REGULATION AND MECHANISM OF PHOSPHOINOSITIDE AND INOSITOL POLYPHOSPHATE METABOLISM IN RAT HEPATOCYTES

by

Mark Allan Seyfred

A DISSERTATION

submitted to Michigan State University
in partial fullfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1984

ABSTRACT

REGULATION AND MECHANISM OF PHOSPHOINOSITIDE AND INOSITOL PHOSPHATE METABOLISM IN RAT HEPATOCYTES

By

Mark Allan Seyfred

Vasopressin stimulated breakdown of phosphoinositides in rat hepatocytes has been demonstrated by a number of workers, however, the subcellular site of this induced breakdown has not been determined. To examine the subcellular site of vasopressin induced breakdown of phosphoinositides, rat hepatocytes were isolated and incubated with $^{32}P_i$ for 90 min. The [γ-^{32}P]ATP specific radioactivity has reached isotopic equilibrium, as well as the rates of ^{32}P incorporation into phosphatidic acid, phosphatidylinositol 4-phosphate (PI-P), and phosphatidylinositol 4,5-bisphosphate (PI-P_2) have approached steady state within this labelling time. Vasopressin, at 200 nM, was incubated with the labeled hepatocytes for 0-10 min. At various times, the cells were fractionated into partially purified plasma membranes, mitochondria, lysosomes, and microsomes using Percoll density gradient centrifugation. The ^{32}P-phospholipid levels were examined in each fraction by deacylating the labeled phospholipids with mild alkali, and then analyzing the ^{32}P-glycerophosphate esters by anion exchange high pressure liquid chromatography. It was found that breakdown of PI-P and PI-P_2, in response to vasopressin, occurs at the

plasma membrane. Further investigation led to the finding that a calcium-dependent polyphosphoinositide-specific phosphodiesterase was responsible for the breakdown of PI-P and PI-P_2. This phosphodiesterase is inactive under normal cellular conditions of ionic strength and calcium concentrations, but can be activated by the addition of vasopressin.

The metabolism of inositol trisphosphate (I-P_3), the product of phosphodiesterase action on PI-P_2 and the proposed second messenger of hormone-induced calcium mobilization, was also examined. It was found that I-P_3 is degraded to form inositol bisphosphate (I-P_2) by a phosphatase, located almost exclusively in the plasma membranes of rat liver. Further phosphatase catalyzed breakdown of I-P_2 occurs primarily in the cytosolic and microsomal fractions. It was determined that I-P_3 phosphatase (I-P_3ase) is a specific enzyme and not a general acid or alkaline phosphatase by pH optimum determination and its insensitivity to phosphatase inhibitors such as LiCl and NaF. The enzyme was inhibited by physiological levels of spermine, but not putrescine or spermidine. This suggest that the activity of I-P_3ase, and thus cytosolic calcium levels, may be associated with the regulation of intracellular spermine concentrations.

To my wife, Linda

ACKNOLEDGEMENTS

I would like to thank Dr. William Wells for his support and guidance during the course of my graduate studies, and the other members of the laboratory, particularly Charles Smith and Jeffrey Nickerson, for their helpful discussion, and also Lynn Farrell for her technical skills. I would also like to express my appreciation to Dr. Rodney Boyer of Hope College for sparking my interest in biochemical research, and to my parents, who provided encouragement and financial support during my educational career. Most of all, I would like to thank my wife, Linda, for her support and patience.

TABLE OF CONTENTS

LIST OF TABLES

Literature Review

Chapter I

Chapter III

LIST OF FIGURES

Literature Review

Chapter I

Chapter II

Chapter III

ABBREVIATIONS

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PG	phosphatidylglycerol
PA	phosphatidic acid
PS	phosphatidylserine
PI-P, DPI	phosphatidylinositol 4-phosphate
$PI-P_2$, TPI	phosphatidylinositol 4,5-bisphosphate
GPC	glycerophosphorylcholine
GPE	glycerophosphorylethanolamine
GPI	glycerophosphorylinositol
GPG	glycerophosphorylglycerol
GP	glycerol phosphate
GPS	glycerophosphorylserine
GPIP	glycerophosphorylinositol 4-phosphate
$GPIP_2$	glycerophosphorylinositol 4,5-bisphosphate
P_i	inorganic phosphate
I-P	myo-inositol phosphate
$I-P_2$	myo-inositol bisphosphate
$I-P_3$	myo-inositol trisphosphate
$I-P_2$ase	myo-inositol bisphosphate phosphatase
$I-P_3$ase	myo-inositol trisphosphate phosphatase

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
TRICINE	N-tris[hydroxymethyl]methyl glycine
MES	2-[N-morpholino]ethanesulfonic acid
BICINE	N,N-bis[2-hydroxyethyl]glycine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid
PMSF	phenylmethylsulfonyl flouride
TCA	trichloroacetic acid
HB	homogenizing buffer
HPLC	high pressure liquid chromatography

Introduction

In many different tissues and cell types, the stimulation of various receptors leads to an increase in the concentration of cytosolic free calcium, and also induces changes in phosphoinositide metabolism (1,2). In rat hepatocytes, the addition of vasopressin increases cytosolic free calcium (3-7) and decreases the level of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI-P), and phosphatidylinositol 4,5-bisphosphate (PI-P_2) (8,9,10). However, neither the subcellular site of the agonist-induced breakdown nor the mechanism of breakdown is known. Therefore, the subcellular site and mechanism of vasopressin-induced breakdown of phosphoinositides in rat hepatocytes was investigated and is described in Chapters 1 and 2.

Chapter 1 describes the methodolgy for the subcellular fractionation of rat hepatocytes, and the analysis of the polar products of mild alkali deacylation of phospholipids by anion exchange high pressure liquid chromatography. The rates of ^{32}P incorporation into the phospholipids of various subcellular fractions isolated from rat hepatocytes are also described. The subcellular site and mechanism of

vasopressin-induced phosphoinositide breakdown are described in Chapter 2. Both of these chapters are preesented in manuscript form as outlined by the Journal of Biological Chemistry and are to be published as separate, but adjoining papers in one of the June or July issues of the Journal. They are reprinted here by permisssion of the publisher.

The role of myo-inositol trisphosphate ($I-P_3$), the product of plasma membrane phosphodiesterase action on $PI-P_2$ (11), as a second messenger for the hormonal mobilization of cellular calcium, has been described in saponin-permeabilized pancreatic acinar cells (12) and rat hepatocytes (13). The cytosolic level of $I-P_3$ is determined by not only its rate of release from $PI-P_2$, but also the rate of its degradation. Therefore, in Chapter 3, the subcellular distribution and properties off a specific myo-inositol trisphosphate phosphatase from rat liver are described. This chapter is also in manuscript form and has been submitted to the Journal of Biological Chemistry for publication.

Literature Review

In this literature review, I would like to review two main topics. The first topic involves cell calcium; its role in modulation of cellular processes, and the mechanisms by which intracellular calcium concentrations are regulated. In the second topic, the biosynthesis and degradation phosphoinositides, and the effect of hormones on these processes, will be described. Also, the effect of phosphoinositides and its metabolites upon various enzymes and intracellular calcium concentrations will be reviewed.

The Effect of Calcium or Calcium-Calmodulin Complexes Upon Cellular Processes - A vast number of enzymes and cellular processes are affected by calcium-calmodulin complexes or by calcium alone. CDP-diacylglycerol-inositol transferase from rat liver (14) and rabbit lung (15) as well as the liver mitochondrial enzymes, pyruvate carboxylase and carbamoyl-phosphate synthetase, are inhibited by calcium (16-18). Microtubule dissassembly is also inhibited by calcium-calmodulin (19). However, a majority of the enzymes and cellular processes are stimulated by calcium or calcium-calmodulin. These include cytosolic phospholipase C

from liver (20,21); phospholipase A_2 from liver (22) and platelets (23); mitochondrial glycerol phosphate dehydrogenase from insect flight muscle (24); and NAD-linked isocitrate dehydrogenase, α-ketoglutarate dehydrogenase (25), and pyruvate dehydrogenase phosphatase (26) from mitochondria. Cyclic AMP phosphodiesterase in brain (27), liver (28), and heart (29) is calcium-calmodulin dependent. Brain adenylate cyclase is stimulated by low concentrations of calcium, but inhibited at higher calcium concentrations (30). Pancreatic islets also contain a calcium-calmodulin sensitive adenylate cyclase (31). Calcium is also involved in enzyme, catecholamine, and hormone secretion in a variety of cell types (32-39).

A number of protein kinases are be dependent upon or activated by calcium or calcium-calmodulin. These include myosin light chain kinase (40) and tryptophan hydroxylase kinase (41). Garrison et al. (42) demonstrated that angiotensin II and vasopressin, hormones which increase intracellular calcium levels without increasing cAMP levels (3), stimulate the phosphorylation of 10-12 cytosolic proteins in rat hepatocytes. Schulman and Greengard (43) have described a calcium-calmodulin protein kinase system in a variety of other tissues. Protein kinase C, the phospholipid-dependent protein kinase which is stimulated by phorbol esters, requires calcium for maximum activity (44,45).

Mg^{2+}-dependent ATPase's are also stimulated by

calcium-calmodulin. The best characterized systems are the sarcoplasmic reticulum (46-49), which may be regulated through the phosphorylation of a 22k dalton membrane protein (phospholamban), and the erythrocyte membrane (Ca^{2+}-Mg^{2+})ATPase (50-53). Other (Ca^{2+}-Mg^{2+})ATPase activities are found in adipocyte plasma membranes (54) and endoplasmic reticulum (55), pancreatic islet plasma membranes (56), heart plasma membranes (57), and microsomes (58-60) and plasma membranes (61,62) from rat liver.

Phosphorylase kinase and glycogen synthase control the degradation and synthesis of glycogen (63). In skeletal muscle, calcium-calmodulin stimulate phosphorylase kinase to activate glycogen phosphorylase and inhibit glycogen synthase (64). In liver, phosphorylase kinase appears to be regulated by phosphorylation of the enzyme by a cAMP-dependent protein kinase, as well as by a calcium-mediated activation (65-67). Phosphorylation of liver glycogen synthase by phosphorylase kinase has also been shown to inhibit glycogen synthase (68).

Molecular Regulation of Intracellular Calcium Concentrations

Cells, in general, contain between 1 and 2 umol calcium per gm wet weight. Most of the total cellular calcium is bound to fixed anionic binding sites of glycoproteins, proteins and phospholipids, with a smaller fraction complexed with mobile anions such as inorganic phosphate (3). In intact tissues, there is a rapidly exchangeable cellular calcium

pool, representing 10-20% of the total cell calcium, which is bound to glycoproteins, glycolipids and mucopolysaccharides on the external cell surface (69). Rapid cell fractionation has shown that 70-80% of the total intracellular calcium is located in the mitochondria of hepatocytes (70,71) and synaptosomes (72). Free calcium concentrations in the mitochondrial matrix is about 16 uM (73). The endoplasmic reticulum appears to account for most of the remainder of the organelle-bound calcium. Cytosolic free calcium in isolated hepatocytes is about 0.2 uM (70), while cytosolic bound calcium is 700-800 times greater (73).

The question now arises as to what maintains the intracellular calcium levels of the cytosol, mitochondria and endoplasmic reticulum.. The concentration of free calcium in the blood is 1.25 mM, while the free calcium concentration in the cytosol of most cells is 0.1-0.2 uM (70,74). Consequently, there is a gradient across the plasma membrane of about 10^4. Two mechanisms have arisen which maintain this calcium gradient. The first is the Na^+-Ca^{2+} exchange or Na^+/Ca^{2+} antiporter of excitatory and secretory cells (75,76). However, studies in liver show no appreciable Na^+-Ca^{2+} exchange (77,78). The second mechanism for calcium efflux is by a unidirectional Mg^{2+}-dependent Ca^{2+}-ATPase, as demonstrated in erythrocytes (50-53), adipocytes (54), pancreatic islets (56), heart (57), and liver (61,62).

The endoplasmic reticulum also contains calcium pumps

similar to those observed in plasma membranes. Calcium transport by the sarcoplasmic reticulum (Ca^{2+}-Mg^{2+})ATPase has been extensively studied in skeletal and cardiac muscle (46-49). A calcium pump in endoplasmic reticulum of liver has also been demonstrated (58-60), but is poorly understood.

Uptake and efflux of calcium in mitochondria occurs by different mechanisms. Uptake of calcium takes place by an electrophoretic uniport mechanism, which is energetically driven by the electrogenic proton pump of the respiratory chain (75,79,80). The efflux carrier is electroneutral and catalyzes the exchange of one Ca^{2+} for two H^+ in liver mitochondria (81), and one Ca^{2+} for two Na^+ in heart and brain mitochondria (79). The V_{max} for calcium uptake is 100 times greater than the V_{max} efflux rate (82,83). It has been suggested that the mitochondria may be responsible for buffering the cytosolic free calcium in intact cells (84).

Agonist-Induced Increases in Intracellular Free Calcium - A wide variety of agonists can act on various tissues and cell types to increase the level of intracellular calcium. The cholinergic agonist, acetylcholine, stimulates an increase in cytosolic free calcium in adrenal medulla (35), pancreas (85), parotid gland (86) and iris smooth muscle (87). Glucose stimulates calcium accumulation in the Islet of Langerhans (39), IgE, concanavalin A or specific antigens act on mast cells to release histamine, with a corresponding

increase in intracellular calcium (88). Cholecystokinin-pancreozymin acting on pancreas (33), thrombin, collagen, and ADP on platelets (37), and 5-hydroxytryptamine (89) acting on blowfly salivary glands, all induce an increase in the level of intracellular calcium. Whether the increase in free cytosolic calcium is due to an increase in the influx or a decrease in the efflux of calcium from the external media, or an increase in calcium efflux from intracellular sites, is still a subject of controversy. In these tissues, most of the evidence points to a depolarization of the plasma membrane leading to an opening of calcium channels and, hence, increased influx of extracellular calcium.

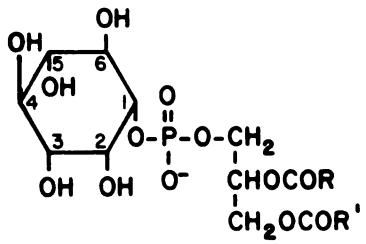
The addition of vasopressin, angiotensin II or α-adrenergic agonists (phenylephrine, epinephrine and norepinephrine) to rat liver increases intracellular calcium levels (3-7). Vasopressin and phenylephrine induce the release of calcium from intracellular stores, probably mitochondria and endoplasmic reticulum (6), and also inhibit calcium transport by plasma membrane vesicles, presumably by inhibiting the (Ca^{2+}-Mg^{2+})ATPase (7). Glucagon, when added at supraphysiological concentrations to rat liver, increases cytosolic free calcium levels (5). The β-adrenergic agonist isoproternol, also increases cytosolic calcium levels when added to mouse kidney cortex slices (90).

Phosphoinositide Biosynthesis and Degradation - In almost

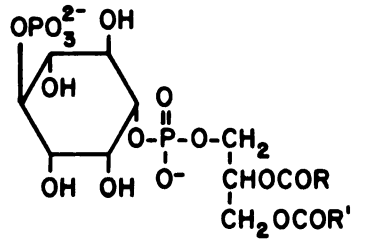
all eucaryotic cells, the predominant inositol lipid is phosphatidylinositol (PI) (Fig. 1). Typical concentrations of this lipid range from 0.5-2.5 umol per gm of tissue, which is equivilant to about 2-12% of the total phospholipids of the cell (91-93). The phosphorylated derivatives of PI, phosphatidylinositol 4-phosphate (PI-P) and phosphatidylinositol 4,5-bisphosphate ($PI-P_2$) (Fig. 1), are also found in a wide range of animal cells (94,95), yeast (96) and higher plants (97), although only in trace quantities (0.1% of the total phospholipids). It is difficult to assess the concentration of the phosphorylated derivatives of PI since they break down rapidly post-mortem, and are difficult to extract quantitatively (94,95). The brain appears to have the highest level of PI-P and $PI-P_2$ at 0.153 umol and 0.083 umol per gm of tissue, respectively, in unmyelinated brain, and 0.219 umol PI-P and 0.280 umol $PI-P_2$ per gm of tissue in myelinated brain (98). Platelets (99) and kidney (98) also appear to be rich sources of polyphosphoinositides (PI-P and $PI-P_2$). Inositol lipids in animal cells possess uncommonly high proportions of stearoyl residue at position 1 and arachidonyl at position 2 (100).

The phosphoinositide biosynthetic and degradative pathways are outlined in Figure 2. The biosynthesis of phosphatidylinositol occurs by two mechanisms, both of which take place in the endoplasmic reticulum (1,101). The first involves the formation of CDP-diglyceride from phosphatidic acid and CTP, catalyzed by CDP-diglyceride synthetase. PI

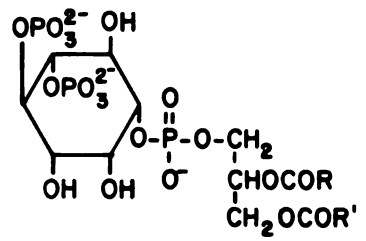
Figure 1. Structure of inositol lipids.



Ptd Ins



Ptd Ins 4-P



Ptd Ins 4, 5-P_2

Figure 1

Figure 2. Pathways for the biosynthesis and degradation of phosphoinositides.

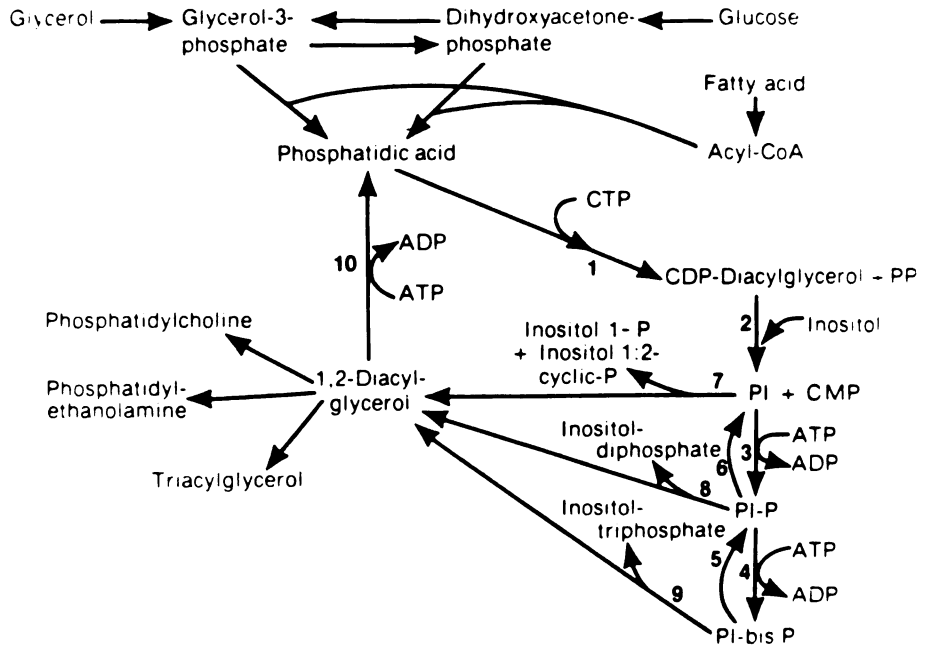

Glycerol
Glycerol-3-phosphate
Dihydroxyacetone-phosphate
Glucose
Fatty acid
Acyl-CoA
Phosphatidic acid
CTP
ADP
ATP
10
1
CDP-Diacylglycerol + PP
2
Inositol
Phosphatidylcholine
Inositol 1-P + Inositol 1:2-cyclic-P
7
PI + CMP
Phosphatidyl-ethanolamine
1,2-Diacyl-glycerol
Inositol-diphosphate
3
6
8
ATP
ADP
PI-P
Triacylglycerol
Inositol-triphosphate
5
4
9
ATP
ADP
PI-bis P

Figure 2

is then formed by the transfer of inositol to CDP-diglyceride to form PI and CTP in a reaction catalyzed by CDP-diglyceride-inositol transferase. PI can also be formed by a base exchange reaction (PI + $I^* \longleftrightarrow PI^*$ + I). PI-kinase, which catalyzes the phosphorylation of PI by ATP to form PI-P, is found in a variety of subcellular fractions and tissues. Among these are lysosomal membranes (102,103), golgi (104), microsomes (102,105-107), nuclear envelopes (102,105) and plasma membranes (102,105,107) of rat liver; and plasma membranes of rat brain, kidney, heart, skeletal muscle, testis, and human erythrocytes (107). PI-P kinase is associated with nuclear envelopes, plasma membranes and microsomes of rat liver (102,105); erythrocyte membranes (108); and is a soluble enzyme in brain (109). In rat liver, the highest specific activity of PI kinase is found in microsomes, while plasma membranes contain the highest specific activity of PI-P kinase. The mitochondria of rat liver possess little or no phosphoinositide or polyphosphoinositide synthetic activity (101,102).

The breakdown of phosphatidylinositol procedes primarily by cleavage of the glycerol-phosphate bond by a phospholipase C activity. PI-specific phospholipase C activity is localized in the cytosolic fraction and requires calcium for maximal activity. This activity exist in a number of tissues including platelets (110,111), rat liver (112,113), sheep seminal vesicle glands (114), iris smooth muscle (115) and rat brain (116). Various reports suggests

that a specific phospholipase A_2 in platelets (117), neutrophils (118), and pancreatic acini (119) acts on PI causing the release of arachidonic acid.

The polyphosphoinositides are degraded by two mechanisms: 1) directly by a phospholipase C activity to form inositol polyphosphates and diacylglycerol, or 2) by action of phosphomonoesterases to form PI which is then degraded. Phosphomonoesterases, which act on PI-P_2, are found in brain (120,121), kidney (122), iris smooth muscle (123), and human erythrocytes (124,125). PI-P_2 phosphatase from brain (121) and kidney (122) hydrolyzes PI-P as well as PI-P_2. A metal independent, PI-P specific phosphatase has been described in human erythrocyte membranes (126) and rat liver nuclear envelopes (127). Polyphosphoinositide phosphodiesterase, like PI phosphodiesterase, requires calcium for optimal activity. This activity exist in brain (128-130), erythrocyte membranes (131,132), platelets (133,134), rat hepatocytes (11) and iris smooth muscle (123). Further descriptions and references concerning the phosphoinositide biosynthetic and degradative enzymes can be found in a recent review by Irvine (135).

Stimuli-Induced Changes in Cellular Phosphoinositide Levels

Hormonal regulation of phosphatidylinositol metabolism was first observed by Hokin and Hokin (136) in pancreas. Since that report, a variety of tissues and cell types have been examined for agonist induced changes in intracellular PI

levels. A list of these tissues and cell types, and the stimuli which induce the "PI response" has been compiled by Michell (1,2) and is shown in Table I. Among these stimuli are vasopressin, angiotensin II and α-adrenergic agonists operating in liver; muscarinic and cholinergic agonist in secretory tissue and smooth muscle; 5-hydroxytryptamine in blowfly salivary glands; thrombin in platelets; and platelet activating factor (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) in platelets (137) and rat hepatocytes (138). Further investigation shows that the metabolism of the polyphosphoinositides is also influenced by the same agents (8-10,139-146). The evidence suggest that these agents stimulate a phosphodiesterase to produce diacylglycerol and the corresponding inositol phosphate (11,134,146-148).

Phosphoinositide Effects on Cellular Function - The possible role of phosphoinositides in cell function can be classified into three catagories: 1) effects on specific enzymes, 2) effects on membrane structure, and 3) phosphoinositide metabolites effect on cellular calcium homeostasis and protein kinase C activity.

Phospholipid-protein interactions are known to affect the activities of many membrane-associated enzymes (149). Acidic phospholipids are associated with the (Na^+-K^+)ATPase protein in isolated microsomal membranes from rabbit kidney (150) and membranes isolated from Torpedo marmorata electric

Table I

Receptor Whose Stimulation Provokes a 'PI response' (PI breakdown or enhanced PI labelling) in Appropriate Target Tissue

Type of Receptor	Tissue
Cholinergic (muscarinic)	Brain, synaptosomes, iris smooth muscle, parotid gland
Adrenergic (α_1)	Hepatocytes, adipocytes, brain, iris smooth muscle, parotid and submaxillary gland
Histamine (H_1)	Smooth muscle, brain
5-Hydroxytryptamine ($5\text{-}HT_1$)	Smooth muscle, brain, blowfly salivary gland
Vasopressin (V_1)	Hepatocytes, smooth muscle
Pancreozymin	Pancreas, smooth muscle
Substance P	Parotid, hypothalmus
Bombesin	Pancreas
Angiotensin II	Hepatocytes
Bradykinin	Endothelia, kidney
Thyrotropin	Thyroid
f-Met-Leu-Phe, phagocyticable particles	Neutrophils, macrophages
Secretagogues (antigens, etc.)	Mast cells
Thrombin, collagen, ADP	Platelets
Membrane depolarization	Smooth muscle, ganglia, synaptosomes
Glucose	Islets of Langerhans

organ (151). Phosphoinositides may be involved in the regulation of canine renal (Na^+-K^+)ATPase (152). The (Ca^{2+}-Mg^{2+})ATPase in erythrocyte membranes (50,153,154) and sarcoplasmic reticulum (155) is stimulated by increased levels of polyphosphoinositides. Studies done by Smith and Wells (156) demonstrated that the nuclear membrane ATPase can be synergistically activated by RNA and polyphosphoinositides. Polyphosphoinositides also activate cholesterol side chain cleavage with purified cytochrome P-450 (157), and inactivate low K_m cAMP phosphodiesterase in rat adipocyte microsomes (158). Phosphatidylinositol has been proposed to act as an uncoupler of β-adrenergic receptors from the remainder of the adenylate cyclase complex (159).

Acidic phospholipids, along with divalent cations are important factors in membrane fusion (160). Sundler and Papahadjoupoulos (161) have proposed that head-group hydration is critical in the fusion process. Thus, the polyphosphoinositides, which are highly hydrated, would tend to block fusion. Indeed, the fusion of chick embryo myoblast, initiated by raising the medium calcium levels, also results in polyphosphoinositide breakdown (162). Pickard and Hawthorne (163) suggested that the breakdown of phosphatidylinositol to form diacylglycerol, promotes the fusion of vesicles and plasma membranes during exocytosis. The lateral mobility of glycoproteins in erythrocyte membranes is increased by the addition of $PI\text{-}P_2$ (164).

In 1975, Michell (1) reviewed phosphatidylinositol metabolism and suggested that phosphatidylinositol breakdown may somehow influence intracellular calcium concentrations. The stimulation of many different types of recceptors in different tissues, brought about PI breakdown and also increased calcium concentrations in the cytosol. PI breakdown does not require the presence of extracellular calcium, is unaffected by the addition of the calcium ionophore A23187 in the presence of calcium, and stimulation of glucagon or β-adrenergic receptors does not provoke PI breakdown. The same agonist, which were involved in stimulating PI breakdown and intracellular calcium elevation, also stimulate polyphosphoinositide breakdown, but at a much faster rate (165). Although the breakdown of polyphosphoinositides is thought not to be regulated by calcium, it does appear to be calcium dependent (8,9,11). Exactly how polyphosphoinositides are involved in cellular calcium homeostasis is just now becoming apparent. Agonists, which stimulate the breakdown of polyphosphoinositides, also inhibit calcium transport in plasma membrane vesicles isolated from rat liver (7). Furthermore, PI-P and PI-P_2 stimulate the (Ca^{2+}-Mg^{2+})ATPase (50,153,154). myo-Inositol trisphosphate (I-P_3), the product of phosphodiesterase action upon PI-P_2, mobilizes intracellular non-mitochondrial calcium stores in saponin-permeabilized pancreatic acinar cells (12) and rat hepatocytes (13). Therefore, phosphodiesterase catalyzed

breakdown of PI-P_2 would inhibit the plasma membrane calcium pump, decreasing the calcium efflux rate out of the cell, and the product, I-P_3, would act to mobilize intracellular calcium stores. The combined result would be to increase cytosolic free calcium concentrations.

Polyphosphoinositide breakdown may also be involved in protein phosphorylation. Jolles et al. (166) demonstrated that corticotropin stimulates both protein phosphorylation and polyphosphoinositide turnover in brain. Thrombin can stimulate polyphosphoinositide turnover in platelets and also phosphorylation of an endogenous 40k dalton protein (167), which results in serotonin secretion. This phosphorylation is catalyzed by a calcium-activated, phospholipid-dependent protein kinase (protein kinase C). Protein kinase C can be activated in-vitro by diacylglcerol (45) suggesting that polyphosphoinositide breakdown, which produces diacylglycerol and elevates cytosolic calcium, activate protein kinase C (168), leading to some cellular response. A similar kinase activity has been described in rat hepatocytes where a 16k dalton protein present in purified plasma membranes and cytosol can be phosphorylated by an endogenous protein kinase C activity (169). This activity is stimulated in intact hepatocytes by the addition of vasopressin.

The turnover of phosphoinositides may be involved in controlling cell growth. It was recently reported that the purified Rous sarcoma virus (RSV) transforming gene product,

$pp60^{v\text{-}src}$, which has tyrosine kinase activity, can also phosphorylate PI, PI-P, and 1,2-diacylglycerol (170). The transforming protein of avian sarcoma virus UR2, $p68^{v\text{-}ros}$, also possesses tyrosine kinase and PI kinase activity (171). Transformation of fibroblast by a temperature sensitive mutant of RSV led to a large increase in the level of ^{32}P-labelled PI-P, PI-P_2 and phosphatidic acid only at the permissive temperature (170). Cells transformed by UR2 showed both an increase in ^{32}P-labelling of PI-P and PI-P_2 relative to other phosphlipids and an accumulation of I-P_2 and I-P_3 (171). The link between phosphoinositide turnover and cell transformation may occur through protein kinase C. The tumor promoting phorbol esters can stimulate protein kinase C activity in the absence of phosphatidylinositol turnover or production of diacylglycerol (172). Thus, the phorbol esters may be imitating the action of diacylglycerol by intercalating into the cell membrane and activating the kinase. The kinase may remain activated for prolonged periods of time since the phorbol esters are very slowly metabolized (173). In contrast, production of diacylglycerol occurs transiently during phosphoinositide turnover in normal cells and disappears rapidly. These reports suggests that the oncogene products contribute to uncontrolled cell division by increasing the availability of the polyphosphoinositides and their metabolites, diacylglycerol and inositol polyphosphates, which in turn may lead to prolonged activation of protein kinase C and

elevation of cytosolic calcium. Further evidence suggesting that inositol lipid metabolism and elevation of cytosolic calcium affects cell proliferation has been reviewed by Michell (174).

References

1. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.

