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The regulation of mammalian adenylyl cyclases by protein kinase C

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THE REGULATION OF MAMMALIAN ADENYLYL CYCLASES BY PROTEIN KINASE C

by

OFER JACOBOWITZ

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

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Abstract

THE REGULATION OF MAMMALIAN ADENYLYL CYCLASES BY PROTEIN KINASE C

by

Ofer Jacobowitz

Advisor: Professor Ravi Iyengar

Many hormones and neurotransmitters regulate target organs through the adenylyl cyclase pathway. Hormone receptors activate the heterotrimeric G protein, G_s, which in turn stimulates the enzyme adenylyl cyclase to produce cAMP. Activation of protein kinase A by cAMP leads to phosphorylation and regulation of metabolic enzymes, ion channels and transcription factors. Many hormones, however, stimulate adenylyl cyclase independently of G_s. For example, angiotensin II induces steroidogenesis in adrenal glands by raising cAMP levels without activating G_s. Nerve growth factor, which does not activate G_s, can also raise cAMP levels. I have investigated the molecular basis for these signal transmission pathways.

Since both angiotensin II and nerve growth factor stimulate protein kinase C (PKC) and stimulation of PKC raises

cAMP levels in some cells, I decided to test whether PKC activation would stimulate any of the six cloned adenylyl cyclases. Using transient transfection of human embryonal kidney cells (HEK-293), I identified adenylyl cyclase 2 (AC2), an isoform abundant in brain and lung, as the isoform most extensively stimulated by PKC. Phorbol 12-myristate, 13-acetate (PMA), a PKC activator, stimulated *basal* adenylyl cyclase activity in AC2-expressing cells. This suggested that AC2 may be phosphorylated through PKC activation in absence of G_s stimulation. To demonstrate phosphorylation, AC2 cDNA was transduced into Sf9 cells by recombinant baculovirus. PMA treatment of AC2-expressing Sf9 cells increased basal activity. This stimulation was blocked by staurosporine. Stimulation was reflected by an increased V_{max} and unaltered K_m of AC2. PMA treatment also potentiated stimulation of AC2 by G_s-α and $\beta\gamma$ subunits. AC2 was epitope-tagged at the N-terminus to permit purification. Tagged-AC2 was also stimulated by PMA treatment of cells. Tagged-AC2 was purified to apparent homogeneity with an anti-epitope antibody affinity column. PMA treatment of ^{32}P-labeled Sf9 cells expressing tagged-AC2 resulted in enhanced ^{32}P incorporation into purified, tagged-AC2. Thus, PKC activation results in G_s-independent stimulation and enhanced phosphorylation of AC2. AC2 may therefore serve as a signal recognition and integration element allowing many external signals to impart some of their cellular effects through the cAMP pathway.

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I wish to thank Ravi Iyengar for his support and guidance throughout the years that we have worked together. I am also grateful to the members of the Iyengar lab for their assistance and fellowship: Upinder Bhalla, Steven Brower, Donna Carty, Jianghao Chen, Jianqiang Chen, Michael DeVivo, Anya Harry, Spencer Kroll, Hai-Wen Ma, Irina Orekhova, Joe Pieroni and Richard Premont. I also thank Jack Green and Ronald Magnusson for their help and advice.

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CHAPTER 1

INTRODUCTION

A variety of extracellular signals such as hormones, neurotransmitters, growth factors, ions, odorants and photons regulate the function of their target cells. The various signals are processed by cellular systems which not only recognize signals, but also regulate the magnitude and duration of single and multiple signals and process them into intracellular messages.

Signal transduction begins by interaction of signals with their specific receptors. For many signals, cell surface receptors transmit the signals by activating G proteins, a group of heterotrimeric guanine nucleotide-binding proteins. Activated G proteins in turn modulate the activity of enzymes or ion channel effectors. Such pathways that utilize G proteins to convert extracellular signals to intracellular messages are called G protein-coupled signaling systems.

Effectors in G protein-coupled systems are cell surface enzymes which generate or mobilize second messengers or ion channels (Hepler and Gilman, 1992; Birnbaumer, 1992). Examples of effector enzymes are adenylyl cyclases, which produce the intracellular messenger cAMP, and phospholipases C, which produce the intracellular messengers inositol trisphosphate and diacylglycerol. Through the generation of intracellular messengers, effectors modulate a large array of enzymatic and structural components of cells.

In recent years, multiple isoforms of effectors with differential organ distribution profiles have been discovered. If all isoforms of an effector produce the same intracellular messenger in response to activation by the same G protein, then it would appear that the observed molecular diversity is of little physiological value. One possible reason for the multiplicity of effectors is that, like receptors and G proteins, effectors too have distinct regulatory functions in signal transduction. Hence the presence of a particular effector subtype may impart unique signal processing capabilities to a cell. In my study, I have focused on the role of subtypes of the effector adenylyl cyclase in signal recognition and integration.

General features of the adenylyl cyclase system:

The hormone-stimulated adenylyl cyclase system is among the most extensively studied G protein-signaling systems. The first three components of the system are plasma membrane proteins. They are the receptor, the heterotrimeric G protein, G_S, and the effector enzyme adenylyl cyclase. Signaling is initiated by agonist occupancy and activation of a receptor. The activated receptor activates the heterotrimeric G protein, G_S, which subsequently stimulates the effector enzyme, adenylyl cyclase. Adenylyl cyclase catalyzes the formation of the intracellular messenger cAMP from ATP. cAMP activates protein kinase A which

phosphorylates many cellular proteins. Target proteins for PKA include metabolic enzymes, such as those involved in glycogenolysis and amino acid biosynthesis and cell-surface ion channels, such as the L-type Ca^{2+} channels. PKA also activates the transcription factor CREB (cAMP Response Element Binding protein) by phosphorylation (Gonzalez & Montminy, 1989, Gonzalez *et al*, 1989). CREB binding to the cAMP response element in the regulatory regions of responsive genes results in modulation of transcription of these genes. Thus external signals that are capable of activating the cAMP pathway can elicit a variety of cellular effects.

G Protein-coupled Receptors:

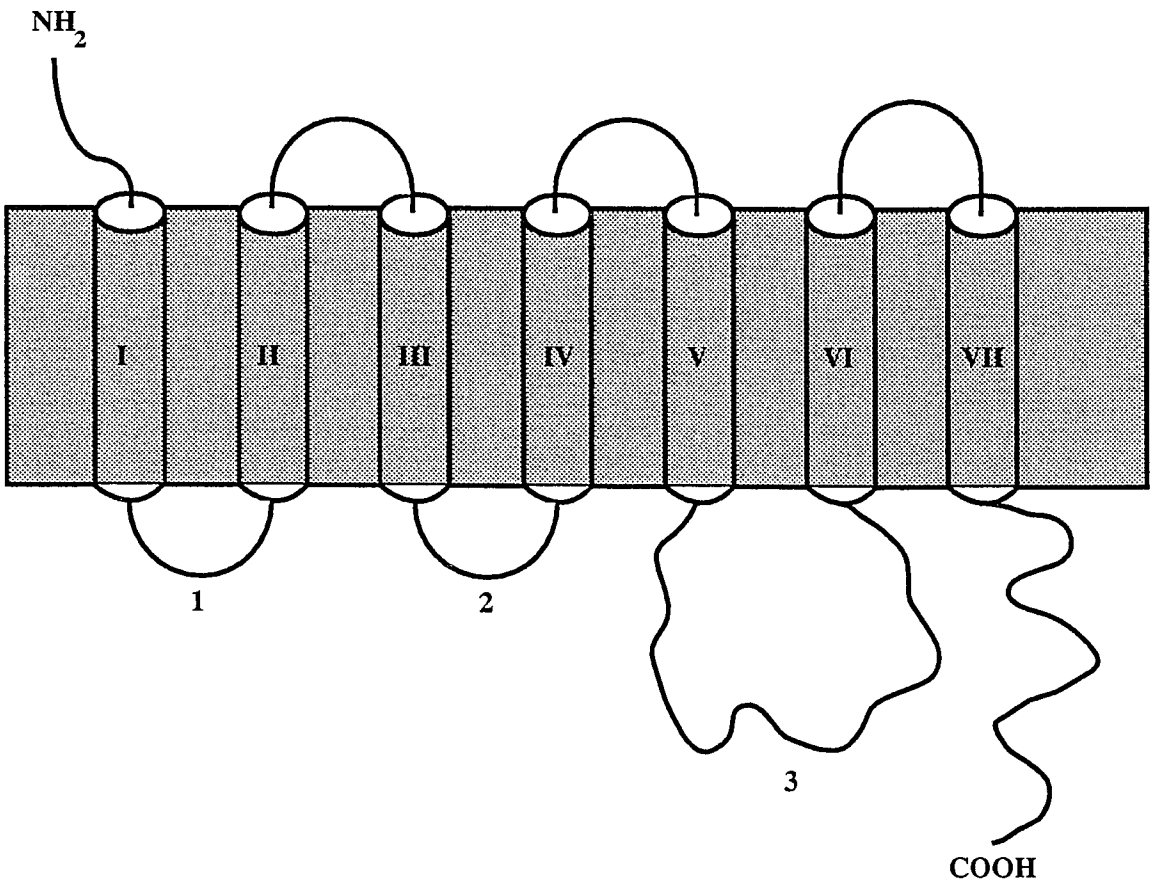
Plasma membrane receptors which regulate the activity of G proteins comprise a large and growing family of receptors. All of these receptors appear to share a common structural motif of seven stretches of hydrophobic amino acids that are thought to comprise membrane spanning regions (Fig. 1-1). The differences in the primary sequence of the presumed transmembrane domains of the various members allow this superfamily of receptors to function as very specific pharmacological targets for a panoply of neurotransmitters, hormones of various classes and vasoactive factors, and to mediate visual, olfactory and some gustatory sensory transduction. The presence of a receptor on a cell determines whether that cell can be a target of particular signals, and the type or subtype of receptor can define the type of response mounted by a cell.

In spite of predicted similarity of secondary structure, the groups of G protein-coupled receptors differ in their amino acid sequence. The main group consists of the receptors for most small neurotransmitters, neuropeptides and some lipid factors (PAF, eicosanoids). A second group consisting of the calcitonin, parathyroid hormone, parathyroid-hormone-related peptide, secretin and VIP receptors represents a divergent group of G protein-coupled receptors (Ishihara *et al*, 1992). The four metabotropic glutamate receptors (Tanabe *et al*, 1992), the parathyroid Ca^{2+}-sensing receptor (Brown *et al*, 1993) and the IGF-

Fig. 1-1. Model of Seven Transmembrane Span G Protein-coupled Receptors:

Schematic diagram of topology of main group seven-transmembrane span receptor. Transmembrane domains are labeled in Roman numerals and intracellular domains are labeled in Arabic numerals.

Figure 1-1. Model of Seven Transmembrane Span G Protein-coupled Receptors



2/mannose-6-phosphate receptors (Okamoto *et al*, 1990) are other unique receptors. The small subgroups are not as well characterized in terms of structure-function relationships as the receptors belonging to the large, structurally similar group of G protein-linked receptors.

The putative *transmembrane* domains are proposed to form the ligand binding domain of some of the subgroups of receptors on the basis of chimerical and mutagenic analyses of α– and β–adrenergic receptors (Dixon *et al*, 1987, Dohlman *et al*, 1988, Kobilka *et al*, 1988, Wong, S. K.-F. *et al*, 1988, Matsui *et al*, 1989). However, some G protein-linked receptors may bind agonist elsewhere. Loosfelt *et al* (1989) cloned variants of the Luteinizing Hormone-human Chorionic Gondotrophin (LH-hCG) receptor lacking the transmembrane regions, suggesting that agonist binding may occur at a large extracellular domain present on the LH-hCG receptor. It is possible that receptors for the glycoprotein hormones, LH-hCG, Follicle Stimulating Hormone (FSH), Thyroid Stimulating Hormone (TSH) and the parathyroid Ca^{2+}-sensing receptor bind their ligands at their unusually large *extracellular* domains (Loosfelt *et al*, 1989, Parmentier *et al*, 1989, Sprengel *et al*, 1990, Brown *et al*, 1993).

The ability to activate G proteins is the defining feature of the "seven transmembrane" receptor family. For the adrenergic and muscarinic acetylcholine receptors, several studies have

ascribed the G protein-coupling function to their third intracellular loop between the fifth and sixth transmembrane domains and to the cytoplasmic tail (Kobilka *et al*, 1988, O'Dowd *et al*, 1988, Leichleiter *et al*, 1990). It remains to be tested if less related receptors in the family also have the specificity of their G protein interaction defined by the third intracellular loop and cytoplasmic tail.

The function of G protein-coupled receptors is to selectively respond to agonists by activation of their cognate G proteins. Agonist-induced activation of G proteins requires the presence of guanine nucleotides (Rodbell *et al*, 1971a), hence the name "G protein". With agonist binding to receptor, a conformational change of the receptor is presumed to occur, allowing the activated complex to catalyze the exchange of GDP for GTP on the α subunit of the G protein, and thereby leading to G protein activation. G proteins can be activated in the absence of receptor by GTP and Mg^{2+}. Receptor occupation by agonist induces G protein activation by lowering the threshold Mg^{2+} concentration required for activation to levels readily obtained within the cell (Iyengar & Birnbaumer, 1981, 1982). Not only do receptors regulate G protein activity, but conversely, G proteins regulate agonist binding to the receptor. By themselves, receptors exist in a low affinity state for agonists, but when coupled to G proteins, receptors bind agonist with a high affinity (Maguire & Gilman, 1976).

G Proteins:

G proteins are heterotrimeric GTPases which couple the agonist-occupied receptor to effectors. Twenty different forms of α subunits of G proteins have been cloned (Simon *et al*, 1991, Birnbaumer, 1992) and most of their functional targets (effectors) are now known. Much of our current understanding of G protein function is derived from studies of the G protein, G_s, which is named for its ability to stimulate the effector adenylyl cyclase.

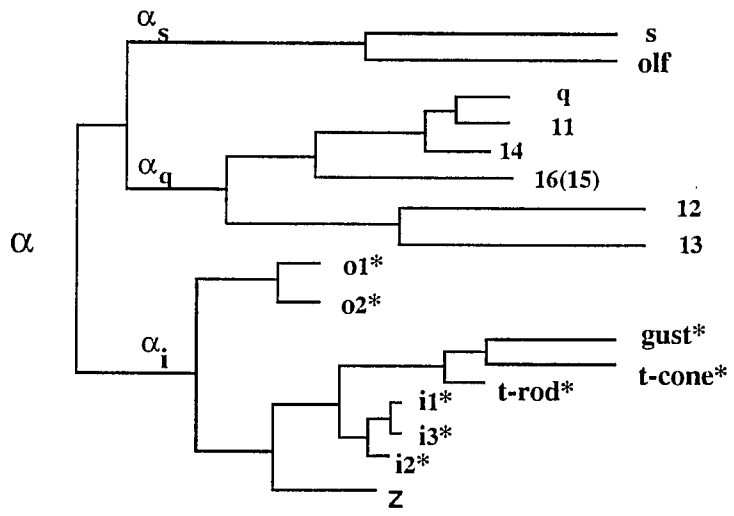
The members of the G protein family are defined by the amino acid sequence of their α subunits (Fig. 1-2). Indeed, most G protein functions are attributed to the α subunits. The α subunits of G proteins bind GTP, possess intrinsic GTPase activity (Fung *et al* 1981, Northup *et al* 1983a) and modulate the function of an effector enzyme or ion channel (Northup *et al*, 1983a, Imoto *et al*, 1988, Ewald *et al*, 1989, Yatani *et al*, 1988, Simonds *et al*, 1989, Moriarty *et al*, 1990, Lowndes *et al*, 1991, Taylor & Exton, 1991a, 1991b, Smrcka *et al*, 1991, Wong *et al*, 1991, Lee *et al*, 1992, Wong *et al* 1992, Wu *et al*, 1992). The α subunits are also functional targets of toxins produced by *Vibrio cholera* and *Bordatella pertussis*. Cholera toxin persistently activates the G protein G_s by ADP-ribosylating α_s, which virtually abolishes the GTPase activity of G_s (Cassel & Selinger, 1977, Cassel & Pfeuffer, 1978). Pertussis toxin functionally uncouples the G proteins G_i, G_o, and G_t from their receptors by ADP-ribosylating their α subunits (Katada & Ui, 1982,

Fig. 1-2. G proteins α subunit phylogeny tree and α subunit function:

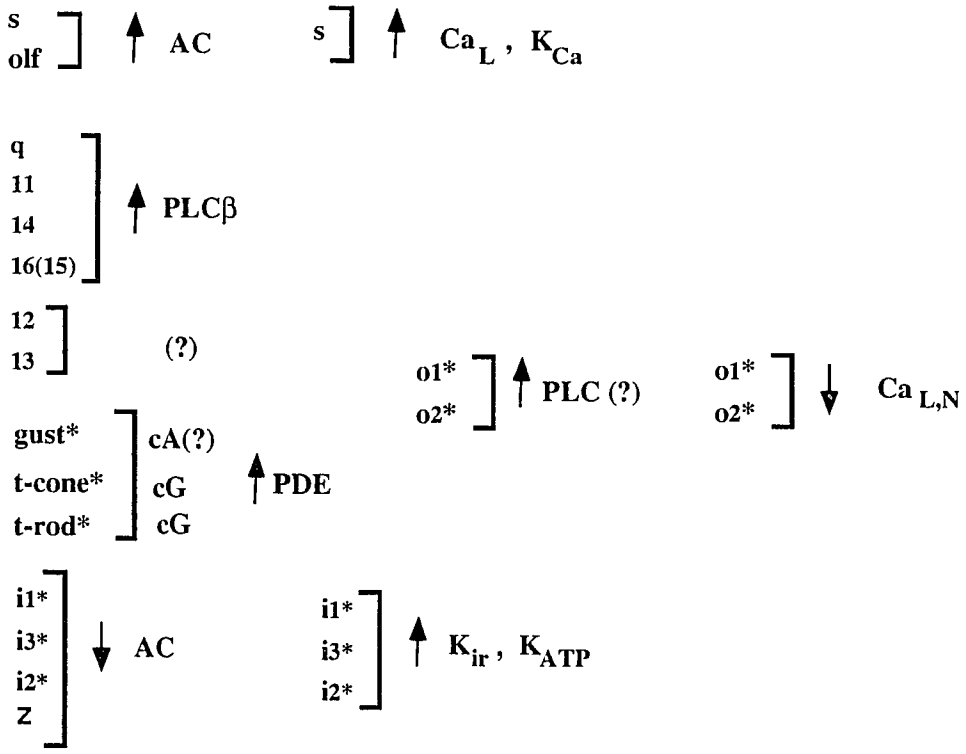
Adapted from Birnbaumer (1992). Asterisks indicate subunit is substrate for pertussis toxin ADP-ribosylation.

Abbreviations: AC, adenylyl cyclase; cA, cyclic AMP; cG, cyclic GMP; gust, gustatory; K_{ATP}, ATP-inhibited potassium channel; K_{ir}, inward rectifying potassium channel; PDE, phosphodiesterase; PLC, phospholipase C.

Figure 1-2. G proteins α subunit phylogeny tree



G proteins α subunit effectors



Van Dop *et al*, 1984, Murayama and Ui, 1984). Though the α subunit is responsible for many functions of a G protein, the $\beta\gamma$ subunit can also modulate effector activity and is required for receptor coupling to G proteins.

The β and γ subunits of G proteins exist as heterodimers that can only be separated under denaturing conditions, hence a $\beta\gamma$ subunit is believed to be the functional form (Hildebrandt *et al*, 1984). $\beta\gamma$ subunits are capable of both passive and active modulation of signaling processes. $\beta\gamma$ subunits are required for receptor coupling to G proteins (Shinozawa *et al*, 1980, Florio & Sternweis, 1985, 1989) and can also promote the deactivation of α subunits (Northup *et al*, 1983b). In recent years, mammalian $\beta\gamma$ subunits have been shown to directly regulate effector function (for reviews see Birnbaumer, 1992, Clapham & Neer, 1993). $\beta\gamma$ subunits can modulate the activity of specific isoforms of adenylyl cyclase (Katada *et al*, 1987, Gao & Gilman, 1991, Tang & Gilman, 1991), phospholipase C-β_2 and β_3 (Camps *et al* 1992, Carozzi *et al*, 1993, Katz *et al*, 1992), β-ARK (β-Adrenergic Receptor Kinase; Pitcher *et al*, 1992) and K^+ channels (Logothetis *et al*, 1987, Kim *et al*, 1993).

There are four known isoforms of the β subunit (Sugimoto *et al*, 1985, Fong *et al* 1986, Fong *et al* 1987, Gao *et al*, 1987a, Gao *et al*, 1987b, Levine *et al* 1990, Von Weizsäcker *et al*, 1992) and six isoforms of the γ subunit (Hurley *et al*, 1984a, Robishaw *et al*, 1989, Gautam *et al*, 1989, 1990, Fisher & Aronson 1992, Cali *et al*, 1992). The utility of this diversity is not

completely understood. $\beta\gamma$ subunits of different composition similarly modulate adenylyl cyclase or PLC-β activity with the exception of $\beta_1\gamma_1$ (transducin $\beta\gamma$) which is significantly less potent than other combinations (Cerione *et al*, 1987, Iñiguez-Lluhi *et al*, 1992, Ueda *et al*, 1994). $\beta\gamma$ subunit diversity may function in G protein receptor coupling-specificity. Using anti-sense oligonucleotides to "knock-out" particular β or γ subtypes in GH_3 cells, Kleuss and coworkers (1992, 1993) implicated β_1, γ_3 and β_3, γ_4 in specifically coupling somatostatin and muscarinic (M_4) receptors, respectively, to modulation of voltage-dependent Ca^{2+} channels. Also, Kisselev & Gautam (1993) demonstrated that γ_1 mediates coupling of transducin to rhodopsin. In contrast to their specificity in coupling to receptors, several $\beta\gamma$ subtypes can associate with the same α subunit. G protein preparations containing the same α subunits are found to be composed of different $\beta\gamma$ subunits from tissue to tissue (Morishita *et al*, 1992). Therefore it is possible that $\beta\gamma$ subtypes can specifically link an α subunit to several different receptors.

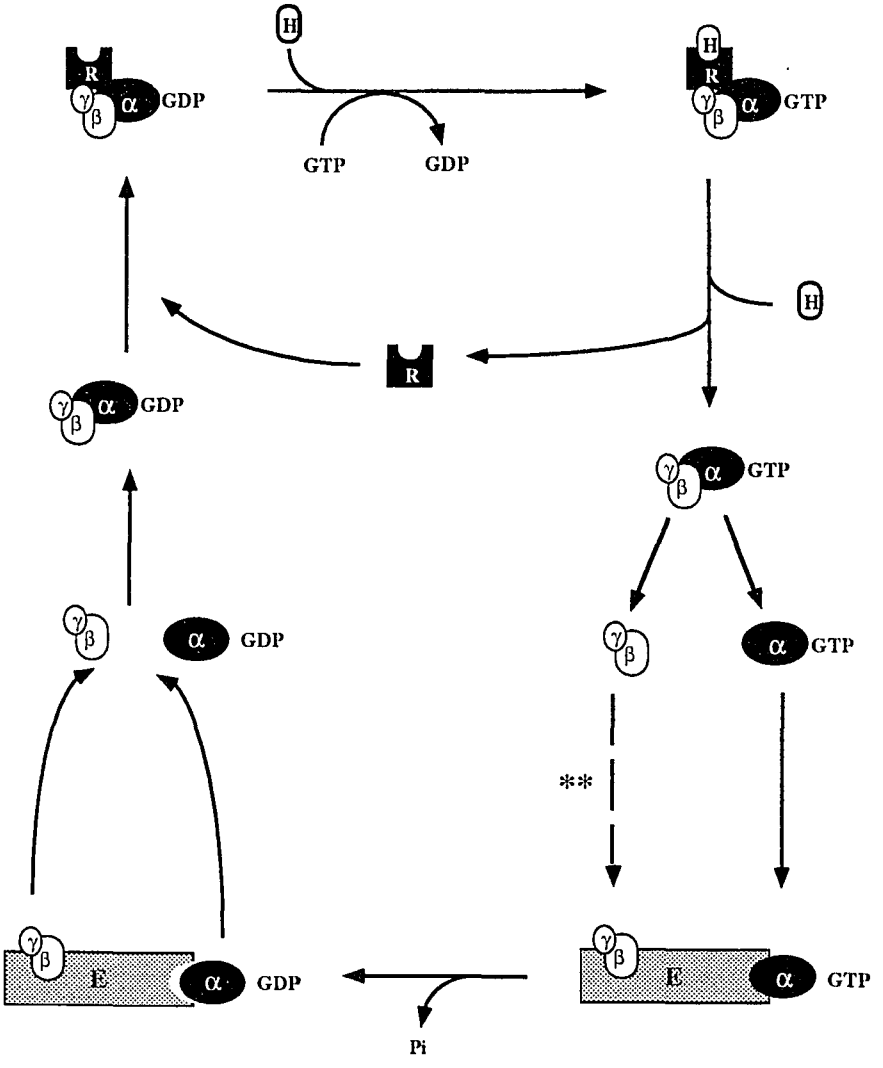
The best characterized models of effector modulation by G proteins are the G_s stimulation of adenylyl cyclase and the G_t (transducin) stimulation of cGMP phosphodiesterase. The G protein activation cycle is illustrated in Fig. 1-3. In the resting state, the G protein heterotrimer exists in a GDP-bound state (Cassel & Selinger, 1978, Fung & Stryer,1980, Northup *et al*, 1983a). With occupation of receptor by agonist, a conformational change is induced in G_s (Codina *et al*, 1984) which reduces its affinity for GDP

Fig. 1-3. G protein activation cycle:

Schematic diagram of G protein activation cycle and effector regulation. Asterisks and dotted lines indicate that βγ regulates the activity of a *subset* of effectors. All effectors are not regulated by both βγ and α subunits.

Abbreviations: α and βγ, α and βγ G protein subunits; E, effector; H, hormone; R, receptor.

Fig. 1-3. G protein activation cycle



(Blume *et al*, 1976, Cassel & Selinger, 1978, Pike & Lefkowitz, 1981, Murayama *et al*, 1984), increases its relative affinity for GTP (Florio & Sternweis, 1989) and increases its affinity for Mg^{2+} (Iyengar & Birnbaumer, 1981, 1982). GDP dissociation is followed by binding of the more abundant guanine nucleotide, GTP (Cassel & Selinger, 1978), and dissociation of the α subunit from $\beta\gamma$ (Howlett & Gilman, 1980 , Fung *et al*, 1981, Northup *et al* 1983a,b, Codina *et al*, 1984, Iyengar *et al*, 1988). These steps result in α subunit activation and release of $\beta\gamma$ subunits which may also regulate effector activity. Since a single agonist-activated receptor can activate multiple G protein molecules (Levitzki & Tolkovsky, 1977, Fung & Stryer, 1980, Pedersen & Ross, 1982), the initial signal is subject to considerable amplification.

Activated α_s subunits stimulate adenylyl cyclase during the period in which GTP is bound to α_s. Thus, deactivation of α_s occurs by conversion of the GTP to GDP through the intrinsic GTPase activity of the α subunit (Cassel & Selinger, 1976, 1977). In the G_S-α system, the GTPase activity serves as a built-in molecular clock which limits the duration of an α subunit's activated state and the subsequent stimulation of adenylyl cyclase. Deactivation of the α_s subunit can be blocked by cholera toxin ADP-ribosylation (Cassel & Pfeuffer,1977, 1978) and specific point mutations in α_s which virtually abolish the GTPase activity (Masters *et al*, 1989). *In vitro,* GTPase resistance is achieved by activation of the α by nonhydrolyzable analogues of GTP (such as GTPγS) or by AlF_4. AlF_4

mimics the terminal phosphate of GTP thus allowing the GDP-bound α_s to be activated (Bigay *et al*, 1985).

Though G_s is considered a single species in the functional model, multiple molecular forms of G_s are known to exist. Both 42 kDa and 52 kDa forms of α_s are widely observed (Northup *et al*, 1980, Mumby *et al*, 1986) and four cDNA forms of α_s are known from molecular cloning (Bray *et al*, 1986, Robishaw *et al*, 1986a, 1986b). The four forms of α_s differ by a fourteen amino acid deletion with two amino acid changes flanking the deletion. From the analysis of the human α_s gene (Kozasa *et al*, 1988), it appears that the four forms arise by multiple splicing of a single transcript. Functional studies of the bacterially expressed isozymes of α_s indicate that both the short and long forms of α_s are capable of stimulating adenylyl cyclase (Graziano *et al* 1987, 1989). The purified recombinant α_{s1} (52 kDa) and α_{s4} (42 kDa) expressed in E. *coli* have comparable catalytic GTPase rates but the rate of GDP dissociation from α_{s1} is three-fold faster than from α_{s4} (Graziano *et al*, 1989). Therefore, α_{s1} may be capable of faster stimulation of adenylyl cyclase. Other possible explanations for the multiplicity of forms of α_s include differential stimulation of specific types of adenylyl cyclase or perhaps differential subcellular compartmentalization.

G Protein Effectors:

G proteins transduce receptor signals by regulating the activity of effectors. Known effectors include adenylyl cyclases, phospholipases C-β, cGMP phosphodiesterases, G protein-coupled receptor kinases (GRKs) as well as the Ca^{2+}_{L}, Ca^{2+}_{N}, and K^{+}_{ir} ion channels. The interaction of activated G protein α subunits with effectors is dependent on the integrity of the associated GTP molecule. Whereas for most effectors the duration of association with the activated G protein is dependent on the α subunit's *intrinsic* GTPase activity, for phospholipase C-β1, the effector itself serves as a GTPase activator protein (GAP) that regulates its own deactivation by stimulating the GTPase of its stimulatory G protein subunit (Berstein *et al*, 1992).

Several effectors are now known to exists in multiple forms. Different isoforms of phospholipases C-β are differentially stimulated by G protein α subunits of the G_q family and βγ subunits. Multiple types of adenylyl cyclases are now also known to exist. The various adenylyl cyclases differ in their regulatory properties as described below.

Adenylyl Cyclases:

Adenylyl cyclases (EC 4.6.1.1) are membrane-bound enzymes which convert MgATP into adenosine 3', 5'-monophosphate (cAMP) and pyrophosphate. Despite their ubiquity in mammalian tissues, adenylyl cyclases have been difficult to isolate due to their extreme lability and low concentration in the plasma membrane. Purification of these enzymes was achieved after the development of appropriate *affinity* chromatography matrix by Pfeuffer and Metzger (1982). The purification of adenylyl cyclases facilitated the cloning of their cDNAs. In the last five years, eight full length and two partial adenylyl cyclase cDNA sequences have been cloned from mammalian tissues. As assessed by mRNA measurements, the different forms of adenylyl cyclases have distinct distribution profiles. They also have unique functional properties. It now appears likely that signal transmission may be subject to regulation at the level of the effector as well.

From functional studies over the last two decades, the existence of at least three forms of mammalian adenylyl cyclases was predicted. A hormone-responsive G_s-sensitive adenylyl cyclase was found in all mammalian tissues and a Ca^{2+}/calmodulin-responsive adenylyl cyclase was discovered in brain tissue (Brostrom *et al*, 1975, Cheung *et al*, 1975). The calmodulin-responsive form was later determined to be G_s-sensitive as well (Smigel, 1986). While these two G_s-sensitive forms were membrane bound, the adenylyl cyclase purified from germ cells by

Braun and Dods (1975) was soluble and refractory to stimulation by G_s. The mammalian adenylyl cyclases in my studies do not include the soluble enzyme since it has not been cloned.

The G_s-sensitive adenylyl cyclases are reversibly stimulated by forskolin, a diterpene antihypertensive from the *Coleus forskohlii* root (Seamon *et al*, 1981). This stimulation can be *direct*, as proposed by Seamon and Daly who observed that forskolin (1-20μM) stimulates adenylyl cyclase activity in G_s-deficient *cyc*$^-$ S49 lymphoma cell membranes (Seamon & Daly, 1981). However in G_s-containing systems, forskolin enhances the G_s-stimulation of adenylyl cyclase at concentrations lower than those required for direct stimulation of adenylyl cyclase (10-100nM) (Clark *et al*, 1982), presumably by stabilizing the G_s-adenylyl cyclase complex. Further support for this idea comes from the observation that G_s activation increases the number of high affinity binding sites (K_d 20nM) for forskolin (Nelson & Seamon, 1986, 1988). The K_d for forskolin binding to membranes of various tissues ranges from 9 nM in rat brain to 100μM in rat lung (Laurenza & Seamon, 1991). Hence, it is possible that forskolin does not bind all adenylyl cyclases with the same affinity. Indeed, the type 2 adenylyl cyclase may not be as sensitive to forskolin as other types (O. Jacobowitz & J. Chen, unpublished observations). At micromolar concentrations, forskolin also binds other plasma membrane proteins (Laurenza *et al*, 1989) such as glucose transporters (Kashiwagi *et al*, 1983), nicotinic acetylcholine receptors (McHugh & McGee 1986), GABA-A receptor (Heuschneider *et al*, 1986),

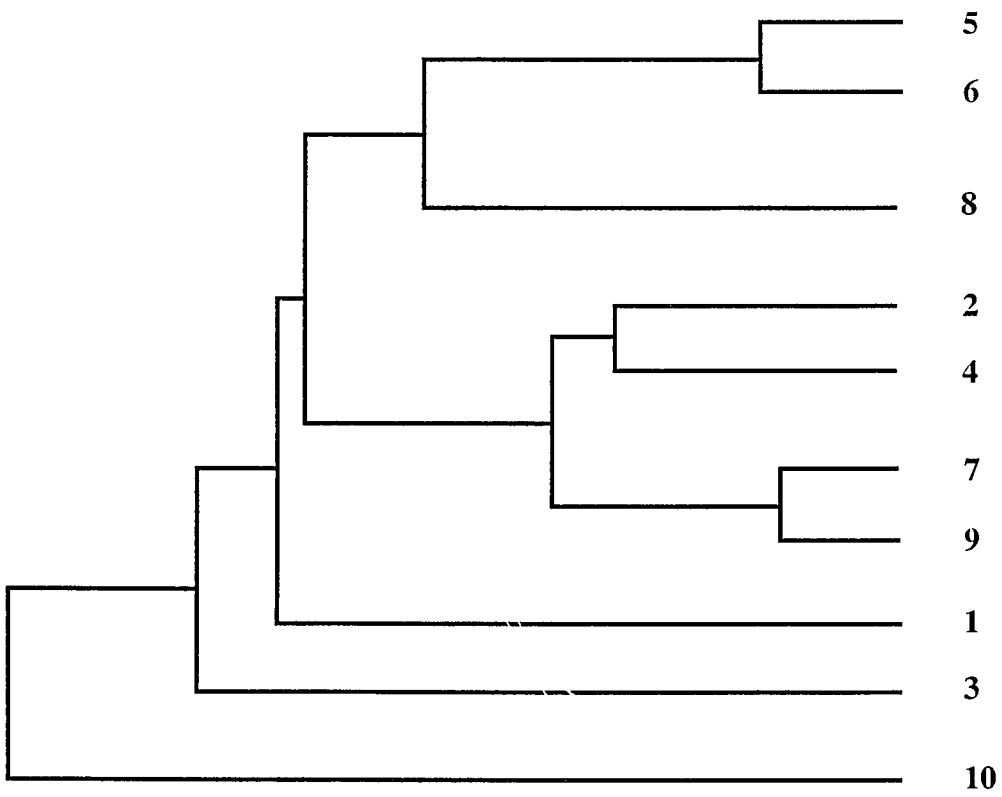
voltage-dependent K^+ channels (Hoshi *et al*, 1988) and P-glycoprotein multidrug transporters (Wadler & Wiernik, 1988, Morris *et al*, 1991). Certain derivatives of forskolin can be used to distinguish between the cAMP-dependent effects of forskolin and those due to interactions with proteins other than adenylyl cyclase (Laurenza *et al*, 1989).

