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Characterization of phosphoinositide hydrolysis in a human endometrial adenocarcinoma cell line (Ishikawa)

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CHARACTERIZATION OF PHOSPHOINOSITIDE HYDROLYSIS IN A HUMAN ENDOMETRIAL ADENOCARCINOMA CELL LINE (ISHIKAWA)

by

Daniel J. Weiss

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

1987

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ABSTRACT

CHARACTERIZATION OF PHOSPHOINOSITIDE HYDROLYSIS
IN A HUMAN ENDOMETRIAL ADENOCARCINOMA CELL LINE

by

DANIEL J. WEISS

Advisor: Erlio Gurpide, Ph.D.

Addition of acetylcholine (ACh) or carbamylcholine (CCh) to suspensions of human endometrial carcinoma cells preincubated with [3H]-myoinositol promoted a rapid rise in levels of radiolabeled inositol mono- (IP1), bis- (IP2), and tris- (IP3) phosphates. The pattern of accumulation of these compounds suggests that the initial stimulated event was hydrolysis of phosphatidylinositol 4,5-bisphosphate. Levels of this phosphoinositide decreased significantly within 30 sec following exposure to CCh while no changes in the levels of phosphatidylinositol 4-monophosphate or phosphatidylinositol were observed for as long as 5 min.

Stimulation of inositol phosphate (IP) accumulation by CCh or acetylcholine (ACh) was concentration-dependent and saturable. The Hill coefficients and EC50's of the concentration-response curves for CCh and ACh indicate interaction with a single class of low-affinity receptor. The Ki for atropine inhibition of these effects identifies the receptor as muscarinic.

Both basal and CCh-stimulated levels of IP3 and IP2, as well as IP1, were increased in the presence of 10 mM LiCl. The effect on IP1 accumulation was observed under isosmotic and hyperosmolar conditions and was concentration-dependent between 1-100 mM LiCl.

Vasopressin, oxytocin, histamine, phenylephrine, and PGF2@ had no apparent effect on IP levels following incubations for up to 1 hr. Estradiol, progesterone and sulfated estrogens were also without effect on IP levels during both long and short term incubations. Phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate inhibited up to 35% of the CCh-stimulated increase in IP accumulation. Triphenylethylene antiestrogens at micromolar concentrations increased IP levels in a concentration-dependent manner but inhibited the increase stimulated by CCh. A plasma membrane perturbing effect of these compounds is suggested by the concurrent loss of ability of the cells to exclude trypan blue.

These studies demonstrate and characterize the plasma membrane signal transduction system involving phosphoinositide hydrolysis coupled to muscarinic receptors in Ishikawa cells. Several features of the transduction events not observed in similar model systems with respect to the actions of Li+ are described. Phorbol esters and triphenylethylene antiestrogens significantly altered phosphoinositide hydrolysis in Ishikawa cells. Suggestions are made concerning the possible presence and physiological role of phosphoinositide hydrolysis in human endometrium.

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To my family: for all your love and support

To Stephanie: you have suffered with me and have believed in me. This is for us.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACh	acetylcholine
CCh	carbamylcholine
CDTA	1,2 diaminocyclohexane N,N,N'N'-tetraacetic acid
cIP3	1:2 cyclic inositol 4,5-bisphosphate
DG	1,2 diacylglycerol
EC50	half-maximal stimulatory concentration
EDTA	ethylenediamine N,N,N',N'-tetraacetic acid
IC50	half-maximal inhibitory concentration
IP	inositol phosphate(s)
IP1	inositol monophosphate
IP2	inositol bisphosphate
IP3	inositol trisphosphate
PDB	phorbol 12,13-dibutyrate
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-monophosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PMA	phorbol 12-myristate, 13-acetate
PPI	phosphoinositides

INTRODUCTION

Investigation of a signal transduction mechanism and of the hormones that activate it in a given system is often preliminary to elucidating the physiological role played by those agents. Several signal transduction processes have been described whereby a hormone binding to its specific receptor, either cytoplasmic, nuclear, or located on the plasma membrane, can initiate different intracellular events. The aim of this dissertation research was to investigate the hydrolysis of plasma membrane inositol phospholipids in an adenocarcinoma cell line derived from a primary human endometrial tumor. Endometrium, the lining of the uterine cavity, plays a major role in events of the female reproductive cycle. Although much is known about the regulation of endometrial physiology by steroid hormones, relatively little information about the role of compounds acting on endometrial plasma membrane receptors is available. In particular the presence of endometrial cholinergic receptors has been suggested but actual demonstration of either specific cholinergic binding or of specific cholinergic functions has not yet been reported. The studies described here are concerned with the characterization of muscarinic activation of phosphoinositide hydrolysis in neoplastic cells derived from human endometrium as well as the regulation of this transduction system by other agents and with its potential physiological role.

I) PHOSPHOINOSITIDE HYDROLYSIS

It is now well established that stimulation of phosphoinositide hydrolysis is a means of signal transduction across the plasma membrane for many hormones, growth factors, and secretagogues. Agonist binding to membrane receptors initiates a process whereby at least three intracellular second messengers, inositol 1,4,5-trisphosphate (1,4,5-IP3), cyclic-1:2 inositol 4,5-trisphosphate (cIP3), and 1,2 diacylglycerol (DG), are produced from cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2). All of these have been implicated in cellular processes.

Elucidation of the Phosphatidylinositol Cycle (PI cycle), the sequence of processes involving phosphorylation of the inositol moiety in PI, phosphoinositide hydrolysis and the subsequent resynthesis of the inositol phospholipids, began with observations in the 1950's of increased [32P]-PO4 labeling of phospholipids, most prominently phosphatidylinositol and phosphatidic acid (PA), in response to hormonal stimulation in pancreatic and brain tissue (Hokin and Hokin, 1953; Hokin et al., 1960). During the 1960's and early 1970's, a large body of information accumulated suggesting that the agonist-stimulated cleavage and subsequent resynthesis of PI was a universal concommittant of Ca2+ flux for hormones

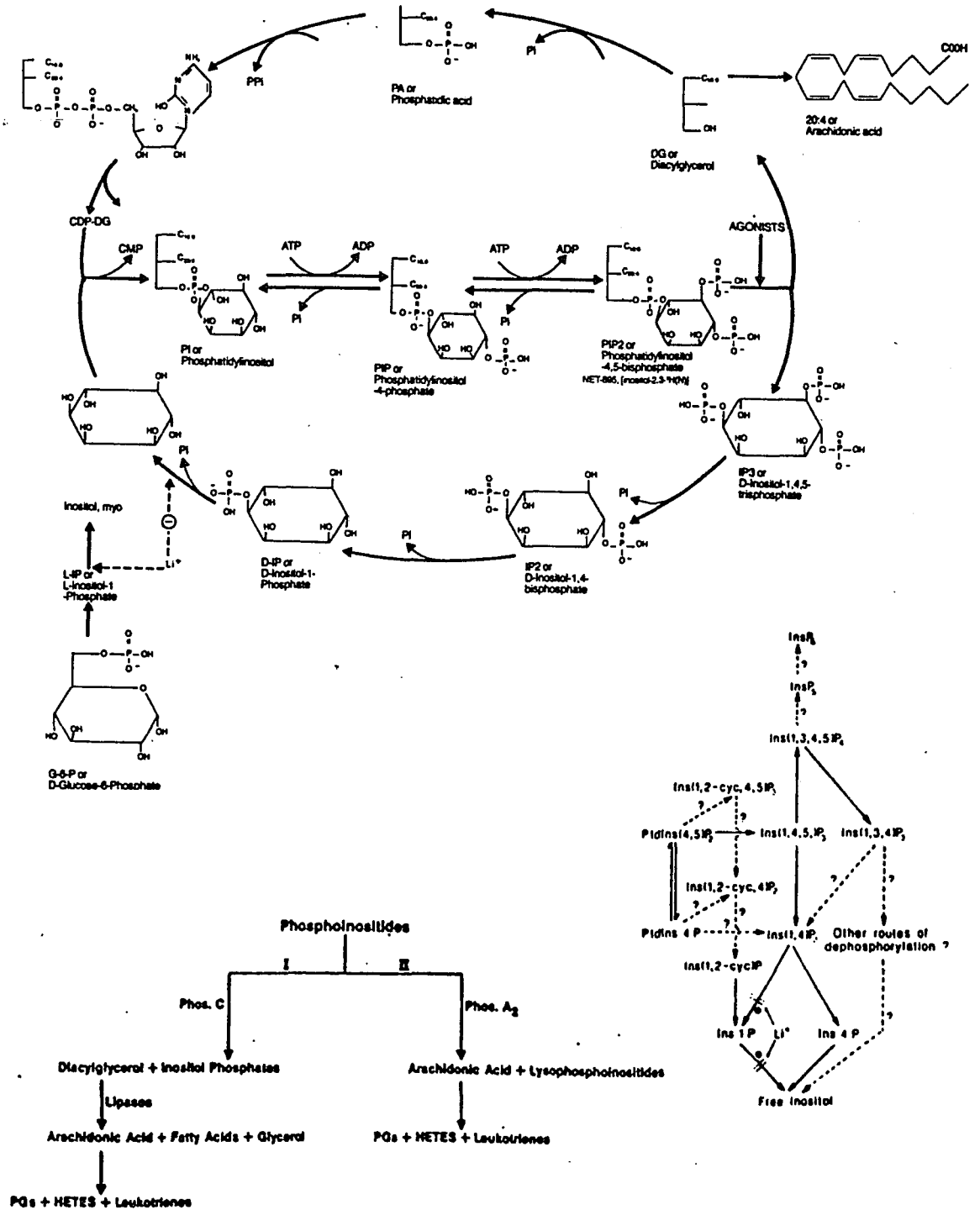
that were believed to mobilize Ca2+ as part of their action (Dawson, 1960; Durell et al., 1969). A review in 1975 (Michell, 1975) summarized the concept of a closed cycle of hormonally stimulated PI cleavage and resynthesis which allowed influx of extracellular Ca2+.

Since that review was published the processes involved in the PI cycle have been further elucidated. Three schemes diagramming current knowledge are presented in Fig. 1. One of the phosphorylated derivative of PI, PIP2, is now known to be the direct target of hydrolysis by a hormonally activated phospholipase C (Berridge, 1983; Fisher et al., 1984; Majerus et al., 1985) although in several systems, direct cleavage of the other phosphorylated derivative of PI, phosphatidylinositol 4-monophosphate (PIP), as well as PI itself, is also suggested (Creba et al., 1983; Emilsson and Sundler, 1984; Haslam et al., 1986). The coupling between agonist occupied receptor and phospholipase C appears to be mediated by a GTP-binding protein, designated Np, in a manner analogous to plasma membrane receptor coupling with adenylate cyclase (Joseph, 1985; Taylor and Merrit, 1986). Np has recently been postulated to decrease the Km of the phospholipase for Ca2+, enabling hydrolysis of PIP2 and PIP at ambient Ca2+ concentration (Smith et al., 1986). In platelets, neutrophils and mast cells (Molski et al., 1984; Nakamura and Ui, 1985; Brass et al., 1986), Np is inactivated by

Fig 1: Phosphatidylinositol Cycle

These schemes outline pathways of phosphoinositide metabolism that have been demonstrated in various systems.

Figure 1


PPi
PA or Phosphatidic acid
PI
COOH
20:4 or Arachidonic acid
DG or Diacylglycerol
CDP-DG
CMP
ATP
ADP
PI
AGONISTS
PI or Phosphatidylinositol
PIP or Phosphatidylinositol -4-phosphate
PIP2 or Phosphatidylinositol -4,5-bisphosphate
IP3 or D-inositol-1,4,5-trisphosphate
IP2 or D-inositol-1,4-bisphosphate
D-IP or D-inositol-1-Phosphate
L-IP or L-inositol-1-Phosphate
Inositol, myo
G-6-P or D-Glucose-6-Phosphate
Phospholnositides
I
II
Phos. C
Phos. A2
Diacylglycerol + Inositol Phosphates
Lipases
Arachidonic Acid + Fatty Acids + Glycerol
PGs + HETES + Leukotrienes
Arachidonic Acid + Lysophosphoinositides
PGs + HETES + Leukotrienes
Ins(1,3,4,5)IP4
Ins(1,2-cyc,4,5)IP3
PtdIns(4,5)IP2
Ins(1,4,5)IP3
Ins(1,3,4)IP3
Ins(1,2-cyc,4)IP2
PtdIns 4 P
Ins(1,4)IP2
Other routes of dephosphorylation ?
Ins(1,2-cyc)P
Ins 1 P
Li+
Ins 4 P
Free Inositol

pertussis toxin suggesting a similarity with Ni, the GTP-binding subunit which mediates inhibition of adenylate cyclase. In other systems, (Masters et al., 1985; Schimmel and Elliott, 1986; Hinkle et al., 1986), however, putative Np is insensitive to pertussis toxin suggesting a heterogeneity of GTP-binding proteins coupled with phospholipase C.

The products of PIP2 hydrolysis include 1,4,5-IP3, 1:2-cyclic 4,5-IP3 and 1,2 diacylglycerol. 1,4,5-IP3 is reported to release Ca+2 from intracellular stores (Streb et al., 1983; Prentki et al., 1984; Berridge and Irvine, 1984) through what appears to be a GTP-binding protein dependent stimulation of Ca2+/K+ exchange across the endoplasmic reticulum membrane (Muallem et al., 1985; Gill et al., 1986). The released Ca2+ can activate a variety of intracellular processes (reviewed in Williamson, 1986). Cyclic IP3 has been found to be as effective as noncyclic IP3 in mobilizing Ca2+ in saponin-permeabilized platelets and is actually more potent in stimulating conductance changes in Limulus photoreceptors (Wilson et al., 1985a). Cyclic IP2 and cyclic IP1 are also known to be produced as a result of hormonal stimulation (Dawson et al., 1971; Wilson et al., 1985b) but their physiological function and precise relationship to the noncyclic inositol phosphates is not clear. 1,4,5-IP3 can be dephosphorylated by a 5-phosphatase, yielding 1,4-IP2 (Storey et al., 1984) and also phosphorylated by a 3-kinase, producing

inositol 1,3,4,5-tetrakisphosphate (IP4) (Irvine et al., 1986). 1,4-IP2 is sequentially cleaved to 1-IP1 and then to myoinositol, which is recondensed with diacylglycerol to form PI (Michell, 1975).

IP4 can be dephosphorylated by a 5-phosphatase yielding 1,3,4-IP3 (Batty et al., 1985). 1,4,5-IP3, 1,3,4-IP3, and IP4 have all been observed to rise rapidly, although with different kinetics, in response to hormonal stimulation in several tissues (Batty et al., 1985; Burgess et al., 1985; Turk et al., 1986). IP4 and 1,3,4-IP3 are relatively weak stimulators of intracellular Ca2+ release and membrane conductance changes and currently are without known function.

Changes in intracellular Ca2+ may differentially affect pathways of inositol phosphate metabolism. Increasing free Ca2+ concentration increased formation of IP4 in both broken cell homogenates and intact RINm5F insulin secretory cells (Wollheim and Biden, 1986). Recent evidence also suggests that phosphorylation of the 5-phosphatase increases its relative activity towards both 1,4,5-IP3 and IP4 (Connolly and Majerus, 1986; Molina y Vedia and Lapetina, 1986). IP5 and IP6 (phytic acid) have also recently been found in GH4 rat pituitary cells (Heslop et al., 1985). Evidence suggests that 1,3-IP2, and possibly other stereoisomers may transiently appear during hormonal stimulation (Michell, 1985; Nahorski and Batty, 1986). None of these stereoisomers or higher forms of inositol

phosphates have been found to be effective in mobilizing intracellular Ca2+ and at present are without a known function.

The cleavage of 1,4-IP2 to IP1 and the cleavage of IP1 to myoinositol are both sensitive to inhibition by Li+ (Hallcher and Sherman, 1980, Thomas et al., 1984). This was a fortuitous discovery which has been used as a tool to study the inositol phosphates and has also suggested a role of the PI cycle in manic-depressive illness for which Li+ is used as a therapeutic agent (Sherman et al., 1981). Recent data in several systems demonstrates selective enhancement of the 1,3,4-IP3 stereoisomer in the presence of Li+, suggesting the presence of a 3-IP3 phosphatase sensitive to inhibition by Li+ (Burgess et al., 1985, Turk et al., 1986).

1,2-Diacylglycerol is known to activate the Ca+2, phospholipid-dependent protein kinase C, originally identified in 1977 (Takai et al., 1977). Protein kinase C is also a cellular receptor for the tumor promoting phorbol esters (Castagna et al., 1982) and a vast literature has accumulated on the role of the PI cycle and protein kinase C in protein phosphorylation and subsequent physiological processes as well as in mechanisms of cell transformation (Nishizuka, 1984, 1986). Interestingly, pretreatment with phorbol ester has been shown to inhibit hormonally-stimulated phosphoinositide hydrolysis in several different systems (Orellana et al., 1985; Lynch et

al., 1985). A role of protein kinase C in desensitization of the PI cycle is strenghthened by evidence that both plasma membrane receptors (Hunter et al., 1984; Leeb-Lundberg et al., 1985) and GTP-binding proteins (Katada et al., 1985) can be phosphorylated and inactivated by protein kinase C. Recent data suggests that this desensitization may be specific for PIP2 and PIP hydrolysis. Direct hormonally-mediated cleavage of PI, known to occur subsequent to that of PIP2 and PIP in several systems (Williamson, 1986; Majerus et al., 1986), is unaffected by phorbol ester pretreatment in rat vascular smooth muscle cells whereas hydrolysis of both PIP and PIP2 are inhibited (Griendling et. al., 1986). The suggested role of direct PI hydrolysis is to provide a continuing source of DG following the transient elevation provided by PIP and PIP2 hydrolysis (Majerus et al., 1986). Protein kinase C mediated phosphorylation of the IP3 5-phosphatase resulting in increased enzymatic conversion of IP3 to IP2 may also contribute to desensitization of responses initiated by PIP2 hydrolysis (Molina y Vedia and Lapetina, 1986). Diacylglycerol also serves as an intermediate in the (re)synthesis of the phosphoinositides and in some systems is a source of arachidonic acid used for the production of prostaglandins, thromboxanes, and leukotrienes (Marcus, 1978; Irvine, 1982; Majerus et al., 1984).