2. Michell, R.H. (1979) Trends Biochem. Sci. 4, 128-131.

3. Williamson, J.R., Cooper, R.H., and Hoek, J.B. (1981) Biochim. Biophys. Acta 639, 243-295.

4. Exton, J.H. (1981) Mol. Cell Endocr. 23, 233-264.

5. Charest, R., Blackmore, P.F., Berthon, B., and Exton, J.H. (1983) J. Biol. Chem. 258, 8769-8773.

6. Joseph, S.K., and Williamson, J.R. (1983) J. Biol. Chem. 258, 10425-10432.

7. Pripic, V., Green, K., Blackmore, P.F., and Exton, J.H. (1984) J. Biol. Chem. 259, 1382-1385.

8. Rhodes, D., Pripic, V., Exton, J.H., and Blackmore, P.F. (1983) J. Biol. Chem. 258, 2770-2773.

9. Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H., and Kirk, C.J. (1983) Biochem. J. 212, 733-747.

10. Thomas, A.P., Marks, J.S., Coll, K.E., and Williamson, J.R. (1983) J. Biol. Chem. 258, 5716-5725.

11. Seyfred, M.A., and Wells, W.W. (1984) J. Biol. Chem., in press. (Chapter I, this thesis)

12. Streb, H., Irvine, R.F., Berridge, M.J., and Schulz, I. (1983) Nature 306, 67-69.

13. Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F., and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077-3081.

14. Fain, J.N. (1982) in Horizons in Biochemisty and Biophysics (Kohn, L.D., ed.), Vol. 6, pp. 237-276, John Wiley, London.

15. Bleasdale, J.E., Wallis, P., MacDonald, P.C., and Johnston, J.M. (1979) Biochim. Biophys. Acta 575, 135-147.

16. McClure, W.R., and Lardy, H.A. (1971) J. Biol. Chem. 246, 3591-3596.

17. Folds, M., and Barritt, G.J. (1977) J. Biol. Chem. 246, 3591-3596.

18. Meijer, A.J., VanWoerkam, G.M., Steinma, R., and Williamson, J.R. (1981) J. Biol. Chem. 256, 3443-3446.

19. Marcum, J.M., Dedman, J.R., Brinkley, B.R., and Means, A.R. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3771-3775.

20. Irvine, R.F., and Dawson, R.M.C. (1980) Biochem. Soc. Trans. 8, 27-30.

21. Takenawa, T., and Nagai, Y. (1981) J. Biol. Chem. 256, 6769-6775.

22. Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1980) J. Biol. Chem. 255, 10227-10231.

23. Wang, P.Y.K., and Cheung, W.Y. (1979) Biochem. Biophys. Res. Commun. 90, 473-480.

24. Kligenberg, M., and Buchholz, M. (1970) Eur. J. Biochem. 13, 247-252.

25. Denton, R.M., and McCormack, J.G. (1980) FEBS Lett. 119, 1-8.

26. Denton, R.M., Randle, P.J., and Martin, B.R. (1972) Biochem. J. 128, 161-163.

27. Kakiuchi, S., Yamazuki, R., Tashima, H., and Menishi, K. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3526-3530.

28. Smoake, J.A., Johnson, L.S., and Peake, G.T. (1981) Arch. Biochem. Biophys. 206, 331-335.

29. Teo, T.S., and Wang, J.H. (1973) J. Biol. Chem. 248, 5950-5955.

30. Brostrum, M.A., Brostrum, C.O., Breckenridge, B.M., and Wolff, D.J. (1978) Adv. Cyclic Nucl. Res. 9, 85-99.

31. Valverde, I., Vandermeers, A., Anjaneyulo, R., and Malaisse, W.J. (1979) Nature (London) 206, 225-227.

32. Berridge, M.J. (1975) Adv. Cyclic Nucl. Res. 6, 1-98.

33. Case, R.M., and Clausen, T. (1973) J. Physiol. 235, 75-102.

34. Douglas, W.W., and Rubin, R.P. (1961) J. Physiol. 159, 40-57.

35. Douglas, W.W. (1966) Pharmacol. Rev. 18, 471-480.

36. Butcher, F.R., and Putney, J.W. (1980) Adv. Cyclic Nucl. Res. 13, 215-250.

37. Feinstein, M.B. (1978) in Calcium in Drug Action,(Weiss, G.B., ed.), Chapter 9, Plenum Press, New York.

38. Gomperts, B.D. (1976) in Receptors and Recognition, (Greave, M.F., and Cuatrecasas, P., eds.) Vol. A2, pp. 43-102, Chapman and Hall, London.

39. Malaisse, W.J., Herchuelz, A., Devis, G., Somers, G., Boschero, A.C., Hutton, J.C., Kawazu, S., Atwater, I.J., Duncan, G., Ribalet, B., and Rojas, E. (1978) Ann. N.Y. Acad. Sci. 307, 562-582.

40. Adelstein, R.S., and Eisenberg, E. (1980) Ann. Rev. Biochem. 49, 921-956.

41. Yamauchi, T., and Hidaka, H. (1979) Biochem. Biophys. Res. Commun. 90, 28-35.

42. Garrison, J.C., Borland, K., Florio, V.A., and Twible, D.A. (1979) J. Biol. Chem. 254, 7147-7156.

43. Schulman, H., and Greengard, P. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5452-5456.

44. Takai, Y., Kishimoto, A., Iwasa, Y., Kowahara, Y., Mori, T., and Nishzuka, Y. (1979) J. Biol. Chem. 254, 3692-3695.

45. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273-2276.

46. Wray, L.H., and Gray, R.R. (1977) Biochim. Biophys. Acta 461, 441-459.

47. LePeuch, C.J., Haiech, J., and Demaille, J.G. (1979) Biochemistry 18, 5150-5157.

48. Katz, S. (1979) Adv. Cyclic Nucl. Res. 11, 303-345.

49. Tada, M., Yamada, M., Ohmori, F., Kuzuya, T., Inui, M., and Abe, H. (1980) J. Biol. Chem. 255, 1985-1992.

50. Carafoli, E., and Zurini, M. (1982) Biochim. Biophys. Acta 683, 279-301.

51. Niggli, V., Adunyah, E.S., Penniston, J.T., and Carafoli, E. (1981) J. Biol. Chem. 256, 395-401.

52. Hinds, T.R., Larson, F.L., and Vincenzi, F.F. (1978) Biochem. Biophys. Res. Commun. 81, 455-461.

53. Larsen, F.L., and Vincenzi, F.F. (1979) Science 204, 306-309.

54. Pershadsingh, H.A., and McDonald, J.M. (1980) J. Biol. Chem. 255, 4087-4093.

55. Black, B., Jarrett, L., and McDonald, J.M. (1980) Biochim. Biophys. Acta 596, 359-371.

56. Pershadsingh, H.A., McDaniel, M.L., Landt, M., Bry, C.G., Lacy, P.E., and McDonald, J.M. (1980) Nature (London) 288, 492-495.

57. Caroni, P., and Carafoli, E. (1981) J. Biol. Chem. 256, 3263-3270.

58. Moore, L., Chen, T.S., Knapp, H.R., Jr., and Landon, E.J. (1975) J. Biol. Chem. 250, 4562-4568.

59. Becker, G.L., Fiskum, G., and Lehninger, A.L. (1980) J. Biol. Chem. 255, 9009-9012.

60. Dawson, A.P. (1982) Biochem. J. 206, 73-79.

61. Chan, K.M., and Junger, K.D. (1983) J. Biol. Chem. 258, 4404-4410.

62.. Lotersztajn, S., and Pecker, F. (1982) J. Biol. Chem. 257, 6638-6641.

63. Roach, R.J., DePaoli-Roach, A.A., Larner, J. (1978) J. Cyclic Nucl. Res. 4, 245-257.

64. Cohen, P. (1978) Current Topics in Cell Regul. 14, 117-196.

65. Krebs, E.G., and Beavo, J.A. (1979) Ann. Rev. Biochem. 48, 923-959.

66. Cohen, P. (1980) in Molecular Aspects of Cellular Regulation, (Cohen, P. ed.) Vol. 1, pp. 255-268, Elsevier-North Holland, Amsterdam.

67. Chrisman, T.D., and Exton, J.H. (1980) J. Biol. Chem. 255, 3270-3273.

68. Imazu, M., Strickland, W.G., Chrisman, T.D., and Exton, J.H. (1984) J. Biol. Chem. 259, 1813-1821.

69. Claret-Berthon, B., Claret, M., and Mazet, J.L. (1977) J. Physiol. 272, 529-552.

70. Murphy, E., Coll, K., Rich, T., and Williamson, J.R. (1980) J. Biol. Chem. 255, 6600-6608.

71. Blackmore, P.F., Dehaye, J.P., and Exton, J.H. (1979) J. Biol. Chem. 254, 6945-6950.

72. Scott, I.D., Akerman, K.E.O., and Nicholls, D.G. (1980) Biochem. J. 192, 877-880.

73. Joseph, S.K., Coll., K., Cooper, R.H., Marks, J.S., and Williamson, J.R. (1983) J. Biol. Chem. 258, 731-741.

74. Marban, E., Rink, T.J., Tsien, R.W., and Tsien, R.Y. (1980) Nature 286, 845-850.

75. Carafoli, E., and Crompton, M. (1978) Current Topics in Membrane Transp. 10, 151-216.

76. Reeves, J.P., and Sutko, J.L. (1980) Science 208, 1461-1464.

77. Lamb, J.F., and Lindsay, R. (1971) J. Physiol. 218, 691-708.

78. Cittadine, A., and Van Rossum, G.D.V. (1978) J. Physiol. 281, 29-43.

79. Carafoli, E. (1979) FEBS Lett. 104, 1-5.

80. Nicolls, D.G., and Crompton, M. (1980) FEBS Lett. 111, 261-268.

81. Fiskum, G., and Lehninger, A.L. (1979) J. Biol. Chem. 254, 6236-6239.

82. Saris, N. -E., and Akerman, K.E.O. (1980) Current Topics in Bioenerg. 10, 103-179.

83. Coll, K.E., Joseph, S.K., Corkey, B.E., and Williamson, J.R. (1982) J. Biol. Chem. 257, 8696-8704.

84. Nicholls, D.G. (1978) Biochem. J. 176, 463-474.

85. Schulz, I. (1980) Am. J. Physiol. 239, 6335-6347.

86. Butcher, F.R., and Putney, J.W. (1980) Adv. Cyclic Nucl. Res. 13, 215-250.

87. Akhtar, R.A., and Abdel-Latif, A.A. (1979) Gen. Pharmacol. 10, 445-450.

88. Cockroft, S., and Gompert, B.D. (1979) Biochem. J. 178, 681-687.

89. Berridge, M.J., and Lipke, H. (1979) J. Exp. Biol. 78, 137-148.

90. Koenig, H., Goldstone, A.D., and Lu, C.Y. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 7210-7214.

91. Rouser, G., Nelson, G.J., Fleister, s., and Simon, G. (1968) in Biological Membranes: Physical Fact and Function, (Chapman, D., ed.) pp. 5-69, Academic Press, London.

92. Galliard, T. (1973) in Form and Function of Phospholipids, (Ansell, G.B., Hawthorne, J.N., and Dawson, R.M.C., eds.) pp. 253-288, Elsevier, Amsterdam.

93. White, D.A. (1973) in Form and Funcction of Phospholipids, (Ansell, G.B., Hawthorne, J.N., and Dawson, R.M.C., eds.) pp. 441-482, Elsevier, Amsterdam.

94. Dawson, R.M.C., and Eichberg, J. (1965) Biochem. J. 96, 634-643.

95. Dittmer, J.C., and Douglas, M.G. (1969) Ann. N.Y. Acad. Sci. 165, 801-814.

96. Steiner, M.R., and Lester, R.L. (1972) Biochim. Biophys. Acta 316, 296-304.

97. Asada, K., Tanaka, K., and Kasai, T. (1969) Ann. N.Y. Acad. Sci. 165, 801-814.

98. Hauser, G., and Eichberg, J. (1973) Biochim. Biophys. Acta 326, 201-209.

99. Cohen, P., Broekman, H.J., Verkley, A., Lisman, J.W.W., and Derksen, A. (1971) J. Clin. Invest. 50, 762-772.

100. Holub, B.J., Kuksis, A., and Thompson, W. (1970) J. Lipid Res. 11, 558-564.

101. Jelsemma, C.L., and Morre, D.J. (1978) J. Biol. Chem. 253, 7960-7971.

102. Seyfred, M.A., Smith, C.D., Farrell, L.E., and Wells, W.W. (1984) in Proceedings of the Chilton Conference on Inositol and Phosphoinositides, (Bleasdale, J.E., Eichberg, J., and Hauser, G., eds.), Humana Press, in press.

103. Collins, C.A., and Wells, W.W. (1983) J. Biol. Chem. 258, 2130-2134.

104. Jergil, B., and Sundler, R. (1983) J. Biol. Chem. 258, 7968-7973.

105. Smith, C.D., and Wells, W.W. (1983) J. Biol. Chem. 258, 9368-9373.

106. Behar-Bannelier, M., and Murray, R.K. (1980) Biochem. J. 187, 147-156.

107. Harwood, J.L., and Hawthorne, J.N. (1969) Biochim. Biophys. Acta 171, 75-88.

108. Fisher, D.B., and Mueller, G.C. (1971) Biochim. Biophys. Acta 248, 434-448.

109. Kai, M., Salwang, J.G., and Hawthorne, J.N. (1968) Biochem. J. 106, 771-801.

110. Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580-587.

111. Chau, L.-Y., and Tai, H.-H. (1982) Biochim. Biophys. Acta 713, 344-351.

112. Takenawa, T., and Nagai, Y. (1981) J. Biol. Chem. 256, 6769-6775.

113. Kemp, P., Hubscher, G., and Hawthorne, J.N. (1961) Biochem. J. 79, 193-200.

114. Hofman, S.L., and Majerus, P.W. (1982) J. Biol. Chem. 257, 6461-6469.

115. Abdel-Latif, A.A., Luke, B., and Smith J.P. (1980) Biochim. Biophys. Acta 614, 425-434.

116. Irvine, R.F. and Dawson, R.M.C. (1978) J. Neurochem. 31, 1427-1434.

117. Billah, M.M., and Lapetina, E.G. (1982) J. Biol. Chem. 257, 11856-11859.

118. Rubin, R.P., Sink, L.E., and Freer, R.J. (1981) Biochem. J. 194, 497-505.

119. Halenda, S.P., and Rubin, R.P. (1982) Biochem. J. 208, 713-721.

120. Dawson, R.M.C., and Thompson, W. (1964) Biochem. J. 91, 244-250.

121. Nijjar, M.s., and Hawthorne, J.N. (1977) Biochim. Biophys. Acta 480, 390-402.

122. Cooper, P.H., and Hawthorne, J.N. (1975) Biochem. J. 150, 537-551.

123. Akhtar, R.A., and Abdel-Latif, A.A. (1978) Biochim. Biophys. Acta 527, 159-170.

124. Roach, P.D., and St. C. Palmer, f.B. (1981) Biochim. Biophys. Acta 661, 323-333.

125. Koutouzov, S., and Marche, P. (1982) FEBS Lett. 144, 16-20.

126. Mack, S.E., and St. C. Palmer, F.B. (1984) J. Lipid Res. 25, 75-85.

127. Smith, C.D., and Wells, W.W. (1984), submitted for publication.

128. Thompson, W., and Dawson, R.M.C. (1964) Biochem. J. 91, 233-236.

129. Van Rooijen, L.A.A., Sequin, E.B., and Agranoff, B.W. (1983) Biochem. Biophys. Res. Commun. 112, 919-926.

130. Griffin, H.D., and Hawthorne, J.N. (1978) Biochem. J. 176, 541-552.

131. Downes, c.P., and Michell, R.H. (1981) Biochem. J. 198, 133-140.

132. Downes, C.P., and Michell, R.H. (1982) Biochem. J. 202, 53-58.

133. Agranoff, B.W., Murthy, P., and Sequin, E.B. (1983) J. Biol. Chem. 258, 2076-2078.

134. Rittenhouse, S.E. (1983) Proc. Natl. Acad. Aci. U.S.A. 80, 5417-5420.

135. Irvine, R.F. (1982) Cell Calcium 3, 295-309.

136. Hokin, M.R., and Hokin, L.E. (1953) J. Biol. Chem. 209, 549-558.

137. Shukla, S.D., and Hanahan, D.J. (1982) Biochem. Biophys. Res. Commun. 106, 697-703.

138. Shukla, S.D., Buxton, D.B., Olson, M.S., and Hanahan, D.J. (1983) J. Biol. Chem. 258, 10212-10214.

139. Akhtar, R.A., and Abdel-Latif, A.A. (1978) J. Pharmacol. Exp. Ther. 204, 655-668.

140. Farese, R.V., Saber, M.A., and Vandor, S.L. (1979) J. Biol. Chem. 254, 6842-6844.

141. Kirk, C.J., Creba, J.A., Downes, C.P., and Michell, R.H. (1981) Biochem. Soc. Trans. 9, 377-379.

142. Weiss, S.J., McKinney, J.S., and Putney, J.W., Jr. (1982) Biochem. J. 206, 555-560.

143. Billah, M.M., and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705-12708.

144. Benabe, J.E., Spry, L.A., and Morrison, A.K. (1982) J. Biol. Chem. 257, 7430-7434.

145. Putney, J.W., Burgess, G.M., Halenda, S.P., McKinney, J.S., and Rubin, R.P. (1983) Biochem. J. 212, 483-488.

146. Berridge, M.S., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983) Biochem. J. 212, 473-482.

147. Rebecchi, M.J., and Gerschengorn, M.C. (1983) Biochem. J. 216, 287-294.

148. Martin, T.F.J. (1983) J. Biol. Chem. 258, 14816-14822.

149. Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 515, 209-237.

150. Roelofsen, B., and Van Linde-Sibenius Trip, M. (1981) Biochim. Biophys. Acta 642, 302-306.

151. Zachowski, A., and Devaux, P.F. (1983) FEBS Lett. 163, 245-249.

152. Lipsky, J.J., and Lietman, P.S. (1980) Antimicrob. Agents Chemother. 18, 532-535.

153. Buckley, J.T., and Hawthorne, J.N. (1972) J. Biol. Chem. 247, 7218-7223.

154. Penniston, J.T. (1982) Ann. N.Y. Acad. Sci. 402, 296-303.

155. Varsanyi, M., Tolle, H.-G., Heilmeger, L.M.G., Dawson, R.M.C., and Irvine, R.F. (1983) EMBO J. 2, 1543-1548.

156. Smith, C.D., and Wells, W.W. (1984), submitted for publication.

157. Kowluru, R.A., George, R., and Jefcoate, C.R. (1983) J. Biol. Chem. 258, 8053-8059.

158. Macaulay, S.L., Kiechle, F.L., and Jarett, L. (1983) Arch. Biochem. Biophys. 225, 130-136.

159. McOsker, C.C., Weiland, G.A., and Zilversmit, D.B. (1983) J. Biol. Chem. 258, 13017-13026.

160. Duzgunes, N., Hang, K., and Papahadjopoulos, D. (1980) in Calcium Binding Proteins: Structure and Function, (Siegel, F.L., Carafoli, E., Krestinger, R.H., MacLennan, D.H., and Wasserman, R.H., eds.) pp. 17-22, Elsevier-North Holland, New York.

161. Sundler, R., and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 649, 743-750.

162. Wakelan, M.J.O. (1983) Biochem. J. 214, 77-82.

163. Pickard, M.R., and Hawthorne, J.N. (1978) J. Neurochem. 30, 145-155.

164. Sheetz, M.P., Febbroriello, P., and Koppel, D.E. (1982) Nature (London) 296, 91-93.

165. Downes, P., and Michell, R.H. (1982) Cell Calcium 3, 467-502.

166. Jolles, J., Zwiers, H., Dekker, A., Wirtz, K.W.A., and Gispen, W.H. (1981) Biochem. J. 194, 283-291.

167. Sano, K., Takai, Y., Yamanishi, J., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010-2013.

168. Nishizuka, Y. (1983) Trends in Biochem. Sci. 8, 13-16.

169. Cooper, R.H., Kobayashi, K., and Williamson, J.R. (1984) FEBS Lett. 166, 125-130.

170. Sugimoto, Y., Whitman, M., Cantley, L.C., and Erikson, R.L. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2117-2121.

171. Macara, I.G., Marinetti, G.V., and Balduzzi, P.C. (1984) Proc. Natl. Acad. Sci. U.S.A., 81, 2728-2732.

172. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkowa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.

173. Blumberg, P.M. (1980) CRC Crit. Rev. Toxicol. 8, 153-234.

174. Michell, R.H. (1982) Cell Calcium 3, 429-440.

CHAPTER I

Subcellular Incorporation of ^{32}P into Phosphoinositides and Other Phospholipids in Isolated Hepatocytes

ABSTRACT

Isolated rat hepatocytes were incubated with ^{32}Pi for various times and then fractionated into plasma membranes, mitochondria, nuclei, lysosomes and microsomes by differential centrifugation and Percoll density gradient centrifugation. The phospholipids were isolated and deacylated by mild alkaline treatment. The glycerophosphate esters were separated by anion exchange high pressure liquid chromatography and assayed for radioactivity. It was found that plasma membranes, mitochondria, nuclei, lysosomes, and microsomes displayed similar rates of ^{32}P incorporation into the major phospholipids, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol and phosphatidic acid. This data suggests that the phospholipids of these organelles are undergoing rapid turnover and replacement with newly synthesized phospholipids from the endoplasmic reticulum.

However, the plasma membrane fraction incorporated ^{32}P into phosphatidylinositol 4-phosphate (DPI) and phosphatidylinositol 4,5-bisphosphate (TPI) at rates 5-10 and 25-50 times, respectively, faster than any of the other subcellular fractions. Although the plasma membrane is the primary site of ^{32}P incorporation into DPI and TPI, this study also demonstrates that significant incorporation of ^{32}P into DPI occurs in other subcellular sites, especially lysosomes.

FOOTNOTES

[1]The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; DPI (diphosphoinositide), phosphatidylinositol 4-phosphate; TPI (triphosphoinositide), phosphatidylinositol 4,5-bisphosphate; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; GPI, glycerophosphorylinositol; GPG, glycerophosphorylglycerol; GP, glycerol phosphate; GPS, glycerophosphorylserine; GPIP, glycerophosphorylinositol 4-phosphate; $GPIP_2$, glycerophosphorylinositol 4,5-bisphosphate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TRICINE, N-tris[hydroxymethyl]methyl glycine; PMSF, phenylmethylsulfonyl fluoride; HB, homogenizing buffer; HPLC, high pressure liquid chromatography.

INTRODUCTION

Hormonal regulation of phosphatidylinositol (PI)[1] metabolism was first observed in the pancreas by Hokin and Hokin (1). Since that report, the influence of hormones upon the phosphatidate-inositide cycle has been studied in a number of tissues and cell types. Agonist induced changes in phosphoinositides and phosphatidic acid have been reported in kidney (2,3), iris smooth muscle (4,5), pancreas (1,6,7), parotid gland (8,9), adrenals (10), adipose tissue (11,12), platelets (13,14), brain (9,15,16), insect salivary glands (9,17) and liver (18-23). However, nearly all these studies measured whole cell phospholipid metabolism and not in specific organelles where the site of hormone action may occur. Evidence suggests that the plasma membrane is the subcellular site of increased PI hydrolysis in response to vasopressin (20,24). However, vasopressin stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (TPI) could not be attributed to any specific subcellular fraction presumably due to the extreme lability of this phospholipid (23). Studies on phosphatidylcholine (PC) and phosphatidylethanolamine (PE) metabolism have suggested regulation by insulin (25), glucagon (25,26), cAMP analogues (27), vasopressin and angiotension (28) in rat hepatocytes. However, the intracellular site of hormone action was presumed to be the endoplasmic reticulum although this was not fully documented.

In this report, a rapid subcellular fractionation was used to prepare microsomes, nuclei, mitochondria, lysosomes and plasma membranes from isolated rat hepatocytes which induced minimal post-homogenization artifacts. Phospholipid metabolism in these fractions was examined by incubating the hepatocytes with ^{32}Pi, isolating the organelles and extracting their component phospholipids. These phospholipids were deacylated and the glycerophosphate esters analyzed by a new method utilizing anion exchange high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials - (^{32}P)Orthophosphate, carrier free, was purchased from ICN. Phospholipid standards were obtained from either Sigma or Serdary Research Laboratories. Glass distilled organic solvents were obtained from MCB. Collagenase (E.C. 3.4.99.5) CLSII was purchased from Worthington. Penicillin-streptomycin solution (10,000 units/ml penicillin, 10 mg/ml streptomycin), MEM non-essential amino acids solution (100X), MEM amino acids solution with 100 mM L-glutamine (50X) and MEM vitamin solution (100X) were obtained from Grand Island Biological Co. CD-Sprague Dawley male rats were acquired from Charles River Laboratories. Roller bottles (490 cm^2) were a product of Corning. Percoll was purchased from Pharmacia. All other chemical were of reagent grade.

Hepatocyte Isolation - Hepatocytes were isolated from 200-250 gm fed male rats by the method of Seglen (29) as modified by Kurtz and Wells (30). The procedure was further modified by providing the perfusion buffer with 10 mM glucose and the normal concentration of MEM non-essential and essential amino acids. Typically, 4.0-6.0 x 10^8 cells of 85-95% viability, as judged by Trypan blue exclusion, were obtained from a single animal. The cells were suspended at a concentration of 1.0-2.0 x 10^6 cells/ml in media modeled after Dulbecco's modified Eagle's medium. This medium contained (per liter)

6.4 g NaCl, 3.7 g $NaHCO_3$, 0.4 g KCl, 0.2 g anhydrous $CaCl_2$, 98 mg anhydrous $MgSO_4$, 0.11 g sodium pyruvate, 1.0 g glucose, 4.8 g HEPES, 15 mg phenol red, 40 μg $FeCl_3$, 14.0 mg $NaH_2PO_4 \cdot H_2O$, 20.0 g bovine serum albumin (Fraction V), 10 ml of penicillin-streptomycin solution, 10 ml of 100X MEM non-essential amino acids solution, 20 ml of 50 MEM essential amino acids solution and 10 ml (100X) MEM vitamin solution at a pH of 7.3. Cell suspensions of 35 ml were placed in 490 cm^2 tissue culture roller bottles which were flushed every 30 min with a 10 sec burst of 95% O_2/5% CO_2, sealed and rotated at 2 r.p.m. at 37°. Under these conditions, the cells remained well suspended and exhibited no loss in viability over a 3 hr period.

Hepatocyte Incubations - Carrier-free ^{32}Pi was added to the cell suspensions at the level of 5-10 μCi/10^6 cells. At various times of incubation up to 120 min, the cells were diluted with 7 volumes of ice-cold homogenizing buffer (HB) which consisted of 250 mM sucrose, 1 mM EDTA, 10 mM NaN_3, 20 mM NaF, 75 mg/l PMSF and 10 mM TRICINE pH 8.0. The cells were pelleted at 75 x g for 5 min at 4°C. The cell pellet was resuspended in 5.0 ml of ice-cold HB.

Cell Homogenization and Subcellular Fractionation - The cell suspension was homogenized and fractionated at 0-4°C as described in Figure 1. After the second homogenization step, cell breakage exceeded 95% as judged by phase contrast microscopy. The stock 45% Percoll solution was prepared in homogenizing buffer (HB). Resuspension of the 1500 x g pellet and subsequent "nuclear" pellets was accomplished by 3 strokes of a tight fitting glass-glass Potter-Elvehjem homogenizer. All other

Figure 1. Flow diagram for the subcellular fractionation of rat hepatocytes. Hepatocytes were isolated and labeled with $^{32}P_i$ for various times as described under "Experimental Procedures". The cells were transferred into 7 volumes of ice cold HB and pelleted at 4°C. The cell pellet was gently resuspended in 5.0 ml of ice cold HB and subjected to the fractionation scheme at 4°C depicted to the right.

SUBCELLULAR FRACTIONATION OF RAT HEPATOCYTES

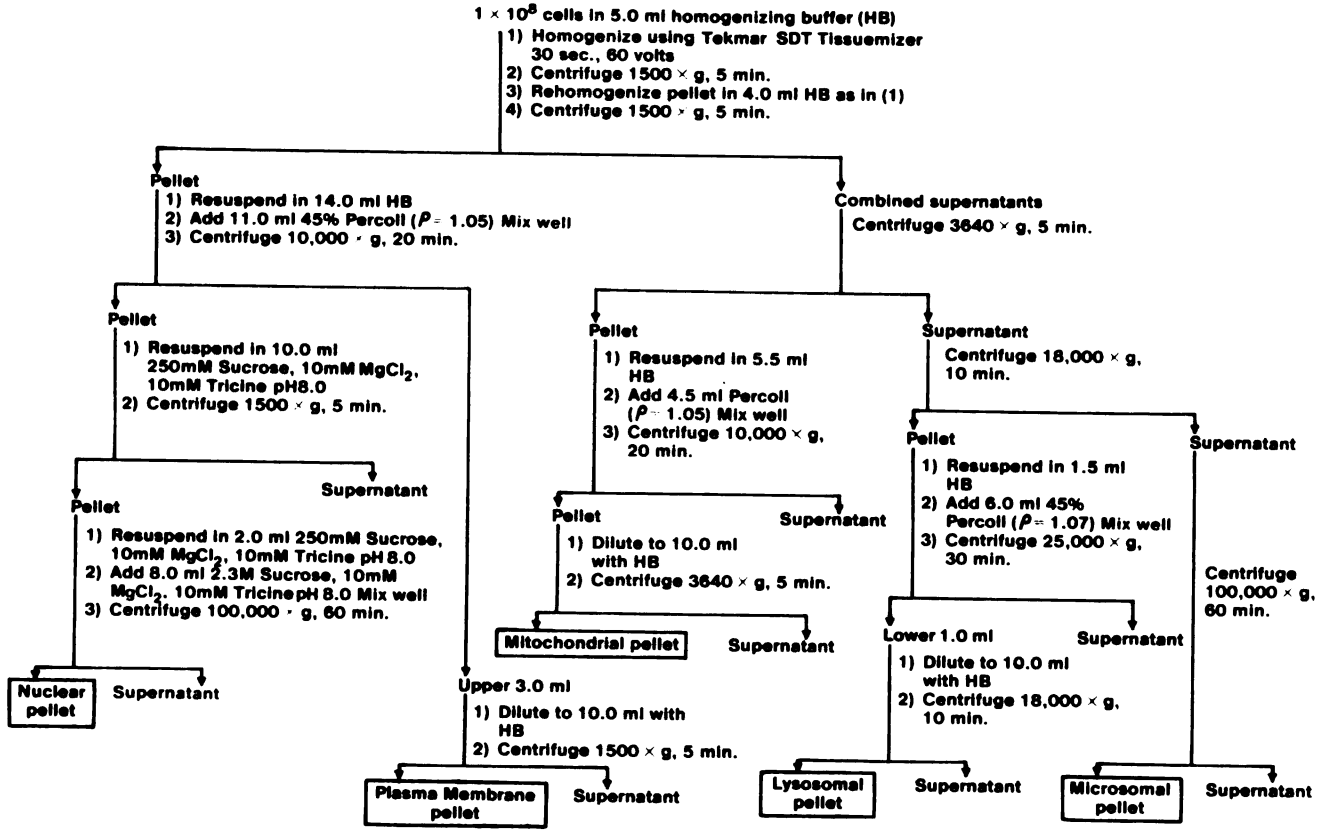


Figure 1

pellets were resuspended using 3 strokes of a Teflon-glass Potter-Elvehjem homogenizer. The final pellets were resuspended in 1.0 ml of HB. Complete fractionation of five groups of cells was accomplished in 3.5-4.0 hrs.