Following Seamon & Daly's proposal that forskolin directly stimulates adenylyl cyclase (1981), Pfeuffer & Metzger (1982) synthesized 7-*O*-hemisuccinyl-deacetyl forskolin-sepharose and used it to purify a 150 kDa adenylyl cyclase from rabbit myocardium (Pfeuffer *et al*, 1985a). Subsequent purifications of adenylyl cyclase from different tissues resulted in different size estimates. A calmodulin-stimulated adenylyl cyclase was resolved from bovine brain by Smigel to 120 kDa apparent molecular weight (1986) and by Pfeuffer *et al* (1985b) to 115 kDa. A rat liver adenylyl cyclase was resolved by Bushfield and coworkers to 130 kDa while porcine liver adenylyl cyclase was resolved to 135 kDa by R. Premont and J. Herberg (unpublished observations). The existence of at least two forms of adenylyl cyclase in the same organ was demonstrated when antibodies raised against the 115 kDa (brain) form bound both a calmodulin-sensitive 115 kDa and a calmodulin-insensitive 150 kDa adenylyl cyclase in bovine brain extracts (Pfeuffer *et al*, 1988). The heterogeneity of molecular weights is consistent with the cloning of multiple adenylyl cyclases.

Fig. 1-4. Mammalian adenylyl cyclases phylogeny tree:

Adapted from R Premont (unpublished Fig.). Adenylyl cyclases were compared for amino acid similarity (conservative substitutions permitted) with the GeneWorks (Intelligenetics) software for the Macintosh. Since complete sequences are not available for all adenylyl cyclase isoforms, the phylogeny tree was generated from comparisons of the conserved region of the C-terminal cytoplasmic tail (see Fig. 1-5).

Figure 1-4. Mammalian adenylyl cyclases phylogeny tree



The cloning of multiple adenylyl cyclase cDNAs revealed an unpredicted diversity of adenylyl cyclase species (Fig. 1-4). Using amino acid sequences from the 120 kDa bovine brain adenylyl cyclase, Krupinski and coworkers (1989) cloned the type 1 adenylyl cyclase cDNA from bovine brain. When expressed in Sf9 cells, the type 1 enzyme was stimulated by G_s and Ca^{2+}/calmodulin (Tang et al, 1991). The cloning of the type 1 enzyme facilitated the discovery of additional types. Oligonucleotide probes corresponding to the type 1 cDNA sequence were used to clone five other full length adenylyl cyclase cDNAs and two partial cDNAs. An additional mammalian adenylyl cyclase was discovered by a random gene cloning method (Genbank access #D25538). The cloned adenylyl cyclases were found to be about 50% similar to each other at the amino acid level.

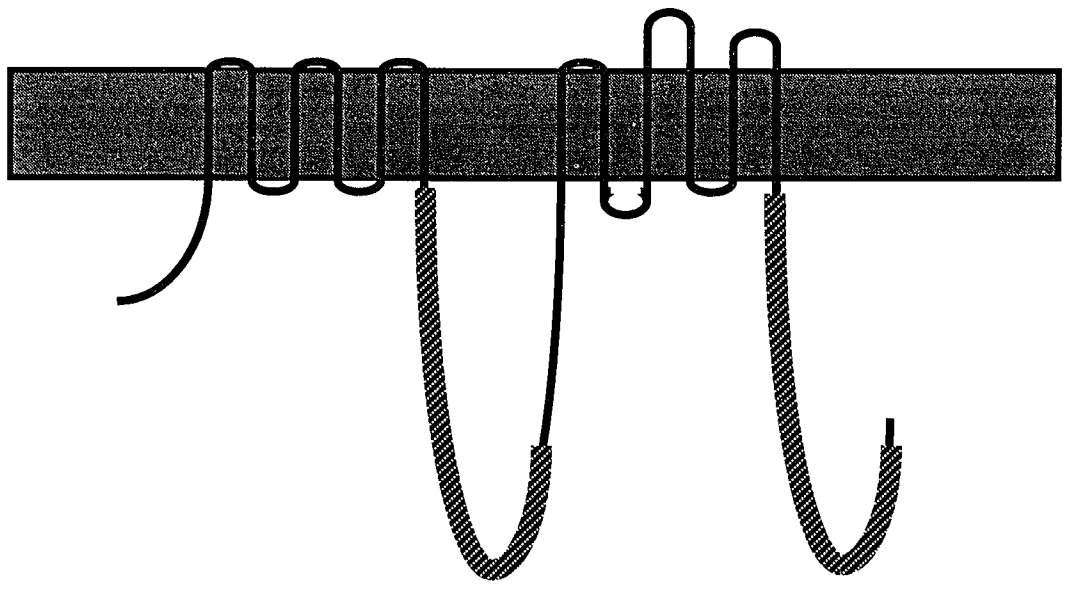
The cloned mammalian adenylyl cyclases are predicted to be integral membrane proteins on the basis of their amino acid hydropathy profiles. Their common predicted structure is quasi-duplicated, consisting of two clusters of six transmembrane spans and two large cytoplasmic domains (Fig. 1-5). Putative N-linked glycosylation sites are present in the sequences, consistent with the identification of adenylyl cyclases as glycoproteins (Pfeuffer et al, 1985a). The catalytic site is presumed to reside in the two large cytoplasmic domains (thick lines) which are not only highly conserved between the adenylyl cyclases but also similar in sequence to the catalytic domains of cloned guanylyl cyclases (Chinkers and Garbers, 1991, Danchin, 1993). The importance of the

cytoplasmic domains is substantiated by studies of the Drosophila mutant, *rutabaga,* which possesses a defect in associative learning and lacks Ca^{2+}/Calmodulin-sensitive adenylyl cyclase activity. The molecular defect associated with the mutant phenotype is a Gly→Arg mutation in the carboxyl cytoplasmic domain of the type 1 adenylyl cyclase of the flies. The calmodulin binding domain of the mammalian version of AC1 was mapped to a domain in the predicted cytoplasmic sequences (Vorherr *et al*, 1993, Wu *et al*, 1993) and studies in our laboratory indicate that a region involved in interaction with $\beta\gamma$ subunits is in the cytoplasmic tail.

Fig. 1-5. Predicted structure of mammalian adenylyl cyclases:

Schematic diagram of adenylyl cyclase topology. Hatched, thick lines indicate regions of high amino acid identity or conservation among the cloned adenylyl cyclases.

Fig. 1-5. **Predicted structure of mammalian adenylyl cyclases**



The highly conserved cytoplasmic domains are similar with each other intramolecularly, but neither domain, expressed separately in Sf9 cells, is sufficient for catalytic activity. Catalytic activity is observed when the two halves are expressed in the same cell (Tang et al, 1991). Half molecules of adenylyl cyclase are indigenous to cardiac tissue (Katsushika *et al*, 1993). Thus one may wonder whether adenylyl cyclases exist as monomers, homodimers or heterodimers. The requirement of two predicted catalytic regions of adenylyl cyclases for activity parallels the requirement for activity of guanylyl cyclases, where dimers of catalytic subunits are necessary.

The cloned adenylyl cyclases may be grouped into six families on the basis of their amino acid similarity (Iyengar, 1993, Premont *et al*, 1993 and Fig. 1-4). Two of the families contain several members. The type 2 family consists of the types 2, 4, 7 and 9 adenylyl cyclases. Type 2 adenylyl cyclase was cloned from rat brain (Feinstein *et al*, 1991) and the type 4 enzyme was cloned from rat testis (Gao & Gilman, 1991). The human analogue of the type two enzyme (a partial clone) was chromosomally localized to 5p15 (Stengel *et al*, 1992). The partial cDNA of the type 7 adenylyl cyclase was cloned from mouse S49 lymphoma cells (Krupinski *et al*, 1992, Premont, R. T., personal comm). The type 9 (tentative assignment) was cloned from human myoblast KG-1 cells (Nomura *et al*, Genbank access #D25538). The type 5 family consists of types 5 and 6 adenylyl cyclases. The type 5 adenylyl cyclase was

cloned from rat liver and kidney (Premont *et al*, 1992a), dog heart (Ishikawa *et al*, 1992) and rat striatum (Glatt & Snyder, 1993). The type 6 adenylyl cyclase was cloned from rat liver and kidney (Premont *et al*, 1992a), dog heart (Katsushika *et al*, 1992), rat heart (Krupinski *et al*, 1992), mouse S49 lymphoma cells (Premont *et al*, 1992b) and mouse/hamster hybrid NCB-20 cells (Yoshimura & Cooper, 1992). The type 1, 3, and 8 enzymes comprise three distinct families. The type 3 adenylyl cyclase was cloned from rat olfactory neuroepithelium (Bakalyar *et al*, 1990) and the type 8 enzyme was cloned from human brain (Parma *et al*, 1991) and rat brain (Krupinski *et al*, 1992, Cali *et al*, 1994). The type 10 enzyme was discovered independently in several laboratories (D. Carty and R Premont, personal communications). The various forms are differentially expressed in tissues.

In the absence of antibodies specific for the various isoforms, the tissue distributions of cloned adenylyl cyclases are currently known at the mRNA level (Table 1-1). Types 1 and 8 are restricted to neuronal (brain) tissue (Krupinski *et al*, 1992). The type 2 family (types 2,4,7,9) however, is distributed in neuronal and non-neuronal tissues. Type 2 is expressed in brain, lung and skeletal muscle (Feinstein *et al*, 1991, Krupinski *et al*, 1992). Type 4 is widely distributed in tissues at low amounts and may be especially rare in brain (Gao & Gilman, 1991, Iyengar *et al*, 1993). Type 7 is widely distributed and may be present in high levels in heart, liver, testes and in low levels in brain tissue (Krupinski *et*

Table 1-1. Mammalian adenylyl cyclase cloning and mRNA distributions

Isozyme	Source	Distribution (mRNA)	Reference
1	Cow brain	neuronal	Krupinski et al, 1989
2	Rat Brain	high in brain, lung	Feinstein et al, 1991
3	Rat olfactory epithelium	widely distributed	Bakalyar et al, 1990
4	Rat testis	widely distributed low in brain	Gao & Gilman, 1991
5	Rat liver, kidney, striatum Dog heart	widely distributed high in striatum, heart	Premont et al, 1992a Ishikawa et al, 1992 Glatt et al, 1993
6	Rat liver, kidney, heart Dog heart Mouse S49 lymphoma cells Mouse/Hamster NCB-20 cells	widely distributed	Premont et al 1992a,b Katsushika et al, 1992 Krupinski et al, 1992 Yoshimura & Cooper, 1992
7	Mouse S49 lymphoma cells	high in heart, liver testes low in brain	Krupinski et al, 1992 R. Premont (*)
8	Human brain Rat brain	neuronal	Parma et al, 1991 Cali et al, 1994
9	Human KG-1 myoblast cells	KG1 cells (?)	Nomura et al, 1993
10	SK-N-SH neuroblastoma cells mouse brain	brain (?)	R. Premont (*) D. Carty (*)

(*) unpublished personal communication

al, 1992, Hellevuo *et al*,1993). Type 7 is also present in S49 lymphoma cells (Krupinski *et al*, 1992). The type 3 adenylyl cyclase, originally thought to be specific to olfactory neurons (Bakalyar & Reed, 1990) is widely distributed within the brain and is also found in peripheral organs (Xia *et al*, 1992, Glatt & Snyder 1993, Hellevuo *et al*, 1993). Both types 5 and 6 are widely distributed (Yoshimura *et al*, 1992, Ishikawa *et al*, 1992, Premont *et al*, 1992a, Katsushika *et al*, 1992, Krupinski *et al*, 1992, Pieroni *et al* 1993), but type 5 is especially prevalent in brain in the corpus striatum (Glatt & Snyder, 1993) and in heart (Ishikawa *et al*, 1992, Pieroni *et al*, 1993) and type 6 (but not 5) is found in S49 lymphoma cells. The distributional diversity of adenylyl cyclases is accompanied by a functional diversity.

The cloned adenylyl cyclases that have been expressed are stimulated by the α subunit of the G protein, G_s (Tang et al, 1991, Feinstein *et al*, 1991, Gao & Gilman, 1991, Taussig *et al*, 1993, Cali *et al*, 1994) and are probably all inhibited by α_i. The stimulation by α_s was relatively simple to demonstrate using recombinant activated α_s, however inhibition by α_i has been more difficult to observe. Using purified α_i, a few groups demonstrated inhibition of adenylyl cyclase activity (Gilman, 1984, Katada *et al*, 1984, Roof *et al*, 1985, Simmoteit *et al*, 1991). α_i was otherwise implicated in inhibition when α_i-specific antibodies blocked the inhibition of adenylyl cyclase (Simonds *et al*, 1989). Recently, inhibition of adenylyl cyclase activity (or cAMP accumulation) by specific types of α_i was observed in cells transfected with the

GTPase-deficient *mutant* α_i cDNAs (Wong *et al*, 1991, Lowndes *et al*, 1991, Hermouet *et al*, 1991, Wong *et al*, 1992) and *in vitro* in assays with recombinant, bacterially expressed α_i (Taussig *et al*, 1993). In our laboratory, using mammalian transfection systems, Chen & Iyengar (1993) have shown that the GTPase-deficient mutant α_i's inhibit types 2, 3, and 6 adenylyl cyclase. Interestingly, inhibition by α_i of only the type 2 adenylyl cyclase is alleviated by treatment of cells with phorbol ester activators of protein kinase C (Chen & Iyengar, 1993). The α subunit of G_z, shown to inhibit cAMP accumulation in a cell transfection system (Wong *et al*, 1992), has not yet been tested for inhibition of specific types of adenylyl cyclase.

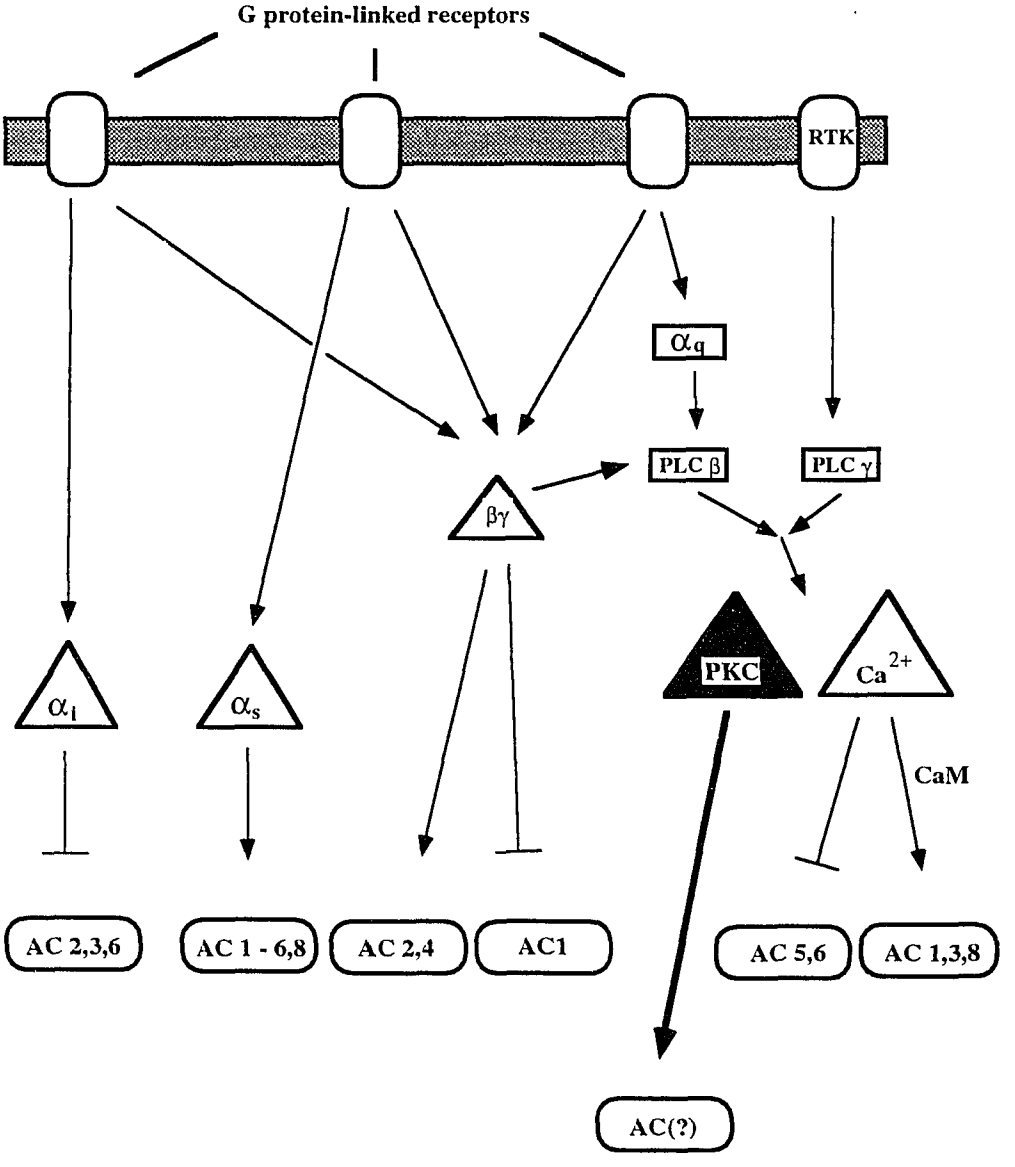
The $\beta\gamma$ subunits of G proteins also regulate adenylyl cyclase function (Fig. 1-6). The Ca^{+2}/calmodulin stimulated activity of the type 1 adenylyl cyclase is prominently inhibited by $\beta\gamma$ subunits at an IC_{50} of 2-3nM (Iñiguez-Lluhi *et al*, 1992, Katada *et al*, 1987, Tang et al, 1991, Tang & Gilman, 1991). However, the Ca^{2+}/calmodulin stimulated activity of the related type 3 adenylyl cyclase is not inhibited by $\beta\gamma$ subunits. Types 5 and 6 adenylyl cyclase are not directly affected by $\beta\gamma$ subunits (Tang & Gilman, 1991, Premont *et al*, 1992a). Types 2 and 4 adenylyl cyclase are *stimulated* by $\beta\gamma$ subunits at an EC_{50} of 5-10nM (Tang & Gilman, 1991, Gao & Gilman, 1991, Federman *et al*, 1992). The $\beta\gamma$ stimulation of types 2 and 4 is most extensive in the presence of activated α_s and occurs at higher concentrations than those required for type 1 inhibition.

Fig. 1-6. Signal integration by mammalian adenylyl cyclases:

Schematic diagram of stimulatory and inhibitory modulators of adenylyl cyclase isoforms. Stimulatory inputs are indicated by pointed arrows while inhibitory inputs are indicated by arrows terminating in a perpendicular line.

Abbreviations: AC, adenylyl cyclase; PKC, protein kinase C; PLC, phospholipase C; RTK, receptor tyrosine kinase

Fig. 1-6. Signal integration by mammalian adenylyl cyclases



Mammalian adenylyl cyclases are also modulated by a variety of small intracellular molecules, some of which differentially affect the cloned adenylyl cyclases. The metal ions, Mg^{2+} and Mn^{2+} modulate adenylyl cyclases at catalytic as well as allosteric sites. The ions stimulate adenylyl cyclase activity allosterically (Somkuti *et al*, 1982) and associated with ATP, form the substrates of adenylyl cyclase. It is not yet clear whether there are differences in sensitivity to Mg^{2+} among the different adenylyl cyclases. Adenylyl cyclases are inhibited by intracellular adenosine (Haslam *et al*, 1972, McKenzie *et al*, 1973, Londos & Wolff, 1977, Johnson & Welden, 1977, Johnson *et al*, 1989) and Ca^{+2}. Inhibition of adenylyl cyclase by Ca^{2+} (0.1-1μM), reported to occur in cardiac tissue and in several cell lines (Cooper & Brooker, 1993), appears to be mediated by direct inhibition of the types 5 and 6 adenylyl cyclases (Ishikawa *et al*, 1992, Katsushita *et al*, 1992, Yoshimura & Cooper, 1992).

In contrast to the *direct* inhibition of the types 5 and 6 adenylyl cyclase by Ca^{2+}, the types 1, 3 and 8 adenylyl cyclase are *indirectly* stimulated by Ca^{2+} by association with Ca^{2+}/calmodulin (Tang *et al*, 1991, Choi *et al*, 1992, Jacobowitz *et al*, 1993, Cali *et al*, 1994). A region of type 1 adenylyl cyclase involved in calmodulin binding was mapped to the less conserved part of the predicted central cytoplasmic loop (Vorherr *et al*, 1993). The stimulation of types 1 and 3 in the presence of Ca^{2+}/calmodulin is of similar extent to that in the presence of α_s and concurrent stimulation by Ca^{2+}/calmodulin and α_s is synergistic or additive.

Thus the relative levels of types 1,3,8 versus the type 5,6 adenylyl cyclases in a cell may influence the magnitude of the cAMP response to intracellular Ca^{2+}.

Crosstalk between the Adenylyl Cyclase and Phospholipid Hydrolysis Pathways

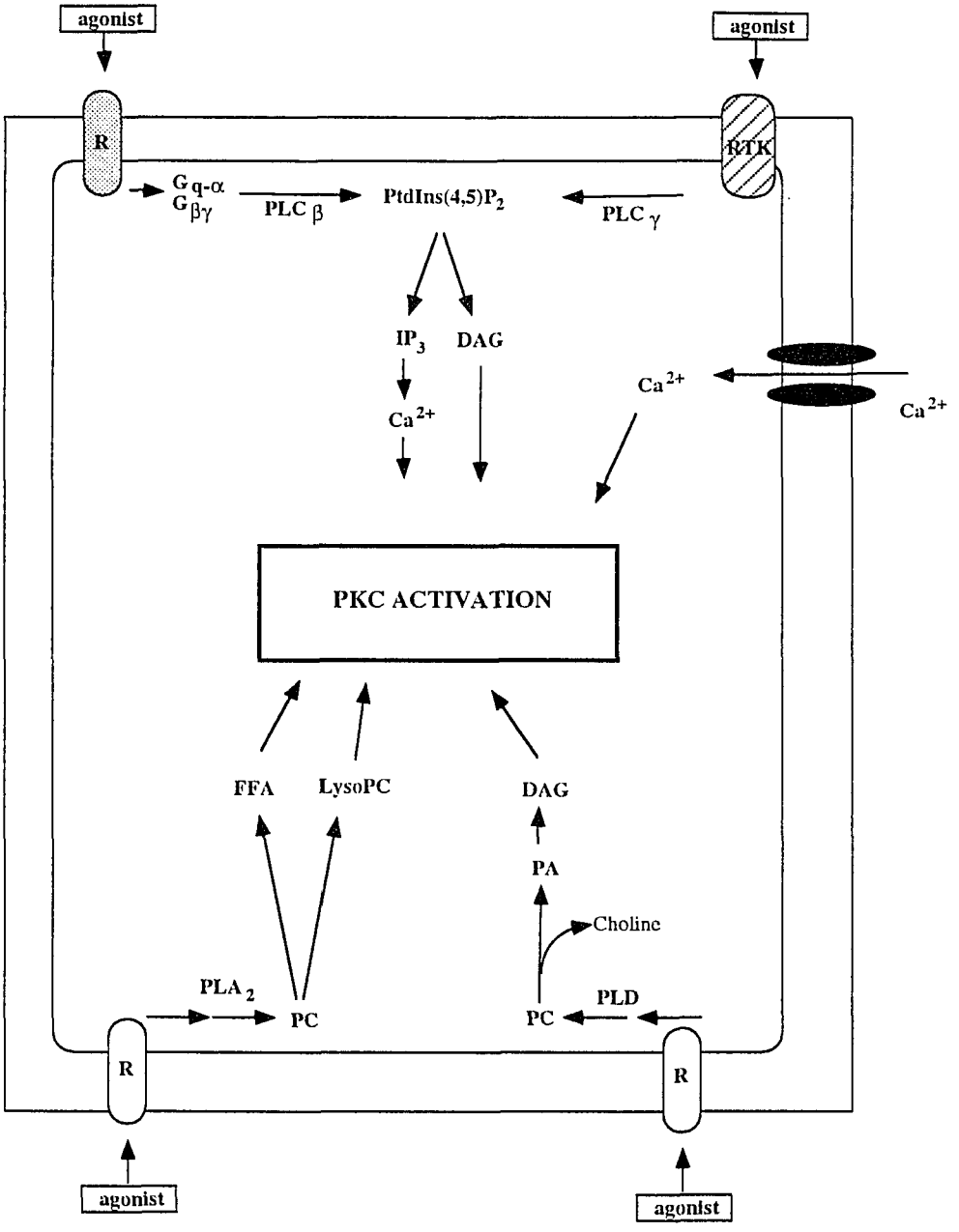
Signaling pathways form complex networks which coordinate cellular processes. Within these networks, different signaling systems interact with and influence each other's signal transmission capabilities. The adenylyl cyclase and phospholipid hydrolysis pathways are capable of interaction or "crosstalk". Their interactions include antagonism, cooperation, potentiation and synergism. With the cloning of multiple members of the adenylyl cyclase effector class of signaling molecules, it is now possible to examine the role of individual adenylyl cyclases as targets of crosstalk.

Signal transduction by the phospholipid hydrolysis pathways can lead to the activation of protein kinase C (PKC) and to mobilization of intracellular calcium, both of which can regulate the adenylyl cyclase pathway (Fig. 1-7). The generation of these mediators is effected by several phospholipases (Asaoka *et al*, 1992a). Phospholipase C-mediated hydrolysis of phosphatidyl inositol 4,5-bisphosphate releases inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which mobilize intracellular calcium and activate most protein kinase C species, respectively. Other phospholipases, acting on phosphatidyl choline can also lead to PKC activation (Fig. 1-7). Phospholipase D (PLD) can activate PKC species through DAG production from phosphatidic acid. Phospholipase A_2 can greatly potentiate the activation of some PKC

Fig. 1-7. Pathways of protein kinase C activation:

Schematic diagram of pathways to protein kinase C activation. Abbreviations: DAG, diacylglycerol; FFA, free fatty acids; IP_3, inositol triphosphate; LysoPC, lysophosphatidyl choline; PC, phosphatidyl choline; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; and PLA_2, phospholipase A_2; PtdIns(4,5)P_2, phosphatidylinositol 4,5 bisphosphate; R, receptor; RTK, receptor tyrosine kinase; R, receptor.

Figure 1-7. **Pathways of protein kinase C activation**



isoforms by generating *cis*-unsaturated fatty acids and lysophosphatidyl choline (Yoshida *et al*, 1992, Asaoka *et al*, 1992b). Protein kinase C can also be activated by tumor promoter phorbol esters such as phorbol 12,13 myristate acetate.

Stimulatory or inhibitory effects of protein kinase C activation on the adenylyl cyclase pathway occur in various systems (Table 1-2). These effects are produced by treatment with hormones or phorbol esters that activate PKC. Several mechanisms may underlie these functional effects. Inhibition is generally mediated by PKC phophorylation of receptors, which attenuates receptor-transduced cAMP production (Sibley *et al*, 1984, Kelleher *et al*, 1984, Johnson *et al*, 1990). Stimulation of adenylyl cyclase systems by PKC is attributed to modification of sites downstream of the receptor.

The state of downstream loci can be assessed through the use of different stimulators of the adenylyl cyclase pathway. Mg^{2+} and Mn^{2+} are used to directly stimulate adenylyl cyclase and guanine nucleotide analogues are used to examine G protein activation of adenylyl cyclase. Forskolin, as previously described, is used as an enhancer of adenylyl cyclase-G_s coupling as well as a direct stimulator of adenylyl cyclase.

The mechanisms for G_s-independent stimulation of cAMP generation by hormones are more complex than those of phorbol esters since hormones which activate PKC through PLC can also

raise the levels of cytosolic calcium and release G protein $\beta\gamma$ subunits. Calcium can increase cAMP levels by several mechanisms. These mechanisms include inhibition of cAMP phosphodiesterase (Verghese *et al*, 1985, Abou-Samra *et al*, 1987), an enzyme which degrades cAMP, and activation of calmodulin (Felder *et al*, 1989), a stimulator of types 1 and 3 adenylyl cyclases. Independently of PKC and calcium fluxes, hormones which release sufficient amounts of G protein $\beta\gamma$ subunits can conditionally stimulate the types 2 and 4 adenylyl cyclases and inhibit the type 1 adenylyl cyclase.

Functional and physical studies have implicated adenylyl cyclase as a target of PKC mediated *sensitization*. Direct stimulation of adenylyl cyclase by Mg^{2+} and Mn^{2+} (basal) in phorbol ester-treated systems resulted in enhancement of adenylyl cyclase activity or cAMP accumulation (Table 1-2). Enhancement of forskolin-stimulated adenylyl cyclase activity is also reported in many systems. Since forskolin affects adenylyl cyclase and its interaction with G_s, the possibility exists that G_s is modified, however no PKC-mediated alterations of G_s have been found to date. In contrast, adenylyl cyclase is thought to be phosphorylated in response to phorbol ester treatment of cells. Yoshimasa *et al* (1987) reported sensitization of adenylyl cyclase activity and phosphorylation of a forskolin-purified 130 kDa protein from phorbol ester treated frog erythrocytes. Simoteit *et al* (1991) demonstrated sensitization and phosphorylation of adenylyl cyclase in phorbol ester-treated human platelets. Simoteit & coworkers also demonstrated that α_i is not likely to be the target of

modification during the sensitization of the adenylyl cyclase system by showing that α_i was not phosphorylated as a result of treatment and that reconstitution with exogenous α_i did not affect the sensitization. In platelets and additional systems, phorbol ester treatment impaired α_i inhibition of adenylyl cyclase (Simoteit *et al* 1991, Katada *et al*, 1985, Nikula & Huhtaniemi 1989).

Table 1-2. Hormonal and phorbol ester crosstalk with the adenylyl cyclase pathway:

Abbreviations: AC, adenylyl cyclase; ACTH, AdrenoCorticoTropic Hormone; AHP, After HyperPolarization; AVP, arginine vasopressin; BK, bradykinin; 2-CA, 2-chloroadenosine; CaM, calmodulin; 5-CT, 5-carboxyamidotrypatamine; CTX, cholera toxin; DA, dopamine; DHG, dihexanoyl glycerol; DOG, 1,2 dioctanoyl-glycerol; EGF, Epidermal Growth Factor; epi., epinephrine; FMLP, formyl methionine leucine phenylalanine; hCG, human chorionic gonadotrophin; 5-HT, serotonin; Mab, monoclonal antibody; NE, norepinepherine; NGF, nerve growth factor; NMDA; OAG, 1-oleoyl, 2-acetylglycerol; OXO, oxotremorine; PDBu, phorbol dibutyrate; PDE, phosphodiesterase; $PGE_1/PGE_2/PGF2\alpha$, prostaglandins $E_1/E_2/F2\alpha$; P_i, phosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PMA, phorbol 12;13 myristate acetate; PTX, pertussis toxin; SS, somatostatin; TGLP, truncated glucagon-like peptide; VIP, vasoactive intestinal peptide.

part., partially; stim., stimulated

Table 1-2: Hormonal and phorbol ester crosstalk with the adenylyl cyclase pathway

System	Treatment	Effect On Adenylyl Cyclase Activity	Effect On cAMP Accumulation	Comment(S)	Reference
Olfactory bulb or bulb membranes (rat)	carbachol (1mM), 15'	↑ basal:50%	↑ basal: 75%	PTX sensitive. βγ stimulation?	Olianas *et al* (1992)
Olfactory receptor cells (frog)	PDBu (0.5μM) or carbachol (50μM) or 5-HT (50μM)		↑ fsk:2-3 fold	PKC dependent partly Ca^{2+}/CaM dependent	Frings (1993)
Anterior pituitary cells (rat)	vasopressin (10nM) or PMA (100nM) 15'		↑ CRF:4-5-fold	not additive with PTX. partly due to PDE inhibition	Abou-Samra *et al* (1987)
1° anterior pituitary cells (rat)	PMA (100nM) 15'		↑ GRF:2-fold ↓ SS inhibition: 50%	not PTX sensitive	Cronin *et al* (1986)
GH_4C_1 pituitary tumor cell homogenates (rat)	purified rat brain PKC		↑ basal: 2 fold	not Ca^{+2}/CaM dependent	Summers *et al* (1988)
235-1 anterior pituitary tumor line (rat)	PMA (100nM), 10'	↑ basal:26% ↑ fsk: 32%	↑ basal:40% ↑ fsk:3.5 fold ↑ PGE_1:2-fold		Summers *et al* (1986)

(Table 1-2: Continued)

GH_3 pituitary tumor cells (rat)	PDBu (500nM)		↑ basal: 65% ↑ CTX: 75% ↓ VIP: 50%	additive to enhancement by PTX	Quilliam *et al* (1985)
	TRH (200nM)		↑ basal: 50% ↑ CTX: 45% ↔ VIP		as above
GH_3 pituitary tumor cell homogenates (rat)	PMA (30μM)	↑ basal:2.5 fold			Brostrom *et al* (1982)
	VIP (30μM)	↑ basal:4 fold		Ca^{+2} dependent	
Pinealocytes (rat)	PMA(100nM)		↑ iso.: 7-fold	coincident with PKC mobilization to plasma membrane. Same max as NE stim.	Sugden *et al* (1985)
Hippocampal slices (rat)	tetanic stimulation (NMDA-R activation) or NMDA (100μM)		↑ tetanic:50% ↑ NMDA:300%	Ca^{+2} dependent	Chetkovich *et al* (1991)
Hippocampal slices	5-CT [$5\text{-}HT_{1A}$-R] (300nM) or baclofen [$GABA_B$-R] (10-30μM)		↑ NE reduction of AHP: 2-3 fold	possible mechanism: βγ stimulation of AC2	Andrade (1993)

(Table 1-2: Continued)

Cerebral cortex (guinea pig)	α-adrenergic stimulation (epinepherine + propranolol, 10μM)		↑ Adenosine: 2 fold		Sattin *et al* (1975)
Cerebral cortical particulate preparations (guinea pig)	PMA (10μM) 10'		↑ fsk and 2-CA: 60-70%		Hollingsworth *et al* (1985b)
Cerebral cortical slices (guinea pig)	NE [α_1-R] or 5-HT [5-HT_2-R] or histamine [H_1-R] (10-100mM)		↑ 2-CA and VIP: 2-3 fold	treatments ↑ accumulation of inositol phosphates	Hollingsworth *et al* (1985a)
Cerebral cortical slices (mouse)	PDBu (1μM), 10'		↑ VIP: 36-100%	not prostaglandin dependent. H7 sensitive	Schaad *et al* (1989)
	baclofen [$GABA_B$] (100μM), 10'		↑ VIP: 3 fold	not prostaglandin dependent. not PDE or H7 sensitive	as above
Brain slice (rat)	PMA (10μM), 15'		↑ iso., NE, 2-CA, PGE_2 and VIP: 2-3 fold	not Ca^{2+}, PDE, or PLA_2 dependent	Karbon *et al* (1986)

(Brain/neuronal/glial)

(Table 1-2: Continued)

Brain slices (rat)	baclofen (100mM), 10'		↑ basal, NE, VIP, histamine, adenosine and iso.: 2-3 fold	Ca^{+2} (and PKC?) dependent	Karbon *et al* (1985)
Neuroectoderm (toad)	PMA (300nM) 30'		↑ fsk:2 fold	PKC activation during neural induction coincides with increase of fsk stim. AC activity	Otte *et al* (1989)
PC12 pheochromocytoma cells (rat)	PMA (100nM) 5'		↑ fsk:3 fold ↑ 2-CA: 30%		Hollingsworth *et al* (1986)
PC-12 pheochromocytoma cells	β-NGF (10ng/ml), 8'		↑ basal:63%		Schubert *et al* (1978)
Retinas (rat)	carbachol (300μM), 2'		↑ basal: 60% ↑ DA: 2-fold	not Ca^{+2} dependent not PDE dependent	Heller Brown *et al* (1981)
1321N1 astrocytoma cells (human)	PMA (1μM), 60'		↑ fsk: 2.5-5 fold ↑ iso.: 50%	PKC dependent not PTX or CTX sensitive. not PDE dependent.	Johnson *et al* (1990)
	carbachol or histamine (100μM), 60'		↑ fsk: 2 fold ↑ iso.:45-105%	as above	as above
1321N1 astrocytoma cells (human)	OXO (100μM), 5'		↓ iso. and PGE_1: 40-70%	Ca^{+2} and PDE dependent	Meeker *et al* (1983)