Physiologically, the products of phosphoinositide

hydrolysis have been linked with processes of secretion, muscle contraction, visual transduction, and cell growth and differentiation. Recently it has also become apparent that glycosylated forms of PI may be important in signal transduction. These derivatives serve both as precursors for unique forms of glycosylated inositol phosphate intracellular messengers (Saltiel et al., 1986; Saltiel and Cuatrecasas, 1986) and as anchors for proteins on the external surface of the plasma membrane that are released from the cell upon hormonal or antigenic stimulation (Low et al., 1986).

II) Muscarinic Receptors and Phosphoinositide Hydrolysis.

Muscarinic agonists have been reported to be involved in phosphoinositide hydrolysis, inhibition of adenylate cyclase, stimulation of guanylate cyclase, alteration of plasma membrane K+ conductance, and most recently, stimulation of calmodulin-dependent cAMP phosphodiesterase (McKinney and Richelson, 1984; Noma, 1986). Activation of phosphodiesterase activity is postulated to occur subsequent to the 1,4,5-IP3-mediated rise in intracellular Ca+2 (Tanner et al., 1986). Increase in guanylate cyclase activity is also thought to be linked with stimulation of PI hydrolysis (Michell, 1975). These processes often occur concurrently but the exact relationship between each transduction event and the connection with physiological response(s) is not yet clear.

The original description of increased phospholipid

radiolabeling in response to hormonal exposure (Hokin and Hokin, 1953) was based on the effects of ACh and CCh. Most of the subsequent early work investigating hormonally-stimulated phospholipid turnover on which theories of a phosphatidylinositol cycle were developed used ACh and other cholinergic agents as stimulants (Durell et al., 1969). Recent work investigating muscarinic stimulation of phosphoinositide hydrolysis has centered on tissues known to be rich in muscarinic receptors and to be regulated by muscarinic influence; brain, heart, smooth muscle, and exocrine glands as well as clonal cell lines derived from these tissues. Some of these are listed in Table 1.

Muscarinic receptors have been divided into classes of either different subtypes or of different affinity states based on both agonist and antagonist binding. Agonists are able to discriminate as many as three binding states corresponding to affinities in the low nanomolar, high nanomolar, and low-mid micromolar ranges respectively (Burgen and Spero, 1968; Birdsall et al., 1978). Data from several groups suggests that stimulation of phosphoinositide hydrolysis involves a low affinity state of the receptor while inhibition of adenylate cyclase involves a higher affinity state (Miller, 1977; Brown and Brown 1984; Fisher et al., 1982). The EC50's for CCh and ACh stimulation of PI hydrolysis in several tissues are shown in Table 2. The similarity of these values suggests

Table 1: Muscarinic Stimulation of PI Turnover

List of selected tissues in which muscarinic stimulation of phosphoinositide hydrolysis has been studied

Tissue	Reference
Brain	
rat	
cortex	Gonzales and Crews, 1984
striatum	Kelly et al., 1985
hippocampus	Janowsky et al., 1984
guinea pig	
cortex, hippocampus, neostriatum	Fisher and Bartus, 1985
Heart	
embryonic chick heart cells	Brown and Brown, 1984
rat atria	Quist, 1982
mouse atria	Brown and Brown, 1983
Secretory Tissues	
rat parotid gland	Aub and Putney, 1985
rat submaxillary gland	Farese et al., 1982
rat lacrimal gland	Evans and Marty, 1986
mouse pancreas	Tennes and Roberts, 1981
bovine adrenal glomerulosa cells	Kojima et al., 1986
Smooth Muscle	
rabbit iris	Akhtar and Abdel-Latif, 1980
canine trachea	Baron et al., 1984
bovine trachea	Grandordy et al., 1986
guinea pig ileum	ChandraSekar and Roufogalis, 1984
rat vas deferens	Egawa et al., 1981
bullfrog stomach smooth muscle cells	Salmon and Honeyman, 1980
Transformed Cell Lines	
PC12 (rat pheochromocytoma)	Vicentini et al., 1985
1321N1 (human astrocytoma)	Hughes et al., 1984
N1E-115 (mouse neuroblastoma)	Kanba et al., 1986

that muscarinic coupling to PI hydrolysis is through the same state of the receptor in different tissues. Reported IC50's for inhibition of adenylate cyclase are generally 1 to 2 orders of magnitude lower (Brown and Brown, 1984; Ehlert, 1985).

Binding properties of the antagonist pirenzepine and related compounds have suggested the presence of two subtypes of muscarinic receptor. High affinity pirenzepine binding sites, denoted M1, are described in cerebral cortex, hippocampus, and peripheral ganglia, whereas low affinity pirenzepine sites, M2, predominate in brainstem, cerebellum, heart, and ileal smooth muscle. Studies with pirenzepine antagonism of PI turnover have yielded conflicting results. In rat forebrain (Gil and Wolfe 1985) and in guinea pig hippocampus and cortex (Fisher et al., 1985) pirenzepine blocks PI hydrolysis with high affinity and cyclase inhibition with low affinity. In embryonic chick heart and 1321N1 astrocytoma cells (Brown et al., 1985), rat brainstem (Lazarino et al., 1985) and guinea pig neostriatum (Fisher et al., 1985), however, PI hydrolysis is blocked by pirenzepine with low affinity. Further resolution of the nature of muscarinic receptor subtypes and their coupling awaits new selective ligands.

Muscarinic receptor mediated inhibition of adenylate cyclase involves Ni, the pertussis toxin-sensitive GTP binding protein apparently common to all receptors inhibiting cyclase (Gilman, 1984). The data at present

Table 2: EC50'S for Stimulation of Inositol Phosphate Accumulation by CCh and Ach

Survey of selected tissues in which EC50's for muscarinic stimulation of phosphoinositide hydrolysis have been reported.

Reference	Tissue	EC50
Brown and Brown, 1984	embryonic chick heart	CCh 20 uM
Janowsky, Labarca, and Paul, 1984	rat hippocampus	CCh 50 uM
Jacobsen, Wusteman, and Downes, 1985	rat cerebral cortex rat parotid gland	CCh 68 uM ACh 29.5 uM
Gonzales and Crews, 1984	rat cerebral cortex	CCh 47 uM ACh 36 uM
Fisher and Bartus, 1985	rat neostriatum rat cerebral cortex	CCh 7 uM CCh 200 uM
Vicentini etal, 1985	PC12 rat pheochromocytoma cells	CCh 20 uM

suggests that the GTP-binding protein (Np) linking muscarinic receptors with PI hydroysis is pertussis toxin-insensitive (Kelly et al., 1985; Masters et al., 1985).

III) The Endometrium

The lining of the uterine cavity is composed of two anatomical and functional layers, the zona basalis and the zona functionalis (Padykula, 1980). The zona functionalis is comprised of surface and glandular epithelial cells and also fibroblast-like stromal cells. In reproductively mature females, the zona functionalis undergo monthly cycling of proliferation, differentiation, and subsequent shedding if fertilization and ovum implantation have not occurred. The zona basalis, composed mostly of stromal cells and blood vessels, but also containing glandular epithelial cells, remains intact during the menstrual cycle and serves as the source for regeneration of the functionalis. These events are considered to be mostly regulated by estradiol and progesterone (Mossman, 1980; Padykula, 1980).

Endometrium is innervated by both adrenergic and cholinergic branches of myometrial nerve fibers. Many of these terminate on blood vessels in the zona basalis, but nerve fiber termination on both epithelial and stromal cells has been described (Jacobson and Nieves, 1961; Kuhnel and Beier, 1976). Histochemical demonstration of endometrial acetylcholinesterase has also been presented

(Coupland, 1962). Apart from proposed regulation of vasomotor function and mucin secretion (Hammarstrom and Sjostrand, 1979)), little is known about the role(s) of endometrial innervation (Bell, 1972).

Vasopressin (Stromberg et al., 1983), oxytocin (Soloff et al., 1977; Fuchs et al., 1985), histamine (Dey et al., 1979), PGE2 (Kennedy et al., 1986), and PGF2@ (Orlicky et al., 1986a) have all been demonstrated to either specifically bind or to stimulate responses in endometrium. Recently, PGF2@ (Orlicky et al., 1986b) and oxytocin (Flint et al., 1986) have been shown to stimulate phosphoinositide hydrolysis in rabbit and sheep endometrial preparations respectively.

Materials and Methods

Ishikawa Cells

The Ishikawa human endometrial adenocarcinoma cell line, derived from a surgically removed primary well-differentiated tumor was established by Nishida et al., (1985) and made available to us by Dr. H. Kuramoto (Kitasato University, Japan). It is the first of several similar lines to demonstrate estrogen-responsiveness. Estradiol-stimulated cell proliferation (Holinka et al., 1986a) and induction of alkaline phosphatase (Holinka et al., 1986b) and DNA polymerase @ (Gravanis and Gurpide, 1986) activities have thus far been demonstrated.

Cells were maintained in minimal essential medium (Eagle) with Earle's salts (MEM, Grand Island Biological Co., Grand Island, NY.) supplemented with 15% fetal bovine serum (FBS, GIBCO), 1% Antibiotic-Antimycotic mixture (10,000 U penicillin, 10 ug streptomycin, 25 ug/ml fungizone, GIBCO). Cells were maintained in 100x20 mm polystyrene tissue culture dishes (Falcon, Becton Dickinson Co., Cockeysville, Md.) and cells were maintained at 37 C in a National Incubator (Fisher Scientific, Pittsburgh, Pa.) in a humidified atmosphere of 5% CO2 - 95% air.

Cell harvesting

When cells reached maximal density, media was removed by aspiration and the cells were washed 3 times with 4 ml/dish Hank's balanced salt solution (HBSS, GIBCO). Four ml of 0.02% EDTA in HBSS was added to each dish and the cells were incubated at 37 C for 10-20 min. The suspended cells were then transferred by gentle pipetting from the dishes to 50 ml polystyrene centrifuge tubes (Corning Scientific, Corning, NY.) containing 4 ml/dish HBSS supplemented with 1.5 mM CaCl2, 0.5 mM MgCl2, and 5.6 mM glucose. After centrifugation (850xg for 5 min), the cell pellets derived from several dishes (usually 10) were pooled in one 50 ml centrifuge tube and washed twice with 50 ml HCMG. The combined pellet was then transferred to a 15 ml polypropylene centrifuge tube (Corning) and washed 1 time with 15 ml HCMG. In experiments in which LiCl was included, the pellet was washed with 15 ml of HCMG supplemented with 10 mM LiCl (HCMGL). The final pellet was resuspended in the desired volume of either HCMG or HCMGL, usually 1ml/dish, and immediately aliquoted into 15 ml polypropylene centrifuge tubes (200 ul/tube). Aliquots were also taken for protein determination by the method of Lowry et al., (1951). The tubes were incubated in a shaking water bath at 37 C for 30-40 min before addition of test compounds. Cell viability after these procedure was >90% as determined by trypan blue dye exclusion.

Phospholipid Extraction and Thin Layer Chromatography

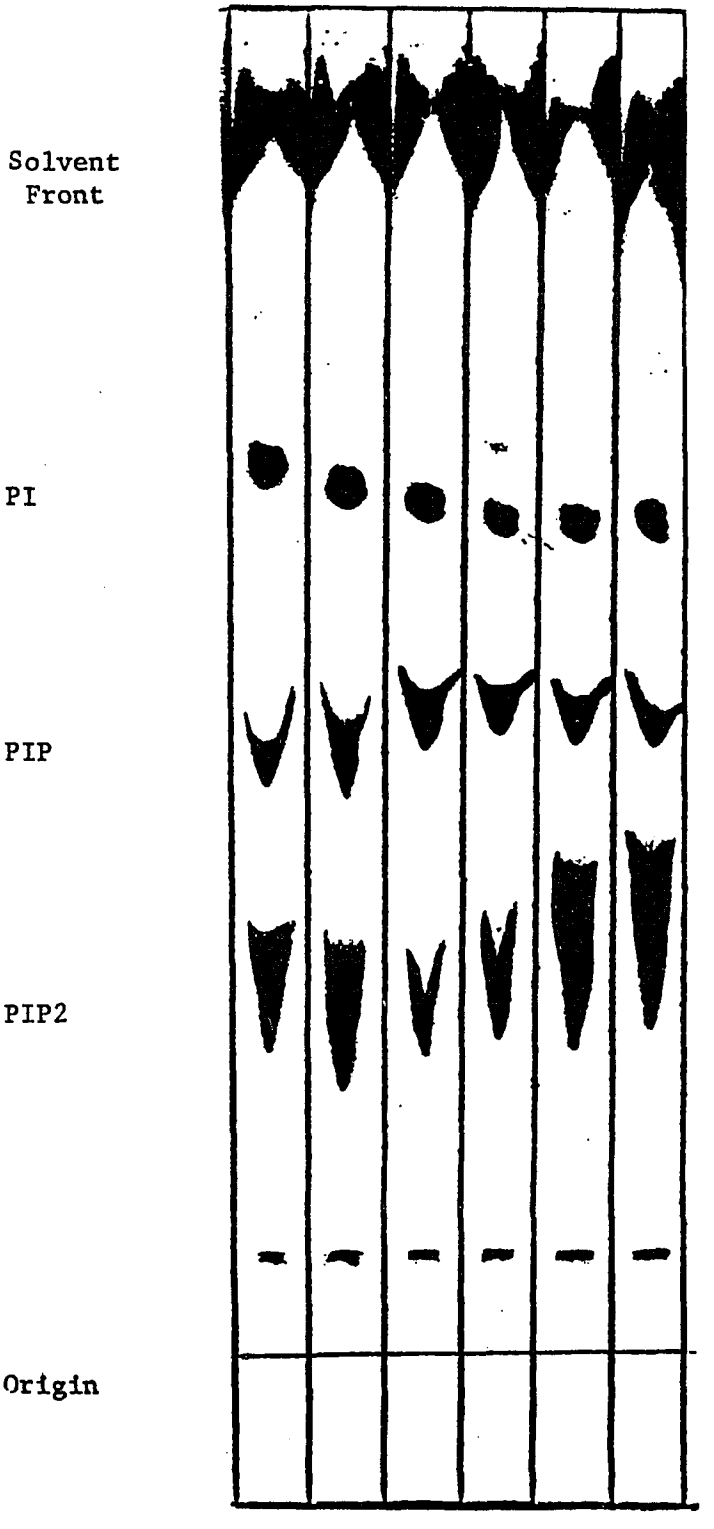
At the end of the test procedure, 2 ml of ice-cold acidified chloroform/methanol (chloroform:methanol:conc.HCl 100:100:1) was added to each tube, immediately vortexed for approximately 5 sec and placed on ice. Five hundred microliters of doubly-distilled H2O was added and each tube vortexed again for 30 sec. Emulsions were broken by centrifugation (2000xg for 5 min) The bottom (organic) layer was tranferred with a pasteur pipet to a 5 ml polypropylene test tube and the remaining aqueous layer was re-extracted with the same solvent mixture. The organic phases were combined and washed once with 1/20 vol of the upper phase of a 1:1 mixture of 1 M KCl and acidified chloroform/methanol. If not processed immediately, samples were stored at -20 C.

The extracts were taken to dryness under N2 and the dried residues were redissolved in chloroform and streaked onto the preadsorbent zone of a silica gel TLC plate (Preadsorbent Prescored Silica Gel HL, Analtech, Newark, Del.). The plate was developed in chloroform:methanol:conc.NH3(28%):H2O (90:90:7:22 with 20 mM CDTA) (Schacht, 1978). This system gives a clear separation of PI, PIP, PIP2, and myoinositol (Rf:0.68, 0.48, 0.27, 0.06 respectively; Fig. 2). Identification of phospholipids present in the samples was based on comigration with commercially available standards (Sigma).

Fig 2: Thin Layer Chromatographic Separation of Phosphoinositides

Separation of PI, PIP, and PIP2 by the chloroform:methanol:conc.NH3 (28%):H2O (90:90:7:22 with 20 mM CDTA) system described in the Methods section is demonstrated.

Figure 2



Confirmation of the identity of the phosphoinositides was suggested by the selective incorporation of [3H]-myoinositol. Recovery of phospholipid standards, determined in preliminary experiments using a lipid phosphorus assay, was > 90%.

Phospholipids were visualized by brief exposure to I2 vapor in a closed glass container. Myoinositol was visualized by spraying a 1% KMnO4/2% Na2CO3 solution onto the plate. Silica from the phospholipid containing areas was scraped directly into separate vials containing scintillation fluid and radioactivity was measured in Beckman LS9000 liqiud scintillation spectrometer to determine the incorporation of [3H]-myoinositol into phosphoinositides. Scraping and counting known amounts of labeled compounds directly spotted onto chromatographic plates demonstrated negligible quenching by the silica gel.

