggregating, precipitating and sedimenting sequentially. Calmodulin, PGE_2 , and $\text{PGF}_{2\alpha}$ led to 240%, 200% and 170% increases in light scattering, respectively, within 10-15 seconds of their additions (Figures 34 A-C). Preincubation of decoated vesicles with 1.0×10^{-5} M parabromophenacylbromide prior to incubation with CaM, PGE_2 or $\text{PGF}_{2\alpha}$ led to significant inhibition of light scattering (Figures 34A-C). This implied that stimulation of endogenous PLA_2 led to increased light scattering. To facilitate interpretation of the light scattering effects, light microscopic observations of decoated vesicles

Figure 32. Decoated vesicles (30 μ g/ml) were incubated with 1mM calcium for 1-5 minutes, after which 0.1 μ g, PLA₂ (Vipera Russellii) (●) or 10 μ g lysolecithin (egg yolk) (■) were added. The arrows indicate the point of addition of PLA₂ or lysolecithin. The decoated vesicles were suspended in 10mM Tris-HCl buffer, pH 6.5 or 9.0, in the PLA₂ and lysolecithin conditions, respectively. The decoated vesicles were adjusted so that their baseline nephelometer readings ranged between 0.45-0.55. Plotted is light scattering measured in nephelometer units as a function of time.

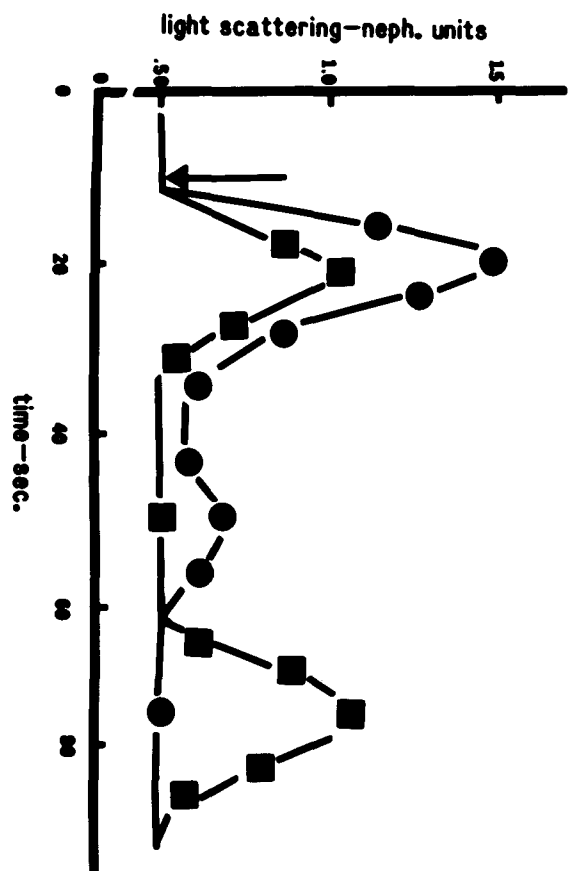
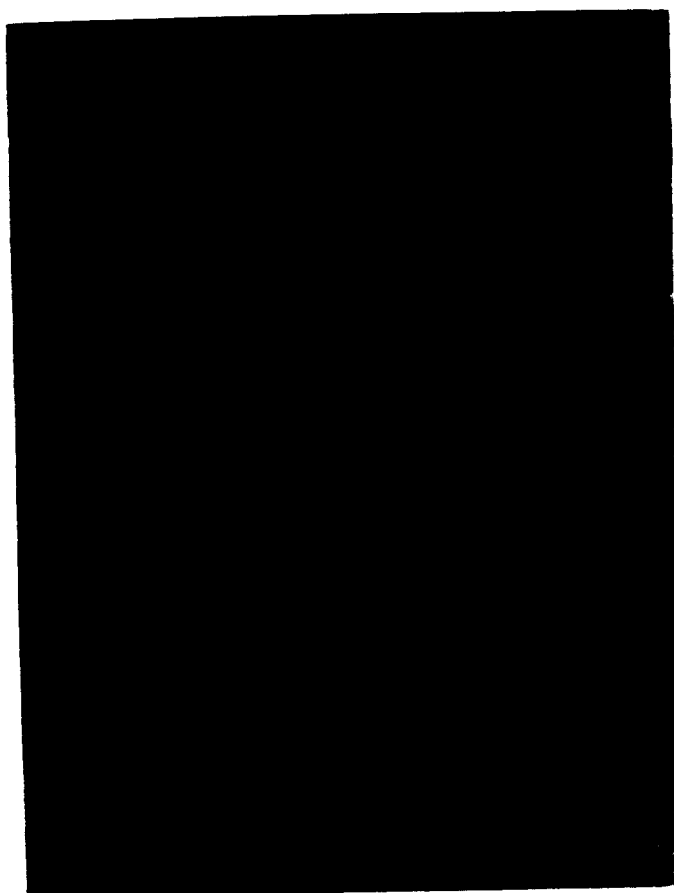


Figure 33. Light microscopy of decoated vesicles (0.75mg/ml) suspended in Tris-HCl buffer, pH 9.0, untreated (A) or treated with 20 μ g lysolecithin (B). Magnification x 4,330.



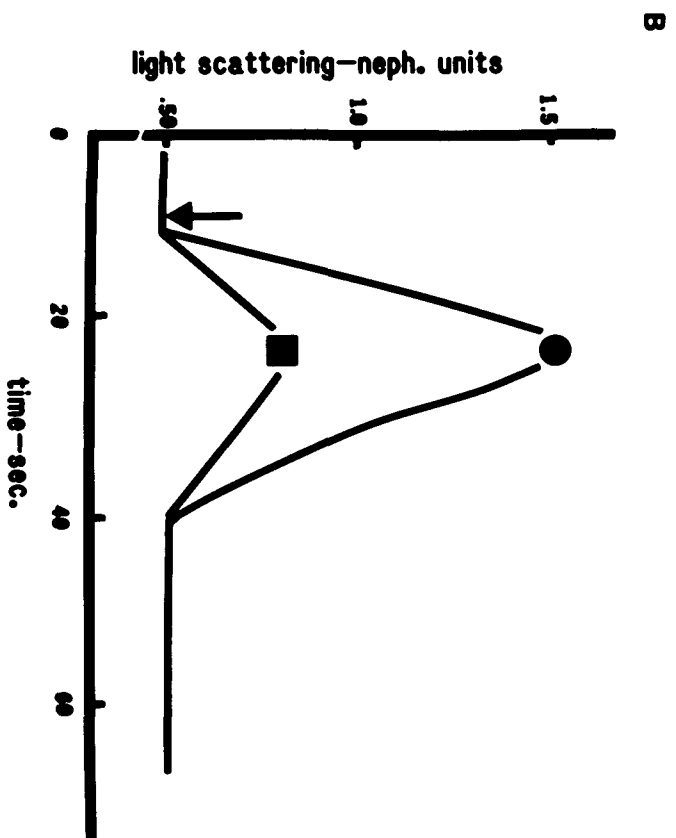
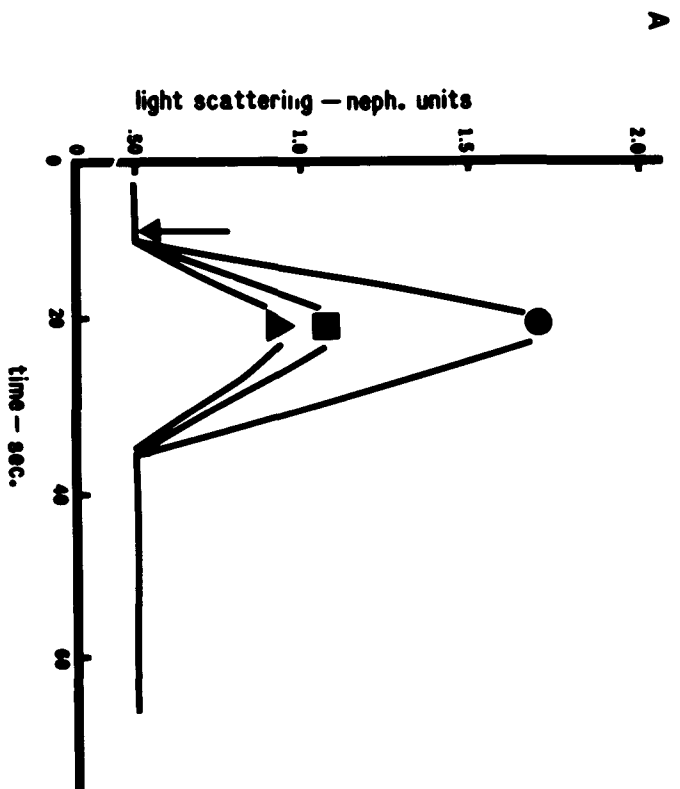
treated under similar conditions as in the light scattering experiments were employed. Addition of PGE_2 or $\text{PGF}_{2\alpha}$ led to an increase in the number of small vesicle aggregates (Figure 35). Calmodulin also increased the number of observable clumps but to a lesser extent (data not shown). Preincubation of decoated vesicles with 1.0×10^{-7} M para-bromophenacylbromide prior to incubation with calmodulin, PGE_2 or $\text{PGF}_{2\alpha}$ inhibited vesicle aggregation, and vesicles so treated were indistinguishable from untreated vesicles. Thus coated vesicle PLA_2 activation was correlated with increased vesicle-vesicle light scattering which was in turn correlated with increased vesicle-vesicle aggregation. An increase in light scattering in decoated vesicles like in synaptic vesicles might mean that an increase of lysis and possible vesicle fusion has occurred.

Light scattering and light microscopic experiments took place at pH 9.0. When vesicles were treated with calmodulin, PGE_2 or $\text{PGF}_{2\alpha}$ at pH 6.5 no increase in light scattering was observed.

Table 10 summarizes the effects of various modulators on coated vesicle PLA_2 activity, light scattering profile and morphology. Stimulation of coated vesicle PLA_2 activity was roughly correlated with vesicle-vesicle aggregation.

The fact that PGE_2 induced a small increase in PLA_2 activity (31%), yet enhanced light scattering and aggregation by 200%, may mean that PGE_2 stimulated aggregation by stimulating PLA_2 as well as stimulating some other membranal system. The fact that PLA_2 is stimulated in coated vesicles and associated with altered membranal changes which lead to vesicle aggregation implies that PLA_2 may function in pinching off of the coated pit and eventual fusion of coated vesicle with other

Figure 34. Light scattering profiles of decoated vesicles incubated with 1.0 μM calmodulin (A), 4.0 nmol $\text{PGF}_{2\alpha}$ (B), or 4.0 nmol PGE_2 (C) at 25°C after preincubation with 1mM CaCl_2 for 1-5 minutes are indicated by a circle (●). Decoated vesicles were suspended in 10 mM Tris-HCl buffer, pH 9.0. Light scattering profiles of decoated vesicles preincubated with 1.0×10^{-5} M parabromophenacylbromide for 30 minutes prior to the addition of the modulators in figures A-C are indicated by a square (■). The light scattering profile of decoated vesicles induced by calmodulin after preincubation with 3.0×10^{-5} M quinacrine for 30 minutes is indicated in figure (A) by a triangle (▲). Decoated vesicles were adjusted so that their baseline nephelometer values ranged from 0.45-0.55 nephelometer units. The arrows in each figure indicate the point of addition of the particular modulator. Light scattering is plotted as function of time.



C

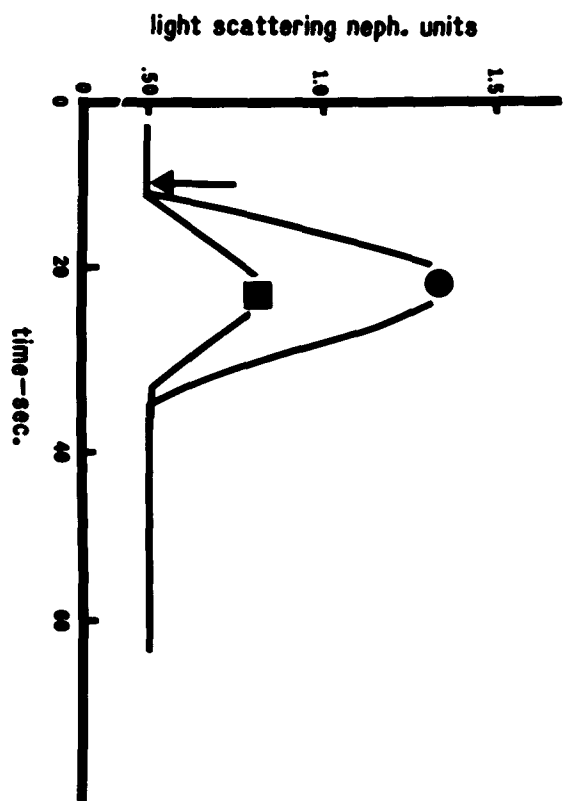


Figure 35. Light microscopy of decoated vesicles (0.75mg/ml) suspended in 10 mM Tris-HCl buffer, pH 9.0, in a total volume of 200 μ l treated with 4.0 nmol PGE₂. Magnification x 4330.



Table 10

Comparison of the effects of a variety of modulators on coated vesicle PLA₂ activity, light scattering profile and morphology as determined by light microscopy. The change in PLA₂ activity induced by the modulator is presented as change in coated vesicle k parameter relative to reactions incubated in the absence of modulators but in the presence of mM CaCl₂. The percent change in light scattering presented is derived from the peak values of light scattering relative to deoated vesicle conditions incubated in the absence of calcium and modulators. Morphological alterations presented are manifestations of degrees of vesicle aggregation which are correlated with the number of (+)'s. (0) = no visible aggregation relative to control conditions.

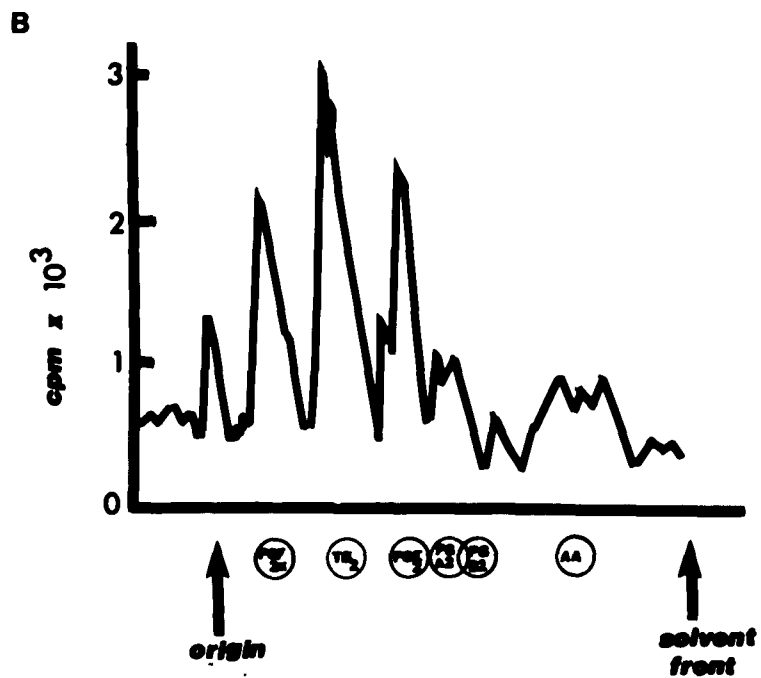
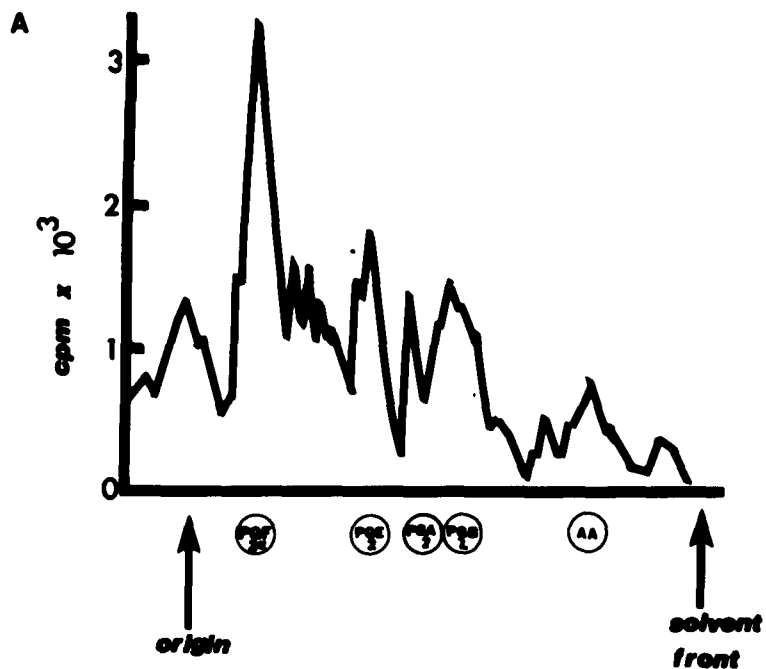
Modulator	PLA ₂ activity stimulation (+)/ inhibition (-)	Light Scattering	Δ Morphology (Lt. Microscopy)
CaM	131 (+)	240 (+)	+
PGE ₂	31 (+)	200 (+)	+++
PGF _{2α}	168 (+)	170 (+)	+++
cAMP	37.5 (-)	0	0
ATP	97 (-)	0	0

organelles or membranes.

5. Synaptic vesicle and coated vesicle prostaglandin synthesis

In view of the finding that both synaptic vesicles and coated vesicles have endogenous PLA₂ activity which theoretically is capable of releasing arachidonic acid, and the fact that certain prostaglandins can influence activity in synaptic and coated vesicles and synaptic plasma membranes, it was of interest to see whether synaptic and coated vesicles were capable of metabolizing arachidonic acid, and if so, to identify the products produced. The radiochromatograph scan obtained of synaptic and coated vesicle extracts after incubation with ¹⁴C-arachidonic acid are illustrated in Figures 36A and 36B, respectively. The peaks obtained in synaptic vesicles co-chromatographed with authentic PGs E₂, F_{2α}, A₂ and B₂ standards. In coated vesicles, the radioactive peaks co-chromatographed with authentic standards of PGE₂, PGF_{2α}, PGA₂, PGB₂ and thromboxane B₂ standards. It appeared that most of arachidonic acid was metabolized, indicating that both coated and synaptic vesicles have active PG synthetase activity. The major products of synaptic vesicles appeared to be PGF_{2α}. PGE₂, PGA₂, and PGB₂ were present in significantly smaller quantities. The major eicosanoid product in coated vesicles appeared to be TxB₂. PGE₂ and PGF_{2α} were synthesized in lesser and in approximately equal quantities. PGA₂ and PGB₂ were present in relatively smaller quantities. A further indication of the presence of active PG synthetase in synaptic vesicles was verified by incubating synaptic vesicles with 1.0×10^{-6} M indomethacin, a PG synthetase inhibitor, in the presence of PLA₂ and observing by phase contrast microscopy a huge increase in the number and size of

Figure 36. Radiometric scan of thin layer chromatogram of synaptic (A) and coated vesicle (B) arachidonic acid metabolites. Prostaglandin and thromboxane standards that co-chromatographed with the peaks are indicated.



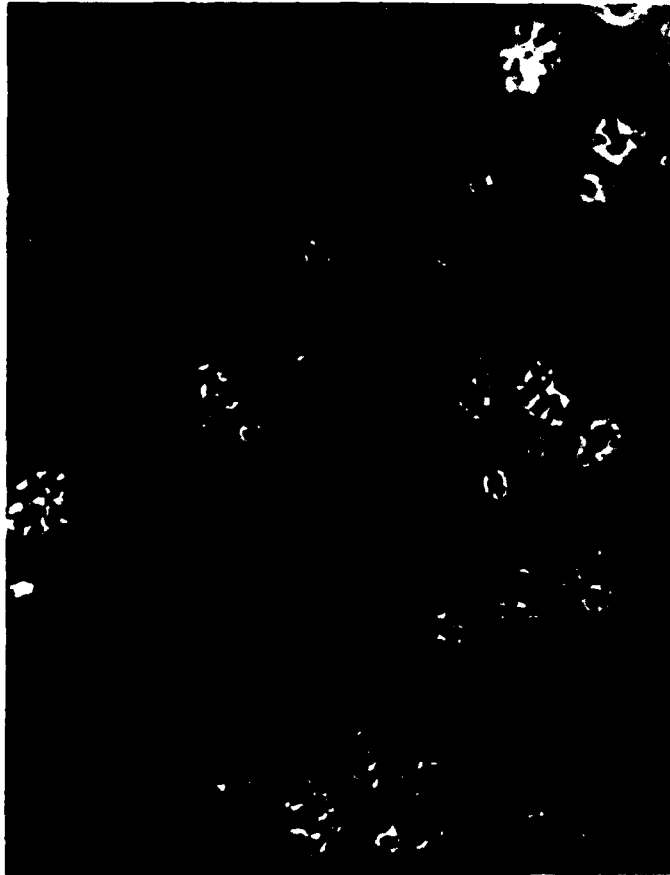
synaptic vesicle aggregates (Figure 37C). This was the greatest degree of aggregation and clumping seen with synaptic vesicles out of all the conditions studied. The size of the vesicle clumps were greater than that observed upon lysolecithin or PLA₂ by treatment (see Results, Figures 25B and C). Apparently the inhibition of PG synthesis potentiated PLA₂ aggregation. Since from the radiochromatography it appeared that PGF_{2α} is the major PG produced by synaptic vesicles, it might be expected that inhibition of PG synthetase would lead to the reverse effect to that observed, because it activates PLA₂ activity and stimulates aggregation. It should be recalled however that PGE₂ inhibited PLA₂ activity and inhibited CaM's potentiation of PLA₂ activity and synaptic vesicle aggregation. It is also possible that PGE₂ can reverse the stimulating effect of PGF_{2α} thereby explaining the stimulatory effect of indomethacin inhibition.

6. Calmodulin association with purified phospholipases A₂

The fact that PLA₂ in different membranes was present and regulated by different modulators prompted the question of whether the effects of the modulators were exerted directly on the membrane PLA₂ or on other membranal components which subsequently activated membranal PLA₂. An extensive analysis of the effect of calmodulin on five different phospholipases A₂ was undertaken. Naja naja PLA₂ was then analyzed in greater detail for interaction with PGE₂, PGF_{2α}, cAMP and cGMP.

The elution profiles of the different phospholipases A₂ on a calmodulin affinity column are illustrated in Figures 38A-E. Both *Crotalus adamanteus* (CA) and *Crotalus durissus terrificus* (CDT) venom PLA₂ were eluted entirely by calcium. EGTA failed to elute either of these

Figure 37. Phase contrast microscopy of synaptic vesicle (0.75mg/ml) suspended in 0.1M MES buffer, pH 6.5, treated with 1.0×10^{-6} M indomethacin for 20 minutes and then by 20 μ g PLA₂ (*Vipera russelli*) at 25°C. Magnification x 4,330.