Using the marker enzymes hexosaminidase (31) for lysosomes, fumarase (32) for mitochondria, alkaline phosphodiesterase I (33) for plasma membrane and glucose 6-phosphatase (34) in the presence of 40 mM L-tartrate (35) for microsomes, the organelle content of the various fractions was determined using the method reported by Leighton et al. (36). Protein was determined by fluorescamine (37) using bovine serum albumin as the standard. DNA was measured using the method of Karsten and Wollenberger (38).

Phospholipid Isolation and Deacylation - The total phospholipid fraction was isolated by a modification of the method of Schacht (39). Briefly, 3.0 ml of methanol:chloroform (2:1 v/v) were added to the resuspended pellets followed by 1.0 ml of 2.4N HCl and 1.0 ml of chloroform, and the contents were vortexed for 15 sec. The resulting two phases were separated by centrifugation. The lower phase was set aside and the upper phase washed first with 2.0 ml chloroform and then with 1.0 ml chloroform. The lower phases were combined and washed with 5.0 ml of methanol:1N HCL (1:1 v/v). The lower phase was removed and evaporated to dryness using a N_2 stream at 37°C. The residue was dissolved in 0.5 ml of methanol:chloroform (3:2 v/v).

The phospholipids were deacylated using the mild alkaline hydrolysis procedure described by Kates (40). A minor modification was made which allowed the pH of the polar fraction to remain slightly

alkaline (pH Ca. 8.0) by using only 0.1 ml of Dowex 50 [H^+]. This modification resulted in increasing the recovery of glycerolphosphorylcholine. The polar fraction was evaporated using a N_2 stream at 37° and dissolved in 100 µl of 20 mM ammonium borate, 100 mM ammonium formate, pH 9.5.

Analysis of Deacylated Phospholipids by High Pressure Anion Exchange Liquid Chromatography - The deacylated products were analyzed by high pressure liquid chromatography (HPLC) on a 250 x 4 mm BioRad Aminex A-27 anion exchange column using a modification of the ion exchange procedure described by Dittmer and Wells (41). A polyphasic gradient beginning with 100 mM ammonium formate, 20 mM ammonium borate, pH 9.5 and ending with 750 mM ammonium formate, 20 mM ammonium borate, pH 9.5 was established by a Beckman Model 342 Gradient Liquid Chromatograph and used to elute the deacylated phospholipids (Fig. 2A). Fractions of 0.78 ml were collected at a rate of 0.6 ml/min in Pyrex tubes. The fractions were either counted by Cerenkov radiation or evaporated overnight at 150°C in a vented hood. The residue which remained in the tubes after evaporation was digested with 0.3 ml of 14% H_2SO_4:12% $HClO_4$ (2:1 v/v) for 90 min at 180°C after placing glass marbles on top. The phosphate content of the fractions was determined by the method of Ames (42) with detection of the reduced phospho-molybdate complex at 820 nm.

Determination of Cellular [γ-^{32}P]-ATP Specific Radioactivity - A 3.5 ml aliquot of cell suspension was removed after various times of ^{32}Pi labeling and placed in 500 µl of ice cold 5% $HClO_4$. The $HClO_4$-treated samples were homogenized using a glass-glass

Potter-Elvehjem homogenizer, placed on ice for 15 min and then centrifuged. The supernatant was removed and neutralized with 2M $KHCO_3$ at 4°C. The $KClO_4$ was pelleted and the supernatant removed. The [γ-^{32}P]-ATP specific radioactivity in the supernatant was determined using the method of Hawkins et al. (43).

RESULTS

Purity of Subcellular Fractions - Subcellular fractions were obtained by using the fractionation scheme outlined in Figure 1 and analyzed for various marker enzymes as summarized in Table I. The plasma membrane fraction was enriched in alkaline phosphodiesterase I activity greater than 4-fold over the whole homogenate. Fumarase activity in the mitochondrial fraction was enriched 2.2-fold over the whole homogenate. In the lysosomal fraction, hexosaminidase was enriched approximately 13-fold over the whole homogenate while glucose 6-phosphatase, in the presence of L-tartrate, was enriched nearly 2.8-fold in the microsomal fraction compared with the whole homogenate. DNA/protein was enriched 3-fold in the nuclear fraction compared with the whole homogenate.

After measuring the contamination of other marker enzymes within each fraction and using the formula described by Leighton et al. (36), the purity of the various fractions was estimated (Table I). The fractionation process resulted in a plasma membrane fraction which consisted of 28% plasma membrane but also contained 57% microsomes. The mitochondrial fraction was composed of 60% mitochondria, 36% microsomes but only 2% plasma membrane. The lysosome fraction contained 19% lysosomes, approximately equal amounts of mitochondria and microsomes but only 2% plasma membranes. The microsome fraction was the purest fraction obtained and was composed of nearly 86% microsomes and 9% plasma membranes.

Table I

Marker enzymes and estimate of fraction purity

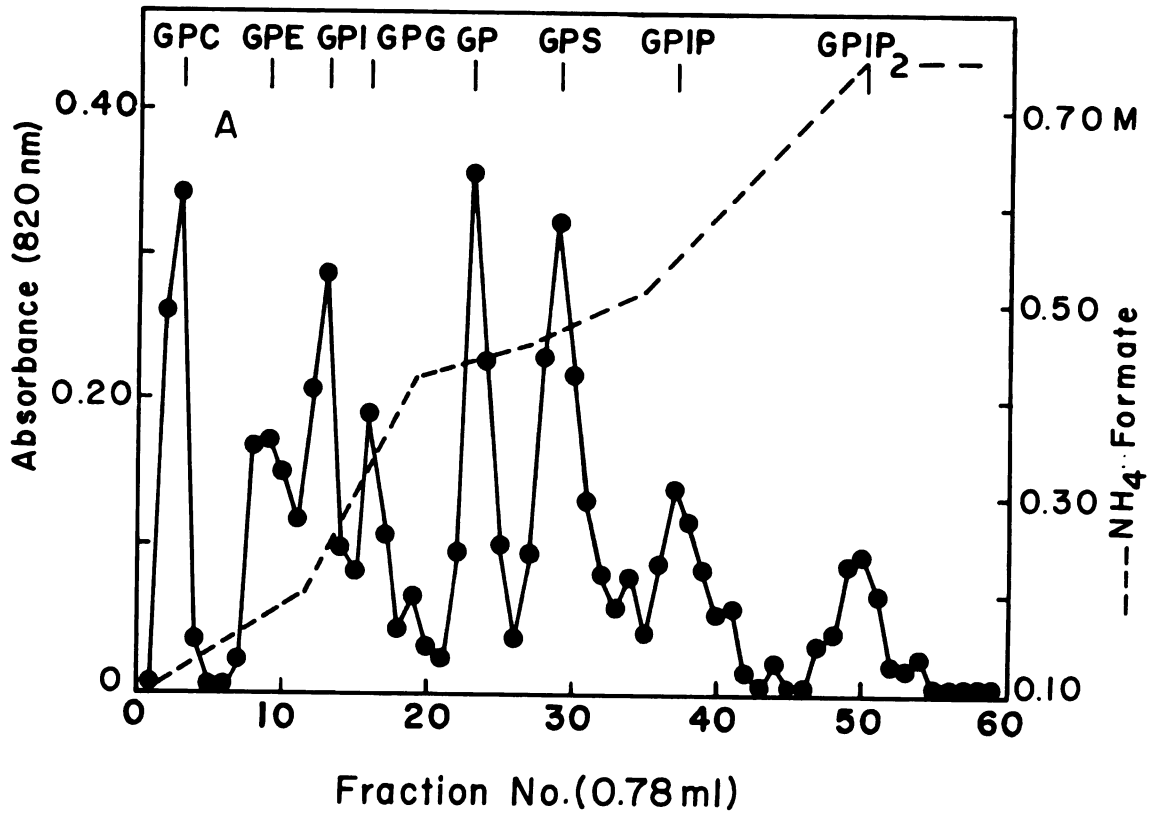
Fractions in the left-hand column refer to the fractions evaluated. The fraction designations above each of the marker enzymes identify the fraction for which that particular marker was assumed to be specific when calculating purity. Units for specific activity are micromoles of p-nitrophenol released/min/mg of protein from p-nitrophenyl-N-acetyl- -D-glucosaminide for hexosaminidase and from p-nitrophenyl-thymidine-5'monophosphate for alkaline phosphodiesterase I; micromoles of fumarate produced from malate/min/mg of protein for fumarase; and micromoles of inorganic phosphate released from glucose 6-phosphate/min/mg of protein for glucose 6-phosphatase. Enrichment of nuclei in the nuclear fraction was estimated by determining the DNA/protein (w/w). The first number in parenthesis gives the enrichment of the specific activity in a given membrane fraction relative to the specific activity of the homogenate (relative specific activity). The second number in parenthesis is the percentage of contamination while the fraction purity is the percentage of contaminating organelles subtracted from 100%. The percentage of contaminating organelles was calculated by the method of Leighton et al. (36). Values are means of four different hepatocyte preparations ± standard deviations.

	Marker Enzymes and Specific Activity						
Fraction	Plasma Membrane, alkaline phosphodiesterase I	Mitochondrion, fumarase	Lysosome, hexosaminidase	Microsome, Glucose 6-phosphatase	Nuclei DNA/protein	Fraction Purity %	Yield %
Homogenate	0.0337 ± 0.0089	0.390 ± 0.031	0.0313 ± 0.0043	0.0542 ± 0.0010	0.23 ± .009		
Nuclear	0.0157 ± 0.0057	0.379 ± 0.160	0.0317 ± 0.0086	0.0443 ± 0.0047	0.72 ± .029		14.7
	(0.48)	(0.97)	(1.01)	(0.82)	(3.13)		
Plasma Membrane	0.147 ± 0.0082	0.102 ± 0.006	0.0301 ± 0.0061	0.0594 ± 0.0089		27.5	8.1
	(4.36)	(0.26)	(0.96)	(1.10)			
		(12.7)	(2.3)	(57.4)			
Mitochondrial	0.0193 ± 0.0045	0.869 ± 0.089	0.0880 ± 0.0240	0.0633 ± 0.0084		60.5	7.0
	(0.57)	(2.23)	(2.81)	(1.17)			
	(2.0)		(3.8)	(33.8)			
Lysosomal	0.0165 ± 0.0035	0.536 ± 0.098	0.416 ± 0.0300	0.0659 ± 0.0132		19.4	4.5
	(0.49)	(1.37)	(13.3)	(1.22)			
	(1.9)	(40.4)		(38.3)			
Microsomal	0.0769 ± 0.0092	0.0665 ± 0.017	0.0157 ± 0.0007	0.152 ± 0.036		86.2	24.6
	(2.28)	(0.16)	(0.50)	(2.80)			
	(8.5)	(4.6)	(0.7)				

Anion Exchange HPLC Analysis of Deacylated Phospholipids - Phospholipid standards were deacylated and subjected to anion exchange HPLC as described under "Experimental Procedures". Eight glycerophosphoryl species were separated including glycerophosphorylinositol 4-phosphate (GPIP) and glycerophosphorylinositol 4,5-bisphosphate ($GPIP_2$) (Fig. 2A). Greater than 95% recovery of deacylated phospholipids was obtained. ^{32}P-labeled phospholipids isolated from rat hepatocyte cell homogenate were similarly deacylated and chromatographed. As shown in Figure 2B, radioactivity corresponded very well with lipid phosphorous with the exception that very little radioactivity was incorporated into PS (glycerophosphorylserine, GPS). Also, there was no detectable lipid phosphorous associated with DPI (GPIP) or TPI ($GPIP_2$), however, there was substantial ^{32}P incorporation into these minor phospholipids.

Estimation of Possible Post-Homogenization Artifacts - Although the subcellular fractionation process was relatively rapid, enzyme inhibitors were present in the buffer, and the fractionation was conducted at 0-4°C, the possibility of large changes in the level of ^{32}P-labeled phospholipids after homogenization did exist. To investigate this possibility, an aliquot of cell homogenate was placed into methanol:chloroform (2:1, v/v) immediately after homogenization. Another aliquot was kept on ice and methanol:chloroform (2:1, v/v) was added following the completion of the subcellular fractionation. The phospholipids were isolated, deacylated and analyzed by anion exchange HPLC as described under "Experimental Procedures". In three different cell preparations, no change in the amount of ^{32}P in PC, PG and TPI

Figure 2. Anion exchange HPLC elution profile of polar deacylated phospholipid products. Phospholipids were isolated from hepatocytes, labeled for 60 min with $^{32}P_i$, deacylated with mild alkali and the polar products were analyzed on an Aminex A-27 anion exchange column as described under "Experimental Procedures".
A. Deacylated phospholipid standards. ●——●, absorbance at 820 nm of reduced phosphomolybdate complexes; ---, ammonium formate concentration. B. Deacylated phospholipids from hepatocyte cell homogenate. O——O, radioactivity as determined by Cerenkov radiation; ●——●, nanomoles lipid phosphorous. The position of elution of polar deacylated standards is indicated.


GPC
GPE
GPI
GPG
GP
GPS
GPIP
GPIP2
A
0.40
0.20
0
Absorbance (820 nm)
0.70 M
0.50
0.30
0.10
---NH4 Formate
0
10
20
30
40
50
60
Fraction No.(0.78 ml)

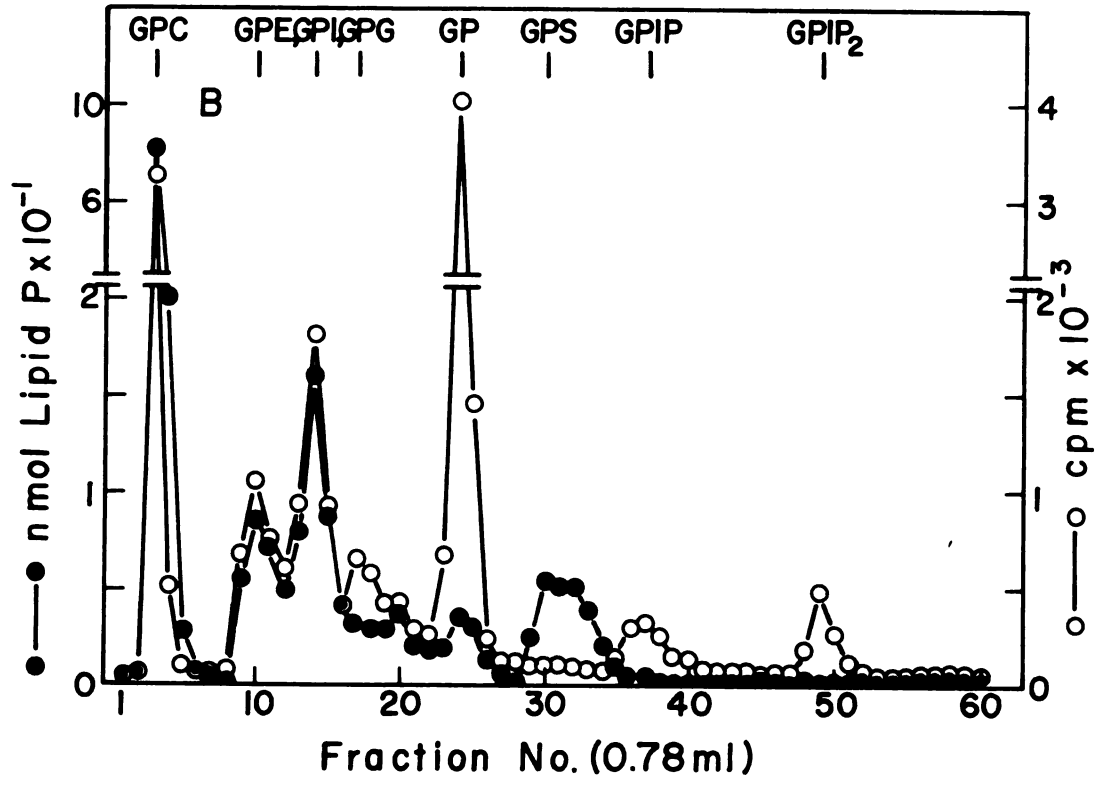

GPC
GPE
GPI
GPG
GP
GPS
GPIP
GPIP2
B
10
6
2
1
0
● n mol Lipid P x 10⁻¹
4
3
2
1
0
o cpm x 10⁻³
1
10
20
30
40
50
60
Fraction No. (0.78 ml)

Figure 2

occurred during fractionation. Only a 1-2% decrease in ^{32}P-labeled PE, PI and PS was observed. PA appeared to increase an average of 7% during fractionation while DPI decreased an average of 13%.

The isolation of purified nuclei require the use of divalent cations, usually Mg^{2+} or Ca^{2+}, to prevent aggregation of nuclei with other subcellular organelles (44). Therefore, a similar experiment was performed to determine if any changes in ^{32}P-labeled phospholipids occurred in the crude nuclear fraction in the presence of Mg^{2+}. After removal of plasma membranes, the crude nuclear fraction was resuspended in 250 mM sucrose, 10 mM $MgCl_2$ and 10 mM Tricine pH 8.0 (Fig. 1). An aliquot was removed and placed in methanol:chloroform (2:1, v/v). Another aliquot was placed on ice and then placed in methanol:chloroform (2:1, v/v) following isolation of the nuclear pellet. Analysis of the deacylated phospholipids showed that large changes in the amount of ^{32}P in PC, PE, PI and PA occurred during the isolation of nuclei in the presence of buffer containing $MgCl_2$ even at 4°C. Only small changes (ca. 5-10%) were seen in the amount of ^{32}P in PG, PS, DPI and TPI (data not shown). Therefore, the incorporation rates of ^{32}P into phospholipids isolated from partially purified nuclei that were measured are only valid for PG, PS, DPI and TPI.

The possibility of exchange of labeled phospholipids between organelles after homogenization was also investigated by mixing ^{32}P-labeled microsomes obtained using the scheme depicted in Figure 1 with unlabeled rat hepatocyte cell homogenate. The homogenate was fractionated by the standard procedure and an analysis of the partially purified fractions revealed 81.5% of the radioactivity was recovered in

the microsomal fraction, 9% in the plasma membrane, 8% in the mitochondrial fraction and 1.4% of the radioactivity was recovered in the lysosome fraction.

Rate of Incorporation of ^{32}P into Phospholipids of Various Subcellular Fractions - Isolated hepatocytes were labeled with ^{32}Pi for various times and fractionated. The phospholipids were isolated, deacylated and analyzed by anion exchange HPLC as described under "Experimental Procedures". The results are shown in Figures 3A-H. The data shown are the levels of ^{32}P in the various glycerophosphate esters which are a measurement of the amount of ^{32}P incorporated into various phospholipids. The rates of ^{32}P incorporation per mg protein into PC, PI, PG, and PA were similar between the lysosomal, microsomal and plasma membrane fractions but slightly slower in the mitochondrial fraction. Similar rates of ^{32}P incorporation into PE were seen in the lysosomal, plasma membrane and mitochondrial fractions but ^{32}P incorporation rates into PE were twice as fast in the microsomal fraction compared with the other subcellular fractions. The nuclear fraction has the fastest rate of ^{32}P incorporation into PS of all the fractions analyzed. However, the most striking result was the rapid and extensive amount of ^{32}P incorporated into DPI and TPI in the plasma membrane fraction compared with the other subcellular fractions. After 30 min of ^{32}Pi labeling, the amount of ^{32}P incorporated per mg protein into TPI was approximately 5 and 2.5 times greater than the amount of ^{32}P incorporated into PI and DPI, respectively, in the plasma membrane fraction. ^{32}P incorporation into DPI was nearly 2 times greater than PI after 30 min of labeling

Figure 3. Time course of ^{32}P incorporation into various phospholipids isolated from subcellular fractions of rat hepatocytes. Hepatocytes were isolated and labeled for various times with $^{32}P_i$ as described under "Experimental Procedures." The ^{32}P-labeled hepatocytes were homogenized and fractionated as described in Figure 1. The phospholipids were isolated from the various subcellular fractions, deacylated with mild alkali and analyzed by anion exchange HPLC as described under "Experimental Procedures." A. Glycerophosphorylcholine; B. Glycerophosphorylethanolamine; C. Glycerophosphorylinositol; D. Glycerophosphorylglycerol; E. Glycerol phosphate; F. Glycerophosphorylserine; G. Glycerophosphorylinositol 4-phosphate; F. Glycerophosphorylinositol 4,5-bisphosphate. O——O, Cell homogenate; □---□, Nuclear fraction; Δ——Δ, Plasma membrane fraction; ●——●, Microsomal fraction; ■——■, Lysosomal fraction; ▲——▲, Mitochondrial fraction, and x---x, [γ-^{32}P]ATP. The results shown are from one of three similar hepatocyte preparations.

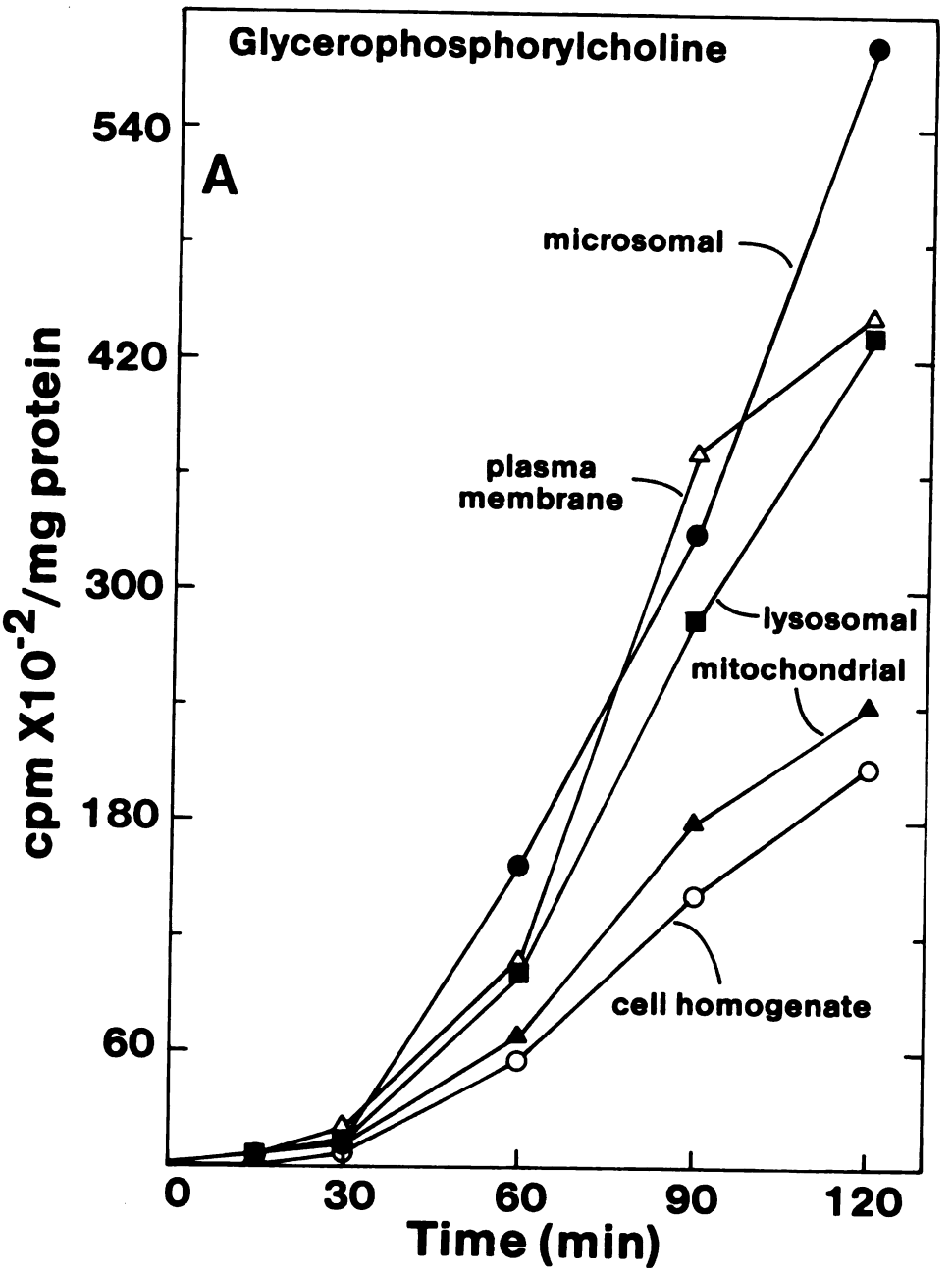

Glycerophosphorylcholine
A
540
420
300
180
60
cpm X10-2/mg protein
microsomal
plasma membrane
lysosomal
mitochondrial
cell homogenate
0
30
60
90
120
Time (min)

Figure 3A

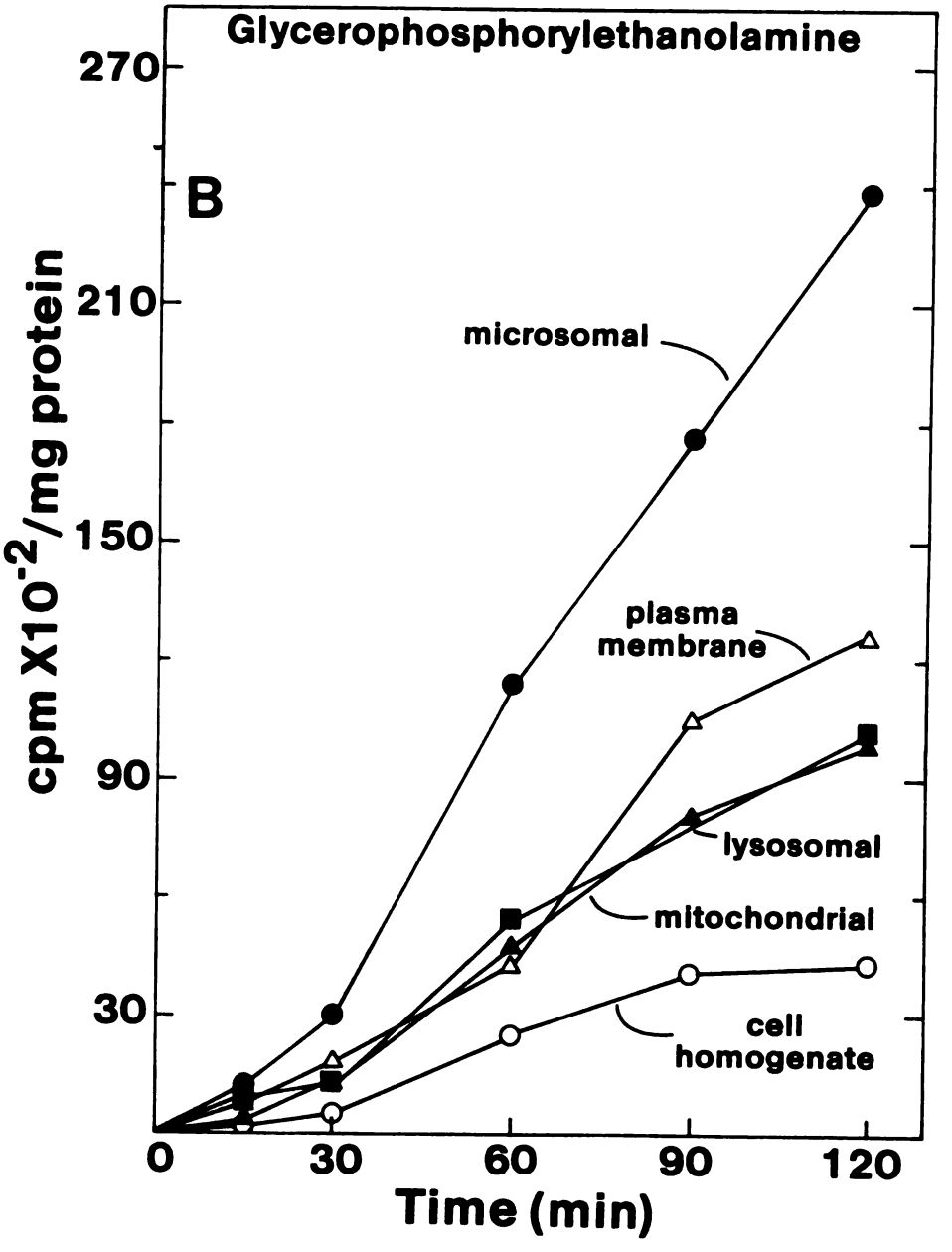

Glycerophosphorylethanolamine
B
270
210
150
90
30
cpm X10-2/mg protein
microsomal
plasma membrane
lysosomal
mitochondrial
cell homogenate
0
30
60
90
120
Time (min)