In order to obtain information about the specific activity of the separated phosphoinositides, radioactive content of PI, PIP, and PIP2 was expressed as dpm/nmol of PI. The amount of PI was measured in another aliquot of the sample was placed on a second plate and developed in chloroform:methanol:glacial acetic acid:H2O (100:75:4:2), a system that separates PI from the other major phospholipids (Thomas et al., 1983). Silica from the area of the plate corresponding to PI was scraped into a 5 ml polypropylene test tube and eluted by adding 2.5 ml of acidified chloroform/methanol and vortexing vigourously for 30 sec.

Following centrifugation (2000xg for 5 min), the supernatant was poured into a 5 ml borosilicate test tube (Fisher 12 x 75 mm) and the pellet was re-extracted. The combined supernatants were taken to dryness under air for assay of lipid phosphorus.

Lipid Phosphorus Assay

The procedure of Chen et al., (1956) as modified by Rebecchi et al., (1983) was used. Phospholipids eluted from TLC plates were dried under air in borosilicate tubes (Fisher 12x75 mm) to evaporate any solvents. Fifty ul of 72% perchloric acid was added to each tube, the tubes capped with marbles and then heated in aluminum blocks (Pierce Reacti-block L) at 180 C for 30 min. After cooling, 300 ul doubly-distilled H20 and 450 ul molybdate reagent (75 ul 6 N H2SO4, 75 ul 2.5% ammonium molybdate, 300 ul 2.5% ascorbic acid) were added to each tube to measure the amount of liberated phosphate. The samples were incubated at 50 C for 1 hr and the developed color was measured at 800 nm in a Beckman DU-7 spectrophotometer. Aliquots of a 1 mM K2HPO4 stock solution were used as standards following the same procedure, without the heating step, adding H20 to obtain a final assay volume of 800 ul. Linearity between 1 and 100 nmoles phosphate was obtained.

Analysis of Inositol Phosphates

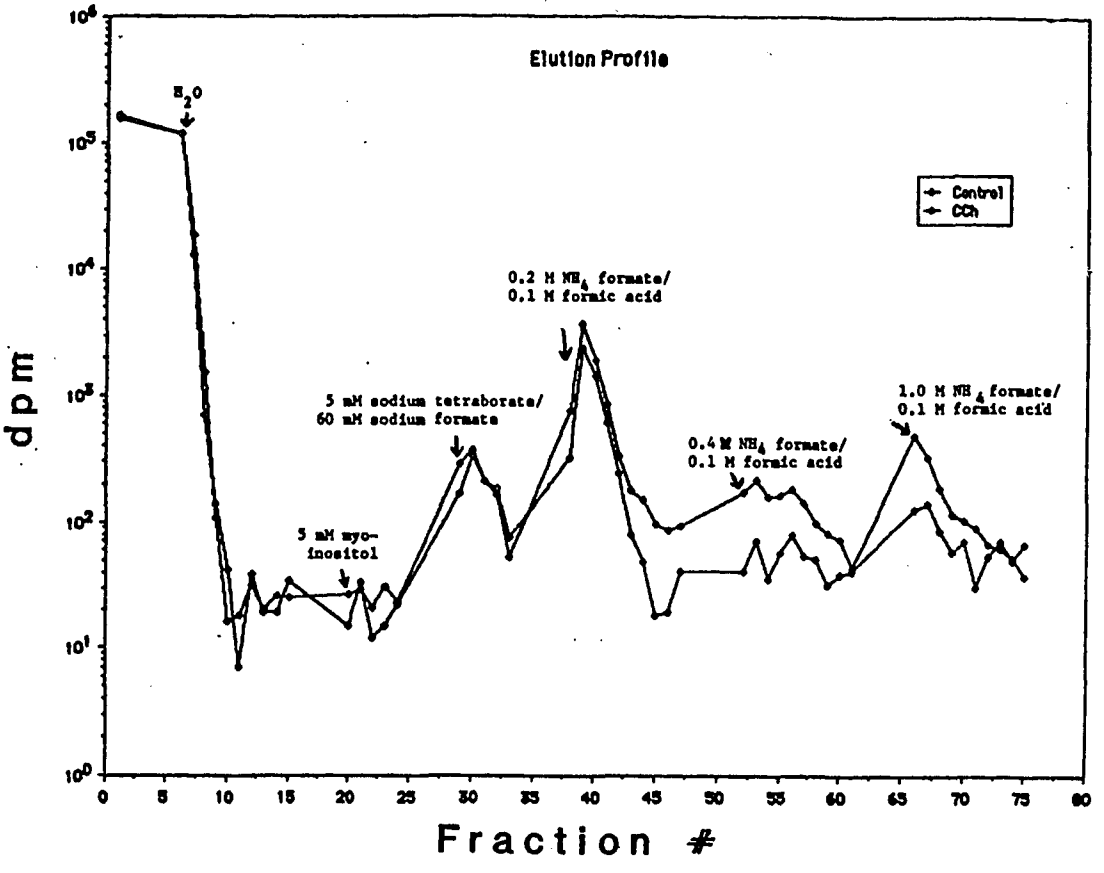
The aqueous phase from the phospholipid extraction was added directly to 5 ml polypropylene pipet tips (Sarstedt) containing 1 ml bed volume of Dowex-1 anion exchange resin

(X-8; formate form) prepared by washing sequentially with H2O, 3 N HCl, and 3 M ammonium formate. Inositol phosphates were eluted in the following fractions (Downes and Michell, 1981; Berridge et al., 1983): 10 ml H2O followed by 5 ml 5 mM myoinositol to remove free inositol; 5 ml of a 5 mM disodium tetraborate/60 mM sodium formate solution to remove cyclic inositol phosphates and glycerophosphoinositols; 10 ml 0.2 M ammonium formate/0.1 M formic acid to remove IP1; 10 ml 0.4 M ammonium formate/0.1 M formic acid to remove IP2; and finally 10 ml 1.0 M ammonium formate/0.1 M formic acid to remove IP3. Flow rates through the columns were about 0.2-0.4 ml/min at atmospheric pressure with less than 6 cm of solvent above the resin. The eluates were collected in 20 ml liquid scintillation counting vials and either aliquots or the whole fraction was mixed with 10 ml scintillation fluid (Dimiscint, National Diagnostics, Somerville, N.J.) for radioactivity measurements. Quenching was monitored by counting blank samples containing each of the eluting buffers. An elution profile is shown in Fig. 3. For examination of total inositol phosphate, the elutions with 0.2 M and 0.4 M ammonium formate were omitted and all the inositol phosphates were eluted with 1.0M ammonium formate/0.1 M formic acid. Attempts to separate IP3 and IP4 using 0.7M ammonium formate/0.1 M formic acid and 1.0 M ammonium formate/0.1 M formic acid respectively were unsuccessful. It was

Fig 3: Elution Profile of Inositol Phosphates from Anion-Exchange Columns

The aqueous fractions from the extraction of Ishikawa cells following 4 min control or CCh stimulation were added to the anion exchange columns and eluted as described in the Methods section. Each fraction represents 1 ml of the eluting buffer. The logarhythmic scale of the Y axis emphasizes the small amount of inositol phosphates produced by Ishikawa cells as compared to free myoinositol.

Figure 3


Elution Profile
H_2O
Control
CCh
0.2 M NH_4 formate/
0.1 M formic acid
5 mM sodium tetraborate/
60 mM sodium formate
1.0 M NH_4 formate/
0.1 M formic acid
0.4 M NH_4 formate/
0.1 M formic acid
5 mM myo-
inositol
dpm
Fraction #

determined that omission of the 5 mM myoinsitol elution step did not alter either the recovery or elution profile of the inositol phosphates and this step was subsequently left out. It was also determined that both the presence of methanol and the acidic pH of the aqueous phase following the extraction procedure did not affect either the elution profile or the recovery of the inositol phosphates (Berridge, et al., 1983) and so the aqueous phase was added directly to the columns without neutralization or evaporation of methanol. Recovery of isotopically labeled precursor and products from columns was >95% as monitored with [3H]-myoinositol and [14C]-IP1 standards run in separate columns. Columns were reused following washing with 3 M ammonium formate and H2O.

Curve Analyses and Statistics

Concentration-response curves were analyzed using the nonlinear regression least squares analysis program FITFUN on the PROPHET computer system (Baig and Reid-Miller, 1980). Concentration-response curves were fit to the equation:

$$IP=IPs-(IPs-IPo)/[(C/E)^{N}+1]$$

where C=hormone concentration, IPo=initial IP level, IPs=maximally stimulated IP level, IP=IP level at any given hormone concentration, E=EC50, and N=Hill coefficient.

Concentration-inhibition curves were fit to the equation:

$$IP=IPi+(IPo-IPi)/[(A/I)^{N}+1]$$

where A=antagonist concentration, IPo=initial IP level, IPi=maximally inhibited IP level, IP=IP level at any given antagonist concentration, I=IC50, and N=Hill coefficient. For both types of analyses, IP refers to either IP1, IP2, or IP3 depending on the data being evaluated. The Ki was calculated from the IC50 using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$Ki=IC50/(1+[d/Kd])$$

where Ki=equilibrium dissociation inhibition constant, IC50=half-maximal inhibitory concentration, d=hormone concentration, and Kd=equilibrium dissociation constant for the hormone (agonist). For the experiments reported here, the EC50 was considered to approximate the Kd.

Statistical analysis was performed using the two-tailed Student's t-test. Data was considered significant if $P<0.05$.

VII. MATERIALS

Compounds obtained from commercial sources included: from Sigma (St. Louis, Mo.), acetylcholine chloride, atropine sulfate, 1,2 diaminocyclohexane N'N'N'N'-tetraacetic acid (CDTA), carbamylcholine hydrochloride, decamethonium bromide, dehydroepiandrosterone sulfate, diethylstilbestrol, dihydrotesterone, dimethylsulfoxide (DMSO), estradiol, estradiol 3-sulfate, estrone, estrone 3-sulfate, ethylenediamine N'N'N'N'-tetraacetic acid (EDTA), formic acid (ammonium salt), histamine dihydrochloride,

myoinositol, phenylephrine hydrochloride, phospholipid standards, phorbol 12-myristate 13-acetate, phorbol 12,13-dibutyrate, 4@-phorbol, progesterone, sodium citrate; from Fisher (Fairlawn, N.J.), L-ascorbic acid, glacial acetic acid, conc. ammonium hydroxide (28%), chloroform, conc. hydrochloric acid (12M), lithium chloride, methanol, perchloric acid (72%), sodium (tetra)borate, from Aldrich (Milwaukee, Wi.), physostigmine (eserine), hexamethonium bromide, from Parke Davis (Morris Plains, N.J.), oxytocin (pitocin), vasopressin (pitressin), from Stuart Pharmaceuticals (Wilmington, De.), tamoxifen citrate, 4-hydroxytamoxifen citrate, from Baker (Phillipsburg, N.J.), Dowex 1X-8 anion-exchange resin (200-400 mesh, Cl-form), from Upjohn (Kalamazoo, Mi.), nafoxidine hydrochloride, and from Merrell National Labs (Cincinnati, Oh.), cis and trans clomiphene citrate. Radiochemicals ([3H]-myoinositol, [14C]-IP1, and [32P]-PO4) were from New England Nuclear (Boston, Mass.) and Amersham (Arlington Heights, Ill.). All compounds were of reagant grade purity. Purity of radiochemicals was checked by thin layer chromatographic analysis after mixing with cold standards.

RESULTS

I) Incorporation of [3H]-Myoinositol into Ishikawa Cell Phosphoinositides.

The uptake of [3H]-myoinositol following its addition (0.5 uCi/ml) to monolayer cultures of Ishikawa cells was initially examined. As shown in Figure 4, radiolabeled myoinositol entered into the cells and was incorporated into the phosphoinositides. Only a small fraction of the total radioactive tracer taken up by the cells accumulated in phospholipid pools: more than 95% of intracellular radioactivity could be recovered as free myoinositol (data not shown). Preliminary experiments also demonstrated that [32P]-PO4 could be taken up and incorporated into the phosphoinositides. This radiolabel proved unsuitable, however, for several of the analytical procedures utilized and was not used in any subsequent studies.

Isotopic steady-state labeling of PI was reached in approximately 30 hrs (Fig. 4A) as observed in other cell types under similar labeling conditions (Rebecchi et al., 1983). [3H]-Myoinositol incorporation into PIP and PIP2 reached a steady-state at approximately 30-40 hrs (Fig. 4B). Incorporation of radiolabel into other phospholipids was negligible. The amounts of radioactivity incorporated into the phosphoinositides at isotopic steady-state, shown in Table 3, reflects their relative intracellular levels. The polyphosphoinositides each constituted approximately 2-3% of the total inositol phospholipid pool, a proportion

Fig 4: Incorporation of [3H]-Myoinositol into Ishikawa cell Phosphoinositides

At time 0, media in petri dishes was replaced with media containing 0.5 uCi/ml [3H]-myoinositol. Dishes were harvested at the indicated times and phospholipids immediately extracted. Phospholipid and lipid phosphorus analyses were as described in the Methods section. Data is expressed as dpm/nmoles PI phosphorus to control for cell density. Values represent mean +/- SEM of triplicate determinations of combined data from 2 experiments.

Figure 4

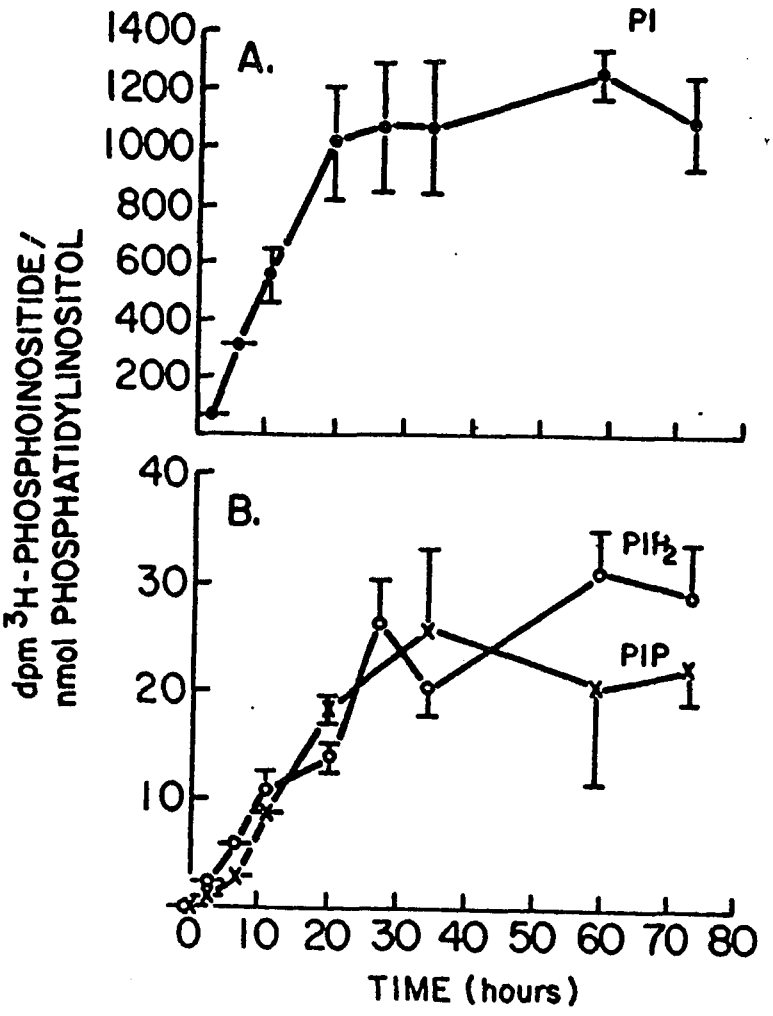

A.
PI
1400
1200
1000
800
600
400
200
B.
PIP2
PIP
40
30
20
10
0 10 20 30 40 50 60 70 80
TIME (hours)
dpm 3H-PHOSPHOINOSITIDE/
nmol PHOSPHATIDYLINOSITOL

TABLE 3: RELATIVE [3H]-MYOINOSITOL INCORPORATION INTO ISHKAWA CELL PHOSPHOINOSITIDES FOLLOWING 34 HR INCUBATION

	dpm/nmole PI phosphorus
PI	1062 ± 231
PIP	25 ± 7
PIP2	20 ± 2

Monolayer cultures of Ishikawa cells were incubated with 0.5 uCi/ml (1.1 X 10 6 dpm/ml) [3H]-myoinositol for 34 hrs, a time at which isotopic steady-state labeling of the phosphoinositides had been attained (Fig 1). Cells were harvested and the phospholipids extracted and separated by thin layer chromatography. The samples were scraped from the chromatography plates and analyzed for radiolabel content as described in the methods section. The area comigrating with the PI standard was also analyzed for lipid phosphorus and samples are expressed as dpm/nmole PI phosphorus. Values represent mean + std dev from triplicate determinations from one of two similar experiments.

that has been observed in other systems (Williamson, 1986). All subsequent experiments were performed following 48 hr incubation of monolayer cultures with [3H]-myoinositol.

II) Phosphoinositide Hydrolysis

A) Effect of Cholinergic Agonists

1) Effect of Carbachol on Phosphoinositide Levels.