(Brain/neuronal/glial)

(Table 1-2: Continued)

	carbachol (100μM), 75'		↑ iso. and PGE_1: 50-100%	not Ca^{+2} dependent	
Platelets (human)	PMA (1μM), 5'	↓ inhibition by endogenous and exogenous α_i. ↔ βγ inhibition		phosphorylation of adenylyl cyclase shown.	Simmoteit *et al* (1991)
Platelet membranes (human)	PMA (1μM), 5'	↑ fsk: 50% ↑ PGE_1:2 fold ↓↓↓ epi. and thrombin inhibition			Jakobs *et al* (1985)
S49 lymphoma cells (mouse)	PMA (5nM), 20'		↑ epi.: 55%	PKC dependent. not PTX sensitive. stimulation dependent on hormone and PMA concentration	Johnson *et al* (1986)
	PMA (50nM), 20'		↑ fsk: 51%	as above	as above
	PMA (0.5μM), 18'		↑ epi.: 75%	as above	as above
S49 cyc^- lymphoma cell membranes (mouse)	PMA (0.5μM), 20'	↑ epi.:30% ↓ GppNHp inhibition of fsk: 60-80%	.	as above	as above

(Hematopoietic cells)

(Table 1-2: Continued)

S49 lymphoma cells (mouse)	PMA (0.1-1μM)	↑ basal, NaF and iso.25-31%	↑ iso.:2 fold ↑ fsk: 50% ↓ SS inhibition: 50%	not PTX sensitive no effect in cyc$^-$ or H21a mutant cells (lacking G_s-AC coupling)	Bell *et al* (1985)
Jurkat T-lymphoma cells (human)	PMA (100nM) 10'		↑ fsk: 2 fold ↑ CTX:2 fold ↓ PGE_2: 50%	blocked by PKC down regulation.	Bihoreau *et al* (1991)
	OKT3 (α-CD3 MAb, 25μg/ml), 10'		↑ fsk: 2 fold ↑ CTX:40%	Ca^{2+} but not PKC dependent	as above
Jurkat T-lymphoma cells (human)	OKT3 (α-CD3 MAb, 6μg/ml)		↑ fsk: 3 fold ↑ 2-CA:7 fold ↑ PGE_2:3-fold	may be mediated by PKC, Ca^{2+} and/or tyrosine kinase	Buc *et al* (1993)
B lymphocytes (nu/nu mouse)	PMA (97nM) 3'		↑ fsk: 2.5 fold ↓ PGE_1: 50%		Wiener *et al* (1989)
	α-IgM Ab (25μg/ml), 10'		↑ fsk: 76% ↓ PGE_1: 30%		as above
CT6, IL2-dependent T cells (mouse)	IL2 (10U/ml)	↓ basal: 67% ↓ PGE_2 (1μM): 70% ↓ NaF: 62%		PKC dependent.	Beckner *et al* (1986)

(Hematopoietic cells)

(Table 1-2: Continued)

	PMA (20nM)	↓ basal: 33% ↓ PGE_2 (1μM): 75% ↓ NaF: 45%		PKC dependent.	as above
IC2 cultured mast cells (mouse)	PMA (1μM) 10'		↑ carbacyclin:8 fold	PKC and CaM-dependent	Sawai *et al* (1993)
HEL erythroleukemia cells (human)	thrombin (2U/ml), 5'		↑ PGE_1 and CTX: 3-fold ↑ PGI_2:2-fold ↑ carbacyclin: 2.5-fold ↑ VIP:1.6-fold ↑ AlF_4: 2.3-fold	not PTX sensitive. partly mimicked by ATP, PMA. blocked by PKC inhibition or down regulation.	Turner *et al* (1992)
Erythrocytes (frog)	PMA (10μM)	↑ GTP, PGE_1, NaF and iso.: 2-fold		purified fsk binding phosphoprotein. Also, PKC phosphorylates bovine brain AC.	Yoshimasa *et al* (1987)
Erythrocytes (frog)	PMA (10μM), 5-60'	↑ PGE_1, isoproterenol, NaF, fsk and Mn^{2+}: 2-3 fold		↔ receptor binding.	Sibley *et al* (1986)
Erythrocytes (duck)	PMA (1μM)	↓ iso.: 38%		↑ βAR Pi incorporation 3-4 fold	Sibley *et al* (1984)
	iso. (10μM)	↓ iso.: 50%		as above	as above

(Table 1-2: Continued)

Erythrocytes (turkey)	PMA (25μg/ml) or iso. (100μM), 30'	↓ iso.: 20-40% ↔ fsk or Fluoride		↑ βAR Pi incorporation 3 fold. ↔ receptor number	Kelleher *et al* (1984)
Reticulocytes (rat)	PMA (1μM), 90'	↑ PGE_1: 3-4-fold ↓ iso.: 50%		Desensitization by ↓ receptor coupling. Sensitization not additive with PTX. PKC dependent.	Yamashita *et al* (1988)
PolyMorphoNuclear leukocytes (human)	FMLP (1μM) 5'		↑ basal:2-3 fold	Ca^{+2} dependent. PDE sensitive	Verghese *et al* (1985)
Myeloid leukemic cells (mouse)	PMA (10ng/ml), 60'		↑ iso.: 2-fold ↑ PGE_2: 2.5 fold	not Ca^{+2} dependent	Simantov *et al* (1982)
Cultured calvaria of nenonates (mouse)	PMA or PDBu (100nM)		↑ PTH, fsk and CTX:2-4 fold	not prostaglandin or PDE dependent	Ransjo *et al* (1991)
UMR 106-01 osteosarcoma cells (rat)	PMA or PDBu (100nM)	↑ fsk and CTX:0.5-3 fold		not prostaglandin dependent	as above
UMR-106 osteosarcoma cell membranes (rat)	PMA (100nM) 15'	↑ PTH:2 fold ↑ NaF, fsk and GppNHp: 40-60%	↑ iso.: 60% ↑ fsk: 68% ↓ PGE_2:48%	not additive with PTX enhancement	Freyaldenhoven *et al* (1992)
UMR-106 osteosarcoma cells (rat)	PMA (1μM), 2-12 hrs		↓ PTH: 50%	PTH-R binding ↓ 20%. PKC dependent.	Ikeda *et al* (1991)

(Table 1-2: Continued)

Fat pads (rat)	PMA (1μM), 30'	↑ Mn^{2+} + fsk: 37% ↑ GppNHp:49%	↑ iso. 85%		de Mazancourt *et al* (1991)
Adipocyte membranes (rat)	rat brain or guinea pig pancreas PKC	↑ basal 2-3-fold		potentiated by Ca^{+2} and PMA. blocked by PKC inhibitors	Naghshineh *et al* (1986)

(Fat)

Trachea smooth muscle (guinea pig)	PMA (100nM)	↑ basal and GppNHp:2 fold			Grady *et al* (1993)
Iris sphincter (cow)	PDBu (100nM)		↑ basal:3-fold ↑ iso.: 40%	not Ca^{2+} or PDE dependent. PKC dependent.	Tachado *et al* (1993)
	PGF2α (25μM), 45'		↑ PGF2α: 55-73%	PKC dependent	as above
Iris sphincter smooth muscle (dog)	carbachol (25μM)		↑ basal:80% ↑ iso.:65% ↑ fsk: 58%	dependent on both PKC and Ca^{2+}	Abdel-Latif *et al* (1992)
Vascular smooth muscle cells (rat)	angiotensin II (100nM) 5'		↑ iso.:11-fold, ↑ VIP:25-fold	not Ca^{+2} dependent	Nabika *et al* (1985)

(Smooth muscle)

(Table 1-2: Continued)

	PMA (10nM) 5'		↑ iso. and VIP:2 fold	not Ca^{+2} dependent	as above
1° aortic smooth muscle cells (rat)	PMA (100nM), 10'		↑ iso.: 30% ↑ fsk 88% ↑ CTX: 80%	PKC dependent. PTX without effect.	Phaneuf *et al* (1988)
Skin fibroblasts (fetal rat)	angiotensin II (100nM) 10'		↑ basal: 100% ↑ iso.: 50% ↑ CTX: 50%	not PG dependent. not PTX sensitive.	Johnson *et al* (1992)
	PMA (1μM) 10'		↑ basal:100%	additive with CTX stimulation	as above
Swiss 3T3 cells (mouse)	PDBu (200nM-5μM) or OAG (100nM), 20'		↑ fsk: 6 fold ↑ CTX: 5-8 fold	PKC dependent. PTX sensitive.	Rozengurt *et al* (1987)
	Vasopressin (100nM) 20'		↑ CTX: 5 fold	PKC dependent.	as above
Swiss 3T3 cells (mouse)	bombesin (1μM) 1 hr		↑ fsk: 5-fold		Issandou *et al* (1990)
	vasopressin (18nM) or PDBu (200nM) 1 hr		↑ fsk: 3-fold		

(Fibroblasts)

(Table 1-2: Continued)

L fibroblasts transfected with m5 Ach-R (mouse)	ATP (30μM), 10-20'	↑ PGE_1-, epi., and fsk: 50-150%		unaffected by 19hr PMA treatment	Johnson *et al* (1991)
	PMA (10nM), 10-20'	↑ PGE_1:150-250%		eliminated by 19hr PMA treatment	as above
	carbachol (30μM) + PMA (10nM), 12'	↑ basal:250%			as above
L fibroblasts transfected with m1 Ach-R (mouse)	carbachol (100μM), 5'		↑ basal: 2-5 fold ↑ PGE_2 and CTX:40%	Ca^{+2}/CaM dependent	Felder *et al* (1989)
	PMA (1μM), 5'		↓ carbachol: 90%		as above
CCL39 fibroblasts (Chinese hamster)	thrombin (~1pM-100pM), 5'		↓ PGE_1 and CTX: ~30%	PTX abolishes decrease.	Magnaldo *et al* (1988)
	thrombin (0.1-10nM), or PBt_2, 5'		↑ CTX: 40-50%	PKC dependent. PTX pretreatment required.	as above

1° adrenal medulla cells (cow)	angiotensin II (100nM), 3'		↑ PGE_1: 4 fold	PKC dependent. not PDE dependent.	Boarder *et al* (1988)

(Fibroblasts)

(Table 1-2: Continued)

	PMA (100nM), 3'		↑ PGE_1: 2 fold		as above
Adrenal cortex cells (fetal cow)	angiotensin II or PMA (100nM) 3 hrs		↑ basal: 2-5 fold	not depdendent on PG production	Bird *et al* (1993)
Adrenocortical cells (cow)	angiotensin II (100nM), 3-6 hrs		↑ basal:5-10 fold		Rainey *et al* (1991)
Adrenal cells (cow)	angiotensin II (1μM), 3'		↑ basal:70% ↑ ACTH: 60%	correlates with steroidogensis	Peytremann *et al* (1973)
CHO (Chinese Hamster Ovary) cells	PMA (100nM), 10'		↓ DA (D_2-R) inhibition of fsk stim. 30%	PKC dependent.	Di Marzo *et al* (1993)
Leydig cells (rat)	PMA (100nM), 3hrs		↑ fsk and CTX: 60-80% ↓ hCG:20-40%	PMA enhancement not additive with PTX	Nikula *et al* (1989)
1° luteal cells (cow)	phospholipase D (2U/ml), 10'	↑ LH/GTP: 4-5 fold			Budnik *et al* (1993b)
	phosphatidic Acid (100μM), 10'	↑ LH/GTP: 2.5-fold			as above
	PMA (10nM), 10'	↑ LH/GTP: 3-fold			as above

(Table 1-2: Continued)

	EGF (10ng/ml), 10'	↑ LH/GTP: 4-fold		EGF stimulates phosphatidic acid formation.	as above
1° luteal cells (cow)	EGF (10ng/ml), 10'	↑ basal:3 fold ↑ fsk: 2.5 fold			Budnik *et al* (1991)

(Epidermis)

Epidermal cells (mouse)	PMA (1μM) 15'		↑ basal or CTX:3-4 fold		Perchellet *et al* (1980)
SCC 12F keratinocyte membranes (human)	PMA (100nM), 15'	↑ basal, iso., and iso./GTP: 2 fold ↑ CTX: 55%		not additive with PTX enhancement. PMA ↑ PTX ADP-ribosylation	Choi *et al* (1988)

(Heart)

Cardiac plasma membranes or partially purified cardiac adenylyl cyclase (rat)	acute ischemia of perfused hearts (5-15')	↑ fsk:30% ↑ part. purified AC:50%		PKC dependent. not α_1-adrenergic dependent.	Strasser *et al* (1992)
	PMA (1μM), 10'	↑ fsk:25%		PKC dependent.	
1° cardiac myocytes (neonatal rat)	Angiotensin II (100nM), 10'		↑ basal:40%		Sadoshima & Izumo (1993)

(Table 1-2: Continued)

Glomeruli and cultured mesangial cells (rat)	BK (100nM-1μM) or PMA (100nM), 5'		↓ or abolished fsk, PGE_2, iso.	PKC dependent.	Bascands *et al* (1993)
Manine-Darby Canine Kidney cells (MDCK)	PMA (100ng/ml) or BK (0.1-10μM), 15'		↔ fsk, basal, PGE_2, glucagon ↓ vasopressin: 57% (PMA), 25% (BK)	not PG dependent. blocked by H7	Friedlander *et al* (1987)
Rat Inner Medullary Collecting Tubule cells (RIMCT)	DOG (100nM) or EGF (10nM)		↔ fsk or CTX ↓ AVP: 60-74% ↔ PGE_2 or iso.	inhibition ablished by PTX treatment.	Teitelbaum (1993)
(RIMCT)plasma membranes	PMA (1μM), 30'	↓ AVP: 77% ↔ fsk			as above
Hepatocytes (Sprague-Dawley rats)	angiotensin, vasopressin, glucagon (10nM each) or PMA (10ng/ml), 5'	↓ glucagon: 2 fold. ↔ fsk		treatments stimulate PLC activity (except PMA). PTX abolishes desensitization.	Murphy *et al* (1987)
Hepatocytes (Sprague-Dawley rats)	PMA (10ng/ml), 15'		↓ glucagon: 31% ↓ GppNHp: 22% ↓ CTX: 45%	↔ receptor number. cAMP independent.	Heyworth *et al* (1984)

(Table 1-2: Continued)

Hepatocytes (Wistar rat)	PMA (1μM), 5'		↓ glucagon: 42%	glucagon dose-reponse shift to rht and lower max.	García-Saínz *et al* (1985)
Hepatocytes (Sprague-Dawley rats)	DHG (10 ng/ml) or glucagon (10nM), 5'	↓ glucagon: 70% ↔ fsk			Newlands *et al* (1991)
HGT-1 gastric cancer cells (human)	PMA (100nM), 10'		↓ histamine or TGLP: 50%. ↑ GIP: 60% ↔ CTX or fsk	PKC dependent. possible uncoupling of histamine-R or TGLP-R. PTX blocked ↑ of GIP stim. activity.	McKenna *et al* (1993)
Parietal cells (rat)	PMA (100nM), 10'	↓ histamine: 70%. ↑ fsk: 20% ↔ NaF		PTX without effect. possible uncoupling. of histamine-R.	Emly *et al* (1992)

(Kidney/Liver/Stomach)

CHAPTER 2

STATEMENT OF PROBLEM

The discovery of multiple types of adenylyl cyclases and their differential distribution profiles has now prompted examination of the integrative properties that specific adenylyl cyclases may confer to particular cells or tissues. It has been reported that adenylyl cyclase can be modulated by signals that activate the protein kinase C pathway. However, depending on the tissue or cell examined, protein kinase C modulates cAMP levels positively, negatively or not at all. To better understand these divergent biological responses I will:

1. Identify the adenylyl cyclase types stimulated by protein kinase C

Since sensitization occurs in diverse systems, it is possible that several or a widely expressed form of adenylyl cyclase is stimulated in response to PKC activation. I will compare all the cloned adenylyl cyclases for their response to PKC activation. The cDNAs of the adenylyl cyclases will be subcloned into pcDNA1, a mammalian-expression vector that directs expression of cDNAs from a cytomegalovirus promoter/enhancer element. Recombinant plasmids will be transfected into HEK-293 cells (Human Embryonal Kidney cells). Transiently transfected cells will be treated with phorbol 12,13-myristate acetate, a cell permeable PKC activator, lysed and assayed for adenylyl cyclase activity.

2. Characterize the adenylyl cyclase activity most extensively stimulated by PKC

Adenylyl cyclases are modulated by a variety of molecules. These include G protein α and $\beta\gamma$ subunits as well as Ca^{2+}, Mg^{2+} and Mn^{2+}. Having identified the specific types of adenylyl cyclase stimulated by PKC, I will determine which activities are altered by PKC. In particular, it would be important to determine whether the basal activities are directly stimulated or whether potentiation occurs for G protein stimulation of the adenylyl cyclase(s). I will also determine whether sensitization affects the V_{max} or K_m of the particular adenylyl cyclase(s).

3. Determine whether stimulation of adenylyl cyclase through activation of protein kinase C is accompanied by phosphorylation of adenylyl cyclase

The simplest predicted mechanism for PKC-mediated sensitization of adenylyl cyclase is direct phosphorylation of the enzyme. I will examine whether PKC activation leads to phosphorylation of the expressed adenylyl cyclase. I will use the Baculovirus-Sf9 cell expression system which is advantageous for a number of reasons. Sf9 cell Baculovirus-mediated expression of foreign genes is easier to perform and is consistently successful compared to the experience with transient transfections. The Sf9-Baculovirus system can achieve higher levels of expression of

exogenous proteins than mammalian systems. Sf9 cells are capable of growing in suspension and therefore can be easily grown in large numbers. The Sf9 cell-expressed enzyme will be identified with adenylyl cyclase-specific antisera. The expressed enzyme will be assayed for activity using various stimulators.

Sf9 cells will be treated with PMA to activate their endogenous PKCs. If the endogenous PKC activity in Sf9 cells can mediate sensitization of the virally expressed adenylyl cyclase, then I will proceed to characterize the functional alteration in terms of different stimulators of adenylyl cyclase. If PMA treatment does not result in enhancement of adenylyl cyclase activity, then I will use rat brain PKC to phosphorylate Sf9 membranes, and then test for stimulation of adenylyl cyclase.

To demonstrate phosphorylation of the exogenous adenylyl cyclase I will need to resolve it from endogenous Sf9 cell adenylyl cyclases. For this purpose I will tag the exogenous enzyme at the N-terminus with FLAG, an eight amino acid epitope and then purify the enzyme with antibody-affinity media against the tag. The FLAG affinity medium, available from IBI-Kodak, has been previously used to purify many proteins expressed in heterologous systems including insect cells.

CHAPTER 3

MATERIALS AND METHODS

A. MATERIALS

Chemicals and Reagents

Gene-Clean and Gene-Clean II kits were from Bio101 (La Jolla, CA). N, N, N', N' tetramethylethylene diamine, N, N' methylene-bis-acrylamide, Tween 20 were from Bio-Rad (Richmond, CA). Dried nonfat milk was from Borden. NuSieve agarose was from FMC (Rockland, ME). Ro 20-1724, G418 (Geneticin™), Sf-900™ media, DMEM media, fetal calf serum, agarose, high molecular weight protein standards (unstained or prestained) were from Gibco BRL (Gaithersburg, MD). Bovine calf serum was from HyClone (Logan, UT). Anti-FLAG™ affinity gel and FLAG peptide were from Kodak-IBI (New Haven, CT). DEAE-dextran was from Pharmacia-LKB (Piscataway, NJ). BaculoGold™ DNA was from Pharmingen (San Diego, CA). Fluorescamine was from Pierce (Rockford, IL). Acrylamide (2X) was from Serva (Paramus, NJ). Phorbol 12-myristate 13-acetate, 4α phorbol 12,13 didecanoate, forskolin (7β acetoxy-1α, 6β, 9α trihydroxy 8-13 epoxy labd-14-en 11 one), *n*-dodecyl β-D maltoside, sodium dodecyl sulfate, Lubrol™, agarose, serum free insect media-1, streptomycin sulfate, penicillin G, dexamethasone, staurosporine, isobutylmethylxanthine, creatine phosphate, phenylmethylsulfonyl fluoride, 1,10 phenanthroline, leupeptin, aprotinin and chloroquine were from Sigma (St. Louis, MO). XL1-Blue™ competent cells were from Stratagene (La Jolla, CA).

Enzymes

Restriction enzymes were from NEB (Beverly, MA) or Promega (Madison, WI). Klenow DNA polymerase, T4 DNA ligase, Calf Intestinal Alkaline Phosphatase were from NEB, or Promega. Taq polymerase™ was from Promega. Pfu polymerase™ was from Stratagene. Trypsin was from Gibco or Sigma. Myokinase (rabbit muscle), creatine phosphokinase (type I, rabbit muscle), calmodulin (bovine brain or testes), β-galactosidase (E. *coli*), phosphorylase b (rabbit muscle), RNAase, and Proteinase K (Tritirachium *album*) were from Sigma.

Radiochemicals

[α^{32}P]ATP, 25 Ci/mmol; [^{32}P]H_3PO_4 ; 2-, 8- [^{3}H]cAMP, 31Ci/mmol; [^{3}H]adenine; translation grade [^{35}S]methionine 1071 Ci/mmol, [α^{32}P]dATP, 650 Ci/mmol were from ICN (Irvine, CA). [α^{35}S]dATP was from New England Nuclear (Boston, MA).

G Proteins

The DNA template for G protein synthesis pAGA-α_{s4}-Q227L was the gift of L. Birnbaumer (Baylor College of Medicine, Houston, TX)

α_{s4}-Q227L was synthesized by J. Codina (Baylor College of Medicine) or by myself using the TnT reticulocyte lysate coupled transcription/translation kit (Promega Corporation). α_{s4}-Q227L DNA template was included at 1μg DNA/50μl reaction. Translation grade [^{35}S]methionine (80 μCi/100μl rxn) and cold methionine (1mM) were included in the reaction.

The concentration of α_{s4} was estimated by percent incorporation of [^{35}S]methionine into TCA precipitable product. Specific incorporation was calculated by subtracting the counts of a mock reaction (no DNA added) from the experimental reaction. Reaction products were analyzed on 10% SDS-PAGE gels.

βγ subunits were purified from bovine brain by D. Carty (Mount Sinai, NY). βγ were stored at a concentration of 1.67μM in a buffer containing 25mM HEPES 7.4, 1mM EDTA and 0.1% Lubrol. When used in adenylyl cyclase assays, βγ subunits were diluted to a final concentration of 100nM.

Antisera and immoblized antibodies

AC-Comm antisera was obtained by Richard Premont from rabbits boosted with the KLH-conjugated peptide NH_2-IGARKPQYDIWGNT-COOH. This peptide corresponds to a C-terminal sequence common to adenylyl cyclases 1-8. Optimal detection of adenylyl cyclase by immunoblotting was achieved at a 1:1000 dilution of the antiserum.

Goat anti-rabbit horseradish peroxidase-conjugated IgG (Calbiochem) was used at 1:10000 dilution. Anti-FLAG M2 mouse antibody (IBI-Kodak) was used at 30μg/ml . Affinity-purified goat anti-mouse horse radish peroxidase-conjugated IgG (Bio Rad) was used at 1:3000 dilution.

FLAG-M2 antibody coupled to agarose was from IBI-Kodak.

B. NUCLEIC ACIDS, VECTORS AND VIRUSES

Oligonucleotides

Oligonucleotides were synthesized by the Brookdale Molecular Biology Center (Mount Sinai, New York) or by myself using an Applied Biosystems DNA synthesizer. All reagents used in the synthesis were purchased from Applied Biosystems.

Construction of vectors for 293 cell transfection

1) pcDNAI-AC1: AC1 was cloned from bovine brain cDNA library into pBSII(KS) by R. Premont except for a 160 base pair GC-rich region at the 5' end of the published coding sequence (Krupinski et al, 1989). This 5' region was to be constructed from oligonucleotides coding for the N terminal amino acid sequence but designed to reduce the GC content of the 5' region. For this purpose, two pairs of complementary oligonucleotides, RP111/RP112 and RP113/RP114, whose sequences are shown below

RP111:

5'CATGGCTGGAGCTCCTCGTGGAAGAGGAGGCGGTGGTGGAGGAGGTGGAGC
GGGCGAATCTGGCGGAGCGGAACGGG-3'

RP112:

3'CGACCTCGAGGAGCACCTTCTCCTCCGCCACCACCTCCTCCACCTCGCCCG
CTTAGACCGCCTCGGCTTGCCCGACGTCC-5'

RP113:

5'CTGCAGGTCCTGGCGGCAGGCGCGGGCTGAGGGCTTGTGATGAGGAATTCG
CGTGTCCTGAACTGGAAGCGTTGTTCCGC-3'

RP114:

3'AGGACCGCCGTCCGCGCCCGACTCCCGAACACTACTCCTTAAGCGCACAGG
ACTTGACCTTCGCAACAAGG-5'

were synthesized. RP111 and RP114 were treated with polynucleotide kinase. Complementary oligonucleotides were annealed, leaving complementary ends and ligated together. However, attempts by R. Premont to ligate the oligos to the remainder of the AC1 sequence were unsuccessful. To complete the sequence I used primers

RP245:

5'-GGGGAAGCTTCCATGGCTGGAGCTCCTCGT-3' and

RP246:

5'-GGGCCCGCGGAACAACGCTTCCAGTT-3'

(1μM each), which flank the 160 base pair region, to amplify the missing sequence from a DNA template consisting of the annealed oligos RP111, 112, 113 and 114 (100pg). Reactions were performed by denaturation for 2'@95°C followed by 35 cycles of [1 min @95°C, 1 min @60°C, 30 sec @72°C] in a total volume of 50μl in the presence of 2mM $MgCl_2$, 200μM dNTPs, 1X Taq buffer and 2 units Taq polymerase. Since RP245 and RP246 contain HindIII and SacII sites

respectively, the 160 base pair product was digested with HindIII and SacII, electrophoresed in 4% NuSieve agarose, excised, spin-eluted and ligated into pBSII (Stratagene). The resulting plasmid was named pBSII(KS)-5'AC1. Recombinants were selected by restriction digests and sequenced from the 5' end through the SacII site. The remainder of the AC1 sequence was ligated as a SacII to SacII fragment into pBSII(KS)-5'AC1. Recombinants were verified by restriction digests. The completed AC1 was then inserted from HindIII to XbaI into pcDNAI (Invitrogen, San Diego, CA).

2) pcDNAI-AC2: pBSII(KS)-AC2 was the gift of Dr R. Reed (Johns Hopkins University, Baltimore, MD). AC2 was inserted into the EcoRI site of pcDNAI by J. Chen (Mount Sinai, NY).

3) pcDNAI-AC3 : pBSII(KS)-AC3 was the gift of Dr R. Reed (Johns Hopkins University, Baltimore, MD). AC3 was inserted into pcDNAI from XbaI to XhoI. Recombinants were verified by restriction digests.

4) pcDNAI-neo-AC4: pBSII(SK)-AC4 was generated by R. Premont. Briefly, AC4 was cloned from rat cDNA library by PCR in three fragments and inserted into pBSII(SK). AC4 was then excised from pBSII(SK) and inserted into pcDNAI-neo from BamHI to XhoI.

5) pcDNAI-AC5: AC5 was cloned by R. Premont and J. Chen from rat liver and kidney. An initiator methionine was created at leu 43 by PCR mutagenesis. AC5 was inserted into pcDNAI from BamHI to XhoI.

6) pcDNAI-AC6: AC6 was cloned by R. Premont and J. Chen from rat liver and kidney. AC6 was inserted into pcDNAI from BamHI to EcoRI.

Construction of vectors for Sf9 cells transfection

pVL1392-AC2: AC2 was excised from pBSII(KS)-AC2 and subcloned into the EcoRI site of pVL-1392. Recombinants were screened for proper orientation by restriction digests.

pVL1392-AC2-FLAG : A FLAG epitope and enterokinase cleavage site were incorporated into pVL-1392-AC2 by PCR mutagenesis. The 5' primer, OJ049

5'TACAAGCGGCCGCATGGACTACAAGGACGACGACGATAAGCGGCGGCGCC GCTACC-3'

was designed to contain a NotI restriction site followed by the codons for an initiator methionine, the FLAG epitope and enterokinase cleavage site (DYKDDDDK) and amino acids 2-6 of AC2. The 3' primer, OJ048, coded for base pairs 258-229 of AC2 and included an NheI restriction site. OJ049 and OJ048 (2μM each) were used to amplify a 215bp fragment from a linearized AC2 template (90pg). Reactions were performed by denaturation for 5 min @95°C followed by 35 cycles of [1 min @95°C, 1 min @55°C, 1 min 15 sec @72°C] in a total volume of 50μl containing 1.5mM $MgCl_2$, 200μM dNTPs, 1X Taq buffer and 2 units Taq polymerase. The 215bp PCR

product was directionally subcloned into pVL1392-AC2 from NotI to NheI. Recombinants were screened by restriction digests and positives were verified by dideoxy sequencing of the insert through the integration sites.

Generation of recombinant baculovirus

I prepared the plasmids for transfection by the alkaline lysis-lithium chloride precipitation method (Sambrook et al, 1989). R. Magnusson (Mount Sinai, NY) individually transfected pVL-1392-AC2, pVL-1392-AC2-FLAG and pVL-1393-AC6 into Sf9 cells along with BaculoGold virus DNA (Pharmingen, San Diego, CA). Recombinant baculoviruses containing the AC2 cDNA or AC6 cDNA were purified by 2 rounds of limiting dilutions and confirmed as recombinant by dot blots. TPO-ΔXS baculovirus (Thyroid Peroxidase or TPO) was constructed in a similar manner by R. Magnusson (Kendler *et al*, 1993).

DNA Sequencing

DNA sequencing was performed on double stranded plasmid DNA, 5μg per reaction. DNA was denatured in 0.2M NaOH for 5' at RT, neutralized in 0.3M Ammonium Acetate (pH 5.2) and precipitated in 3 volumes ethanol. Either [α^{35}S]dATP or [α^{32}P]dATP were used as label. Sequencing reactions were performed using Sequenase™ (modified T7 DNA polymerase, USB) according to the protocol supplied by USB. Briefly, DNA was heat-denatured and

annealed to 1 pmol primer. Extension and termination reactions were performed for 5 min each at 37° C. Products were denatured at 70° C and immediately chilled in an ice bath prior to loading on 6 or 8% polyacrylamide/7M Urea/TBE gels. Gels were run at 1500 V and dried prior to autoradiography. Sequence was determined manually. Compressions were resolved by 7-deaza-dGTP-, dITP-based sequencing or by sequencing of the complementary strand.

C. CELL CULTURE

293 cell culture and media

293 cells were obtained from ATCC. Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated horse serum, penicillin and streptomycin. For splitting, cells were washed with HBSS to remove serum protease inhibitors and treated with trypsin-EDTA for two min or until cells began detaching from plates. Proteolysis was terminated with addition of complete medium. Vigorous pipetting was performed to disperse cells for counting purposes.

293 cell transfections

The DEAE-dextran method was used to transfect adenylyl cyclase cDNAs into 293 cells. Typically $3X10^6$ cells from subconfluent dishes were seeded onto 10 cm dishes (Falcon) and grown overnight. Transfection was carried out for two hours with 5μg supercoiled vector DNA/10^6 cells in MEM or DMEM supplemented with 10% *fetal* calf serum, 400μg/ml DEAE-dextran and 100μM chloroquine. After incubating in the transfection mix for two hours, the media was aspirated and cells were shocked for 2 minutes with 10% DMSO in phosphate bufferred saline (PBS). Subsequently, the cells were *gently* washed with PBS and incubated overnight with MEM or DMEM supplemented with 10% *fetal* calf

serum and antibiotics. After the overnight incubation, cells were trypsinized and seeded at 30,000-60,000 cells/well in a 24 well plate (Falcon). To insure accuracy in counting, cells were counted in a small volume and then diluted for plating. Adenylyl cyclase activity was assayed after an additional overnight incubation.

Sf9 cell culture and media

Sf9 cells were obtained from the Mount Sinai Baculovirus Expression Core Facility (Dr. Ron Magnusson).

Sf9 cells were grown in Sf-900 medium (Gibco) or serum free insect culture medium (Sigma) at 27-28°C. Cells were infected with recombinant baculoviruses at a multiplicity of infection of about one. Infection was visually scored. Cells were harvested 2-4 days post infection. When treated with phorbol esters, PMA or 4α-phorbol-12,13-didecanoate (PDD) were added to the culture medium at 1μM (or at indicated concentration) and cells were subsequently incubated for 25-35 minutes. When staurosporine was included in the treatment, cells were pretreated with 1μM staurosporine for 15 min prior to phorbol ester or DMSO additions.

For ^{32}P labeling experiments, Sf9 cells were grown adherent to T80 flasks (Nunc) or in suspension in 1L stirrer bottles (Bellco Glass, Vineland, NJ). When grown in suspension, cells were stirred at 65-90 RPM. Cells were collected at 48 hours post

infection and washed with a phosphate deficient formulation of Grace's media consisting of 26.7g/L D-sucrose, 0.7g/L D-glucose, 1.1g/L NaCl, 1.3g/L $CaCl_2 \cdot 2H_2O$, 2.3g/L $MgSO_4 \cdot 7H_2O$, 2.2g/L KCl, 0.35g/L $NaHCO_3$, 0.6g/L L-glutamine, 1X BME amino acids and 4g/L MES at pH 6.2. 3.5-7.5x10^8 cells/group were incubated in 30 mls of phosphate deficient media for one hour and subsequently with 0.5mCi/ml $[^{32}P]H_3PO_4$ for 3 hours. Phorbol ester treatments were performed as above.

Sf9 membrane preparation

Sf9 cells harvested 2-4 days post infection. After treatments, cells were pelleted at 4°C and washed with lysis buffer (20mM NaHEPES, pH 8.0, 4mM EDTA, 150mM NaCl, 20mM Na phosphate, 20mM NaF, 10mM β-glycero-phosphate, 2mM DTT, 1-2μg/ml aprotinin, 2-4μg/ml leupeptin, 1mM phenanthroline, 1mM phenylmethylsulfonyl fluoride). Cells were lysed either by N_2 cavitation at 600 p.s.i. for 30 minutes or by Dounce homogenization (25-35 strokes). Supernatant from a low speed spin (1000xg, 10min) was centrifuged at 100000xg for 30 min-60 min to obtain the membrane fraction. The pellet was resuspended in 10mM HEPES, pH 8.0, 1mM EDTA, 200mM Sucrose, 2mM DTT and protease inhibitor cocktail to a final concentration of 3-5 mg protein/ml.

D. ASSAYS

Adenylyl cyclase assays in 293 cells

293 cells were lysed in a hypotonic solution of 100µl of 10mM NaHEPES pH 8.0, 2mM EDTA pH 8.0 and 500µM phenylmethylsulfonyl fluoride (freshly prepared). Before lysis the medium was aspirated and cells were washed with 500µl of lysis buffer. The lysis was enhanced by vigorous shaking of the plates. Assay components were subsequently added in 50µl for a final volume of 150µl in the wells.

Adenylyl cyclase activity was measured by the conversion of [α-^{32}P]ATP to [^{32}P]cAMP. Typically, assays were performed for 15 minutes at 32°C in the presence of 30mM NaHEPES pH 8.0, 2-3mM EDTA pH 8.0, 500µM phenylmethylsulfonyl fluoride, 0.5 mM isobutylmethylxanthine, 20mM creatine phosphate, 100µg/ml creatine phosphokinase, 10µg/ml myokinase, 0.1mM [α-^{32}P]ATP (~1000cpm/pmol), 1mM [^{3}H]cAMP (~60,000cpm) and 12mM $MgCl_2$. When Ca^{2+}/calmodulin-stimulated activity was measured, the mix also contains 0.2 mM EGTA, 0.25mM $CaCl_2$ and 2.5 µM calmodulin. All assays were done with triplicate wells. Reactions were terminated by addition of 100µl of 1% SDS, 10mM cAMP and 40mM ATP.

cAMP was purified according to the method of Solomon *et al* (1974). Samples were applied to acidified Dowex DE-52 columns. Samples were washed with 1ml, 2ml dH_2O and then eluted with 4.5 mls into neutral alumina columns. Alumina columns were washed with 2ml of 0.1M imidazole pH 7.5. cAMP was eluted with application of an additional 3 ml of imidazole and was counted in a scintillation counter. [^{3}H]cAMP counts were used to normalize for variation between columns. Adenylyl cyclase activities were expressed as pmol cAMP generated per 10^6 cells per 15 min assay.