Figure 3B

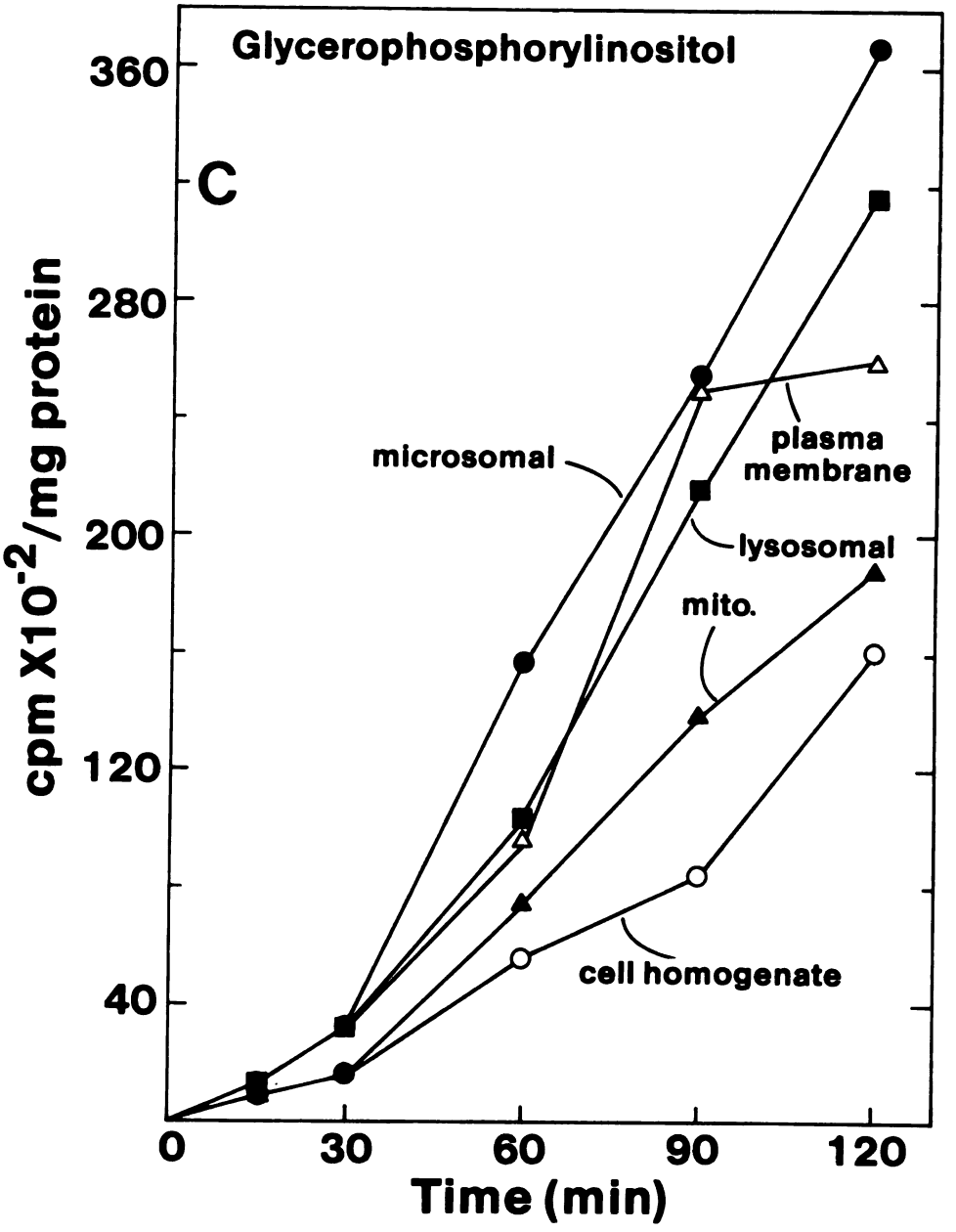

Glycerophosphorylinositol
C
360
280
200
120
40
cpm X10-2/mg protein
microsomal
plasma membrane
lysosomal
mito.
cell homogenate
0
30
60
90
120
Time (min)

Figure 3C

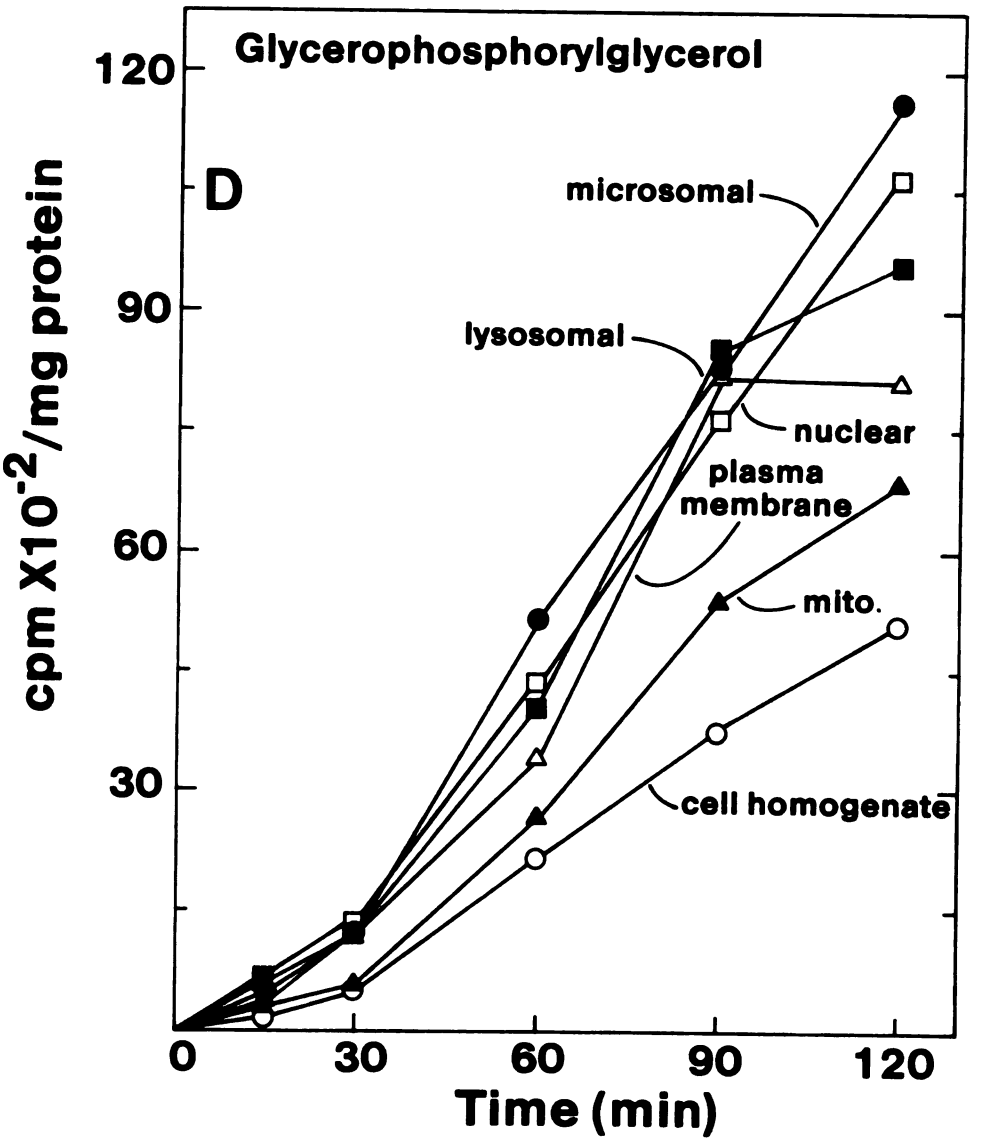

Glycerophosphorylglycerol
D
120
90
60
30
0
30
60
90
120
Time (min)
cpm X10-2/mg protein
microsomal
lysosomal
nuclear
plasma membrane
mito.
cell homogenate

Figure 3D

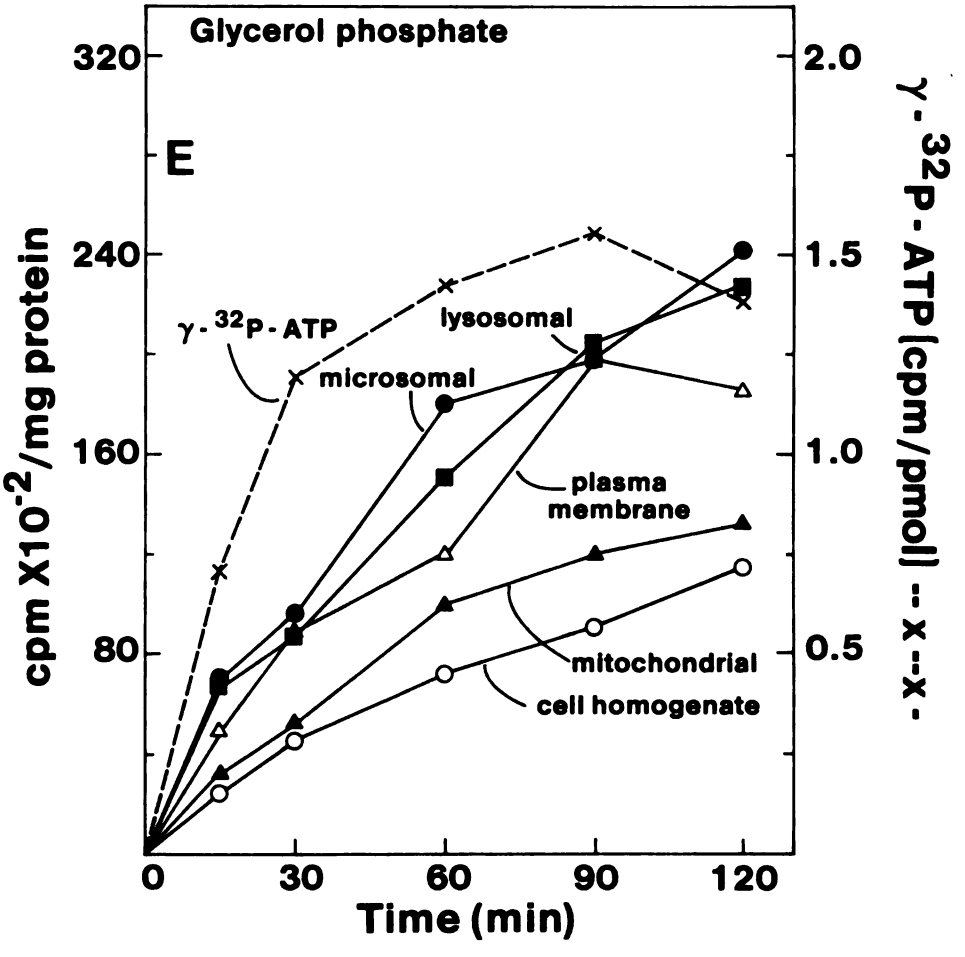

Glycerol phosphate
E
320
240
160
80
2.0
1.5
1.0
0.5
0
30
60
90
120
Time (min)
cpm X10-2/mg protein
γ-32P-ATP [cpm/pmol] --x--x-
γ-32P-ATP
lysosomal
microsomal
plasma membrane
mitochondrial
cell homogenate

Figure 3E

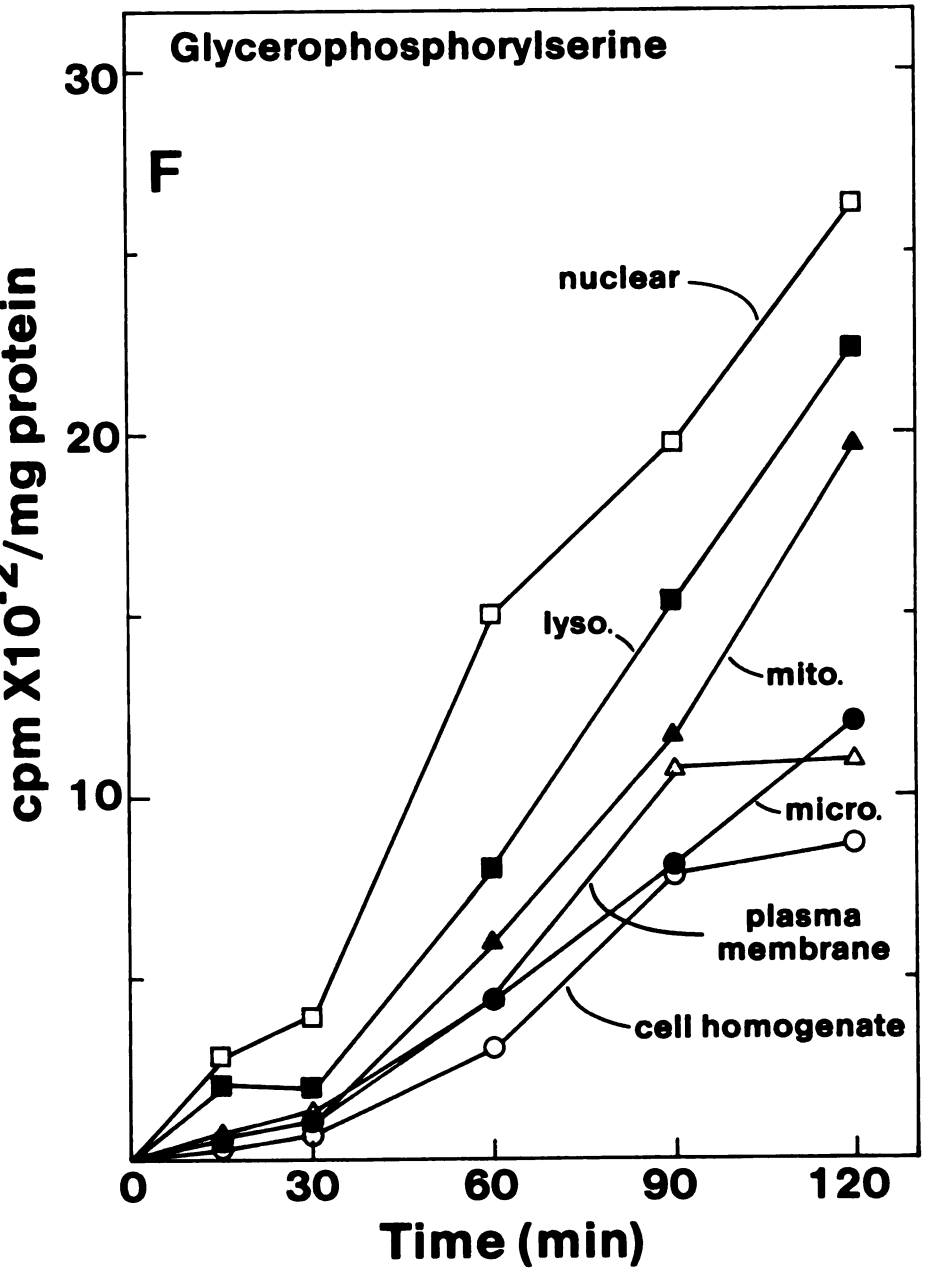

Glycerophosphorylserine
F
30
20
10
cpm X10-2/mg protein
nuclear
lyso.
mito.
micro.
plasma membrane
cell homogenate
0
30
60
90
120
Time (min)

Figure 3F

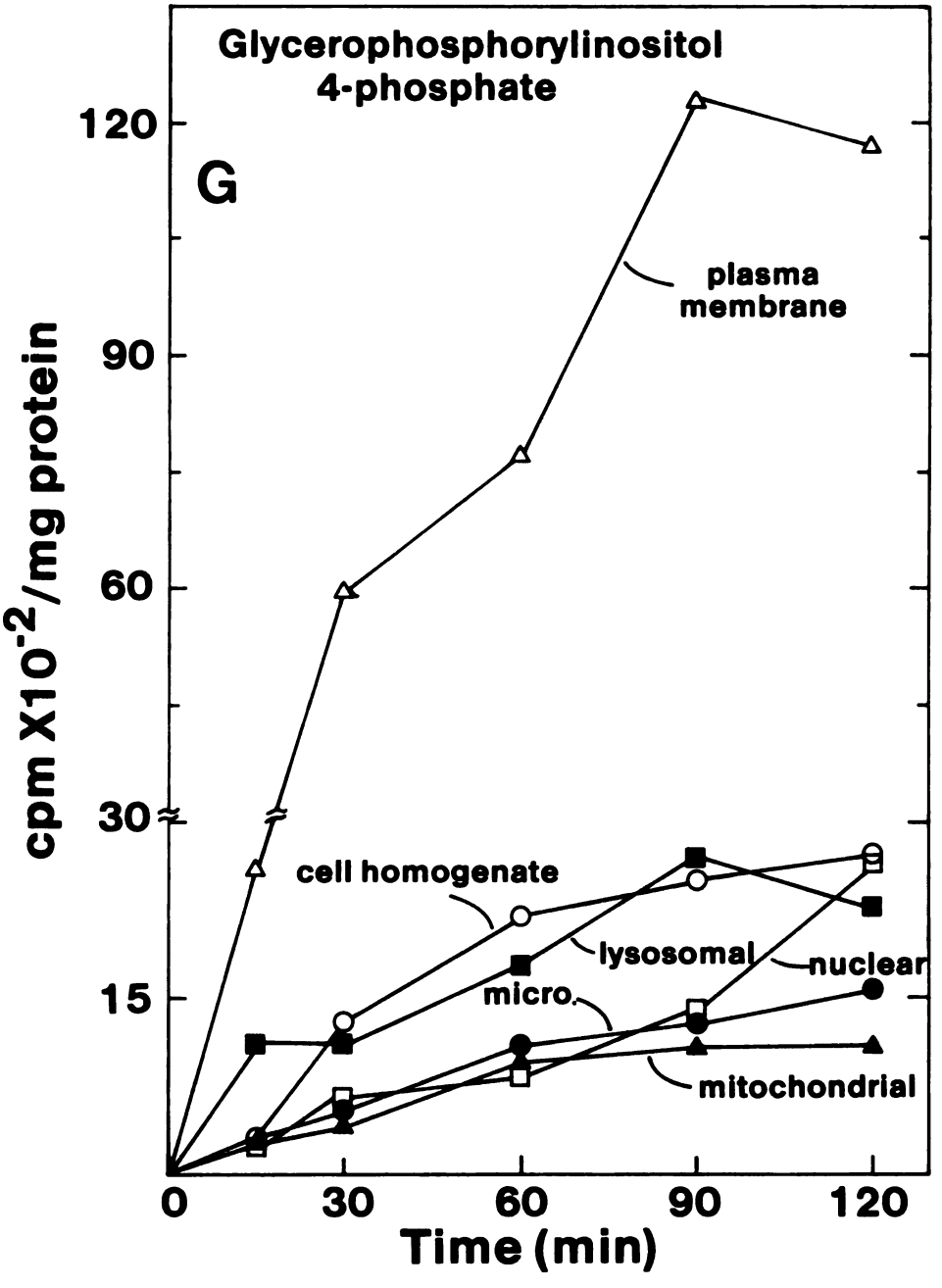

Glycerophosphorylinositol
4-phosphate
G
120
90
60
30
15
cpm X10-2/mg protein
plasma
membrane
cell homogenate
lysosomal
nuclear
micro.
mitochondrial
0
30
60
90
120
Time (min)

Figure 3G

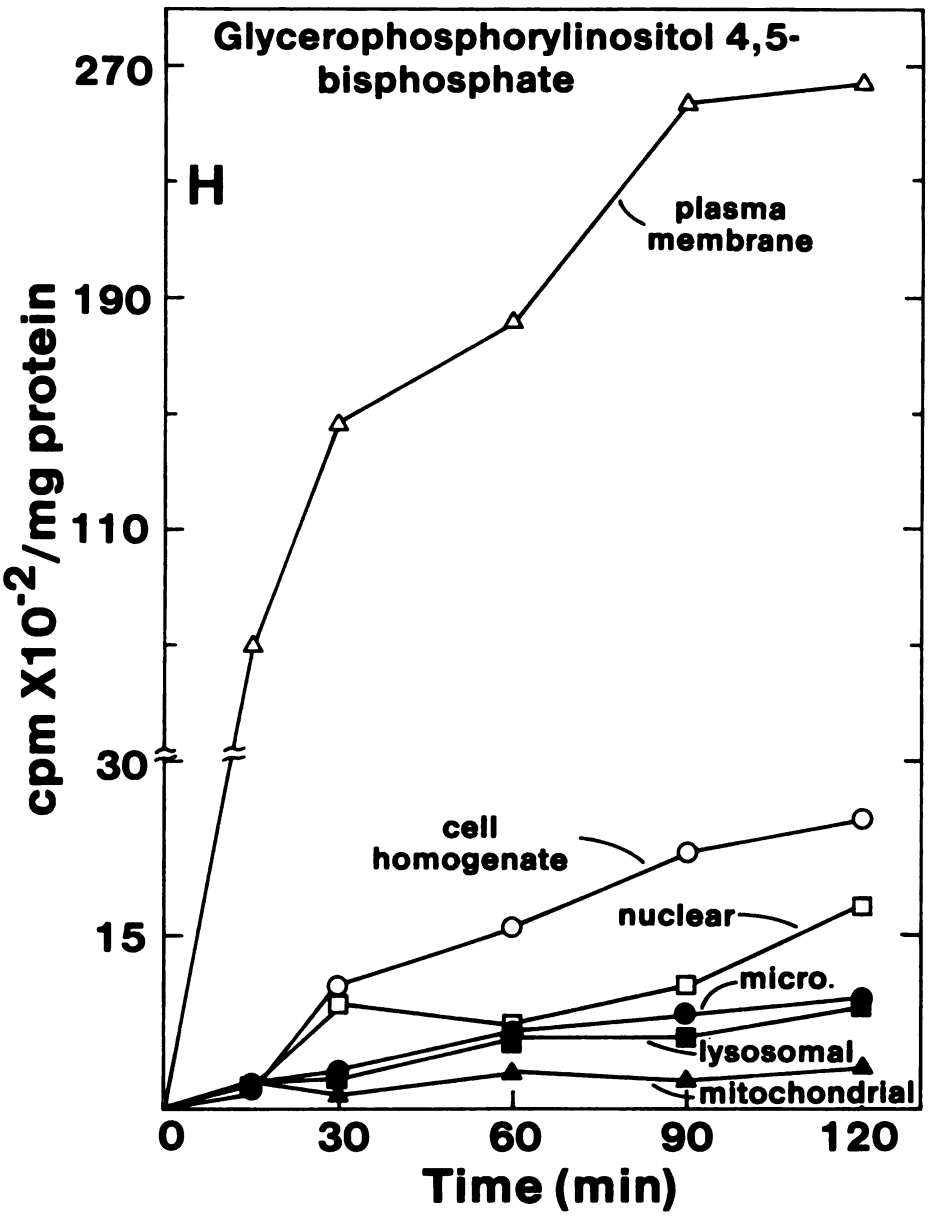

Glycerophosphorylinositol 4,5-bisphosphate
H
270
190
110
30
15
cpm X10-2/mg protein
plasma membrane
cell homogenate
nuclear
micro.
lysosomal
mitochondrial
0
30
60
90
120
Time (min)

Figure 3H

with ^{32}Pi and only slightly less than the amount of ^{32}P incorporated into PA in the plasma membrane fraction.

Analysis of cell homogenate ^{32}P-labeled phospholipids illustrates that after 120 min of ^{32}Pi labeling, the rates of ^{32}P incorporation into PC, PE, PI, PG, and PS were still increasing. However, the incorporation of ^{32}P into PA, DPI and TPI approached a steady state, reflecting the specific radioactivity of [γ-^{32}P]-ATP which reached steady state after 60-90 min of ^{32}Pi labeling.

Table II illustrates the high level of ^{32}P per mg protein in DPI (GPIP) and TPI ($GPIP_2$) in the plasma membrane compared with the other organelles after 90 min. The lysosomal fraction had 20% while the other fractions had approximately 10% of the amount of ^{32}P in DPI in the plasma membrane fraction. The amount of ^{32}P-labeled TPI in the nuclear, lysosomal, mitochondrial and microsomal fraction was only 1-4% of the amount of ^{32}P-labeled TPI isolated from the plasma membrane fraction.

Attempts to chemically measure the mass of GPIP and $GPIP_2$ by phosphate analysis were unsuccessful because of the low quantity of DPI and TPI in the various subcellular fractions.

Table II

Distribution of ^{32}P incorporation into DPI and TPI of various subcellular fractions.

Hepatocytes were labelled with $^{32}P_i$ for 90 min and processed as described in Figure 3. Incorporation of ^{32}P into DPI (GPIP) and TPI ($GPIP_2$) per mg of protein in each subcellular fraction was measured. The data reported are the percentage of ^{32}P incorporation/mg protein in the nuclear, mitochondrial, lysosomal and microsomal fractions compared with the plasma membrane fraction. Values are the mean of three different hepatocyte preparations ± standard deviations.

	Plasma Membrane %	Nuclear %	Mitochondrial %	Lysosomal %	Microsomal %
GPIP	100	10.1 ± 2.3	9.3 ± 0.74	20.1 ± 3.1	10.5 ± 1.2
$GPIP_2$	100	4.1 ± 1.6	1.4 ± 0.31	2.8 ± 0.97	3.3 ± 1.0

DISCUSSION

In this study, we used a rapid subcellular fractionation method to prepare plasma membrane, mitochondrial, lysosomal, microsomal and nuclear fractions that are enriched by at least two-fold based on the fraction's marker enzyme (mitochondrial-fumarase) or as much as 13-fold (lysosomal-hexosaminidase). The yields of organelles, although as low as 4.5% for the lysosomes, were adequate to accurately measure ^{32}P incorporation into various phospholipids. Using Percoll instead of sucrose as the density gradient centrifugation medium, greatly decreased the time required to obtain the mitochondrial, plasma membrane, nuclear and lysosomal fractions. Thus, the procedure allows one to process a number of samples in a relatively short period of time. The enzyme inhibitors present in the homogenizing buffer in combination with decreased temperature and rapid fractionation prevented major changes in ^{32}P-phospholipid levels during fractionation. Changes in ^{32}P-labeled PC, PE, PI and PA occurred in the crude nuclear fraction probably due to the presence of Mg^{2+} in the buffer which is a known cofactor for many of the enzymes involved in the metabolism of these phospholipids (45). Only small changes were observed in ^{32}P-labeled PG, PS, DPI and TPI. Determination of the rate of ^{32}P incorporated into PC, PE, PI and PA must await the development of an alternative procedure to isolate nuclei, for example, by using divalent cations other than Mg^{2+}.

Very little phospholipid exchange occurred between the microsomes and other organelles during the fractionation. The small percentage of radioactivity recovered in the plasma membrane, mitochondrial and lysosomal fractions was probably due to contamination of these fractions with added labeled microsomes.

There are a number of techniques available for the analysis of phosphoinositides. These include chromatography on formaldehyde treated paper (46) or oxalate impregnated silica gels (47), silica gel chromatography using multi-component solvent systems (2,15,41,48,49) and affinity chromatography on immobilized neomycin columns (39). Although polyphophoinositides can be analyzed quite well using these systems, the ability to separate other phospholipids that may be present in the mixture is limited. Quite often it is necessary to use a second or third solvent system to completely analyze all the phospholipids in the mixture (10). Normal pressure anion exchange chromatography was also used to separate deacylated phospholipids, but it was either very time consuming (41) or analysis of only the deacylated phosphoinositides was reported (21,23). The anion exchange HPLC procedure used in this study separated all the major deacylated phospholipids including those of polyphosphoinositides within 70 minutes. Recovery of the deacylated phospholipids from the anion exchange column exceeded 95% and the compounds remained in solution during the separation making it easier to determine radioactivity or phosphate levels as compared to working with silica gel or cellulose scrapings. However, one obvious drawback of the analysis procedure is that it gives no information on the level of lyso-phospholipids present in the mixture but rather analyzes them together with diacyl-phospho-

lipids. In addition, sphingomyelin and alkenoxy and alkoxy ether phospholipids are omitted from analysis.

The rates of synthesis and degradation of phospholipids would influence the amount of ^{32}P incorporation into phospholipids. Synthesis of major phospholipids has been attributed to the microsomal fraction of rat hepatocytes (45,50). Of the major biosynthetic phospholipid enzymes, only glycerol-3-phosphate phosphatidyltransferase in the mitochondria (45) and diglyceride kinase in the cytoplasm (51) are found primarily outside of the endoplasmic reticulum. Phosphatidylinositol kinase is now known to be present in a number of subcellular sites including the plasma membrane (52,53), Golgi (54), lysosomal membranes (55), microsomes (53,56) and nuclear envelopes (53). DPI kinase is associated with nuclear envelopes, plasma membranes and microsomes of rat liver (53) and is a soluble enzyme in brain (57). Various phospholipases are present in virtually all subcellular organelles (58) including a phosphatidylinositol-specific phospholipase C (59) which is predominantly located in the cytosol. Therefore, specific subcellular locations for breakdown of specific phospholipids has not been established. This present study demonstrates that the microsomal fraction has the highest rates of ^{32}P incorporation per mg protein into phospholipids with the exception of PS, DPI and TPI. This finding agrees well with published reports that the endoplasmic reticulum contain the highest level of phospholipid biosynthetic enzymes compared with other subcellular fractions (46). Nuclear envelopes are reported to have a high level of PS biosynthetic enzymes, second only to the endoplasmic reticulum (45), which is in agreement with this study which shows that the nuclear fraction has the highest

rate of ^{32}P incorporation per mg protein into PS compared with to the other subcellular fractions.

The similarity of ^{32}P incorporation rates into the major phospholipids between the microsomal, plasma membrane and lysosomal fractions (Fig. 3) may be due to similar turnover rates or possibly due to contamination of the lysosomal and plasma membrane fractions with microsomes. However, the two-fold higher ^{32}P incorporation rate into PE seen in the microsomal fraction compared with the plasma membrane or lysosomal fractions would argue against contamination causing the similar incorporation rates between the fractions. Therefore, the presence of ^{32}P-labeled phospholipids in the plasma membrane and lysosomal fractions in the absence of their synthetic enzymes is probably due to breakdown of existing phospholipids and rapid translocation of newly synthesized phospholipids from the microsomes to the other organelles via transport proteins (60) or some other process (61,62) such as membrane flow in the case of plasma membranes (63). The lower rates of ^{32}P incorporation into the major phospholipids in the mitochondrial fraction compared with the microsomal fraction is probably due to slower turnover of the mitochondrial phospholipids. Bygrave (64) reported that microsomal phosphatidylcholine turns over at twice the rate of mitochondrial phosphatidylcholine.

Despite the multiple sites within the cell where PI kinase and DPI kinase have been reported (52-56), this study indicates that the plasma membrane has the fastest rate of ^{32}P incorporation into polyphosphoinositides per mg protein of all the subcellular fractions analyzed. The rate of ^{32}P incorporation per mg protein was 5-10 and 25-50

times faster into DPI and TPI, respectively, in the plasma membrane fraction compared with the other subcellular fractions. This would suggest that an active phospholipase C or phosphomonoesterase which acts on polyphosphoinositides is present in the plasma membrane of rat hepatocytes. Such enzymes are present in erythrocyte membranes (65,66). The very low amounts of ^{32}P incorporated into TPI in other subcellular fractions compared with the plasma membrane fractions may reflect the plasma membrane contamination in these fractons which ranged from 2-9% (Table I). It is interesting to note that although plasma membranes composed 9% of the microsomal fraction as determined by alkaline phosphodiesterase I activity (Table I), ^{32}P incorporation into TPI in the microsomal fraction is only 3% of that seen in the plasma membrane fraction (Table II). This suggests that turnover of TPI may occur in a specific region of the plasma membrane which preferentially pellets under the influence of lower g-forces. Contamination by plasma membranes cannot account for the ^{32}P incorporation into DPI in the other subcellular fractions suggesting that DPI turnover may occur in parts of the cell other than the plasma membrane. Of particular interest is the lysosome where the rate of ^{32}P incorporation is only 20% of the plasma membrane rate but nearly 2-fold greater than the rate seen in other subcellular fractions.

Although the amount of DPI and TPI present in various subcellular fractions is still unknown, this study provides evidence that the plasma membrane may contain the most metabolically active pool of DPI and TPI. Published reports which describe hormonal effects on ^{32}P-TPI metabolism in the whole hepatocyte (18-23), therefore,

closely reflect changes in plasma membrane ^{32}P-TPI levels. However, analysis of ^{32}P-DPI metabolism in the whole hepatocyte may not accurately reflect changes in ^{32}P-DPI levels in the plasma membrane because of previously unaccounted contribution of ^{32}P-DPI from other subcellular organelles especially lysosomes.