Within 30 sec of exposure of suspended cells to 1 mM CCh in the presence of 10 mM LiCl, the levels of PIP2 consistently decreased to approximately 35% below control values and remained at this level for at least 5 min (Fig. 5). No consistent change in levels of radiolabeled PIP or PI were noted during this period, a finding confirmed in two similar experiments. Comparison of control and stimulated phosphoinositide levels at 30 sec following CCh exposure, as well as concurrent measurement of IP3 and IP2, is shown in Table 4. Significant increases in IP3 and IP2, 72 and 121%, respectively, were observed at this time. These results indicate that hydrolysis of PIP2 is one of the earliest effects of CCh on Ishikawa cell phosphoinositides.

2) Time Course of Carbachol-Stimulated Inositol Phosphate Accumulation: Effect of LiCl

The temporal pattern of inositol phosphate accumulation following exposure to CCh in the presence or absence of 10 mM LiCl was next investigated. The effect of lithium was examined to assess any qualitative changes in the

Fig 5: Carbachol-Stimulated Changes in Phosphoinositide Levels

Ishikawa cells were incubated in suspension with 1 mM CCh (closed figures) or H20 (open figures) for the indicated times. Following extraction, the phospholipids were separated by T.L.C. and phosphoinositides were eluted to determine radioactivity by liquid scintillation counting. Values represent means +/- S.D. from one of two similar experiments. Means at each time point were compared using Students' t-test.

Figure 5

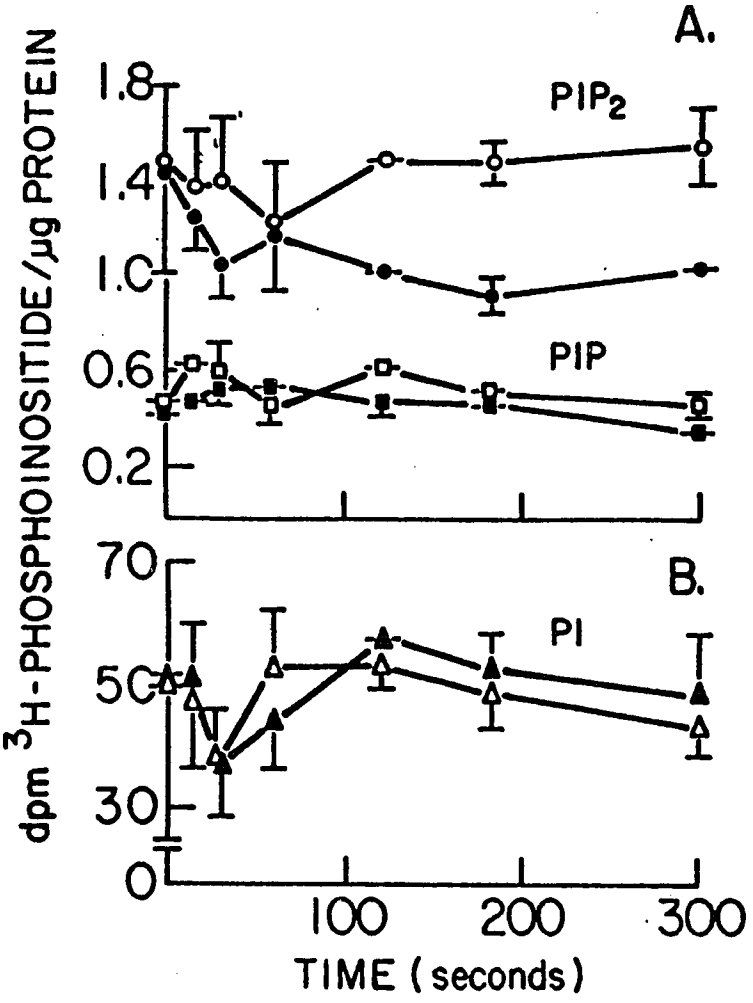

A.
PIP2
PIP
B.
PI
1.8
1.4
1.0
0.6
0.2
70
50
30
0
100
200
300
TIME (seconds)
dpm 3H-PHOSPHOINOSITIDE/µg PROTEIN

TABLE 4:

Change in Levels of Phosphoinositides and Inositol Phosphates Following 30 sec Exposure to CC

	dpm/ug protein		
	Control	CCh	% Change
PIP2	625 ± 33	448 ± 38	-35% *
PIP	273 ± 17	243 ± 24	-11%
PI	17,100 ± 1500	16,100 ± 2400	-6%
IP3	670 ± 41	1156 ± 27	+72% **
IP2	738 ± 132	1635 ± 189	+121% *

Ishikawa cells in suspension were exposed to H2O (control) or CCh in the presence of 10 mM LiCl for 30 sec and the reaction terminated by addition of chloroform:methanol:conc.HCl (100:100:1). The inositol phosphate-containing aqueous phase was placed on a Dowex X-8 anion-exchange column and the individual inositol phosphates eluted as described. The phospholipids were separated by thin layer chromatography and the areas comigrating with PIP2, PIP, and PI standards were scraped and analyzed for radiolabel content as were the anion-exchange column eluates. Data represents mean + dev for triplicate determinations from one of two simila experiments. (* $p<.005$; ** $p<.001$)

characteristics of inositol phosphate generation occuring in addition to the quantitative enhancement of inositol phosphate levels expected from inhibition of inositol phosphate phosphatase(s).

Within 30 sec of CCh exposure, concentrations of IP3 and IP2 began to rise, reaching peak levels within 2 min, and remaining elevated for 10-20 min before returning towards basal (Fig. 6A,B,D,E). In the presence of LiCl (10 mM), however, both IP3 and IP2 remained slightly elevated above basal levels at 60 min (Fig. 6D,E). In three separate experiments, the range of maximal increases of IP3 and IP2 levels relative to control values were 2.7-4.0 and 3.0-6.3 fold, respectively in the presence of LiCl and 1.5-2.6 and 2.0-3.6 fold in the absence of LiCl.

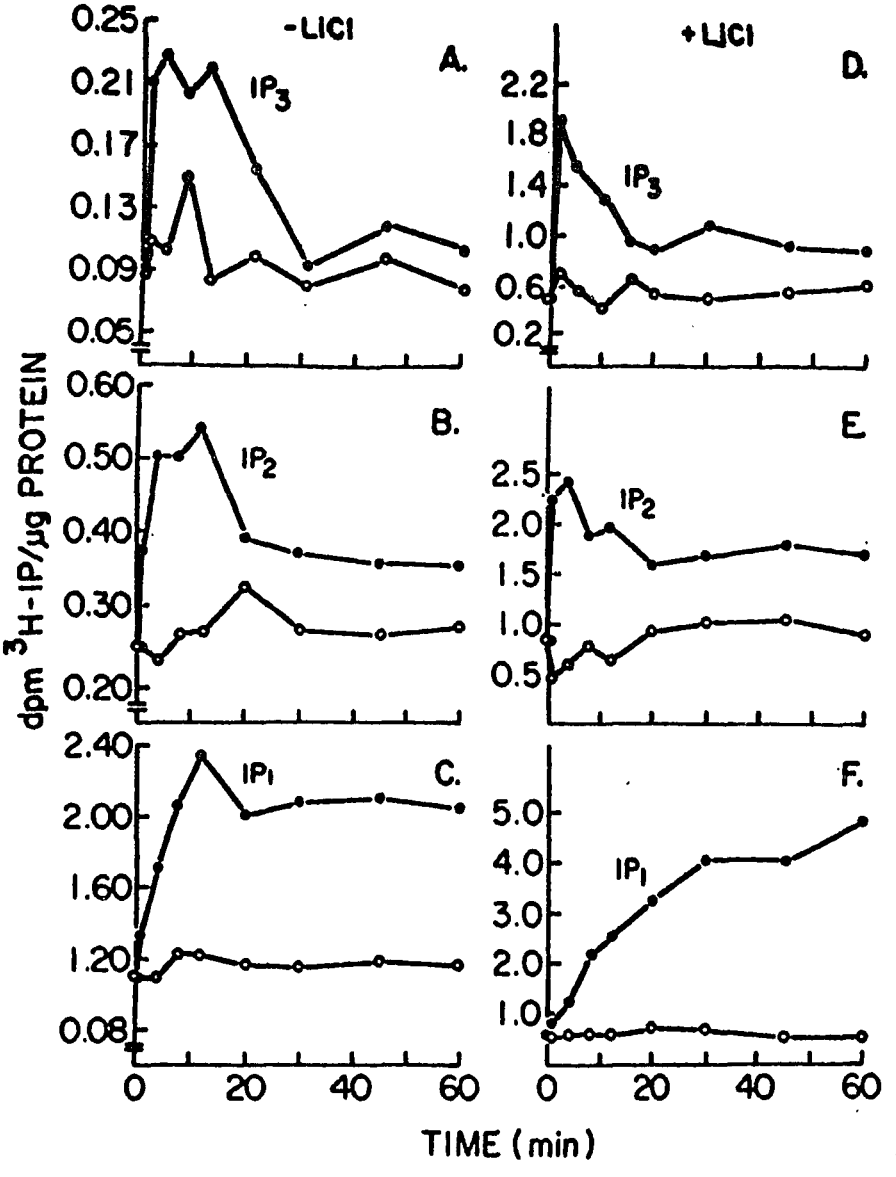
Inositol monophosphate accumulation was not detected until 1-3 min following exposure to CCh (Fig 6C,F). In the absence of LiCl, IP1 levels reached a maximum at about 10 min and remained nearly constant for at least 60 min at values approximately 2 fold higher than basal level. In contrast, IP1 continued to accumulate in the presence of LiCl reaching levels 4.2-5.8 fold higher than basal at 60 min. The initial rate of IP1 accumulation was approximately equal under both conditions.

The peak levels of radiolabeled IP3 and IP2 in the absence of LiCl were 2 to 8 fold lower than in the presence of LiCl under both basal and CCh-stimulated conditions

Fig 6: Time Course of Carbachol-Stimulated Inositol Phosphate Accumulation: Effect of LiCL

Ishikawa cells were incubated in suspension without (A-C) or with (D-F) 10 mM LiCl for the indicated times with 1 mM CCh (●) or H2O (○). Reactions were terminated and inositol phosphates separated as described in the Methods section. Values represent averages of duplicate determinations from one of three similar experiments with and without LiCl.

Figure 6


-LICl
+LICl
A.
B.
C.
D.
E.
F.
IP3
IP2
IP1
dpm 3H-IP/µg PROTEIN
TIME (min)
0.25
0.21
0.17
0.13
0.09
0.05
0.60
0.50
0.40
0.30
0.20
2.40
2.00
1.60
1.20
0.08
2.2
1.8
1.4
1.0
0.6
0.2
2.5
2.0
1.5
1.0
0.5
5.0
4.0
3.0
2.0
1.0
0
20
40
60

(range from 3 separate experiments). The relative basal levels of IP1, IP2, and IP3 shown in Fig. 6 were similar in the presence of LiCl and absence of LiCl; 10:1.7:1 and 12:2.7:1, respectively.

A more detailed analysis of the early pattern of CCh-stimulated inositol phosphate accumulation in the presence of 10 mM LiCl is presented in Fig. 7. IP3 and IP2 rose above basal levels within 15 sec following CCh addition. At 5 min, both were still elevated but had returned towards basal by 60 min. Inositol monophosphate did not increase above basal until 1-5 min after CCh exposure and was increasingly elevated at 60 min. Control levels for each inositol phosphate did not change during the time period examined (not shown). These results are consistent with those presented in Fig. 6 and further demonstrate that IP3 and IP2 are rapidly generated in response to CCh.

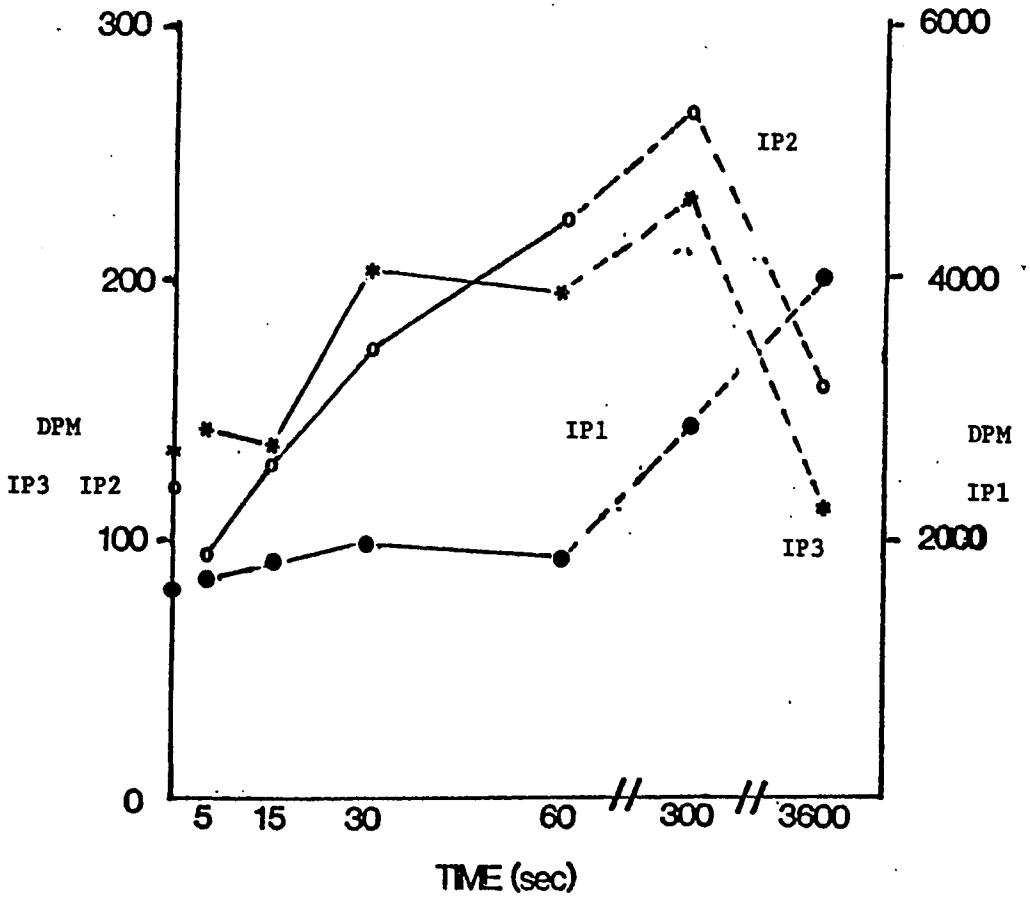
3) Concentration Dependence of the Effect of LiCl on IP1 Accumulation

Lithium was found to increase intracellular levels of IP1 in a concentration-dependent manner. In one series of experiments, LiCl was added to the modified Hank's salt solution (HCMG) to obtain concentrations ranging from 10-6 M to 10-1 M. After 60 min exposure to 1 mM CCh, the effect

Fig 7: Rapid Generation of Inositol Phosphates by Carbachol

Ishikawa cells were incubated in suspension, in the presence of 10 mM LiCl, with CCh for the times indicated. Reactions were terminated and inositol phosphates separated as described in the Methods section. Basal inositol phosphate levels are shown on the axes. Experiment shown represents one of three similar experiments.

Figure 7


300
200
100
0
6000
4000
2000
DPM
IP3
IP2
IP1
IP2
IP3
DPM
IP1
5
15
30
60
300
3600
TIME (sec)

of Li+ on IP1 accumulation was evident at concentrations greater than 1 mM and continued to increase throughout the concentration range tested (Fig. 8A). Results of parallel control experiments using increasing concentrations of NaCl in the presence of 1 mM CCh (Fig. 8A) indicated that this was not an osmotic effect but was specific for the Li+ cation.

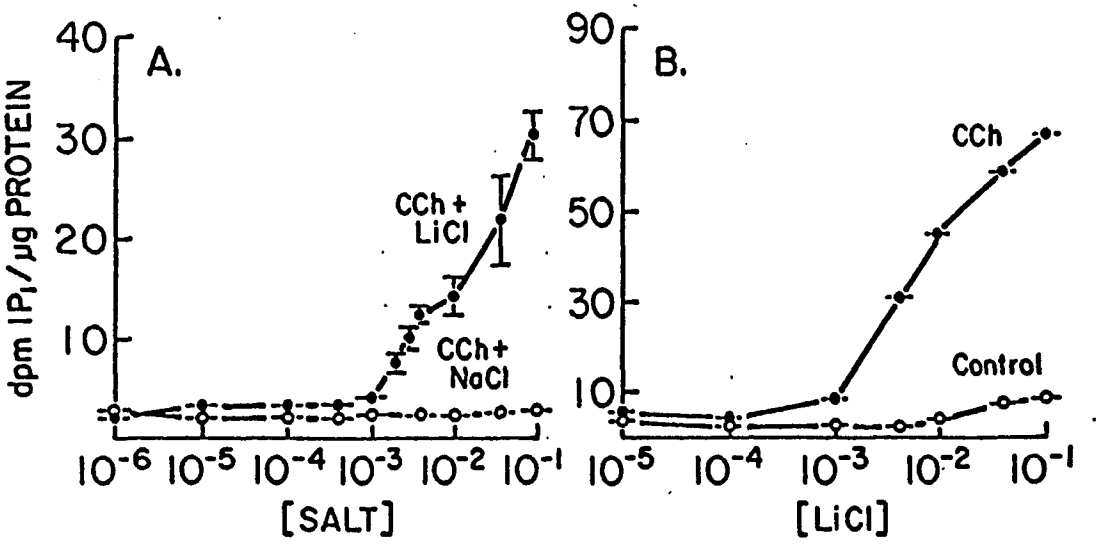
Experiments conducted in analogous solutions in which NaCl was isosmotically replaced with LiCl produced similar results. During CCh exposure, increased IP1 levels were observed at LiCl concentrations greater than 1 mM; raising the LiCl concentration to levels as high as 0.1 M continued to augment the accumulation of IP1. In the absence of CCh, basal IP1 levels were increased by LiCl at concentrations greater than 10 mM but to a lesser extent than with CCh stimulation. In three similar experiments, the maximal stimulation produced by LiCl was up to 3.3 fold the level in its absence.