Adenylyl cyclase assays in Sf9 membranes

Adenylyl cyclase activity was measured by the conversion of [α-^{32}P]ATP to [^{32}P]cAMP. Typically, assays were performed for 15 minutes at 32°C in the presence of 25mM NaHEPES pH 8.0, 1.3mM EDTA pH 8.0, 1mM phenylmethylsulfonyl fluoride, 1mM 1,10 phenanthroline, 0.5 mM isobutylmethylxanthine, 20mM creatine phosphate, 100μg/ml creatine phosphokinase, 10μg/ml myokinase, 0.1mM [α-^{32}P]ATP (~2000-3000 cpm/pmol), 1mM [^{3}H]cAMP (~10,000cpm) and 6.3 mM $MgCl_2$. Usually, 2-5 μg of membrane protein were used per tube. All assays were done with triplicate tubes. Assays were terminated and cAMP was determined as above for 293 cell assays. Adenylyl cyclase activities were expressed as pmol cAMP generated per mg protein per minute.

Protein determination

Determination of protein was performed by the method of Lowry et al (1951), or by fluorescamine fluoresence, measured at 380 nm excitation, 470 nm emission. BSA was used a standard for all determinations.

E. SDS-PAGE SILVER STAINING AND IMMUNOBLOTTING

Protein samples were electrophoresed through 8 or 15cm SDS-polyacrylamide gels at 150 V by the method of Laemmli (1970). 6% gels were used for adenylyl cyclase resolution while 10% gels were used for G protein subunit analysis. Stacking gels were 3-5% acrylamide. In all gels acryl:bis ratio was 30:0.4. Gels were fixed in 5:5:1 (methanol:water:acetic acid) and stained with silver (Wray et al,1981), or in 0.1% Coomassie blue. For immunoblotting, gels were not fixed, and transferred to nitrocellulose. For autoradiography, gels were soaked in 10% acetic acid/10% glycerol, vacuum dried, and exposed to Kodak X-AR film. Densitometry of gels or films was performed using the Hoeffer GS-300 scanning densitometer on an IBM-PC running Hoeffer GS-350 software.

For immunoblotting, proteins were transferred to nitrocellulose membranes (Hybond C, Amersham) according to Towbin et al (1979). Transfer buffer contained 25mM Tris, 192mM glycine, 20% methanol at 35 V for 16 hrs or 80 V for one hour. Membranes were blocked in 1X PBS, 5% dried nonfat milk (Borden), 1M glycine, 5% fetal calf serum for 30 min at RT or overnight at 4°C. Blocked membranes were washed 3 times for 5 min in 1X PBS, 0.1% dried nonfat milk, 1% fetal calf serum, 0.1% Tween 20 and subsequently exposed to antisera diluted in wash buffer for 3-16 hrs. Membranes were then washed 3 times for 5 min in wash buffer and finally in 0.05% Tween 20/1X PBS (T-PBS). Horseradish

peroxidase-conjugated goat anti-rabbit antibody was added in T-PBS at 1:10000 dilution and incubated with membranes for 1-2 hrs. Horseradish peroxidase-conjugated goat anti-mouse antibody was added in T-PBS at 1:3000 dilution and incubated with membranes for 1-2 hrs. Membranes were washed 3 times for 15 min in T-PBS with vigorous shaking. Chemiluminescent detection was performed using the ECL kit and HyperFilm (Amersham) or Reflection film (NEN).

F. IMMUNOAFFINITY PURIFICATION OF AC2-FLAG

4-5mg of Sf9 membrane protein per sample were pelleted for 30 min at 60000xg and resuspended in 1 ml of 150mM NaCl, 5mM EDTA, 20mM HEPES pH 8.0, 20% glycerol, 1mM EGTA and 0.8% dodecyl maltoside. The suspension was gently shaken at 4°C for 90 min. Supernatants (60000xg, 30 min) containing the solubilized adenylyl cyclase were incubated with 15-20µl of solubilization buffer pre-equilibrated Anti-FLAG M2 affinity gel (Kodak) for 3 hours at 4°C. The gel was washed 2-3 times with solubilization buffers containing 0.8%, 0.4% and 0.05% dodecyl maltoside. F-AC2 was eluted by incubation with 0.1M glycine pH 3.0 for 5-10 min in a volume of 90µl. Eluates were immediately neutralized with 10 µl of 1M NaHEPES pH 8.0 and rapidly frozen on dry ice/acetone for subsequent analysis on SDS-PAGE.

CHAPTER 4

PHORBOL ESTER-INDUCED STIMULATION OF SPECIFIC TYPES OF ADENYLYL CYCLASE IN 293 CELLS

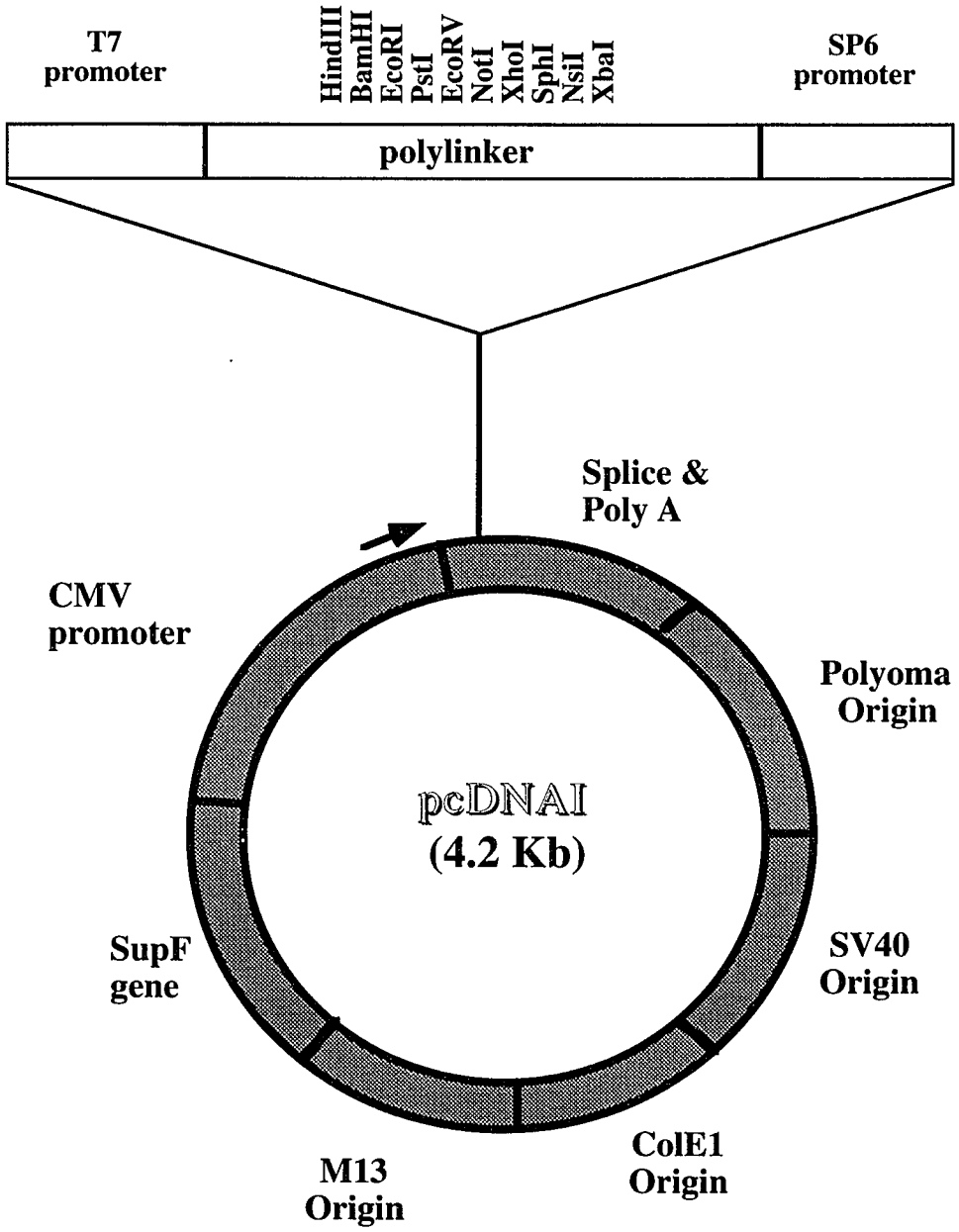
Adenylyl cyclases isoforms 1-6 were expressed in 293 cells and tested for stimulation by PMA treatment of cells. In this chapter, adenylyl cyclase 2 was identified as the isoform most extensively responsive to PMA treatment of cells. Adenylyl cyclase isoforms 1 and 3 activities were only only moderately affected by PMA treatment while isoforms 4, 5 and 6 were unaffected. Hence, in this chapter, I first present data from experiments on the adenylyl cyclase 2 isoform and then the data for the other isoforms.

4.1) Construction of vectors for mammalian cell transfections

To express the six cloned adenylyl cyclases, their corresponding cDNAs were individually inserted into the pcDNAI vector. A map of pcDNAI is shown in Fig. 4-1.

For replication in bacteria, pcDNAI contains a ColE1 high copy-number origin and a SupF suppresser tRNA gene for ampicillin selection. The SupF suppresser tRNA permits translation of an episomal ampicillin resistance gene present in the bacteria. For mammalian expression, pcDNAI contains a cytomegalovirus (CMV) promoter/enhancer element as well as splicing and polyadenylation elements. The splicing and polyadenylation elements, placed 3' to the cDNA insert, are useful for enhancing expression and half-life of mRNAs. Adenylyl cyclase cDNAs were excised from pBSII with the appropriate restriction enzymes and subcloned into the pcDNAI polylinker site. Recombinants were screened by restriction digests for proper orientation and partially sequenced. Details are provided in the Materials and Methods section.

Figure 4-1. **Map of pcDNAI mammalian expression vector**

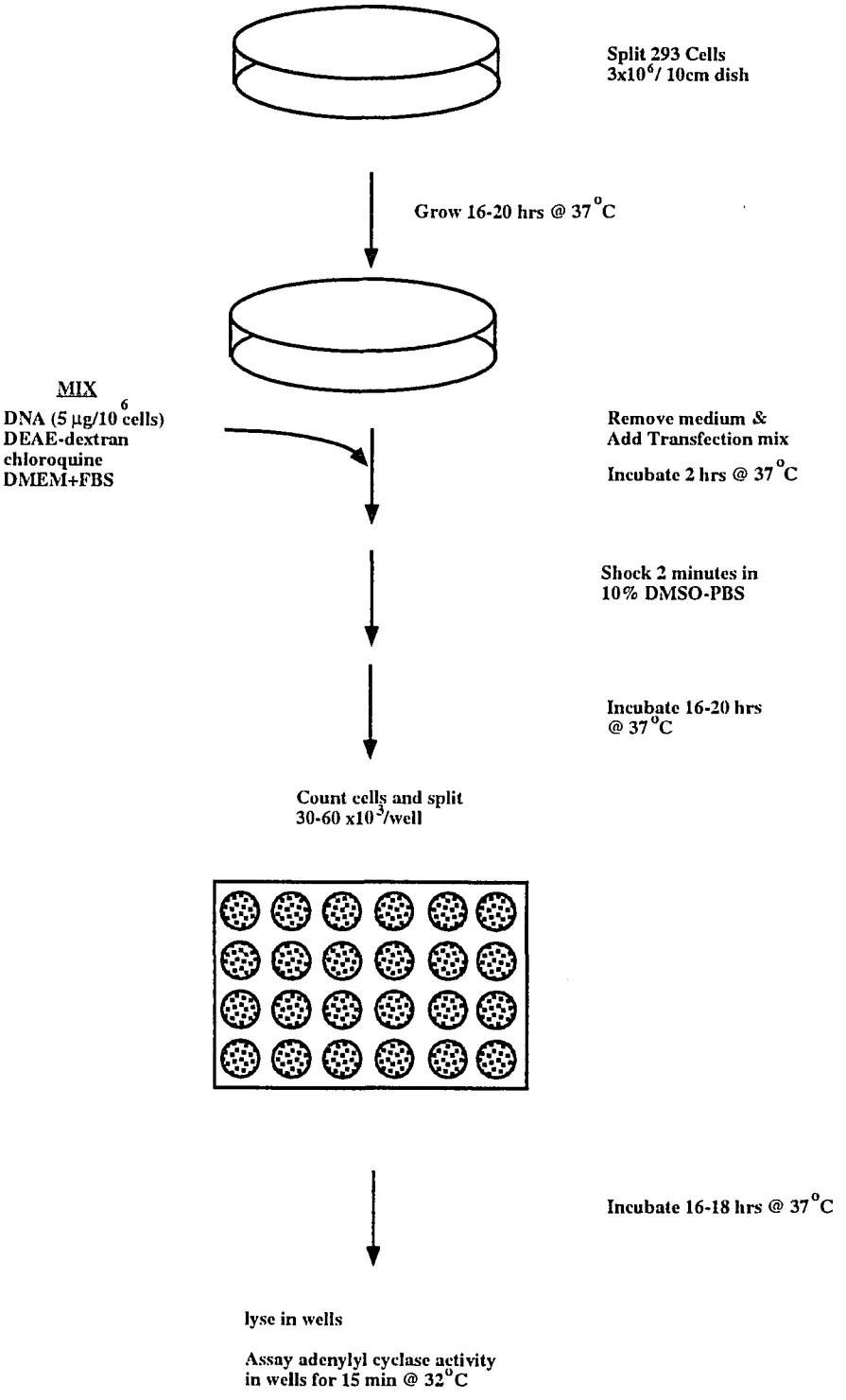


4.2) Transfection of pcDNAI constructs into HEK-293 cells

For transfection, plasmid DNA was prepared from transformed bacteria by the alkaline lysis method. Typically, DNA yields were about four-fold lower than those of standard bacterial plasmid preparations. The DNA was analyzed by TAE-agarose gel electrophoresis against known amounts of λHindIII DNA to quantitate transfection-competent supercoiled DNA and to verify of the integrity of the DNA.

As illustrated in Fig. 4-2, HEK-293 cells were split and allowed to recover overnight before transfection. Transfection was performed in 10cm tissue culture dishes. Medium was removed and the cells were incubated for two hours in a mix containing DNA, DEAE-dextran and chloroquine in DMEM supplemented with 10% FCS. DEAE-dextran was used as the transfection reagent for its binding to DNA and adherence to cells. Chloroquine, a lysosomotropic agent, was included in the medium in order to inhibit lysosomal degradation of internalized DEAE-dextran-DNA complexes. Fetal calf serum (FCS) was included in media during and subsequent to transfection in order to enhance survival of transfected cells. After the incubation cells were shocked for 2 min in 10% DMSO to enhance transfection. Surviving cells were subsequently allowed to recover overnight. The next day cells were counted and plated on a 24 well plate. Cell counting was performed in a small volume to insure accuracy. Cells were

Figure 4-2. Transfection method of HEK-293 cells



incubated overnight to ensure their attachment in the wells prior to assay.

Adenylyl cyclase activity was assayed in the individual wells. The medium was removed and cells were rapidly washed once in 500µl hypotonic buffer. Cells were lysed by incubation in 100µl of hypotonic solution and assay components were added in an additional 50µl. Adenylyl cyclase activity was assayed for 15 min at pH 8.0, 32°C. An ATP regenerating system was provided in the assay mix to maintain the ATP concentration. The assay was terminated by addition of 1% SDS and 40mM unlabeled ATP. cAMP product was purified over Dowex and alumina columns. Inter-column variation was normalized by monitoring for [^{3}H]cAMP recovery. All points were determined in triplicate.

4.3) Effects of PMA treatment on type 2 adenylyl cyclase-transfected HEK-293 cells

The type 2 adenylyl cyclase, kindly provided by R. Reed, was subcloned into pcDNAI as described in Materials and Methods. 293 cells were transfected with pcDNAI, or pcDNAI containing the type 2 adenylyl cyclase (pcDNAI-AC2) and assayed 48 hours later. Transfection of the type 2 adenylyl cyclase resulted in a 4-fold enhancement of basal adenylyl cyclase activity (Fig. 4-3, top, hatched bars). Forskolin-stimulated adenylyl cyclase activity was increased only about 50% in the AC2 transfected group. When treated with 1μM PMA for 15 min prior to lysis and assay, pcDNAI vector-transfected cells had, on average, a 30% increase in basal and forskolin-stimulated adenylyl cyclase activity (Fig. 4-3, left panels). In contrast, in AC2-transfected cells PMA treatment resulted in a three-fold increase in basal adenylyl cyclase activity and a two-fold increase in forskolin-stimulated activity (Fig. 4-3, right panels). In assays from twenty other transfections PMA enhanced basal adenylyl cyclase activity 2.5 to 4.5 fold in the type 2-transfected cells.

To confirm that the PMA-mediated increase in type 2 adenylyl cyclase activities was mediated by specific activation of protein kinase C, a 4-α phorbol ester incapable of activating protein kinase C, 4α phorbol 12,13 didecanoate (PDD), was tested on the transfected cells. Treatment of vector-transfected or type 2 adenylyl cyclase-transfected cells with PDD resulted in adenylyl

cyclase activities comparable to those of untreated cells (Fig. 4-4, hatched and white columns). In contrast, PMA-treated cells increased type 2 adenylyl cyclase activities two to three-fold over untreated or PDD-treated cells.

Figure 4-3. PMA treatment of AC2-transfected HEK-293 cells:

HEK-293 cells were transfected with pcDNAI or pcDNAI-AC2. Cells were treated for 15 min with 1μM PMA (black bars) or DMSO vehicle (hatched bars), lysed and assayed as described under "Materials and Methods". Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-3. PMA treatment of AC2-transfected HEK-293 cells

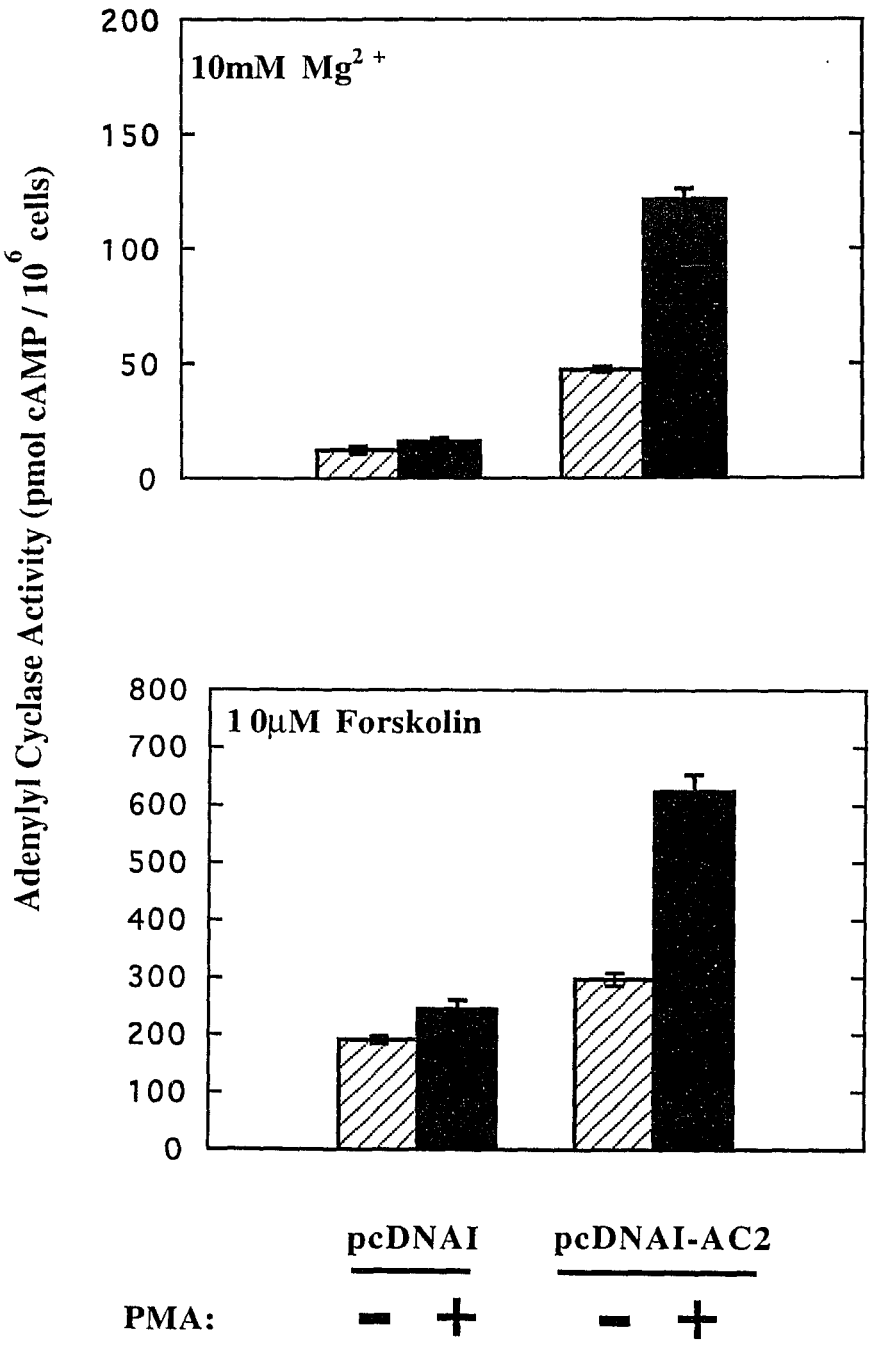
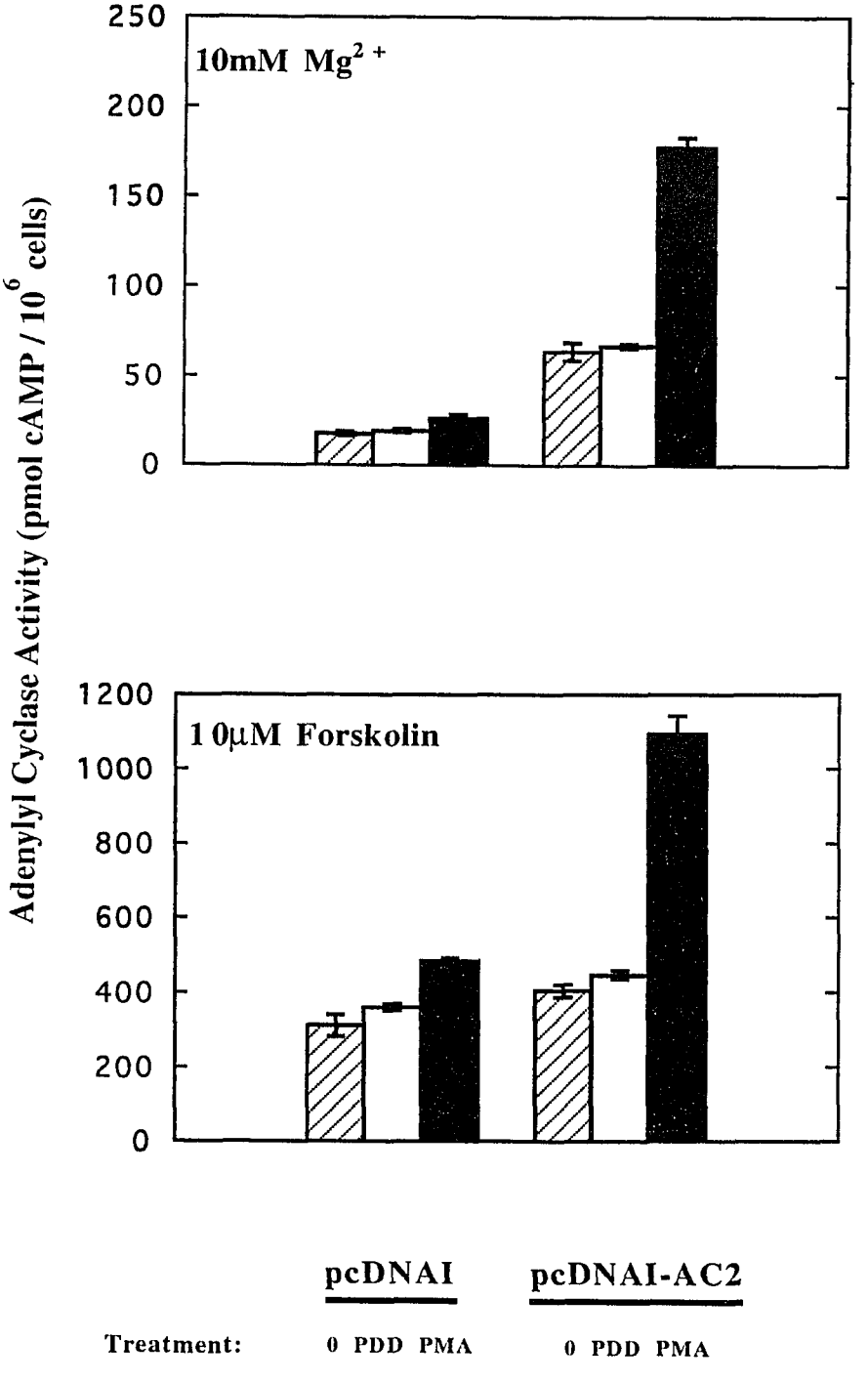


Figure 4-4. PMA and PDD treatment of AC2-transfected HEK-293 cells:

HEK-293 cells were transfected with pcDNAI or pcDNAI-AC2. Cells were treated for 15 min with 1μM PMA (black bars), 1μM PDD (white bars), or DMSO vehicle (hatched bars), lysed and assayed as described under "Materials and Methods". Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-4. PMA and PDD treatment of AC2-transfected HEK-293 cells



I next determined which activities of the type 2 adenylyl cyclase were enhanced by Protein kinase C activation. The basal activity (assayed at 3mM Mg^{2+}), the most extensively increased activity, was elevated 2.5-fold (Fig. 4-5, upper left panel). Activation of the type 2 adenylyl cyclase by Mn^{2+} was enhanced by about 50%. G protein-stimulated activities were enhanced by about 30% as assessed by Fluoride and GTPγS stimulation (Fig. 4-5, bottom panels). Forskolin stimulation of the type 2 enzyme, as previously shown (Fig. 4-3, 4-4), was enhanced about two fold. Similar increases for these stimulators were seen in three other experiments.

Since the basal, Mg^{2+}-stimulated activity was the most extensively increased activity, I then determined the Mg^{2+} concentration dependence of the PMA effect. Specifically, it was important to determine whether the enhancement was most pronounced at high Mg^{2+} concentrations compatible with G_s activation (e.g. 15mM), or at low Mg^{2+} concentrations (e.g. 3mM) where most of the G_s would not be activated. The basal adenylyl cyclase activity of the type 2 enzyme was enhanced at all Mg^{2+} concentrations tested (Fig. 4-6) with the greatest increases (350%) occurring at low Mg^{2+} concentrations and the smallest (74%) occurring at 15mM Mg^{2+}.

Finally, I tested the effect of PMA treatment on hormonally-stimulated adenylyl cyclase activity in AC2-transfected 293 cells. For this purpose, the lysed cells were

Figure 4-5. Effect of PMA treatment on Mg^{2+}, Mn^{2+}, Fluoride and GTPγS-stimulated adenylyl cyclase activities of type 2 adenylyl cyclase-transfected cells:

HEK-293 cells were transfected with pcDNAI or pcDNAI-AC2. Cells were treated with 1μM PMA (black bars) or DMSO vehicle (hatched bars), lysed and assayed in the presence of the indicated ligands as described under "Materials and Methods". Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-5. Effect of PMA treatment on Mg^{2+}, Mn^{2+}, Fluoride and GTPγS-stimulated adenylyl cyclase activities of type 2 adenylyl cyclase-transfected cells

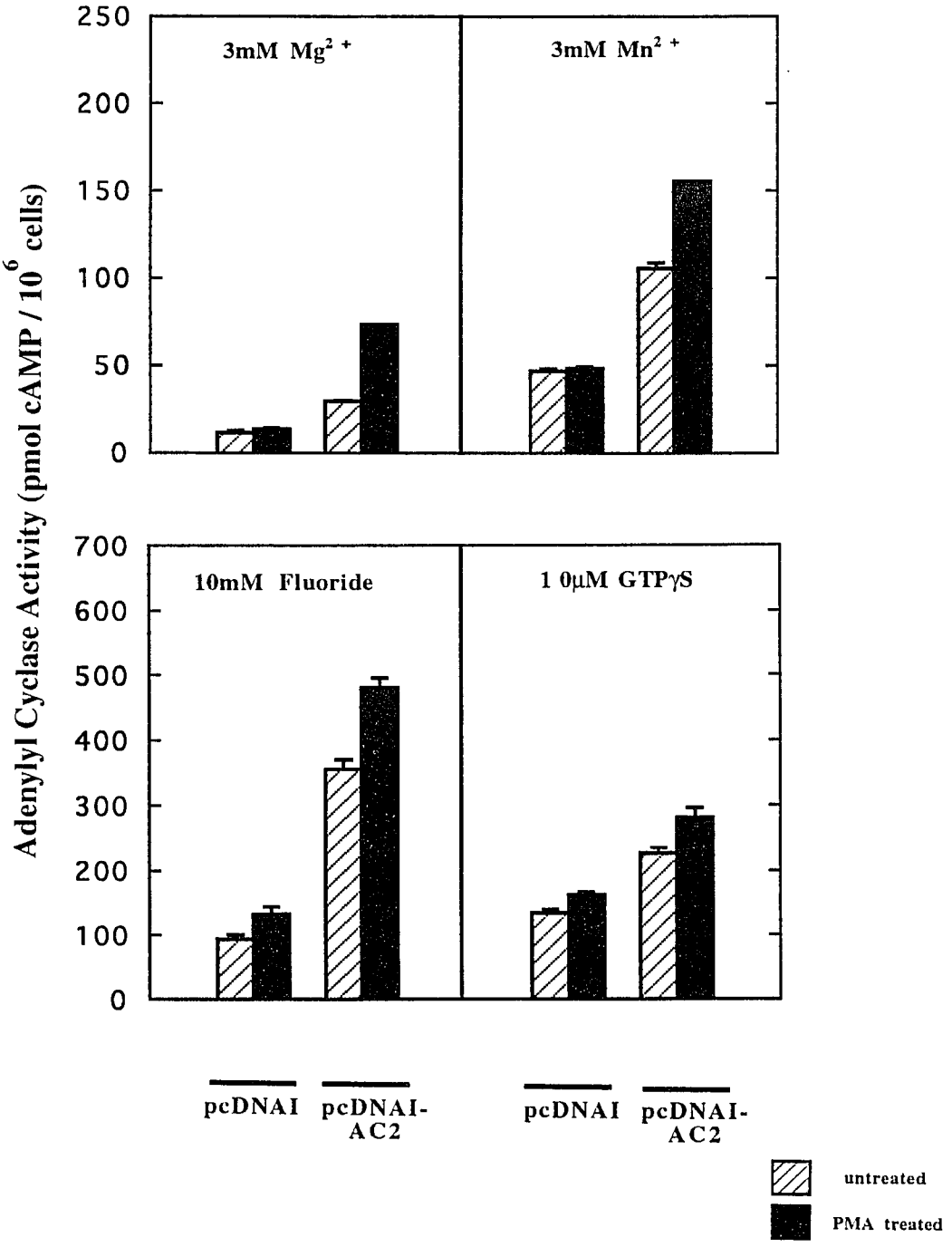
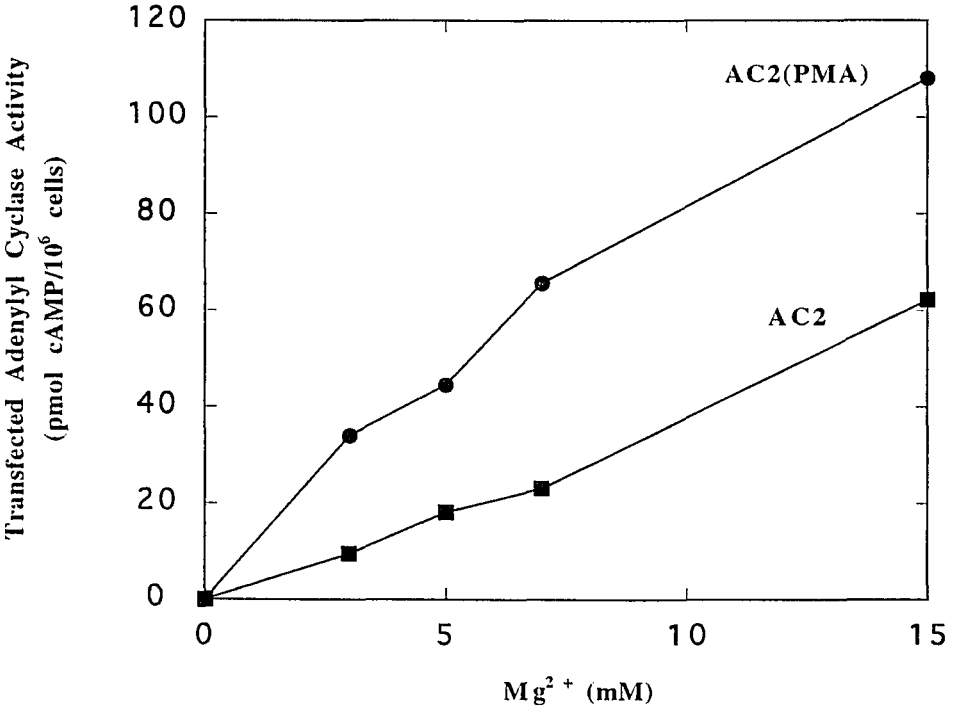


Figure 4-6. Mg^{2+} dependence of PMA effect on type 2 adenylyl cyclase:

HEK-293 cells were transfected with pcDNAI or pcDNAI-AC2. Cells were treated with 1μM PMA or DMSO vehicle, lysed and assayed in the presence of the indicated concentrations of Mg^{2+}. Free Mg^{2+} was estimated by subtraction of 2mM EDTA from the total added Mg^{2+}. The activities from pcDNAI-transfected cells were subtracted from the corresponding values of pcDNAI-AC2-transfected cells to obtain the values shown. Hence the activity is labeled "transfected adenylyl cyclase activity". (●), activity from PMA-treated cells; (■), activity from DMSO-treated cells. Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-6. **Mg^{2+} dependence of PMA effect on type 2 adenylyl cyclase**

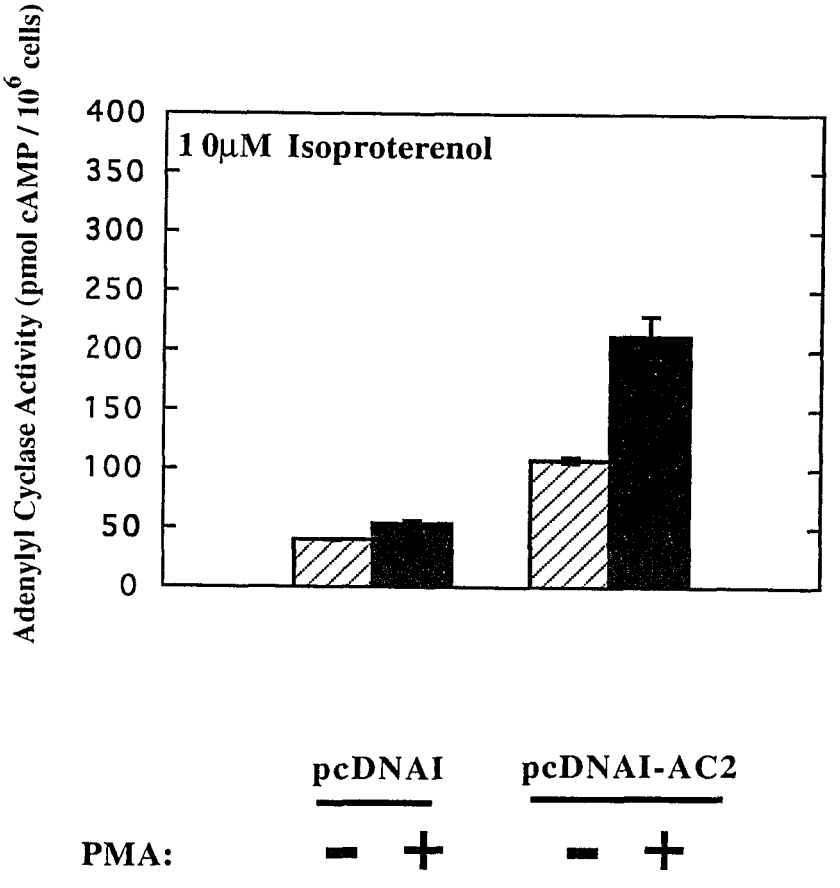


assayed for adenylyl cyclase activity in the presence of the β-adrenergic agonist isoproterenol Mg^{2+} and GTP. Stimulation with isoproterenol tripled the adenylyl cyclase activity of pcDNAI-transfected 293 cells and doubled the activity of pcDNAI-AC2-transfected cells (Fig. 4-3 and 4-7). Hence, the transfected type 2 enzyme could functionally integrate into the hormonally-stimulated adenylyl cyclase pathway in HEK-293 cells. When the cells were treated with PMA, the isoproterenol-stimulated adenylyl cyclase activity of pcDNAI-transfected cells was increased by about 30%. In pcDNAI-AC2-transfected cells, however, isoproterenol-stimulated activity was doubled by PMA treatment. Therefore, the PMA-mediated increase in the type 2 enzyme's activity also occurs by upstream activation of the adenylyl cyclase pathway through hormone-receptor interaction.