Acknowledgements - We wish to acknowledge the technical assistance of Lynn Farrell, and Carol Fenn and Theresa Fillwock for preparation of the manuscript.

REFERENCES

1. Hokin, M.R., and Hokin, L.E. (1953) J. Biol. Chem. 209, 549-558.
2. Benabe, J.E., Spry, L.A., and Morrison, A.K. (1982) J. Biol. Chem. 257, 7430-7434.
3. Meltzer, V., Weinseb, S., Bellorin-Font, E., and Hruska, K.A. (1982) Biochem. et Biophys. Acta. 712, 258-267.
4. Akhtar, R.A., and Abdel-Latif, A.A. (1978) J. Pharmacol. Exp. Ther. 204, 655-668.
5. Akhtar, R.A., and Abdel-Latif, A.A. (1980) Biochem. J. 192, 783-791.
6. Halenda, S.P., and Rubin, K.P. (1982) Biochem. J. 208, 713-721.
7. Putney, J.W., Burgess, G.M., Halenda, S.P., McKinney, J.S., and Rubin, R.P. (1983) Biochem. J. 212, 483-488.
8. Weiss, S.J., and Putney, J.W. (1981) Biochem. J. 194, 463-468.
9. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvie, R.F. (1983) Biochem. J. 212, 473-482.
10. Farese, R.V., Saber, M.A., and Vandor, S.L. (1979) J. Biol. Chem. 254, 6842-6844.
11. Farese, R.V., Larson, R.E., and Saber, M.A. (1982) J. Biol. Chem. 257, 4042-4045.
12. Honeyman, T.W., Strohsnitter, W., Scheid, C.R., and Schimmel, R.J. (1983) Biochem. J. 212, 489-498.

13. Billah, M.M., and Lapetina, E.G. (1982) J. Biol Chem. 257, 12705-12708.

14. Neufield, E.J., and Majerus, P.W. (1983) J. Biol. Chem. 258, 2461-2467.

15. Jolles, J., Zwiers, H., Dekker, A., Wirtz, K.W.A., and Gispen, W.H. (1981) Biochem. J. 194, 283-291.

16. Fisher, S.K., and Agranoff, B.W. (1981) J. Neurochem. 37, 968-977.

17. Berridge, M.J. (1983) Biochem. J. 212, 849-858.

18. Tolbert, M.E.M., White, A.C., Aspry, K., Cutts, J., and Fain, J.N. (1980) J. Biol. Chem. 255, 1938-1944.

19. Billah, M.M., and Michell, R.H. (1978) Biochem. J. 182, 661-668.

20. Kirk, C.J., Michell, R.H., and Hems, D.A. (1981) Biochem. J. 194, 155-165.

21. Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H., and Kirk, C.J. (1983) Biochem. J. 212, 733-747.

22. Shukla, S.D., Buxton, D.B., Olson, M.S., and Hanahan, D.J. (1983) J. Biol. Chem. 258, 10212-10214.

23. Rhodes, D., Prpic, V., Exton, J.H., and Blackmore, P.F. (1983) J. Biol. Chem. 258, 2770-2773.

24. Lin, S.H., and Fain, J.W. (1981) Life Sci. 29, 1905-1912.

25. Geelen, M.J.H., Groener, J.E.M., De Haas, C.G.M., Wisserhof, T.A., and Van Golde, L.M.G. (1978) FEBS Lett. 90, 57-60.

26. Geelen, M.J.H., Groener, J.E.M., De Haas, C.G.M., and Van Golde, L.M.G. (1979) FEBS Lett. 105, 27-30.

27. Pelech, S.L., Pritchard, P.H., and Vance, D.E. (1982) Biochim. Biophys. Acta 713, 260-269.

28. Alemany, S., Varela, I., and Mato, J.M. (1982) Biochem. J. 208, 453-457.

29. Seglen, P.O. (1976) Methods Cell Biol. 13, 29-83.

30. Kurtz, J.W., and Wells, W.W. (1981) J. Biol. Chem. 256, 10870-10875.

31. Sellinger, O.Z., Beaufay, H., Jacques, P., Doyen, A., and DeDuve, C. (1960) Biochem. J. 74, 450-456.

32. Hill, R.L., and Bradshaw, R.A. (1969) Methods Enzymol. 13, 96-99.

33. Aronson, N.N., Jr., and Touster, O. (1974) Methods Enzymol. 31, 90-102.

34. DeDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmens, F. (1955) Biochem. J. 60, 604-617.

35. Brightwell, R., and Tappel, A.L. (1968) Arch. Biochem. Biophys. 124, 333-343.

36. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S., and DeDuve, C. (1968) J. Cell Biol. 37, 482-512.

37. Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220.

38. Karsten, U., and Wollenberger, A. (1977) Anal. Biochem. 77, 464-470.

39. Schacht, J. (1981) Methods Enzymol. 72, 626-631.

40. Kates, M. (1972) in Techniques of Lipidology: Isolation, Analysis and Identification of Lipids pp. 558-559. North-Holland Publishing Co., Amsterdam.

41. Dittmer, J.C., and Wells, M.A. (1969) Methods Enzymol. 14, 519-523.

42. Ames, B.N. (1966) Methods Enzymol. 8, 115-118.

43. Hawkins, P.T., Michell, R.H., and Kirk, C.J. (1983) Biochem. J. 210, 717-720.

44. Tata, J.R. (1974) Methods Enzymol. 31, 253-262.

45. Jelsema, C.L., and Morre, D.J. (1978) J. Biol. Chem. 253, 7960-7971.

46. Wagner, H., Lissau, Ä., Holzl, J., and Horhammer, L. (1962) J. Lipid Res. 3, 177-180.

47. Gonzalez-Sastre, F., and Folch-Pi, J. (1968) J. Lipid Res. 9, 532-533.

48. Hauser, G., Eichberg, J., and Gonzalez-Satre, F. (1971) Biochim. Biophys. Acta. 248, 87-95.

49. Volpi, M., Yassin, R., Naccache, P.H., and Sha'afi, R.I. (1983) Biochem. Biophys. Res. Commun. 112, 957-964.

50. McMurray, W.C., and Magee, W.L. (1972) Annu. Rev. Biochem. 41, 129-160.

51. Kanoh, H., and Akesson, B. (1978) Eur. J. Biochem. 85, 225-232.

52. Harwood, J.J., and Hawthorne, J.N. (1969) Biochim. Biophys. Acta 171, 75-88.

53. Smith, C.D., and Wells, W.W. (1983) J. Biol. Chem. 258, 9368-9373.

54. Jergil, B., and Sundler, R. (1983) J. Biol. Chem. 258, 7968-7973.

55. Collins, C.A., and Wells, W.W. (1983) J. Biol. Chem. 258, 2130-2134.

56. Behar-Bannelier, M., and Murray, R.K. (1980) Biochem. J. 187, 147-156.

57. Kai, M., Salway, J.G., and Hawthorne, J.N. (1968) Biochem. J. 106, 791-801.

58. van den Bosch, H. (1974) Annu. Rev. Biochem. 43, 243-277.

59. Takenawa, T., and Nagai, Y. (1981) J. Biol. Chem. 256, 6769-6775.

60. Wirtz, K.W.A. (1974) Biochim. Biophys. Acta. 344, 95-117.

61. Yatke, M.P., and Kennedy, E.P. (1983) Biochemistry 22, 1497-1507.

62. Sleight, R.G., and Pagano, R.E. (1983) J. Biol. Chem. 258, 9050-9058.

63. Morre', D.J. (1977) Cell Surface Rev. 4, 1-83.

64. Bygrave, F.L. (1969) J. Biol. Chem. 244, 4768-4772.

65. Downes, C.P., and Michell, R.H. (1981) Biochem. J. 198, 133-140.

66. Koutouzov, S., and Marche, P. (1982) FEBS Lett. 144, 16-20.

CHAPTER II

Subcellular Site and Mechanism of Vasopressin Stimulated Hydrolysis of Phosphoinositides in Rat Hepatocytes

Abstract

The intracellular site of vasopressin induced phosphoinositide breakdown in rat hepatocytes was investigated. After 45 sec of vasopressin treatment of hepatocytes prelabeled with $^{32}P_i$, the levels of ^{32}P-labeled phosphatidylinositol 4-phosphate (PI-P) and phosphatidylinositol 4,5-bisphosphate (PI-P_2) in the plasma membrane decreased by approximately 40%, then gradually returned to near control levels after 10 min of treatment. Only small changes in the levels of [^{32}P]PI-P and [^{32}P]PI-P_2 were observed in the other subcellular fractions, and were attributed to contamination of these fractions by plasma membranes. The level of ^{32}P-labeled phosphatidylinositol (PI) in the plasma membrane decreased by 15% after 45 sec of vasopressin treatment and then increased above control levels at later times while ^{32}P-labeled phosphatidic acid (PA) levels in the plasma membrane gradually increased to 2-fold greater than control after 5 min of treatment.

Using ^{32}P-labeled plasma membranes obtained from prelabeled hepatocytes, it was found that PI-P and PI-P_2 were rapidly degraded by a calcium-dependent polyphosphoinositide-specific phosphodiesterase. The enzyme was activated by physiological concentrations (200nM) of free calcium when assayed at low ionic strength, but the calcium requirement shifted to micromolar concentrations under iso-osmotic, intracellular-like, ionic conditions. Addition

of vasopressin (200nM) to the [^{32}P]plasma membranes stimulated the breakdown of 20% of the [^{32}P]PI-P_2 present in the plasma membranes in 1 min when assayed under iso-osmotic conditions in the presence of 2mM $MgCl_2$ and approximately 200nM free calcium. This data suggests that the phosphoinositide-specific phosphodiesterase is not active under normal cellular conditions, but is activated upon the addition of vasopressin to the intact cell.

Footnotes

[1]The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PA, phosphatidic acid; PI-P, phosphatidylinositol 4-phosphate; PI-P_2, phosphotidylinositol 4,5-bisphosphate; I-P, inositol phosphate; I-P_2, inositol bisphosphate; I-P_3, inositol trisphosphate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TRICINE, N-tris[hydroxymethyl]methyl glycine; PMSF, phenylmethylsulfonyl flouride; EGTA, ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HPLC, high pressure liquid chromatography.

[2]Seyfred, M.A., and Wells, W.W., manuscript in preparation.

Introduction

In many tissues, there is an association between stimuli induced changes in phosphatidylinositol (PI) metabolism and cellular calcium (1,2). Further investigation has shown that the metabolism of the polyphosphoinositides, phosphatidylinositol 4-phosphate (PI-P) and phosphatidylinositol 4,5-bisphosphate (PI-P_2), is also influenced by the same agents (3-10). In rat hepatocytes, calcium mobilizing hormones such as vasopressin, angiotensin II and epinephrine enhance PI breakdown and subsequent resynthesis with phosphatidic acid as a key intermediate in this process (11-14). These studies have been extended to demonstrate that there is a decrease in ^{32}P-labeled PI-P and PI-P_2 after treatment of prelabeled hepatocytes with calcium mobilizing hormones (15-17).

The correlation between phosphoinositide (PI, PI-P, and PI-P_2) breakdown and increased levels of intracellular calcium has led to speculation that decreases in the plasma membrane concentrations of phosphoinositides could affect calcium channels (1,18, but see 19) or some other calcium transport system such as the plasma membrane (Ca^{2+}-Mg^{2+})-ATPase (20-22). Alternatively, products of the metabolism of phosphoinositides may serve as calcium mobilizing agents. Inositol trisphosphate (I-P_3), which is released by phosphodiesterase action on PI-P_2, has been shown to release calcium from non-mitochondrial intracellular stores in

permeabilized rat pancreatic acinar cells (23).

Despite the proposed important role of phosphoinositide metabolism in calcium homeostasis, the intracellular site of stimulated phosphoinositide metabolism in response to calcium mobilizing hormones in the hepatocyte has not been fully characterized. Increased PI hydrolysis in response to vasopressin has been attributed to plasma membranes (11,24), but the plasma membrane fractions were either highly contaminated with other organelles (11) or the response much slower (24) than observed in intact cells. Additional studies using isolated plasma membrane preparations required very high levels of norepinephrine (25) or very long incubation times (30 min) in the presence of detergent to observe significant PI hydrolysis (26). An attempt to localize the intracellular site of PI-P_2 hydrolysis in response to vasopressin was not successful presumably due to the extreme lability of this inositol lipid (15).

In a previous report (27), we characterized the incorporation of ^{32}P into phospholipids including polyphosphoinositides in plasma membranes, mitochondria, lysosomes and microsomes isolated from prelabeled rat hepatocytes. It was found that the plasma membrane fraction contained the highest level of ^{32}P-labeled PI-P and PI-P_2 per mg protein of all the fractions examined. In the present report, we have extended those studies and have examined the subcellular location of vasopressin induced phosphoinositide breakdown. Also, ^{32}P-labeled plasma

membranes isolated from prelabeled hepatocytes were used to investigate the enzymes involved in the degradation of polyphosphoinositides.

Experimental Procedures

Hepatocyte Incubations - Hepatocytes were isolated from fed male CD-Sprague Dawley rats by a modification of the method described by Seglen (28). The preparation procedures and the incubation conditions were described previously (27). After 90 min of incubation with $^{32}P_i$, the hepatocytes were treated with 200nM vasopressin for 0-10 min. At various times, the hepatocyte suspensions were diluted with 7 volumes of ice cold homogenizing buffer which consisted of 250mM sucrose, 1mM EDTA, 10mM $NaPP_i$, 20mM NaF, 75mg/l PMSF and 10mM TRICINE, pH 8.0.

Subcellular Fractionation of Hepatocytes and Phospholipid Analysis - The hepatocytes were fractionated by differential centrifugation and Percoll density gradient centrifugation into plasma membranes, mitochondria, lysosomes and microsomes as previously described (27).

The phospholipids in the various fractions were isolated by extraction with acidic chloroform:methanol as described by Schacht (29). The phospholipids were deacylated using the mild alkaline conditions reported by Kates (30) except that two 10 min incubations of the phospholipid fraction with 0.1N NaOH at room temperature were used instead of one 15 min incubation. This led to more consistant and increased recovery of glycerophosphorylinositol 4-phosphate and glycerophosphorylinositol 4,5-bisphosphate without risking further breakdown of the

more alkali-labile glycerophosphorylcholine and glycerophosphorylinositol. The glycerophosphate esters were separated by high pressure liquid chromatography (HPLC) on a Bio-Rad Aminex A-27 anion exchange column as previously described (27) using a polyphasic gradient beginning with 20mM ammonium borate, 100mM ammonium formate, pH 9.5 and ending with 20mM ammonium borate, 850mM ammonium formate, pH 9.5. The HPLC system was automated using an ISCO ISIS autosampler and an ISCO Foxy fraction collector.

Phospholipid Degradation Studies Using ^{32}P-Labeled Plasma Membranes - ^{32}P-labeled plasma membranes were isolated in the presence of 1mM EDTA and in the absence of 20mM NaF from hepatocytes that had been incubated for 90 min with $^{32}P_i$ as previously described (27). The omission of NaF had no effect on the level of any of the ^{32}P-labeled phospholipids (data not shown). The final pellet was resuspended at a concentration of 1-2 mg protein/ml using 10 strokes of a Teflon-glass Potter-Elvehjem homogenizer in either 20mM Na-HEPES, 0.5mM Na-EGTA, pH 7.4 or 120mM KCl, 20mM Na-HEPES, 0.5mM Na-EGTA, pH 7.4. Typically, 75 ul of plasma membrane suspension was added to 425 ul of assay mix containing 20mM Na or K-HEPES, 0.5mM Na or K-EGTA, pH 7.4 plus other additions which had been preincubated at 37^{o}C for at least 15 min. The suspensions were vortexed and incubated at 37^{o}C in a Dubnoff water bath shaking at 60 oscillations per min. The reactions were stopped by the addition of 1.5 ml chloroform:methanol (1:2).

The phospholipids were isolated, deacylated and analyzed as described above. In addition, the methanol:HCl phases from the phospholipid extractions were saved and evaporated under a N_2 stream at 37°C. The residues were dissolved in 3 ml of water and neutralized using 2-3 ul of conc. NH_4OH. The solutions were loaded on anion exchange columns containing 0.5 ml of Dowex 1 (x8; 200-400 mesh; formate form) in Pasteur pipets. The columns were washed with 10 ml of 60mM ammonium formate and the radioactive phosphate esters were then sequentially eluted and analyzed as described by Downes and Michell (31).

Other Methods - The specific radioactivity of [γ-^{32}P]ATP was determined by the method described by Hawkins et al. (32). Protein was determined by fluorescamine (33) using bovine serum albumin as the standard. ^{32}P radioactivity was determined by Cerenkov radiation. Free calcium levels were established with a calcium-EGTA buffer system using $K_a=3.7x10^7\ M^{-1}$ to calculate the concentration of free calcium (34). Statistical significance was determined by Student's t-test.

Materials - [Arg]Vasopressin (grade VI) and Dowex 1 (x8; 200-400 mesh; Cl^-form) were obtained from Sigma. The sources of all other reagents and materials were described previously (27).

Results

Effect of Vasopressin Treatment of Rat Hepatocytes on ^{32}P-Labeled Phospholipids Isolated from Various Subcellular Organelles - It was previously shown (27) that after 90 min of incubation with $^{32}P_i$, hepatic intracellular [γ-^{32}P]ATP reached isotopic equilibrium. ^{32}P-labeled PA, PI-P and PI-P_2 in all subcellular fractions analyzed were also at isotopic equilibrium, but the ^{32}P incorporation rate into all other phospholipids was still increasing. Therefore, changes in [^{32}P]PA, PI-P or PI-P_2 represents changes in the total mass of the phospholipid. To investigate the effect of vasopressin treatment of rat hepatocytes on the phospholipid levels of various subcellular fractions, hepatocytes were incubated with $^{32}P_i$ for 90 min, and then treated with 200nM vasopressin for various times up to 10 min. The cells were fractionated into plasma membranes, mitochondria, lysosomes and microsomes and the ^{32}P-phospholipid levels in these fractions determined as described in "Experimental Procedures". The results are shown in Figures 1A-E. The ^{32}P-phosphatidylcholine (PC) levels in all the various subcellular fractions except mitochondria increased significantly after 2 min of vasopressin treatment, but returned to near control levels after 10 min (Fig. 1A). Similiar results were also seen with ^{32}P-labeled phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (data not shown). In contrast,

Figure 1. Effect of Vasopressin Treatment of Rat Hepatocytes on ^{32}P-Labeled Phospholipids Isolated from Various Subcellular Fractions. Hepatocytes were incubated with $^{32}P_i$ for 90 min and then treated with vasopressin for an additional 0-10 min. Partially purified plasma membranes, mitochondria, lysosomes and microsomes were obtained and the levels of ^{32}P-phospholipids determined as described in "Experimental Procedures". A. Phosphatidyl-choline; B. Phosphatidylinositol; C. Phosphatidic acid; D. Phosphatidylinositol 4-phosphate; E. Phosphatidylinositol 4,5-bisphosphate. ●, Cell homogenates; ■, Plasma membranes; ▲, Mitochondria; ○, Lysosomes; □, Microsomes. The results shown are the mean percentage changes versus untreated samples obtained from three or four different hepatocyte preparations. *, $p < 0.10$; **, $p<0.05$; ***, $p<0.01$.

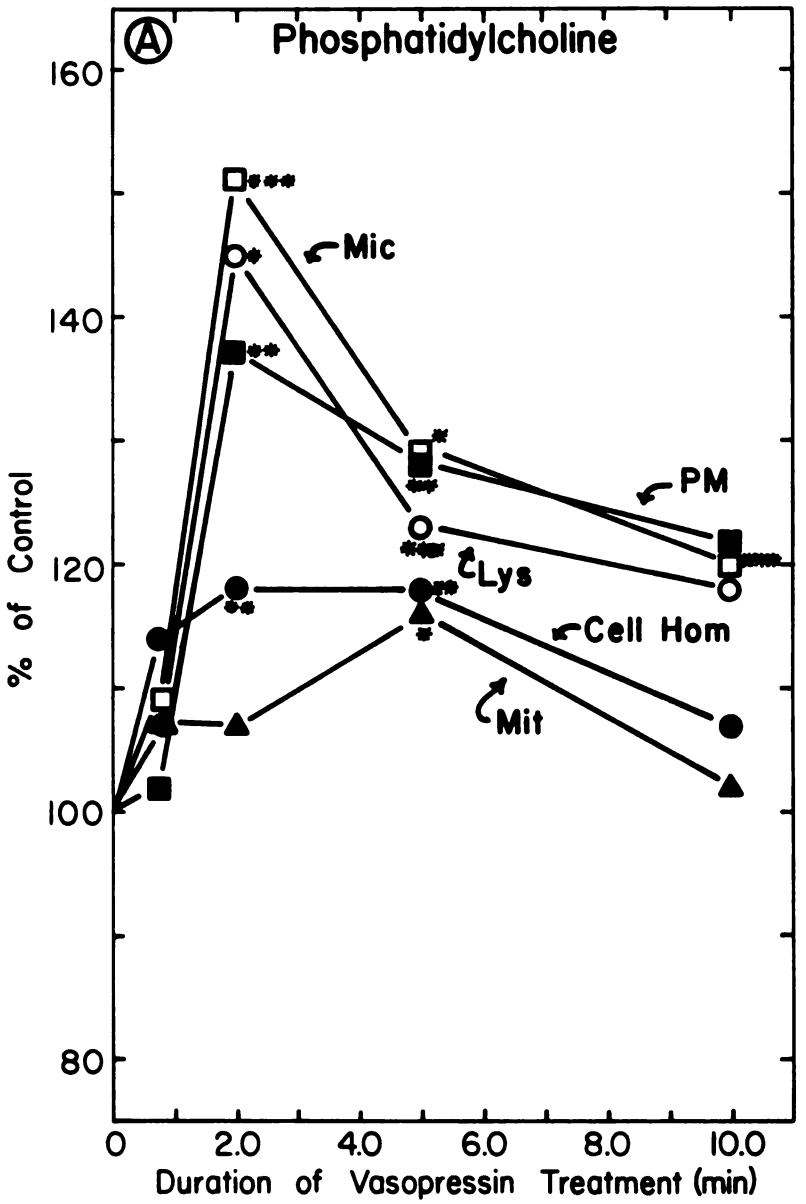

Ⓐ Phosphatidylcholine
160
140
120
100
80
% of Control
Mic
PM
Lys
Cell Hom
Mit
0
2.0
4.0
6.0
8.0
10.0
Duration of Vasopressin Treatment (min)

Figure 1A

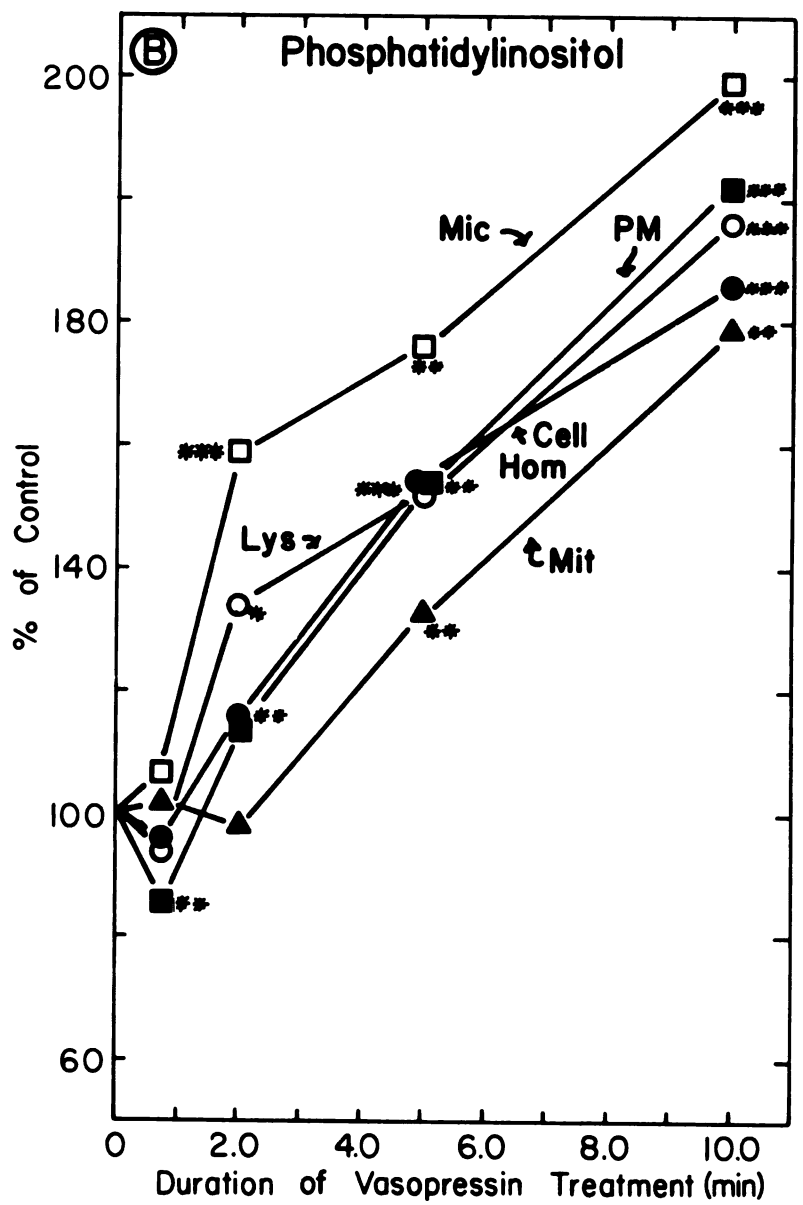

B
Phosphatidylinositol
200
180
140
100
60
% of Control
Mic
PM
Cell
Hom
Lys
Mit
0
2.0
4.0
6.0
8.0
10.0
Duration of Vasopressin Treatment (min)

Figure 1B

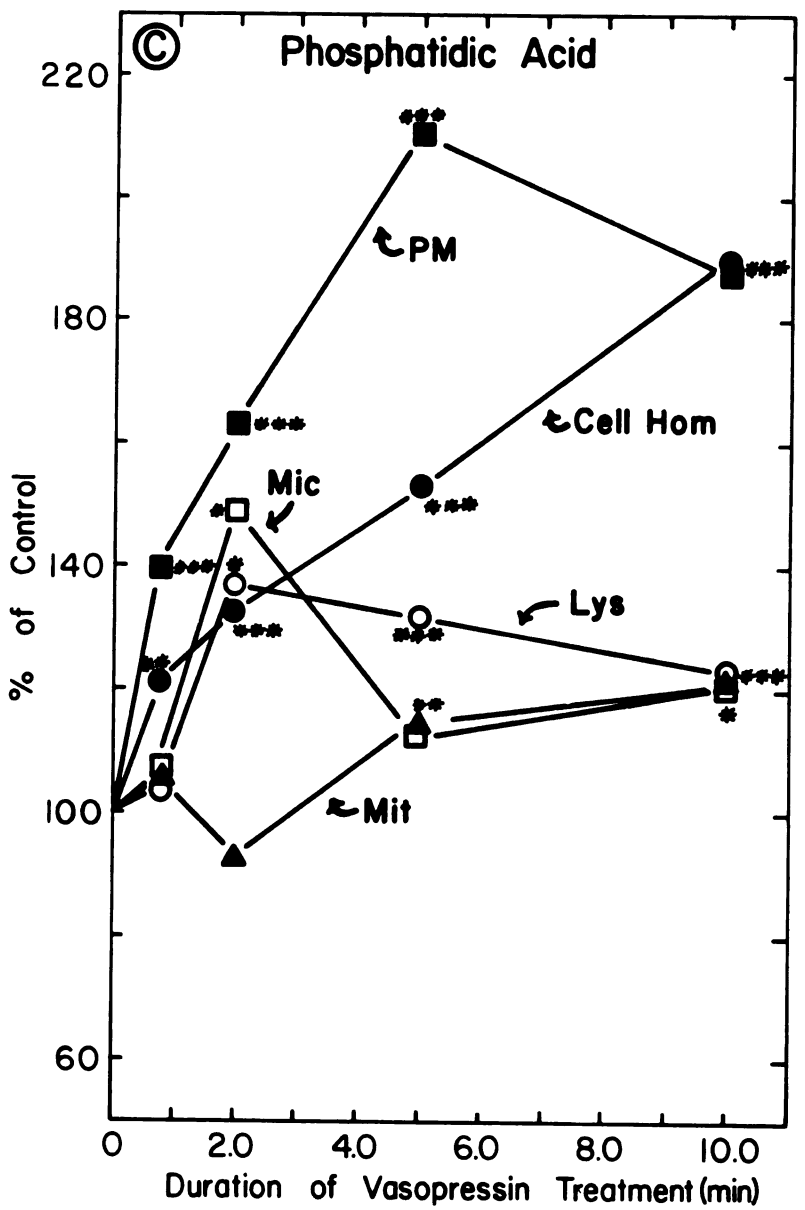

©
Phosphatidic Acid
220
180
140
100
60
% of Control
PM
Cell Hom
Mic
Lys
Mit
0
2.0
4.0
6.0
8.0
10.0
Duration of Vasopressin Treatment (min)