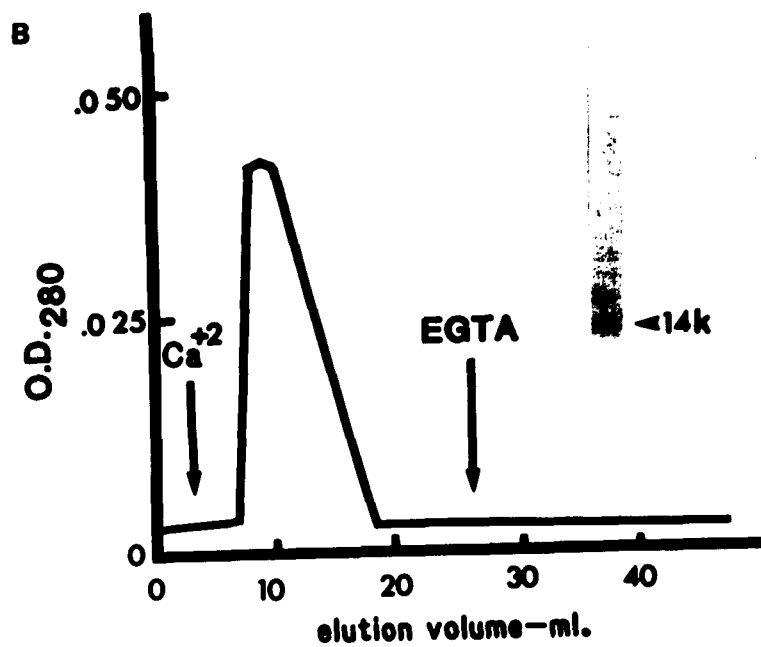
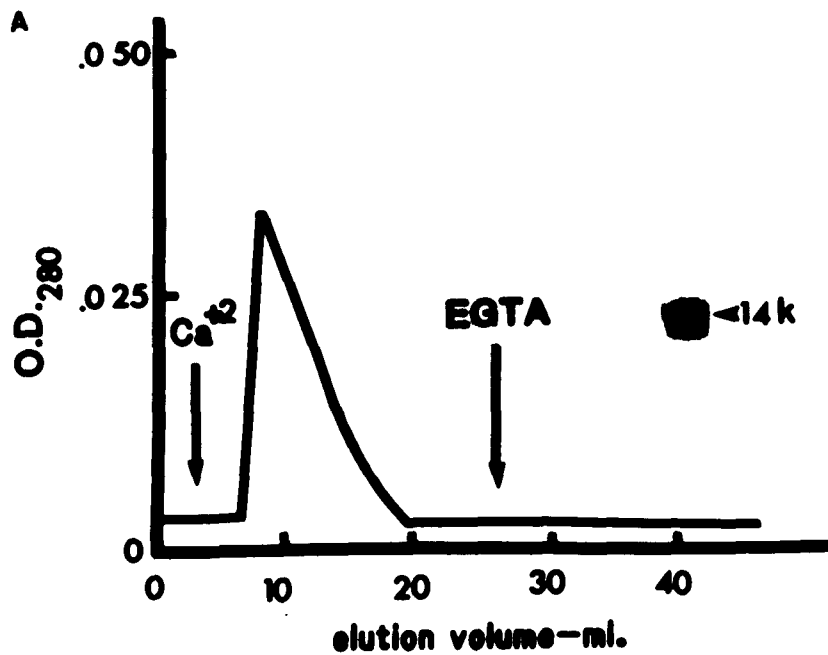


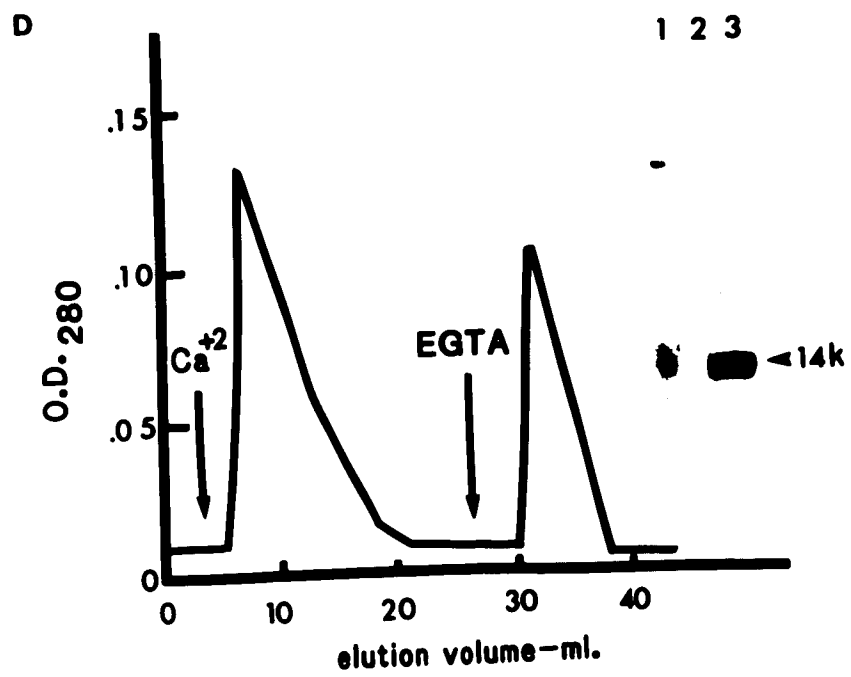
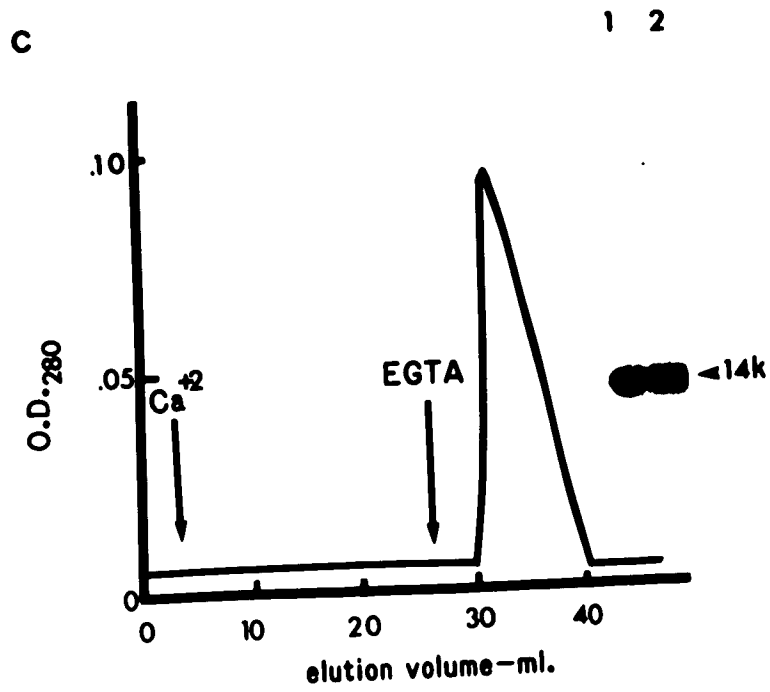
enzymes. This indicated that these two phospholipases A_2 did not bind to the calmodulin affinity column in a Ca^{2+} dependent manner. The predominant polypeptide in both enzymes was the 14K monomer. The dimer was present in lower quantities. Bee venom phospholipase A_2 eluted entirely with EGTA indicating that it bound to the calmodulin column in a calcium dependent manner (Figure 38C). The insert in Fig 38C shows that the bee venom PLA_2 consisted of one single band of M_r 14K. *Naja naja* PLA_2 also eluted entirely with EGTA indicating that it bound to the calmodulin affinity column in a calcium dependent manner (Figure 38D). Polypeptide analysis revealed that a protein impurity of M_r 68K was separated from the enzyme in the calcium eluate. A small fraction was retained with the enzyme (Figure 38D). Figure 39E is a representative elution profile of porcine pancreatic PLA_2 (P_T). Most of the enzyme was eluted by calcium and a small fraction eluted by EGTA. The protein composition of the enzyme prior to chromatography and the protein composition in the calcium and EGTA eluates all consisted of one polypeptide of M_r 14K. Porcine pancreatic Phospholipase A_2 from several different batches were passed through the column. The percent of protein loaded that eluted with EGTA from the column varied from 0-19.8% as determined by Lowry protein determinations.

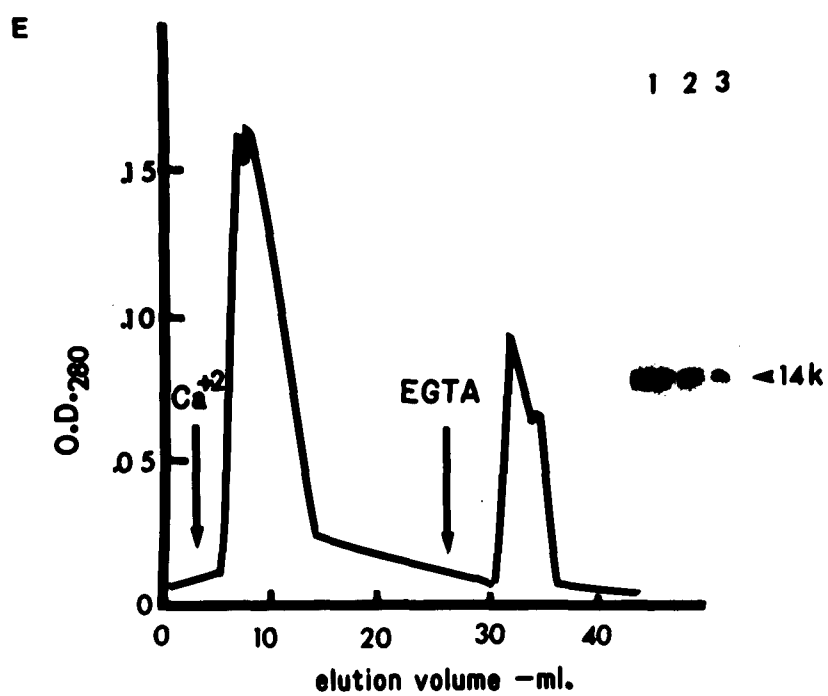
a. PLA_2 activity

The kinetic parameters of the 5 different phospholipases A_2 incubated with $1mM$ Ca^{2+} in the absence or presence of $7.0\mu g$ CaM appear in Table 11. Calmodulin did not significantly affect the K_m or V_{max} of CA PLA_2 . Calmodulin did however slightly decrease the K_m of CDT PLA_2 leading to a 21% increase in the substrate conversion rate of the enzyme. Calmodulin lowered both the K_m and the V_{max} of bee venom PLA_2

Figure 38. Elution profiles of phospholipases A₂ from CDT venom (A), CA venom (B), Bee venom (C), Naja naja venom (D) and porcine pancreas (E) chromatographed on a calmodulin affinity column. The enzymes (0.5-1.0 mg) were incubated on the column for 30-60 minutes. Enzymes or proteins binding to the column were eluted with 0.1M Tris buffer, 1mM MgCl₂ and 1mM CaCl₂ (Ca²⁺). Enzymes binding to the column were eluted with 0.1M Tris, 1mM MgCl₂, and 10 mM EGTA (EGTA). The pH of the buffers was adjusted to the pH optimum of the enzymes, viz. pH 8.0, 8.5, 8.9, 8.9, 8.9 for pancreatic, bee venom, Naja naja, CA, and CDT phospholipases A₂, respectively. The polypeptide composition of the enzymes are inserted. In Figure 38A and 38B, polypeptide composition of the single eluate is depicted. In Figure 38C, lanes 1 and 2 correspond to the protein composition before and after chromatography. In Figure 38D, lanes 1, 2 and 3 represent protein composition of the enzyme prior to chromatography and in the Ca²⁺ and EGTA eluates, respectively. In Figure 38E, lanes 1, 2 and 3 represent the protein composition of P_T, P_C and P_E, respectively.







leading to a more significant decrease in the K_m and an overall 62% stimulation of the substrate conversion rate of the enzyme. Calmodulin significantly lowered the K_m of *Naja naja* PLA₂ without affecting the V_{max} leading to an overall 208% stimulation of the substrate conversion rate.

The kinetic parameters of the pancreatic enzyme prior to calmodulin affinity chromatography and of the pancreatic enzymes found in the calcium and EGTA eluates are tabulated in Table 12. For convenience I named these three enzymes species P_T (Pancreatic PLA₂ total), P_C (Pancreatic PLA₂ eluted by Ca^{2+}), and P_E (Pancreatic PLA₂ eluted by EGTA). Calmodulin significantly lowered the K_m of P_C PLA₂ leading to a 179% increase in the substrate conversion rate. Calmodulin induced a slight increase in the V_{max} of P_C leading to a 17% increase in enzymatic activity. Calmodulin decreased both the K_m and V_{max} of P_E decreasing the K_m more significantly leading to an overall 209% stimulation of the substrate conversion rate. It was apparent that P_T had the greatest substrate conversion rate in the presence of calmodulin. The substrate conversion rate of P_T in the absence of calmodulin was approximately equal to that of P_E in the presence of calmodulin. P_E PLA₂ in the absence of calmodulin had the lowest substrate conversion rate.

b. DMS-crosslinking

DMS crosslinking of CA PLA₂ under different conditions is depicted in Figure 39A. A polypeptide of M_r 29K corresponding to the M_r of a PLA₂ dimer was present in the uncrosslinked and Ca^{2+} or Ca^{2+} -calmodulin crosslinked conditions. The monomer was the predominant species. Calmodulin did not promote dimerization of this enzyme nor was calmodulin crosslinked to the enzyme. EGTA slightly inhibited dimerization.

Table 11

Kinetic parameters of PLA₂s from four different sources in the presence or absence of calmodulin (CaM).

PLA ₂ Source	Condition	K _m (μM)	V _{max} nmol arach.acid/ pgm/min	k (pgm ⁻¹ .min ⁻¹) (*pgm ⁻¹ .sec ⁻¹)	r	% Calmodulin Stimulation
Bee venom	-CaM	36.0	29.0	0.13*	0.98	62.0
	+CaM	12.0	15.3	0.21*	0.99	
Crotalus Durissus Terrificus venom	-CaM	14.0	0.0020	0.0014	0.99	21.0
	+CaM	12.0	0.0020	0.0017	0.94	
Crotalus Adamanteus venom	-CaM	6.4	0.0015	0.0023	0.97	0
	+CaM	9.6	0.0019	0.0020	0.99	
Naja Naja venom	-CaM	16.0	0.0020	0.0013	0.99	208.0
	+CaM	5.0	0.0020	0.0040	0.94	

Phospholipase A₂ (1.0 pgm) was incubated with 0.2-2.8 nmol 1-[¹⁴C]-β-arachidonyl phosphatidylcholine for 10 minutes in the presence of 1 mM Ca²⁺ or 1 mM Ca²⁺ and 7.0 μg calmodulin in a final volume of 100 μl. Naja Naja, CDT and CA PLA₂s were incubated at 25°C. Bee venom PLA₂ was incubated at 37°C. Kinetic parameters were calculated from Lineweaver-Burk plots.

Table 12

Kinetic parameters of pancreatic PLA₂ species in the presence or absence of calmodulin (CaM)

Pancreatic PLA ₂	Condition	K _m (μM)	V _{max} nmol arach.acid/ pgm/min	k (pgm ⁻¹ .min ⁻¹)	r	% Calmodulin Stimulation
P _T	-CaM	21.0	0.0080	0.067	0.98	179.0
	+CaM	1.2	0.0060	0.240	0.86	
P _C	-CaM	11.0	0.0320	0.029	0.99	17.0
	+CaM	14.0	0.0417	0.034	0.99	
P _E	-CaM	100.0	0.2200	0.022	0.99	209.0
	+CaM	4.4	0.0300	0.068	0.95	

PLA₂ (1.0 pgm) was incubated with 0.2-2.8 nmol 1-[¹⁴C]-β-arachidonyl-phosphatidylcholine for 10 minutes in the presence of 1 mM Ca²⁺ or 1 mM Ca²⁺ and 7.0 μg calmodulin in a total volume of 100 μl for 10 minutes at 37°C. Kinetic parameters were calculated from Lineweaver-Burk plots.

It has previously been suggested that CA PLA₂ is active as a dimer, and is a dimer at the time of enzymatic hydrolysis and that dimerization is not calcium dependent. It is possible that dimerization of CA PLA₂ does not occur in regions of lysine-lysine interactions between the monomers and therefore dimerization was not detected by the method employed. However the slight decrease of dimerization detected by this procedure may imply that CA PLA₂ dimerization may be calcium dependent to a limited extent.

DMS crosslinking of CDT PLA₂ under a variety of conditions is demonstrated in Figure 39B. In the absence of crosslinking, the enzyme exists as polypeptide monomers with M_r 12K and 14K, the latter polypeptide being the predominant monomer. In addition, polypeptides of M_r 27K and 32K were also present which may correspond to dimer complex formation between the 12K-14K and 14K-14K monomers, respectively. Crosslinking in the presence of calcium revealed an additional protein of M_r 44 (Figure 39B). This protein corresponded to the approximate theoretical M_r of a PLA₂ trimer (consisting of 3 x 14K monomers).

The formation of CDT dimers and trimers is calcium dependent in that their formation was inhibited by EGTA. In the presence of 1mM calcium and 7.0 µg calmodulin, an additional two proteins of M_rs 36K and 47K are apparent (Figure 39B). These polypeptides correspond to PLA₂ monomer-CaM protein complexes (theoretical M_r: 14 + 17 = 31K) and to PLA₂ dimer-CaM complexes (theoretical M_r: 28 + 17 = 45K). In the presence of CaM, the PLA₂ dimer and trimer complexes in the presence of calcium alone were diminished in intensity. The association of CDT PLA₂ with calmodulin was entirely calcium dependent. In the absence of calcium, PLA₂-calmodulin complexes were not formed (figure 39B). CDT

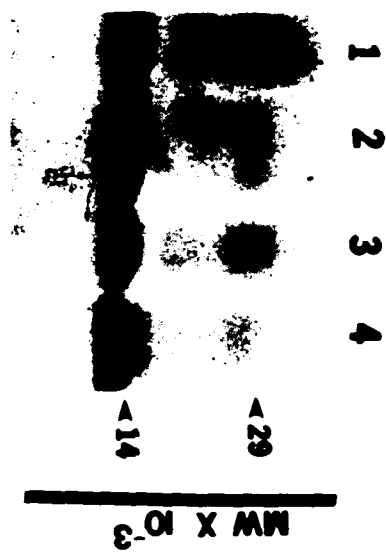
PLA₂ may not have bound to the calmodulin affinity column because the affinity between CDT and CaM may be low. It is apparent from the cross-linking experiments, however, that the affinity of the enzyme for calmodulin is sufficient so that they are at least within 11 Å from each other and are capable of interacting with each other. The fact that the enzyme is calcium dependent and stimulated by calmodulin implied that calcium in part stimulated CDT-PLA₂ by promoting PLA₂ polymerization which is somehow more conducive to substrate hydrolysis. Calmodulin may stimulate the enzyme by favoring PLA₂-CaM formation. Apparently association of CaM with PLA₂ may change the conformation of the PLA₂ enzyme so that it has a higher affinity for the substrate (note decrease in K_m , Table 11).

The PLA₂'s from *Crotalus atrox* and *Crotalus adamanteus* venoms have been sequenced. Their amino acid sequences are almost identical. The sequence of CDT to date has not been elucidated. Because CDT is in the *Crotalus* PLA₂ class, which has been designated as group II, it is likely that the amino acid sequence of PLA₂ from *Crotalus atrox* and CDT venoms are highly similar. It would be of great interest to sequence the CDT PLA₂ and to compare the amino acid sequence to elucidate the mechanism whereby calmodulin binds to CDT venom PLA₂ but not to *Crotalus Adamanteus* PLA₂.

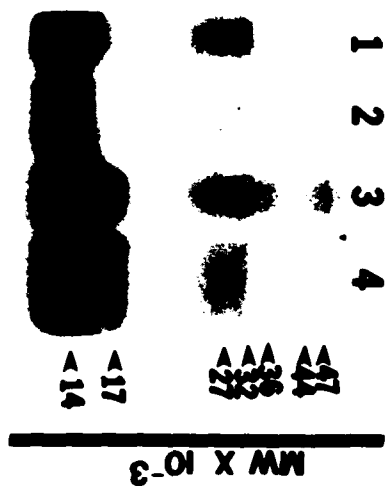
The effects of various conditions on PLA₂ bee venom DMS-crosslinking are demonstrated in Figure 39C. In the presence of calcium, proteins of M_r 14K and 35K are discernible. It is likely that these proteins represent monomers and dimers, respectively. Dimerization of this PLA₂ is dependent on calcium to a limited extent in that EGTA inhibits dimer formation. The monomer is the predominant species. In

Figure 39. Polypeptide composition of DMS-crosslinked phospholipase A₂ obtained from CA venom (A), CDT venom (B), bee venom (C), Pancreatic_T (D), Pancreatic_C (E), and Pancreatic_G (F) as determined by 5-15% SDS gel analysis. PLA₂ (20 µg) was incubated with 0.42 µg/ml DMS (dissolved in 20 mM Triethanolamine, pH 8.5) for 30 minutes in a total volume of 60 µl 1mM imidazole, pH 6.5. The reactions were stopped with 14 µl phenol red (10% SDS and 1 mM β-Mercaptoethanol). PLA₂'s were crosslinked in the presence of 1mM CaCl₂ (lane 1), 1mM EGTA (lane 2), 1mM CaCl₂ and 7 µg Calmodulin (lane 3), 1mM EGTA and 7 µg calmodulin (lane 4). Gels were stained with Coomassie Blue.

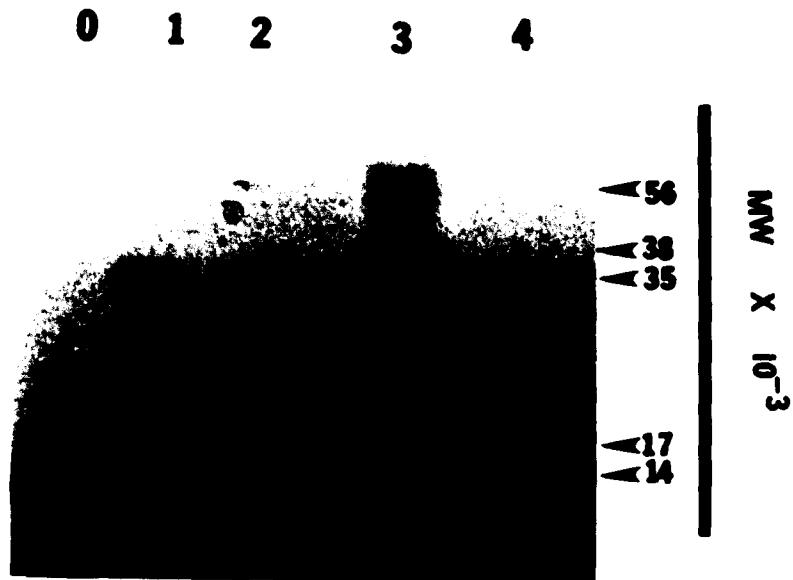
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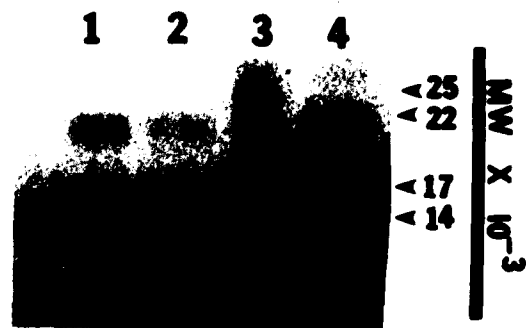
B



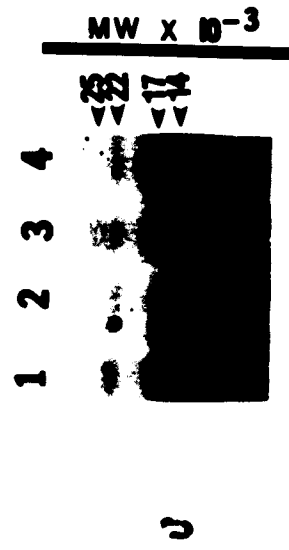
C



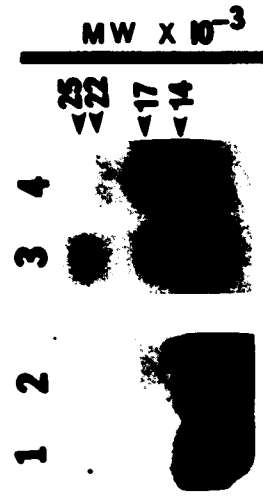
D



E



F



the presence of calcium and calmodulin, two additional polypeptides of M_r 38K and 56K were detected (Figure 39C). The M_r s corresponded to M_r s of PLA₂ monomer-CaM and PLA₂ dimer-CaM protein complexes, respectively. PLC₂-CaM protein complex formation was entirely calcium dependent. In the absence of calcium these protein complexes were not formed.

The DMS crosslinking patterns of Pancreatic PLA₂ enzymes P_T, P_C and P_E are shown in Figure 39D. In the presence of calcium, P_T exhibited polypeptides of M_r s 14K and 22K, which corresponded in M_r to monomers and dimers, respectively. The monomer was the predominant form of the enzyme. Dimer formation was not calcium dependent in that EGTA did not inhibit its formation (Fig. 39D). In the presence of calcium and calmodulin an additional polypeptide of M_r 25K was observed (Fig. 39D) corresponding in M_r to a pancreatic PLA₂ monomer-calmodulin protein complex. This complex was calcium dependent in that EGTA inhibited its formation (Figure 39D). Almost identical DMS crosslinking patterns in the presence of calcium and calmodulin were obtained for the P_C PLA₂. P_C formed a complex with calmodulin. This indicated that this PLA₂ was not sufficiently separated from P_E PLA₂ or that it bound to calmodulin with a different affinity than did the P_E PLA₂.

DMS crosslinking of P_E PLA₂ under a variety of conditions is presented in Figure 39E. In the presence of calcium or EGTA, a polypeptide corresponding in M_r to a dimer was formed. In the presence of Ca²⁺ and calmodulin, P_E dimers were not formed. Instead monomer P_E PLA₂-CaM protein complexes were preferentially formed. In the presence of calmodulin and EGTA (Figure 39E), P_E dimers were present and P_E-CaM complex formation was completely inhibited. Thus P_E-CaM protein complexing was entirely calcium dependent.

It has previously been postulated that bee venom and pancreatic PLA₂ enzymes exist and are active only as monomers. This study refutes this viewpoint and reveals that indeed dimers of these species are formed. Calmodulin stimulation of both these enzymes along with the finding that PLA₂ monomer-CaM protein complexes were formed implied that the association of PLA₂ with CaM may lead to a structural conformational change of PLA₂ that has a greater catalytic potential.