The synergistic effect of LiCl on the increase in IP1 stimulated by CCh was quantitatively similar whether LiCl was added hypertonically or isotonically. Under hypertonic conditions, addition of 10 mM LiCl alone resulted in a 2-fold increase in basal IP1 level after 60 min. Carbachol addition (1 mM) in the absence of LiCl resulted in up to a 2.5 fold increase in IP1 levels. When CCh was tested in the presence of 10 mM LiCl, either in hypertonic or isotonic solutions, IP1 levels rose up to

Fig 8: Effect of LiCl on IP1 Accumulation

Ishikawa cells were incubated in suspension with LiCl or NaCl 30 min prior to exposure to CCh for 60 min. Reactions were terminated and the inositol phosphates separated as described in the Methods section. The curves in A represent CCh stimulated IP1 levels with increasing amounts of LiCl or NaCl added hypertonically to the buffer. In B, LiCl was added isosmotically, replacing NaCl present in the HCMG buffer. The curves in B represent basal and CCh-stimulated inositol phosphate accumulation. Values shown are means +/- S.D. of triplicate determinations for one of three similar experiments for both hyperosmotic and isosmotic salt addition.

Figure 8


A.
B.
dpm IP1/µg PROTEIN
40
30
20
10
90
70
50
30
10
CCh + LiCl
CCh + NaCl
CCh
Control
10-6 10-5 10-4 10-3 10-2 10-1
10-5 10-4 10-3 10-2 10-1
[SALT]
[LiCl]

9.2 fold. Similarly, no difference in the maximal enhancement of CCh-stimulation by LiCl (100 mM) was observed between hypertonic (4.7-9.5 fold) and isotonic (8.8-13.5 fold) conditions.

B) Characterization of Muscarinic Response

1) Concentration Dependence of the Effects of CCh and ACh on Inositol Phosphate Accumulation.

Acetylcholine and carbamylcholine were both found to increase the intracellular levels of tritiated total inositol phosphate (consisting mostly of IP1) in a concentration-dependent, saturable manner following 60 min exposure of suspended Ishikawa cells in the presence of 10 mM LiCl (Fig. 9A,B). The observed EC50's of 26.5 +/- 4.8 uM for CCh and 3.5 +/- 1.6 uM for ACh (Table 5) are similar to the values reported for cholinergic stimulation of PI turnover in other systems (Table 2). Non-linear regression curve fitting using the program FITFUN of the PROPHET computer system yielded Hill coefficients of 1.02 +/- 0.25 for CCh and 0.91 +/- 0.04 for ACh. The observed coefficients, which approximate unity, suggest that the effects of ACh and CCh on the phosphatidylinositol system can be described by simple mass action equations involving a single class or subtype of receptor.

Included also in Fig. 9 is a concentration-response curve corresponding to the effect of ACh in the presence of the acetylcholinesterase inhibitor physostigmine (10 uM). No significant effect on either the EC50 or the Hill

Fig 9: Concentration-Response Curves for CCh and ACh. Ishikawa cells in suspension were incubated for 60 min with increasing concentrations of CCh (A), ACh (B), or ACh + 10 uM physostigmine (C). Reactions were terminated and inositol phosphates separated as described. The curves were analyzed by least-squares nonlinear regression curve fitting as described in the methods. Values represent means of triplicate determinations from one of 5 (CCh), 3 (ACh), or 3 (ACh + 10 uM physostigmine) similar experiments.

Figure 9

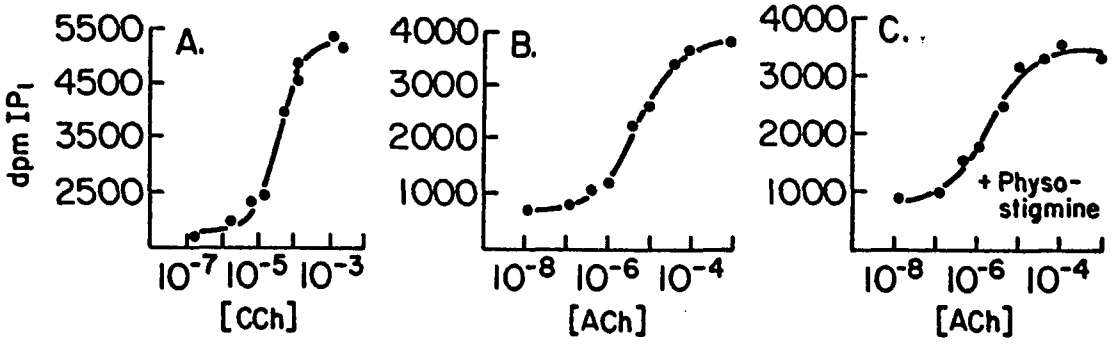

dpm IP1
5500
4500
3500
2500
A.
10-7 10-5 10-3
[CCh]
4000
3000
2000
1000
B.
10-8 10-6 10-4
[ACh]
4000
3000
2000
1000
C.
+ Physo-
stigmine
10-8 10-6 10-4
[ACh]

Table 5:

Regression Analyses of Concentration-Response and Concentration-Inhibition Curves

Concentration-response and concentration-inhibition curves were analyzed using the nonlinear regression least-squares analysis program FITFUN on the PROPHET computer system as described in the Methods section. The Ki's for the concentration-inhibition curves were calculated from the computer-generated IC50's using the Cheng-Prusoff equation. Values represent means +/- S.E. from 3 similar experiments for each condition except for the 4 min atropine inhibition and CCh concentration-response without LiCl experiments where the values represent results of one of 2 similar experiments.

Concentration-Response

	EC50 (uM)	Hill Coefficient	
CCh (1 hr total IP)	26.5 ± 4.8	1.02 ± 0.25	(n=3)
CCh w/o LiCl (1 hr total IP)	9.4	0.97	(n=2)
CCh (4 min IP3)	31.4 ± 19.4	1.02 ± 0.53	(n=3)
CCh (4 min IP2)	45.7 ± 14.7	0.92 ± 0.04	(n=3)
CCh (4 min IP1)	11.7 ± 2.0	0.78 ± 0.31	(n=3)
ACh (1 hr total IP)	3.5 ± 1.6	0.91 ± 0.04	(n=3)
ACh + 10 uM physostigmine (1 hr total IP1)	2.7 ± 1.6	0.98 ± 0.18	(n=3)

Concentration-Inhibition

	Ki (nM)	Hill Coefficient	
Atropine (1 hr CCh-stim total IP)	1.6 ± 1.3	0.71 ± 0.09	(n=3)
Atropine (4 min CCh stim IP3)	0.49	0.94	(n=2)
Atropine (4 min CCh stim IP2)	1.92	0.90	(n=2)
Atropine (4 min CCh stim IP1)	2.20	1.07	(n=2)

coefficient for ACh stimulation of IP1 accumulation was noted with physostigmine (Fig. 9C, Table 5) indicating lack of demonstrable acetylcholinesterase activity in Ishikawa cells incubated under these conditions.

ACh and CCh were of equal efficacy (data not shown). Studies including both agonists demonstrated that saturating concentrations of each produced approximately the same increase in inositol phosphate accumulation (ranging from 3 to 15 fold the basal level in a given experiment).

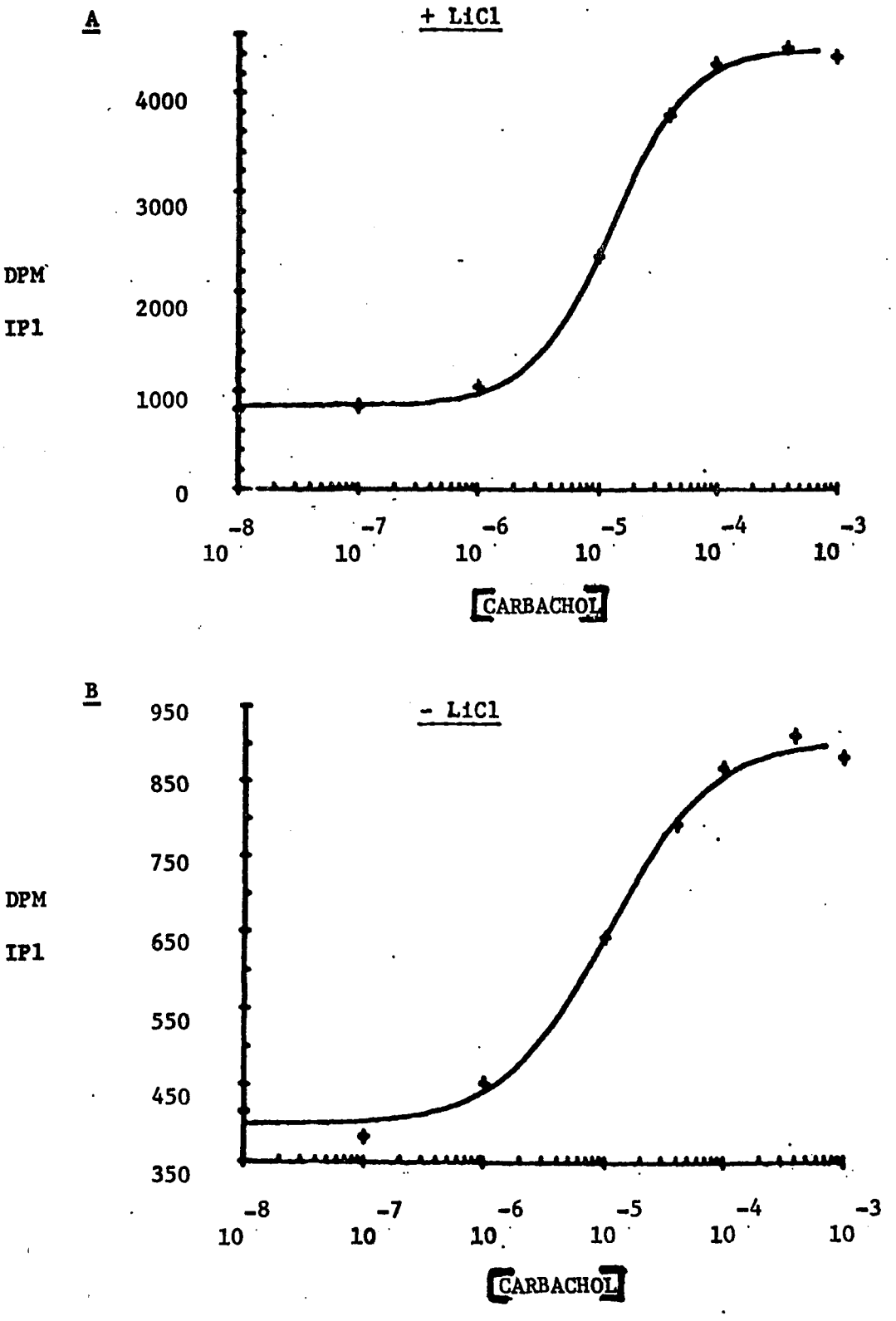
2) Effect of LiCl on Concentration-Response Curves to CCh

Since lithium influenced the pattern of inositol phosphate generation in response to CCh (Figs. 6 and 8), it is possible that at least part of LiCl's action may be on the initial hormone-dependent steps of phosphoinositide hydrolysis. To examine potential roles in the processes through which the muscarinic agonists generate inositol phosphate, several concentration response curves for CCh were simultaneously performed in the presence and absence of 10 mM LiCl. As seen in Fig. 10, the accumulation of inositol phosphate in the absence of LiCl was concentration-dependent and saturable. The EC50 of CCh-stimulation was approximately 1/3 that for concentration-response curves carried out in the absence of LiCl. The Hill coefficient, however, was similar to that in the presence of LiCl ie., approximately equal to 1 (Table 5). Both the maximal stimulation induced by CCh

Fig 10: Concentration-Response Curve to CCh in the Presence and Absence of 10 mM LiCl

Ishikawa cells were incubated in suspension for 60 min with increasing concentrations of CCh in either the presence (A) or absence (B) of 10 mM LiCl. Reactions were terminated and inositol phosphates separated as described in the Methods section. The curves were analyzed by least-squares nonlinear regresion analysis as described. Values represent means of triplicate determinations from one of 2 separate experiments.

Figure 10


A
+ LiCl
4000
3000
2000
1000
0
DPM
IP1
10^-8
10^-7
10^-6
10^-5
10^-4
10^-3
[CARBACHOL]
B
- LiCl
950
850
750
650
550
450
350
DPM
IP1
10^-8
10^-7
10^-6
10^-5
10^-4
10^-3
[CARBACHOL]

(2-3 fold) and the basal levels of inositol phosphate were also lower in the absence of LiCl, consistent with the findings of Figs. 6 and 8.

Apart from the slight decrease in EC50 and the decrease in maximal fold stimulation, the characteristics of the concentration-response for CCh-stimulated inositol phosphate (IP1) generation do not appear to be dependent on the presence of LiCl.

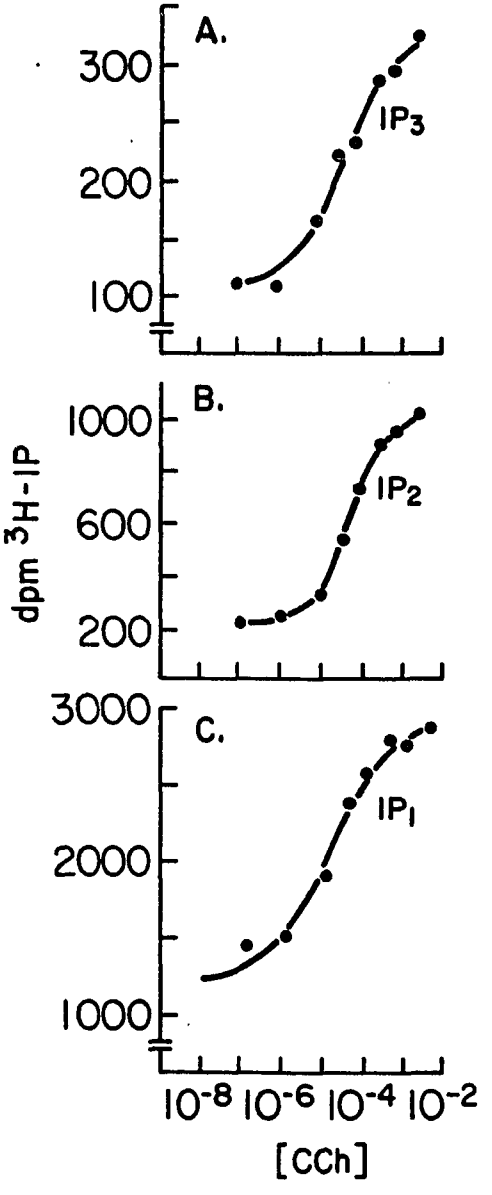
3) Concentration-Response of Individual Inositol Phosphates to CCh

To assess whether the accumulation of the individual inositol phosphates demonstrated similar concentration-response characteristics to those for total IP accumulation, studies examining the simultaneous production of each after 4 min exposure to CCh were performed (Fig. 11). This incubation period was chosen because, as demonstrated in Figs. 6 and 7, all three inositol phosphates were significantly elevated at that time. For each of the inositol phosphates, there was a saturable, concentration-dependent accumulation following exposure to increasing amounts of CCh. The respective EC50's and Hill coefficients, shown in Table 5 do not appreciably differ from that for total inositol phosphate accumulated at 60 min.

Fig 11: Concentration-response for CCh-stimulation of IP3, IP2, and IP1 Accumulation Measured at 4 min.

Ishikawa cells in suspension were incubated for 4 min with increasing concentrations of CCh. Inositol phosphates were analyzed as described in the Methods section. Curves were analyzed using least-squares nonlinear regression analysis as described. Values represent means of triplicate determinations from one of three similar experiments.

Figure 11


A.
300
200
100
IP3
B.
1000
600
200
IP2
dpm 3H-IP
3000
2000
1000
C.
IP1
10-8 10-6 10-4 10-2
[CCh]

4) Effect of Cholinergic Antagonists

The stimulatory effect of CCh was inhibited by atropine but not by nicotinic antagonists. Inhibition by atropine of the total inositol phosphate accumulation stimulated by 60 min exposure to CCh was concentration-dependent, saturable, and complete at approximately 1 uM (Fig. 12). Maximal concentrations of atropine had no effect on basal values. Regression analysis of 3 similar experiments demonstrated an average IC50 of approximately 10 nM corresponding to an approximate Ki of 1.6 +/- 1.3 nM as determined by using the Cheng-Prusoff equation (Table 5). The Ki, however, is only an approximation because the Cheng-Prusoff transformation is dependent on a Hill coefficient of unity. The average Hill coefficient for atropine inhibtion observed in Ishikawa cells was 0.71 +/-0.09.

Atropine also inhibited, in a concentration-dependent, saturable manner, accumulation of the individual inositol phosphates following 4 min exposure to CCh (Fig. 13). Observed Hill coefficients and calculated Ki's from one of two similar experiments are shown in Table 5. The respective Ki's are again indicative of interaction with muscarinic receptors. In contrast to that for atropine inhibition of total inositol phosphate accumulated following 1 hr CCh exposure, the Hill coefficients for inhibition of IP1, IP2, and IP3 accumulated following 4 min CCh exposure are all close to unity.