Figure 4-7. Effect of PMA treatment on isoproterenol-stimulated adenylyl cyclase activity in type 2 adenylyl cyclase-transfected HEK-293 cells:

HEK-293 cells were transfected with pcDNAI or pcDNAI-AC2. Cells were treated with 1μM PMA (black bars) or DMSO vehicle (hatched bars), lysed and assayed in the presence of 10μM isoproterenol and GTP as described under "Materials and Methods". Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-7. Effect of PMA treatment on isoproterenol-stimulated adenylyl cyclase activity in type 2 adenylyl cyclase-transfected HEK-293 cells



4.4) Effects of PMA treatment on types 1 and 3 adenylyl cyclase-transfected HEK-293 cells

Types 1 and 3 adenylyl cyclase were subcloned into pcDNAI. The type 1 adenylyl cyclase was cloned from a bovine brain cDNA library by R. Premont (Jacobowitz et al, 1993) except for the GC-rich 5' terminus. R. Premont then unsuccessfully attempted to construct the 5' end from synthetic oligonucleotides which were annealed, kinased and ligated together. These synthetic oligonucleotides were designed to reduce GC content while preserving the amino acid sequence. To complete the AC1 sequence, I PCR-amplified the 5' end of AC1 from a template consisting of these annealed and ligated synthetic oligonucleotides. The construction scheme of pcDNAI-AC1 is illustrated in Fig. 4-8. AC3 was a kind gift of R. Reed and was subcloned into pcDNAI as described under Materials and Methods.

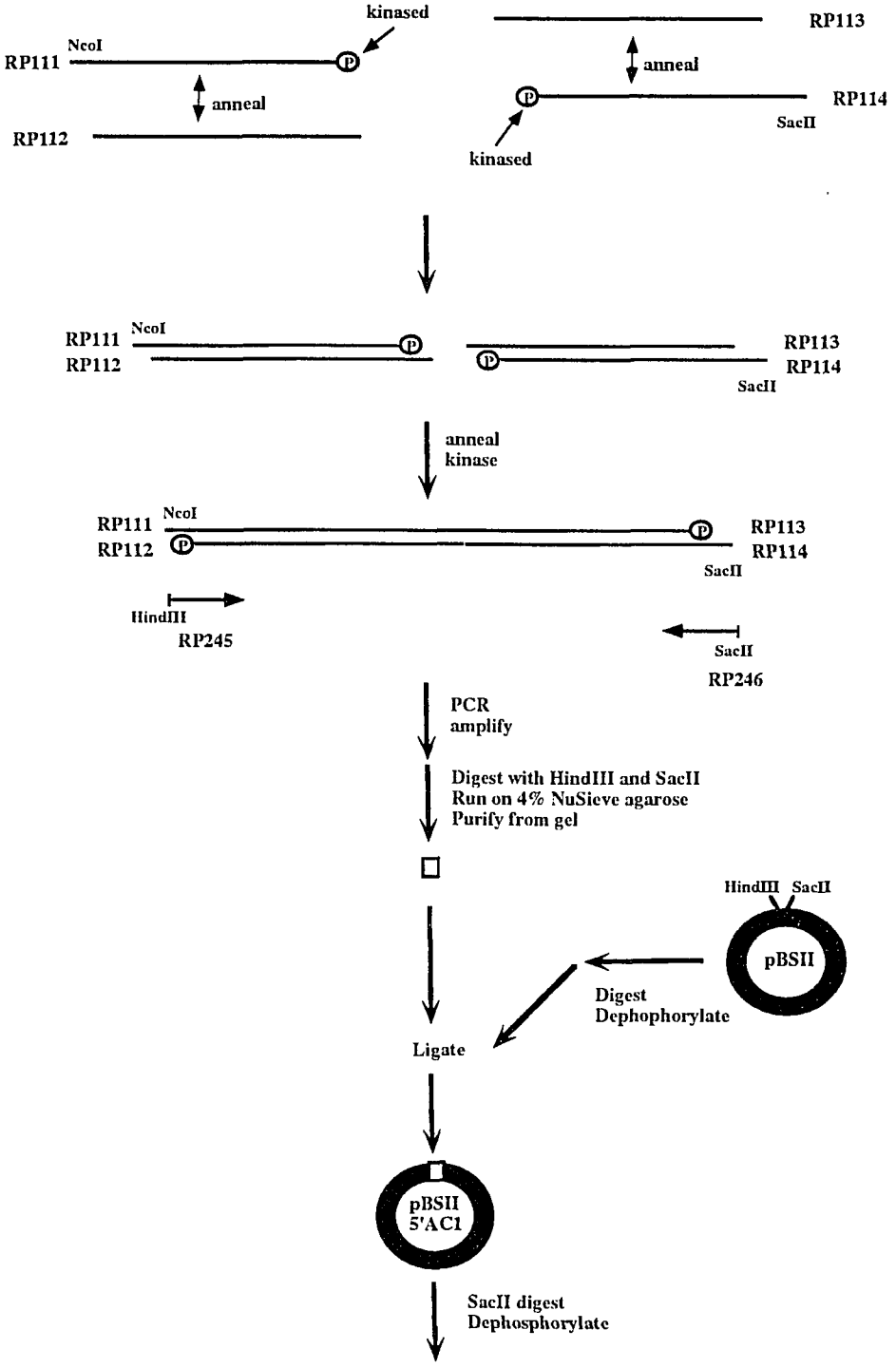
Types 1 and 3 adenylyl cyclases were reported to be stimulated by Ca^{2+}/CaM. Expression of the type 1 enzyme in HEK-293 cells resulted in adenylyl cyclase activity stimulated 2.7-fold by Ca^{2+}/CaM (Fig. 4-9, upper panels). Surprisingly, neither the basal nor the forskolin-stimulated activities were increased in type 1 adenylyl cyclase-transfected cells (Fig. 4-9, left panels). Similar results were obtained in six other independent transfections. Treatment with PMA enhanced the Ca^{2+}/CaM-stimulated activity of AC1-transfected cells by about 80%, typically. Expression of the type 1 adenylyl cyclase did not result

Figure 4-8. Construction of pcDNAI-AC1:

The construction of pcDNAI-AC1 is described under "Materials and Methods".

Briefly: To construct the 5' end of AC1, oligonucleotides RP113, 114, 111 and 112 were annealed pairwise and kinased. The pairs were annealed and ligated together to form a PCR template. Primers RP245 and RP246 were used to amplify the sequence of the annealed oligonucleotides. The PCR product was digested with HindIII and SacII and subcloned into pBSII to generate pBSII-5'AC1. The remainder of the AC1 sequence [AC1(3')] was then subcloned into the SacII site of pBSII-5'AC1 to form pBSII-AC1. The completed AC1 was then excised and subcloned from HindIII to XbaI in pcDNAI.

Figure 4-8. Construction of pcDNAI-AC1



(*Figure 4-8.* Construction of pcDNAI-AC1)

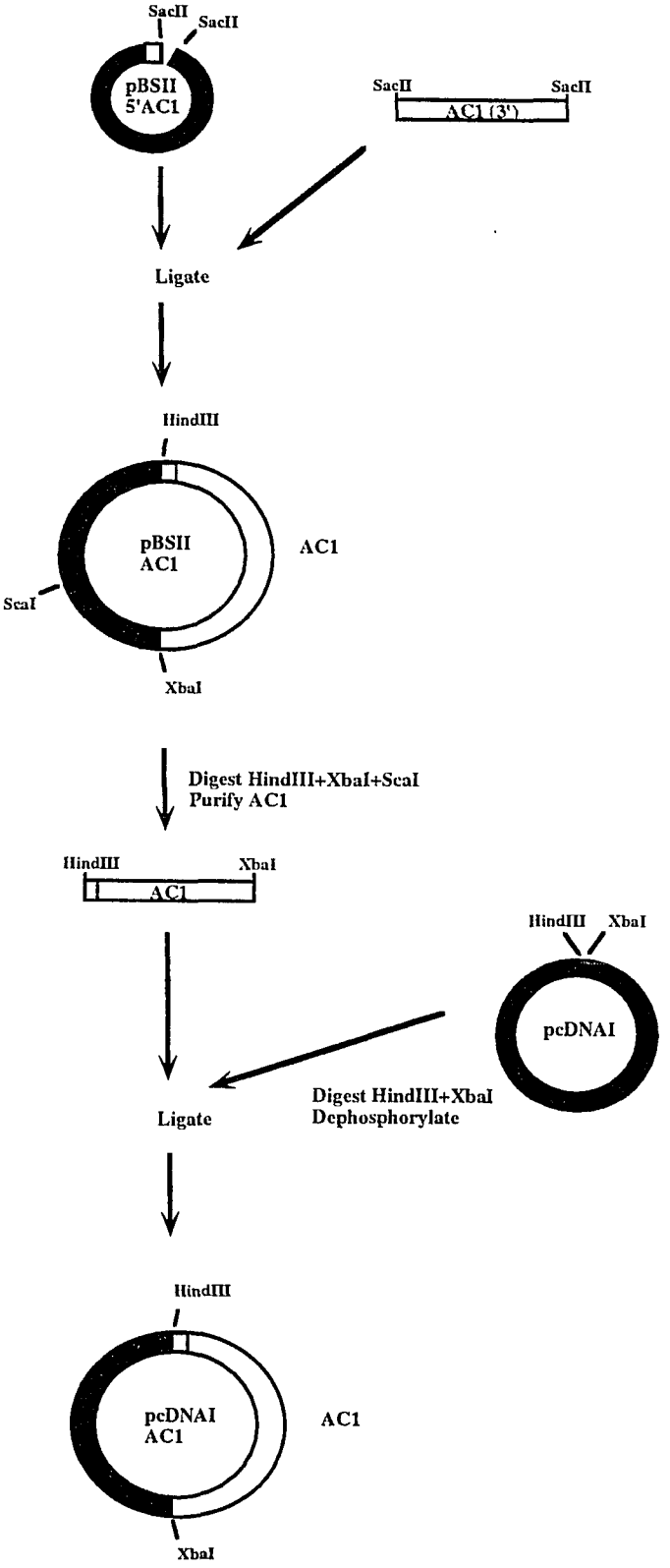
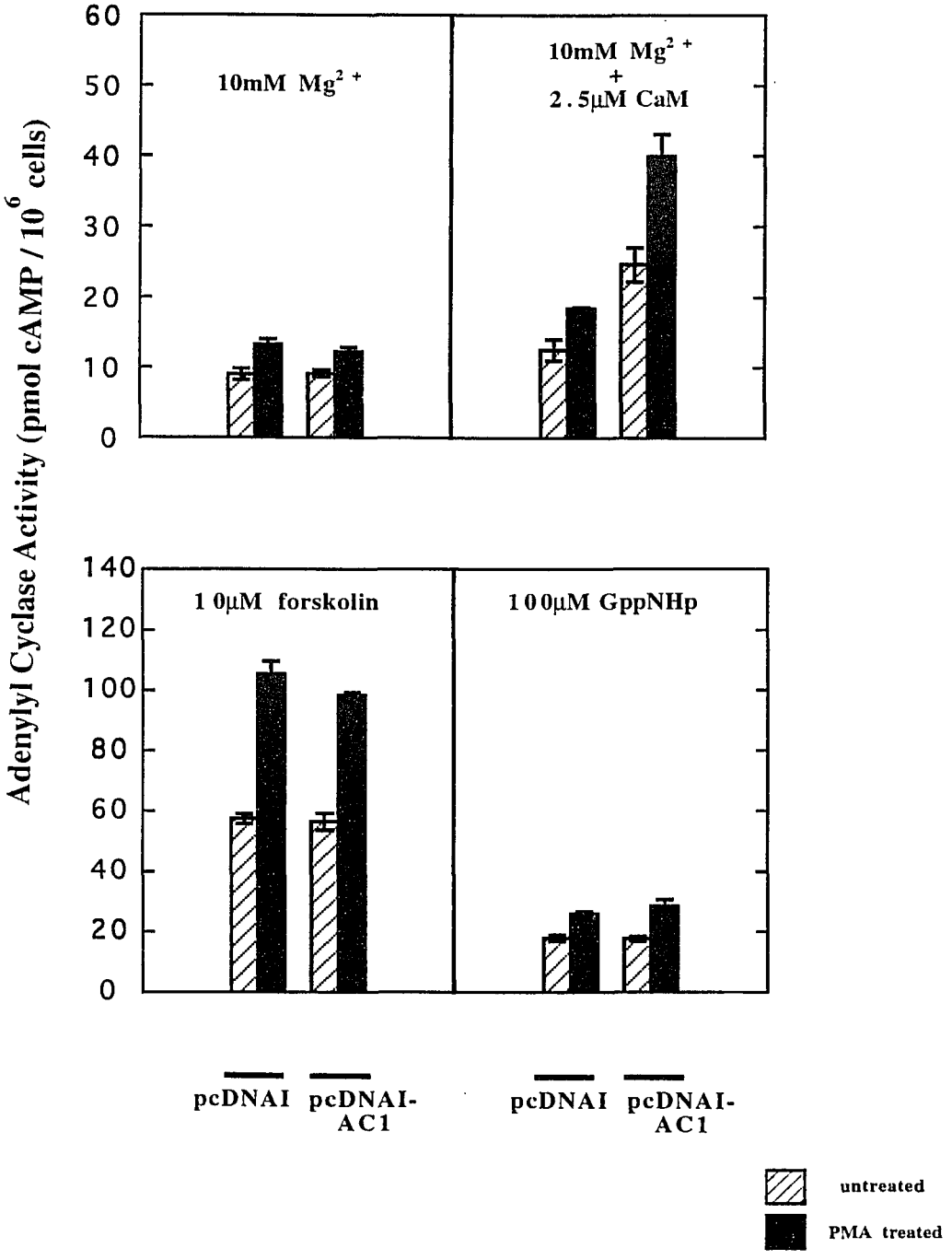


Figure 4-9. PMA treatment of type 1 adenylyl cyclase-transfected HEK-293 cells:

HEK-293 cells were transfected with pcDNAI or pcDNAI-AC1. Cells were treated with 1μM PMA (black bars) or DMSO vehicle (hatched bars), lysed and assayed in the presence of the indicated ligands as described under "Materials and Methods". When present, CaM was 2.5μM and estimated free Ca^{2+} was 50μM. Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-9. PMA treatment of type 1 adenylyl cyclase-transfected HEK-293 cells



in enhancement of the Gpp(NH)p-stimulated activity (Fig. 4-9, bottom panels). This activity was similarly enhanced by PMA treatment in vector and AC1 transfected cells. Thus PMA appears to enhance the Ca^{2+}/CaM-stimulated activity of the type 1 enzyme.

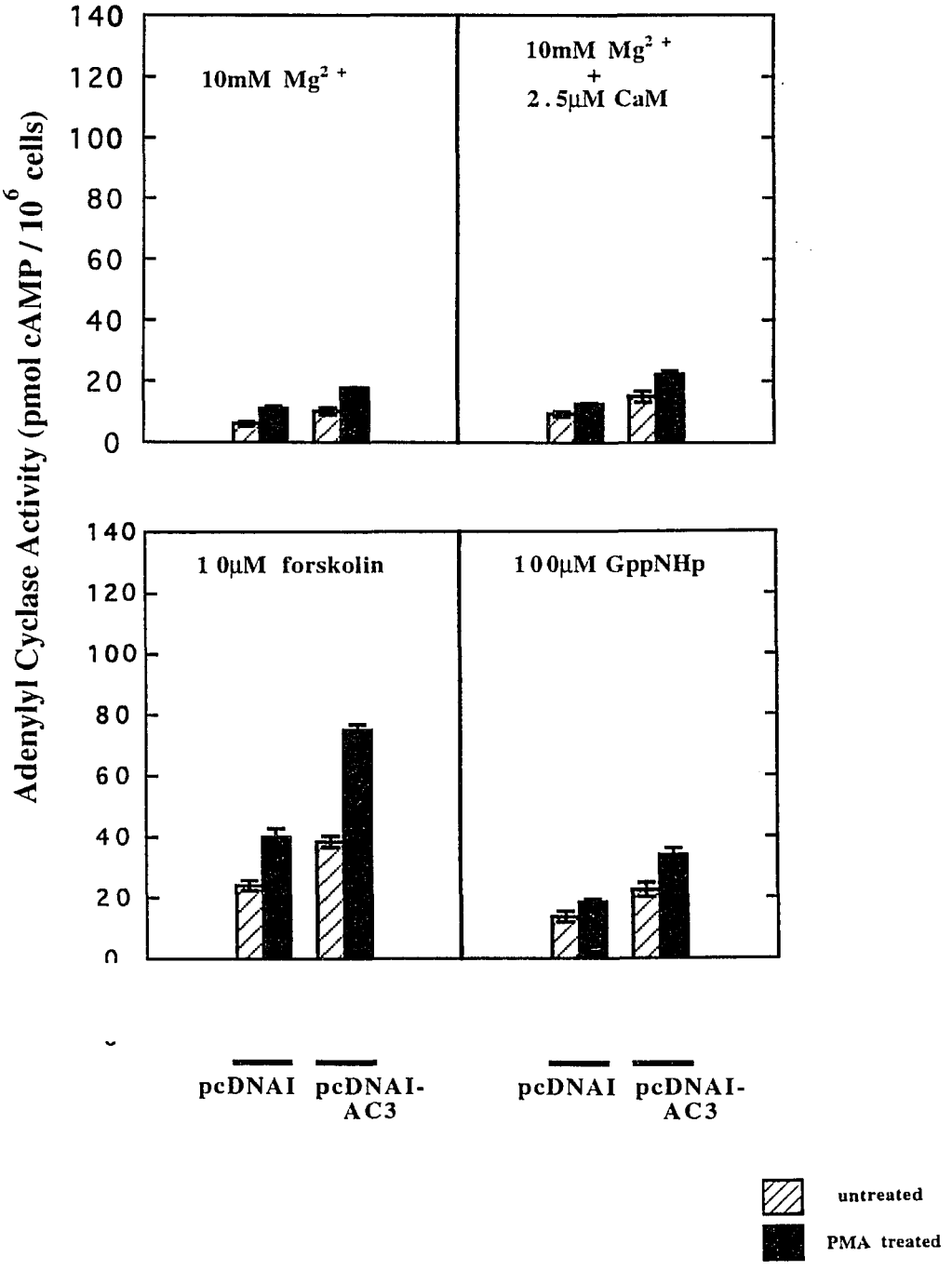
Expression of the type 3 adenylyl cyclase in HEK-293 cells resulted in a small (40%) but significant increase in basal adenylyl cyclase activity (Fig. 4-10, top panel). This activity was stimulated 50% by Ca^{2+}/CaM treatment. Similar results were obtained in three other independent transfections. Forskolin and Gpp(NH)p-stimulated activities were also increased with expression of the type 3 enzyme (Fig. 4-10, bottom panels). With PMA treatment, basal, Ca^{2+}/CaM and Gpp(NH)p-stimulated activities were moderately enhanced and forskolin-stimulated activity was doubled in AC3-transfected cells.

The types 4, 5 and 6 adenylyl cyclases were also tested for PKC-dependent effects on their activity. The type 4 adenylyl cyclase is highly homologous in sequence with the type 2 enzyme (Fig. 1-3) and is likewise stimulated by G protein $\beta\gamma$ subunits. The types 5 and 6 enzymes comprise a family of mammalian adenylyl cyclases divergent from the type 2 enzyme. The types 4, 5 and 6 enzymes were expressed in HEK-293 cells (Fig. 4-11). Expression of these three isozymes resulted in moderately elevated basal and forskolin-stimulated adenylyl cyclase activities. The data in Fig. 4-11 are taken from three separate transfections. When an AC2 group was included in the above transfections, prominent elevation

Figure 4-10. PMA treatment of type 3 adenylyl cyclase-transfected HEK-293 cells:

HEK-293 cells were transfected with pcDNAI or pcDNAI-AC3. Cells were treated with 1μM PMA (black bars) or DMSO vehicle (hatched bars), lysed and assayed in the presence of the indicated ligands as described under "Materials and Methods". When present, CaM was 2.5μM and estimated free Ca^{2+} was 50μM. Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-10. PMA treatment of type 3 adenylyl cyclase-transfected HEK-293 cells

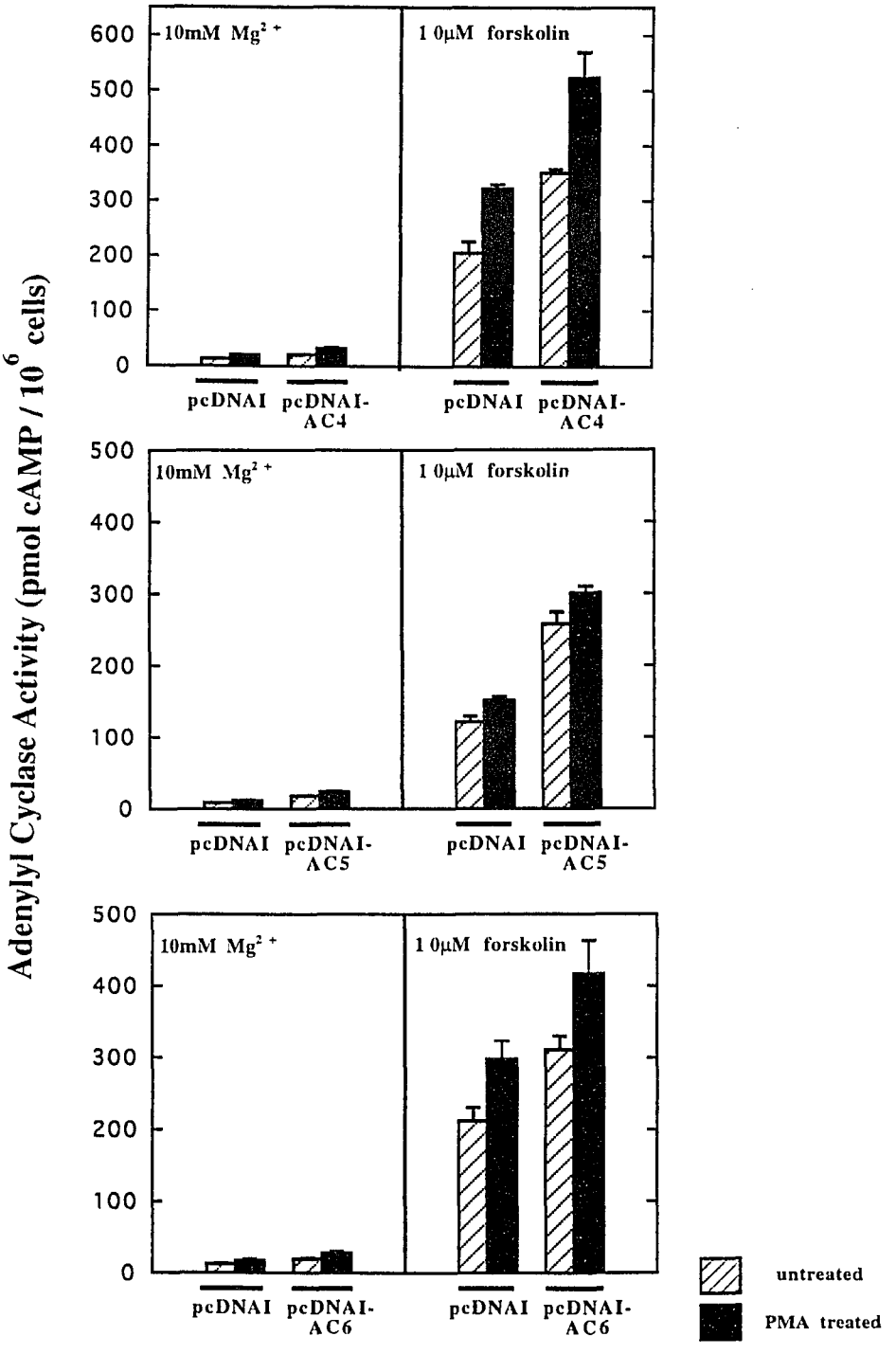


of the basal activity was always seen. PMA treatment did not result in an enhancement of the adenylyl cyclase activities of the types 4, 5 and 6 enzymes different from that of the vector-transfected group.

Figure 4-11. PMA treatment of type 4, 5 and 6 adenylyl cyclase-transfected HEK-293 cells:

HEK-293 cells were transfected with pcDNAI, pcDNAI-AC4, pcDNAI-AC5, or pcDNAI-AC6. Cells were treated with 1μM PMA (black bars) or DMSO vehicle (hatched bars), lysed and assayed in the presence of the indicated ligands as described under "Materials and Methods". The data shown are taken from three separate experiments. Values are mean of triplicate determinations. Coefficient of variance was less than 10%.

Figure 4-11. PMA treatment of type 4, 5 and 6 adenylyl cyclase-transfected HEK-293 cells



CHAPTER 5

PHORBOL ESTER-INDUCED STIMULATION OF ADENYLYL CYCLASE 2 IN SF9 CELLS

5.1) Construction of baculovirus vector for Sf9 cell expression

Using the HEK-293 cell mammalian expression system, I identified adenylyl cyclase 2 as the isozyme most extensively stimulated by PKC activation among isozymes 1-6. To facilitate further characterization of this effect and determine whether phosphorylation of the adenylyl cyclase was involved, I used the baculovirus-driven expression of adenylyl cyclase 2 in insect Sf9 cells.

For expression in Sf9 cells, the adenylyl cyclase 2 cDNA was inserted into the genome of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). AcMNPV is an insect cell-specific vector used for expression of exogenous proteins in insect cells (O'Reilly et al, 1992, pp 285-296). Recombinant AcMNPV-AC2 was constructed in two steps. First, AC2 was subcloned into the transfer plasmid vector pVL-1392. Then, pVL-1392-AC2 and AcMNPV DNA (BaculoGold™ DNA, Invitrogen) were transfected into Sf9 cells where by homologous recombination the AC2 baculovirus was generated.

pVL-1392 is a DNA plasmid derived from pUC8, a plasmid carrying a high copy number origin and an ampicillin resistance gene for replication and selection in bacteria. pVL-1392 also contains the AcMNPV polyhedrin region which includes an

essential AcMNPV gene (ORF 1629) flanking the polyhedrin promoter and the multiple cloning site (MCS). The polyhedrin promoter directs high-level expression of exogenous cDNAs during the very late phase of AcMNPV infection. The polyhedrin region and ORF 1629 also allow for homologous recombination with the BaculoGold™ DNA and for complementation of a lethal deletion in the AcMNPV BaculoGold™ DNA. By homologous recombination with pVL1392-AC2, both the essential gene of ORF 1629 and AC2 can be inserted into the AcMNPV genome to generate infectious recombinant progeny.

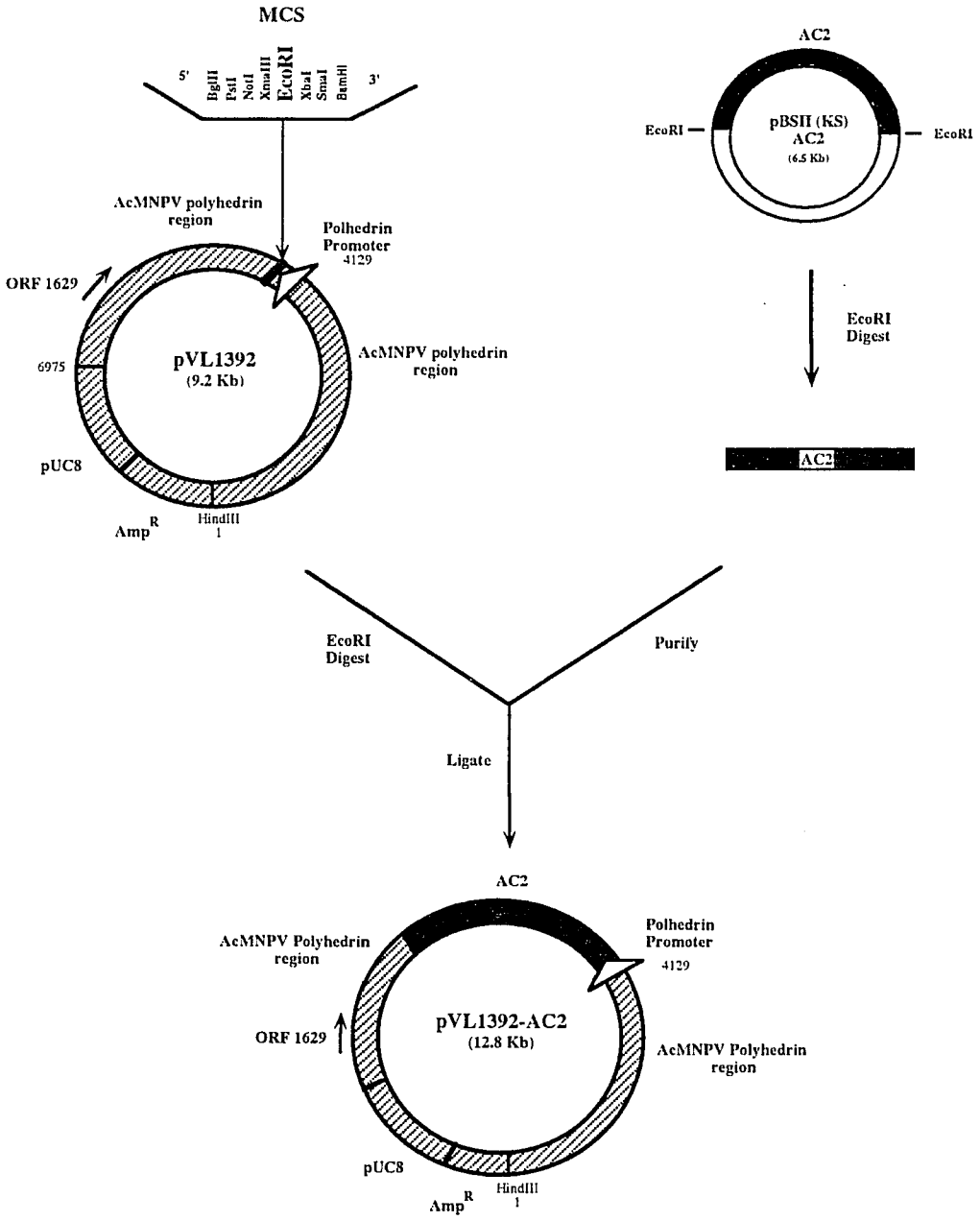
The construction and map of pVL-1392 is illustrated in Fig. 5-1. AC2 was excised from pBSII-AC2 and subcloned into the EcoRI site of the MCS of pVL-1392. The recombinant plasmid was analysed by restriction digests and limited sequencing. pVL-1392-AC2 was transfected along with BaculoGold™ AcMNPV DNA into Sf9 cells. Recombinant viruses were cloned by two rounds of limiting dilution and checked for the presence of the AC2 cDNA by hybridization to a full-length AC2 DNA probe (R. Magnusson).

Fig. 5-1. Construction and map of pVL-1392-AC2:

pVL1392 was digested with EcoRI and dephosphorylated with calf intestine alkaline phosphatase. AC2 was excised from pBSII-AC2 by EcoRI digest. pVL1392 and pBS-AC2 digest products were run on 0.8% and 1% agarose gels respectively and the appropriate DNA bands were purified by centifugation of gel slices through siliconized glass wool followed by phenol extraction and ethanol precipitation. AC2 and pVL1392 were ligated and then transformed into E. *coli*. Recombinants were identified by EcoRI digestion.

pVL1392 is a pUC8 derivative plasmid containing AcMNPV polyhedrin region sequences. The large white arrow represents the polyhedrin promoter and the direction of transcription. The polyhedrin gene is encoded by bases 4093-4825. ORF1629 (Open Reading Frame) is encoded by bases 6491-4864 and is transcribed in a direction opposite to that of the polyhedrin gene (black arrow). Amp^R denotes sequences coding for an ampicillin resistance gene.