7. Interaction of calmodulin, PGF_{2α}, PGE₂, cAMP, and cGMP with Naja naja phospholipase A₂.

Detailed analysis of PLA₂ obtained from Naja naja PLA₂ revealed that calmodulin induced a dose dependent increase of PLA₂ activity (Figure 40). Maximum activity was obtained using 10⁻⁶ M calmodulin. In the absence of calmodulin, PGF_{2α} stimulated PLA₂ in a dose dependent manner (Figure 41). Maximum activation was achieved at a concentration of 10⁻⁷ M PGF_{2α} leading to a 63.6% increase above baseline values. In the presence of 1 μM calmodulin, PGF_{2α} led to a dose dependent increase of PLA₂ activity above baseline calmodulin activation values. Maximal stimulation was achieved at 10⁻⁵ M PGF_{2α} leading to a 106% stimulation above baseline CaM activation levels (Figure 41). PGE₂ in the absence of CaM induced a 50% maximal inhibition of PLA₂ at a concentration of 10⁻⁹ M (Figure 41). PGE₂ also inhibited the stimulating effect of CaM on the enzyme in a dose dependent manner. PGE₂ inhibition of CaM stimulation was not achieved below values of 10⁻⁵ M. Thus PGF_{2α} and PGE₂ had diametrically opposed effects on PLA₂ both in the presence and absence of calmodulin. PGF_{2α} stimulated whereas PGE₂ inhibited Naja naja PLA₂.

Because it has previously been hypothesized that the action of PGE₂

Figure 40. *Naja naja* PLA₂ activity plotted as a function of calmodulin concentration. 1.0 pgm *Naja naja* PLA₂ was incubated with 1 mM CaCl₂ and 1.85 mmol ¹⁴C-arachidonyl-phosphatidylcholine and incubated for 10 minutes at 25°C in 100 mM Tris-HCl buffer, pH 8.9. The reaction was stopped by the addition of 300 µl ethanol.

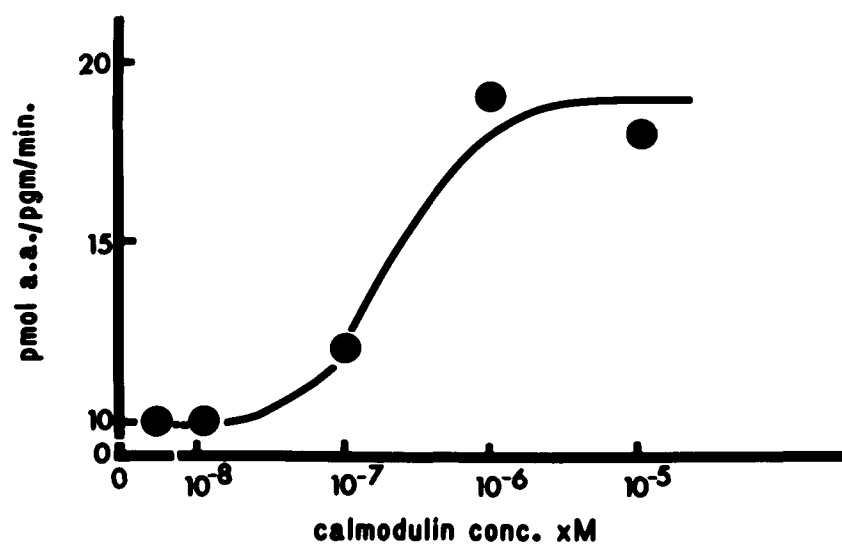
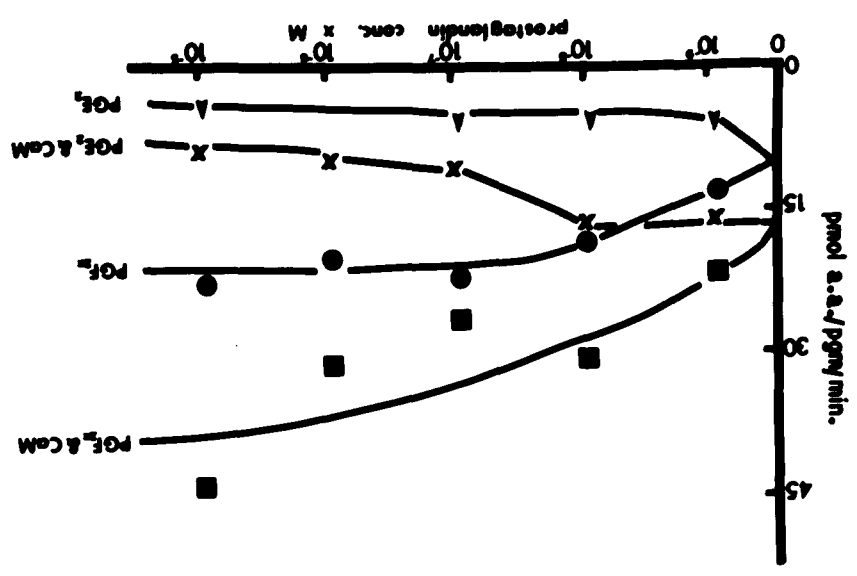


Figure 41. *Naja naja* PLA₂ activity plotted as a function of PGE₂ or PGF_{2α} concentration in the presence of 1 mM CaCl₂, or 1 mM CaCl₂ and 1 μM calmodulin. The origin of the curves represents the baseline values of enzymatic activity in the presence or absence of calmodulin viz. 11.0 pmol/a.a./pgm/min. in the absence of calmodulin and 17.0 pmol/a.a./pgm/min. in the presence of calmodulin. The conditions of incubation and processing are identical to that described in Figure 40.



and $\text{PGF}_{2\alpha}$ are mediated via interaction with adenylate and guanylate cyclases, respectively, which generate cAMP and cGMP, respectively, it was of interest to see what the effects of cAMP and cGMP were on *Naja naja* PLA_2 .

Cyclic AMP in the absence of calmodulin inhibited PLA_2 activity in a dose dependent manner achieving maximum inhibition of 29% at a concentration of 10^{-7} M (Figure 42A). Cyclic AMP also inhibited the CaM stimulation of the enzyme in a dose dependent manner reaching maximal inhibition of 63.5% at a concentration of 10^{-5} M (Figure 42A). Cyclic GMP inhibited PLA_2 in the absence of CaM in a dose dependent manner reaching a maximum of 53.6% inhibition at a concentration of 10^{-5} M (Figure 42B). Furthermore, cGMP inhibited the calmodulin stimulating effect of PLA_2 maximally by 68.2% at 10^{-5} M (Figure 42B). The activity of the enzyme in the presence of calmodulin at this concentration of cAMP was equivalent to its activity with calcium in the absence of CaM.

Thus the effects of both cAMP and cGMP were very similar, implying that both may be directly interacting with the enzyme at a common binding site.

DMS crosslinking of *Naja naja* PLA_2 in the presence of calcium are displayed in Figure 43. In the presence of calcium, polypeptides of M_r 14K and 21K were observed (Figure 43) which corresponded in M_r to monomers and dimers, respectively. The monomer constitutes 77% of the protein and the dimer constitutes 23% of the protein as determined by gel densitometry.

Dimerization was slightly inhibited by EGTA (Figure 43). When DMS crosslinking was performed with calcium in the presence of cAMP, cGMP or PGE_2 , the monomer:dimer ratio remained unchanged in each of these con-

Figure 42. *Naja naja* venom PLA₂ plotted as a function of cAMP (A) or cGMP concentration (B) in the absence ● or presence (■) of calmodulin. The origin of the curves represents the baseline values of enzymatic activity in the presence or absence of calmodulin. The conditions of incubation and processing are identical to that described in Figure 40.

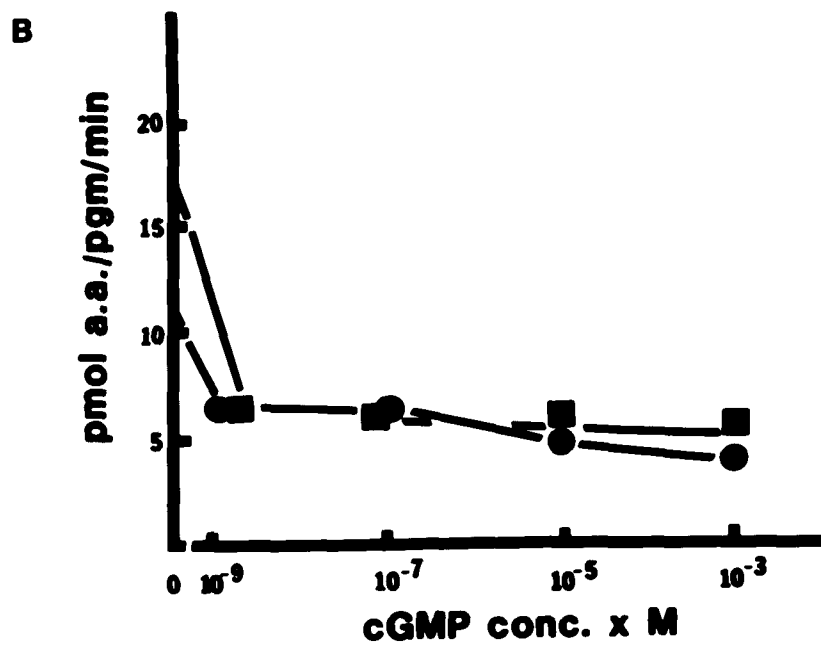
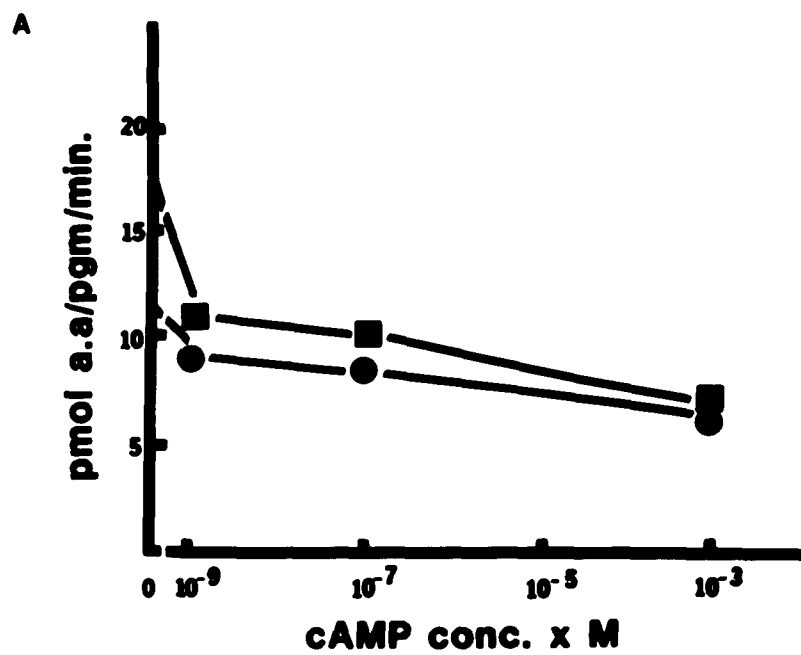
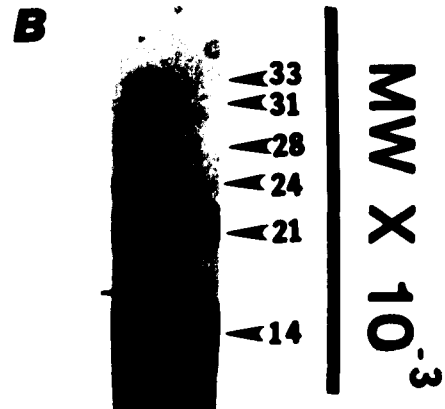
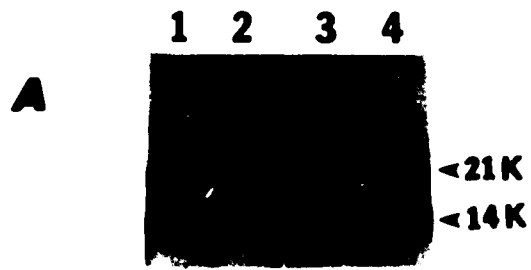


Figure 43. Polypeptide composition of *Naja naja* PLA₂.

A) DMS crosslinked in the presence of 1 mM CaCl₂ (Lane 1), 1mM EGTA (Lane 2), 1 mM calcium and 10 µg PGF_{2α} (Lane 3) or 1 mM EDTA and 10 µg PGF_{2α} (Lane 4).

B) Photographic enlargement of 43A, Lane 3 specifying molecular weights of the higher order polymers.



ditions. DMS crosslinking with $\text{PGF}_2\alpha$ in the presence of calcium led to the appearance of polypeptides with M_r s 14K, 21K, 24K, 28K, 31K and 33K, respectively. It is likely that these M_r s corresponded to the PLA_2 subunits, respectively, of monomers, dimers, trimers, tetramers, pentamers and hexamers. In the presence of $\text{PGF}_2\alpha$ and 1mM EGTA only monomers and dimers could be observed. Dimerization was slightly inhibited and polymers above dimers were absent (Figure 43).

Although the M_r s mentioned do not correspond to the theoretical M_r s of the higher order polymers, the existence of protein bands upon DMS-crosslinking of a single monomer polypeptide implies that the higher M_r species in all likelihood correspond to higher order polymers. It is likely that when PLA_2 monomers are cross-linked, they are crosslinked one to another in such a manner that they form an elongated rod-like structure that is capable of penetrating very deeply into the gel. This would explain why a trimer formed by 3 x 14K monomers (theoretical M_r 42K) migrates in the 24K region. The percent composition of each PLA_2 protein subunit, as determined by densitometry, consisted of 63% monomers, 15.7% dimers, 7.2% trimers, 3.0% tetramers, 2.2% pentamers, and 3.2% hexamers. It is apparent that the increase of PLA_2 activity induced by $\text{PGF}_2\alpha$ in the presence of calcium may in part be responsible for the dose-dependent increase in PLA_2 activity induced by $\text{PGF}_2\alpha$. PGE_2 , cAMP and cGMP did not alter polymerization of the enzyme. Therefore it is likely that these modulators independently inhibited PLA_2 by either changing the conformation of the existing monomers or dimers decreasing their catalytic potential without decreasing their polymerization potential. Alternatively, these modulators may have bound to the substrate and thereby inhibited its hydrolysis. Neither of these

modulators shifted the equilibrium of polymerization in the backward direction, i.e., the percent monomers and dimers always remained constant.

Upon DMS crosslinking in the presence of calcium and calmodulin, polypeptides migrating in the regions of 10K, 14K, 19K, 20K, 21K, 24K, 30K and 36K were observed (Figure 44). It is known from the crosslinking studies performed in the absence of calmodulin (above) that the 14K and 21K polypeptides correspond in M_r to PLA_2 monomers and dimers, respectively. The 30K M_r polypeptide probably corresponds to a PLA_2 -monomer-CaM protein complex. The identities of the other polypeptides await further identification. The polypeptides do not appear when PLA_2 is cross-linked in the absence of calmodulin.

The effects of cAMP, cGMP, and PGE_2 in the presence of calcium alone, however, cannot be attributed to a change in subunit formation. It is possible that the decrease in activity by these modulators is induced by their individual binding to PLA_2 in its monomer or dimer state and conformational changes they induce such that substrate/enzyme interactions are impaired, rather than dimerization. Alternatively, these modulators may inhibit substrate binding to the enzyme and thereby inhibit substrate hydrolysis. In the presence of CaM, the modulators cAMP, cGMP, PGE_2 , and $PGF_{2\alpha}$ individually or in combination did not lead to significant alteration in PLA_2 -CaM complex formation as judged by Coomassie Blue staining intensity. Future studies with radioactive PLA_2 and/or CaM may allow a more accurate quantitation of the effects of these modulators on PLA_2 -CaM complex formation.

Thus it appears that PLA_2 is directly modulated by each of these effectors and that PLA_2 may have binding sites for all of them.

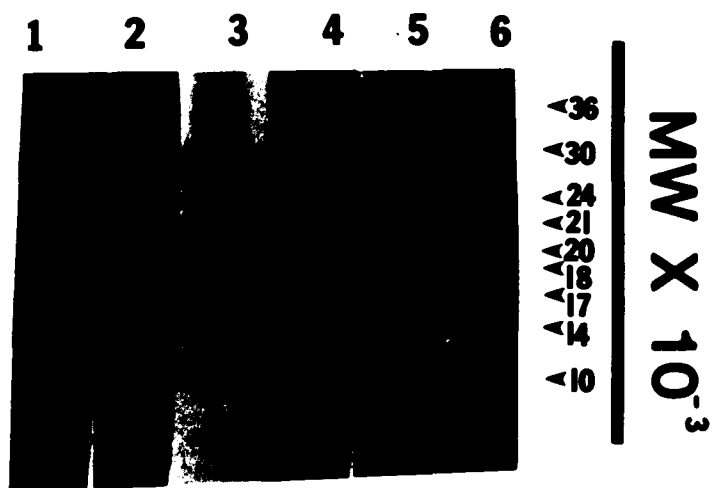
PLA₂ activity in the presence of calcium and calmodulin is reduced to the level of activity present in the absence of calmodulin by both cAMP and cGMP. Thus these modulators apparently may have inhibited PLA₂-CaM formation and may have also changed individual monomer protein conformation impairing enzyme-substrate interaction.

Because PGE₂ and PGF_{2α} induced diametrically opposed effects on PLA₂ activity, it is possible that there exist separate binding sites for these two modulators on the enzyme. Because cAMP and cGMP induced highly similar modulating enzymatic effects, the assumption was made that both nucleotides bind to a common nucleotide binding site on *Naja naja* PLA₂.

Further evidence for the modulation of purified phospholipases A₂ by prostaglandins came from morphological studies on both the light and electron microscopic levels. Preincubation of synaptic vesicles with 10⁻³ - 10⁻⁶ M parabromophenacylbromide, 4.0 nmol PGA₂, 4.0 nmol PGB₂, or 4.0 nmol PGE₂ led to complete inhibition of the ability of exogenous PLA₂ (*Vipera russelli*) to induce vesicle-vesicle clumping and aggregation. There was no morphological distinction between synaptic vesicles treated under these conditions and of untreated synaptic vesicles (Figures not presented). Preincubation of synaptic vesicles with PGE₁, however, did not completely inhibit the ability of PLA₂ to induce vesicle-vesicle aggregation (Figure 45). The effects of these PG's were exerted on the exogenous PLA₂. Inhibition of endogenous PLA₂ would not contribute to the ability of exogenous PLA₂ to hydrolyze vesicle phosphatidylcholine.

Electron microscopy revealed that preincubation of a mixture of synaptic vesicles and synaptic plasma membrane with 4.0 nmol PGE₂ or

Figure 44. Polypeptide composition of *Naja naja* PLA₂ DMS crosslinked in the presence of 1 mM Ca²⁺ and 7.0 µg calmodulin (Lane 1), 1 mM Ca²⁺, 7.0 µg CaM and 10 µg PGF_{2α} (Lane 2). 1mM Ca²⁺, 7.0 µg CaM and 10 µg PGE₂ (Lane 3), 1mM Ca²⁺, 7.0 µg CaM, 1mM cAMP (Lane 4), 1 mM Ca²⁺, 7.0 µg CaM and 1 mM cGMP (Lane 5). 1mM Ca²⁺, 7.0 µg CaM, 1 mM cGMP, 1 mM cAMP, 10 µg PGE₂ and 10 µg PGF_{2α} (Lane 6).



PGE₁ prior to incubation with 10 µg PLA₂ (Naja naja) prevented PLA₂ induced membranal lysis (Figures 46A and 46B). Preincubation with similar concentrations of PGA₂ or PGB₂ did not inhibit the PLA₂ effect (Figures 46C and 46D). This implied that the hydroxyl group of the cyclopentane ring in position 11 was the active group in interacting with Naja naja PLA₂ and inhibiting its stimulation and subsequent membranal lysis.

8. Synaptic and coated vesicle protein kinase activities

The phosphorylation pattern of synaptic vesicle proteins under various conditions is shown in Figure 47. In the presence of Ca²⁺ there is a very low level of phosphorylation. In the presence of Ca²⁺ and calmodulin, proteins with M_rs of 55K and 53K were phosphorylated. However, in the presence of trifluoperazine, a calmodulin inhibitor, a 60% decrease in phosphorylation was noted. Thus phosphorylation in synaptic vesicles appeared Ca²⁺-dependent, and because an exogenous source of protein kinase was not added, it was concluded that synaptic vesicles have an endogenous Ca²⁺/calmodulin-dependent kinase. Incubation of synaptic vesicles with cAMP induced phosphorylation of proteins with the following approximate M_rs: 175K, 100K, 80K, 57K, 55K, 53K, 40K, and 30K. A difference in the protein phosphorylation pattern induced by cAMP, as compared to Ca²⁺/calmodulin, implied that synaptic vesicles also have endogenous cAMP-dependent kinase activity. The proteins of 55K and 53K M_rs were common substrates for both types of kinases. Densitometric analysis revealed that the latter protein was phosphorylated equally by cAMP and Ca²⁺/calmodulin.

Preincubation of synaptic vesicles with anti-clathrin IgG in the

Figure 45. Light microscopy of synaptic vesicles (0.75 $\mu\text{g/ml}$) in a total volume of 200 μl pretreated with 4.0 nmol PGE_1 for 20 minutes prior to treatment with 25 μg PLA_2 (Viperra Russellii). Magnification x 4330.



Figure 46. Electron microscopy of synaptic vesicles and presynaptic plasma membrane mixed in a 1:1 protein ratio and pretreated with 4.0 nmol: (A) PGE₂; (B) PGE₁; (C) PGA₂ or (D) PGB₂ for 30 minutes prior to incubation with 10 µg Naja naja PLA₂ at 25°C. The membranes were suspended in 0.1 M MES, pH 6.5. Magnification x 120,000.





presence of Ca^{2+} and calmodulin effected no change in degree of protein phosphorylation. However, preincubation with anti-30K protein caused a dose dependent decrease in phosphorylation of both aforementioned proteins (Figure 48). Therefore, the calmodulin-dependent kinase in synaptic vesicles may be the 30K protein doublet. Reduction of phosphorylation by the anti-30K protein IgG cannot be attributed to non-specificity of the antibody reaction since anti-clathrin IgG did not affect phosphorylation. When synaptic vesicles were preincubated with the anti-30K protein antibodies in the presence of cAMP, phosphorylation of the different proteins was reduced 15-95%.

The phosphorylation pattern of coated vesicles induced by various conditions is demonstrated in Figure 49. In the presence or absence of Ca^{2+} , phosphorylation is at a very low baseline level. In the presence of Ca^{2+} and calmodulin, proteins with M_r s of 175K and 55K were phosphorylated. Incubation with trifluoperazine diminished phosphorylation. Cyclic-AMP did not activate protein phosphorylation in coated vesicles. When coated vesicles were preincubated in the presence of Ca^{2+} and calmodulin with anti-clathrin IgG, densitometric analysis revealed no reduction in phosphorylation. If under the same conditions the coated vesicles were preincubated with anti-30K protein IgG, phosphorylation of both proteins partially diminished. When the de-coated vesicles were incubated in the presence of $[\gamma^{32}\text{p}]\text{-ATP}$ and Ca^{2+} , Ca^{2+} and calmodulin, or Mg^{2+} and cAMP, phosphorylation was not detected under any of these conditions.

Figure 47. Autoradiogram of synaptic vesicle proteins incubated in the presence of [γ ^{32} P]-ATP. Gels were analyzed by 5-15% SDS-PAGE.

Incubation conditions include: lane 1 - Ca^{2+} ; lane 2 - Ca^{2+} and calmodulin (CAM); lane 3 - Ca^{2+} , calmodulin and trifluoperizine (TFP); lane 4 - Ca^{2+} , calmodulin and affinity-purified anti-clathrin IgG; lane 5 - Ca^{2+} , calmodulin and anti-30K protein IgG; lane 6 - Mg^{2+} and cAMP; lane 7 - Mg^{2+} , cAMP- and affinity-purified anti-30K protein IgG.