Fig 12: Atropine Inhibition of CCh-Stimulated Inositol Phosphate Accumulation.

Ishikawa cells in suspension were incubated for 10 min with increasing concentrations of atropine prior to the addition of 300 uM CCh. Reactions were terminated after 60 min and inositol phosphates analyzed as described. Curves were analyzed using least-squares nonlinear regression analysis and the Ki calculated using the Cheng-Prusoff equation as described in the Methods section. Values represent means of triplicate determinations from one of 3 similar experiments.

Figure 12

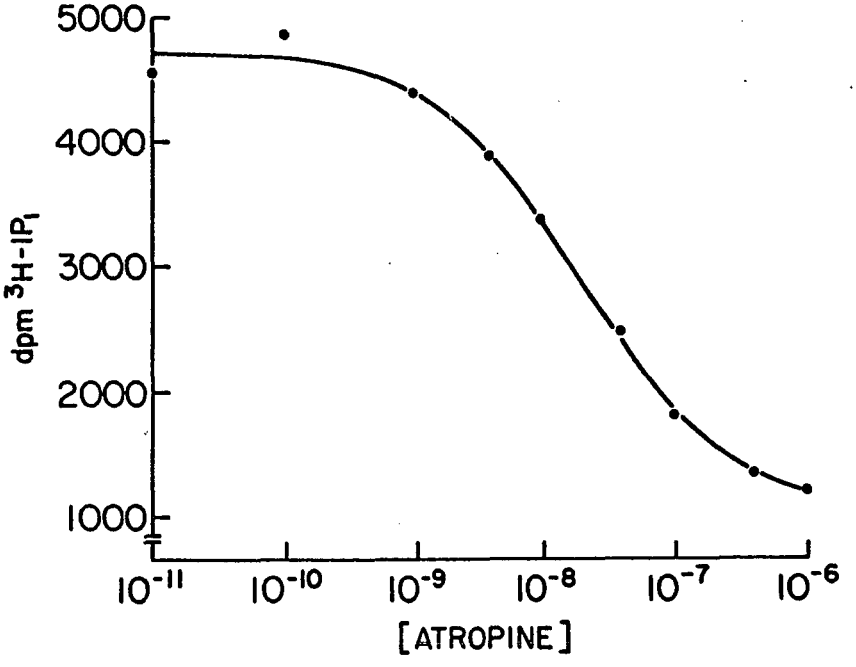

5000
4000
3000
2000
1000
dpm ^{3}H-IP$_1$
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
[ATROPINE]

Fig 13: Atropine Inhibition of 4 min CCh-Stimulated IP Accumulation

Ishikawa cells in suspension were incubated for 10 min with increasing concentrations of atropine prior to the addition of 300 uM CCh. Reactions were terminated after 4 min and inositol phosphates analyzed as described. Curves were analyzed using least-squares nonlinear regression analysis and the Ki calculated using the Cheng-Prusoff equation as described in the methods section. Values represent means of triplicate determinations from one of two similar experiments.

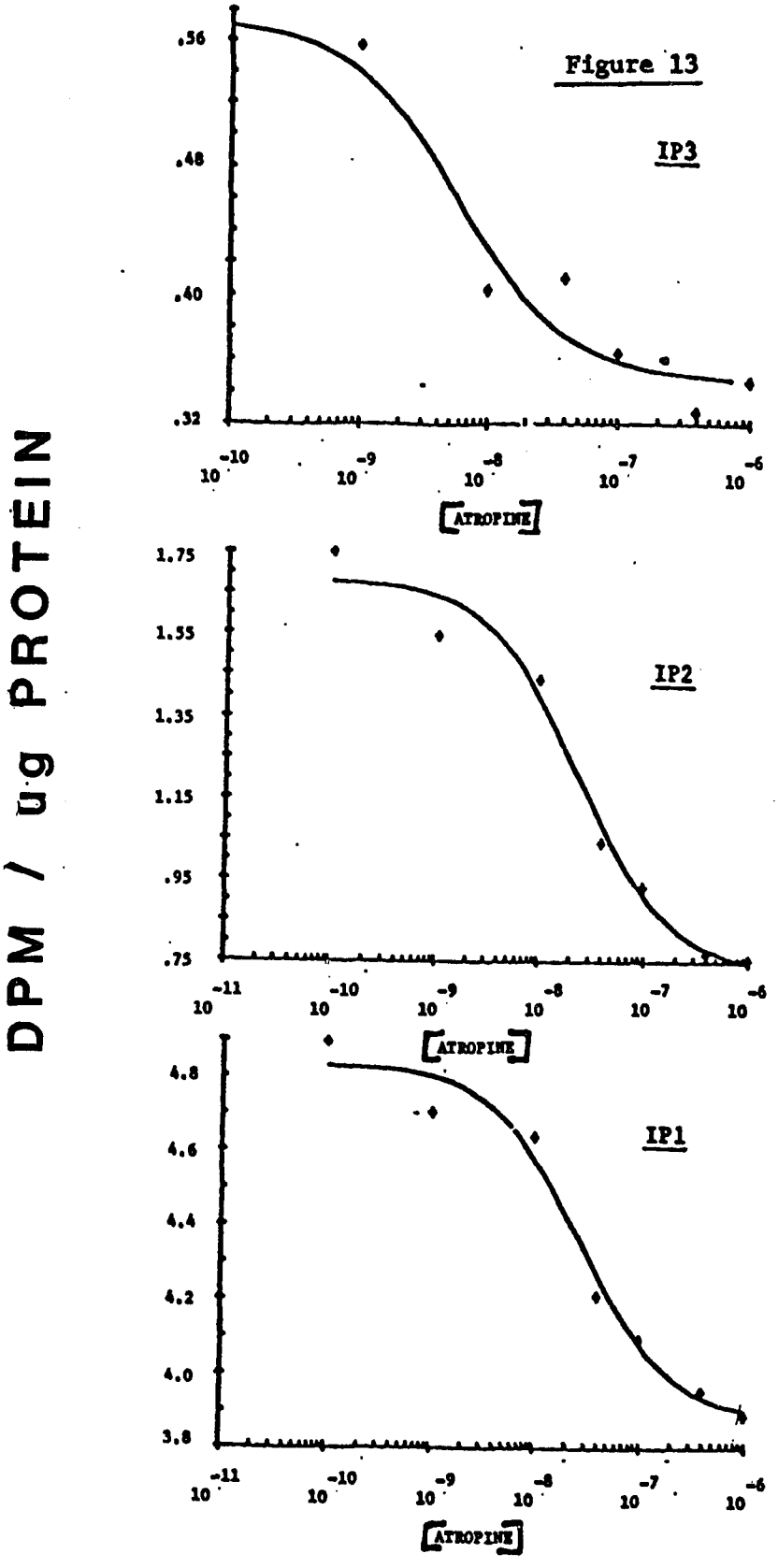
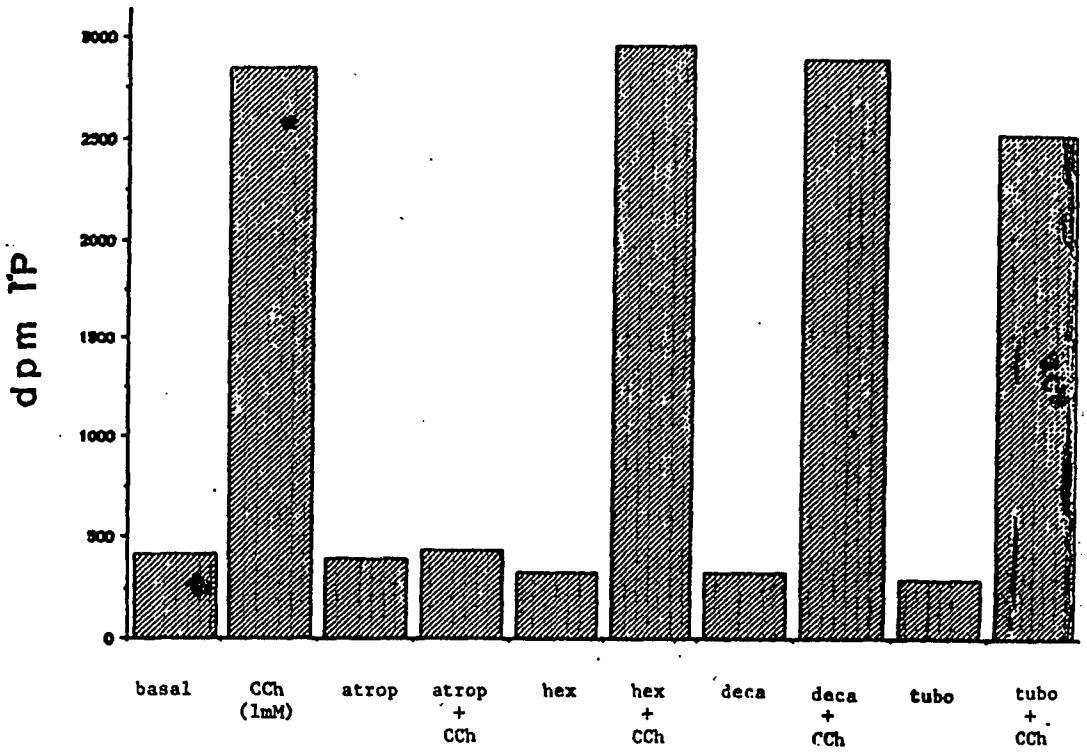

DPM / ug PROTEIN
.56
.48
.40
.32
10^-10
10^-9
10^-8
10^-7
10^-6
[ATROPINE]
IP3
1.75
1.55
1.35
1.15
.95
.75
10^-11
10^-10
10^-9
10^-8
10^-7
10^-6
[ATROPINE]
IP2
4.8
4.6
4.4
4.2
4.0
3.8
10^-11
10^-10
10^-9
10^-8
10^-7
10^-6
[ATROPINE]
IP1

Figure 13

Fig 14: Effect of Nicotinic Antagonists on Basal and CCh-Stimulated Inositol Phosphate Accumulation

Ishikawa cells in suspension were incubated with 100 uM concentrations of hexamethonium, decamethonium, or d-tubocurarine or with 1 uM atropine for 10 min and then subsequently incubated for 60 min with or without 1 mM CCh. Values shown on bar graph represent mean of triplicate determinations for each condition from one of 2 similar experiments.

Figure 14


dpm ³P
3000
2500
2000
1500
1000
500
0
basal
CCh (1mM)
atrop
atrop + CCh
hex
hex + CCh
deca
deca + CCh
tubo
tubo + CCh

The nicotinic antagonists hexamethonium (10-4 M), decamethonium (10-4 M), and d-tubocurarine (10-4 M) had no effects on either basal or stimulated inositol phosphate levels following 60 min exposure to CCh (Fig. 14) further confirming the muscarinic nature of the observed effects.

C) Effects of Other Plasma Membrane Acting Agents on Ishikawa Cell PI Turnover

1) Agonists

Since many agents have been demonstrated to stimulate phosphoinositide hydrolysis, we were interested in whether other than muscarinic effect could be observed in Ishikawa cells. Vasopressin, oxytocin, histamine, and PGF2@ have all been observed to either specifically bind or to initiate various cellular processes in endometrium. Phenylephrine was also assessed because of suggestions of adrenergic innervation of endometrium (Hammarstrand and Sjostrand, 1979). All of these agents have been demonstrated to stimulate phosphoinositide hydrolysis through their relevant receptors in other systems. As shown in Table 6, no effect on inositol phosphate accumulation in Ishikawa cells was observed for any of these substances following exposure for up to 60 min. The concentrations used for each agent were those that had been shown to stimulate phosphoinositide hydrolysis elsewhere. The lack of effect in Ishikawa cells may reflect absence of the relevant receptors or lack of proper coupling between receptor occupation and phosphoinositide hydrolysis.

Table 6: Effect of other agents on Ishikawa cell inositol phosphate accumlation

Ishikawa cells in suspension were incubated for 60 min, except where noted, with the listed agents in the presence of 10 mM LiCl. The reactions were stopped and inositol phosphates separated as described in the Methods section. Ethanol or DMSO alone consistently raised both basal and CCh-stimulated IP values from 10-50%. Vasopressin, oxytocin, PGF2@ and estradiol values were corrected for vehicle (ethanol) stimulation. Single experiments with 1 mM histamine and with 1 uM concentrations of progesterone, dihydrotestosterone, and dehydroepiandrosterone sulfate also showed no difference from control values. Single experiments examining estradiol + CCh, estradiol sulfate + CCh, estrone sulfate + CCh, oxytocin + CCh, and PGF2@ + CCh also showed no difference from control values. For each agent, mean +/- S.E. of determinations from several investigations is shown with n signifying the total number of experimental points. The total number of experiments in which each agent was evaluated is also indicated.

		% of basal IP		Expts
PGF2@	1.2 uM	109 ± 6	(n=6)	2
	12 uM	103 ± 7	(n=5)	2
Oxytocin (30 min)	100 nM	103 ± 13	(n=6)	2
Vasopressin	100 nM	107 ± 7	(n=5)	2
Phenylephrine	1 mM	110 ± 5	(n=5)	2
Estradiol	1 uM	92 ± 17	(n=5)	2
Estradiol 3-sulfate	1 uM	103 ± 13	(n=6)	2
Estrone 3-sulfate	1 uM	110 ± 12	(n=6)	2

D) Other Compounds that Modulate Inositol Phosphate Accumulation in Ishikawa Cells

1) Phorbol Esters

Preliminary experiments from other ongoing projects in the laboratory had suggested that phorbol esters affected activities of certain enzymes in Ishikawa cells notably alkaline phosphatase (Holinka et al., 1986b) and DNA polymerase @ (Gravanis and Gurpide, 1986). It thus became of interest to evaluate any discernible intracellular effects of these compounds. Figure 15 shows the inhibition of CCh-stimulated total inositol phosphate accumulation resulting from incubation of the cells for 10 min with increasing concentrations of PMA and PDB prior to 60 min exposure to CCh. For both compounds, inhibition was concentration-dependent; initially apparent at concentrations greater than 10 fM and maximal at approximately 1 nM with IC50's of approximately 2 pM and 7 pM respectively for PMA and PDB. The maximal inhibition produced by either compound was about 35%. The IC50's and extent of inhibition observed in Ishikawa cells are comparable to similar effects of phorbol esters on hormonally stimulated phosphoinositide hydrolysis in many other systems (Labarca et al., 1984; Lynch et al., 1985; Kanba et al., 1986). Preincubation of Ishikawa cells with PMA for up to 30 min had no further effect on the degree of inhibition observed (data not shown). Neither phorbol ester had any effect on basal IP level although the DMSO

vehicle caused a slight increases in both basal and CCh-stimulated IP.

The non-tumor promoting ester, 4@-phorbol, is known to be unable to activate protein kinase C or to stimulate protein kinase C-mediated events (Nishizuka, 1984). In accordance, no effect of 2.7 nM 4@ phorbol, a concentration slightly higher than that which produced maximal effect by PMA and PDB, was observed on CCh-stimulated IP accumulation in Ishikawa cells (Fig. 15).

2) Steroid Hormones

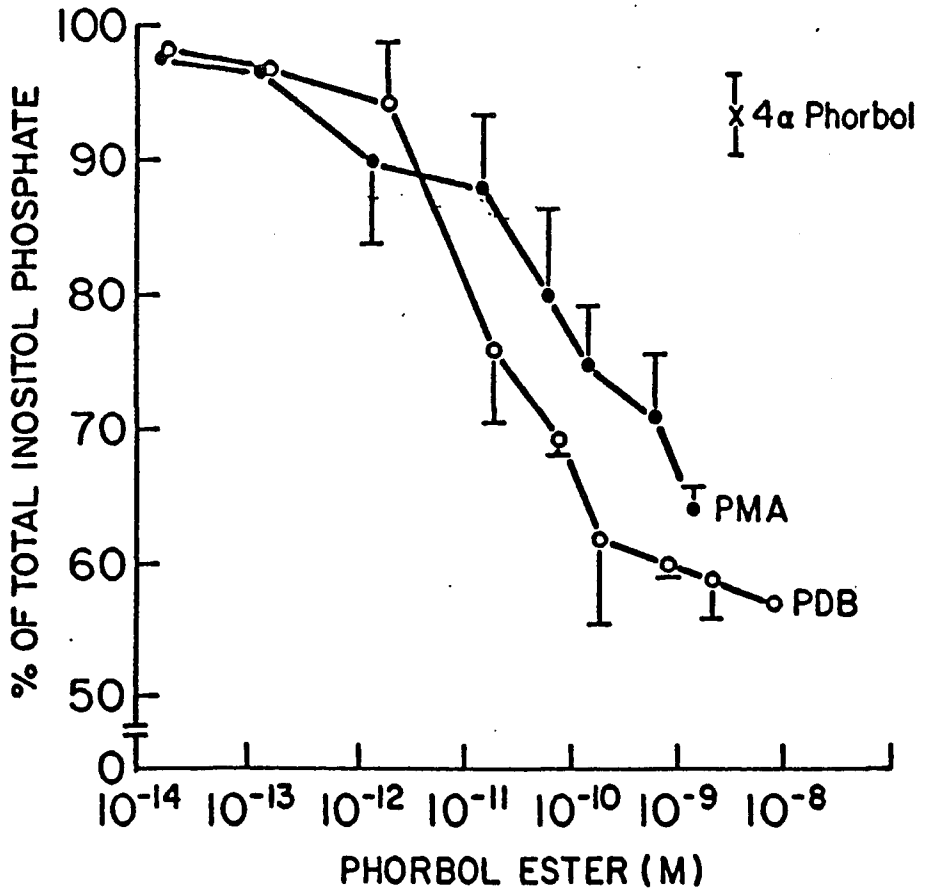
At present, evidence for the interaction of steroid hormones with phosphoinositide hydrolysis is scant. There is, however, a surprisingly large amount of information demonstrating the influence of steroids on other plasma membrane signal transduction processes, most notably the adenylate cyclase system. The Ishikawa cell line, the first of several clonal endometrial adenocarcinoma lines to respond to estrogen, is potentially an ideal system in which to examine the effects of steroid hormones on other second messenger processes.