Fig. 5-1. Construction and map of pVL-1392-AC2



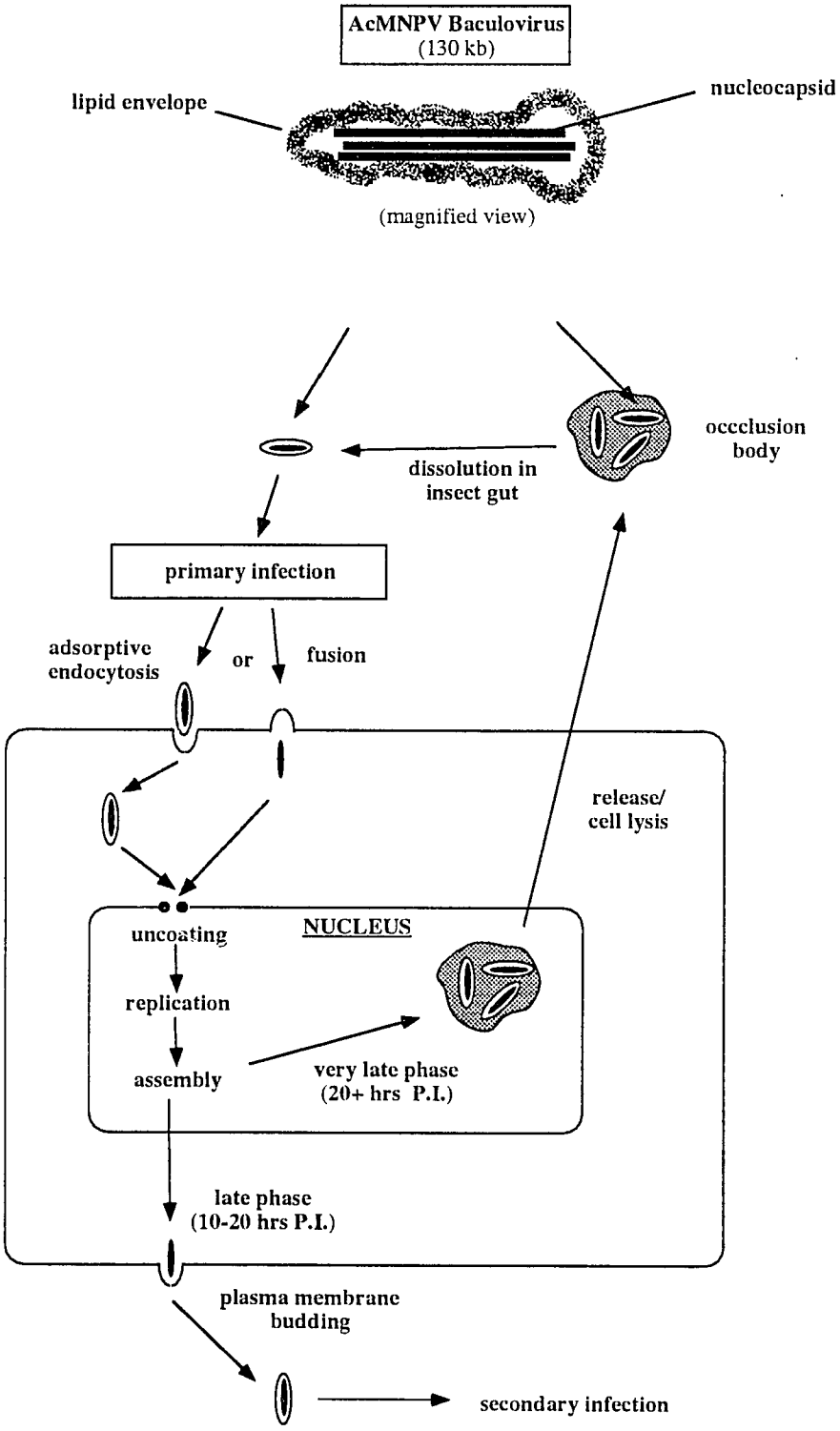
5.2) Expression of adenylyl cyclase 2 in Sf9 cells

The baculovirus/Sf9 cell system was used to express adenylyl cyclase 2. Baculoviruses are double-stranded enveloped DNA viruses with a narrow host range limited to arthropods. The family name *baculo* refers to their rod shaped nucleocapsids. The AcMNPV baculovirus life cycle is illustrated in Fig. 5-2. Baculoviruses enter insect cells by adsorptive endocytosis or fusion. After entry into the cytoplasm, nucleocapsids interact with nuclear pores to enter the nucleus and uncoat. The early phase of infection (6 hrs Post Infection or P.I.) is characterized by viral gene expression and host cytoplasmic and nuclear rearrangements. During the late phase (6-24 hrs P.I.), viral DNA replication, late gene expression and logarithmic virion production take place. Nucleocapsids assemble in the nucleus and bud from the plasma membrane. In the very late phase (20+ hrs P.I.), nucleocapsids become enveloped in the nucleus and are embedded in multiples within occlusion bodies, made of a crystalline matrix composed almost entirely of polyhedrin. Cell lysis begins about 3 days post infection, releasing accumulated occlusion bodies. In the natural infection cycle of AcMNPV, insect larvae ingest occlusion bodies in their food. Polyhedrin is solubilized in the alkaline mileu of the insect gut and virions fuse with midgut cells. After replication in the midgut, new virons bud into the hemolymph and therby gain access to other cells in the insect. While important in the preservation and transmission of AcMNPV *in vivo*, the polyhedrin

Figure 5-2. Life cycle of AcMNPV

AcMNPV virions are composed of multiple nucleocapsids enveloped within a lipid membrane (top of figure, "magnified view"). As occlusion bodies dissolve in the insect gut, AcMNPV virions are released and primary infection is initiated. Virus entry occurs through adsorptive endocytosis or fusion with the plasma membrane. Nucleocapsids are transported to the nucleus where they uncoat. At 10-20 hours P.I. (Post Infection) AcMNPV virions are rapidly produced and virion budding occurs through the plasma membrane. Beyond 20 hours P.I., virions acquire their lipid envelope from nuclear membranes and are encasulated in occlusion bodies, composed almost entirely of polyhedrin. With death of the host insect, occlusion bodies are released to the environment where they can be ingested by another insect to initiate the next round of infection.

Figure 5-2. **Life cycle of AcMNPV**



gene is not required for the infection cycle in tissue culture. Hence, in the construction of the AC2 recombinant baculovirus, the polyhedrin gene was mutated and used to permit homologous recombination with pVL-1392-AC2. The polyhedrin promoter was used to express AC2 since during infection with wild type virus, polyhedrin protein can account for 30-50% of cell protein.

The baculovirus system was selected for its capacity for high level expression of exogenous genes. While it was not possible to predict the levels of expression of AC2, the baculovirus system has been used to express other mammalian membrane-spanning proteins in levels superior to those achieved in mammalian expression systems. Baculovirus-mediated expression is also easier to perform and reproduce consistently compared with transient transfection of mammlian cells. Furthermore, Sf9 cells can be grown in suspension, thereby facilitating large-scale production of the desired protein.

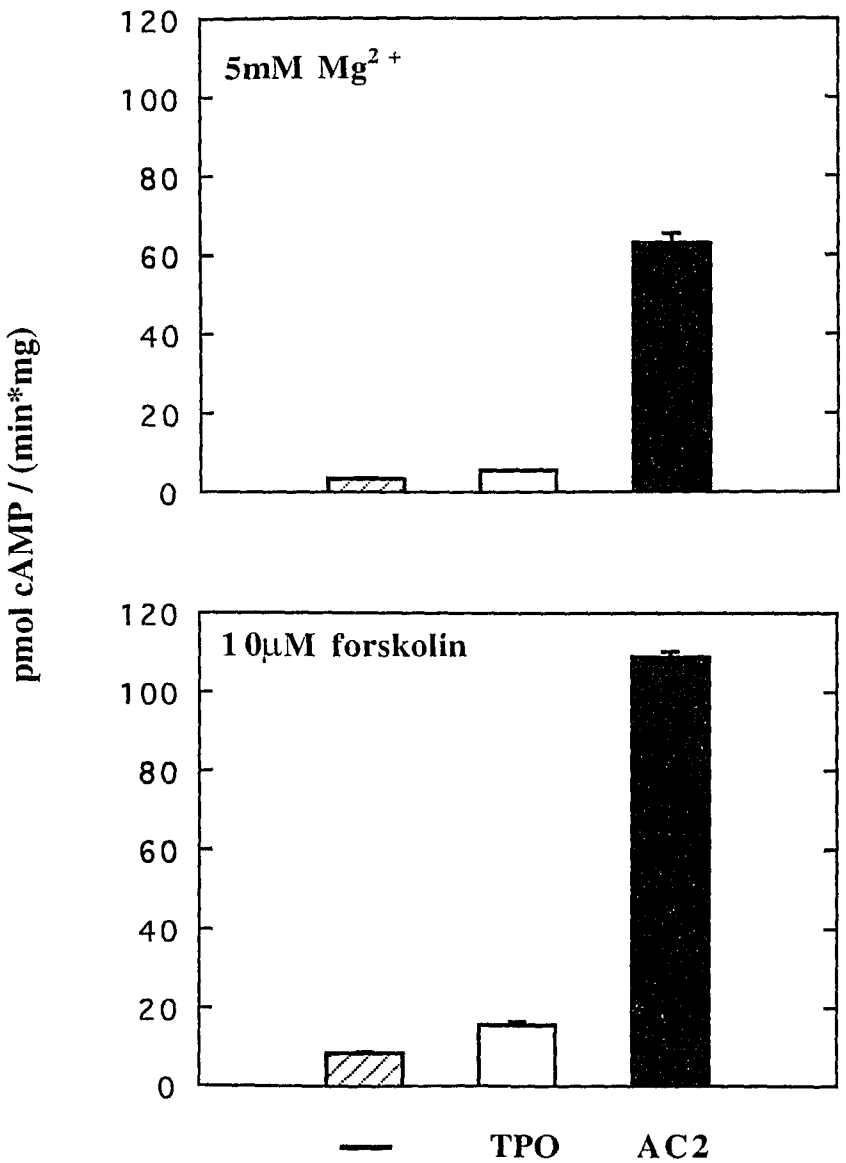
Expression of AC2 in Sf9 cells resulted in 8-25 fold increase of the basal adenylyl cyclase activity in Sf9 membranes as compared to uninfected cells or cells infected with baculovirus containing the thyroid peroxidase gene (Fig. 5-3, top). Forskolin-stimulated activities were elevated from 3-15 fold in AC2 expressing membranes, typically about 7 fold (Fig. 5-3, bottom). The extensive stimulation of adenylyl cyclase activities in the AC2-expressing Sf9 cell membranes indicated that the type 2 enzyme was produced in a functional form and properly

compartmentalized in the Sf9 cells. Furthermore, fold stimulation of adenylyl cyclase activities was greater in AC2-expressing Sf9 cells than in mammalian HEK-293 cells (compare with Fig. 4-3).

Figure 5-3. Adenylyl cyclase activities in AC2-expressing Sf9 membranes:

Basal and forskolin-stimulated adenylyl cyclase activities were measured in the presence of 5mM Mg^{2+} in membranes of uninfected (hatched bars), TPO baculovirus-infected (white bars), or AC2-baculovirus-infected (black bars) Sf9 cells. Assays were performed for 15 min as described under "Materials and Methods". Abbreviations: TPO, thyroid peroxidase; AC2, adenylyl cyclase 2.

Figure 5-3. Adenylyl cyclase activities in AC2-expressing Sf9 membranes



5.3) Protein kinase C dependence of PMA enhancement of adenylyl cyclase 2 activity in Sf9 cells

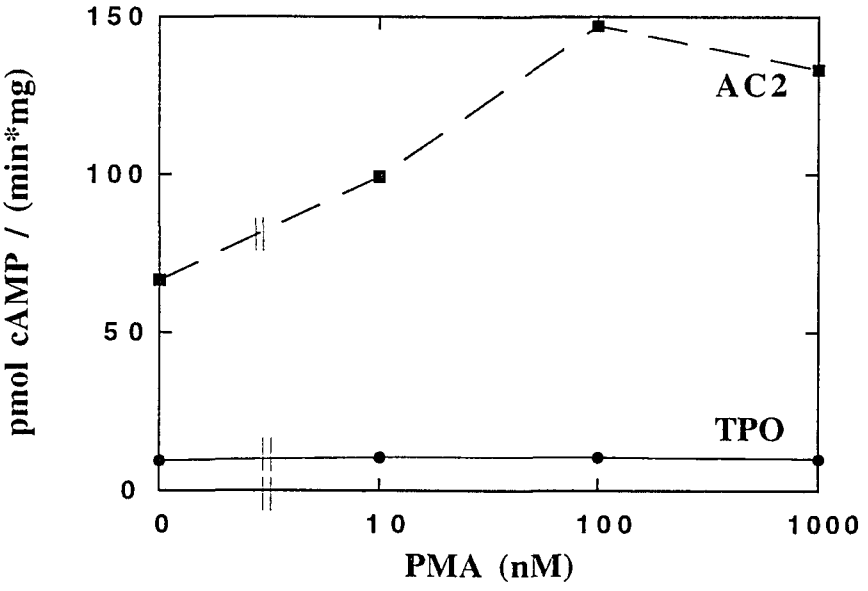
Sf9 cells are capable of performing most mammalian post-translational modifications including serine/threonine and tyrosine phosphorylation (O'Reilly et al 1992, pp 216-236). Hence, I reasoned that PMA treatment of Sf9 cells may reproduce the enhancement of AC2 activity observed in HEK-293 cells. If the Sf9 cells or AcMNPV baculovirus did not contain a PMA-responsive isozyme capable of enhancing the type 2 activity, Sf9 membranes would have been treated with rat brain PKC.

Sf9 cells were treated with the phorbol ester PMA to activate their endogenous protein kinase(s) C and test the effect on adenylyl cyclase 2 activity. In Fig. 5-4, the effect of PMA dosage on basal AC2 activity in Sf9 membranes is displayed. The greatest enhancement (2.5 fold) of AC2 activity was attained by 100nM PMA. Thus, both the HEK-293 PKC isozyme(s) and the Sf9 or AcMNPV PKC(s) were capable of enhancing AC2 activity. In subsequent experiments, PMA was used at 1μM to ensure maximal effects.

Figure 5-4. Effect of PMA dosage on AC2 basal activity in Sf9 membranes:

Basal adenylyl cyclase activities was measured in the presence of 5mM Mg^{2+} in membranes of TPO baculovirus-infected (●) or AC2-baculovirus-infected (■) Sf9 cells. Cells were treated with the indicated concentrations of PMA for 20 minutes. Assays were performed as described under "Materials and Methods".

Figure 5-4. Effect of PMA dosage on AC2 basal activity in Sf9 membranes



The specificity of PMA activation of PKC in Sf9 cells was tested against treatment with the inactive 4α phorbol ester, phorbol 12, 13 didecanoate (PDD). PMA treatment of Sf9 cells resulted in 2.5-3 fold enhancement of AC2 activities in membranes compared to untreated or PDD-treated cells (Fig. 5-5). Thus only the PKC activator, PMA, was capable of stimulating AC2 activity. Furthermore, staurosporine, which inhibits PKC, abolished the PMA-mediated enhancement of AC2 activity (Fig. 5-6).

Taken together, the PMA dose dependence, PDD independence and staurosporine inhibition of the increase in AC2 activity are consistent with protein kinase C activation mediating the enhancement of AC2 function.

Figure 5-5. Effect of PMA or PDD treatment on AC2 activity in Sf9 membranes:

Basal and forskolin-stimulated adenylyl cyclase activities were measured in the presence of 5mM Mg^{2+} in membranes of AC2-baculovirus-infected Sf9 cells. Cells were treated for 20 minutes with 1μM PMA (black bars), 1μM PDD (white bars), or DMSO vehicle (hatched bars) prior to preparation of membranes. Assays were performed as described under "Materials and Methods".

Figure 5-5. Effect of PMA or PDD treatment on AC2 activity in Sf9 membranes

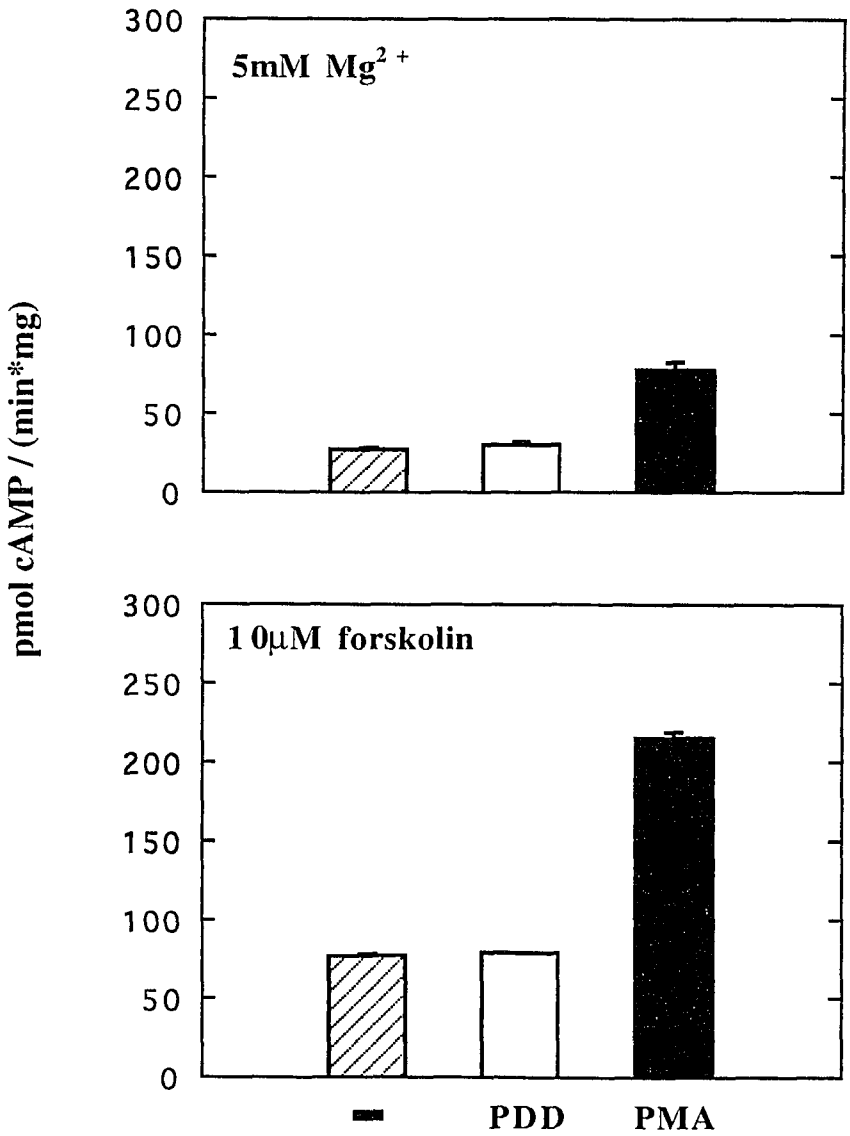
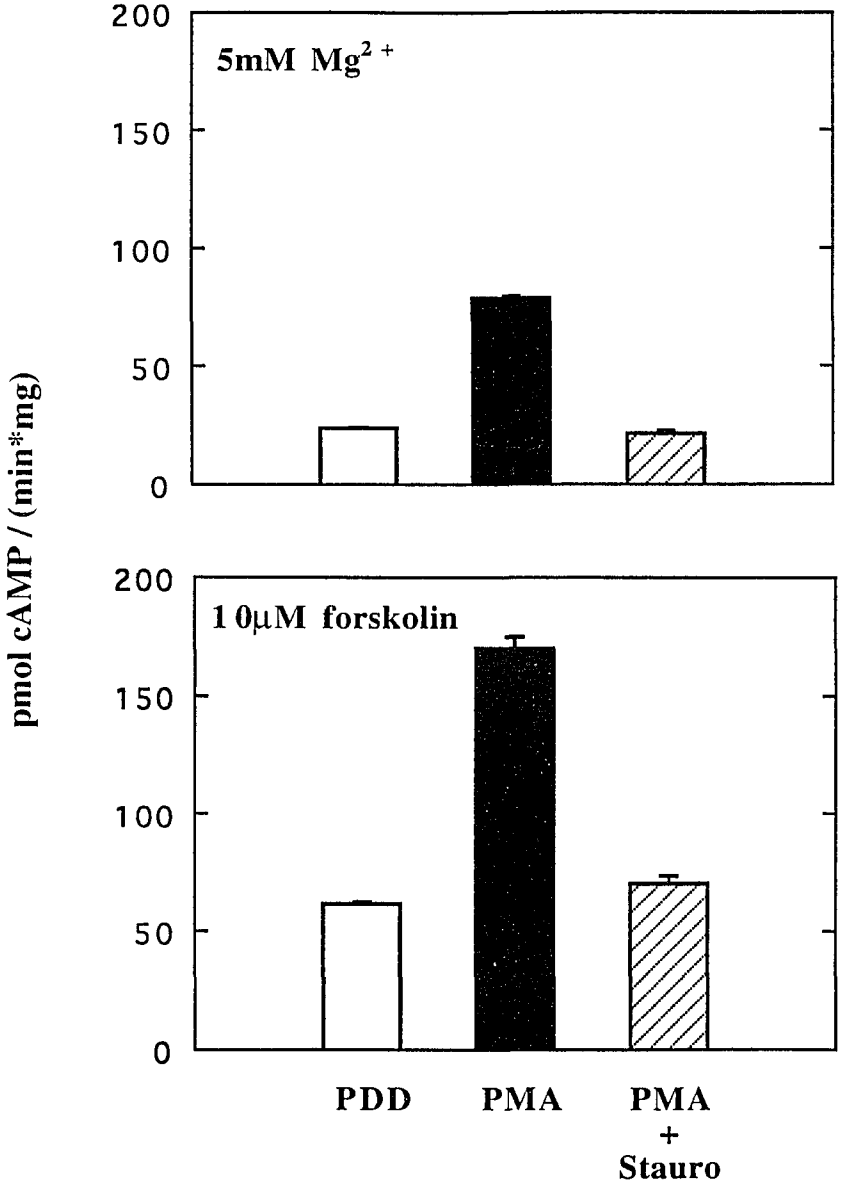


Figure 5-6. Effect of staurosporine on PMA-mediated enhancement of AC2 activity in Sf9 membranes:

Basal and forskolin-stimulated adenylyl cyclase activities were measured in the presence of 5mM Mg^{2+} in membranes of AC2-baculovirus-infected Sf9 cells. Cells were treated for 20 minutes with 1μM PDD (white bars), 1μM PMA (black bars) or a combination of 1μM PMA and 1μM staurosporine (hatched bars). For the PMA + staurosporine group, cells were incubated for 15 minutes with 1μM staurosporine before the twenty minute incubation in 1μM PMA (PMA+stauro). Assays were performed as described under "Materials and Methods".

Figure 5-6. Effect of staurosporine on PMA-mediated enhancement of AC2 activity in Sf9 membranes



5.4) Adenylyl cyclase 2 can be independently stimulated by activation of PKC

Independent stimulators of adenylyl cyclase activity, such as G_S-α or forskolin activate adenylyl cyclase by increasing its V_{max}. To determine whether PKC activation can also independently stimulate AC2, I examined whether PKC activation alters the V_{max} and/or K_m of the type 2 adenylyl cyclase. The V_{max} and K_m of AC2 in untreated and PMA-treated membranes were determined by assaying basal adenylyl cyclase activity in AC2-expressing Sf9 membranes in the presence of 20-500 μM ATP. The Lineweaver-Burke transformation of the data from this experiment is presented in Fig. 5-7. As determined from the linear transformation, the V_{max} of AC2 increased 2.5 fold as a result of PMA treatment of the cells (from 49.2 to 126.8 pmol cAMP/(mg*min). The K_m was virtually unaltered, decreasing by 20% with PMA treatment of the cells. These results are mechanistically similar to those obtained for other stimulators of adenylyl cyclase activity.

Stimulation of AC2 by PKC activation was tested over the range of 0.5 mM to 20mM Mg^{2+} (Fig. 5-8, top panel). The greatest fold stimulation, about three fold, was observed at low Mg^{2+} concentrations at which most of the G_s proteins are not activated. With increasing Mg^{2+} concentrations, basal activities in both untreated and PMA-treated membranes increased, but fold stimulation due to PMA treatment decreased (Fig. 5-8, bottom

Figure 5-7. Effect of PMA treatment on V_{max} and K_m of AC2 in Sf9 membranes:

Basal adenylyl cyclase activity was assayed in membranes of AC2-baculovirus-infected cells. Cells were treated with 1μM PMA "AC2(PMA)" or without PMA "AC2". Adenylyl cyclase activity was assayed as described under "Materials and Methods" in the presence of 5mM Mg^{2+} and 20-500μM ATP. The figure represents a Lineweaver-Burke linear transformation of the data. *Inset*: V_{max} and K_m determined from the slopes and Y intercepts of the lines according to

$$1/V = (1/V_{max}) + (K_m / V_{max}) * (1/[S])$$

where [S] is the ATP concentration and V is the empirically determined velocity..

Figure 5-7. Effect of PMA treatment on V_{max} and K_m of AC2 in Sf9 membranes

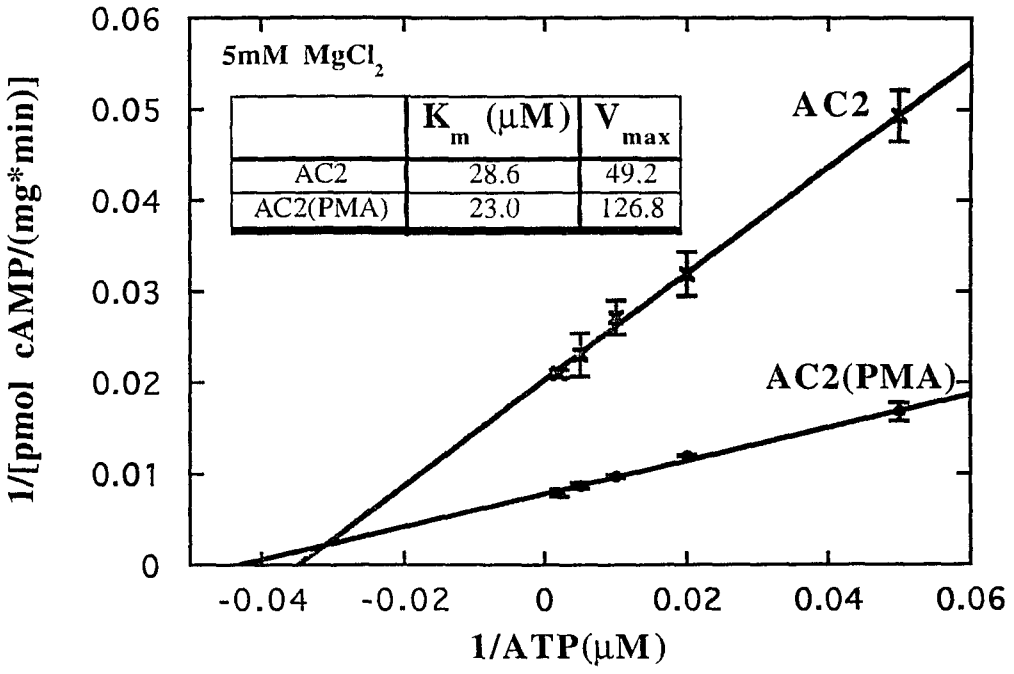
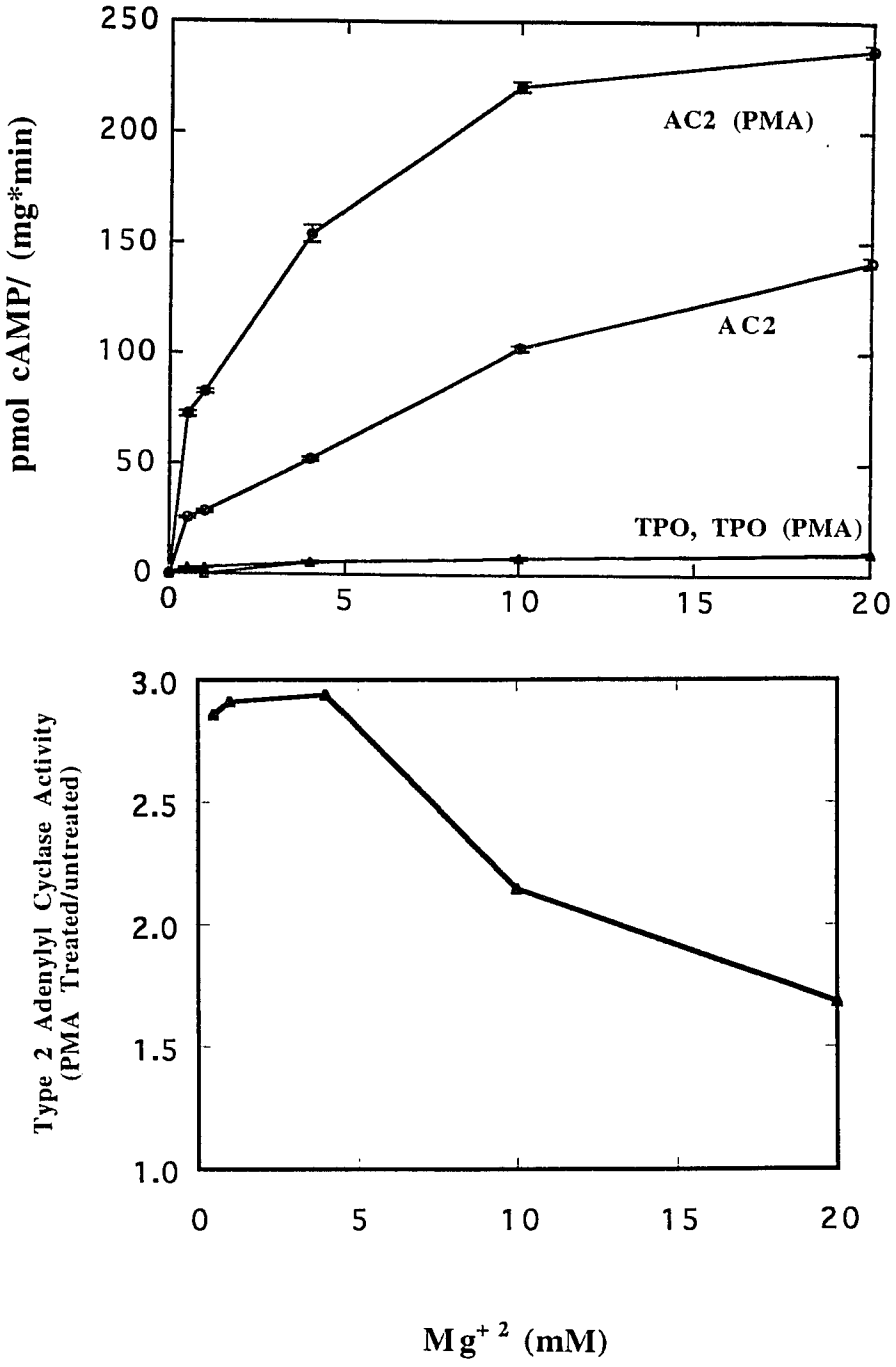


Figure 5-8. Effect of Mg^{2+} concentration on enhancement of AC2 activity by PMA treatment:

Basal adenylyl cyclase activity was assayed in membranes of TPO-baculovirus-infected and AC2-baculovirus-infected cells over the indicated Mg^{2+} concentrations. Cells were treated with or without 1μM PMA. Adenylyl cyclase activity was assayed as described under "Materials and Methods" and plotted (top panel). The ratio of the activity in PMA-treated to untreated AC2 membranes was plotted against each Mg^{2+} concentration (bottom panel).

Figure 5-8. Effect of Mg^{2+} concentration on enhancement of AC2 activity by PMA treatment



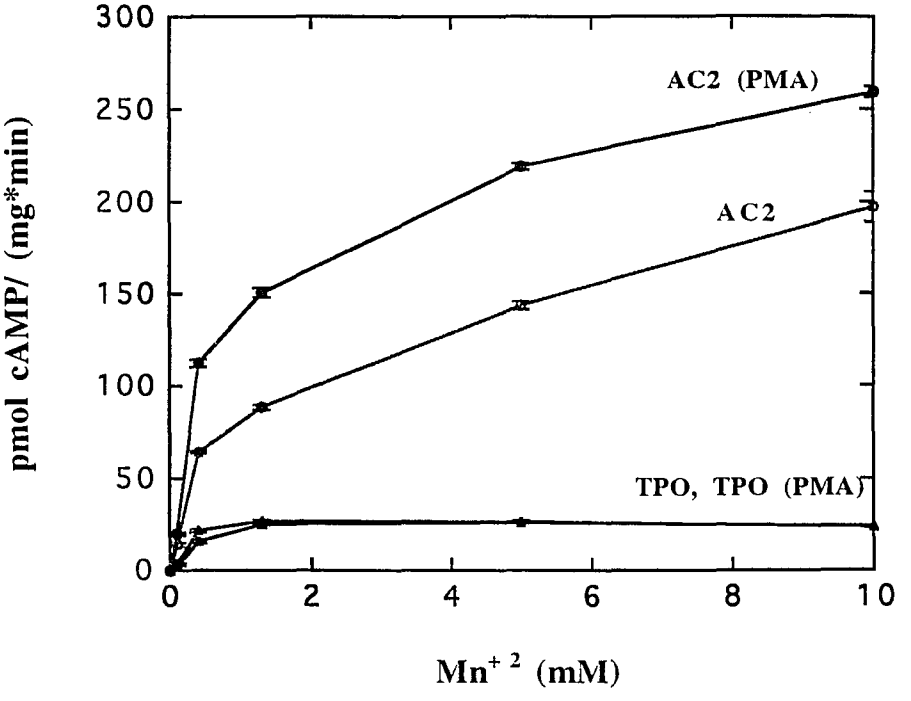
panel). These data are similar to those obtained in AC2-transfected HEK-293 cells.

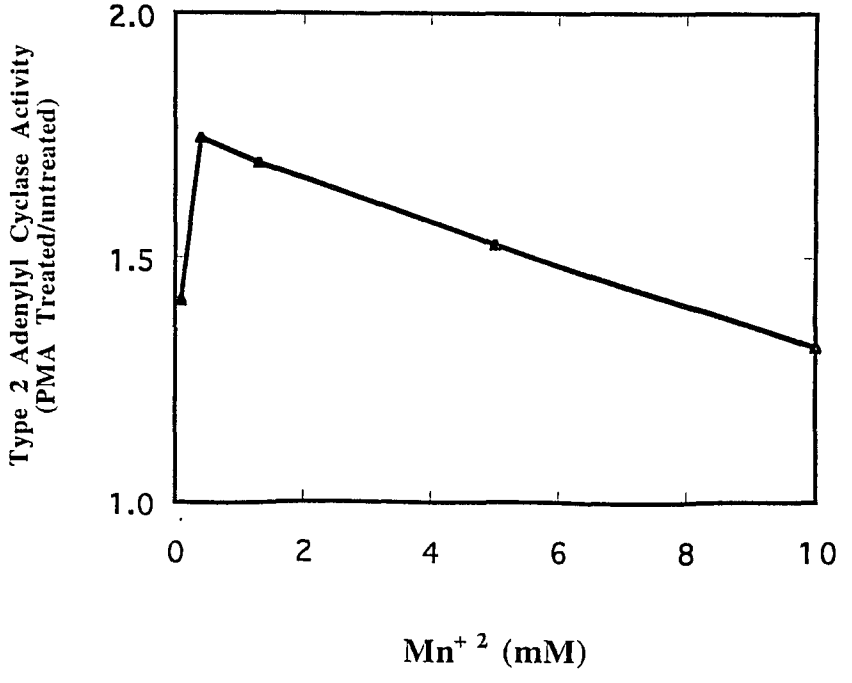
The allosteric stimulation of adenylyl cyclase by Mn^{2+} has been used to assess catalytic activity of the enzyme. PMA treatment of Sf9 membranes resulted in 1.5-1.75 fold enhancement of the Mn^{2+}-stimulated activity (Fig. 5-9). Since the stimulation of adenylyl cyclase by Mn^{2+} is direct, the enhancement of AC2 activity by PKC activation appears to be due to a modification of adenylyl cyclase 2 itself.

Figure 5-9. Effect of Mn^{2+} concentration on enhancement of AC2 activity by PMA treatment:

Mn^{2+}-stimulated adenylyl cyclase activity was assayed in membranes from TPO-baculovirus-infected and AC2-baculovirus-infected cells over the indicated Mn^{2+} concentrations. Cells were treated with or without 1μM PMA. Adenylyl cyclase activity was assayed as described under "Materials and Methods" and plotted (top panel). The ratio of the activity in PMA-treated to untreated AC2 membranes was plotted against each Mn^{2+} concentration (bottom panel).

Figure 5-9. Effect of Mn^{2+} concentration on enhancement of AC2 activity by PMA treatment





5.5) PKC activation potentiates G protein stimulation of adenylyl cyclase 2

In the previous section, it was shown that PKC activation can independently stimulate AC2, as assayed by direct stimulation with Mg^{2+} and Mn^{2+}. Next, I examined whether adenylyl cyclase 2 can integrate signals routed through PKC with the G_s pathway. Hence, I tested the effects of PKC activation on G protein stimulation of adenylyl cyclase 2. In Fig. 5-10, the effects of PMA treatment on the Mg^{2+}, fluoride and forskolin-stimulated activities of AC2-expressing and TPO-expressing Sf9 membranes are compared. Expression of AC2 in Sf9 cells resulted in prominent elevation of adenylyl cyclase activities over activities in the TPO-expressing group. PMA treatment resulted in 2-3 fold enhancement of forskolin and Mg^{2+}-stimulated AC2 activities and a 50-60% elevation of the fluoride-stimulated adenylyl cyclase activity. At the concentrations used, forskolin stimulated AC2 more extensively than fluoride in untreated membranes but in PMA-treated membranes AC2 was stimulated to the same extent by fluoride and forskolin. By contrast in TPO-expressing cells, fluoride was a more extensive stimulator of Sf9 adenylyl cyclase activity than (10μM) forskolin.

Fluoride is an activator of heterotrimeric G proteins and not a specific activator of G_s. Thus, in order to assess the effect of PKC activation on G_s stimulation of AC2, G_s-α Q227L was synthesized *in vitro* and reconstituted with Sf9 membranes. The

Q227L mutant G_S-α (α_S^*) has a very low GTPase activity compared to wildtype α_S. Thus, the persistently activated, α_S^* would readily stimulate adenylyl cyclase in the presence of GTP. In addition, G_S stimulation of AC2 could now be assessed with defined concentrations of the recombinant *mammalian* α_S.