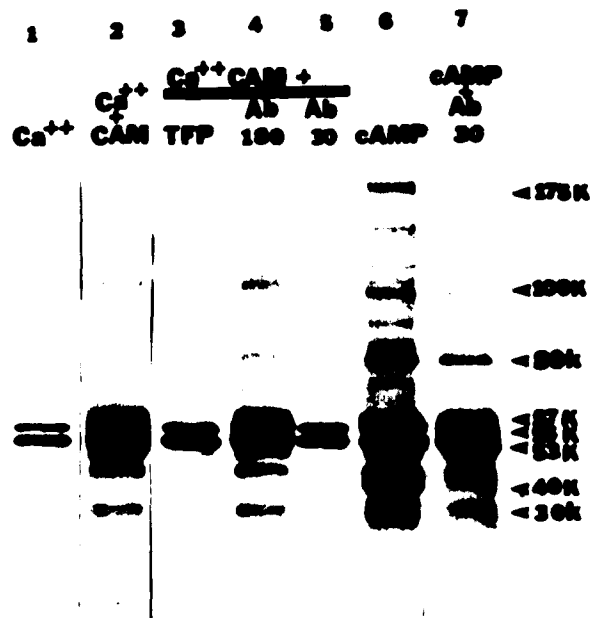


Figure 48. Percent [γ ³²P] incorporation into the major calmodulin-mediated phosphorylated protein bands of M_r 55K (■) and 53K (●) plotted as a function of anti-30K IgG concentration. Phosphorylation in the presence of Ca²⁺ and calmodulin and in the absence of anti-30K protein IgG was taken as 100% phosphorylation. Percent [γ ³²P] incorporation was estimated from densitometric scans by integration of the protein band peak areas.

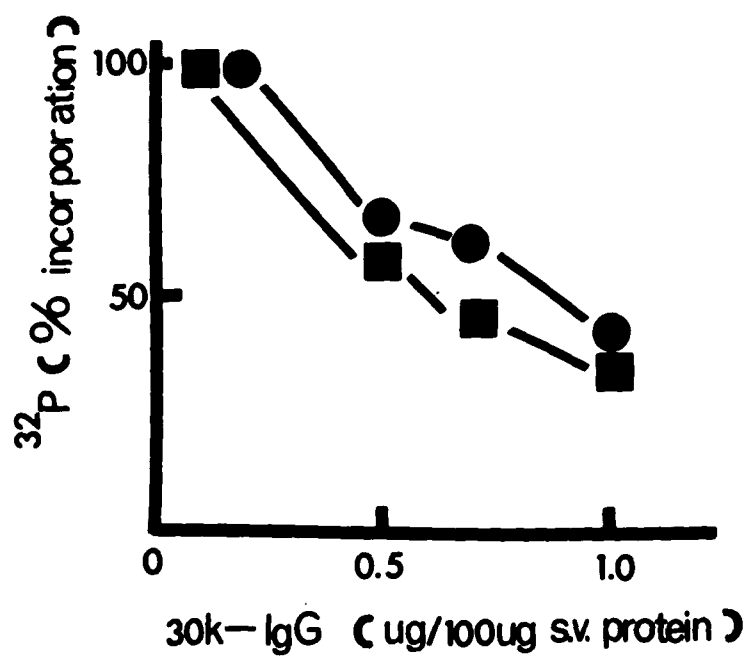
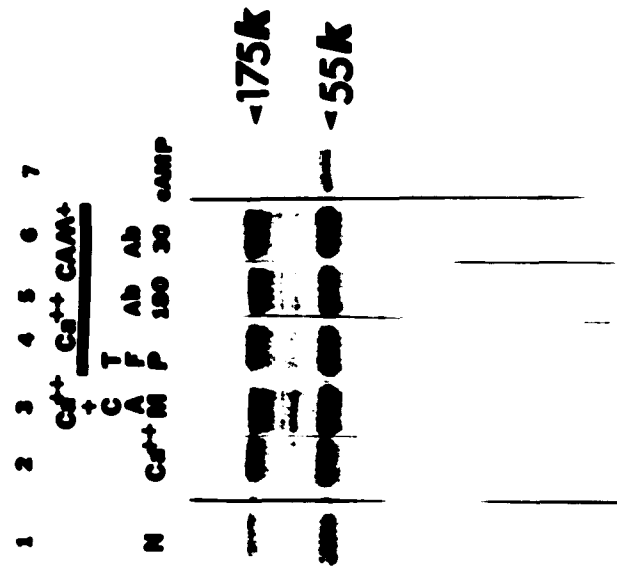


Figure 49. Autoradiogram of coated vesicle proteins incubated in the presence of [γ ³²P]-ATP. Gels were analyzed by 5-15% SDS-PAGE.

Incubation conditions included: lane 1 - no Ca²⁺ or Mg²⁺; lane 2 - Ca²⁺; lane 3 - Ca²⁺ and calmodulin; lane 4 - Ca²⁺, calmodulin and trifluoperazine (TFP), lane 5 - Ca²⁺, calmodulin and affinity-purified anti-clathrin IgG; lane 6 - Ca²⁺, calmodulin and anti-30K protein IgG; lane 7 - Mg²⁺ and cAMP.



9. Synaptic and coated vesicle $[Ca^{2+}-Mg^{2+}]$ -ATPase activities

Figures 50 and 51 illustrate the effect of calmodulin on synaptic and coated vesicle $[Ca^{2+}-Mg^{2+}]$ -ATPase activities, respectively. $[Ca^{2+}-Mg^{2+}]$ -ATPase activity in both vesicle types was linear for the first ten minutes of incubation and declined steadily over the next 30 minutes. At each time point calmodulin stimulated $[Ca^{2+}-Mg^{2+}]$ -ATPase activity. Calmodulin maximally stimulated $[Ca^{2+}-Mg^{2+}]$ -ATPase by 50% at the 45 minute time point. The amount of calcium sequestered by the vesicles was calculated by correlating the amount of ^{45}Ca which co-eluted with vesicle protein as determined by optical density scanning at 280 nm. Scanning the elution profile of vesicles eluting from the Sephadex G-50 column revealed that most of the synaptic vesicles were eluted from the column. It was found that calmodulin optimally stimulated calcium uptake in synaptic vesicles by 140% from 30 to 72 pmol Ca^{2+} /mg protein/hour and by 80% in coated vesicles from 320 to 580 pmol/mg/min. The value of calmodulin stimulation, however, varied within a certain range. It is possible that the variability may be due to the capability of different vesicle samples to be eluted from the Sephadex G-50 column. Calmodulin appeared to have a greater stimulating effect on calcium uptake than on $[Ca^{2+}-Mg^{2+}]$ -ATPase activity. The procedure used for calcium uptake determinations may have been better able to detect the effect of calmodulin. Alternatively, calmodulin may be activating an enzyme, or altering a protein conformation other than the $[Ca^{2+}-Mg^{2+}]$ -ATPase which could result in increased vesicle permeability not directly related to $[Ca^{2+}-Mg^{2+}]$ -ATPase or Ca^{2+} uptake activities.

Figure 50. $[Ca^{2+}+Mg^{2+}]$ -ATPase activity. ATP hydrolysis was measured by the release of $[\gamma^{32}P]$ from ATP. Denoted are synaptic vesicles incubated with (■) or without (●) calmodulin.

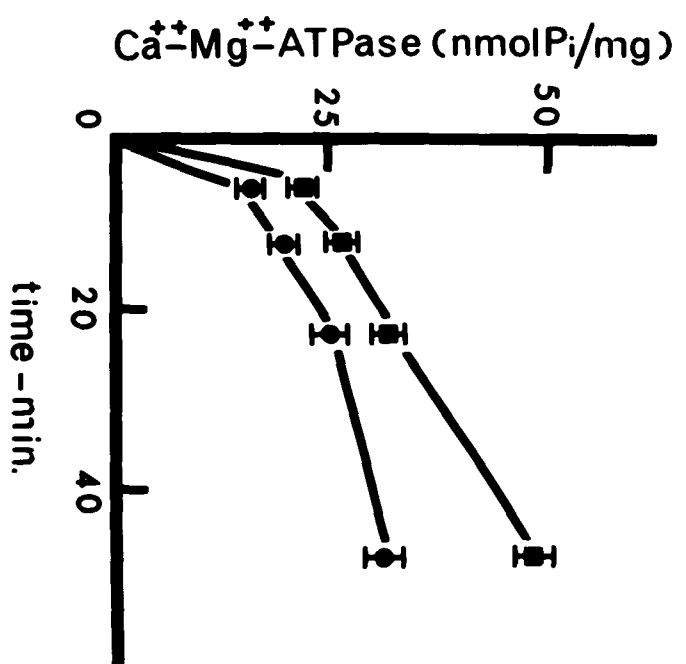
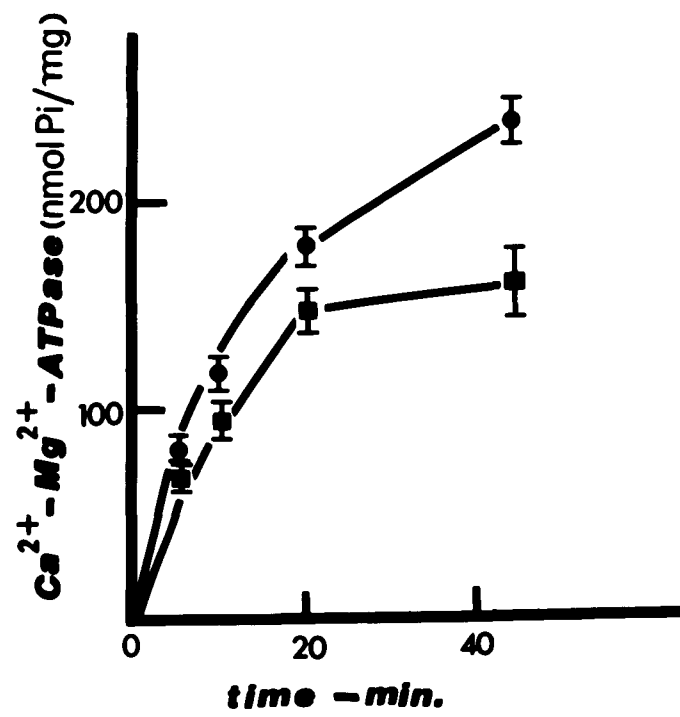


Figure 51. $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase activity. ATP hydrolysis was measured by the release of " γ $^{32}\text{P}_1$ " from ATP. Denoted are coated vesicles incubated with (●) or without (■) calmodulin.



D. DISCUSSION

1. Calmodulin association with brain synaptic and coated vesicles

The fact that multiple proteins from both synaptic and coated vesicles bound to a calmodulin affinity column in a calcium dependent manner lends credence to the interpretation of the curved Scatchard plot of synaptic and coated vesicle calmodulin binding as being indicative of the existence of multiple calmodulin binding sites in both synaptic and coated vesicles.

The major calmodulin binding proteins of synaptic vesicles consisted of protein doublets with M_r s 55K and 30K. The major calmodulin binding proteins of coated vesicles consisted of one singlet of M_r 100K and two doublets of M_r 55K and 30K. Protein doublets of M_r s 55K and 30K from both vesicle types bound to camodulin. These proteins may be identical proteins common to both vesicle types and may account for the similar camodulin affinity values in synaptic and coated vesicles (see Results - Table 1).

The data indicated that calmodulin was associated with proteins of the membrane bilayer of coated vesicles rather than with the coat lattice. This was determined in several ways. First radioimmunoassay revealed that removal of the coat lattice from the coated vesicle did not decrease calmodulin content. Second, clathrin, the predominant coat protein, did not bind to the calmodulin affinity column. The origin of the difference between the calmodulin content of coated vesicles reported here and in the study by Linden et al. (815) is unclear.

The following observations support the concept that the 30K M_r protein doublet that bound to calmodulin in both synaptic and coated

vesicles is an endogenous calcium/calmodulin dependent protein kinase:

1) When the protein was purified from coated vesicles, it bound entirely to calmodulin; 2) Affinity purified antibodies raised against the purified protein inhibited the Ca^{2+} /calmodulin dependent protein kinase in synaptic vesicles in a dose-dependent manner (to be discussed in greater length later on in this section); and 3) Immunoelectronmicroscopy of the affinity purified antibodies labeled synaptic and coated vesicles but did not label the cytoplasm or the presynaptic plasma membrane in axon terminals of the rat brain cerebellum (816).

The protein subunits of the $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase in sarcoplasmic reticulum have been reported to have M_r s of 100K and 55K (798). Because calmodulin activated $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase in both synaptic and coated vesicles, it is possible that the 100K singlet in coated vesicles and the 55K doublet in both coated and synaptic vesicles that bound to calmodulin may be components of endogenous $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase. Because $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase activity was higher in coated vesicles the 100K putative component of the $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase may be present in relatively higher quantities in coated vesicles. Alternatively, the 100K singlet in coated vesicles may be an endogenous coated vesicle transglutaminase. Platelet transglutaminase has been demonstrated to be activated approximately fivefold by calmodulin and to bind to a calmodulin affinity column (E. Puszkin, unpublished observations). It is also possible that both transglutaminase and $[\text{Ca}^{2+}\text{-Mg}^{2+}]$ -ATPase enzymes in coated vesicles bind to calmodulin and comigrate with each other in SDS gels.

It is possible that the 55K calmodulin binding protein doublets in both synaptic and coated vesicles may be alpha and beta tubulin.

Tubulin has been demonstrated to be endogenous to synaptic vesicles (767) and has been demonstrated to bind to calmodulin (772).

Because synaptic and coated vesicle PLA₂ activities were stimulated by calmodulin, it is likely that calmodulin must bind to these endogenous PLA₂s for stimulation to occur. Assuming amino acid sequence homology of vesicle PLA₂ to other PLA₂s thus far purified, its M_r is probably 14K. A protein with this M_r has not been detected by Coomassie blue stained SDS PAGE gels in either synaptic or coated vesicles before or after calmodulin affinity chromatography. Because PLA₂ usually constitutes less than 0.1% of the total membrane protein content (817), it is unlikely that this protein would be visualized in a PAGE gel stained by Coomassie blue. This point emphasizes the distinct possibility of the existence of calmodulin binding proteins in synaptic and coated vesicles that may be present in low protein concentrations that may not be detected by the methods employed in this study.

2. Membrane PLA₂

Previous studies have revealed that human and rat brain homogenate PLA₂ activity have pH optima of 5.5 and 5.0, respectively, and are not Ca²⁺ dependent (412,413). Because lysosomal PLA₂ has a pH optimum in the acidic range and is not Ca²⁺ dependent (416), it is likely that the PLA₂ activity predominant in the above homogenates (412,413) was of lysosomal origin. Goracci et al. demonstrated that brain mitochondrial PLA₂, which comprises 63% of PLA₂ activity in brain homogenate (416), has a pH optimum of 8.4 and is inhibited by Ca²⁺. Bazan, Jr. (415) demonstrated that brain microsomes have predominantly PLA₁ rather than PLA₂ activity. Brain glial PLA₂ activity has a pH optimum of 8.0

(418). Based on a comparison of the PLA₂ characteristics of synaptic and coated vesicle, and synaptic plasma membrane preparations to PLA₂ activities found in other brain membranal systems, it is unlikely that PLA₂ activity present in the brain membranal PLA₂s in this study represents an artifact of fractionation, or that they are significantly contaminated by PLA₂ derived from neuronal microsomes, mitochondria, lysosomes or glial membranes. All the PLA₂s from other sources which could possibly have contaminated these preparations have different Ca²⁺ and/or pH dependencies.*

Exogenously added PLA₂ and lysolecithin independently induced an increase in synaptic and coated vesicle light scattering. The addition of Ca²⁺ in the presence of either calmodulin, PGF_{2α} or cAMP and ATP induced a synaptic and coated vesicle light-scattering profile comparable to that produced by exogenous PLA₂. These effects were probably due in part to the activation of endogenous synaptic PLA₂ because quinacrine and/or parabromophenacylbromide diminished them.

Although quinacrine has been demonstrated to inhibit calmodulin (818) as well as PLA₂, it is unlikely that calmodulin was the site of quinacrine inhibition because endogenous synaptic vesicle calmodulin was completely removed from the vesicles as determined by RIA. Although it

*In addition, recent studies in progress (Schook, W., Andres, A., and Hua, E., unpublished observations) while this thesis was being prepared showed that the synaptic vesicles employed throughout this study did not have detectable or significant amounts of brain synaptic plasma membrane, microsomes, lysosomes, Golgi or soluble cytoplasmic material as indicated by enzymatic marker studies. In addition, synaptic vesicles prepared by an alternative procedure (Zizapel, Brain Research) also had Ca²⁺/calmodulin dependent PLA₂. Furthermore, at the conditions under which PLA₂ was assayed, brain lysosomes, Golgi, microsomes and cytosolic material did not have PLA₂ activity as responsive to calmodulin stimulation compared to synaptic vesicles prepared by the two alternative procedures.

is possible that quinacrine or parabromophenacylbromide may have non-specific effects other than PLA₂ inhibition, the fact that both these compounds independently produced the same effects implied that PLA₂ inhibition was the predominant action of these compounds in this system.

Individually ATP inhibited synaptic vesicle PLA₂ by 50 percent and cyclic AMP activated the enzyme by 50%. However cAMP in conjunction with ATP activated PLA₂ by 200%. The difference in individual versus combined effects of these nucleotides on PLA₂ activity may be due to an involvement of endogenous synaptic vesicle protein kinase. As will be discussed in detail later in this section, 100 μ M cAMP stimulated phosphorylation of a synaptic vesicle protein with a M_r of 40K, as well as many other proteins.

As mentioned in the introduction, Hirata reported that lipomodulin, a 40K protein found in neutrophils and synaptic plasma membrane (518,851), inhibits PLA₂. Upon phosphorylation by Ca²⁺ or cyclic AMP, however, lipomodulin's inhibitory effect is suppressed (518). It is possible that the phosphorylated 40K protein in synaptic vesicles is lipomodulin and that the stimulation induced by cAMP and ATP on synaptic vesicle PLA₂ is affected by way of phosphorylation of this protein. Although Ca²⁺ inhibits cAMP-dependent protein kinases, it is possible that this protein kinase is dependent on both Ca²⁺ and cAMP, as appears to be the implication in another brain protein kinase system (819). Neither Ca²⁺ nor calcium and calmodulin induced phosphorylation of this protein.

Table 1 compares the different characteristics of the 3 different membranal PLA₂'s studied.

Table 1

A comparison of the different characteristics of synaptic vesicle, coated vesicle, and synaptic plasma membrane PLA₂ activities.

Membrane	Ca ²⁺ conc.	pH	V _{max} (nmol a.a./ mg/hr)	K _m (μM)	k
	optimum (M)	optimum			
Synaptic vesicle	1.0 x 10 ⁻⁸	9	2.0	60.0	.0028 (mg ⁻¹ /min ⁻¹)
Synaptic plasma membrane	1.0 x 10 ⁻⁷ - 1.0 x 10 ⁻⁸	7	98.0	60.0	.1300 (mg ⁻¹ /min ⁻¹)
Coated vesicle	1.0 x 10 ⁻⁸	9	22.6	3.5	.5300 (mg ⁻¹ /min ⁻¹)

In all the membranal PLA₂ activities assayed, the kinetic parameters presented in Table 1 represent activities of endogenous membranal PLA₂ that hydrolyzed exogenous phosphatidylcholine. It should be emphasized that the values presented are mere reflections of the enzymatic activity that could be expressed in vivo. Specifically, PLA₂ activity was expressed in terms of activity/mg membrane protein. PLA₂ usually constitutes less than 0.1% of the total membrane protein (817). PLA₂ activity/mg purified enzyme would therefore be approximately 1000 times higher than that presented in Table 1. It should also be emphasized that the amount of endogenous phosphatidylcholine that is present in synaptic and coated vesicles has been reported to be 32.3% and 42% of the total vesicle lipid content respectively (166,253). Thus, in all the PLA₂ assays, exogenous and endogenous phosphatidylcholine were competing for enzyme interaction. Because the amount of endogenous substrate is considerable, the amount of competition is in all likelihood also significant. Taking these factors into consideration, the PLA₂ relative V_{max}'s of the different membranes may be approximately 10³-10⁴ times higher per enzyme than the values presented in Table 1.

The various pH optima of the different membranal PLA₂s may reflect the pH conditions at which PLA₂ is oriented within the membrane to optimally hydrolyze exogenous substrate. The pH optimum may not necessarily reflect the in vivo pH optima of these enzymes.

In comparing relative activities of the three enzymes in Table 1, it is apparent that coated vesicles have the highest affinity for the substrate and the highest relative enzymatic activity. Synaptic plasma membranes and synaptic vesicles have the second highest and lowest

activities, respectively. Synaptic plasma membrane PLA₂ activity is 4.6×10^1 times that of synaptic vesicle PLA₂. Coated vesicle PLA₂ is 4.1 times that of synaptic plasma membrane PLA₂ and 1.89×10^1 times greater than synaptic vesicle PLA₂.

Morphological and biophysical studies correlated PLA₂ activity in all 3 membrane preparations with membranal lysis, and altered morphology and vesicle-vesicle aggregation. The strategy developed was to observe the effects of exogenous PLA₂ and lysolecithin on these parameters and to compare them to the effects on these parameters by modulators which stimulated endogenous PLA₂ activity. Those modulators which did stimulate PLA₂ activity induced similar biophysical and morphological alterations as did exogenous PLA₂. These functional correlations along with the fact that the PLA₂ inhibitors, parabromophenacylbromide and quina-
crine (530-532, 520), diminished the modulators' ability to induce biophysiological and morphological alterations buttressed the hypothesis that endogenous PLA₂ was at least partly involved in these effects.

The in vivo physiological manifestations of PLA₂ stimulation in synaptic vesicles and synaptic plasma membrane could be the induction of membranal lysis in both these membranes and subsequent synaptic vesicle-synaptic plasma membrane fusion. Once synaptic vesicle-synaptic plasma membrane fusion has occurred, the activation of endogenous coated pit and/or synaptic plasma membrane PLA₂ on both sides of the coated pit membrane could lead to membranal lysis and eventual detachment of the coated pit from the plasma membrane. Although vesicle fragments reseal naturally, it is quite possible that coated membrane PLA₂ activation may function in the fusion of the free coated pit membrane ends to each other to form a coated vesicle.

It is necessary to reevaluate the physiological significance of the different calcium concentration optima of the phospholipases A_2 in the different membranes and their relative activities in light of their distinct functions. Synaptic plasma membrane PLA_2 has the lowest calcium concentration optimum of all 3 membranal phospholipases A_2 ranging from 10^{-7} - 10^{-8} M. The calcium concentration of axoplasm in the unstimulated axon terminal ranges from 10^{-8} - 10^{-7} M (184). This may mean that synaptic plasma membrane PLA_2 in the resting neuron may be very active. Synaptic plasma membrane PLA_2 may have the most varied function of all three membranal phospholipases A_2 studied in this work. It has been demonstrated that exogenous PLA_2 inhibits neurotransmitter and sodium uptake into the axon terminal and inhibits ligand binding to synaptic membranes (400, 468-471, 478). Presumably the exogenous PLA_2 is exerting these effects by acting on the synaptic plasma membrane.

Because endogenous synaptic plasma membrane- PLA_2 is active at Ca^{2+} concentrations of 10^{-7} - 10^{-8} M, which is within the calcium concentration range of both unstimulated and stimulated axon terminals, synaptic plasma membrane- PLA_2 may function in synaptic vesicle-synaptic plasma membrane fusion in the stimulated cell and in the regulation of axon terminal neurotransmitter uptake, ion fluxes, and neurotransmitter binding to synaptic plasma membrane in the resting cell. As long as reacylation of lysophosphoglycerides occurs by acetyltransferases in synaptic plasma membrane after PLA_2 activation (820), lysis of synaptic plasma membrane would not occur nor would neurotransmitter be released in excess.

It is quite possible that synaptic vesicles have the lowest activity of all 3 membranes studied because it is this membranal enzyme which

might critically control neurotransmitter release. If the synaptic vesicles had a high relative activity they might fuse with each other, with membranal areas other than at the active site, or in an unregulated manner with the presynaptic plasma membrane. It is therefore possible that the relatively low PLA₂ activity in synaptic vesicles is a mechanism which ensures the controlled regulation of synaptic vesicle-synaptic plasma membrane fusion. For synaptic vesicle-synaptic plasma membrane fusion to occur, it is possible that both synaptic vesicle and synaptic plasma membrane phospholipases A₂ must be activated and that PLA₂ activity is relatively higher in synaptic plasma membrane because synaptic plasma membrane is the immobile membrane and because the synaptic vesicles can be propelled into it.