Figure 1C

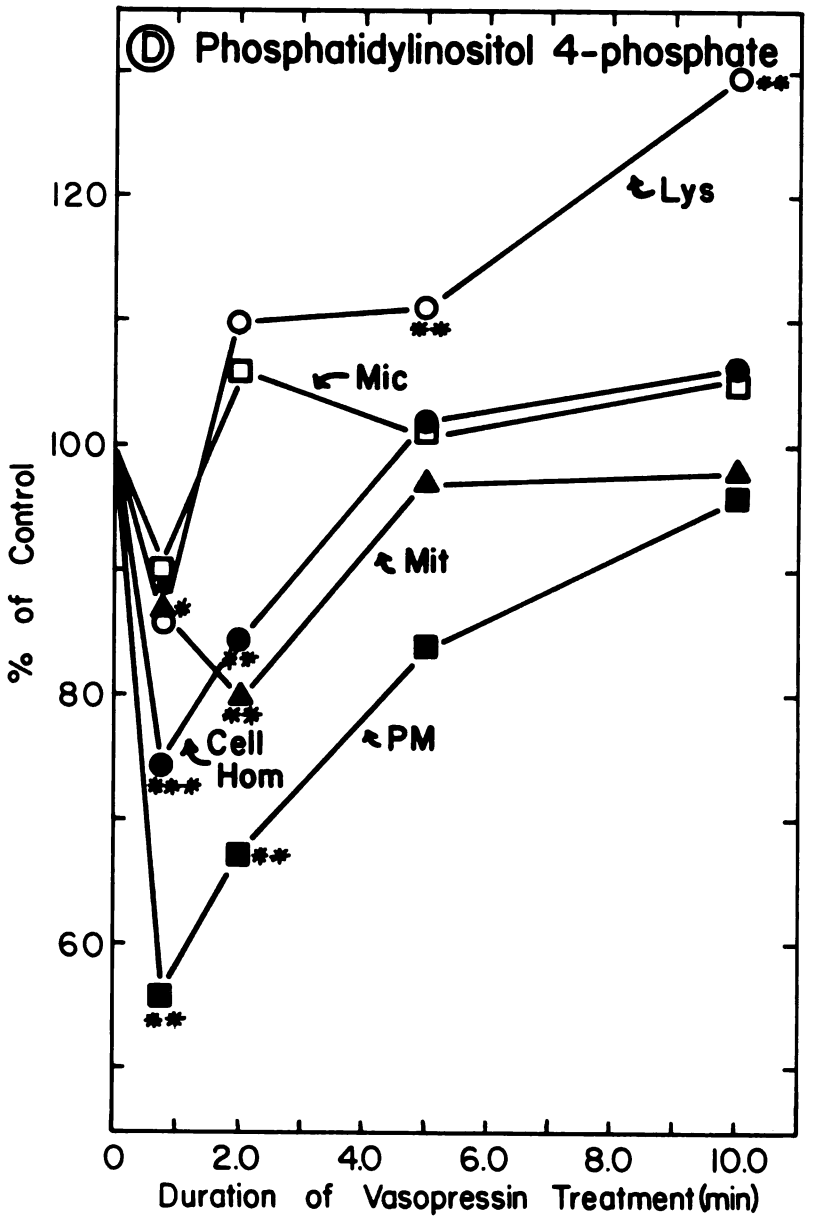

Ⓓ Phosphatidylinositol 4-phosphate
120
100
80
60
% of Control
Lys
Mic
Mit
PM
Cell
Hom
0
2.0
4.0
6.0
8.0
10.0
Duration of Vasopressin Treatment (min)

Figure 1D

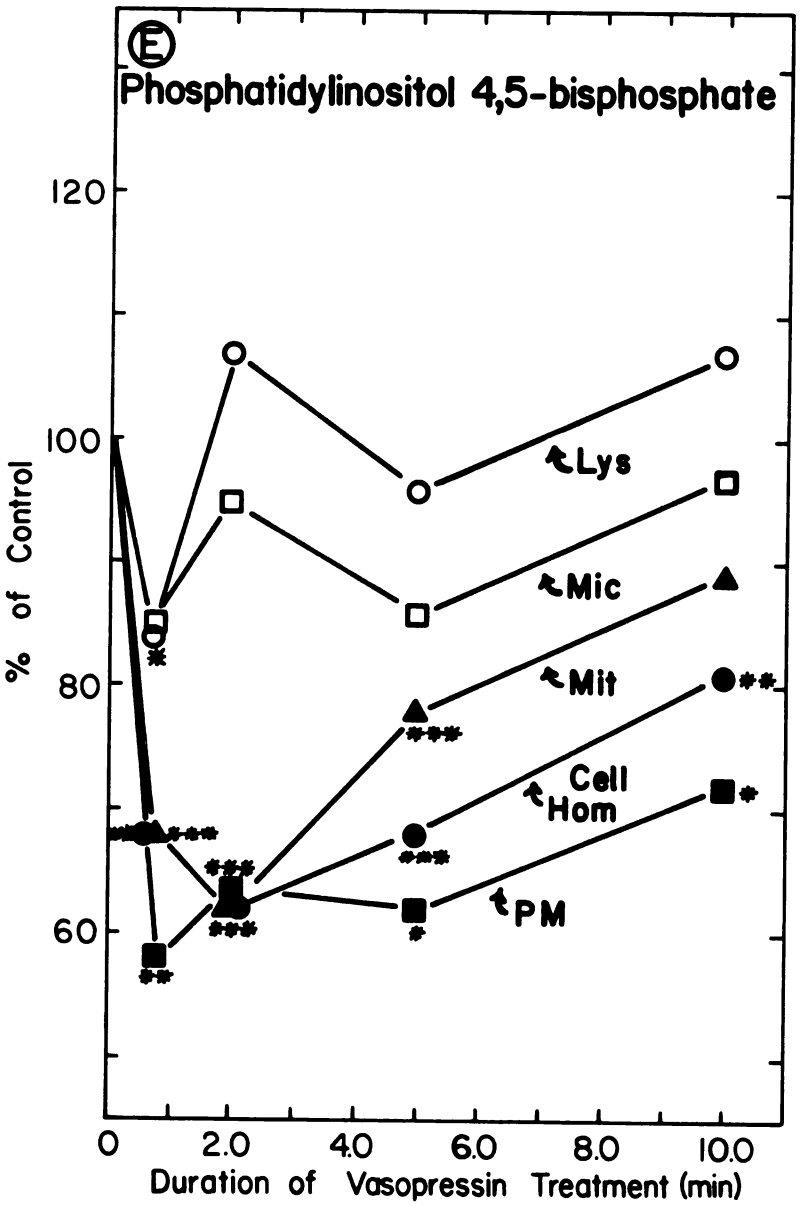

E
Phosphatidylinositol 4,5-bisphosphate
120
100
80
60
% of Control
Lys
Mic
Mit
Cell Hom
PM
0
2.0
4.0
6.0
8.0
10.0
Duration of Vasopressin Treatment (min)

Figure 1E

the ^{32}P-labeled PI levels decreased 15% ($p<0.05$) in the plasma membrane fraction after 45 sec of vasopressin treatment (Fig. 1B). No significant changes in [^{32}P]PI levels were observed in any other subcellular fraction after 45 sec of vasopressin treatment, however, longer treatment times led to an increase in [^{32}P]PI levels above control in all fractions analyzed. The amount of ^{32}P-labeled PA in the plasma membrane fraction increased 40% after only 45 sec of vasopressin treatment and continued to increase to 210% of control levels after 5 min of treatment (Fig 1C). The levels of [^{32}P]PA were also elevated in the cell homogenate and other subcellular fractions in response to vasopressin, but the increases were much smaller and the response much slower than observed in the plasma membrane fraction.

The amount of ^{32}P-labeled PI-P in the plasma membrane fraction decreased by 44% after 45 sec of incubation with vasopressin, but returned to near control levels after 10 min of treatment (Fig 1D). A similar response was seen in the cell homogenate, but the decrease in [^{32}P]PI-P was only approximately one-half as great as seen in the plasma membrane fraction. A significant decrease in [^{32}P]PI-P levels was also observed in the mitochondrial fraction in response to vasopressin, but the response was not maximal until after 2 min of treatment and the decrease was only 20%. This response may simply be a reflection of the plasma membrane contamination of the mitochondrial fraction since the amount of [^{32}P]PI-P per mg of protein in mitochondria

was approximately 10% of the [^{32}P]PI-P level in plasma membranes and plasma membranes make up approximately 2% of the mitochondrial fraction protein (27).

Analysis of the ^{32}P-labeled PI-P_2 levels in the various subcellular fractions showed that they decreased after 45 sec of vasopressin treatment in the plasma membrane, mitochondrial and lysosomal fraction as well as in the cell homogenate (Fig. 1E). The decrease in the amount of [^{32}P]PI-P_2 ranged from 42% in the plasma membrane to only 16% in the lysosomal fraction. After 10 min of vasopressin treatment, the level of [^{32}P]PI-P_2 in plasma membranes remained depressed by 22% compared with control level while [^{32}P]PI-P_2 levels in the other subcellular fractions were not significantly different from control levels. As with [^{32}P]PI-P, the vasopressin stimulated decrease in [^{32}P]PI-P_2 levels observed in the other subcellular fractions may simply be due to plasma membrane contamination of the other fractions. The plasma membrane contamination ranged from 2-8% of the other fractions protein and the amount of [^{32}P]PI-P_2 per mg of protein found in the other fractions was only 1-4% of the level observed in the plasma membrane fraction (27).

There was no change in the cellular [γ-^{32}P]ATP specific radioactivity during the course of vasopressin treatment in three different hepatocyte preparations (data not shown).

Time Course of the Degradation of ^{32}P-Labeled Plasma Membrane Phospholipids: Effect of Calcium - Studies with intact hepatocytes indicated that the plasma membrane was the site of vasopressin stimulated hydrolysis of phosphoinositides. To further characterize phosphoinositide breakdown in plasma membranes, assays using ^{32}P-labeled plasma membranes prepared from hepatocytes which were incubated with $^{32}P_i$ were used. Figure 2A describes the time course of the breakdown of ^{32}P-labeled PA, PI, PI-P, and PI-P_2 in the presence or absence of calcium at low ionic strength (20mM Na-HEPES, 0.5mM Na-EGTA, pH 7.4). No significant change in the level of [^{32}P]PI was observed over the 300 sec incubation time in the presence or absence of calcium. The levels of ^{32}P-labeled PC, PE, and PG also did not change. The amount of [^{32}P]PA linearly decreased, independent of calcium, by 13% within 120 sec. In contrast, hydrolysis of [^{32}P]PI-P and PI-P_2 was stimulated approximately 10-fold in the presence of 110uM free calcium after 1 min of incubation. Very rapid rates of hydrolysis of [^{32}P]PI-P and PI-P_2 occurred in the first 30 sec of incubation, and within 100 sec, 50% of the amount of [^{32}P]PI-P and PI-P_2 present in the plasma membrane fraction was hydrolyzed in the presence of 110uM free calcium. In the absence of calcium, only 5-10% of the [^{32}P]PI-P and PI-P_2 was broken down within the first 120 sec of incubation, but increased to approximately 40% after 300 sec of incubation at 37^{o}C.

Figure 2. Time Course of the Degradation of ^{32}P-Labeled Plasma Membrane Phospholipids: Effect of Calcium. ^{32}P-labeled plasma membranes were prepared from prelabeled hepatocytes and incubated at 37°C for various times in the presence or absence of 110uM free calcium at low ionic strength. The levels of ^{32}P-labeled PI, PA, PI-P, $PI-P_2$, I-P, $I-P_2$, $I-P_3$ and $^{32}P_i$ were determined as described in "Experimental Procedures". A. ^{32}P-labeled phospholipids- 0,● PI; □,■ PA;△,▲ PI-P; ▽,▼ $PI-P_2$. B. ^{32}P-labeled inositol phosphates and $^{32}P_i$- 0,● I-P and/or P_i; □,■ $I-P_2$; △,▲ $I-P_3$. Open symbols- absence of calcium; closed symbols- presence of 110uM free calcium. Typical levels of ^{32}P-labeled compounds (in c.p.m.) obtained at t=0: 1362, PI; 1453, PA; 929, PI-P; 4008, $PI-P_2$; 11,160, I-P and/or P_i; 1287, $I-P_2$; 1961, $I-P_3$. The results shown are the mean percentage changes of duplicate samples from two or three different hepatocyte preparations. S.E.M. ranged from 5-15%.

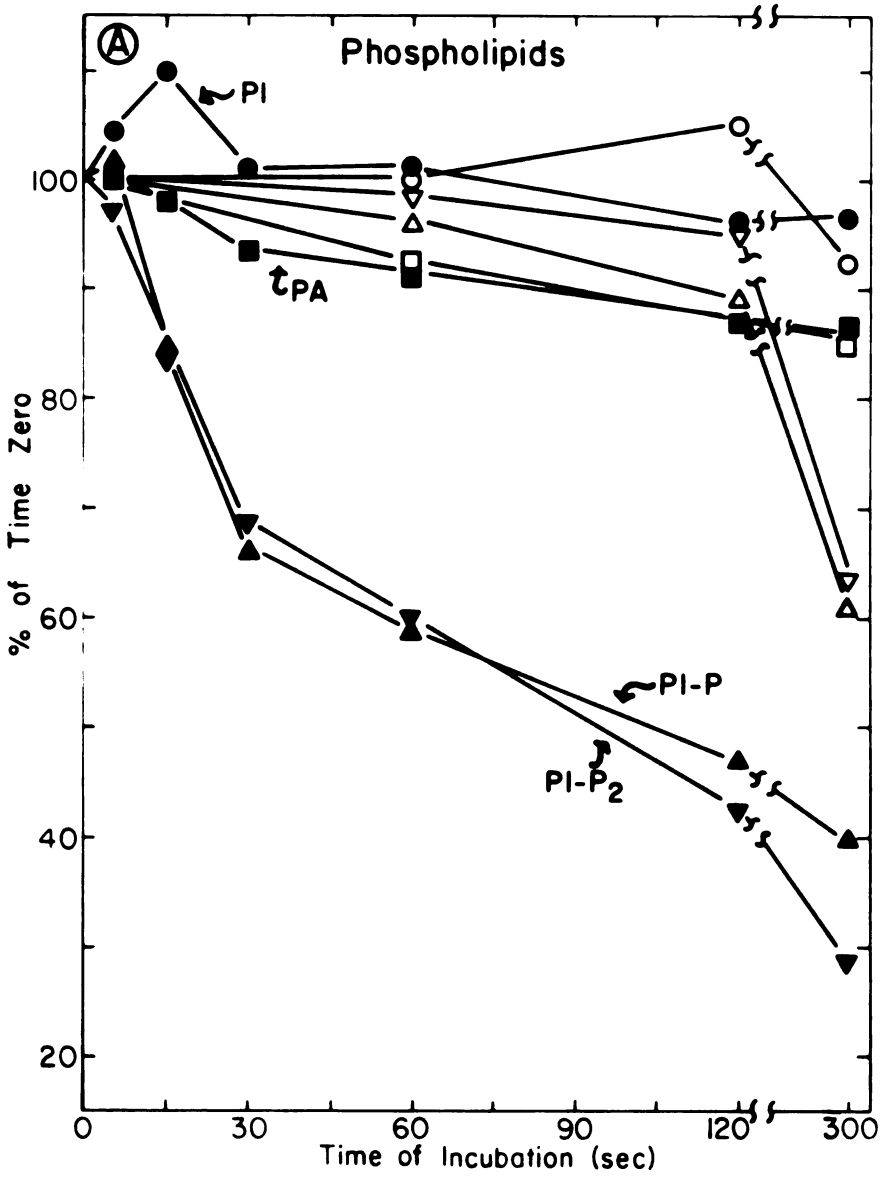

Ⓐ
Phospholipids
PI
100
80
60
40
20
% of Time Zero
tPA
PI-P
PI-P2
0
30
60
90
120
300
Time of Incubation (sec)

Figure 2A

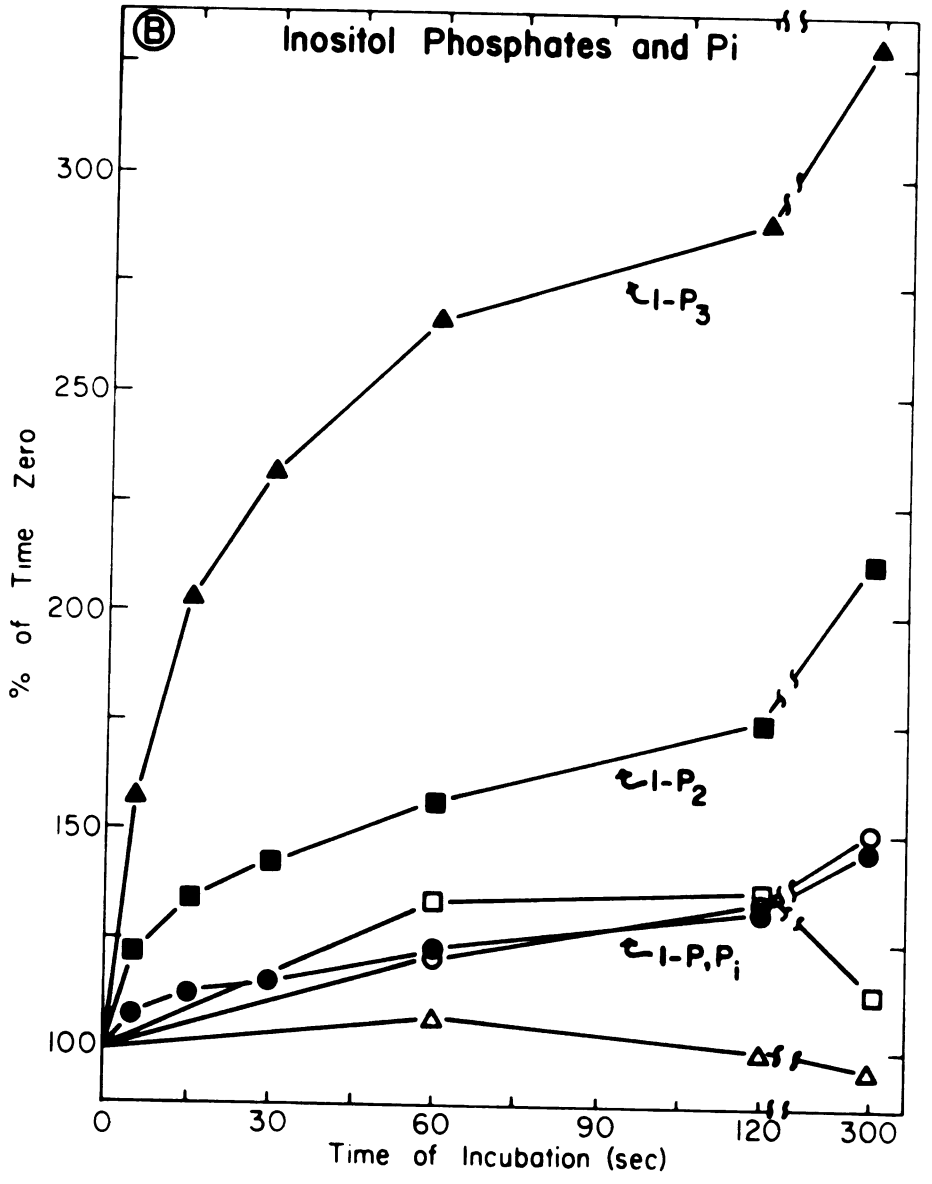

B
Inositol Phosphates and Pi
300
250
200
150
100
% of Time Zero
I-P3
I-P2
I-P, Pi
0
30
60
90
120
300
Time of Incubation (sec)

Figure 2B

Figure 2B describes the rate of ^{32}P-labeled inositol trisphosphate (I-P_3), inositol bisphosphate (I-P_2), inositol phosphate (I-P) and $^{32}P_i$ released into the water soluble fraction in the presence or absence of calcium. [^{32}P]I-P and $^{32}P_i$ which cannot be separated from one another by the anion exchange method increased by 50% after 300 sec of incubation in the presence or absence of calcium. There are five possible sources for the radioactivity which would co-elute with [^{32}P]I-P and $^{32}P_i$: 1) non-enzymatic release of $^{32}P_i$ which was non-covalently bound to membranes present in the plasma membrane fraction; 2) a calcium independent protein phosphatase releasing $^{32}P_i$; 3) a calcium independent phospholipid phosphomonoesterase acting on ^{32}P-labeled PA, PI-P and PI-P_2 releasing $^{32}P_i$; 4) a calcium independent PI-P and PI-P_2 specific phosphodiesterase which would release [^{32}P]I-P_2 and [^{32}P]I-P_3 which was then rapidly dephosphorylated to $^{32}P_i$ and [^{32}P]I-P; and 5) release from membranes of some other ^{32}P-labeled water soluble component possessing a negative 2 charge. The calcium independent breakdown of ^{32}P-labeled PA, PI-P and PI-P_2 supports the presence of a phospholipid phosphomonoesterase in the plasma membrane preparation. The small amount of [^{32}P]I-P_2 produced iin the absence of calcium suggests the presence of a phospholipid phosphodiesterase acting on PI-P and/or PI-P_2 but not on PI since there was no decrease in the level of [^{32}P]PI. However, the main contributor to the radioactivity recovered in the P_i and I-P fraction was probably $^{32}P_i$

released from non-phospholipid sources since the level of radioactivity which elutes in this region was very high at time=0 and the decrease in radioactivity of the phospholipids in the absence of calcium was only 35% of the amount recovered in the P_i and I-P region (see fig. 2 legend). The addition of 1mM spermine to the assay mix completely inhibited the calcium independent breakdown of ^{32}P-labeled PA, PI-P and PI-P_2 but had no effect on the calcium stimulated breakdown of ^{32}P-labeled PI-P and PI-P_2. Spermine caused the level of $^{32}P_i$ and/or [^{32}P]I-P to be reduced to 120% of t=0 after 120 sec of incubation in the presence or absence of calcium. [^{32}P]I-P_2 and I-P_3 levels were identical after 120 sec of incubation with 1mM spermine in the absence of calcium as seen at t=0 (data not shown).

Figure 2B also illustrates the calcium dependent release of water soluble ^{32}P-labeled I-P_2 and I-P_3 indicating that ^{32}P-labeled PI-P and PI-P_2 were hydrolyzed by a calcium dependent phosphodiesterase. A 2.5 to 3 fold increase in the amount of [^{32}P]I-P_3 released into the water soluble fraction was seen within 60 sec of incubation with 110uM free calcium. Release of [^{32}P]I-P_2 was also stimulated by calcium but to a lesser extent.

Magnesium (0.5mM or 2mM) could not substitute for calcium nor did it affect the calcium dependent phosphodiesterase activity. The addition of 1 mg of cytosol protein had no effect on the calcium dependent phosphodiesterase activity nor did cytosol affect any of the

^{32}P-phospholipid levels when added alone or in the presence of vasopressin or magnesium during a 2 min incubation (data not shown). Figure 2B is somewhat misleading due to the background radioactivity levels of the fractions collected. However, all of the radioactivity lost from ^{32}P-labeled PI-P and PI-P_2 by the action of the calcium dependent phosphodiesterase could be accounted for in the ^{32}P-labeled I-P_2 and I-P_3 fractions (data not shown).

Dose Response of Calcium on Plasma Membrane Polyphosphoinositide Phosphodiesterase Activity: Effect of Ionic Strength - ^{32}P-labeled plasma membranes were incubated for 1 min at 37^{o}C under various free calcium concentrations at low and normal (iso-osmotic) ionic strength. The activity of the polyphoinositide phosphodiesterase was monitored by measuring the levels of ^{32}P-labeled PI-P, PI-P_2, I-P_2 and I-P_3. The results of these experiments are shown in Figures 3A and B. Incubation of the ^{32}P-labeled plasma membranes in 20mM Na-HEPES, 0.5mM Na-EGTA, pH 7.4 (low ionic strength) resulted in maximal ^{32}P-labeled PI-P and PI-P_2 breakdown at a free calcium concentration of 8.4uM with half-maximal hydrolysis observed at 100nM free calcium. Similar results were observed when [^{32}P]I-P_3 release into the water soluble fraction was measured. There was very little change in phosphodiesterase activity when assayed over the range of 200nM to 100uM free calcium, suggesting that the phosphodiesterase activity was dependent on calcium, but not regulated by changes in calcium

Figure 3. Dose Response of Calcium on Plasma Membrane Polyphosphoinositide Phosphodiesterase Activity: Effect of Ionic Strength. ^{32}P-labeled plasma membranes were prepared from prelabeled hepatocytes and incubated for 1 min at 37°C at various free calcium concentrations under conditions of low (20mM Na-HEPES, 0.5mM Na-EGTA, pH 7.4) and iso-osmotic ionic strength (120mM KCl, 20mM Na-HEPES, 0.5mM Na-EGTA, pH 7.4). The levels of ^{32}P-labeled PI-P, PI-P_2, I-P_2 and I-P_3 were determined as described in "Experimental Procedures". A. ^{32}P-labeled polyphosphoinositides- □,■ PI-P; ○,● PI-P_2. B. ^{32}P-labeled inositol phosphates- □,■ I-P_2; ○,● I-P_3. Open symbols- iso-osmotic ionic strength; closed symbols- low ionic strength. Typical levels of ^{32}P-labeled compounds (in c.p.m.) obtained at zero free calcium: iso-osmotic ionic strength- 1531, PI-P; 7047, PI-P_2; 1963, I-P_2; 2132, I-P_3; low ionic strength- 1485, PI-P; 6416, PI-P_2; 1783, I-P_2; 2206, I-P_3. The results shown are the mean percentage changes of duplicate samples from three different hepatocytes preparations. S.E.M. ranged from 5-15%.

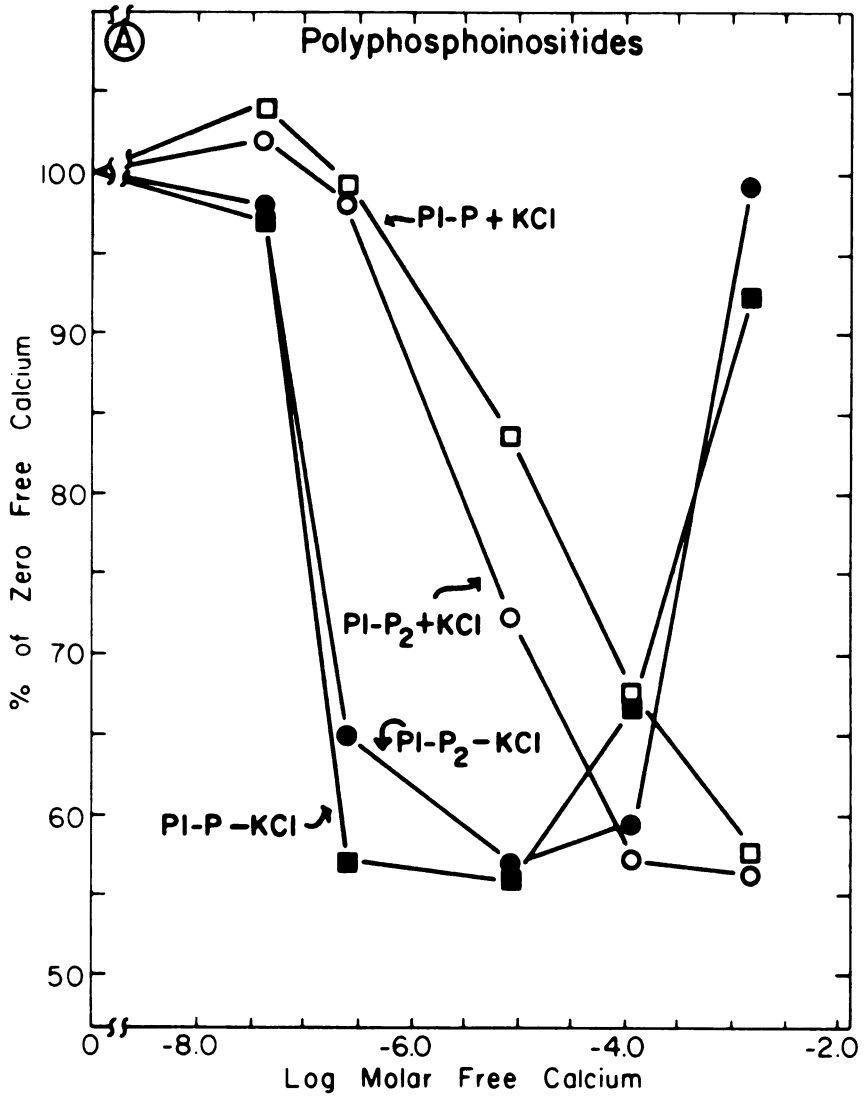

Polyphosphoinositides
PI-P + KCl
PI-P2+KCl
PI-P2-KCl
PI-P-KCl
% of Zero Free Calcium
100
90
80
70
60
50
0
-8.0
-6.0
-4.0
-2.0
Log Molar Free Calcium

Figure 3A

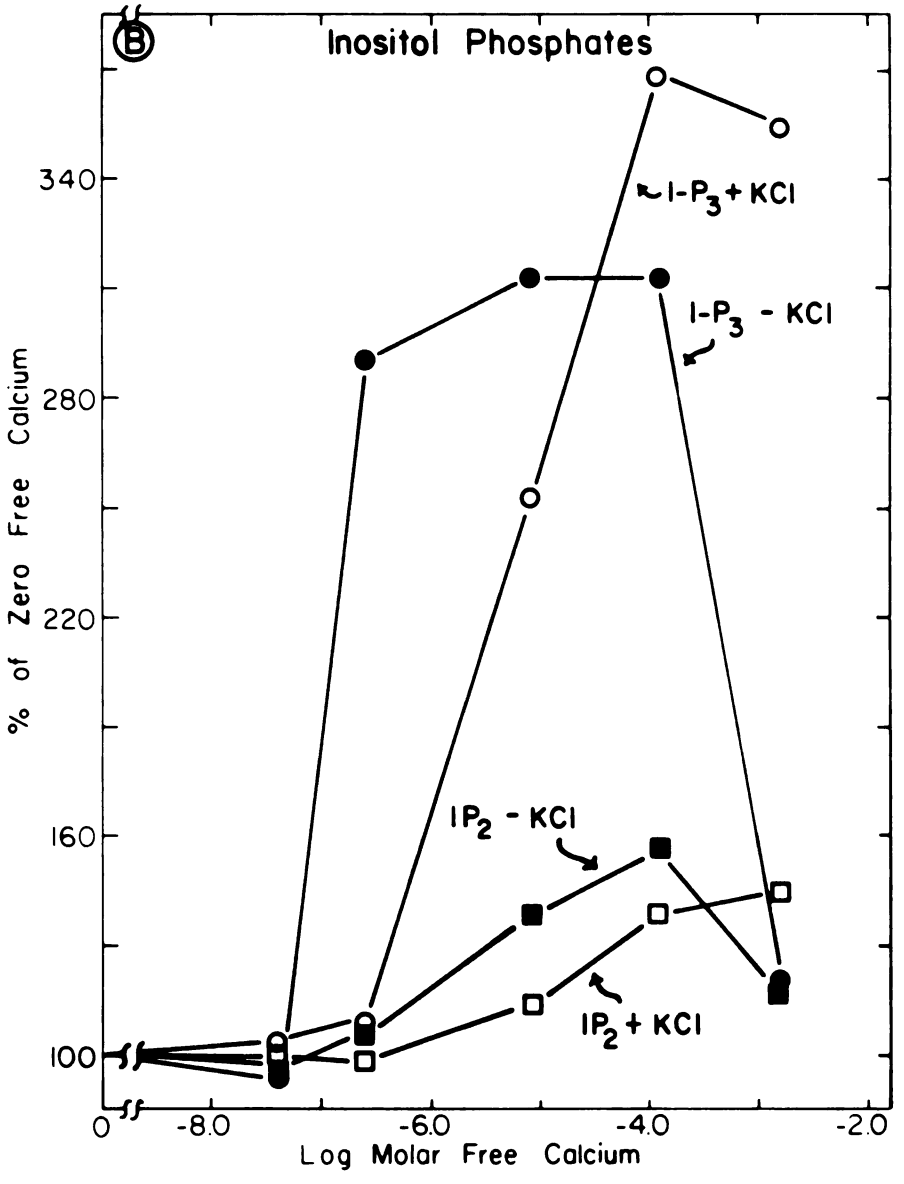

B
Inositol Phosphates
340
280
220
160
100
0
% of Zero Free Calcium
I-P3 + KCl
I-P3 - KCl
IP2 - KCl
IP2 + KCl
-8.0
-6.0
-4.0
-2.0
Log Molar Free Calcium

Figure 3B

concentration. The level of [^{32}P]I-P_2 was maximal at 110uM free calcium with half-maximal production observed at 5uM free calcium. Higher levels of free calcium (1.5mM) inhibited the phosphodiesterase.