Q227L-α_S was synthesized from cDNA template by myself or J. Codina (Baylor College of Medicine) using the TnT kit (Promega). Briefly, cDNA template for α_S^* was transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate in one reaction. [^{35}S]methionine was included in order to quantitate and monitor the α_S^* protein synthesized. The reaction product was run on a 10% SDS-PAGE gel. Fig. 5-11 is an autoradiogram of the dried gel. A 42kDa band, corresponding to the size of α_{S4} is seen in the appropriate lane.

The effect of PMA treatment on stimulation of AC2 by α_S^* was examined (Fig. 5-12). α_S^* stimulated adenylyl cyclase 2 activity in Sf9 membranes up to 4 fold over basal activity. Maximal stimulation was attained at 5nM α_S^*. PKC activation resulted in 2.5-3.5 fold potentiation of α_S^* stimulation of adenylyl cyclase 2. Potentiation was more prominent at α_S^* concentrations below 5nM.

Adenylyl cyclase 2 can be synergistically stimulated by α_S and G protein $\beta\gamma$ subunits. The effect of PKC activation on the synergistic stimulation of AC2 by α_S and G protein $\beta\gamma$ subunits

Figure 5-10. Effect of PMA treatment on Mg^{2+}, fluoride and forskolin-stimulated AC2 activities:

Basal, fluoride, and forskolin-stimulated adenylyl cyclase activities were measured in the presence of 5mM Mg^{2+} in membranes of TPO-baculovirus-infected and AC2-baculovirus-infected Sf9 cells. Cells were treated for 20 minutes with 1μM PMA (black bars) or DMSO vehicle (hatched bars) prior to preparation of membranes. Assays were performed as described under "Materials and Methods".

Figure 5-10. Effect of PMA treatment on Mg^{2+}, fluoride and forskolin-stimulated AC2 activities

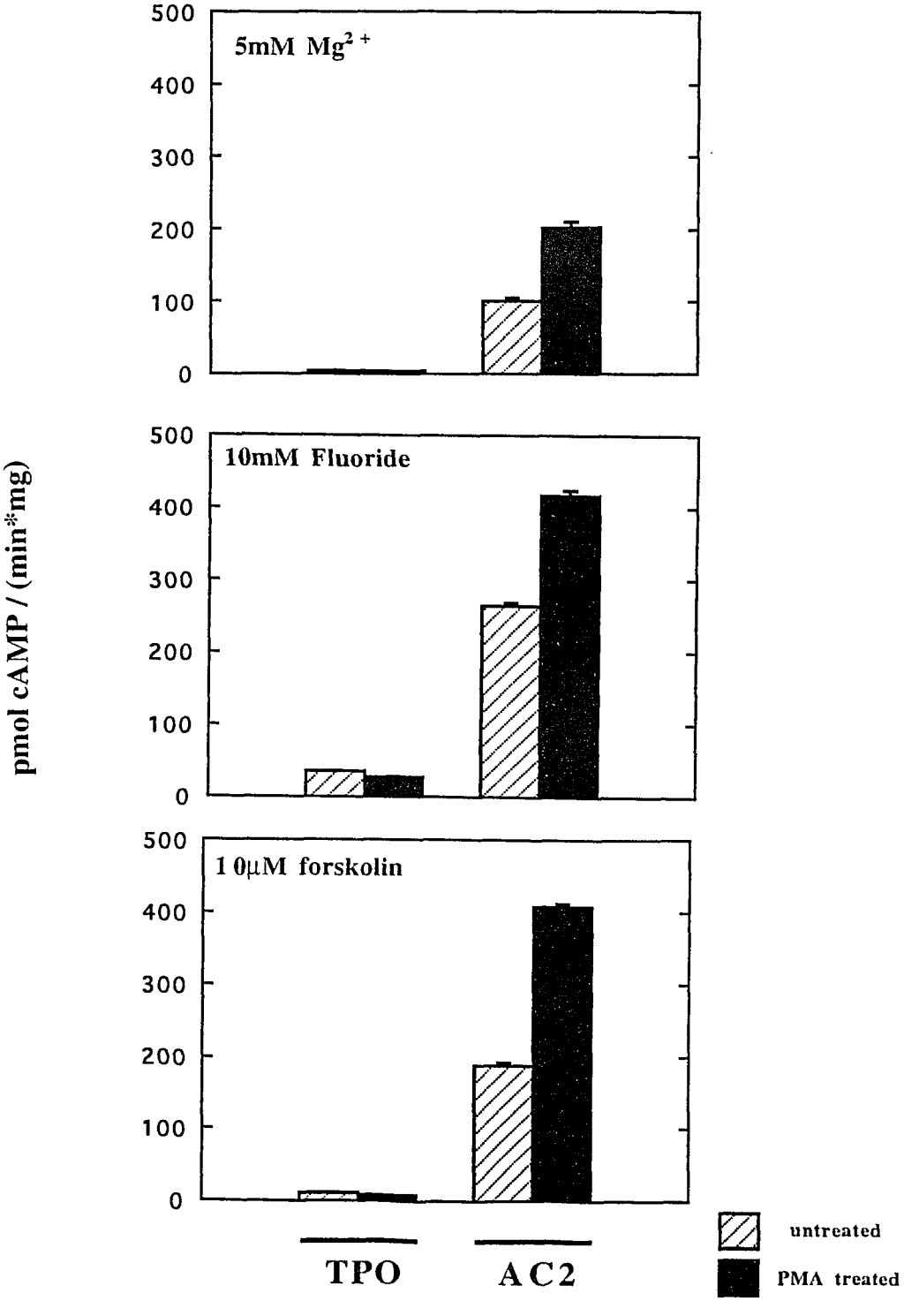


Figure 5-11. Electrophoretic resolution of *in vitro* synthesized Q227L-α_s^*:

The cDNA for Q227L-α_{s4}^* was transcribed and translated according to the TnT coupled transcription/translation kit from Promega. One μg DNA was used per 50μl of total reaction volume. [^{35}S]methionine (40μCi/50μl reaction) and methionine (1mM) were included in the reaction mix. Incubation was performed at 30°C for 2 hrs after which products were aliquoted and frozen at -70°C. Fifteen μl of each sample were analyzed by SDS-PAGE on a 10% gel. The gel was vacuum dried and exposed to X-AR film for 48 hrs.

(-), no DNA included in reaction.
(α_s^*), Q227-α_{s4} DNA included in reaction

Figure 5-11. **Electrophoretic resolution of *in vitro* synthesized Q227L-α_s*

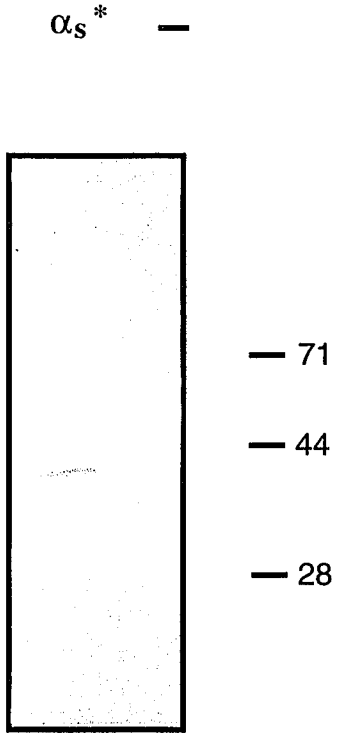
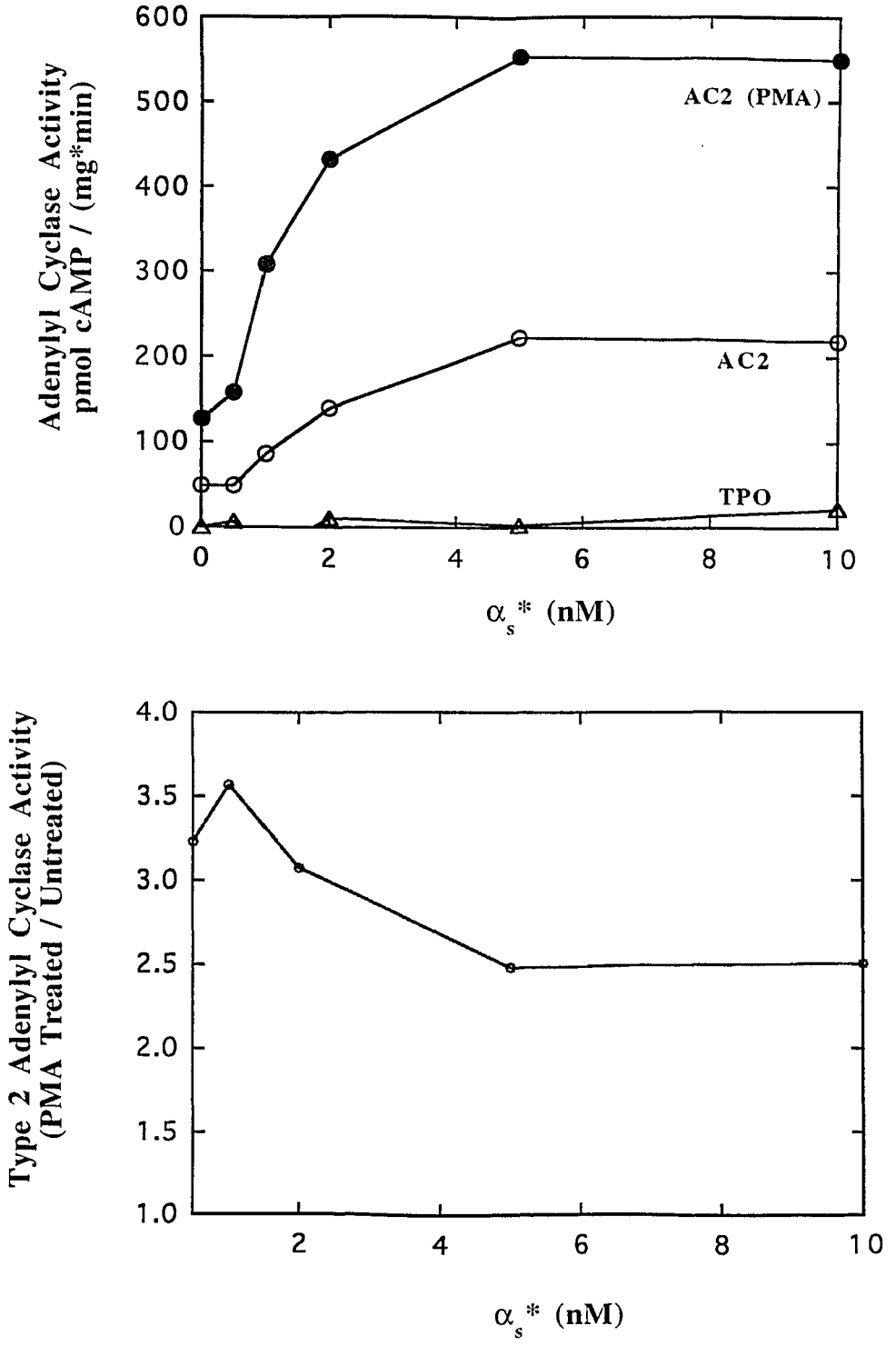


Figure 5-12. Effect of PMA treatment on stimulation of AC2 by α_s^*:

Q227-α_s^*-stimulated adenylyl cyclase activity was assayed in membranes from TPO-baculovirus-infected and AC2-baculovirus-infected cells over the indicated α_s^* concentrations. Cells were treated with or without 1μM PMA prior to membrane preparation. Adenylyl cyclase activity was assayed in the presence of 5mM Mg^{2+} as described under "Materials and Methods". The activities from membranes stimulated with reticulocyte lysate product from mock (no DNA added) TnT reactions were subtracted from the α_s^*-stimulated activities to obtain the values shown (top panel). The ratio of the activity in PMA-treated to untreated AC2 membranes was plotted at each α_s^* concentration (bottom panel).

Figure 5-12. **Effect of PMA treatment on stimulation of AC2 by α_s***



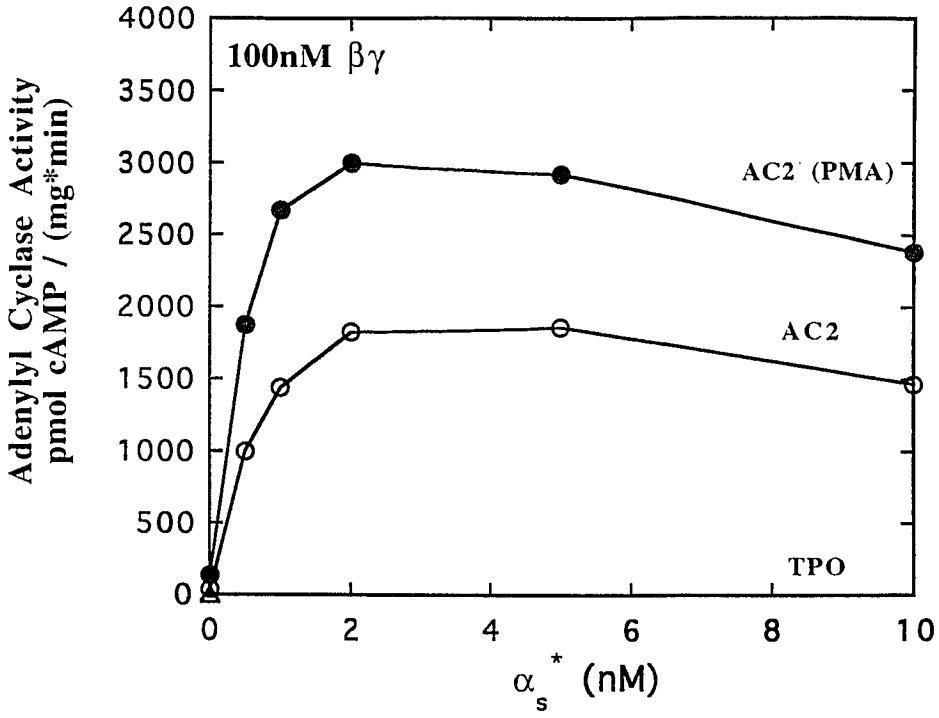
was examined (Fig. 5-13). $\beta\gamma$ subunits from bovine brain (D. Carty, Mount Sinai) were used at 100nM concentration for maximal effects. Adenylyl cyclase activity in AC2-expressing membranes was stimulated 7-20 fold with $\alpha_s^*+\beta\gamma$ over stimulation with only α_s^*. The greatest fold stimulation occurred at the lowest α_s^* concentrations tested. PMA-treated membranes were stimulated 4-12 fold with $\alpha_s^* + \beta\gamma$ as compared to α_s^* stimulation alone. PMA treatment of the expressed AC2 resulted in about 60-90% enhancement of the $(\alpha_s^* + \beta\gamma)$-stimulated activity. The greatest enhancements were observed at the lowest α_s^* concentrations.

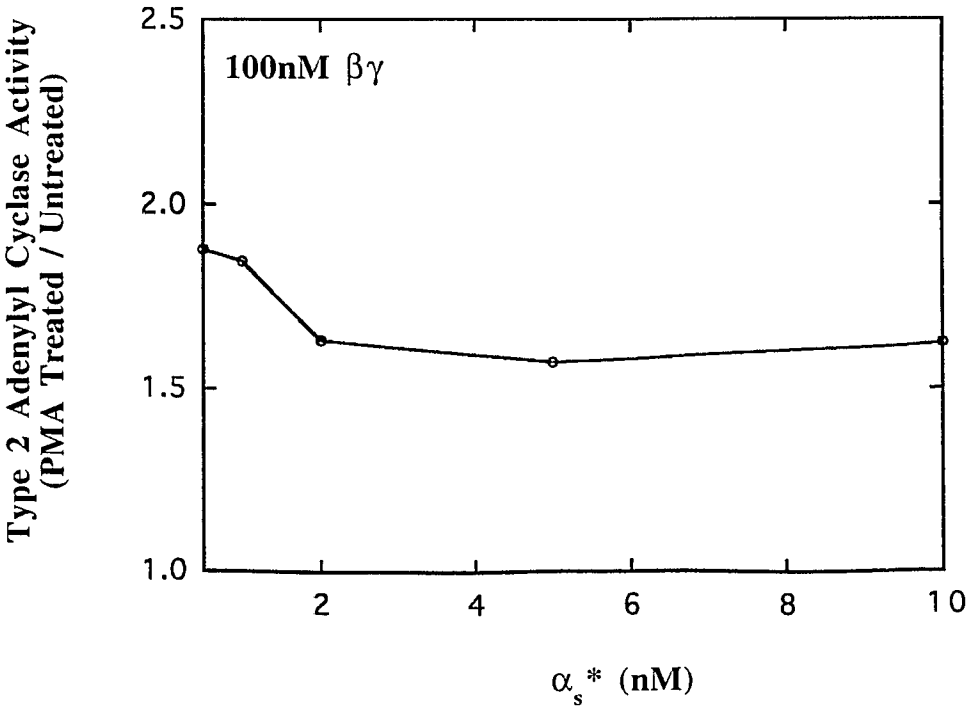
The effect of PKC activation on adenylyl cyclase 2 G protein-stimulated activity was examined above. PKC activation potentiated G protein stimulation of AC2 whether assessed by fluoride stimulation of Sf9 cell G proteins or stimulation with recombinant, mammalian, persistently-activated α_s. The potentiation was most pronounced (3 fold) for stimulation with recombinant α_s at low nM concentrations. Synergistic stimulation of AC2 by α_s^* and $\beta\gamma$ was also enhanced by PKC activation. Hence, Adenylyl cyclase 2 appears to be a locus at which G_s-routed and PKC-routed signals may be integrated.

Figure 5-13. Effect of PMA treatment on stimulation of AC2 by $\alpha_s^* + \beta\gamma$:

Adenylyl cyclase activity was assayed in membranes from TPO-baculovirus-infected and AC2-baculovirus-infected cells over the indicated α_s^* concentrations in the presence of 100nM bovine brain G protein $\beta\gamma$ subunits. Cells were treated with or without 1μM PMA prior to membrane preparation. Adenylyl cyclase activity was assayed in the presence of 5mM Mg^{2+} as described under "Materials and Methods". The activities from membranes stimulated with reticulocyte lysate product from mock (no DNA added) TnT reactions were subtracted from the $\alpha_s^* + \beta\gamma$-stimulated activities to obtain the values shown (top panel). The ratio of the activity in PMA-treated to untreated AC2 membranes was plotted at each α_s^* concentration (bottom panel).

Figure 5-13. Effect of PMA treatment on stimulation of AC2 by $\alpha_s^*+\beta\gamma$





CHAPTER 6

PURIFICATION AND ANALYSIS OF ADENYLYL CYCLASE 2 FROM PHORBOL ESTER-TREATED SF9 CELLS

6.1) Expression of adenylyl cyclase 2 in Sf9 cells

The simplest explanation for the enhancements of adenylyl cyclase 2 activity depicted in chapters 4 and 5 is that the adenylyl cyclase 2 isoform was phosphorylated in response to activation of protein kinase C. To assess whether phosphorylation occurred, I attempted to isolate the adenylyl cyclase 2. expressed in Sf9 cells by baculovirus infection.

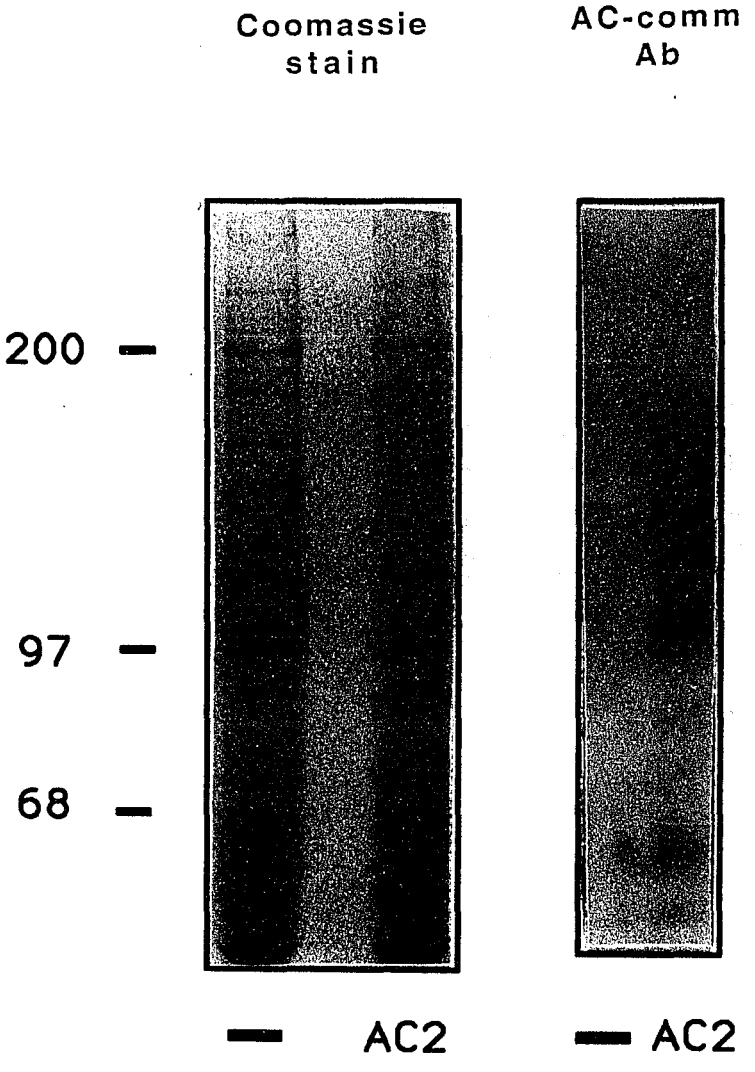
Expression of AC2 in Sf9 cells resulted in a robust elevation of adenylyl cyclase activities in membranes (Fig. 5-3), though adenylyl cyclase 2 was not expressed at a level sufficient for detection by Coomassie staining of Sf9 membrane proteins (Fig. 6-1, left panel). Expression of adenylyl cyclase 2 was confirmed by Western blotting of Sf9 membrane proteins with AC-comm, an antiserum made against a peptide sequence common to all the cloned adenylyl cyclases (Fig. 6-1, right panel). AC-comm specifically recognized a 106 kDa band in AC2-expressing but not in TPO-expressing Sf9 membranes. Therefore, it appears that AC2 was not expressed at sufficiently high levels by recombinant baculovirus infection to permit characterization of its phosphorylation state without extensive purification.

Figure 6-1. Expression of AC2 in Sf9 membranes:

Coomassie stain (left panel): 100μg of membrane protein from TPO-baculovirus (-) and AC2-baculovirus (AC2) infected cells were electrophoresed through a 7% SDS-polyacrylamide gel and stained in 0.1% Coomassie blue.

AC-comm (right panel): Immunoblot of membrane protein from TPO-baculovirus (-) and AC2-baculovirus (AC2) infected cells. 10μg of membrane protein were electrophoresed through a 7% SDS-polyacrylamide gel, transfered to nitrocellulose and probed with AC-comm antisera, made against a peptide sequence common to all adenylyl cyclases. Immunoblot procedures and AC-comm sequence are described in "Materials and Methods".

Figure 6-1. Expression of AC2 in Sf9 membranes



6.2) Construction of FLAG-tagged adenylyl cyclase 2 (F-AC2)

Purification of adenylyl cyclase 2 by conventional methods such as forskolin affinity chromatography could have resulted in co-purification of endogenous Sf9 adenylyl cyclases, thereby potentially confounding the interpretation of the phosphorylation study results. Hence, I decided to tag the adenylyl cyclase 2 isoform with FLAG, an eight amino acid epitope (DYKDDDDK) which could then be used to specifically purify AC2 by immunoaffinity chromatography on anti-FLAG agarose (Kodak-IBI).

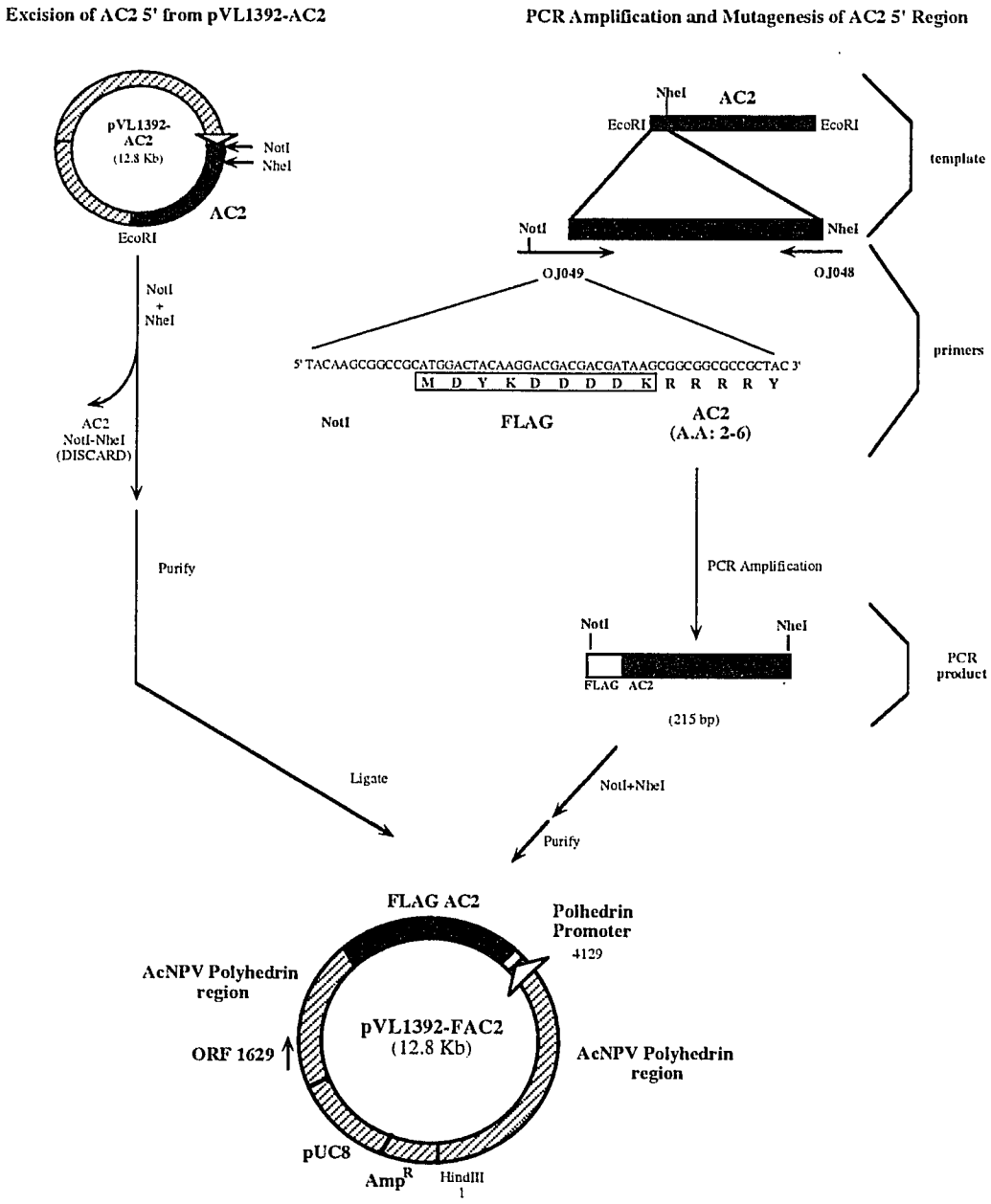
The FLAG epitope was inserted by site-directed mutagenesis into pVL-1392-AC2 immediately after the initiator methionine codon of AC2 (Fig. 6-2). For this purpose, the PCR primer OJ049 was designed to contain a NotI restriction site and the codons for methionine, the FLAG epitope, and amino acids 2-6 of AC2. The reverse primer, OJ048, spanned the NheI restriction site in the AC2 sequence. Using OJ049 and OJ048 for PCR amplification, I obtained a 215 bp product which was inserted in place of the NotI to NheI segment (the 5' end) of AC2 in pVL-1392-AC2. The resultant plasmid was named pVL-1392-FAC2. The pcr product and its insertion sites in pVL-1392-FAC2 were sequenced to verify that no errors had been made. Recombinant baculovirus was obtained as before by transfection of Sf9 cells with pVL-1392-FAC2 and BaculoGold DNA followed by clonal isolation of virus (R. Magnusson).

Figure 6-2. Construction of pVL1392-FAC2:

Excision of AC2 5' from pVL1392-AC2: pVL1392-AC2 was digested with NotI and NheI to excise the 5' region of AC2. The DNA was then dephosphorylated with calf intestine alkaline phosphatase and run on a 0.8% agarose gel. pVL1392-AC2 (-5') was purified by centrifugation of the gel slice through siliconized glass wool followed by phenol extraction and ethanol precipitation of the DNA.

PCR amplification and mutagenesis of AC2 5' region: The 5' region of AC2 was amplified with primers OJ048 and OJ049 whose sequences contain an NheI site and the FLAG epitope codons, respectively. The PCR product (215 bp) was digested with NheI and NotI, run on a 1.8% agarose gel and purified as above. The purified DNA was ligated into pVL1392-AC2 (-5') from NotI to NheI. Recombinants were screened by restriction digests and verified by sequencing the insertion sites.

Figure 6-2. Construction of pVL1392-FAC2



6.3) Expression and purification of FLAG-tagged adenylyl cyclase 2 in Sf9 cells

The FLAG-tagged AC2 (F-AC2) was expressed in Sf9 cells. Though the eight amino-acid FLAG was placed at the N-terminus of adenylyl cyclase 2, a region not conserved among the cloned mammalian adenylyl cyclases, it was still possible that addition of the FLAG epitope would be deleterious to the enzyme's function. Thus, the basal activity of AC2 and AC2-expressing Sf9 membranes were compared. Expression of F-AC2 resulted in adenylyl cyclase activity in Sf9 membranes comparable to that of AC2 (Fig. 6-3). I also tested whether free FLAG peptide would affect adenylyl cyclase 2 activity in Sf9 membranes (Fig. 6-4). FLAG peptide did not affect adenylyl cyclase activity even at the highest concentration tested (100 μM).

Since incorporation of the FLAG epitope did not interfere with the enzymatic activity of adenylyl cyclase 2, I then attempted to purify the FLAG-tagged isoform. Sf9 cells infected with the F-AC2 baculovirus were collected at two days post infection and membranes were prepared from the cells. The membranes were extracted with 0.8% dodecyl maltoside at a protein concentrations of 4-5 mg/ml. The extracted material was clarified at 100,000 x g and supernatant was incubated with M2 anti-FLAG affinity matrix. The matrix was washed and bound material was eluted with 0.1M glycine, pH 3.0. The eluate was analysed by SDS-PAGE followed by silver staining or western

Figure 6-3. Adenylyl cyclase activities in AC2 and FLAG-tagged AC2-expressing Sf9 membranes:

Basal adenylyl cyclase activities was measured in the presence of 5mM Mg^{2+} in membranes of TPO baculovirus-infected (white bars), wild-type AC2-baculovirus-infected (black bars), or FLAG-tagged AC2-baculovirus infected (hatched bars) Sf9 cells. Assays were performed for 15 min as described under "Materials and Methods".

Figure 6-3. Adenylyl cyclase activities in AC2 and FLAG-tagged AC2-expressing Sf9 membranes

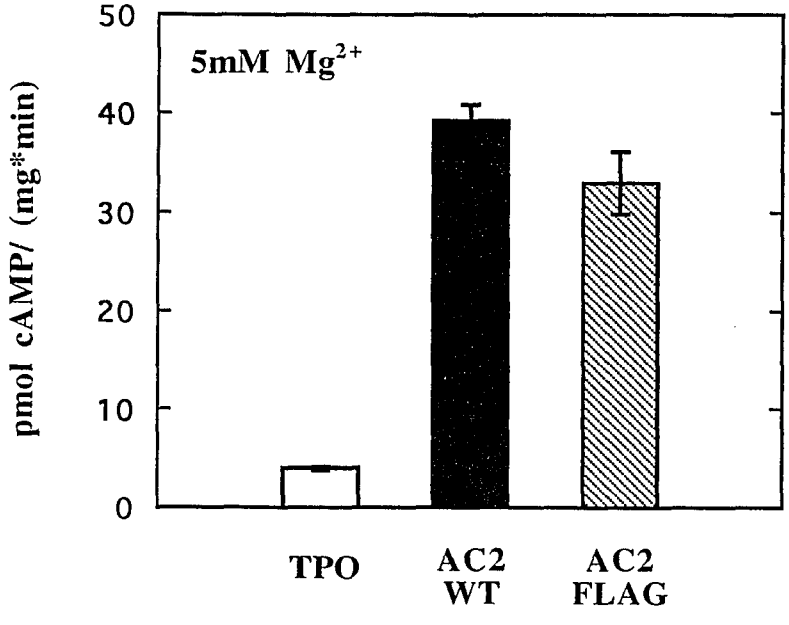
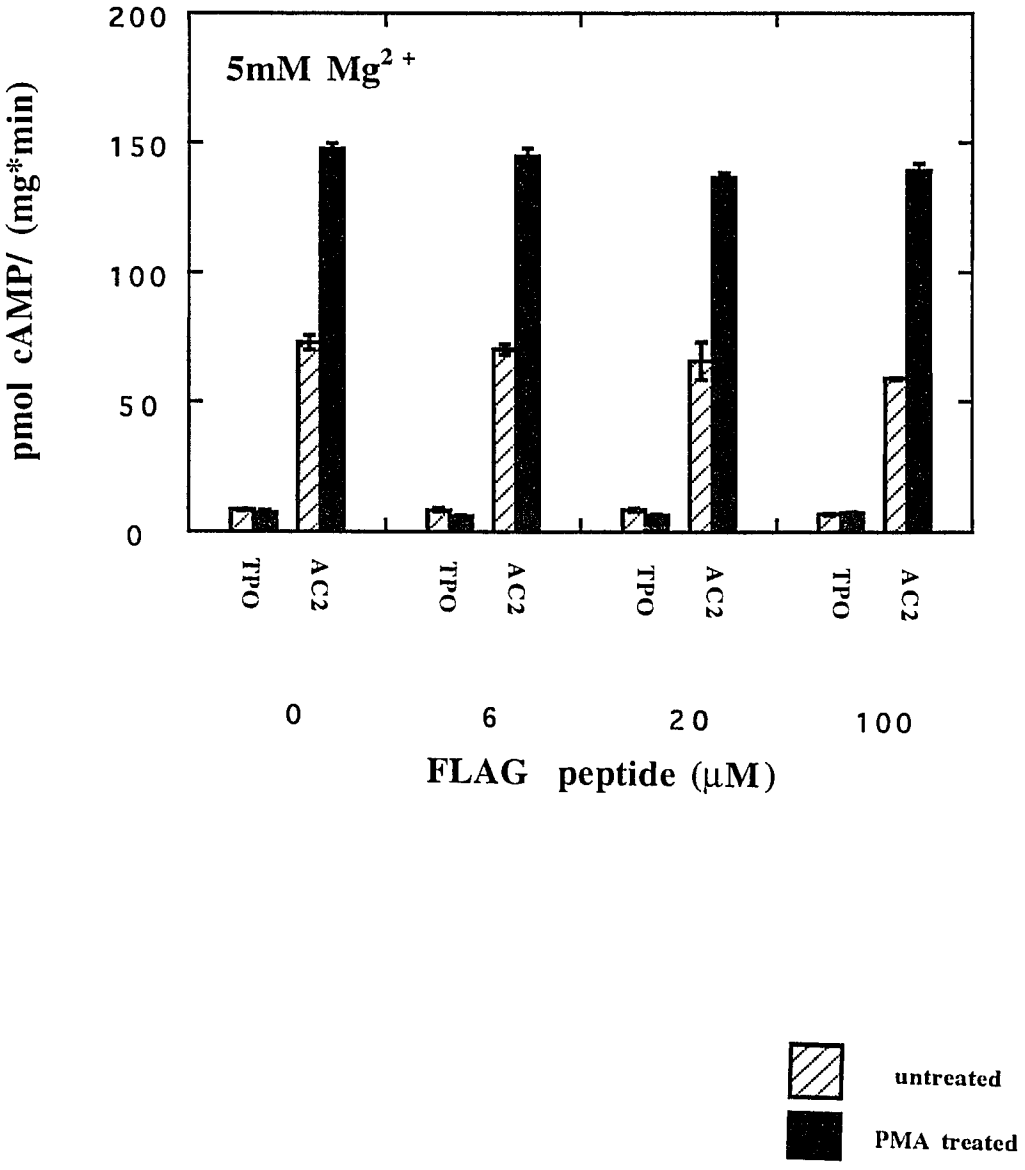


Figure 6-4. Effect of FLAG peptide on adenylyl cyclase 2 activity in Sf9 membranes:

Basal adenylyl cyclase activities was measured in the presence of 5mM Mg^{2+} and the indicated concentration of FLAG peptide in membranes of TPO baculovirus-infected or FLAG-tagged AC2-baculovirus infected Sf9 cells. Cells were treated for 20 minutes with 1μM PMA (black bars) or DMSO vehicle (hatched bars) prior to preparation of membranes. Assays were performed for 15 min as described under "Materials and Methods".