The Ca²⁺ concentration optima of the three different membranal PLA₂'s range from 10⁻⁷-10⁻³ M (Discussion - Table 1). In all cases PLA₂ activity declines beyond mM concentrations of calcium (Results - Figs. 18, 27, 30). Within the stimulated axon terminal as calcium increases from 10⁻⁶-10⁻³ M at the release sites, synaptic plasma membrane-PLA₂ and synaptic vesicle-PLA₂, which have Ca²⁺ concentration optima of 10⁻⁷ and 10⁻⁵ M, respectively, may be sequentially activated leading to synaptic vesicle-synaptic plasma membrane fusion. At 10⁻³ M Ca²⁺, the peak Ca²⁺ concentration at the site of neurotransmitter release (182), coated pit PLA₂ is maximally stimulated and subsequent endocytosis of synaptic vesicle membranes by the coated pit/vesicle ensues. Concomitantly at this calcium concentration, synaptic plasma membrane-PLA₂ activity declines precipitously thus leading to a decline in the rate of synaptic vesicle-synaptic plasma membrane fusion. Beyond 1mM Ca²⁺, coated vesicle-PLA₂ activity declines precipitously (Results - Fig. 30) and

beyond 5 minutes of incubation, activity ceases to be linear with time (Results - Fig. 31). Synaptic vesicle and synaptic plasma membrane PLA₂ activities in comparison are linear for 60 and 80 minutes respectively (Results - Figs. 19 and 20). It is possible that the relatively high activity of coated vesicle-PLA₂ may be a reflection of the high concentration of PLA₂ in this organelle which may be a compensatory mechanism to allow coated vesicles to be maximally active in a very restricted time and calcium concentration range.

In all three membranes, PLA₂ activity declines beyond the mM Ca²⁺ concentration range. This phenomenon is not readily explainable. This phenomenon may reflect an artifact of the PLA₂ assay which measured the amount of exogenous substrate hydrolyzed, in that Ca²⁺ concentration may alter the degree of exposure of membranal PLA₂ to exogenous substrate. This phenomenon cannot be definitively explained unless these parameters are obtained with PLA₂ purified from each of these membranes.

It is very likely that the coat of coated vesicles protects this organelle from random membrane fusion by preventing interaction with other membranes and subsequent activation of its endogenous PLA₂. Coated vesicles have been observed to fuse artifactually with endoplasmic reticulum in axon terminals (123) and to fuse with lysosomes and other membranes in other cell types (297). In all these cases the region of coated vesicle-membrane fusion is at coated vesicle membranal loci which are partially decoated. The fact that the relative activity of PLA₂ in coated vesicle is so high implies that uncoated vesicles would fuse very rapidly. Thus the coating would prevent coated vesicle membrane fusion induced by this highly active endogenous PLA₂ by preventing contact with other membranes. Another mechanism of

controlling excess fusion of free floating uncoated vesicles is having a relatively low vesicle PLA₂ activity and a relatively high PLA₂ activity in the immobilized membrane with which the vesicle fuses. This is the case in synaptic vesicle-synaptic plasma membrane fusion.

This study confirms previous studies demonstrating that exogenous PLA₂ can create lysis of synaptic vesicles, synaptic membranes and other plasma membranes (371, 382, 384). In addition, the aggregation of decoated vesicles by exogenous lysolecithin and PLA₂ are in line with previous studies where application of exogenous PLA₂ to axon terminals led to an increased production of coated vesicles (465). These studies are also in line with those studies demonstrating that quinacrine, parabromophenacylbromide and chlorpromazine inhibit coated pit/coated vesicle mediated endocytosis (466).

It is of interest that under baseline conditions of membranal PLA₂ activity in the absence of any modulators, calcium by itself did not significantly induce synaptic or decoated vesicle interactions or membranal alterations. Only those modulators which stimulated PLA₂ significantly in these membranes induced significant observable changes in the parameters studied. Those modulators which either had no effect or inhibited membranal PLA₂ activities did not lead to any significant biophysical or morphological changes. This may not mean that calcium, in vivo, in the absence of any of the modulators studied does not lead to fusion or other membranal phenomena. Under these in vitro conditions, significant stimulation of membranal PLA₂ might have been necessary to observe membranal alterations by the methods employed.

A comparison of the relative effects of the modulators studied on synaptic vesicle, synaptic plasma membrane and coated vesicle PLA₂

activities is presented in Table 2. In all three membrane types calmodulin and $\text{PGF}_{1\alpha}$ were always stimulatory, and ATP was always inhibitory. PGE_2 inhibited synaptic vesicle and synaptic plasma membrane PLA_2 and stimulated coated vesicle PLA_2 . The regulation of coated vesicle PLA_2 is in this manner different from the regulation of synaptic vesicle and synaptic plasma membrane PLA_2 . This different regulation buttresses the contention that the different membrane preparations were not significantly cross-contaminated. Cyclic-AMP stimulated synaptic vesicle- PLA_2 but inhibited both synaptic plasma membrane and coated vesicle PLA_2 . These studies confirmed the findings of Wong and Cheung that calmodulin stimulates membranal PLA_2 (319). $\text{PGF}_{1\alpha}$ in general has been shown to stimulate neurotransmission (625). PGE_2 which acts as a sedative and inhibits neurotransmission (625) inhibited synaptic vesicle and synaptic plasma membrane PLA_2 . It is possible that the opposing effects of $\text{PGF}_{1\alpha}$ and PGE_2 on neurotransmission may be exerted via their interaction with synaptic vesicle and synaptic plasma membrane PLA_2 . Recently other investigators have reported that activation of synaptosomal PLA_2 by calcium induces neurotransmitter release (821). It has also been demonstrated that stimulation of endogenous adrenal chromaffin granule PLA_2 leads to catecholamine release (822, 823). Thus far no effects of prostaglandins on coated pit endocytosis have been reported. Coated vesicle PLA_2 is the only PLA_2 of the three membranes which was stimulated by PGE_2 . It is possible that because coated vesicle PLA_2 may function in a limited range of conditions, it may be important to continually amplify its activity.

It is interesting to note that ATP inhibits all the membranal

Table 2

Comparison of the effects of various modulators on synaptic vesicle (SV), coated vesicle (CV) and synaptic plasma membrane (SPM) PLA₂ activities.

Modulator	% Stimulation (+)/Inhibition (-) of PLA ₂ activity		
	SV	SPM	CV
Calmodulin	350 (+)	638 (+)	131 (+)
PGF _{2α}	700 (+)	70 (+)	168 (+)
PGE ₂	70 (-)	8 (-)	31 (+)
cAMP	50 (+)	36 (-)	38 (-)
ATP	50 (-)	70 (-)	98 (-)

PLA₂'s studied. ATP and other purines have been demonstrated to inhibit neurotransmitter release (775). This inhibition may be attributed in part to inhibition of synaptic vesicle and synaptic plasma membrane PLA₂. No effects of ATP on coated vesicle endocytosis have thus far been reported. The stimulation and inhibition of cAMP in different membranal PLA₂s is in line with past literature that cAMP has different effects on different membranal PLA₂s (459, 460, 317).

Cyclic AMP and ATP in the presence of calcium and magnesium potentiated synaptic vesicle-PLA₂ activity. This phenomenon was not observed with synaptic plasma membrane-PLA₂ activity.

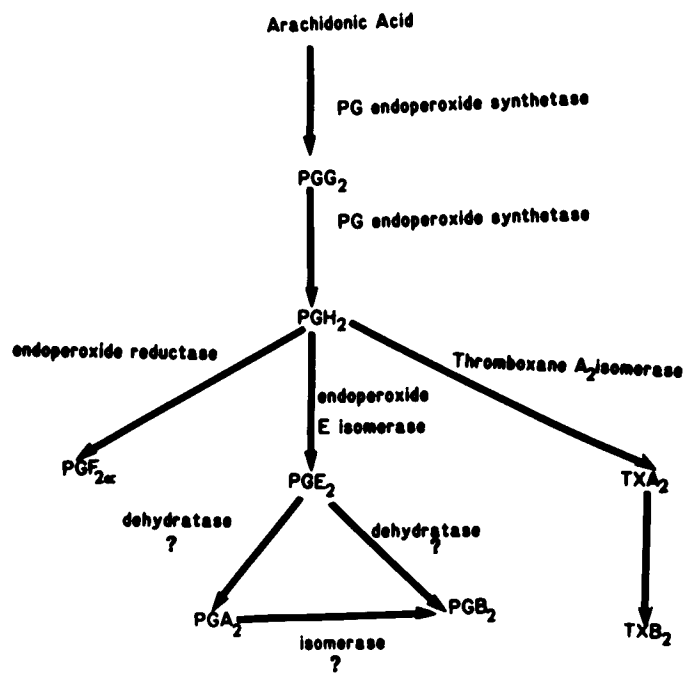
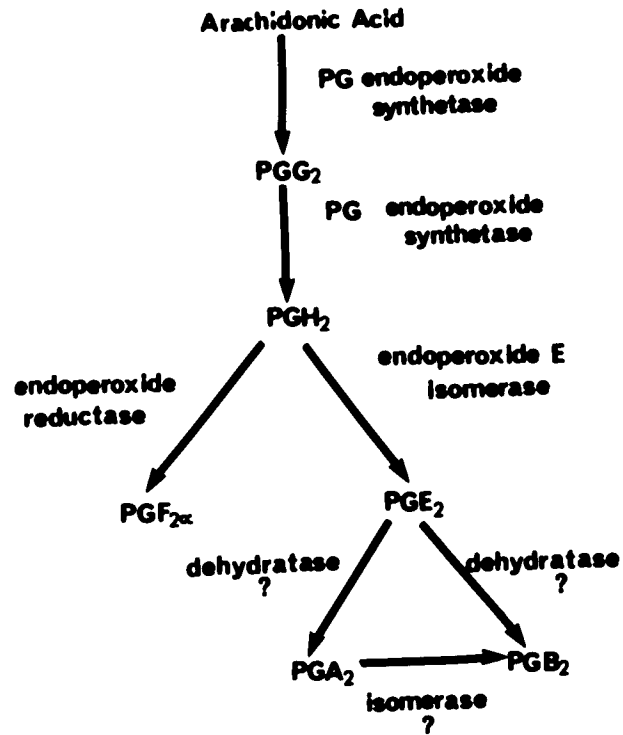
3. Synaptic and coated vesicle prostaglandin and Thromboxane synthetase

The proposed metabolic pathway of arachidonic acid in brain synaptic vesicles is outlined in Figure 1. Arachidonic acid is oxygenated by prostaglandin synthetase to PGG₂, a 15-hydroperoxy prostaglandin endoperoxide, and then reduced by the same enzyme to PGH₂, 15-hydroxy prostaglandin endoperoxide. The highly unstable PGH₂ is converted to PGF_{2α} or PGE₂ by endoperoxide reductase or prostaglandin E isomerase, respectively. Prostaglandin E₂ may be converted to PGA₂ or PGB₂ by dehydrogenases. However, this is not certain since these metabolites are converted from PGE₂ by non-enzymatic means as well (582,583).

The possible metabolic pathway of arachidonic acid in coated vesicles is shown in Figure 2. This pathway is similar to that found in synaptic vesicles except that coated vesicles also are capable of synthesizing thromboxane A₂, which is highly labile (T 1/2 = 30 seconds) and is converted to thromboxane B₂. PGF_{2α}, PGE₂, and TxB₂ have all been demonstrated to be synthesized in the brain. It has been demonstrated

Figure 1. Proposed metabolic pathway of arachidonic acid in synaptic vesicles.

Figure 2. Proposed metabolic pathway of arachidonic acid in coated vesicles.



that synaptosomes store and synthesize PGE_2 and $\text{PGF}_{2\alpha}$ (824). Previous studies have demonstrated that prostaglandins of the one series are located in synaptic vesicles but are not synthesized there (600). My data would confirm the fact that prostaglandins are present and are synthesized in axon terminals and that these vesicular organelles are capable of synthesizing prostaglandins. It is of interest to note that synaptic vesicles are postulated to arise from either perikaryonal or axon terminal endoplasmic reticulum (155,156), membranes where PG synthetase is most highly concentrated and commonly located. Thus it is not surprising, considering the origin of synaptic vesicles that they are capable of synthesizing prostaglandins. Coated vesicles which regenerate synaptic vesicles are thereby also derived from endoplasmic reticulum and are capable of synthesizing PGs. It is evident therefore that both coated and synaptic vesicles have endogenous PLA_2 which can hydrolyze phosphatidylcholine to arachidonic acid and lysolecithin. The arachidonic acid which is released can be metabolized to prostaglandins. In synaptic vesicles PGE_2 feedback inhibits, and $\text{PGF}_{2\alpha}$ feedback stimulates PLA_2 . In coated vesicles both PGE_2 and $\text{PGF}_{2\alpha}$ feedback stimulate PLA_2 . It would be of interest to study the effect of TxB_2 on coated vesicle PLA_2 *. The importance of PG feedback in axon terminal regulation will be emphasized later on in this section.

It has been postulated that PGE_2 may inhibit neurotransmission by either preventing calcium influx into the axon terminal or promoting calcium efflux out of the terminal (646, 647). A recent study, however, has demonstrated that PGE_2 does not influence calcium flux across membranes (825). The only other alternate hypothesis explaining the

* Due to the high cost of TxB_2 this study was not performed.

molecular actions of PGE_2 and $\text{PGF}_{2\alpha}$, respectively, is that they stimulate adenylate and guanylate cyclases, respectively (688). It is possible that PGE_2 and $\text{PGF}_{2\alpha}$ may regulate neurotransmission in addition by their effects on synaptic vesicle and synaptic plasma membrane PLA_2 .

This study has demonstrated that a variety of modulators can stimulate or inhibit membranal PLA_2 . These modulators have variable effects on the different membranal PLA_2 activities. Is the variability due to the intrinsic differences of the different enzymes? Do the modulators directly affect membranal PLA_2 by binding to the enzyme or indirectly affect PLA_2 activity by binding to another membranal component which then influences membranal PLA_2 ? Because of the difficulty in purifying PLA_2 from membranes, the answer to these questions was sought by assaying several purified phospholipases A_2 .

4. Purified phospholipases A_2

It was found that calmodulin bound to and activated 4 out of 5 purified PLA_2 s. This was the first demonstration of calmodulin activation of purified PLA_2 s. CA PLA_2 was the only enzyme that did not bind to calmodulin. CDT PLA_2 formed monomer-calmodulin and dimer-calmodulin protein complexes. In the presence of calcium alone, this PLA_2 formed dimers and trimers. Most PLA_2 s in the *Crotalus* group have highly homologous amino acid sequences (547,561). No doubt the precise difference of amino sequences between CA and CDT venom PLA_2 s might lead to elucidation of the precise amino acid region that calmodulin binds to.

Although it has been reported that these two PLA_2 s are active only as dimers and higher order aggregates, cross-linking did not indicate that the dimers or higher order aggregates were the predominant

species. The predominant species was the monomer. This may be due to the fact that DMS crosslinks lysines (808). It is possible that the site of PLA₂-PLA₂ interaction is not in the vicinity of PLA₂ lysines and therefore dimerization may not have been detected by the methods employed.

It was found that pancreatic PLA₂ exists as 2 species. One species binds to calmodulin and one does not. The species have identical molecular weights as determined by SDS PAGE. It is unknown at this stage what the physical-chemical differences between these 2 species are. Previously calmodulin binding and non-calmodulin binding isoenzymes of adenylate cyclase have been separated by calmodulin affinity chromatography (719).

Although the in vitro association of CaM and pancreatic PLA₂ was established, it is of interest to ask whether there is any in vivo physiological significance to CaM-pancreatic PLA₂ interaction. An in vivo physiological significance can only be assumed if calmodulin has access to the enzyme. PLA₂ exists as a proenzyme in the pancreas (530). Upon hormonal stimulation of the pancreas the proenzyme is extruded into the lumen of the intestine. The terminal sequence is subsequently trypsinized yielding the active enzyme. There are two possible sites of calmodulin interaction with pancreatic PLA₂: 1) the secretory granule in the pancreatic islet cells and 2) the intestinal lumen. Calmodulin is associated with the brush border of intestinal villi (826). However, calmodulin has not been reported to exist in the lumen of the intestine. It is possible that calmodulin is associated with proenzyme PLA₂ in these secretory granules. Future immunocytochemistry of the pancreas would elucidate this matter. Figure

3 presents a hypothetical model for in vivo Ca^{2+} /calmodulin PLA_2 interactions. At low Ca^{2+} concentrations when calmodulin can not be activated, the 2 species P_C and P_E exist independently. DMS cross-linking demonstrated that when P_T or P_C or P_E were crosslinked by themselves in all cases dimers were present, and that dimerization was slightly stimulated by Ca^{2+} . This implied that in the absence of calmodulin activation, dimers of $\text{P}_\text{C}-\text{P}_\text{C}$, $\text{P}_\text{E}-\text{P}_\text{E}$ and $\text{P}_\text{C}-\text{P}_\text{E}$ coexist. When P_E is crosslinked in the presence of Ca^{2+} and calmodulin, $\text{P}_\text{E}-\text{P}_\text{E}$ dimers are not formed, only $\text{P}_\text{E}-\text{CaM}$ protein complexes are formed. Thus in the presence of Ca^{2+} and CaM, $\text{P}_\text{C}-\text{P}_\text{C}$, $\text{P}_\text{C}-\text{P}_\text{E}$, $\text{P}_\text{E}-\text{CaM}$ protein complexes exist as well as P_C and P_E monomers. Because P_T PLA_2 in the presence of Ca^{2+} and CaM has the highest activity of all the other conditions, it is apparent that formation of all the above mentioned PLA_2 protein complexes leads to optimal pancreatic PLA_2 activity.

All the PLA_2 s that bind to calmodulin have branched pathways, i.e. branch 1 - in the presence of calcium, PLA_2 polymerization occurs; branch 2 - in the presence of calcium and calmodulin, PLA_2 -CaM protein complexes are formed.

These results indicate that calmodulin has a direct effect on PLA_2 and that when calmodulin stimulated brain membranal PLA_2 , the stimulation is in all likelihood due to direct PLA_2 stimulation.

Calcium has previously been hypothesized to activate PLA_2 by binding to the enzyme and inducing conformational changes (532) and by facilitation of nucleophile deprotonation (553). These mechanisms of calcium activation may operate to activate PLA_2 in the absence of calmodulin. The mechanism of calcium activation of PLA_2 must now be reexamined in the light of the enzyme's association with calmodulin. It

Figure 3. Hypothetical model of the possible in vivo calcium-calmodulin pancreatic PLA₂ interactions. Explanations are found in the text.

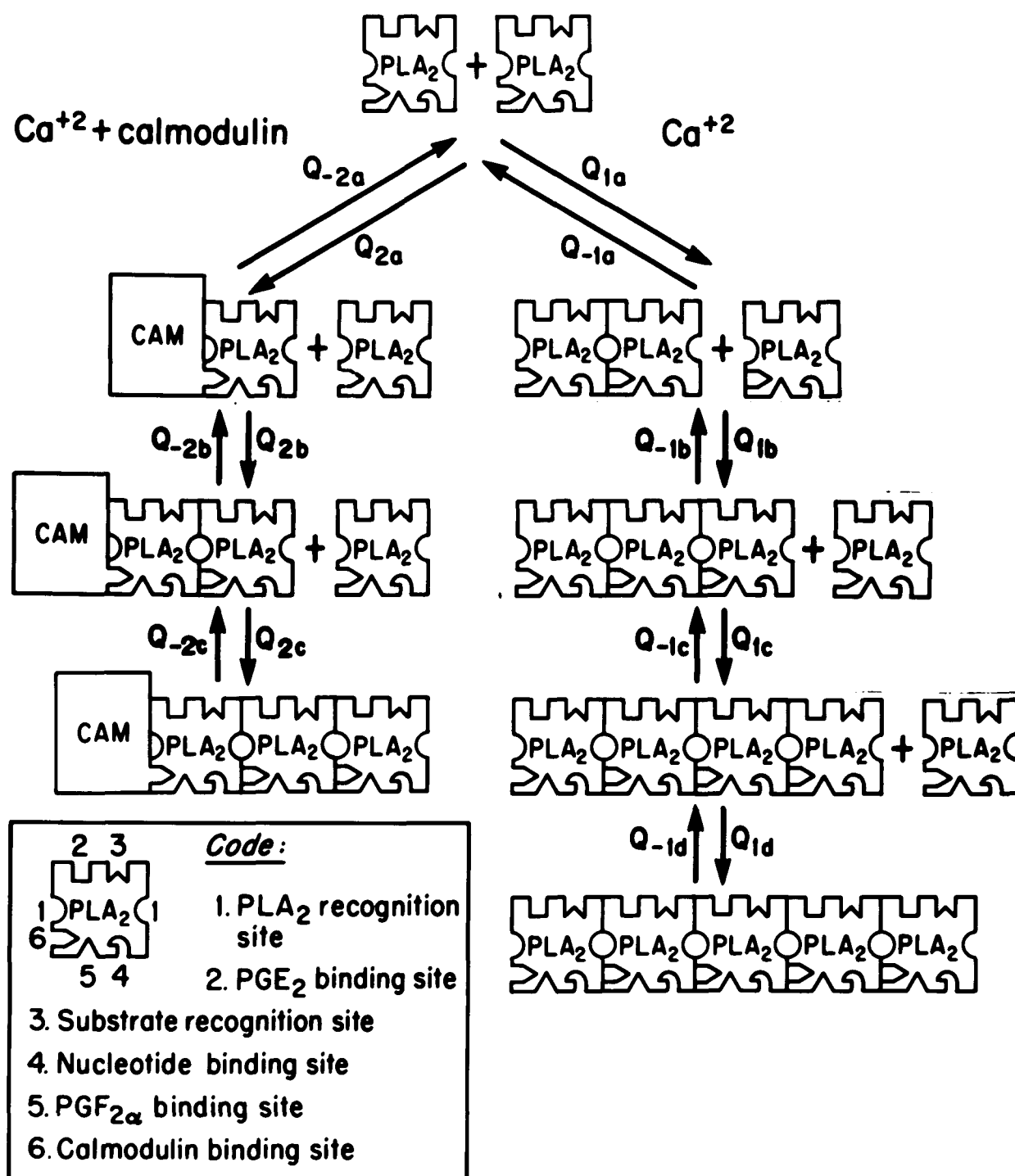
would be of great importance to crystallize the PLA₂-Calmodulin complexes of the various PLA₂ types in order to elucidate the precise mechanism of calmodulin activation.

Figure 4 is a schematic model of hypothesized PLA₂ polymerization pathways, based on DMS crosslinking studies. It is apparent that there are two possible pathways that PLA₂ subunits may undergo. In the presence of calcium alone, PLA₂ may polymerize to higher order polymers. The polymerization and depolymerization of the enzyme subunits, i.e., the forward and reverse reactions, are probably in relative equilibrium. In the absence of any modulators, the forward reaction is slightly favored. In the presence of Ca²⁺ and CaM, monomer-CaM, dimer-CaM and trimer-CaM complexes are formed. In the absence of modulators, the reverse reaction is favored. It is unlikely that two calmodulins bind per one PLA₂ monomer because it has been demonstrated that when bee venom PLA₂ was crosslinked to calmodulin by a photoactive crosslinking reagent which was attached to CaM, only a PLA₂-monomer-CaM complex was formed (Larry Shapiro, unpublished observations). If there were 2 calmodulins bound/monomer, this structure would have been detected by the above procedure. It is likely that the same mode of calmodulin interaction operates with *Naja naja* PLA₂.

Classically both cAMP and cGMP have been demonstrated to exert metabolic effects only via stimulation of protein kinases (703). PLA₂ is the only other enzyme to date reported to be directly controlled by cAMP and cGMP. It is of interest to draw several parallels between protein kinases and PLA₂. Cyclic-AMP and cGMP often stimulate the same protein kinase. PLA₂ is another enzyme which can be regulated by both these nucleotides which produce similar effects on the same enzyme.

Figure 4. PLA₂ polymerization pathways. Proposed scheme of PLA₂ branched polymerization pathway. $Q_{1a}-Q_{1d}$ are the quantity of PLA₂ polymer formation in the presence of calcium. $Q_{-1a}-Q_{-1d}$ is the quantity of monomer depolymerization which is in equilibrium with $Q_{1a}-Q_{1d}$. $Q_{2a}-Q_{2c}$ are the quantity of PLA₂-CaM protein complex formation in the presence of calcium and calmodulin. $Q_{-2a}-Q_{-2c}$ is the quantity of CaM PLA₂ dissociation in the presence of calcium and calmodulin. The structure of PLA₂ presented is not meant to correspond to the actual molecular structure obtained by x-ray crystallography. The PLA₂ monomers and dimers are drawn for heuristic convenience with structural regions corresponding to a substrate recognition site, PLA₂-PLA₂ recognition site, and PGE₁, PGF_{2α} and cyclic nucleotide recognition sites.

PHOSPHOLIPASE A₂ POLYMERIZATION PATHWAYS



Whereas binding of cAMP to cAMP dependent protein kinases leads to dissociation of the protein kinase subunits (730) and subsequent enzyme activation, cAMP and cGMP in a similar sense may lead to dissociation of PLA₂ polymers. However in this situation it inhibits rather than activates the enzyme.

It is also of interest to note that both calmodulin and cAMP, two key cellular regulators, bind to and regulate PLA₂. Calcium/calmodulin activates this enzyme and cAMP directly inhibits the enzyme as well as inhibits the calmodulin stimulation of PLA₂. Thus PLA₂ is a common focal point for both calmodulin and cyclic nucleotides which exert opposing effects on PLA₂. Thus a new possible enzymatic interaction for cyclic nucleotides has been demonstrated. Incidentally, both protein kinases and PLA₂ may be key enzymes in presynaptic chemical neurotransmission. This concept will be extensively discussed later on in this section.