Incubation of the ^{32}P-labeled plasma membranes under iso-osmotic conditions established by 120mM KCl, 20mM Na-HEPES, 0.5mM Na-EGTA, pH 7.4 shifted the phosphodiesterase requirement for calcium to the right. Maximal [^{32}P]PI-P_2 hydrolysis and [^{32}P]I-P_3 production was observed at 110uM free calcium with half-maximal activity observed at 5uM free calcium. [^{32}P]PI-P hydrolysis and [^{32}P]I-P_2 production did not plateau even at 1.5mM free calcium, but did reach the maximum levels of hydrolysis and production observed at low ionic strength at this calcium concentration. No inhibition of phosphodiesterase activity was observed at 1.5mM free calcium under iso-osmotic conditions.

Assaying the phosphodiesterase activity under iso-osmotic conditions at 110uM free calcium did not greatly affect the rate of polyphosphoinositide hydrolysis compared with the hydrolysis rate observed at low ionic strength (data not shown). Maintaining the iso-osmotic condition with NaCl instead of KCl and using the K^+ salts of HEPES and EGTA also did not change the concentration of calcium required for polyphosphoinositide phosphodiesterase activity (data not shown).

Effect of Vasopressin on ^{32}P-Labeled Phosphoinositide Levels in Isolated Plasma Membranes - ^{32}P-labeled plasma membranes were incubated in 120mM KCl, 20mM Na-HEPES, 0.5mM EGTA, pH 7.4, 2mM $MgCl_2$ and approximately 240nM free calcium for 1 min at 37°C in the presence or absence of 200nM vasopressin. Analysis of the ^{32}P-labeled phospholipids showed that the level of ^{32}P-labeled PI-P_2 decreased 21.5% ($p<0.001$) in the presence of vasopressin compared with control while no change in ^{32}P-labeled PI, PA or PI-P was observed. The corresponding production of [^{32}P]I-P_3 and also [^{32}P]I-P_2 was observed in response to vasopressin. [^{32}P]I-P_2 was produced as a result of phosphatase action on [^{32}P]I-P_3 which was stimulated by 2mM $MgCl_2$ (data not shown).[2] Phosphodiesterase activity in the presence of 110uM free calcium was not further stimulated by the addition of vasopressin (data not shown).

Discussion

In this study, we have examined the intracellular site of phosphoinositide hydrolysis in response to vasopressin. It was found that the plasma membrane levels of ^{32}P-labeled PI-P and PI-P_2 decreased approximately 40% while ^{32}P-PI decreased 15% after a 45 sec incubation of prelabeled hepatocytes with vasopressin. These results are in good agreement both in terms of time and extent of response with the data reported on whole cell [^{32}P]phosphoinositide changes in response to vasopressin (12,16,17). ^{32}P-labeled PA levels increased rapidly in the plasma membrane probably due to a phosphodiesterase acting on PI, PI-P and/or PI-P_2 forming diacylglycerol which was rapidly phosphorylated using endogenous [γ-^{32}P]ATP and diglyceride kinase. The increases in ^{32}P-labeled PC, PE, PG, and PI observed in virtually all the subcellular fractions after 2 min of vasopressin treatment reflected only very small changes in the total mass of these phospholipids (data not shown). Only a small portion (1-3%) of the total hepatocyte cellular pool of these phospholipids becomes labeled after 90 min of incubation with $^{32}P_i$ (17), so a two-fold increase in the level of ^{32}P-labeled phospholipids represents only a 3-5% increase in mass. The microsome fraction demonstrated the largest increase in [^{32}P]PC, PE, PG, and PI possibly as a result of mobilization of diglyceride and [^{32}P]PA from the

plasma membrane to the endoplasmic reticulum, where the biosynthesis of these phospholipids occurs. The time lag observed between the increase of [^{32}P]PC, PE, PG, and PI levels in the microsome fraction and polyphosphoinositide breakdown in the plasma membrane fraction, along with the transient increase in the level of [^{32}P]PA in the microsome fraction after 2 min of vasopressin treatment supports this conclusion. [^{32}P]PC, PE, PG, and PI could then be mobilized to other parts of the cell.

Using ^{32}P-labeled plasma membranes isolated from prelabeled hepatocytes, PI-P and PI-P_2 were broken down by a membrane bound polyphosphoinositide specific phosphodiesterase to form ^{32}P-labeled I-P_2 and I-P_3. The reaction was rapid with 15% of the ^{32}P-labeled PI-P and PI-P_2 broken down within 15 sec under optimal conditions. The enzyme was specific for polyphosphoinositides since no breakdown of [^{32}P]PI was observed under any condition. Very little ^{32}P-labeled PI-P and PI-P_2 hydrolysis and ^{32}P-labeled I-P_2 and I-P_3 release was observed in the absence of calcium, but the phosphodiesterase activity was stimulated 10-fold in the presence of micromolar concentrations of free calcium. The enzyme appears to prefer PI-P_2 to PI-P as evidenced by the lower calcium requirement for hydrolysis of PI-P_2 under iso-osmotic conditions. Although the requirement for calcium in the breakdown of PI-P and PI-P_2 in rat hepatocytes contradicts many of the reports of vasopressin stimulated PI-P and PI-P_2 breakdown in cells

depleted of calcium by EGTA, and the lack of polyphosphoinositide breakdown induced by the calcium ionophore A23187 (11,13,16), there is supporting evidence that at least a minimal amount of calcium is required for polyphosphoinositide breakdown in response to vasopressin (14,15,17). Hydrolysis of polyphosphoinositides by a calcium stimulated phosphodiesterase has been demonstrated in brain (35), platelets (36), and erythrocyte membranes (37).

The studies on the dose response of calcium on the polyphosphoinositide phosphodiesterase demonstrated that half-maximal hydrolysis of PI-P and PI-P_2 could be obtained at 100nM free calcium when assayed at low ionic strength. However, under conditions which simulate intracellular K^+ and Na^+ concentrations, the calcium requirement for phosphodiesterase activity shifted markedly to the right. Half-maximal phosphodiesterase activity on PI-P_2 was then observed at 5uM free calcium. Similar effects of ionic strength on the erythrocyte membrane polyphosphoinositide phosphodiesterase have been reported (38). Since hepatocyte intracellular calcium concentrations range from 0.2 - 0.6uM (39,40), it is questionable whether the polyphosphoinositide phosphodiesterase is active under normal intracellular conditions. Basal turnover of polyphosphoinositides may occur instead via a phosphomonoesterase whose presence is suggested by the data in Figure 2. A metal-independent phospholipid phosphomonoesterase has been described in

erythrocyte membranes (41) and rat liver nuclear envelopes (42).

However, perturbations in the lipid and/or protein micro-environment surrounding the phosphodiesterase, may result in shifting the phosphodiesterase requirement for calcium toward normal intracellular concentrations. Trauble (43) reported that when a lipid membrane is fully ionized (pH>7), the membrane's transition temperature is increased upon increasing the ionic strength of the surrounding environment. Therefore, in-vitro, decreasing the ionic strength surrounding the membrane would probably make it more fluid, and may result in less constraints on the phosphodiesterase, thus lowering it's requirement for calcium. It has also been shown that norepinephrine and angiotensin II can increase the lipid fluidity, as measured by diphenylhexatriene fluorescence polarization, of plasma membranes isolated from rat liver (44). We have shown that the addition of vasopressin to plasma membranes incubated under ionic conditions similar to the in-vivo intracellular environment, stimulated the breakdown of 20% of the [^{32}P]PI-P_2 present in the plasma membrane fraction in 1 min. The amount of [^{32}P]PI-P_2 breakdown was less than observed during a 1 min incubation of intact cells with vasopressin suggesting that some other factor may have been lost during isolation of the plasma membranes. Alternatively, an intact cytoskeleton connnected to the plasma membrane may be necessary to invoke the maximum changes in the plasma

membrane structure which occurs during hormone-receptor binding and subsequent phosphodiesterase activity.

This study is the first demonstration that the plasma membrane is the location of polyphosphoinositide breakdown in response to vasopressin treatment of rat hepatocytes. The breakdown proceeds by the action of a calcium-dependent, polyphosphoinositide-specific phosphodiesterase. This enzyme can be activated by incubation of isolated plasma membranes with vasopressin under intracellular-like ionic conditions. The increase in intracellular calcium may then procede by inhibiting the calcium pumping activity of the plasma membrane (Ca^{2+}-Mg^{2+})ATPase (20-22) and/or by releasing $I\text{-}P_3$ from $PI\text{-}P_2$ which acts to mobilize endoplasmic reticulum calcium stores (23).

References

1. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.
2. Michell, R.H. (1979) Trends Biochem. Sci. 4, 128-131.
3. Akhtar, R.A., and Abdel-Latiff, A.A. (1978) J. Pharmacol. Exp. Ther. 204, 655-668.
4. Farese, R.V., Saber, M.A., and Vandor, S.L. (1979) J. Biol. Chem. 254, 6842-6844.
5. Kirk, C.J., Creba, J.A., Downes, C.P., and Michell, R.H. (1981) Biochem. Soc. Trans. 9, 377-379.
6. Weiss, S.J., McKinney, J.S., and Putney, J.W., Jr. (1982) Biochem. J. 206, 555-560.
7. Billah, M.M., and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705-12708.
8. Benabe, J.E., Spry, L.A., and Morrison, A.K. (1982) J. Biol. Chem. 257, 7430-7434.
9. Putney, J.W., Burgess, G.M., Halenda, S.P., McKinney, J.S., and Rubin, R.P. (1983) Biochem. J. 212, 483-488.
10. Berridge, M.S., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983) Biochem. J. 212, 473-482.
11. Kirk, C.J., Michell, R.H., and Hems, D.A. (1981) Biochem. J. 194, 155-165.
12. Litosch, I., Lin, S.H., and Fain, J.N. (1983) J. Biol. Chem. 258, 13727-13732.

13. Billah, M.M., and Michell, R.H. (1979) Biochem. J. 182, 661-668.

14. Pripic, V., Blackmore, P.F., and Exton, J. (1982) J. Biol. Chem. 257, 11323-11331.

15. Rhodes, D., Pripic, V., Exton, J.H., and Blackmore, P.F. (1983) J. Biol. Chem. 258, 2770-2773.

16. Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H., and Kirk, C.J. (1983) Biochem. J. 212, 733-747.

17. Thomas, A.P., Marks, J.S., Coll, K.E., and Williamson, J.R. (1983) J. Biol. Chem. 258, 5716-5725.

18. Michell, R.H. (1982) Nature 296, 492-493.

19. Hawthorne, J.N. (1982) Nature 295, 281-282.

20. Buckley, J.T., and Hawthorne, J.M. (1972) J. Biol. Chem. 247, 7218-7223.

21. Penniston, J.T. (1982) Ann. N.Y. Acad. Sci. 402, 296-303.

22. Carafoli, E., and Zurini, M. (1982) Biochim. Biophys. Acta 683, 279-301.

23. Streb, H., Irvine, R.F., Berridge, M.J., and Schulz, I. (1983) Nature 306, 67-69.

24. Lin, S.H., and Fain, J.N. (1981) Life Sci. 29, 1905-1912.

25. Harrington, C.A., and Eichberg, J. (1983) J. Biol. Chem 258, 2087-2090.

26. Wallace, M.A., Randazzo, P., Li, S.Y., and Fain, J.N. (1982) Endocrinology 111, 341-343.

27. Seyfred, M.A., and Wells, W.W. (1984), J. Biol. Chem., in press. (Chapter I, this thesis)

28. Seglen, P.O. (1976) Methods Cell Biol. 13, 29-83.

29. Schacht, J. (1981) Methods Enzymol. 72, 626-631.

30. Kates, M. (1972) in Techniques of Lipidology: Isolation, Analysis and Identification of Lipids pp. 558-559, North-Holland Publishing Co., Amsterdam.

31. Downes, C.P., and Michell, R.H. (1981) Biochem. J. 198, 133-140.

32. Hawkins, P.T., Michell, R.H., and Kirk, C.J. (1983) Biochem. J. 210, 717-720.

33. Bohlen, P., Stein, S., Dairmain, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220.

34. Pires, E.M.V., and Perry, S.V. (1977) Biochem. J. 167, 137-146.

35. Van Rooijen, L.A.A., Sequin, E.B., and Agranoff, B.W. (1983) Biochem. Biophys. Res. Comm. 112, 919-926.

36. Rittenhouse, S.E. (1983) Proc. Nat. Acad. Sci. 80, 5417-5420.

37. Allan, D., and Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277-286.

38. Downes, C.P., and Michell, R.H. (1982) Biochem. J. 202, 53-58.

39. Joseph, S.K., and Williamson, J.R. (1983) J. Biol. Chem. 258, 10425-10432.

40. Charest, R., Blackmore, P.F., Berthon, B., and Exton, J.H. (1983) J. Biol. Chem. 258, 8769-8773.

41. Mack, S.E., and St. C. Palmer, F.B. (1984) J. Lipid Res. 25, 75-85.

42. Smith, C.D., and Wells, W.W. (1984) Submitted to Arch. Biochem Biophys for publication.

43. Trauble, H. (1976) in Structure of Biological Membranes, (Abrahamsson, S., and Pascher, I., eds.) pp. 509-550, Plenum Press, New York.

44. Burgess, G.M., Giraud, F., Poggioli, J., and Claret, M. (1983) Biochim. Biophys. Acta 731, 387-396.

CHAPTER III

Characterization of myo-Inositol Trisphosphate Phosphatase in Rat Liver Plasma Membranes

Abstract

myo-Inositol trisphosphate ($I-P_3$) has been previously demonstrated to act as a second messenger for the hormonal mobilization of intracellular calcium in rat liver. In this study, the breakdown of $I-P_3$ by a phosphatase activity was characterized. Using partially purified subcellular fractions, it was found that myo-inositol trisphosphate phosphatase ($I-P_3$ase) specific activity was highest in the plasma membrane fraction, while myo-inositol bisphosphate phosphatase specific activity was highest in the cytosolic and microsomal fractions. The plasma membrane $I-P_3$ase was Mg^{2+}-dependent with optimal activity observed at 0.5-1.5 mM free Mg^{2+}. The enzyme had a neutral pH optimum suggesting that it was neither an acid or alkaline phosphatase. Neither LiCl nor NaF inhibited the $I-P_3$ase activity. However, both L-cysteine and dithiothreitol stimulated the activity 2-fold. Spermine inhibited the $I-P_3$ase activity by 50%, while putrescine and spermidine had litle or no effect. These data suggest that cellular $I-P_3$ levels may not be regulated by compartmentalization within the cell, but rather by some other agents affecting the enzyme and/or the substrate such as spermine.

Footnotes

[1]The abbreviations used are: PI, phosphatidylinositol; PI-P, phosphatidylinositol 4-phosphate; PI-P_2, phosphatidylinositol 4,5-bisphosphate; I-P, myo-inositol phosphate; I-P_2, myo-inositol bisphosphate; I-P_3, myo-inositol trisphosphate; I-P_2ase, myo-inositol bisphosphate phosphatase; I-P_3ase, myo-inositol trisphosphate phosphatase; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-[N-Morpholino]ethanesulfonic acid; BICINE, N,N-bis[2-Hydroxyethyl]glycine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N'-tetraacetic acid; TCA, trichloroacetic acid.

Introduction

In rat hepatocytes, calcium mobilizing hormones such as vasopressin, angiotensin II and epinephrine enhance phosphatidylinositol (PI)[1] breakdown (1-4) as well as decrease the level of ^{32}P-labeled phosphatidylinositol 4-phosphate (PI-P) and phosphatidylinositol 4,5-bisphosphate ($PI-P_2$) in prelabeled rat hepatocytes (5-8). Previous studies in this laboratory (8) demonstrated that the plasma membrane is the site of increased polyphosphoinositide (PI-P and $PI-P_2$) breakdown in response to vasopressin. A calcium-dependent polyphosphoinositide-specific phosphodiesterase activity in partially purified plasma membranes was described, which was responsible for the agonist stimulated breakdown of polyphosphoinositides.

The correlation between phosphoinositide (PI, PI-P, and $PI-P_2$) breakdown and increased levels of intracellular calcium, suggests that phosphoinositides or products of the metabolism of phosphoinositides may somehow influence the various mechanisms which act to modulate intracellular calcium. Indeed, it has been postulated that decreases in the concentration of plasma membrane phosphoinositides could affect calcium channels (9,10, but see 11) or some other calcium transport system such as the plasma membrane (Ca^{2+}-Mg^{2+})ATPase (12-14). Alternatively, myo-inositol trisphosphate ($I-P_3$), the water soluble product released from $PI-P_2$ by action of the calcium-dependent

polyphosphoinositide phosphodiesterase, releases calcium from non-mitochondrial intracellular stores in saponin-permeabilized rat hepatocytes (15) and pancreatic acinar cells (16).

$I\text{-}P_3$ is rapidly degraded in rat hepatocytes (15), blowfly salivary glands (17) and human erythrocyte membranes (18), presumably by a phosphatase-type reaction. However, only the erythrocyte membrane myo-inositol trisphosphatase ($I\text{-}P_3$ase) has been characterized. Therefore, in light of the possible important role of $I\text{-}P_3$ as a second messenger for the hormonal mobilization of intracellular calcium in rat liver, we have further characterized the $I\text{-}P_3$ase activity in rat liver.

Experimental Procedures

Hepatocyte Incubations and ^{32}P-Labeled Plasma Membrane Isolation - Hepatocytes were isolated from fed male CD-Sprague Dawley rats by a modification of the method described by Seglen (19). The preparation procedures, incubation conditions and the isolation of ^{32}P-labeled plasma membranes were previously described (8,20).

Isolation of Subcellular Organelles - Plasma membranes were prepared from rat liver as previously described (21), with the following modifications: 10 mM KH_2PO_4, pH 7.5 was used instead of 1 mM $NaHCO_3$; the 1500xg pellets were resuspended to 200 ml; and the 100xg/1000xg layer was resuspended to 70 ml. Mitochondria, microsomes and cytosol were prepared from a single rat liver by standard differential centrifugation.

Preparation of ^{32}P-labeled I-P_2 and I-P_3 - Utilizing the presence of an endogenous Ca^{2+}-dependent polyphosphoinositide-specific phosphodiesterase in rat hepatocyte plasma membranes (20), ^{32}P-labeled I-P_2 and I-P_3 were isolated by incubating ^{32}P-labeled plasma membranes (4-5 mg protein) with 20 mM Na-HEPES, 0.5 mM Na-EGTA, pH 7.4 and 110 uM free Ca^{2+} in a final volume of 10 ml for 10 min at 37^{o}C. The reactions were terminated by the additon of 1 ml of 25 mM Na-EGTA, pH 7.4 and placed on ice. The sample was centrifuged at 4^{o}C for 20 min at 15,000xg to pellet the plasma membrane fragments. The supernatant was removed and loaded onto an anion-exchange column containing 2 ml of

Dowex 1 (x8; 200-400 mesh; formate form). The column was washed with 15 ml of 60 mM ammonium formate and the radioactive phosphate esters sequentially eluted as described by Downes and Michell (22) using 20 ml of 0.1 M formic acid, 0.2 M ammonium formate to elute P_i and myo-inositol phosphate (I-P), 35 ml of 0.1 M formic acid, 0.4 M ammonium formate to elute myo-inositol bisphosphate ($I-P_2$), and 25 ml of 0.1 M formic acid, 1.0 M ammonium formate to elute $I-P_3$. The fractions corresponding to $I-P_2$ and $I-P_3$ were pooled and acidified by batchwise treatment with Dowex 50 (H^+). The resins were pelleted and the supernatants lyophilized to remove the formic acid. The residues were dissolved in 1-1.5 ml of water.

To further characterize the isolated [^{32}P]$I-P_2$ and $I-P_3$, the products were examined by paper chromatography for 20 hrs in 1-propanol:conc. NH_4OH:water (5:4:1) (23,24) and the chromatogram exposed to x-ray film. The [^{32}P]$I-P_2$ product separated into two radioactive components. One component contained 33% of the radioactivity and had a R_f relative to $^{32}P_i$ (R_{P_i}) of 1.29. The second component had a R_{P_i} of 0.79, which corresponds identically to inositol bisphosphate (24). The [^{32}P]$I-P_3$ product also separated into two radioactive components. One component had a R_{P_i} of 0.80, analogous to inositol bisphosphate, but comprised only 10% of the total radioactivity. The other component had a R_{P_i} of 0.57, which is very close to the published R_{P_i} for inositol trisphosphate of 0.59 (24). There was not enough [^{32}P]$I-P_2$

or $I-P_3$ isolated, in terms of mass, for any further chemical analysis.

Assay for the Degradation of [^{32}P]$I-P_2$ and $I-P_3$ - The standard assay to determine the rate of [^{32}P]$I-P_2$ and $I-P_3$ breakdown consisted of preincubating 50-100 ug of protein from either the purified plasma membrane fraction or some other fraction in 250 ul of 50 mM Na-HEPES, 1.0 mM Na-EDTA, pH 7.4 for 2 min at 37°C. Various additions were made (metals, effectors) and the incubations continued for an additional 10 min. [^{32}P]$I-P_2$ or $I-P_3$ was added (3000-4000 cpm) to give a final volume of 500 ul and the reactions continued for various times at 37°C in an oscillating water bath. The reactions were terminated by adding 500 ul of ice-cold 20% trichloroacetic acid (TCA). The precipitates were pelleted by centrifugation and the supernatants extracted four times with 1 ml diethyl ether to remove the TCA and then neutralized with 2 ul of conc. NH_4OH. The phosphate esters were separated on 0.4 ml Dowex 1 (x8; 200-400 mesh; formate form) columns as described by Downes and Michell (22) into P_i/I-P, $I-P_2$ and $I-P_3$ fractions.

Other Methods - Marker enzyme analysis on the subcellular fractions were performed using alkaline phosphodiesterase I as a marker for plasma membranes (25), fumarase for mitochondria (26), and glucose 6-phosphatase in the presence of 40mM L-tartrate for microsomes (27,28). Protein was determined by fluorescamine (29) using bovine serum albumin as the standard. ^{32}P radioactivity was determined by

Cerenkov radiation. Free magnesium concentrations were calculated according to the method described by Bartfai (30).

Materials - The sources of all reagents and materials were previously described (8,20).

Results

Breakdown of Endogenously Produced [^{32}P]I-P_3 by Partially Purified Rat Hepatocyte Plasma Membranes - As previously described (8), when ^{32}P-labeled plasma membranes isolated from rat hepatocytes were incubated with micromolar concentrations of free calcium, [^{32}P]PI-P and PI-P_2 were cleaved by a phosphodiesterase producing [^{32}P]I-P_2 and I-P_3, respectively. If 2 mM $MgCl_2$ was present in the reaction mix, there was no change in the rate of [^{32}P]PI-P_2 or PI-P breakdown, but there was a decrease in the level of [^{32}P]I-P_3 produced, concurrent with an increase in [^{32}P]I-P_2 and [^{32}P]I-P/P_i. This observation is more adequately described in Figure 1. Adding 110 uM free calcium to [^{32}P]plasma membranes released [^{32}P]I-P_3 from [^{32}P]PI-P_2. Na-EGTA (0.5 mM) was added to block further [^{32}P]I-P_3 release. When 2 mM $MgCl_2$ was added to the same sample, the level of [^{32}P]I-P_3 was reduced by 50% within 2 min with corresponding increases in [^{32}P]I-P_2 and [^{32}P]I-P/P_i. Thus, it appears that endogenously produced [^{32}P]I-P_3 is broken down by a magnesium-dependent phosphatase. However, due to the contamination of the [^{32}P]plasma membranes with other organelles, primarily microsomes (20), the subcellular location of I-P_3 phosphatase (I-P_3ase) could not be established. Also, there was a very high background of ^{32}P radioactivity in the [^{32}P]I-P/P_i fraction making quantitation difficult. For these reasons. it was decided

Figure 1. Endogenous Mg^{2+}-dependent I-P_3ase Activity in Partially Purified Plasma Membranes Isolated from Rat Hepatocytes. ^{32}P-Labeled plasma membranes (100 ug protein) were preincubated in 20 mM Na-HEPES, 0.5 mM Na-EGTA, pH 7.4 for 5 min at 37°C. At time=0, $CaCl_2$ was added to give a final concentration of 110 uM free calcium in a total volume of 500 ul. The reaction was allowed to continue for 5 min, and then 10 ul of 25 mM Na-EGTA, pH 7.4 was added to stop the phosphodiesterase activity. The samples were further incubated for 3 min at 37°C in order to observe any non-metal dependent breakdown of [^{32}P]I-P_2 or I-P_3. $MgCl_2$ (2 mM final concentration) was added and the samples incubated at 37°C for 2 min. The reactions were stopped by the addition of 1.5 ml of methanol:chloroform (2:1) and the samples processed as previously described (8). The water soluble phosphate esters were separated by anion exchange chromatography (22). The results shown are the mean values of duplicate samples from one of three similar [^{32}P]-labeled hepatocyte plasma membrane preparations.

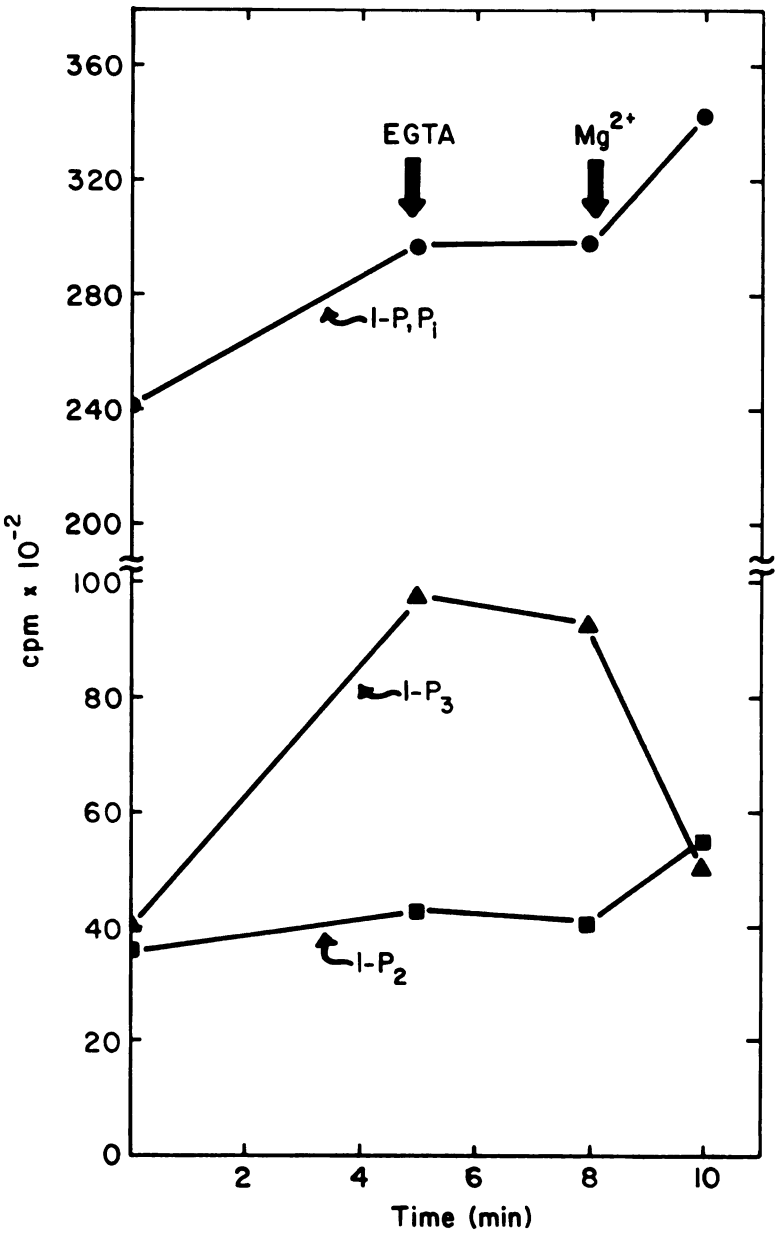

360
320
280
240
200
100
80
60
40
20
0
cpm x 10^{-2}
EGTA
Mg^{2+}
I-P, P_i
I-P_3
I-P_2
2
4
6
8
10
Time (min)

Figure 1

to synthesize [^{32}P]I-P_3 and I-P_2 from ^{32}P-labeled plasma membranes and use the labeled inositol polyphosphates as substrates to determine the subcellular distribution of I-P_2ase and I-P_3ase using purified subcellular fractions and also further characterize the phosphatase activities.

Subcellular Distribution of I-P_2ase and I-P_3ase Activities - Partially purified plasma membranes, mitochondria and microsomes were prepared as described in "Experimental Procedures," and assayed for various marker enzymes along with an aliquot of cell homogenate and cytosol. The results are shown in Table I. The plasma membrane fraction was enriched 11-fold in alkaline phosphodiesterase activity, while the mitochondrial and microsomal fractions were enriched 2-fold in their respective marker enzymes, fumarase and glucose 6-phosphatase. There was little organelle marker enzyme activity in the cytosolic fraction suggesting good integrity of the isolated organelles.

These fractions were assayed, as described in "Experimental Procedures," for their ability to degrade [^{32}P]I-P_2 and I-P_3, and the results are described in Table II. The I-P_3ase activity was found almost exclusively in the plasma membrane fraction, while I-P_2ase activity was found to be more ubiquitous with the highest specific activity observed in the cytosolic and microsomal fractions. Since the plasma membrane exhibited the highest specific activity for I-P_3ase, all further experiments were done using

Table I

Marker Enzyme Analysis

Subcellular fractions were analyzed for marker enzymes as described in "Experimental Procedures." The results shown are the mean ± standard deviations of duplicate samples from two different preparations for each experiment. The relative specific activities of each marker enzyme, defined as the specific activity of the enzyme in the fraction/specific activity of the enzyme in the cell homogenate, is given in parenthesis.