Figure 6-4. Effect of FLAG peptide on adenylyl cyclase 2 activity in Sf9 membranes



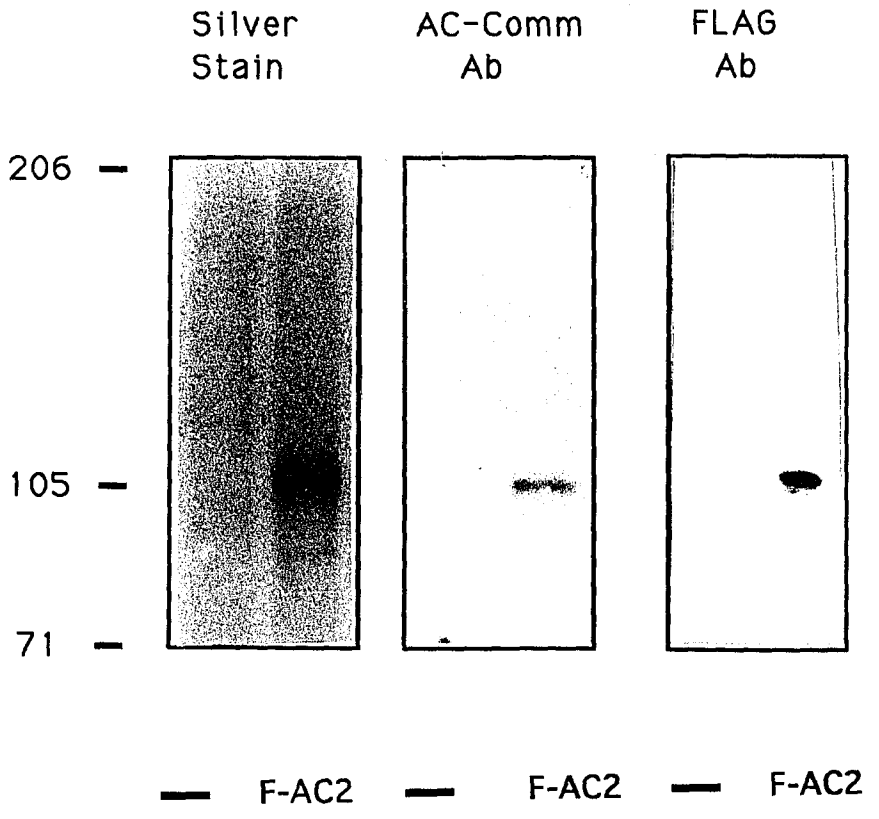
blotting.

The eluate migrated as a 106 kDa band on a silver-stained polyacrylamide gel (Fig. 6-5, left panel). To establish the identity of the eluate, I analyzed it by Western blotting with antisera specific for a conserved region of the cloned mammalian adenylyl cyclases (AC-Comm, Fig. 6-5, middle panel) and with an anti-FLAG monoclonal antibody (Fig. 6-5, right panel). Both AC-comm and the anti-FLAG monoclonal antibody recognized a 106 kDa band in the eluate thus confirming its identity as FLAG-tagged AC2.

Figure 6-5. Characterization of the anti-FLAG affinity matrix eluate:

Membranes from TPO-baculovirus (-) or FLAG tagged AC2-baculovirus (F-AC2) infected cells were extracted with 0.8% dodecyl maltoside and incubated with M2 anti-FLAG affinity matrix for 3 hours. The matrix was washed and bound material was eluted with 0.1M glycine, pH 3.0. The eluates was resolved by SDS-PAGE, 25µl per sample. *Left panel:* Silver stained profile. *Middle and Right Panels:* Immunoblots with AC-comm antiserum (1:1000 dilution) or M2 anti-FLAG antibody (30µg/ml), respectively. Molecular weight markers in kDa are shown. For detailed procedures consult "Materials and Methods".

Figure 6-5. **Characterization of the anti-FLAG affinity matrix eluate**



6.4) Purification and analysis of FLAG-tagged AC2 from ^{32}P-labeled, PMA-treated Sf9 cells

With the FLAG-tagged adenylyl cyclase 2 successfully purified, I tested whether PMA treatment would result in phosphorylation of F-AC2. First, I confirmed that PMA treatment of F-AC2 expressing cells would also result in stimulation of adenylyl cyclase activity. In Fig. 6-6, PMA treatment of Sf9 cells resulted in a 2-3 fold enhancement of the FLAG-tagged adenylyl cyclase 2 activities, comparable to that of AC2

I next analyzed whether F-AC2 was phosphorylated in response to PMA treatment of Sf9 cells. The experimental method is outlined in Fig. 6-7. Sf9 cells were harvested 2-3 days post-infection. In order to efficiently label Sf9 cells with ^{32}P phosphate, I prepared a phosphate-free media formulation based on Grace's medium and incubated the cells in the media supplemented with 0.5 mCi ^{32}P/ml for three hours. Cells were then treated for thirty minutes with PMA, lysed by N_2 cavitation and membranes were prepared. Adenylyl cyclase activities of the membranes are shown in Fig. 6-6. Membranes were extracted in 0.8% dodecyl maltoside, extracts were clarified and supernatants were incubated with the M2 anti-FLAG matrix. The eluates were analyzed by SDS-PAGE followed by silver stain and autoradiography (Fig. 6-8). PMA treatment resulted in a 3-fold increase of ^{32}P incorporation into F-AC2. The enhanced incorporation was confirmed in three independent experiments.

Figure 6-6. Effect of PMA treatment on adenylyl cyclase activities of FLAG-tagged AC2 in Sf9 membranes:

Basal and forskolin-stimulated adenylyl cyclase activities was measured in the presence of 5mM Mg^{2+} in membranes of TPO baculovirus-infected or FLAG-tagged AC2-baculovirus infected Sf9 cells. Cells were incubated with ^{32}P (0.5mCi/ml) for 3 hours and then treated for 30 minutes with 1μM PMA (black bars) or DMSO vehicle (hatched bars) prior to preparation of membranes. Assays were performed for 15 min as described under "Materials and Methods".

Figure 6-6. Effect of PMA treatment on adenylyl cyclase activities of FLAG-tagged AC2 in Sf9 membranes

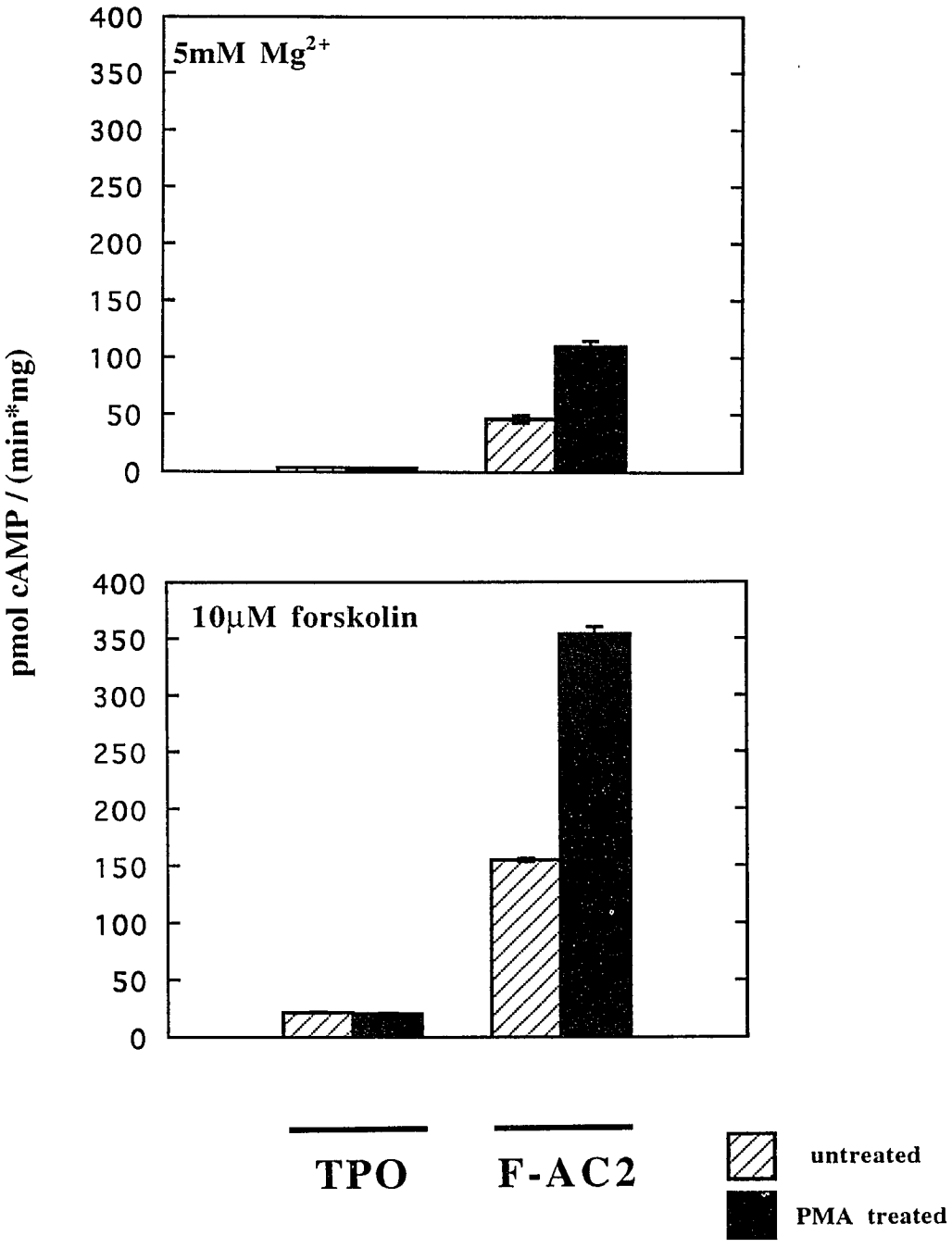
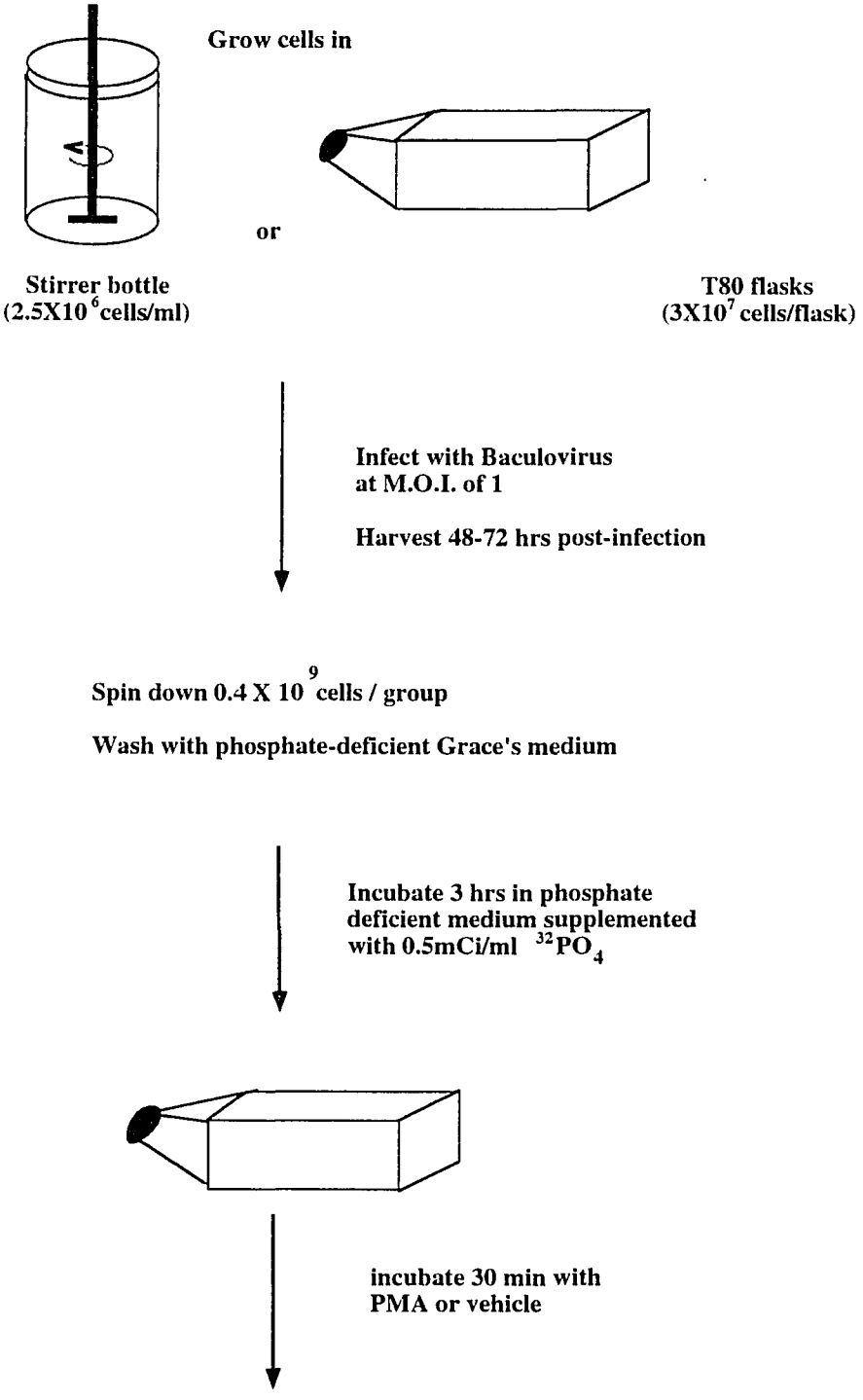


Figure 6-7. Method for purification of FLAG-tagged AC2 from Sf9 cells



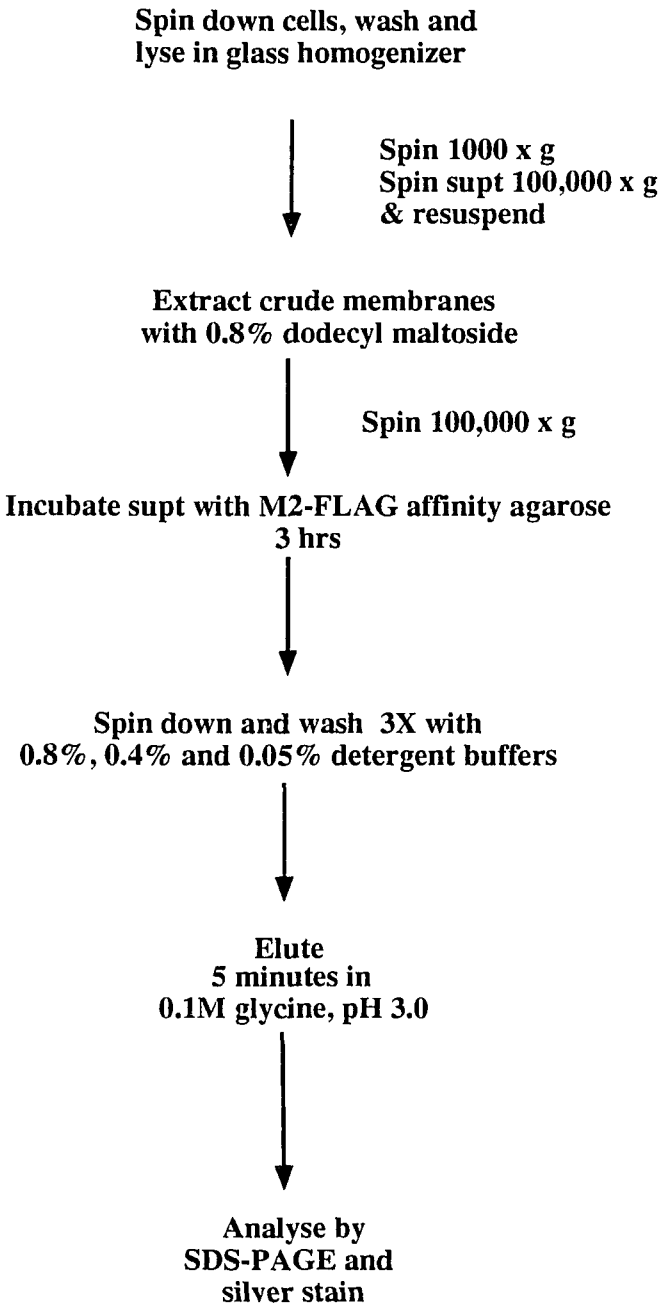
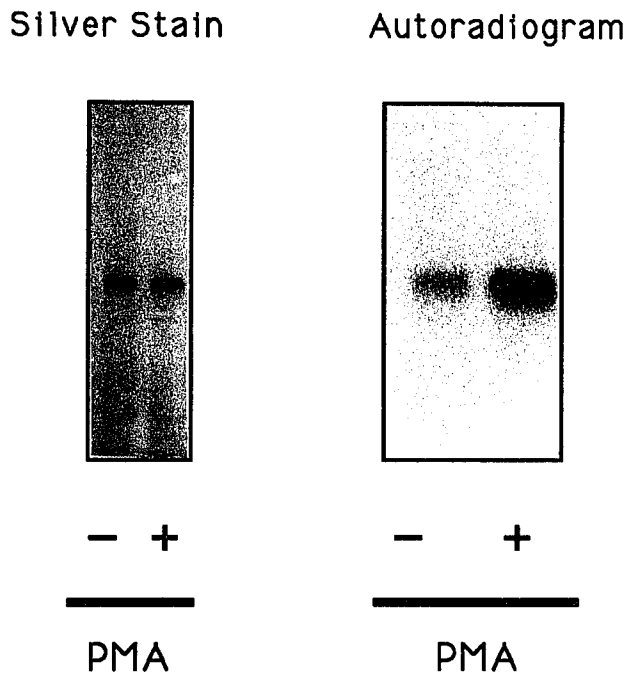

Spin down cells, wash and lyse in glass homogenizer
Spin 1000 x g
Spin supt 100,000 x g
& resuspend
Extract crude membranes with 0.8% dodecyl maltoside
Spin 100,000 x g
Incubate supt with M2-FLAG affinity agarose 3 hrs
Spin down and wash 3X with 0.8%, 0.4% and 0.05% detergent buffers
Elute 5 minutes in 0.1M glycine, pH 3.0
Analyse by SDS-PAGE and silver stain

Figure 6-8. Characterization of FLAG-tagged AC2 from ^{32}P-labeled, PMA-treated Sf9 cells:

Sf9 cells infected with FLAG-tagged-AC2 baculovirus were incubated with ^{32}P (0.5mCi/ml) for 3 hours and then treated for 30 minutes with 1μM PMA or DMSO vehicle prior to preparation of membranes. Membranes were extracted and F-AC2 was purified on anti-FLAG antibody affinity matrix. 30μl aliquots were resolved on 6% SDS-polyacrylamide gels. *Left Panel:* Silver stained profile. *Right Panel:* autoradiogram (9 day) of dried gel.

Figure 6-8. **Characterization of FLAG-tagged AC2 from ^{32}P-labeled, PMA-treated Sf9 cells**



CHAPTER 7

DISCUSSION

Many hormones and neurotransmitters regulate their target organs' function through the adenylyl cyclase pathway. Upon binding hormone agonists, receptors activate the heterotrimeric G protein, G_s, which subsequently stimulates adenylyl cyclase to produce the second messenger cAMP. Thus, a sequence of protein-protein interactions leads to the generation of cAMP.

Until recently, only two isoforms of mammalian, hormone-regulated adenylyl cyclases were recognized: a calmodulin-stimulated isoform in brain tissue, and a calmodulin-insensitive isoform, which is ubiquitously distributed. In the last five years, however, with the cloning of eight full-length cDNAs of mammalian adenylyl cyclases and two partial cDNAs, it is now apparent that there is much diversity within the adenylyl cyclase family of enzymes.

All the cloned adenylyl cyclases are stimulated by G_s and can thus conduct signals from G_s-coupled receptors. This has been determined by reconstitution with exogenous α_s, activation of endogenous G_s by nonhydrolyzable GTP analogues or fluoride, or by hormonal activation of G_s-coupled receptors. It remains to be determined, however, whether specific adenylyl cyclases are differentially stimulated by α_s and whether the four α_s isoforms differentially stimulate specific adenylyl cyclase isozymes.

Though all cloned adenylyl cyclases are stimulated by the same signal transducer, G_s, specific isoforms are capable of

recognition of signals from other receptors and hence, signal integration. The type 1 adenylyl cyclase, for example, is stimulated by Ca^{+2}/CaM while the type 6 adenylyl cyclase is inhibited at low micromolar, physiogically relevant Ca^{+2} concentrations. The type 2 isoform is not regulated by Ca^{+2}, but is conditionally stimulated by G protein $\beta\gamma$ subunits. The signal transduction properties of particular organs or cells can now be correlated with those of the adenylyl cyclase isoforms which they express. Thus, calmodulin stimulates adenylyl cyclase activity in rat brain, where adenylyl cyclase 1 is abundant. Bradykinin, by stimulating Ca^{+2} mobilization, inhibits adenylyl cyclase activity in NCB-20 cells (Boyajian *et al*, 1991) which express adenylyl cyclase 6. 5-$HT1_A$ and $GABA_B$ agonists, though they activate G_i-linked receptors, enhance the response to norepinepherine in rat hippocampus (where adenylyl cyclase 2 is abundant) presumably by releasing G protein $\beta\gamma$ subunits (Andrade, 1993). Hence, the properties of particular adenylyl cyclase isozymes allow for crosstalk with other signaling pathways at the level of the effector.

The phospholipid hydrolysis pathways, which activate protein kinase C, can also regulate adenylyl cyclase activity (Table 1-2, chapter 1). Depending on the system examined, inhibitory, stimulatory or no effects on adenylyl cyclase activity are found. In hepatocytes, protein kinase C activation desensitizes glucagon-stimulated adenylyl cyclase activity by rapid uncoupling of the glucagon receptor from G_s (Heyworth *et al*, 1984, García-Saínz *et*

al, 1985, Murphy *et al*, 1987). This desensitization of receptor-stimulated adenylyl cyclase activity by protein kinase C is believed to occur through protein kinase C phosphorylation of receptors, as demonstrated for the β-adrenergic receptor (Sibley *et al*, 1984, Kelleher *et al*, 1984).

Stimulatory effects of protein kinase C on adenylyl cyclase activity have been documented in many cell types and organs including smooth muscle, brain, ovaries, adrenal glands, fat and hematopoietic cells (Table 1-2). These stimulatory effects were induced by direct activation of PKC by phorbol esters or through hormonal stimulation of receptors which activate PKC. Thus, stimulation of G_q-coupled receptors such as the angiotensin II (Peytremann *et al*, 1973), muscarinic (Frings, 1993) and α_1-adrenergic (Sugden *et al*, 1985) receptors, which activate PKC, resulted in cAMP production. Stimulation of the NGF (Schubert *et al*,1978), EGF (Budnik *et al*, 1991, 1993b) and B cell antigen (Wiener *et al*, 1989) receptors, which signal through tyrosine phosphorylation and activate PKC also resulted in cAMP production. The added capacity to stimulate cAMP production allows these receptors to elicit biological responses that are regulated by several distinct intracellular pathways. For example, in the pineal gland, norepinepherine stimulation of cAMP production through α_1-adrenergic receptors, in concert with β-adrenergic receptor stimulation, is believed to be important in the maintenance of circadian rhythms (Takahashi, 1993). In the adrenal gland, angiotensin II can independently induce or potentiate ACTH-

mediated steroidogenesis through PKC-dependent increases in cAMP levels (Peytremann *et al*, 1973, Bird *et al*, 1993).

To identify the adenylyl cyclase isozymes that are stimulated by protein kinase C, I expressed the cDNAs for isozymes 1-6 individually in HEK-293 cells. The adenylyl cyclase 2 isoform was prominently stimulated by treatment of cells with PMA, a phorbol ester activator of protein kinase C, while smaller enhancements were observed for the types 1 and 3 adenylyl cyclases. In particular, the basal activity of adenylyl cyclase 2 was most enhanced, rather than the G protein-stimulated activities. Given that PMA best stimulated the basal activity and that the presence of particular isoforms of adenylyl cyclase determined whether PMA treatment stimulated adenylyl cyclase activity in 293 cells, the locus of PKC integration with adenylyl cyclase appears to be the effector adenylyl cyclase, itself.

The stimulation of adenylyl cyclase 2 by PKC was further characterized in Sf9 cells. Phorbol ester treatment of cells expressing adenylyl cyclase 2 also resulted in prominent elevation of the basal activity. Since only phorbol ester activators of PKC enhanced the basal adenylyl cyclase activity and since the enhancement was abolished by staurosporine, an inhibitor of PKC, the enhancement of adenylyl cyclase 2 activity was mediated by PKC. Interestingly, activation of PKC in both human 293 cells and insect Sf9 cells stimulated the type 2 adenylyl cyclase. Since PKC-mediated stimulation of adenylyl cyclase 2 was conserved

from invertebrates to vertebrates, it is likely to be a physiologically relevant mechanism for signal integration.

The PKC activation appears to be an independent mode of stimulation of adenylyl cyclase 2. In Sf9 and 293 cells, the basal, Mg^{2+}-dependent activity was elevated about three fold by PKC activation, with the most prominent elevations occurring at Mg^{2+} concentrations in the physiological range. Likewise, stimulation by Mn^{2+}, another divalent cation capable of directly stimulating adenylyl cyclase, was also enhanced by PKC activation. Since other stimulators of adenylyl cyclase such as forskolin and G_s-α increase the V_{max} of adenylyl cyclase, I tested the effect of PKC activation on the V_{max} and K_m of adenylyl cyclase 2. The PKC-mediated increase in adenylyl cyclase 2 activity was reflected by an increased V_{max} of the Mg^{2+}-dependent activity and unaltered K_m. Thus, the PKC enhancement of adenylyl cyclase 2 activity was similar to that induced by other independent stimulators.

PKC activation also potentiated stimulation of adenylyl cyclase 2 activity by both endogenous and exogenous G proteins. Endogenous G_s activation by fluoride or nonhydrolyzable GTP analogues in both 293 and Sf9 cells resulted in enhancement of adenylyl cyclase activity, though more extensively in Sf9 cells. Stimulation of adenylyl cyclase 2 with specific concentrations of recombinant, persistently activated α_s was also tested. PKC activation potentiated adenylyl cyclase stimulation by the exogenous α_{s4}^*, especially at the concentrations below 2nM.

Hence, like forskolin, PKC activation can potentiate G_s stimulation of adenylyl cyclase. Synergistic stimulation of adenylyl cyclase 2 by G protein α and $\beta\gamma$ subunits was also potentiated by PKC activation.

The simplest mechanism for PKC-mediated stimulation is direct phosphorylation of adenylyl cyclase 2. To verify whether phosphorylation occurred, the type 2 isoform was expressed in Sf9 cells. Expression of adenylyl cyclase 2 resulted in robust elevations in adenylyl cyclase activity, but the enzyme was not expressed at levels permitting its visualization by staining of membrane proteins (Fig. 6-1). Hence, to demonstrate phosphorylation of adenylyl cyclase 2, extensive purification was necessary. In order to purify the expressed adenylyl cyclase 2, an eight amino-acid epitope was added to the N-terminus of adenylyl cyclase 2 and the epitope-tagged enzyme was purified by immuno-affinity chromatography against the tag. By virtue of the epitope, adenylyl cyclase 2 was specifically purified from endogenous Sf9 adenylyl cyclases by immuno-affinity chromatography, thereby permitting unequivocal determination of its phosphorylation state. PKC activation resulted in stimulation and enhanced phosphorylation of the type 2 adenylyl cyclase in ^{32}P-labeled cells.

This represents a new mechanism by which signals are directed into the cAMP pathway. Unlike other stimulators, which regulate adenylyl cyclase activity through protein-protein association, protein kinase C activation leads to stimulation of

adenylyl cyclase through covalent modification. When stimulated by PKC activation, adenylyl cyclase 2 can be synergistically stimulated by G protein subunits. Hence, adenylyl cyclase 2 is also a locus at which signal convergence and integration may occur. Adenylyl cyclase 2 and possibly other members of the adenylyl cyclase 2 family may thus serve as signal recognition and integration elements that allow a variety of external signals to impart some of their cellular effects through the cAMP pathway.

Covalent modification of cell-surface, G protein effectors may provide a G protein-independent route for signal transmission to specific isoforms of these G protein-modulated effectors. In a defined cell type, such crosstalk could occur between protein kinase C and adenylyl cyclase, depending on the presence and abundance of adenylyl cyclase 2 or adenylyl cyclase 2-like isoform of adenylyl cyclase.

Potential Future Applications

Adenylyl cyclase 2 is stimulated by activation of protein kinase C. Thus the presence of adenylyl cyclase 2 in a particular cell may allow hormones which activate protein kinase C, whether through G_q-coupled receptors or receptor tyrosine kinases, to elevate cAMP levels in that cell. Potentially, heterologous expression of the type 2 adenylyl cyclase could be used in the therapy of disease states in which elevated basal cAMP levels or altered stimulation of cAMP production may be beneficial.

Expression of the type 2 adenylyl cyclase in breast tissue may be of benefit in the treatment of breast cancer. The oncogene erbB2, or neu, a member of the EGF receptor tyrosine kinase family, was discovered as an amplified gene in human breast cancers (King *et al*, 1985). In fact, erbB2 is amplified or overexpressed in 20-30% of breast cancers and overexpression of the tyrosine kinase erbB2 is a negative prognostic indicator in node-negative and node-positive cancers (Slamon *et al*, 1987, 1989, Gullick *et al*, 1991, Patterson *et al*, 1991). Furthermore, tyrosine kinase activity is also elevated in breast cancers (Ottenhoff-Kalff *et al*, 1992).

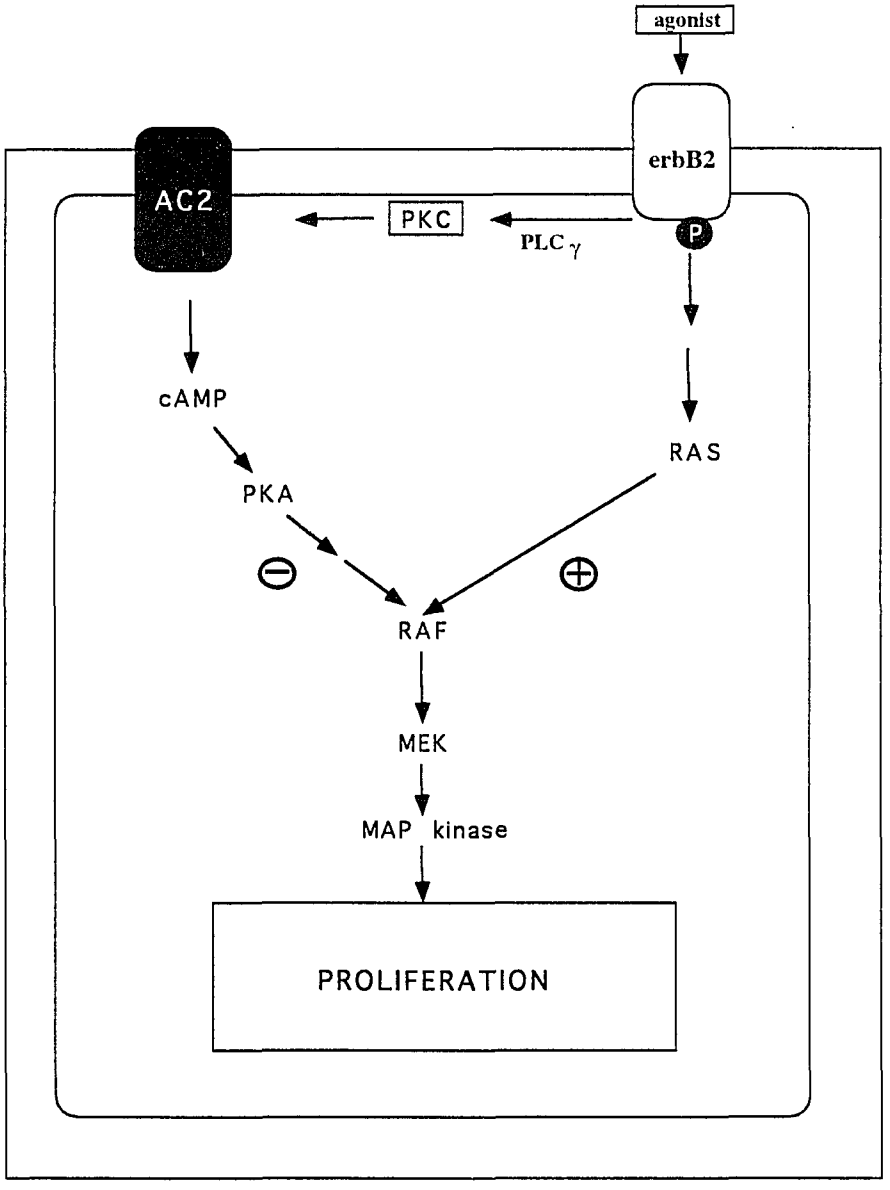
Tyrosine kinase receptors are believed to induce cell proliferation through downstream activation of MAP kinases (Mitogen Activated Protein kinases, see Egan & Weinberg, 1993). However, elevation of cAMP levels can suppress MAP-kinase

activity and cell proliferation (Graves *et al*, 1993, Chen & Iyengar, 1994). Since studies in progress in our laboratory indicate that elevation of cAMP levels in MCF-7 breast cancer cells suppresses DNA synthesis (J. Chen, unpublished), expression of adenylyl cyclase 2 in breast cancer cells may suppress proliferation through elevation of the *basal* cAMP level. In addition, tyrosine kinase receptors such as erbB2 may have a diminished capacity to induce cell proliferation since they would activate protein kinase C and therefore stimulate adenylyl cyclase 2. Thus, through indirect stimulation of adenylyl cyclase 2, tyrosine kinases may be deprived of their cell proliferative effects in a breast cancer cell (Fig 7-1).

Figure 7-1. Proposed convergence of tyrosine kinase and adenylyl cyclase signaling in breast cancer cells:

Abbreviations: AC2, adenylyl cyclase 2; MAP, Mitogen Activated Protein Kinase; MEK, MAP kinase kinase; P, phosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.

Figure 7-1. **Proposed convergence of tyrosine kinase and adenylyl cyclase signaling in breast cancer cells**



Adenylyl cyclase 2 could potentially be of benefit in the treatment of asthma with the advent of appropriate gene therapy delivery methods or adenylyl cyclase isoform-selective drugs. Asthma is an inflammatory state characterized by episodic bronchoconstriction of hyper-responsive airways. Treatment of asthma is currently symptomatic, aimed at relieving the bronchocontriction. Mild to moderate asthma can be managed with β_2-adrenergic agonists which elevate cAMP levels and dilate bronchi. The β_2-adrenergic agonists become ineffective however, due to β-adrenergic receptor desensitization. It is not yet known whether the type 2 adenylyl cyclase is expressed in human bronchial smooth muscle. If adenylyl cyclase 2 is not expressed in the smooth muscle, then novel expression of adenylyl cyclase 2 in smooth muscle may provide an effective cAMP generator which would bypass the desensitization at the receptor level. If adenylyl cyclase 2 is expressed in human lung, in accordance with the mRNA distribution in rat, then development of adenylyl cyclase isoform-selective drugs could provide similar therapeutic benefits.

CHAPTER 8

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