The effect of various gonadal steroids and steroid metabolites was first evaluated following short-term exposure. Incubation of cells suspended in HCMGL buffer for 60 min to estradiol, estrone, progesterone, dihydrotesterone, dehydroepiandosterone sulfate, estradiol 3-sulfate, or estrone 3-sulfate at physiological or supraphysiological concentrations had no effect on inositol

Fig 15: Phorbol Ester Inhibition of CCh-Stimulated IP1 Accumulation.

Ishikawa cells in suspension were incubated with either increasing concentrations of PMA or PDB or with 2.7 nM 4@ phorbol 10 min prior to the addition of 1 mM CCh. After 60 min the reactions were terminated and the inositol phosphates separated as described in the Methods section. The DMSO vehicle slightly enhanced both basal and CCh-stimulated inositol phosphate levels. Values represent mean +/- S.D. of triplicate determinations from one of 3 (PMA), or 2 (PDB, 4@ phorbol) similar experiments.

Figure 15


100
90
80
70
60
50
0
% OF TOTAL INOSITOL PHOSPHATE
4α Phorbol
PMA
PDB
10-14 10-13 10-12 10-11 10-10 10-9 10-8
PHORBOL ESTER (M)

phosphate accumulation (Table 6). Preincubating Ishikawa cells for 60 min with estradiol, estradiol 3-sulfate, or estrone 3-sulfate followed by 60 min exposure to 1 mM CCh did not affect the degree of stimulation by CCh.

Longer exposure to steroids was next considered. In a single experiment, cells grown for 2 passages in medium containing serum that had been pretreated with activated charcoal to remove endogenous steroids (Holinka et al., 1986a) were incubated for 24 h in monolayer culture with the stripped medium to which either estradiol (10-8 M) or progesterone (10-6 M) had been added. Estradiol had no effect on either basal or 1 mM CCh-stimulated total inositol phosphate level. Progesterone slightly decreased both basal and stimulated IP level (data not shown), however, the magnitude of CCh stimulation was unaffected by progesterone. The degree of CCh stimulation was similar to that observed in cells grown in stripped serum-containing medium that had not been exposed to steroids and also to cells grown in normal serum containing medium.

3) Triphenylethylene Antiestrogens

Among the compounds reported to influence protein kinase C activity, recent findings have demonstrated that micromolar concentrations of triphenylethylene antiestrogens can act, in vitro, as protein kinase C inhibitors (O'Brian et al., 1986). These compounds were of particular interest with respect to Ishikawa cells since they had been shown, in other ongoing laboratory

investigations, to antagonize certain estrogen-dependent responses. The results of Fig. 15 demonstrating phorbol ester inhibition of the CCh-stimulated accumulation of total inositol phosphate implied the presence of a functional protein kinase C in Ishikawa cells. This observation suggested that Ishikawa cells were potentially a good system in which to evaluate, in vivo, the effects of antiestrogens on protein kinase C activity.

In preliminary experiments Ishikawa cells were incubated in suspension with tamoxifen (100 uM) or PMA (1 nM) for 10 min followed by 60 min incubation with CCh. Tamoxifen was found to produce the same effect as PMA. Carbachol-stimulated total IP levels were reduced to approximately the same degree by the above concentrations of tamoxifen and PMA. 4-Monohydroxytamoxifen (100 uM) also produced a similar inhibition. Unlike PMA, however, tamoxifen increased the basal IP level about 2.5 fold. These results suggested that, in contrast to the protein kinase C inhibition observed in vitro, tamoxifen appeared to produce an in vivo stimulation. The increased basal IP levels, however, suggested that tamoxifen may have other actions with respect to phosphoinositide hydrolysis. In particular, another triphenylethylene antiestrogen, clomiphene, had been shown to bind to muscarinic receptors from guinea pig brain homogenates (Ben-Baruch et al., 1982), suggesting that tamoxifen may be directly stimulating inositol phosphate accumulation through

muscarinic receptors in Ishikawa cells.

The concentration-dependent effects of tamoxifen on both basal and CCh-stimulated total inositol phosphate accumulation were next evaluated. In the concentration range 10-150 uM, tamoxifen stimulated a concentration-dependent increase in total IP level and simultaneously inhibited the increase elicited by 60 min exposure to a saturating amount of CCh (1 mM) (Fig. 16). The EC50 and IC50 for the stimulatory and inhibitory effects respectively were both approximately 20 uM. Since tamoxifen at concentrations greater than 150 uM is not completely soluble in aqueous buffer, it could not be determined if either of these effects were saturable. The same increase in IP level was obtained with 150 uM tamoxifen alone or with 150 uM tamoxifen plus 1 mM CCh. Similar observations were produced by the related compounds clomiphene and nafoxidine. Interestingly, in 2 preliminary experiments, atropine, in the concentration range 0.1 nM-1 uM, while having no effect on basal IP levels, increased by up to 50% the stimulation produced by tamoxifen (data not shown).

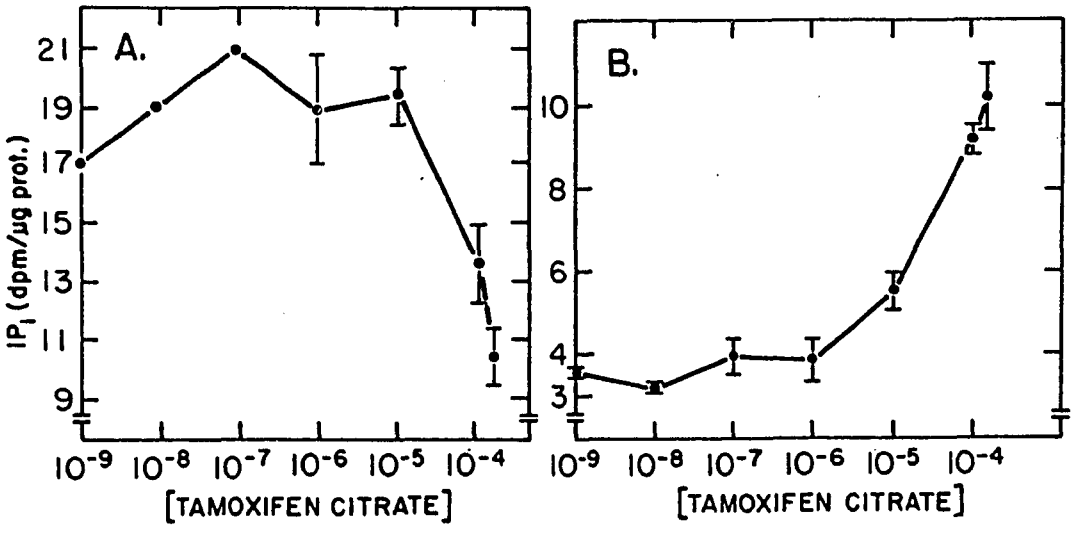
Evaluation of cell viability during antiestrogen exposure using trypan blue dye exposure, however, demonstrated that the cells rapidly (within 4 min of exposure) lost the ability to exclude trypan blue. This observation and the similarity in IP levels reached with maximal tamoxifen alone to those reached with maximal

inhibition of CCh-stimulated levels suggests that tamoxifen and the other above-mentioned compounds act as membrane perturbants, ie., detergents, in the micromolar range.

Fig 16: Effect of tamoxifen on basal and CCh-stimulated IP levels.

In A, Ishikawa cells in suspension were incubated with increasing concentrations of tamoxifen citrate for 60 min. In B, tamoxifen citrate was added 10 min prior to the addition of 1 mM CCh and subsequently incubated for 60 min. Reactions were terminated and inositol phosphates separated as described. Similar results were obtained using either ethanol or DMSO as vehicle for tamoxifen. Both solvents slightly increased basal and CCh-stimulated IP accumulation. Sodium citrate had no effect on either basal or CCh-stimulated IP levels. Similar concentrations of the antiestrogens clomiphene and nafoxidine also stimulated basal IP levels while inhibiting CCh-stimulated levels. Values represent means +/- S.D. of triplicate determinations from one of three similar experiments.

Figure 16


A.
B.
21
19
17
15
13
11
9
10
8
6
4
3
IP_1 (dpm/µg prot.)
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[TAMOXIFEN CITRATE]
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[TAMOXIFEN CITRATE]

DISCUSSION

The data obtained characterize muscarinic cholinergic stimulation of phosphoinositide hydrolysis in Ishikawa cells and is generally consistent with the large body of evidence linking muscarinic receptors with this signal transduction process. There are several points of interest to be emphasized, however, both with respect to the events of phosphoinositide hydrolysis in Ishikawa cells and to the various agents that modulate inositol phosphate accumulation in these cells, including the effects of Li+.

Biochemical and Pharmacological Characterization of Muscarinic Stimulation of Phosphoinositide Hydrolysis

The pattern of phosphoinositide hydrolysis and inositol phosphate accumulation in response to CCh (Figs. 5-7) indicates that the earliest stimulated event was hydrolysis of PIP2. In most cells studied, stimulation of PIP2 breakdown by exogenous agents is transient: levels of PIP2 return to control levels within 1-20 min (Creba et al., 1983; Kirk et al., 1984, Majerus et al., 1986). The return of IP3 towards control values by 10-20 min in Ishikawa cells (Fig. 6) suggests that PIP2 hydrolysis has ceased by this time and that amounts of the phosphoinositide will have returned to basal levels. Longer observation of the pattern of inositol phospholipid hydrolysis in Ishikawa cells, however, is necessary.

The lack of significant change in PIP and PI levels (Fig. 5) suggests that neither compound was directly cleaved during this time period by phospholipase C and that IP2 and IP1 originate from stepwise dephosphorylation of IP3. Where observed, hydrolysis of PIP is generally rapid, occurring within 1 min, while hydrolysis of PI is somewhat slower but is usually evident within 5 min (Kirk et al., 1984; Majerus et al., 1986). Rapid resynthesis of both PI and PIP, however, could obscure changes in their levels. A rapid cycling of the inositol phospholipids during stimulation is suggested by the observation that the increase in radiolabeled IP3 at 30 sec was larger than the net decrease in tritiated PIP2 at that time (Table 4). However, since phosphoinositide-specific phospholipase C is capable of cleaving all three inositol phospholipids in in vitro preparations although at different optimal Ca+2 concentrations for each substrate (Wilson et al., 1984), and since direct cleavage of PI to IP1 as well as cleavage of PIP to IP2 in vivo have been demonstrated (Abdel-Latif, 1986; Majerus et al., 1986), it could not be ascertained whether the delay of 1-3 min in IP1 accumulation following CCh-stimulation (Fig. 6C,F) was due to a delayed direct hydrolysis of PI or to a slow dephosphorylation of IP3 and IP2.

Alternatively, cleavage of select hormonally-responsive pools of phosphoinositides may be obscured if these pools are small relative to the total cellular phosphoinositide

content. Evidence for such pools has been presented in rabbit platelets (Vickers and Mustard, 1986) and rat mammary tumor cells (Koreh and Monaco, 1986), however, it is unclear whether similar pools will be found in all systems.

The contributions of these different pathways to the formation of the inositol phosphates is a question which has confounded investigation of the PI cycle (reviewed in Williamson, 1986). Hormonally stimulated changes in inositol phospholipid content can be effected by action of, among others, phospholipases C and A2 and by various kinases and phosphomonoesterases acting on the inositol headgroup of the phospholipid (Abdel-Latif, 1986). Careful, detailed kinetic analyses (Emilsson and Sundler, 1984; Imai and Gershengorn, 1986), however, and the possibility of selective involvement of GTP binding proteins in hydrolysis of each phosphoinositide (Haslam et al., 1986) may help establish the relative role of each metabolic route. The developement of selective inhibitors for each of these pathways would also greatly facilitate their investigation.

Recent work in GH3 pituitary cells (Imai and Gershengorn, 1986) and in cultured rat aortic smooth muscle cells (Griendling et al., 1986) suggests that direct cleavage of PI to IP1 is mediated independently of the cleavage of PIP2 to IP3. Phosphatidylinositol hydrolysis is dependent upon the increased intracellular Ca+2

resulting from hydrolysis of PIP2. The rise in intracellular Ca+2 resultant from the action of IP3 on the endoplasmic reticulum is transient, lasting only 3-4 min, if not supplemented by influx of extracellular Ca+2 triggered by an as yet unclear mechanism (reviewed in Williamson, 1986). The proposed role of the delayed PI breakdown is to provide a continuing source of DG for protein kinase C activation or for arachidonic acid liberation following the transient rise in DG resultant from PIP2 hydrolysis (Williamson and Hansen, 1987). Heterogeneity of processes contributing to IP1 formation in Ishikawa cells is further suggested by the phorbol ester studies. It has been postulated that the mechanism by which phorbol esters inhibit phosphoinositide hydrolysis involves a protein kinase C-mediated phosphorylation and subsequent inactivation of either plasma membrane receptors (Leeb-Lundberg et al., 1985) or of GTP-binding protein (Np) (Katada et al., 1985). The data of Griendling et al., (1986) in cultured rat smooth muscle cells indicates that this desensitization may be specific for PIP2 and PIP hydrolysis while direct cleavage of PI is unaffected. If so, this suggests that the 35% maximal inhibition of CCh-stimulated IP accumulation produced by PMA and PDB in Ishikawa cells reflects the contribution of PIP2 and PIP hydrolysis followed by dephosphorylation of IP3 and IP2 to the formation of IP1 (Fig. 15). The remaining 65% would represent the amount of

IP1 produced by another mechanism, most likely direct cleavage of PI.

There are, however, several alternative explanations for this observation. Phorbol esters may only produce a partial activation of protein kinase C in Ishikawa cells. A fully active kinase, on the other hand, may only produce a partial desensitization of CCh-stimulated phosphoinositide hydrolysis. Another measure of protein kinase C activity, ie. phosphorylation of an Ishikawa cell protein or other metabolic effect, would help distinguish between these options.

Lithium has several actions in Ishikawa cells not previously described in other systems. The increased levels of IP3 and IP2 observed 60 min after exposure to CCh in the presence of LiCl (Fig. 6D,E) may be explained by inhibition of degradation pathways. IP2 phosphatase activity, as well as that of IP1 phosphatase, can be blocked by LiCL (Storey et al., 1984; Drummond, 1987). Inhibition of IP3 phosphatase <u>in vivo</u> has also been suggested (Drummond et al., 1983; Thomas et al., 1984) although direct studies on IP3 hydrolysis yielded no evidence of Li+ inhibition (Seyfred et al., 1984; Connolly et al., 1986). Batty and Nahorski (1985) have found, however, that concentrations of LiCl up to 1 mM could inhibit the generation of IP3 in rat cerebral cortex slices stimulated with CCh for 60 min although basal IP3 levels increased at the higher concentrations. Steady-state

radiolabeling conditions were not used in these experiments, however, so it is unclear what the effect of LiCl on IP3 levels represents.

Alternatively, the increase in IP3 and IP2 may be explained by the enhancement of certain inositol phosphate isomers in the presence of LiCl. These isomers are generated by the different routes of metabolism that the inositol phosphates have been found to undergo in different systems. The role of each isomer, however, remains to be clarified (Michell, 1985; Drummond, 1987). Burgess et al., (1986) and Turk et al., (1986) have found that Li+ selectively augments the accumulation of inositol 1,3,4-trisphosphate as well as inositol 1,4-bisphosphate and inositol 1-monophosphate. Inositol 1,4,5-trisphosphate levels are not affected. Whether this reflects a greater sensitivity to inhibition by Li+ of the phosphatases acting on the ester at C-3 than at C-5 remains to be demonstrated. Different inositol phosphate isomers were not examined in Ishikawa cells.

The original report of Hallcher and Sherman (1980) using a partly purified IP1 phosphatase showed a 50% inhibition of enzymatic activity at 0.8 mM Li+. Investigations of whole cell and tissue preparations have shown maximal effects on IP1 accumulation obtained at 10-20 mM LiCl concentration (Berridge et al., 1982) and as a

consequence, 10 mM LiCl is usually included in reaction mixtures where inositol phosphates are to be examined. In Ishikawa cells, however, the effect of LiCl was not leveling even at concentrations as high as 100 mM both under isosmotic and hyperosmotic conditions (Fig. 8). The almost linear dependence of IP1 accumulation on LiCl concentrations greater than approximately 10 mM is unexplained. The similarity of effects under hypertonic and isotonic solutions suggests that permeability of Ishikawa cells for Li+ is not a factor. A stereoisomer of IP1 (ie., inositol 3-, 4-, or 5-monophosphate) whose degradation is less sensitive to inhibition by Li+ is possible. These stereoisomers have been described to exist but are without known function (Michell, 1986; Drummond, 1987). Ishikawa cells may contain, alternatively, an isozyme of inositol 1-monophosphate phosphatase less sensitive to Li+ inhibition. Purification or partial purification of the enzyme would help resolve this issue. Similar examination of the concentration-dependent effects of LiCl on IP2 and IP3 accumulation would also prove interesting.