Fraction	Alk. Phosphodiesterase I umoles/min/mg	Glucose 6-P'ase nmoles/min/mg	Fumarase umoles/min/mg
Experiment I:			
Cell Homogenate	0.0682 ± .0082 (1.0)	0.854 ± .132 (1.0)	0.194 ± .001 (1.0)
Mitochondrial	0.0087 ± .0012 (0.13)	0.779 ± .035 (0.91)	0.380 ± .080 (1.96)
Microsomal	0.109 ± .0016 (1.60)	1.67 ± .062 (1.95)	0.101 ± .002 (0.52)
Cytosolic	0.0046 ± .0012 (0.07)	0.068 ± .001 (0.08)	0.129 ± .015 (0.67)
Experiment II:			
Cell Homogenate	0.139 ± .0255 (1.0)	1.25 ± .0035 (1.0)	0.292 ± .052 (1.0)
Plasma Membrane	1.54 ± .231 (11.1)	0.921 ± .035 (0.74)	0.054 ± .008 (0.74)

Table II

Subcellular Distribution of $I-P_2$ and $I-P_3$ Phosphatase Activity

Subcellular fractions were prepared as described in "Experimental Procedures," and assayed for $I-P_2$ and $I-P_3$ phosphatase activities using 100 ug of fraction protein and 1.5 mM free Mg^{2+}. The reactions were initiated by the addition of [^{32}P]$I-P_2$ or [^{32}P]$I-P_3$ and continued for 10 min at 37°C. The reactions were stopped with 20% TCA and the samples processed as described in "Experimental Procedures." Results are the mean ± standard deviation of duplicate samples from two different preparations.

		^{32}P-Radioactivity (cpm)			
Fraction	Substrate	$I-P$, P_i	$I-P_2$	$I-P_3$	Total
Cell Homogenate	$I-P_2$	1132 ± 90	1638 ± 9		2770
	$I-P_3$	526 ± 19	57 ± 4	3037 ± 20	3620
Plasma Membrane	$I-P_2$	759 ± 87	2577 ± 106		3336
	$I-P_3$	1353 ± 17	694 ± 2	1830 ± 85	3877
Mitochondrial	$I-P_2$	492 ± 168	2625 ± 25		3117
	$I-P_3$	234 ± 22	0	3330 ± 180	3564
Microsomes	$I-P_2$	1113 ± 122	1967 ± 151		3080
	$I-P_3$	438 ± 39	76 ± 5	3065 ± 65	3579
Cytosolic	$I-P_2$	1626 ± 207	1209 ± 63		2835
	$I-P_3$	224 ± 22	0	3352 ± 68	3576

purified plasma membranes as the source of I-P_3ase activity.

Metal Requirement for Plasma Membrane I-P_3ase Activity - As shown in Figure 1, Mg^{2+} was required in the assay mix to observe the endogenous breakdown of [^{32}P]I-P_3. Using purified plasma membranes as the source of I-P_3ase activity, the metal requirement for I-P_3 breakdown was investigated. It was found that calcium and manganese at concentrations of either 1.0 or 5.0 mM (approximately 0.5 and 4.5 mM free metal, respectively) gave only 30% of the activity observed using magnesium at the same concentrations (data not shown). No breakdown of [^{32}P]I-P_3 was observed using the polyamines putrescine, spermidine or spermine at 1.0 or 5.0 mM (data not shown).

The dose response of magnesium on I-P_3ase activity is shown in Figure 2. I-P_3ase activity was maximal between 0.5-1.5 mM free Mg^{2+} with inhibition of I-P_3ase activity observed at higher free Mg^{2+} concentrations.

Time Course of the Breakdown of [^{32}P]I-$P_3$by Plasma Membranes The rate of [^{32}P]I-P_3 breakdown by 50 ug of purified plasma membrane protein is shown in Figure 3. A somewhat linear rate of [^{32}P]I-P_3 breakdown was observed over the first 20 min of incubation at 37^{o}C with concurrent production of [^{32}P]I-P_2 and [^{32}P]I-P/P_i. Approximately 65% of the [^{32}P]I-P_3 was degraded after 60 min of incubation.

Effect of pH on Plasma Membrane I-P_3ase Activity - The effect of pH on the breakdown of [^{32}P]I-P_3 by plasma membranes was investigated to determine if the I-P_3ase

Figure 2. Dose Response of Magnesium on Plasma Membrane I-P_3ase Activity. I-P_3ase activity of purified plasma membranes was assayed as described in "Experimental Procedures," using 50 ug of plasma membrane protein and incubating for 20 min at 37^{o}C at various free Mg^{2+} concentrations. The results shown are the mean values of triplicate samples. The standard error of the mean ranged from 5-10%.

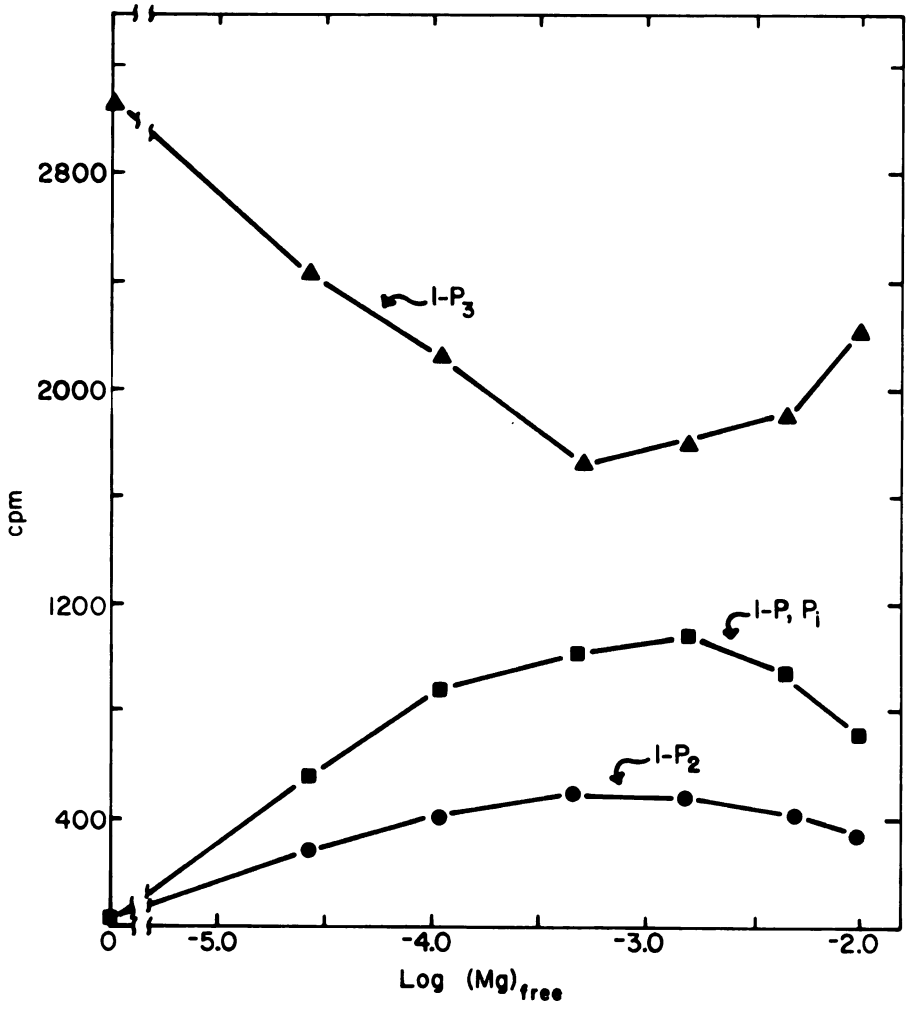


Figure 2

Figure 3. Time Course of I-P_3 Breakdown. I-P_3 breakdown was assayed as described in "Experimental Procedures," using 50 ug of plasma membrane protein and a free magnesium concentration of 1.5 mM. The results shown are the mean values of triplicate samples. The standard error of the mean ranged from 5-10%.

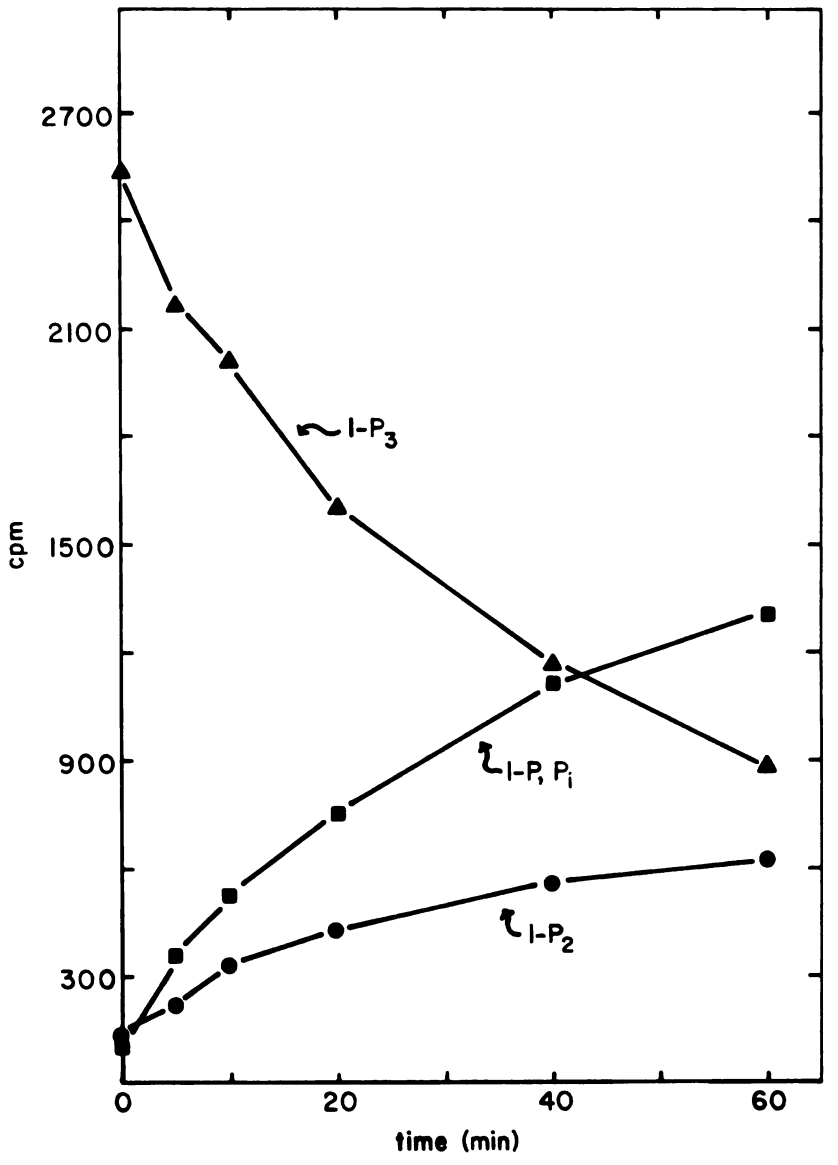


Figure 3

activity was the result of either an alkaline or acid phosphatase activity, or a separate enzyme activity. As shown in Figure 4, very litle activity was observed below a pH of 6.0 or above a pH of 8.5. Although the amount of free Mg^{2+} varies depending upon the pH of the environment, the pH range used in this study would only vary the free Mg^{2+} concentration between 1.0 mM and 1.5 mM. Very little change in I-P_3ase activity was observed over the range of 0.5-1.5 mM free Mg^{2+} (see Fig. 2). Maximal activity was observed at a pH of 7.5 suggesting that I-P_3ase is a unique enzyme activity and not the result of a general acid or alkaline phosphatase acting on I-P_3.

Effect of Various Agents on Plasma Membrane I-P_3ase Activity

Various agents were preincubated for 10 min at 37°C with isolated plasma membranes. The I-P_3ase activity was then assayed as described in "Experimental Procedures." The results are shown in Table 3. NaF, an inhibitor of acid phosphatase (31), protein phosphatase (32,33) and glucose 6-phosphatase (34) had no effect on the I-P_3ase activity at either 0.5 or 5.0 mM. LiCl, which inhibits myo-inositol 1-phosphatase in rat brain (35) and rat mammary gland (36), had no effect on I-P_3ase activity at 1.0 mM concentration and slightly increased the activity at 10 mM. Both L-cysteine and dithiothreitol (0.5 mM) stimulated the I-P_3ase activity approximately 2-fold. Only slightly more stimulation was observed using either compound at 5.0 mM. Putrescine and spermidine, at 0.2 and 2.0 mM, either had no

Figure 4. Effect of pH on the Plasma Membrane I-P_3ase Activity. I-P_3 ase activity was assayed as described in "Experimental Procedures," using 50 ug of plasma membrane protein and a free magnesium concentration which ranged from 1.0 mM at pH 9.5 to 1.5 mM at pH 4.5. The buffers used to extablish the various pH's were: Na-acetate, pH 4.5-5.5; Na-MES, pH 6.0,6.5; Na-HEPES, pH 7.0-8.0; and Na-BICINE, pH 8.5-9.5. The samples were incubated for 20 min at 37^{o}C. The results shown are the mean values of triplicate determinations. The standard error of the mean ranged from 5-10%.

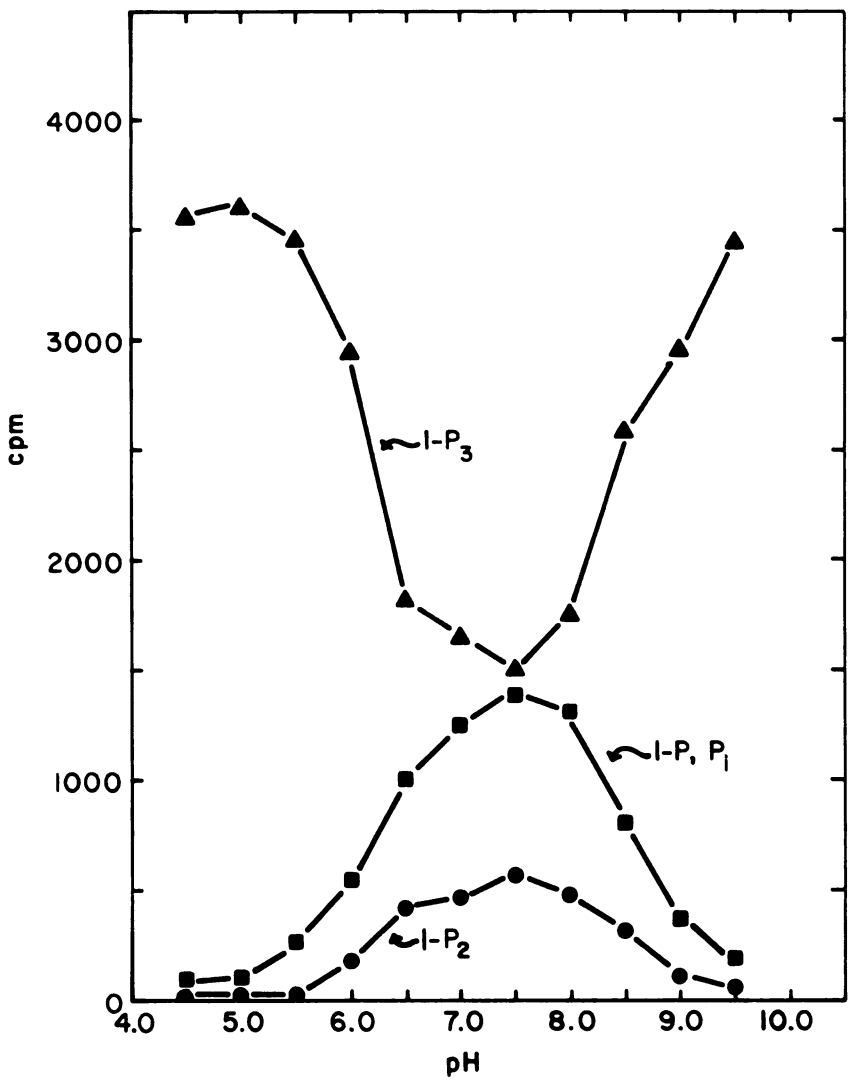


Figure 4

Table III

Effect of Various Agents on I-P_3 Phosphatase Activity

Agent was preincubated for 10 min at 37°C with the assay mix described in "Experimental Procedures" which contains 50 ug of plasma membrane protein and 1.0 mM free Mg^{2+}. The reactions were initiated by the addition of [^{32}P]I-P_3 and continued for 10 min. The reactions were stopped with TCA and the samples were processed as described in "Experimental Procedures." The results are expressed as the mean ± standard deviation of duplicate samples from two different experiments.

Agent	Conc.(mM)	^{32}P-Radioactivity (cpm) I-P, P_1	I-P_2	I-P_3
None	-	894 ± 35	297 ± 14	2017 ± 24
L-Cysteine	0.5	1782 ± 18	531 ± 14	1029 ± 0
	5.0	1862 ± 28	616 ± 79	796 ± 98
Dithiothreitol	0.5	1744 ± 127	487 ± 36	1135 ± 143
	5.0	1893 ± 116	501 ± 25	884 ± 44
NaF	0.5	1040 ± 37	272 ± 4	1834 ± 47
	5.0	1042 ± 9	316 ± 10	1817 ± 53
LiCl	1.0	943 ± 71	269 ± 2	2052 ± 56
	10.0	1230 ± 221	277 ± 12	1823 ± 173
Putrescine	0.2	907 ± 54	287 ± 17	1934 ± 47
	2.0	1143 ± 33	322 ± 24	1679 ± 76
Spermidine	0.2	1163 ± 34	350 ± 2	1773 ± 22
	2.0	1102 ± 6	233 ± 25	1917 ± 29
Spermine	0.2	911 ± 38	273 ± 27	1949 ± 34
	2.0	435 ± 5	73 ± 5	2727 ± 5
2,3-Bisphospho-glycerate	0.2	675 ± 4	174 ± 20	2340 ± 60
	2.0	292 ± 7	73 ± 14	2850 ± 83

effect or only slightly stimulated the I-P_3ase activity. Spermine, at 0.2 mM, had no effect on the activity, but 2.0 mM spermine inhibited I-P_3ase approximately 50%. 2,3-Bisphosphoglycerate, which acts as a competitive inhibitor of I-P_3ase in human erythrocyte membranes (18), also inhibited the I-P_3ase of rat hepatocyte plasma membranes by 24% and 67% at 0.2 and 2.0 mM, respectively.

Discussion

The recent reports of myo-inositol trisphosphate acting as the second messenger for hormonal mobilization of cellular calcium (15,16) has increased the need for understanding the enzymes involved in polyphosphoinositide metabolism. In this study, we have characterized the properties of a myo-inositol trisphosphate phosphatase in rat liver. [^{32}P]I-P_2 and I-P_3 were obtained from partially purified plasma membranes containing ^{32}P-labeled PI-P and PI-P_2 by activating the Ca^{2+}-dependent polyphosphoinositide-specific phosphodiesterase (8) with 110 uM free calcium, and then isolating the phosphate esters released into the aqueous media by anion exchange chromatography. The radiochemical purity of the isolated [^{32}P]I-P_2 and I-P_3 was 66% and 90%, respectively. Using these substrates, it was found that the I-P_3ase activity is located almost entirely in the plasma membrane, while I-P_2ase activity is located primarily in the cytosolic and microsomal fractions. I-P_3ase has a pH optimum of 7.5 and is not inhibited by other phosphatase inhibitors such as NaF or LiCl. This evidence, along with the difference in subcellular distribution of the I-P_2ase and I-P_3ase, suggest that the I-P_3ase activity is the result of a specific enzyme and not a general alkaline or acid phosphatase, nor a non-specific protein phosphatase or myo-inositol 1-phosphatase acting on I-P_3.

The I-P_3ase requires magnesium for activity and is maximally activated at 0.5-1.5 mM, which is well within the range of *in-vivo* intracellular magnesium levels (37). I-P_3ase is stimulated by L-cysteine and dithiothreitol suggesting a role for sulfhydryl groups in the catalytic properties of the enzyme. Downes et al. (18) demonstrated that 2,3-bisphosphoglycerate acted as a competitive inhibitor of the I-P_3ase activity in erythrocyte membranes. A similar level of inhibition by 2,3-bisphosphoglycerate was seen when tested on I-P_3ase in rat liver plasma membranes. This suggest that the enzyme may have a particular affinity for pairs of vicinal monoester phosphates.

The presence of an I-P_3ase activity in rat liver invokes a second level of control between the calcium-mobilizing agonist binding to the receptor and the increased level of intracellular calcium. Release of I-P_3 from PI-P_2 is regulated through a calcium-dependent polyphosphoinositide-specific phosphodiesterase, which appears to be linked either directly or indirectly to the hormone binding to the receptor (8). However, regulation of I-P_3ase activity, and thus cellular I-P_3 levels, remains undefined. Subcellular compartmentalization does not directly regulate I-P_3 levels since the production of I-P_3 and the I-P_3ase activity reside in the plasma membrane. Thus for I-P_3 to act as a second messenger for the hormonal mobilization of intracellular calcium, the *in-vivo* rate of I-P_3 synthesis must exceed the rate of its breakdown at the

plasma membrane. Figure 1 demonstrates that $I-P_3$ breakdown is actually faster than its synthesis in isolated plasma membrane fragmentts. Therefore, one must postulate that inhibitors of the $I-P_3$ase activity located at or near the plasma membrane may inactivate the $I-P_3$ase during calcium-mobilizing agonist activation of the phosphodisterase, in-vivo. Data in this report suggest that spermine, at near physiological levels (38), inhibits $I-P_3$ase activity. The mode of this inhibition is not clear. Yip and Balis (37) demonstrated that polyamine-polyphosphate complexes can act as enzyme inhibitors. Furthermore, it was shown that 2,3-diphosphoglycerate-spermine complex acts as an uncompetitive inhibitor of 2,3-diphosphoglyceric acid phosphatase. Wells and Smith (39) have shown that spermine has the highest affinity of all the polyamines for $PI-P_2$, and three times higher affinity for $PI-P_2$ than calcium. Studies on the regulation of intracellular polyamine levels have focussed primarily on examining the effects on ornithine decarboxylase, the rate limiting enzyme in the polyamine biosynthetic pathway (40). Ornithine decarboxlyase activity is regulated by cAMP (41,42), presumably through a cAMP-dependent protein kinase. These studies show stimulation of ornithine decarboxylase 1-4 hrs following the elevation of cAMP. Therefore, a cAMP-type mechanism for increasing intracellular spermine levels would be too slow to affect calcium mobilization, which occurs only seconds after agonist binding. Thus, the in-vivo

regulation of myo-inositol trisphosphate phosphatase and the possible role of spermine remains unknown.

References

1. Kirk, C.J., Michell, R.H., and Hems, D.A. (1981) Biochem. J. 194, 155-165.
2. Litosch, I., Lin, S.H., and Fain, J.N. (1983) J. Biol. Chem. 258, 13727-13732.
3. Billah, M.M., and Michell, R.H. (1979) Biochem. J. 182, 661-668.
4. Pripic, V., Blackmore, P.F., and Exton, J.H. (1982) J. Biol. Chem. 257, 11323-11331.
5. Rhodes, D., Pripic, V., Exton, J.H., and Blackmore, P.F. (1983) J. Biol. Chem. 258, 2770-2773.
6. Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H., and Kirk, C.J. (1983) Biochem. J. 212, 733-747.
7. Thomas, A.P., Marks, J.S., Coll, K.E., and Williamson, J.R. (1983) J. Biol. Chem. 258, 5716-5725.
8. Seyfred, M.A., and Wells, W.W. (1984) J. Biol. Chem., in press. (Chapter II, this thesis)
9. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.
10. Michell, R.H. (1982) Nature 296, 492-493.
11. Hawthorne, J.N. (1982) Nature 295, 281-282.
12. Buckley, J.T., and Hawthorne, J.N. (1972) J. Biol. Chem. 247, 7218-7223.
13. Penniston, J.T. (1982) Ann. N.Y. Acad. Sci. 402, 296-303.

14. Carafoli, E., and Zurini, M. (1982) Biochim. Biophys. Acta 683, 279-301.

15. Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F., and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077-3081.

16. Streb, H., Irvine, R.F., Berridge, M.J., and Schulz, I. (1983) Nature 306, 67-69.

17. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983) Biochem. J. 212, 473-482.

18. Downes, C.P., Mussat, M.C., and Michell, R.H. (1982) Biochem. J. 203, 169-177.

19. Seglen, P.O. (1976) Methods Cell Biol. 13, 29-83.

20. Seyfred, M.A., and Wells, W.W. (1984) J. Biol. Chem, in press. (Chapter I, this thesis)

21. Emmelot, P., Bos, C.J., Van Hoeven, R.P., and Van Blitterswijk, W.J. (1974) Methods Enzymol. 31, 75-90.

22. Downes, C.P., and Michell, R.H. (1981) Biochem. J. 198, 133-140.

23. Desjobert, A., and Petek, F. (1956) Bull. Soc. Chim. Biol. 38, 871-874.

24. Allan, D., and Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277-286.

25. Aronson, N.N., Jr., and Touster, O. (1974) Methods Enzymol. 31, 90-102.

26. Hill, R.L., and Bradshaw, R.A. (1969) Methods Enzymol. 13, 96-99.

27. DeDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmens, F. (1955) Biochem. J. 60, 604-617.

28. Brightwell, R., and Tappel, A.L. (1968) Arch. Biochem. Biophys. 124, 333-343.

29. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220.

30. Bartfai, T. (1979) in Advances in Cyclic Nucleotide Research, (Brooker, G., Greengard, P., and Robison, G.A., eds.) Vol. 10, pp 219-241, Raven Press, New York.

31. Igarashi, M., and Hollander, V.P. (1968) J. Biol. Chem. 243, 6084-6089.

32. Maeno, H., and Greengard, P. (1972) J. Biol. Chem. 248, 8295-8305.

33. Khandelwal, R.L. (1977) Biochim. Biophys. Acta 485, 379-390.

34. Swanson, M.A. (1950) J. Biol. Chem. 184, 647-649.

35. Hallcher, L.M., and Sherman, W.R. (1980) J. Biol. Chem. 255, 10896-10901.

36. Naccarato, W.F., Ray, R.E., and Wells, W.W. (1974) Arch. Biochem. Biophys. 164, 194-201.

37. Yip, L.C., and Balis, M.E. (1980) Biochemistry 19, 1849-1856.

38. Tabor, H., and Tabor, C.W. (1973) Anal. Biochem. 55, 457-467.

39. Wells, W.W., and Smith, C.D. (1984) Fed. Proc., in press.

40. Williams-Ashman, H.G., Janne, J., Coppoc, G.L., Geroch, M.E., and Schenone, A. (1971) Adv. Enzyme Regul. 10, 225-245.

41. Kudlow, J.E., Rae, P.A., Gutmann, N.S., Schimmer, B.P. and Burrow, G.N. (1980) Proc. Natl. Acad. Sci. 77, 2767-2771.

42. Byus, C.V., and Russell, D.H. (1975) Science 187, 650-652.

SUMMARY

The plasma membrane is believed to be the subcellular site of vasopressin-induced polyphosphoinositide breakdown, however, it has not been adequately documented. In Chapter II of this thesis, it was demonstrated that the plasma membrane is the subcellular site of polyphosphoinositide breakdown in response to vasopressin. Using plasma membrane fragments, it was found that the breakdown was catalyzed by a calcium-dependent, polyphosphoinositide-specific phosphodiesterase. At low ionic strength, the phosphodiesterase was maximally activated at 200 nM free calcium, while at physiological ionic strength, the calcium requirement increased to 110 uM free calcium for maximal activity. This would suggest that under normal in-vivo conditions, the phosphodiesterase is inactive and normal turnover of polyphosphoinositides would probably proceed by a kinase-phosphomonoesterase pathway. However, addition of vasopressin to plasma membrane fragments incubated in buffer containing 200 nM free calcium at physiological ionic strength, activated the phosphodiesterase. Under these conditions, only phosphatidylinositol 4,5-bisphosphate ($PI-P_2$) was broken down, suggesting that in-vivo $PI-P_2$ is the preferred substrate for vasopressin-induced

phosphodiesterase activity. Although these studies were conducted using only vasopressin, the result could be extended to include other calcium-mobilizing hormones. It is reported in the literature that vasopressin acts similarly in the rat hepatocyte as other calcium-mobilizing hormones such as angiotensin II, epinephrine and phenylephrine.

The mechanism by which vasopressin activates the phosphodiesterase is unknown. One could postulate that the binding of hormone to receptor induces a change in the membrane structure allowing calcium to have easier access to the enzyme. A more reasonable mechanism would involve a conformational change in the enzyme upon hormone binding to the receptor, which would then decrease the amount of calcium needed to activate the enzyme. Obviously, more information concerning the nature of the hormone-receptor and receptor-enzyme interactions is necessary. This could be obtained by purifying the enzyme and the vasopressin receptor, and studying their physical properties. The purified proteins could then be used to prepare antibodies, which could be used to probe for the topical relationship of the enzyme and the receptor in the plasma membrane.

The final studies presented in this thesis are perhaps the most interesting and important in terms of the role of phosphoinositides in calcium homeostasis. As was described in Chapter II of this thesis, plasma membranes contain a calcium-dependent, polyphosphoinositide-specific

phosphodiesterase. This enzyme is activated under physiological conditions of calcium and ionic strength, by the calcium-mobilizing hormone, vasopressin. The product of the phosphodiesterase activity acting on PI-P_2, myo-inositol trisphosphate (I-P_3), has been demonstrated in saponin-permeabilized cells to release calcium from non-mitochondrial stores. These two pieces of evidence strongly suggest that I-P_3 may act as a second messenger for the hormone-induced mobilization of intracellular calcium. Therefore, the metabolism of I-P_3 may be very important in regulating intracellular calcium levels.

In Chapter III of this thesis, an I-P_3-specific phosphatase (I-P_3ase) was described in purified plasma membranes. This phosphatase required physiological levels of magnesium for optimum activity, displayed a neutral pH optimum for activity, and was not inhibited by phosphatase inhibitors such as NaF or LiCl. The I-P_3ase activity was confined almost exclusively to plasma membranes, whereas myo-inositol bisphosphate phosphatase was more ubiquitous, with its highest specific activity in the microsomal and cytosolic fractions. The I-P_3ase activity was found to be inhibited by physiological levels of spermine, but not by spermidine or putrescine.

The regulation of I-P_3ase is very important in light of the proposed role of I-P_3 as the second messenger for hormone-induced mobilization of intracellular calcium. The level of I-P_3 does not appear to be regulated by subcellular

compartmentalization. In fact, the plasma membrane contains by far the highest level of I-P_3 synthetic and degradative activity. This would suggest that an inhibitor of the I-P_3ase activity must be localized at or near the plasma membrane in order for adequate levels of I-P_3 to be released. Spermine, at physiological concentrations, was found to inhibit I-P_3ase. Therefore, the regulation of intracellular spermine levels may be linked to I-P_3ase activity and intracellular calcium levels. Alternatively, the calcium mobilizing ability of I-P_3 may be an artifact of the saponin-permeabilized cell system.

Further work on the role of phosphoinositides in calciumm homeostasis and cell differentiation will undoubtedly prove to be both controversial and exciting.