5. Speculations on the evolutionary importance of PLA₂

PLA₂ is present in many eukaryotic membranes and has been detected in bacterial and yeast membranes (444, 445). It is likely that PLA₂ initially functioned in membrane metabolism and may have been one of the many metabolic enzymes of key importance to the survival of unicellular organisms. Other key metabolic enzymes, e.g., phosphofructokinase, are multiallosteric enzymes (827), i.e., they are controlled by a multiplicity of modulators. PLA₂ is also modulated by a multiplicity of modulators. The importance of PLA₂ secondary and incidental to lipid metabolism may have initially resided in its control of membrane permeability and maintenance of osmotic pressure.

In multicellular organisms PLA₂ control of membrane permeability by alteration of the lipid annulus around a variety of proteinaceous ion channels may have led to modulation of ion fluxes and hence played a role in cell-cell communication.

In neurons cell-cell communication is highly refined. Because of PLA₂'s ability to polymerize and to be modulated by a host of ubiquitous cellular effectors, its functions in neuronal membranes may have evolved to regulate lipid metabolism, membrane permeability and ion fluxes, as well as receptor-synaptic plasma membrane binding affinity, neurotransmitter release and uptake. The fact that PLA₂ is composed of multiple identical subunits and can be controlled by so many modulators demonstrates its diversity and complexity. A modulator of PLA₂ may either alter the enzymes conformation such that it can form higher order polymers and/or more favorably interact with and hydrolyze substrate. The fact that PLA₂ can be regulated by its own metabolic byproducts, the prostaglandins, indicates that the enzyme is finely regulated and its activity constantly monitored.

The importance of PLA₂ in neuronal control may reside in the fact that it can be directly modulated by modulators of such key importance in cellular viability such as calcium, calmodulin and cyclic nucleotides. The important functions of PLA₂ in brain may have developed throughout phylogeny as a result of these properties.

6. Synaptic and coated vesicle protein kinases

In this study evidence was presented that coated vesicles have an endogenous Ca²⁺/CaM dependent protein kinase activity and that synaptic

vesicles have endogenous Ca^{2+} /CaM and cAMP dependent activities.* Calmodulin stimulated phosphorylation of synaptic vesicle proteins with M_r s of 55K and 53K which have been previously observed to be phosphorylated by Ca^{2+} /calmodulin and implicated in neurotransmitter release. Cyclic-AMP also stimulated phosphorylation of proteins, in the 55K and 53K molecular weight ranges, in addition to several other polypeptides. A cAMP-dependent protein kinase has also been implicated in facilitation of transmitter release from axon terminals (828).

The inhibition of calcium/calmodulin dependent protein kinase activity by the anti-30K antibody implied that the antibody may have bound directly to the protein kinase itself which has a M_r of 30K thereby inhibiting its activity. Alternatively it is possible that the antibody bound to a 30K M_r protein in close proximity to the kinase and somehow inhibited enzyme-substrate interaction. This however is unlikely because it has been demonstrated that 30K polypeptides in both synaptic and coated vesicles bind to calmodulin. Because calmodulin binds to 30K M_r polypeptides in these vesicles and because antibodies elicited against 30K M_r polypeptides common to both vesicle types inhibited Ca^{2+} /calmodulin-dependent protein kinase activity, it is likely that both calmodulin and anti-30K IgG interact with the same 30K

* While this thesis was being prepared and typed, Pauloin et al. (Nature 298:574-576, 1982) reported the presence of cyclic nucleotide- Ca^{2+} independent protein kinase activity in bovine brain coated vesicles. Experiments performed by Dr. Schook and Dr. A. Andres during this time revealed that coated vesicles purified by glass bead chromatography also possess endogenous Ca^{2+} /CaM dependent protein kinase activity. Ca^{2+} /CaM protein kinase activity was only detectable if β -ME was included in the initial preparation buffers. Elimination of β -ME from these buffers led to results similar to that reported by Pauloin et al. (1982). The unregulated protein kinase activity of coated vesicles detected under the conditions of Pauloin et al. may therefore not reflect in vivo physiological conditions.

protein polypeptides and that a protein in this M_r region is the protein kinase.

It is possible that the Ca^{2+} /calmodulin dependent protein kinase in synaptic and coated vesicles may be the 30K M_r protein doublet which has previously been referred to as clathrin light chains (265, 266).

Because the antibodies were elicited to a gel fragment containing the 30K protein doublet associated with clathrin and not to the purified isolated 30K doublet proteins, it is possible that a protein with a similar M_r comigrated with the clathrin associated proteins and it is this protein which is the protein kinase. Both synaptic and coated vesicles manifested inhibition of phosphorylation when preincubated with anti-30K IgG. The smaller level of inhibition by the antibody in coated vesicles as compared to synaptic vesicles may be due to steric hindrance of membrane antigen-antibody interaction due to the surrounding clathrin basket in coated vesicles.

Phosphorylation studies performed with affinity purified antibodies elicited to the lower M_r protein of the 30K polypeptide doublet failed to inhibit synaptic vesicle or coated vesicle protein phosphorylation (W. Schook and A. Andres, unpublished observations). This implied that the higher M_r polypeptide of this doublet is present in both synaptic and coated vesicles and may be the common Ca^{2+} /calmodulin protein kinase in synaptic and coated vesicles.

The fact that extraction of the 30K protein complex from coated vesicles led to complete depletion of calmodulin-dependent phosphorylation further suggested that the 30K protein doublet represented the Ca^{2+} /calmodulin-dependent protein kinase. SDS PAGE revealed that along with the 180K and 30K polypeptides extracted from the coated vesicle,

there were several other minor proteins. In addition there may also be other minor protein bands that were extracted and not visualized by Coomassie blue staining. The possibility exists that one of these minor proteins may be associated with Ca^{2+} /calmodulin dependent protein kinase activity.

It is also possible that extraction of the 30K polypeptides may have damaged the decoated vesicle and thereby affected possible protein kinase activity. However, it should be noted that another coated vesicle enzyme, $[\text{Ca}^{2+}\text{Mg}^{2+}]\text{-ATPase}$, was not affected by the same extraction procedure.

Inhibition of cAMP-dependent phosphorylation by the anti-30K IgG in synaptic vesicles may be explained by one of the following possibilities: a) antibodies directly inhibited both the Ca^{2+} /calmodulin- and cAMP-dependent protein kinases — which could occur if both the Ca^{2+} /calmodulin-dependent kinase and catalytic subunit of the cAMP-dependent protein kinase have the same M_r ; this implies that antibodies may have been raised against both kinases; b) the regulatory subunit of the cAMP-dependent protein kinase and the catalytic subunit of the calmodulin-dependent protein kinase may be the same protein; c) the antibodies may be inhibiting only the Ca^{2+} /calmodulin-dependent protein kinase and phosphorylation by cAMP and Ca^{2+} /calmodulin may be dependent upon each other. The finding that cAMP phosphorylated proteins in the region of 30K in synaptic vesicles supports all three possibilities: i.e., either the cAMP-dependent protein kinase and/or the Ca^{2+} /calmodulin/cAMP-dependent protein kinase are undergoing autophosphorylation and/or are phosphorylating the Ca^{2+} /calmodulin-dependent kinase. Isolation of 30K protein polypeptides from heat-treated

clathrin preparations and from both synaptic and coated vesicles by calmodulin affinity chromatography again supports all three possibilities.

The 30K protein doublet in both synaptic and coated vesicle membranes was detected ultrastructurally by immunocytochemistry (S. Puzskin, unpublished observations). The protein was prominently localized in synaptic and coated vesicles. Neither the cytosol nor the presynaptic plasma membrane were significantly immunoreactive. The finding that coated vesicles only have Ca^{2+} /calmodulin-dependent, but not cAMP-dependent phosphorylation implied that the 30K protein is associated with a Ca^{2+} /calmodulin and not a Ca^{2+} /calmodulin/cAMP- or a cAMP-dependent protein kinase, and that inhibition of the cAMP dependent kinase in synaptic vesicles by this antibody is due to an interdependence of the Ca^{2+} /calmodulin and cAMP dependent protein kinases. The possibility also exists that both synaptic and coated vesicles have a common endogenous protein kinase that is regulated differently in both vesicle types.

The 80K M_r protein in synaptic vesicles phosphorylated by cAMP, but not by Ca^{2+} /calmodulin has the same M_r as a specific synaptic vesicle protein demonstrated to be phosphorylated either by calcium (829) or cAMP (830). The fact that this protein was not phosphorylated in the presence of calcium and calmodulin implied that this synaptic vesicle protein although phosphorylated by certain other cytosolic and particulate axon terminal calcium/calmodulin-dependent protein kinases apparently is not a substrate for endogenous synaptic vesicles Ca^{2+} /calmodulin-dependent protein kinase.

The 55K and 53K substrates phosphorylated by Ca^{2+} /calmodulin in

synaptic vesicles have been demonstrated to be endogenous synaptic vesicle α and β Tubulin. Tubulin is a substrate for cytoplasmic calcium/calmodulin dependent protein kinase (769). It is possible that the 55K and 53K proteins phosphorylated by cAMP in synaptic vesicles may also be α and β tubulin and represent common substrates for the calcium/calmodulin and cAMP dependent protein kinases. Tubulin has been demonstrated to be endogenous to synaptic plasma membrane as well (768). It is possible that the 55K M_r protein phosphorylated by Ca^{2+} /calmodulin in coated vesicles may be a tubulin.

These data are in agreement with that of Kelly et al., who have demonstrated that a protein of approximate M_r 55K is phosphorylated by cAMP in synaptic vesicles (831).

A 40K M_r protein in the synaptic vesicle preparation was a substrate for cAMP dependent protein kinase. A similar protein has been phosphorylated in synaptosomes by Ca^{2+} , but inhibited by Mg^{2+} (832). Hirata et al. (518) described a protein phosphorylated by either Ca^{2+} or cAMP in neutrophils with a M_r of 40K that is a modulator of phospholipase A_2 named lipomodulin. Because conditions which activated cAMP-dependent protein phosphorylation in synaptic vesicles decreased synaptic vesicle phospholipase A_2 activity, it is possible that this protein is a lipomodulin-like protein.

The phosphorylation of synaptic membrane proteins previously has been demonstrated to be stimulated by Ca^{2+} (748), Ca^{2+} and calmodulin (750) or cAMP (829) and implicated in neurotransmitter exocytosis. Protein phosphorylation is believed to induce conformational changes thereby exerting subsequent physiological effects (833).

7. Synaptic and coated vesicle $[Ca^{2+}-Mg^{2+}]-ATPase$

This study confirmed the findings of others that synaptic and coated vesicles have endogenous $[Ca^{2+}-Mg^{2+}]-ATPase$ activity (787,788). The level of $[Ca^{2+}-Mg^{2+}]-ATPase$ activity in coated vesicles is approximately 5 times higher than that in synaptic vesicles. Coated vesicles sequester approximately 5×10^3 times as much calcium as do synaptic vesicles. Coated vesicles may therefore play a more important role in calcium sequestration than synaptic vesicles in axon terminals.

Although both mitochondria and endoplasmic reticulum sequester calcium in axon terminals (780,783), the importance of their calcium sequestration in regulating transmitter release is questionable. When calcium is injected into axon terminals it remains very close to the site of injection (177). In addition, calcium channels are concentrated in the active zone region of the plasma membrane and thus calcium entry is restricted to the active zone (12,178,179). Mitochondria and endoplasmic reticulum as judged by electron microscopy are rather plentiful but are quite far away from the active zone (12,128,162,171). Thus mitochondria and endoplasmic reticulum may play a very important role in total cell calcium ion regulation. However, their role in regulation of neurotransmitter release is probably limited because of their considerable distance from the release site. Synaptic plasma membrane has $[Ca^{2+}-Mg^{2+}]-ATPase$ that participates in Ca^{2+} extrusion from axon terminals (783-786).

Synaptic vesicle and coated vesicle $[Ca^{2+}-Mg^{2+}]-ATPase$, although sequestering a relatively smaller level of calcium compared to endoplasmic reticulum or mitochondria, may be of key importance in the immediate regulation of synaptic vesicle exocytosis and coated vesicle

endocytosis by sequestering Ca^{2+} in the immediate milieu of the vesicles.

8. Speculations on the diverse effects of calcium on neurotransmitter release

It has been demonstrated that calcium promotes and Mg^{2+} antagonizes membrane fusion (33, 34). There are several points of convergence which should be recognized. Calcium activates calmodulin which in turn can activate PLA_2 , protein kinases, adenylate cyclase, and transglutaminase. In addition, calcium may directly bind to and activate one or more of these enzymes. It should be noted that in many circumstances Mg^{2+} has been found to inhibit binding of calcium to calmodulin, although this area is still controversial (341). Mg^{2+} has been demonstrated to inhibit PLA_2 (481). On the other hand, divalent cations such as barium and strontium in certain instances can replace calcium in inducing neurotransmitter release (38,40). These same ions can activate PLA_2 (550,551) and actomyosin (760,761). Therefore, studying the effects of a variety of cations on several presynaptic enzymes and calmodulin may facilitate elucidation of the relationship between these divalent cations and neurotransmitter release.

9. Unified theory of presynaptic chemical neurotransmission

By reviewing the literature presented in the Introduction and the data presented in the Results it becomes apparent that a multiplicity of presynaptic events may lead to neurotransmitter release. How are these events related to one another? How can these events lead to the release of neurotransmitter within 200 μsec ? This section attempts to answer

these questions and to present a unified theory of presynaptic chemical neurotransmission to account for the ongoing modulation of synaptic vesicle fusion, neurotransmitter release and coated pit/vesicle retrieval of synaptic vesicle membrane.

a. Synaptic vesicle-synaptic plasma membrane fusion

The enzymatic cascades hypothesized to be involved in neurotransmitter release are illustrated in Figure 5. This figure will constantly be referred to throughout this section.

At the resting state of the neuron, axon terminal calcium concentration ranges between 10^{-8} - 10^{-7} M (184). The electric field crossing the plasma membrane upon its depolarization changes the conformation of the calcium channel monomers and redistributes the charge of their inner surfaces making them negative (174,179). In this way membrane depolarization allows calcium to move through the calcium channels into the axon terminal. Because the calcium channels are concentrated at the presynaptic terminal (174,175), calcium enters the terminal at the active site and is distributed throughout the 200A layer of cytoplasm closest to the presynaptic membrane (182,203).

If calcium enters only at the active zones and is not uniformly distributed throughout the cytoplasm but rather is preferentially associated with negatively charged surfaces, then the internal calcium concentration at the release site during the peak of neurotransmitter release reaches 10^{-6} - 10^{-5} M (189).

The incoming calcium current translocates synaptic vesicles to the presynaptic membrane. Because synaptic vesicles cannot move freely in the viscous gel matrix (204), a propelling force must operate to translocate them the required 50-100A toward the membrane. Such a force is

actomyosin (763,764). The incoming calcium binds to and activates calmodulin which is soluble and/or associated with synaptic vesicles. Activated calmodulin then may activate myosin light chain kinase leading to phosphorylation of myosin. Only when myosin is phosphorylated can actin activate myosin Mg^{2+} -ATPase. The hydrolysis of ATP by this ATPase supplies the energy for actomyosin which is associated with synaptic vesicles and synaptic plasma membranes to contract and subsequently bring about the propulsion of synaptic vesicles to the presynaptic membrane (Figure 5, Enzyme Cascade I).

The actomyosin system probably works in conjunction with the pre-synaptic grid (148) and synaptopores (173) to orient the vesicle in a precise manner with respect to the synaptic plasma membrane. It is likely that the vesicle is propelled and oriented in the grid so that a certain highly specific pole or region of the synaptic vesicle is oriented and aligned with a highly specialized region of the synaptic plasma membrane, the synaptopore, for fusion to occur between these 2 membranes. In stimulated axon terminals, synaptic vesicles have not been observed to fuse with each other. The results presented here reveal, however, that synaptic vesicles when extracted from the axon terminal can associate with each other, aggregate, and possibly fuse. The fact that synaptic vesicles do not fuse with each other in vivo implies that they are prohibited from self-association by the viscous matrix which separates them and that actomyosin contraction directs the synaptic vesicles toward the synaptic grid-synaptopore region and not to other synaptic vesicles. Although in vitro synaptic vesicle-vesicle fusion occurs, it does not do so within the microsecond or even millisecond time range. This firstly implies that intraaxonal

mechanisms outside the synaptic vesicle machinery are necessary for μsec fusion to occur and that secondly a specific orientation of different membranal regions is necessary for μsec fusion. In vivo fusion of the synaptic vesicles with the synaptic plasma membrane does not occur in regions of the presynaptic membrane other than at the synaptopore. The concept will be developed later on in this discussion that the rate of the enzymatic cascades involved in the fusion process may be amplified when the enzymes concerned are geographically positioned in an optimal manner. The propulsion of synaptic vesicles by actomyosin into the presynaptic grid, a morphological structure of considerable strength, holds the vesicle in place and in direct contact with the synaptopore throughout the duration of the fusion process for a maximal rate of fusion to occur. Once the synaptic vesicles and synaptic plasma membranes are optimally juxtaposed, a series of interconnected enzymatic cascades all simultaneously stimulated by Ca^{2+} /calmodulin operate to regulate synaptic vesicle fusion to the presynaptic plasma membrane. Ca^{2+} /calmodulin activation of both synaptic vesicle and synaptic plasma membrane PLA_2 leads to hydrolysis of phosphatidylcholine and the subsequent formation of lysolecithin and arachidonic acid (Figure 5, Cascade IV). Lysolecithin incorporates into the bilayers of synaptic plasma membranes and synaptic vesicles producing a transition from the bimolecular leaflet to radially oriented molecules (407) leading to fusion of these 2 separate membranes. There are several ongoing enzymatic processes which modulate the amount of lysolecithin produced and therefore the amount of synaptic vesicle-synaptic plasma membrane fusion and neurotransmitter release. Acetyltransferase catalyzes the transfer of fatty acid to lysolecithin leading to the formation of

phosphatidylcholine (820). At present it is unknown what the concentration of acetyltransferase is in axon terminal membranes or what its properties and possible modulators are.

The free fatty acids that are released from synaptic vesicles and synaptic plasma membranes as a consequence of PLA_2 hydrolysis can be metabolized by endogenous synaptic vesicle prostaglandin synthetase to $PGF_{1\alpha}$ and PGE_2 . These eicosanoid products may continually monitor neurotransmitter release by modulating synaptic vesicle and synaptic plasma membrane PLA_2 activities. PGE_2 can feedback inhibit and $PGF_{1\alpha}$ can feedback stimulate PLA_2 . PGE_2 links the PLA_2 -PG synthetase cascade (Fig. 5, Cascade IV) to another simultaneous ongoing enzymatic cascade, the adenylate cyclase/cAMP protein kinase cascade (Fig. 5, Enzyme Cascade III) which can independently induce neurotransmitter release. Ca^{2+} /calmodulin and released PGE_2 activate presynaptic membranal adenylate cyclase (in the inner surface of the plasma membrane) leading to the conversion of ATP to cAMP. Ca^{2+} /calmodulin further controls cAMP levels by activating cytoplasmic phosphodiesterase which converts cAMP to AMP. Cyclic AMP at sufficient concentration reciprocally interconnects enzyme cascades III and IV (Figure 5) by 2 different mechanisms: 1) cAMP may directly activate synaptic vesicle PLA_2 and directly inhibit synaptic plasma membrane PLA_2 ; 2) cAMP may activate cAMP dependent protein kinase in both synaptic vesicles and synaptic plasma membrane. The phosphorylation of synaptic vesicle lipomodulin would lead to relaxation of lipomodulin PLA_2 inhibition and subsequent stimulation of synaptic vesicle PLA_2 . Thus PGE_2 potentiates the formation of cAMP which in turn can either potentiate or inhibit the formation of PGE_2 . The overall inhibition or stimulation would depend on which of the above

mentioned cAMP dependent pathways is more dominant. The phosphorylation of synaptic vesicle and synaptic plasma membrane proteins by cAMP dependent protein kinase leads to changes in their conformations which are conducive to synaptic vesicle-synaptic plasma membrane fusion thereby leading to neurotransmitter release (Figure 5, Enzyme Cascade III). A protein phosphatase would modulate neurotransmitter release by dephosphorylation of these proteins (730) and subsequent inhibition of fusion.

Simultaneous activation of synaptic vesicle and synaptic plasma membrane Ca^{2+} /CAM dependent protein kinases also leads to the phosphorylation of synaptic vesicle and synaptic plasma membrane proteins altering their conformational changes and thereby inducing fusion (Cascade II, Figure 5). Again, a protein phosphatase would modulate this process by the dephosphorylation of proteins and hence inhibition of synaptic vesicle fusion.

My results have indicated that the inhibition of calcium/calmodulin kinase activity by specific antibodies directed against the Ca^{2+} /calmodulin protein kinase also inhibit to a certain extent the cAMP dependent kinase implying that these two kinases are somehow dependent on one another or interconnected. Thus we see that the Ca^{2+} /CAM protein kinase cascade (II) is reciprocally connected to the cAMP protein kinase cascade (III) which in turn is reciprocally connected to the PLA_2 cascade (IV). Therefore modulation of any one of these 3 cascades can simultaneously affect the other two cascades in varying degrees. The stimulation of $[\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}]$ in synaptic vesicles, coated vesicles, and synaptic plasma membranes by Ca^{2+} /calmodulin constantly monitors the levels of calcium in the axon terminal (Cascade V) thereby modulating

enzyme cascades I-IV, and hence neurotransmitter release.

It should be noted that cascades I, II, III and V all control the levels of axon terminal ATP and as a consequence modulate synaptic vesicle and synaptic plasma membrane PLA_2 (Cascade IV). Phospholipase A_2 in turn has been demonstrated to activate $[Ca^{2+}-Mg^{2+}]-ATPase$ in red blood cells. It is possible that a similar phenomenon occurs in the brain. It therefore appears that enzyme cascades I-V, which are simultaneously activated by Ca^{2+}/CaM , upon increase of axon terminal calcium are parallel and interconnected enzyme cascades which constantly modulate (amplify/depress) each other. The overall activation of enzyme cascades I-IV leads to synaptic vesicle-synaptic plasma membrane fusion and subsequent neurotransmitter release.

Once neurotransmitter is released into the synaptic cleft, it binds to the post-synaptic receptors and induces an eventual change in post-synaptic membrane permeability and hence establishes communication between one neuron and the next (720). The newly released neurotransmitter can also modulate presynaptic neurotransmission by binding to presynaptic neurotransmitter receptors (5). Neurotransmitter binding to presynaptic receptors might lead to the activation of methyltransferases I and II (Enzyme cascade VI) leading to conversion of synaptic plasma membrane phosphatidylethanolamine to phosphatidylcholine (508). This would activate synaptic vesicle and synaptic plasma membrane PLA_2 (Enzyme cascade IV) by (a) presenting more substrate to PLA_2 for hydrolysis, by (b) increasing the membrane fluidity and thereby bringing phosphatidylcholine and PLA_2 in closer juxtaposition and by (c) increasing membranal permeability leading to influx of calcium and hence activation of enzyme cascades I-V (Figure 5).

Activation of methyltransferases (Enzyme cascade VI) therefore modulates the amount of neurotransmitter released which consequently modulates activation of methyltransferases. In this manner the methyltransferase cascade is reciprocally related to all the other enzyme cascades. In addition, the activation of PLA_2 by methyltransferase leads to changes in the lipid annulus in a variety of neurotransmitter receptors leading to inhibition of neurotransmitter receptor binding and consequently inhibition of methyltransferase activities. Binding of neurotransmitter to receptors also leads to coupling of the neurotransmitter receptor complex to presynaptic adenylate cyclase (Enzyme cascade VII, Figure 5) leading to the generation of cAMP (704). As a consequence of neurotransmitter binding to receptors, cAMP can activate protein phosphorylation of certain presynaptic membranal proteins discussed above. In addition, cAMP activates phosphorylation of nuclear proteins and enzymes leading to inhibition of neurotransmitter synthesis and consequently inhibition of neurotransmitter release (5).

cAMP can also activate the phosphorylation of certain ion channels, e.g., the potassium channel (834) leading to influx of potassium and hence continued stimulation of neurotransmitter release (Figure 5, Enzyme Cascade VII). Because cAMP apparently can have a variety of stimulating and inhibiting effects, the overall effect of cAMP probably depends on the compartmentalization of the various cAMP dependent protein kinases within the axon terminal. Automodulation of an axon terminal by its own neurotransmitter may be affected by neurotransmitter-receptor induced increase of cAMP in the vicinity of the nucleus thereby leading to inhibition of neurotransmitter synthesis and hence inhibition of neurotransmitter release (703). Activation of neurotransmitter release

from an axon terminal by a neurotransmitter from another axon terminal may lead to activation of adenylate cyclases/protein kinases in the vicinity of the K^+ channel thereby leading to influx of K^+ and an increase in neurotransmitter release (834). Stimulation of adenylate cyclases by calcium/calmodulin from the inner side of the axon terminal may lead to activation of synaptic vesicle and synaptic plasma membrane protein kinases and subsequent phosphorylation of proteins leading to neurotransmitter release. It should be noted that enzyme cascade VII (Figure 5) is also reciprocally interconnected with all the other cascades, because its activation modulates the amount of transmitter that can be released which in turn can bind to presynaptic receptors and activate methyltransferases (Enzyme cascade VI) which is reciprocally interconnected with enzyme cascades I-V.