The observations that both basal and CCh-stimulated levels of IP3, IP2, and IP1 as well as the degree of stimulation were enhanced in the presence of 10 mM LiCl (Fig. 6) contrast with the data of Thomas et al., (1984) in rat hepatocytes and with that of Huang and Detwiler (1986) in human platelets. Both of these groups found that basal

inositol phosphate levels were unaffected by the presence of Li+. Jackowski et al., (1986), however, working with virally transformed and untransformed mink lung epithelial cells found that following 30 min exposure to Li+, IP3 levels were augmented only in transformed cells whereas IP1 and IP2 levels increased in both types of cells. Several other investigations have shown evidence of increased rates of PI turnover in transformed cells as compared to their normal counterparts (Fleischman et al., 1986; Preiss et al., 1986; Langeland et al., 1986). None of these, however, examined the effects of LiCl. Viral oncogene product regulation of PI turnover has been proposed as a mechanism of cell transformation (Berridge, 1984). One of the products of the ras oncogene shares considerable functional homology with GTP-binding proteins and a PI kinase activity has been reported to coelute with the src oncogene tyrosine kinase (Macara et al., 1984). The functional relevance of lithium to effects mediated by the phosphatidylinositol cycle, however, is just beginning to emerge (Drummond, 1987) and so the role of lithium with respect to modulation of phosphoinositide hydrolysis especially concerning potential events of tranformation remains to be established. Investigation of phosphoinositide hydrolysis and the effects of LiCl in epithelial cells isolated from normal endometrium (Schatz et al, 1985), however, might help determine whether the effects observed in Ishikawa cells are a manifestation of a

transformed state or, rather, reflect events peculiar to PI turnover in endometrial tissue.

Pharmacologic characterization of the effects of cholinergic agents (Figs. 9-14, Table 5) clearly establish the muscarinic identity of the receptor mediating the actions of CCh described above. Furthermore, the EC50's for the effects of CCh and ACh (Table 5) are consistent with prior suggestions that muscarinic stimulation of phosphoinositide hydrolysis involves a low affinity state or subtype of the receptor (Miller, 1977; Fisher et al., 1982; Brown and Brown, 1984).

The similarity of concentration-dependent increases in IP3, IP2, and IP1 levels following 4 min exposure to CCh (Fig. 11) can be interpreted as independent stimulation of hydrolysis of each phosphoinositide. Alternatively, if the hydrolysis of PIP2 to IP3 is rate limiting as compared to the dephosphorylation of IP3 to IP2 and IP1, similar concentration-response curves may result. As previously discussed, differentiating between each possibility would require more sophisticated experimental approaches.

The implication of (1,4,5-) IP3 as a stimulator of intracellular Ca+2 release from endoplasmic reticulum suggests that comparison of concentration-response curves for IP3 generation and for either intracellular Ca+2 mobilization or the resultant cellular response should be characterized by similar pharmacologic parameters.

Grandordy et al., (1986) found, however, that the concentration-response curve for CCh-stimulated contraction in bovine tracheal smooth muscle was to the left of those corresponding to both inositol phosphate generation and that for agonist binding. Maximal contraction was obtained with <20% receptor occupation and with <30% maximal inositol phosphate generation. Similar results were obtained with @-adrenergic response in rabbit aorta (Villalobos-Modina et al., 1984).

Investigations in noncontractile tissues have produced like findings. Stimulation of rat hepatocytes by CCh, vasopressin, phenylephrine, and glucagon showed good correlation between binding and IP3 generation (reviewed in Williamson and Hansen, 1987). There was a poor correlation, however, with intracellular Ca+2 release and a subsequent intracellular effect (activation of glycogen phosphorylase) both of which were more sensitive to hormonal activation. Additionally, the magnitude and temporal pattern of IP3 accumulation and of Ca+2 mobilization differed with each agonist. Some of the heterogeneity may be attributable to different patterns of IP3 stereoisomer and of IP4 generation and also to different receptor content for each agent (Williamson and Hansen, 1987).

The significance of the slight decrease in EC50 for

generation of total inositol phosphate following 60 min exposure to CCh in the absence of LiCl (Fig. 10) is unclear. Similar investigation has not yet appeared in the literature. The unchanged Hill coefficient indicates that the mode of interaction of CCh with the muscarinic receptor ie; simple competitive binding, was unchanged. Lithium is known to affect other cellular processes, including adenylate cyclase activity (Ebstein et al., 1978) and cGMP formation (Kanba et al., 1986), and it is conceivable that alteration of one of these processes may affect interaction of CCh with the muscarinic receptor. Alternatively, the lowered EC50 may reflect the slightly decreased osmolarity resulting from absence of hypertonic LiCl addition. Comparison of concentration-response curves in the presence or absence of isotonically added LiCl may yield a more accurate assessment of any effect on the initial steps in phosphoinositide hydrolysis. Comparison also of the concentration-dependent generation of IP3 in the presence or absence of LiCl would be interesting.

Although concentration-inhibition curves for antagonism of muscarinic-stimulated IP1 or total inositol phosphate accumulation in rat and guinea pig brain preparations have been presented (Miller, 1977; Fisher and Bartus, 1985; Gil and Wolfe, 1985; Jacobson et al., 1985) and atropine is known to inhibit PIP2 degradation and IP3 formation in rat parotid cells (Aub and Putney, 1985), the data presented in Fig. 13 is the first detailed pharmacologic examination of

the inhibtion by atropine of IP2 and IP3 accumulation stimulated by cholinergic agents. As with the data of Fig. 11 showing concentration-dependent accumulation of each inositol phosphate, atropine inhibition of IP3, IP2, and IP1 accumulation can be interpreted as either effects on independent muscarinic action on hydrolysis of each phosphoinositide or as inhibition of the hydrolysis of PIP2 where the hydrolysis is the rate-limiting step.

The inhibition by atropine of the total inositol phosphate accumulation following 60 min CCh exposure (Fig. 12), instead of 4 min as shown in Fig. 13, is problematic. The Hill coefficient of 0.71 +/-0.09 (Table 5) is less than expected for simple competitive antagonism of a single class or subtype of receptor. The Hill coefficients of approximate unity for atropine inhibition of accumulation of each inositol phosphate following 4 min CCh exposure (Fig. 11) indicate that the initial effect of atropine can be described with equations of simple competitive inhibition. At 60 min, however, a more complex description may be necessary. Shallow Hill slopes generally reflect either negative cooperativity or interaction with multiple receptor subtypes or states. None of the other pharmacologic characterizations, however, support either of these possibilities and at present, the low Hill coefficient remains unexplained.

Effects of Other Agents on Ishikawa Cell Phosphoinositide Hydrolysis

The lack of effect of other plasma-membrane acting agents listed in Table 6 on Ishikawa cell phosphoinositide hydrolysis was remarkable considering both the endometrial responses and the stimulation of PI turnover reported to be induced in other systems by these agents. To date, two other studies have documented hormonally stimulated phosphoinositide hydrolysis in endometrial tissues. In one, prostaglandin F2@ (PGF2@)-stimulated inositol phosphate accumulation was demonstrated in cultured rabbit endometrial cells (Orlicky et al., 1986a) and a role of PI turnover in cell proliferation was postulated since PGF2@ also stimulated DNA synthesis and cell growth in this system (Orlicky et al., 1986b). Carbachol also stimulated inositol phosphate accumulation suggesting the presence of cholinergic receptors in the rabbit endometrium. The lack of PGF2@ effect, over a similar concentration range, on inositol phosphate accumulation in Ishikawa cells likely represents absence of receptors for PGF2@ since human endometrial preparations have low or undetectable levels of binding sites for PGF2@ (Hofmann et al., 1985).

Oxytocin has been shown to stimulate PI turnover in slices of ovine endometrium with a time course comparable to the oxytocin-stimulated production of PGF2@ (Flint et al., 1986). Oxytocin is known to bind to human endometrium (Soloff et al., 1977; Fuchs et al., 1985) and thus

evaluation of phosphoinositide hydrolysis in normal endometrium may indicate whether lack of response in Ishikawa cells is peculiar to this cell line. Similar investigation of stimulation of phosphoinositide hydrolysis by vasopressin, histamine, and phenylephrine in normal endometrium may evidence a response not observed in Ishikawa cells.

No interaction of gonadal steroids with transduction systems affecting phosphoinositide hydrolysis could be demonstrated in Ishikawa cells (Table 6) even though steroid hormones are known to affect several aspects of membrane transduction in other systems. Both increases and decreases in adrenergic receptor content have been documented (Rosen et al., 1984; Bottari et al., 1986) as have changes in receptors for other hormones (Gershengorn et al., 1979; Ichida et al., 1983; Mukku and Stancel, 1985) in response to estradiol. Dexamethasone and estradiol can modulate activity of GTP-binding proteins (Kirchick and Birnbaumer, 1983; Rodan and Rodan, 1986) as well as the adenylate cyclase catalytic subunit (Pecquery et al., 1986). Cyclic AMP-dependent protein kinase and cAMP phosphodiesterase activities have also been shown to be modulated by estradiol (Liu, 1984; Etingof et al., 1984). Phospholipase A2 activity is known to be regulated by steroid hormones in reproductive tissues from several species (Dey et al., 1982; Downing and Poyser, 1983; Bonney, 1984). These effects generally require a long-term

steroid exposure (>12 hr) and are believed to occur through classical mechanisms of steroid hormone action. Progesterone, however, has been demonstrated to have a direct inhibitory effect on adenylate cyclase in Xenopus oocyte plasma membranes (Sadler and Maller, 1984; Finidori-Lepicard et al., 1981). Rapid estradiol stimulation of adenylate cyclase (within 20 min) in human endometrial cells (Bergamini et al., 1985) and progesterone regulation of dopaminergic receptors in rat pituitary membranes (Bression et al., 1986) are also suggested to result from direct steroid action in the plasma membrane. Additionally, in membranes prepared from human amnion, sulfated estrogens stimulated a 2 to 3 fold increase in phospholipase A2 activity (Saitoh et al., 1984). Similar investigation using three sulfated steroids, estradiol 3-sulfate, estrone 3-sulfate, and dehydroepiandrosterone sulfate, over a like concentration range did not evidence any effect on phospholipase C activity in Ishikawa cells (Table 6). In contrast, estradiol has been shown to act on these cells by increasing alkaline phosphatase and DNA polymerase @ activities (Holinka et al, 1986b; Gravanis and Gurpide, 1986), and in influencing proliferation (Holinka et al, 1986a).

Steroid effects on phosphoinositide hydrolysis are less well documented. Long-term exposure of cultured human embryonic fibroblasts to dexamethasone was found to increase rates of PI synthesis and degradation (Grove et

al., 1983). The estrogen receptor in MCF-7 human breast cancer cells has been suggested to contain PI and PIP kinase activities (Baldi et al., 1986). Recent work by Freter et al., (1986) demonstrated a 70% increase of total inositol phosphate content in MCF-7 cells exposed to estradiol for 24 hr.

In contrast to the data of Freter et al., (1986), however, investigation of 24 hr exposure of Ishikawa cells to similar concentrations of estradiol and progesterone evidenced no change in either basal or CCh-stimulated total radiolabeled inositol phosphate content. The similarity of basal levels and of response to CCh in cells grown either under normal incubation conditions (medium containing 15% FBS) or grown for two passages with stripped serum is interesting and suggests that any factor present in fetal calf serum that is removed by the stripping procedure has no effect on phosphoinositide hydrolysis in Ishikawa cells. It is not entirely clear, however, which growth factors, mitogens, or other potential regulatory substances are removed by the stripping procedure (Holinka et al., 1986a).

The time of incubation with steroid hormones is an important determinant for observing steroid-dependent effects. Individual steroid actions may take several days to manifest or may occur within a few hours and then subside. The effects may also be subtle and it is conceivable that the measure of inositol phosphate levels

in Ishikawa cells may not reflect steroidal influence. Ishikawa cells may also have lost responsiveness demonstrable in normal endometrium. More detailed analysis of the behavior of each of the inositol phospholipids and inositol phosphates in response to steroid exposure in both Ishikawa cells and in normal endometrium may reveal affects not discerned in the experiments described. The lack of obvious response should not serve as a deterrent to further investigation.

Triphenylethylene antiestrogens at micromolar concentrations have been shown to interact with muscarinic (Ben-Baruch et al., 1982), histaminergic (Brandes et al., 1986), and dopaminergic (Hiemke et al., 1984) receptors as well as to inhibit calmodulin-activated cAMP phosphodiesterase, myosin light chain kinase, (Lam, 1984), and protein kinase C (O'Brian et al., 1986) activities. These actions are independent of binding to estrogen receptors or to previously characterized microsomal "antiestrogen binding sites" and are thought to be related to the amphiphilic cationic character of this type of antiestrogen since phenothiazines, compounds of similar structural characteristics, mimic these effects (Jordan, 1984). Tamoxifen at concentrations greater than 10 uM, however, is generally cytocidal within 2-3 days of continuous exposure (Lippman et al., 1976; Coezy et al., 1982). The rapid uptake of trypan blue uptake by Ishikawa cells demonstrates that alteration of the plasma membrane

occurs even more quickly. The increase in inositol phosphate seen at this and higher tamoxifen concentrations as well as the inhibition of CCh-stimulated IP accumulation may thus be an unexplained, nonspecific consequence of plasma membrane disruption. The enhancement of total inositol phosphate levels by tamoxifen in the presence of atropine may also be considered to reflect plasma membrane perturbation. A general model of cell disruption and necrosis, involving activation of Ca2+-dependent enzymes following plasma membrane derangement and subsequent influx of extracellular Ca2+ (Farber, 1982), also supports the hypothesis that triphenylethylene antiestrogens can act as detergents. A similar conclusion has been reached concerning the reported effects of chlorpromazine on phosphoinositide metabolism in human platelets (Opstvedt et al., 1986). The pharmacological relevance of effects of tamoxifen or chlorpromazine at 10-100 uM concentrations on inositol phosphate accumulation (Leli and Hauser, 1986) and phosphoinositide metabolism (Tallant and Wallace, 1985) as well as other reported effects of these compounds in the micromolar range should therefore be evaluated cautiously.

Conclusions

The presence of muscarinic receptors linked to hydrolysis of inositol phospholipids has been demonstrated in the Ishikawa human endometrial adenocarcinoma cell line. Pharmacological characterization of this response shows that it is similar to muscarinic stimulation of phosphoinositide hydrolysis in other systems and may be mediated by a low affinity form of the muscarinic receptor.

Kinetic characterization of phosphoinositide hydrolysis and inositol phosphate accumulation in Ishikawa cells suggests that the earliest event is a phospholipase C mediated cleavage of PIP2 to IP3 and DG. It was not possible, however, to ascertain whether PI and PIP were directly cleaved. Support for a delayed direct hydrolysis of PI to IP1 and DG following CCh stimulation was provided by the partial inhibition of IP1 accumulation observed when PMA or PDB was added to the medium. Identification of selective inhibitors of the various enzymatic reactions which constitute the PI cycle would facilitate the study of these reactions.

Except for the actions of LiCl, the events demonstrated in Ishikawa cells are consistent with observations on phosphoinositide hydrolysis in other systems. The unexplained lack of complete inhibition of IP1 accumulation by quite high concentrations of Li+ has not been reported before. Purification of IP1 phosphatase from Ishikawa cells and comparison with IP1 phosphatase from normal human

endometrium would help determine if an isozyme less sensitive to the effects of Li+ is present in the transformed cells..

The increased basal levels of inositol phosphates in the presence of LiCl was also unexpected. In addition to actions on IP3 and IP2 phosphatases, Li+ may also affect other enzymes or processes that regulate PI turnover in Ishikawa cells, as suggested by the lowering of the EC50 for CCh concentration-response curves in the absence of LiCl. The hypothesis that the effect of Li+ on basal levels of inositol phosphate and diacylglycerol is a characteristic of transformed cells could be tested in studies of epithelial cells of normal endometrium.

There is evidence for interaction between transduction systems for steroid hormones and plasma membrane-acting hormones. This may become one of the more exciting aspects of studies of hormonal action. Although no effects of steroid hormones on Ishikawa cell PI turnover were noted, the experimental designs used may not have been completely appropriate. Given the suggested role of the PI cycle and the known roles of steroid hormones in cell proliferation and differentiation, it is not unreasonable to expect interplay between the two transduction systems especially in tissues where steroid influence is particularly notewhorthy.

The recent demonstration of inositol phosphate accumulation stimulated by PGF2@ and CCh in rabbit

endometrial cells, by oxytocin in sheep endometrial slices, and our finding of effects of cholinergic agents on human endometrial adenocarcinoma cells suggests that the PI cycle and agents that affect it also may play a physiological role in human endometrium. Studies to date of hormonal regulation of endometrial function have emphasized the roles of steroid hormones. Given the complex events of the human menstrual cycle and the functions of the endometrium during pregnancy and parturition, as well as the prevalence of endometrial cancer and other disease states, elucidation of nonsteroidal regulation of endometrial physiology will be of great interest.

The Ishikawa human endometrial adenocarcinoma cell line is presented as a new model system for study of the PI cycle. The discrepant actions of Li+ suggest that these cells may have features of interest not found in other model systems. It is also hoped that the studies presented herein serve as a basis for the investigation of the regulation and physiological roles of phosphoinositide hydrolysis and of cholinergic influence in human endometrium.

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