It can be observed that the same modulator, e.g., cAMP can have different effects on the same enzyme in different membranes, e.g., it activates synaptic vesicle PLA_2 and inhibits synaptic plasma membrane PLA_2 and leads to protein phosphorylation which can either stimulate or inhibit neurotransmitter release. The overall effect of a single modulator would be determined by which enzyme cascade is the one predominantly activated in the particular axon terminal.

It was noted in the introduction that PGs have variable effects on neurotransmission. This may be attributed in vivo to the multiple effects of PGE_2 on PLA_2 and adenylate cyclase. PGE_2 inhibits synaptic vesicle and synaptic plasma membrane PLA_2 and stimulates adenylate cyclase. The latter phenomenon leads to increased cAMP levels which leads to further activation of PLA_2 and subsequent production of lysolecithin, PGE_2 and $PGF_{2\alpha}$. Thus inhibition of PLA_2 by PGE_2 leads to

Figure 5. Scheme of events postulated to be involved in SV-SPM fusion and subsequent neurotransmitter release from axon terminals. Roman numerals represent individual enzyme cascades referred to in the text. The following abbreviations were employed.

CaM = calmodulin; ATP = adenosine triphosphate; ADP = adenosinediphosphate; sv = synaptic vesicle; spm = synaptic plasma membrane.

Enzyme cascade I: MLCK = myosin light chain kinase

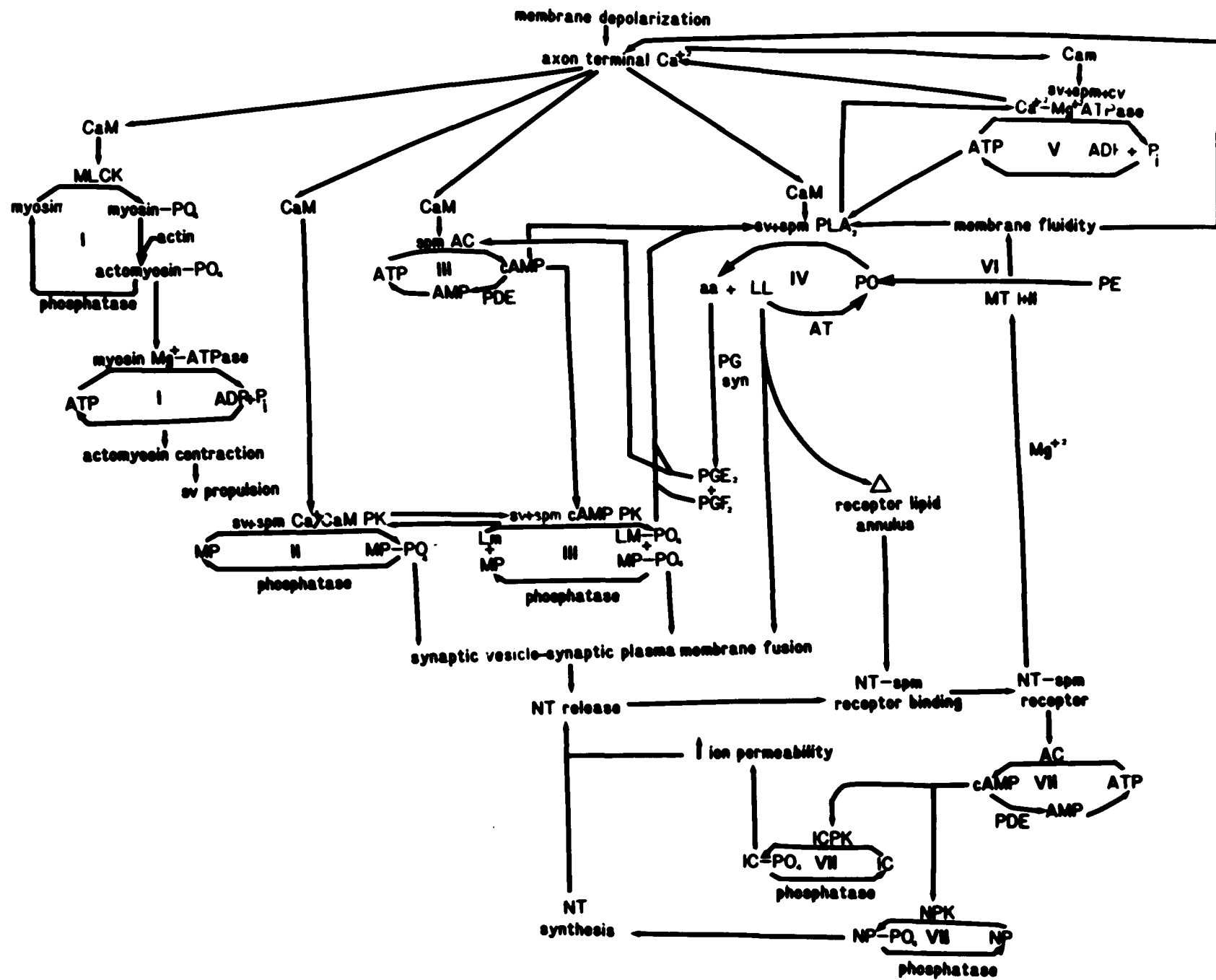
Enzyme cascade II. Ca^{2+} /CaM PK = Calcium/calmodulin dependent protein kinase, MP = membrane proteins

Enzyme cascade III. cAMP = 3'-5' cyclic adenosine monophosphate, PDE = phosphodiesterase, AMP = 5'-adenosine monophosphate, PK = protein kinase, MP = membrane proteins, LM = lipomodulin.

Enzyme cascade IV: PLA_2 = Phospholipase A_2 , aa = arachidonic acid, PG = prostaglandin, Δ = change in, LL = lysolecithin, PC = phosphatidylcholine, NT = neurotransmitter, PG syn = Prostaglandin synthetase, AT = acetyltransferase.

Enzyme cascade VI: MT I and II = methyltransferases I and II, PE = phosphatidylethanolamine.

Enzyme cascade VII: ICPK = ion channel protein kinase, NPK = nucleus related protein kinase, IC = ion channel, NP = nucleus proteins.



inhibition of neurotransmitter release. However, the simultaneous activation of adenylate cyclase leads to stimulation of PLA_2 by cAMP and increased synthesis of $PGF_{2\alpha}$ and lysolecithin and subsequent stimulation of neurotransmission.

Because multiple interconnected enzymatic cascades are involved in neurotransmitter release it becomes apparent that a single modulator such as PGE_2 may have effects on multiple systems which in vitro have diametrically opposed effects. In vivo these diametrically opposed effects are controlled by the temporal situation and thus modulate neurotransmitter release. The multiple number of enzyme cascades that prostaglandins are capable of modulating may explain why exogenous prostaglandins have such variable effects on neurotransmitter release.

Recently the term "synarchy" has been introduced by Rasmussen to describe the mutual effects of Calcium and cAMP in various metabolic and cellular events (835). This study confirms that calcium and cAMP are synarchic with respect to activation of synaptic vesicle protein kinases and PLA_2 . Figure 6 illustrates the synarchy of Ca^{2+} and cAMP with respect to protein kinase activation in synaptic vesicles. The activation of Ca^{2+} /calmodulin and cAMP dependent protein kinase independently lead to phosphorylation of synaptic vesicle membrane proteins which lead to neurotransmitter release. Synarchy is evident in two aspects of these cascades. As mentioned previously, the activation by Ca^{2+} and cAMP of the kinases is interdependent as evidenced by the fact that antibody inhibition of the Ca^{2+} kinase inhibits the cAMP kinase. Also two of the synaptic vesicle substrates phosphorylated by the 2 protein kinases with M_r s 55K and 53K are identical. These two substrates may be α and β tubulin whose conformational changes may lead

to synaptic vesicle-synaptic plasma membrane fusion (769).

Figure 7 illustrates the synarchy between Ca^{2+} and cAMP with respect to PLA_2 . Ca^{2+} /calmodulin activates PLA_2 leading to the production of lysolecithin and arachidonic acid. The latter product can subsequently be converted to PGE_2 which can then lead to activation of adenylate cyclase and generation of cAMP. Cyclic AMP in turn can increase PLA_2 activity in synaptic vesicles or inhibit PLA_2 activity in synaptic plasma membranes and thereby modulate its own axon terminal levels. Because cAMP inhibits purified PLA_2 it is likely that the universal effect of cAMP on PLA_2 is inhibition. Because most PLA_2 s are homologous to a certain extent it is likely that synaptic vesicle and synaptic plasma membrane PLA_2 are highly similar. Stimulation of synaptic vesicle PLA_2 by cAMP in synaptic vesicles may be due to stimulation of cAMP dependent protein kinases by virtue of its interconnection with synaptic vesicle PLA_2 via lipomodulin.

In light of the fact that a host of enzymatic cascades may synergistically control neurotransmitter release, it is apparent that the inhibition of any one of these cascades may severely alter neurotransmitter release. It is no wonder therefore that trifluoperazine, a calmodulin inhibitor, has been reported to inhibit secretion and neurotransmitter release (359) in view of the fact that calmodulin activates many of the enzyme cascades that lead to neurotransmitter release.

It is also apparent why disruption of actomyosin by actin disrupters, disruption of tubulin (a substrate protein for synaptic vesicle Ca^{2+} /CaM and cAMP dependent protein kinases) by tubulin disrupters, inhibition of PLA_2 by quinacrine and parabromophenacylbromide and inhibition of methyltransferase all individually lead to inhibition of

Figure 6. Ca^{2+} -cAMP synarchy in modulation of synaptic vesicle protein kinase activity. M_r of proteins that are phosphorylated in synaptic vesicles are indicated. Abbreviations employed include: CaM = calmodulin, PK = protein kinase, R_2C_2 = Regulatory subunits₂-Catalytic subunits₂.

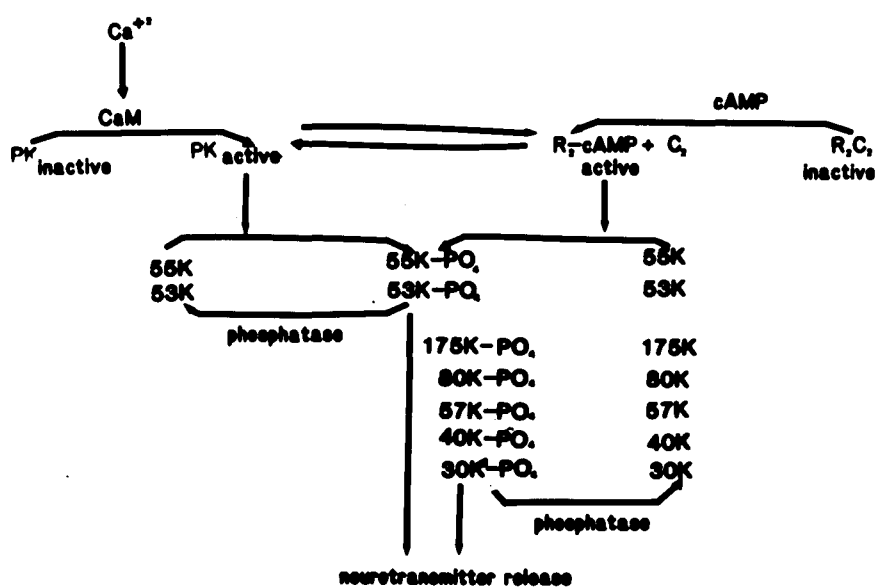


Figure 7. Ca^{2+} -cAMP synarchy in modulation of synaptic vesicle PLA_2 .

Abbreviations employed include:

CaM = calmodulin

AC = adenylate cyclase

PLA_2 = unactivated phospholipase A_2

PLA_2^Δ = activated PLA_2

cAMP = 3'-5' cyclic adenosine Triphosphate

ATP = adenosine triphosphate

AMP = Adenosine monophosphate

PDE = phosphodiesterase

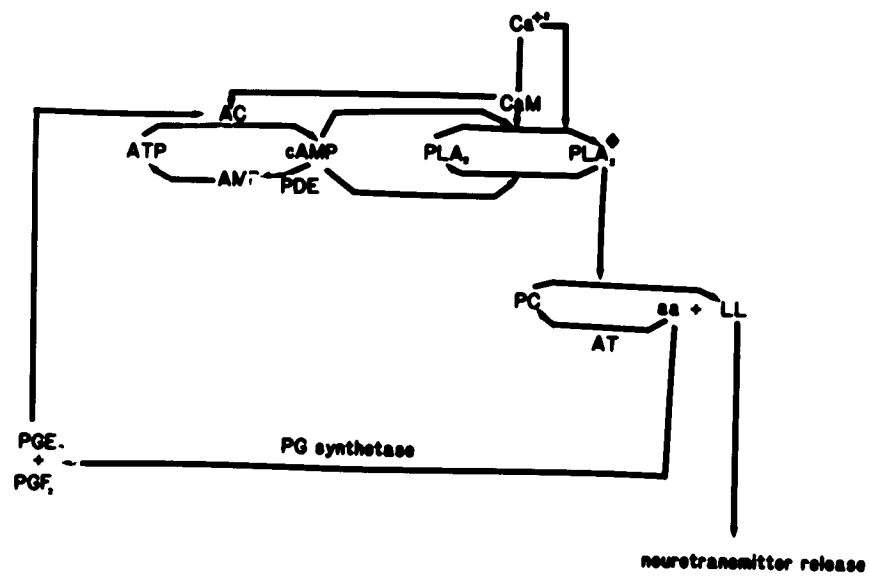
PC = phosphatidylcholine

LL = lysolecithin

aa = arachidonic acid

AT = acetyltransferase

PG = prostaglandin



secretion and/or neurotransmitter release.

The fact that PLA₂ has been demonstrated to be modulated by a variety of key cellular modulators in this study and by at least 30 other modulators including neurotransmitters in previous studies (Introduction - Table 2) makes it a most important enzyme to be reckoned with in terms of understanding the neurotransmitter release process.

PLA₂'s superiority as a regulator of important metabolic processes from the phylogenetic point of view must again be emphasized. The fact that it is composed of multiple identical subunits and is modulated by the extent of its own polymerization and complexing with calmodulin implies that it is an enzyme that can sense minute changes in its environment. Modulation of key enzymes by subunit association and dissociation is a common theme which runs throughout nature. The same general mechanism is involved in the modulation of cAMP/dependent protein kinases. It is of no surprise that both these highly developed enzymes are involved in such a key process as neurotransmitter release.

The involvement of enzymes in neurotransmitter release has previously been criticized as not being fast enough to account for release of neurotransmitter within 100-200 μ sec (182). According to Stadtman and Chock (836) enzyme cascades serve as multiplier systems with respect to kinetic parameters, amplify catalytic potentials and signals, and are capable of responding to a variety of allosteric interactions in a flexible manner. The multiplier effect of enzyme cascades is due to the fact that the rate of product formation in the last stage of the cycle has a multiplicative effect on all the rate constants of all the cascades (836). The ability of cascade systems to respond to a variety of

effectors, according to Stadtman and Chock, is due to the fact that the cascades are comprised of many different enzymes which can be a target for different modulators. Enzyme cascades can sense fluctuations in the concentration of many different modulators and their interactions lead to automatic adjustments in the activities of the converter enzymes and thereby determine the steady state levels of covalent modification. Because of these properties enzyme cascades are suitable for the regulation of key metabolic enzymes. Stadtman and Chock maintain that enzymatic cascades might play an important role in neuronal function. They specifically refer to the protein kinase cascade in post-synaptic membranes. Because such minor physiochemical perturbations lead to regulation of nerve function, they maintain that cascades are particularly well suited for neurochemical functions such as neurotransmitter synthesis and release. They maintain that:

The fact that nerve-cell membrane proteins are phosphorylated and dephosphorylated in response to electrical and hormonal stimuli suggests that interconvertible enzyme cascades play an active role in neurochemical events. Indeed, enzyme cascades possess many characteristics that make them attractive candidates for key roles in the regulation of neurochemical processes. Because they are multiplier systems with respect to rate functions and act as signal amplifiers with respect to biochemical and/or biophysical perturbations, they can generate an almost explosive increase in catalytic activity in response to minute stimuli. In addition, enzyme cascades can function as highly sophisticated metabolic integration

systems; by means of allosteric interactions with one or more of the component enzymes, cascades can continuously monitor fluctuations in the concentrations of a multitude of allosteric effectors and adjust the specific activities of the ultimate interconvertible enzyme according to its biological necessity. Finally, cascades can, under extreme but physiologically feasible situations, serve as biological switches that can be turned on or off, intermittently, for temporal "all-or-none" control of key biological activities. (836)

Based on their theoretical calculations the activation of merely an adenylate cyclase/protein kinase cascade can operate in a 10^{-3} second time scale. Although this is sufficient time for neurotransmitter induced post-synaptic membrane increased permeability, it is not fast enough to account for neurotransmitter release from axon terminals.

We must at this point recall that seven types of enzymatic cascades are all going on simultaneously within the axon terminal. Enzyme cascades I-V (Figure 5) go on in the activated neuron. Enzyme cascades V and VII probably go on in activated and resting neurons. Although in the active neuron cascades I-V are most operative, in the resting state in the absence of calmodulin activation, there may exist a steady state level of baseline enzymatic activity.

It must be emphasized that the primary enzyme cascades involved in neurotransmitter release (I-IV, Fig. 5) can be activated synergistically and simultaneously and are interdependent on one another. Thus these cascades may be considered parallel interconnecting enzyme cascades. Chock and Stadtman have calculated that there is an exponential linear

relationship between the number of steps in an enzymatic cascade and its amplification potential (11). The simultaneous activation of a series of parallel enzymatic cascades could be envisioned as the activation of a polycyclic enzymatic cascade with "n" number of steps. Therefore if activation of enzymatic cascade III (Fig. 5) by itself can occur within 10^{-3} secs, then surely if this cascade is interconnected to 6 other enzymatic cascades, neurotransmitter release could occur in 10^{-6} seconds or less, which is within the required time limit for neurotransmitter release. In addition to the synergist activation of parallel enzymatic cascades, also in operation are a variety of non-enzymatic mechanisms which could further amplify the enzymatic effects. At the area of synaptic vesicle-synaptic plasma membrane apposition, calcium may reduce the electrostatic surface charge of both membranes thereby bringing about more direct apposition, inducing a phase transition or phase separation in the membranes which leads to crystallization of the outer membrane bilayers (205). The condensation of these membrane regions would result in instability of the membranes and make them more susceptible to fusion (205)

Calcium may also directly serve as an ionic link, bridging ionic groups of the synaptic vesicles to the synaptic plasma membrane (218,219), decrease the energetic barriers between the two membranes (220) and/or lead to calcium-dithiolate formation which could lead to further apposition and fusion (221).

Once the synaptic vesicle is brought into relative proximity of the synaptic plasma membrane, key synaptic vesicle and synaptic plasma membrane glycoproteins may facilitate membrane attachment and interaction leading to apposition and fusion (773).

The fact that calcium alone does not induce neurotransmitter release supports the hypothesis that calcium/calmodulin stimulation of enzymes promotes neurotransmitter release. At this point it must be recalled that fusion of synaptic vesicle to synaptic plasma membrane must occur in discrete areas. Stadtman and Chock have shown that in cyclic cascades an even greater enzyme rate amplification is achieved by proper positioning of the multienzyme complex (11). Such a situation also occurs in mammalian pyruvate dehydrogenase (11). The function of the presynaptic grid and synaptopores must be to perfectly align the various enzymes in both the synaptic vesicle and synaptic plasma membrane with each other to further amplify the enzymatic cascade and lead to enhancement of transmitter release. Thus a series of enzymatic cascade types that can be optimally oriented by presynaptic morphological structures to obtain maximal geographical positioning may explain how synaptic vesicle-synaptic plasma membrane fusion can occur in 10^{-6} seconds or less.

The fact that an adenylate cyclase/protein kinase cascade can only operate within 10^{-3} secs (836) emphasizes and corroborates the idea that more than 1 enzyme cascade is involved in neurotransmitter release and that multiple interconnected enzymatic cascades are necessary to bring about the fastest cell-cell communication that is presently known in biological systems.

b. Coated pit/coated vesicle retrieval of synaptic vesicles

After synaptic vesicles fuse with the synaptic plasma membrane and intermix with it, synaptic vesicle particles translocate laterally within the synaptic plasma membrane and are subsequently retrieved by the coated pit/coated vesicle system. There is a soluble pool of

clathrin heavily concentrated at the axon terminal (269). These clathrin triskelions are in equilibrium with clathrin triskelions bound to coated pits and vesicles. How does clathrin recognize and insert into the synaptic vesicle membrane to become a coated pit? What governs the reversible nature of clathrin-membrane recognition? At the peak of neurotransmitter release Ca^{2+} might lead to calmodulin activation of Ca^{2+} /CaM dependent protein kinase which phosphorylates proteins with M_r s of 175K and 55K. The phosphorylation of these proteins would lead to changes in their conformations. The transient conformational changes might allow clathrin to recognize and insert into them thereby leading to the creation of a coated pit.

Calmodulin has been implicated in the recruitment of clathrin to membranes to form coated pits (360). The Ca^{2+} /calmodulin-dependent phosphorylation of membrane proteins may be the mechanism by which this is accomplished.

Transglutaminases cross-link proteins into the coated pit by catalyzing the formation of E (γ -glutamyl)-lysine bonds between proteins and coupling of amines and diamines to the γ -carbonyl residues of glutamine (776). This has been confirmed by the inhibition of coated pit/vesicle-mediated endocytosis in the presence of transglutaminase inhibitors (777). Once selected proteins are cross-linked, clathrin may invaginate the phosphorylated 175K and 55K M_r proteins which possibly are located at regular intervals between the specific synaptic vesicle proteins destined for endocytosis. Transglutaminase crosslinking would assure that all the proteins form a discrete entity. This would mean that clathrin would have to recognize only the 175K and 55K M_r synaptic vesicle proteins. The other synaptic vesicle proteins would be selec-

tively retrieved in the coated pit by virtue of transglutaminase cross-linking. Selectivity of synaptic vesicle proteins included in the pit would be mediated, therefore, mostly by transglutaminase activity and, to a limited extent, by clathrin recognition of proteins. The fact that coated vesicles do not have cAMP-dependent protein kinase activity, whereas synaptic vesicles do, implies that the synaptic vesicle cAMP-dependent protein kinase is one protein complex that is not selectively retrieved by coated vesicles. The mechanism of transglutaminase protein selectivity remains to be resolved. Others have demonstrated that a 100K protein is necessary for insertion of clathrin triskelions into coated pits/vesicles (266). A transglutaminase of M_r 100K that is Ca^{2+} /calmodulin dependent has been demonstrated in platelets (E. Puszkun, unpublished observations). My results indicate the existence of a coated vesicle calmodulin binding protein with a M_r of 100K. It is conceivable that this 100K protein may be a Ca^{2+} /calmodulin dependent transglutaminase and upon its activation cross-links the coated pit proteins stabilizing the entire cross-linked coated pit complex and thereby facilitates clathrin binding to the membrane. The inhibition of coated vesicle morphology by metabolic inhibitors (249,297) suggests that ATP plays an important role in coated vesicle function. The role that ATP plays in coated vesicle function may be in the protein kinase mediated transfer of its gamma phosphate to coated membrane proteins.

After clustering of the proteins and the formation of the coated pit, the coated pit invaginates, pinches off and becomes a coated vesicle. Although Kanaseki and Kadota's non-enzymatic hypothesis (228) that the conversion of coated pit hexagons into pentagons causes an increased curvature and creates a sufficient force to pinch off the

coated pit from the membrane is documented by electron microscopy (134), it is uncertain what initiates this transformation and what the nature of this force is.

Calmodulin activation of PLA_2 in the region of the coated pit on both sides of the membrane and subsequent release of lysolecithin could create membranal nicks and perturbations thereby leading to detachment of the coated pit from the rest of the plasma membrane. Presynaptic plasma membrane PLA_2 activation may function in the resealing of the free ends remaining at the membrane. Coated vesicle PLA_2 may also function in the resealing of the detached coated pit into a coated vesicle. The contribution of PLA_2 to this process is unknown.

Activation of coated vesicle and synaptic plasma membrane PLA_2 leads to release of arachidonic acid and its consequent metabolism by coated vesicle-PG and Thromboxane synthetase to PGE_2 , $PGF_{1\alpha}$, and TxB_2 . PGE_2 and $PGF_{1\alpha}$ serve to modulate PLA_2 and facilitate formation of coated pits by amplifying coated vesicle PLA_2 . Cyclic-AMP, which is generated by synaptic plasma membrane adenylate cyclase, and axon terminal ATP serve to inhibit this process of coated pit/vesicle formation. The regulation of plasma membrane area is therefore modulated by coated vesicle and synaptic plasma membrane PLA_2 . Prostaglandins stimulate and cAMP and ATP inhibit coated vesicle PLA_2 , respectively.

The generation of an acidic milieu by PG's and Tx's would increase local H^+ ion concentration. This increase in H^+ , along with the increase in local calcium, facilitates clathrin-clathrin interaction in the coated pit and vesicle leading to the formation of the continuous coat structure (181, 222).

Once the coated vesicle forms, electron microscopic observations

have revealed that the coat disassembles from the coated vesicle, and the denuded vesicle is subsequently transformed into a synaptic vesicle (134). Because clathrin association and dissociation is reversible, it is possible that when calcium concentration decreases, the protein kinase is no longer maximally stimulated. Additionally, a protein phosphatase might be activated to hydrolyze phosphates from previously phosphorylated coated vesicle proteins leading to the return of the protein's original conformation which does not recognize clathrin, thereby leading to clathrin dissociation from the membrane. It is of interest to note that a cytoplasmic factor in conjunction with ATP has been found to lead to dissociation of coats from vesicles (837). Protein phosphatases are usually soluble and cytoplasmic (704). Recently a brain phosphatase dependent on ATP has been characterized (838). It is possible that this soluble factor leading to the dissociation of coats from coated vesicles is a protein phosphatase.

Although there are specific high affinity neurotransmitter uptake systems (839), the invagination of coated pits and subsequent coated vesicle formation may lead to additional uptake of neurotransmitter from the synaptic cleft. Until the content of neurotransmitter in coated vesicles is determined, the contribution of this pathway to neurotransmitter conservation will remain unresolved.

Figure 8 summarizes all the enzymatic cascades postulated to be involved in coated pit/coated vesicle regeneration of synaptic vesicles.

Figure 9 is a schematic model emphasizing the possible functional significance of vesicle protein kinase activities.

Figure 8. Scheme of events hypothesized to be involved in coated pit and coated vesicle formation. The following abbreviations were employed:

SPM = synaptic plasma membrane

AC = adenylate cyclase

PDE = phosphodiesterase

AMP = Adenosine monophosphate

CV = coated vesicles

PLA₂ = Phospholipase A₂

PG = prostaglandin

Tx = thromboxane

AT = acetyltransferase

PK = protein kinase

MP = membrane proteins

cp = coated pit

CaM = calmodulin

cAMP = 3'-5' cyclic adenosine monophosphate

ATP = adenosine triphosphate

PC = phosphatidylcholine

LL = lysolecithin

aa = arachidonic acid

+ = stimulation

- = inhibition

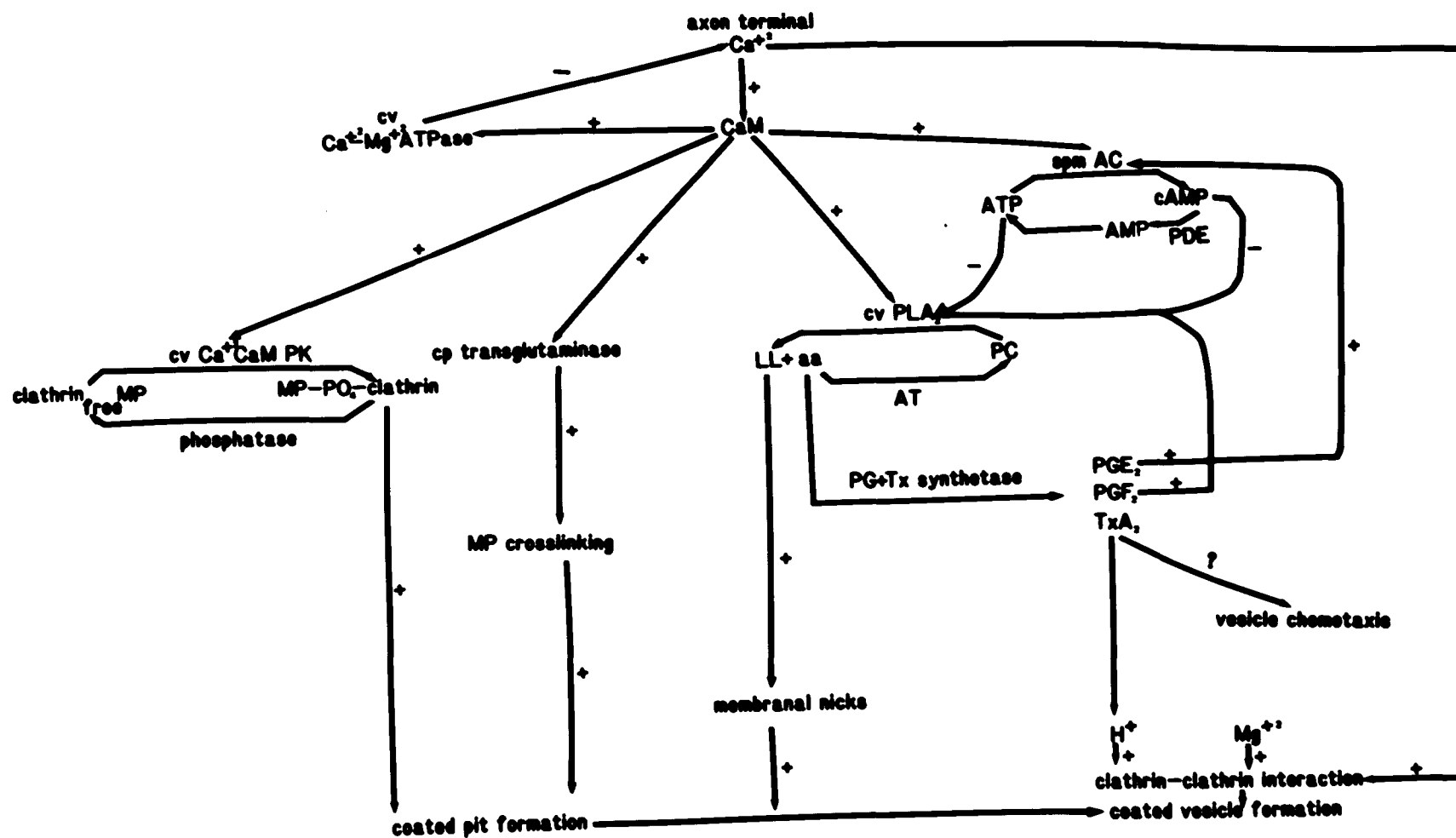
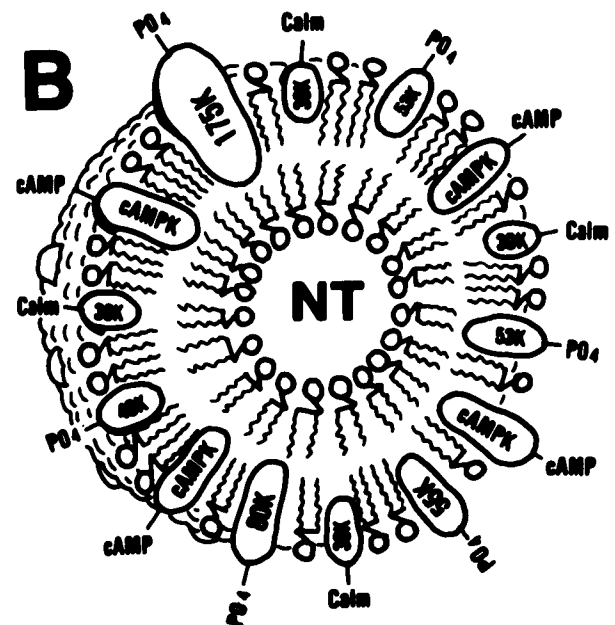
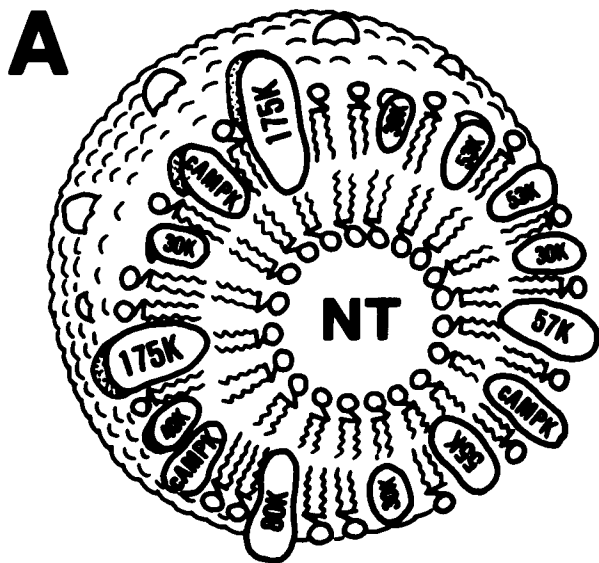
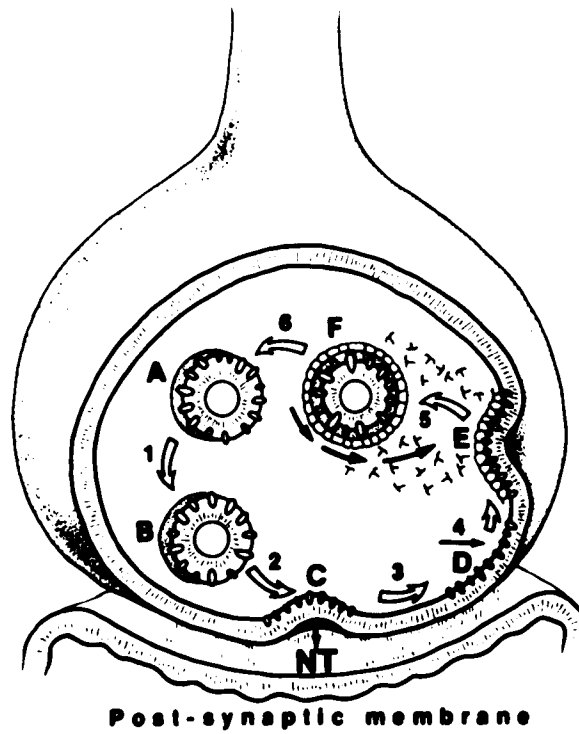
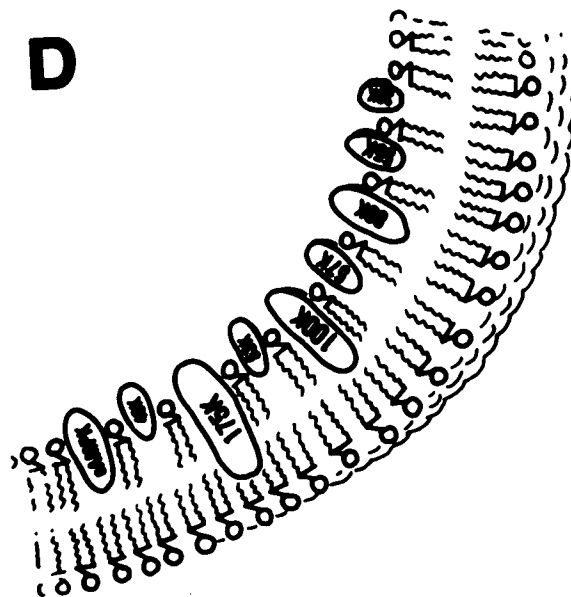
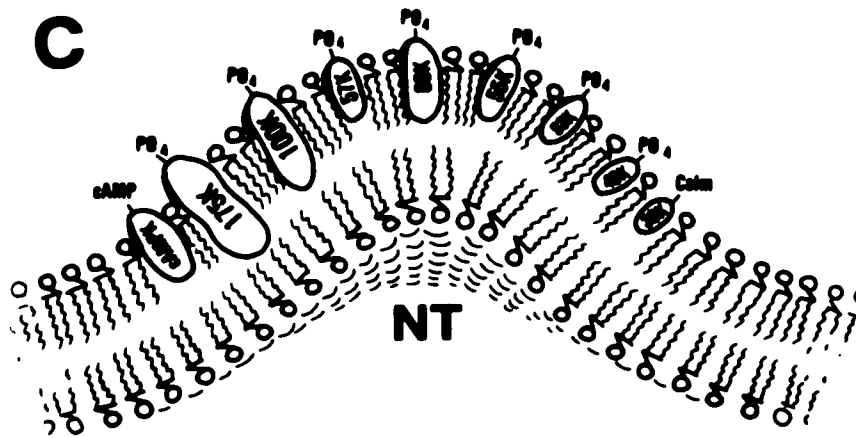
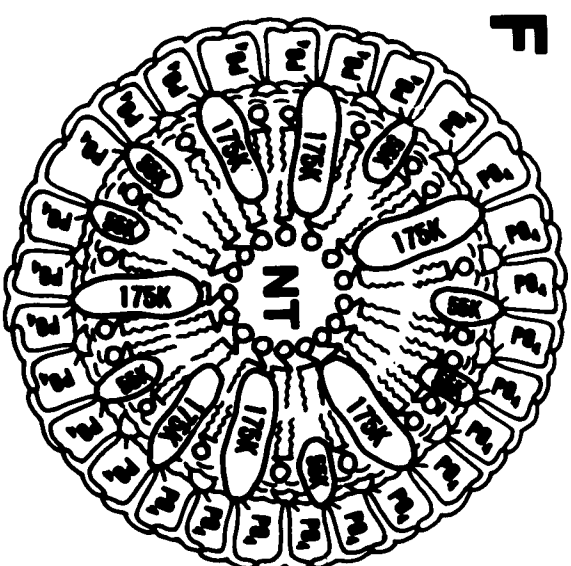
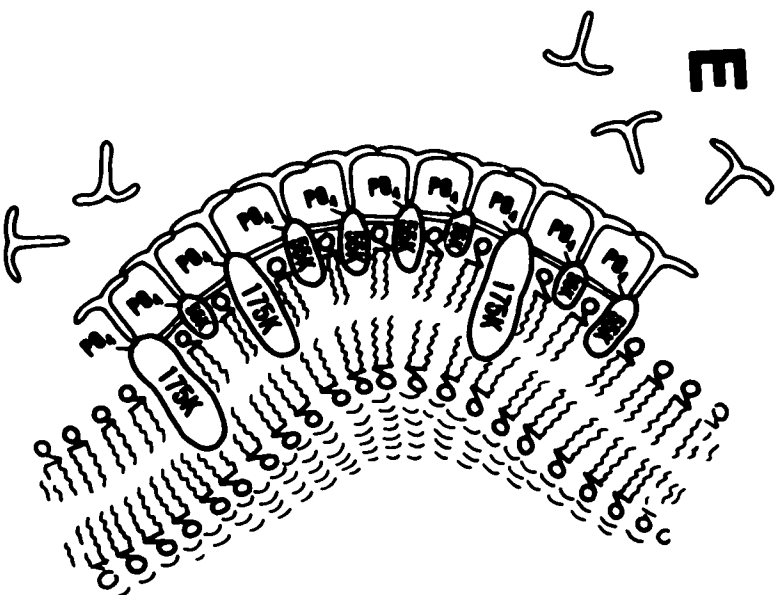


Figure 9. Model of the possible functional significance of synaptic and coated vesicle protein kinase activity. Enlarged details of steps A-F are inserted. Insert A illustrates a synaptic vesicle prior to calcium influx into the axon terminal. The cAMP dependent kinase (cAMP K) and the Ca^{2+} /CaM dependent kinase (30K) as well as a variety of protein kinase substrates with M_r s 175K, 80K, 57K, 55K, and 53K are shown inserted in the lipid bilayer of the vesicle. Insert B illustrates an activated synaptic vesicle with Calmodulin (Calm) bound to the 30K protein kinase, and cAMP bound to the cAMP dependent protein kinase. The phosphorylated protein substrates are also depicted. Insert C illustrates a synaptic vesicle that has fused with the synaptic plasma membrane. Insert D illustrates proteins of the synaptic vesicle integrated in the synaptic plasma membrane, prior to their selective reuptake. Insert E illustrates the insertion of clathrin triskelions (Y) into the phosphorylated coated pit proteins of M_r 175K and 55K after activation of coated pit Ca^{2+} /CaM dependent protein kinase. It is postulated that clathrin inserts into the proteins only in their phosphorylated state. Insert F illustrates a coated vesicle formed from the invaginated coated pit. It is postulated that the integrity and

existence of the coat depends on the phosphorylated state of the 175K and 55K protein substrates. During Step 1 there is an increase in Ca^{2+} and cAMP concentration. During Step 2 the synaptic vesicle is propelled to and fuses with the presynaptic plasma membrane. During Step 3 there is a decrease in Ca^{2+} and cAMP concentration, and synaptic vesicle proteins intermix with the presynaptic plasma membrane proteins. During Step 4 there is a local increase in calcium ion concentration activation of membrane Ca^{2+} /CaM dependent protein kinase and transglutaminase activities with resultant cross-linking of specific SV proteins destined for retrieval, insertion of soluble cytoplasmic clathrin triskelions into the membrane with the resultant formation of a coated pit. During Step 5, as a result of several processes discussed in the text, the coated pit invaginates, pinches off and forms a coated vesicle. During Step 6 there is a decrease in local calcium concentration, activation of coated vesicle protein phosphatase resulting in the shedding of the clathrin triskelions from the vesicle and eventual transformation of the coated vesicle into a synaptic vesicle. The clathrin triskelions join the cytoplasmic soluble pool. NT = neurotransmitter.







10. Vesicle Mobility

Vesicle mobility can be inhibited by cytochalasin B and colchicine, actin and tubulin disrupters, respectively (840-842). Both synaptic and coated vesicles have proteins with M_r s corresponding to actin and tubulin. Whether endogenous vesicle contractile proteins or cytoplasmic contractile proteins impinging on synaptic and coated vesicles are involved in their movement is not clearly established. It is interesting to speculate on the role of prostaglandins and thromboxanes produced by these vesicles with respect to vesicle mobility.

Whole cells, e.g., neutrophils and leukocytes, undergo chemotaxis, i.e., they move in response to a chemical gradient. The basis of directional movement involves (a) the interaction of a chemoattractant with the plasma membrane; (b) an altered transmembrane potential; (c) changes in cyclic nucleotide metabolism; and (d) oxygen uptake as well as PLA_2 activation (843). Polarity of cells is established by reorganization of cytoskeletal contractile elements (843). Both synaptic and coated vesicles undergo directed movement. Synaptic vesicles move to the plasma membrane and coated vesicles away from the plasma membrane. In other cells, coated vesicles must move from the golgi to the plasma membrane or shuttle to lysosomes (844, 845).

Contractile elements are involved in moving these vesicles, as inhibition of these movements by contractile protein disruptors indicates. Still, questions remain. How is directionality of vesicle movement established? Are vesicles capable of chemotaxis? All the necessary elements required for chemotaxis are present, i.e., PLA_2 , protein kinase, ion transport potential and even cytoskeletal elements. It is of interest that TxB_2 and various prostaglandins are

produced by and are chemotactic for leukocytes, polymorphonuclear and other cells (698). Is it possible that one of the functions of prostaglandins and thromboxanes generated by synaptic and coated vesicles is to interact with other vesicles and/or other organelles/membranes and initiate vesicle chemotaxis? Perhaps selective activation of actin, myosin, and tubulin on one pole of the vesicle or polar clustering of these proteins could bring about directionality of vesicle movement.

After clathrin dissociates from the coated vesicle, synaptic vesicles are thereby regenerated. There are many unexplained phenomena in this recycling process. If coated vesicles are retrieved by synaptic vesicles, why do so many of the synaptic and coated vesicle enzymes and their properties differ from each other? There may be several explanations for this phenomenon. By some unknown mechanism synaptic vesicles that are newly transformed from coated vesicles far away from the active zone may be continually transformed so that by the time they reach the active zone they have the full characteristics of synaptic vesicles. If there is a limited amount of protein synthesis occurring in the axon terminal, it may perhaps function in transformation of coated vesicles to synaptic vesicles. It must also be recognized that coated and synaptic vesicles obtained in this study are a heterogeneous population. Both synaptic and coated vesicles are heterogeneous in that they come from a variety of axon terminals. The coated vesicles in addition may arise from perikaryon regions as well as from the axon terminal. Coated vesicles from these different sites of origin may have different characteristics.

E. REFLECTIONS ON THE PAST AND THOUGHTS ON THE FUTURE

It has been approximately 90 years since Locke (19) observed that calcium is necessary for the release of neurotransmitter from peripheral axon terminals. Since then the role of calcium has been established at almost all chemical transmitting synapses.

It is probably true of most scientific endeavors that although it is difficult to make the initial observation, it may be equally as difficult if not more difficult to understand the mechanism behind the observed phenomenon. Today 90 years after the initial observation of the effect of calcium on promoting neurotransmitter release, the mechanism of calcium induced transmitter release is still unresolved and remains controversial. This controversy is attested to by the abundance of theories produced throughout the years, some of which have been mentioned in the Introduction of this work.

The hypothesis I presented in this work provides one more theory which attempts to elucidate the mechanism of calcium-induced neurotransmitter release. Within the context of the past literature and the results obtained during the course of this study, this hypothesis to me seems logical and tenable. Further experiments will prove whether this hypothesis will or will not withstand the test of time. Whether it does or does not, I think in the very least it is a useful model to use as a starting point for future experiments.

Interdisciplinary experiments can probably yield information on the true in vivo functions of various enzymatic cascades in the transmitter process. It has already been demonstrated by Castellucci et al. (828) that the injection of cAMP dependent protein kinase can modulate neuro-

transmitter release concomitantly with behavior. Similarly by looking at other possible presynaptic enzymatic cascades it may be possible to understand certain general aspects of brain function. The mechanism of anesthetic action, for example, has been attributed to inactivation of PLA₂ (513-515). In light of the involvement of PLA₂ in neurotransmitter release, this remains a tenable hypothesis.

Recently the pharmacological agent piracetam has been used in combination with choline to lead to memory improvement (846). Choline is converted to acetylcholine, a neurotransmitter associated with memory (847). Piracetam has many effects including the ability to enhance cerebral neuronal activity, and may deplete hippocampus ACh levels by enhancing neurotransmitter release (848,849). Looking at Table 2 (Introduction), it should be recognized that piracetam is an activator of brain PLA₂. It is quite possible that after choline is converted to acetylcholine and stored in synaptic vesicles, piracetam may activate SV and SPM PLA₂, facilitating the release of the newly formed transmitter leading to improvement of memory. The defect in various Alzheimer patients may be related to a lack of acetylcholine as well as some pathology in the neurotransmitter release process which pharmacological PLA₂ activation may somehow ameliorate.

In the hope of immunologically identifying lipomodulin in synaptic vesicles, I obtained sera from a variety of systemic lupus erythematosus (SLE) patients reported to have anti-lipomodulin antibodies (850). Upon incubation of these sera with synaptic vesicles after which kinase assays were performed, I noticed that some of the sera completely inhibited Calcium/CaM kinase activity. SLE is manifested by a variety of clinical defects (851). By obtaining sera from SLE patients with CNS

and non-CNS manifestations of the disease, it was noted that a preponderance (not all) of SLE patients with a diversity of neuropsychiatric manifestations which included psychosis, depression and organic brain syndrome inhibited Ca^{2+} /CaM protein kinase activity. Most of the SLE patients without CNS manifestations did not inhibit this protein kinase. Upon performing similar experiments with sera from psychotic patients not suffering from SLE, it was observed that approximately 50% of non-SLE psychotics inhibited this kinase activity. To identify the inhibiting factor, the serum from one psychotic patient with SLE was passed through a protein A column. Only the IgG fraction of the serum inhibited calcium/calmodulin dependent protein kinase activity, implying that the serum factor in this one patient was an IgG antibody. This preliminary study is of course fraught with pitfalls. The different patients may have been on different medications and they may have been at different stages of the disease. Furthermore, it is unknown how clinically diverse this protein kinase inhibiting effect is and whether IgG antibodies are responsible for inhibition of protein kinase activity in all the patient sera tested. Surely many questions remain to be answered. Nevertheless, this study demonstrates that using the hypothesis presented in this work as a preliminary working model may help to eventually elucidate abnormalities in brain function. A pathological diversion of any one of the many multiple enzymatic cascades in the axon terminal may lead to profound alteration in brain function.

It is hoped that this work has provided a tentative working model for the future study of presynaptic mechanisms involved in neurotransmitter release and the retrieval of synaptic vesicle membrane.

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