PHOSPHOINOSITIDE METABOLISM IN CULTURED CELLS OF NEURONAL AND GLIAL ORIGIN

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Dedicated to my mother Anne Morris

to whom I owe everything

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Abstract

Phosphoinositides are quantitatively minor phospholipids which are part of an intracellular signal tranduction system with broad effects upon cell function. Phosphoinostide metabolism, especially as relating to availibility of phosphoinositides for participation in signal transduction, was investigated in two cultured cell lines (N1E-115 mouse neuroblastoma, and C6 rat glioma).

The hypothesis that the small portion of cellular phosphoinositide participating in signal transduction might be preferentially recycled within the plasma membrane was tested. The subcellular distribution of all enzymes mediating phosphoinositide turnover was assessed in a purified plasma membrane fraction. A small but significant proportion of PtdIns(4,5)P_2-specific phospholipase C was located in the plasma membrane but only two of the five enzymes required to replace PtdIns(4,5)P_2 (DAG kinase and PtdIns(4)P 5-kinase) also were present. CTP:phosphatidate cytidylyltransferase and PtdIns synthase were located exclusively in a microsomal fraction containing enriched levels of endoplasmic reticulum markers. Thus, DAG from agonist-stimulated cleavage of PtdIns(4,5)P_2, or phosphatidic acid formed from it, must be transferred to the endoplasmic reticulum for conversion to PtdIns. Accordingly, PtdIns resynthesis is not required for phosphoinositide-mediated signal transduction. Plasma membrane of N1E-115 cells is incapable of forming PtdIns from 1-acyl-lysoPtdIns. Microsomal 1-acyl-lysoPtdIns synthase activity is most active with 20:4-CoA. Therefore, the PtdIns deacylation/reacylation cycle does not take place in the plasma membrane and formation of archidonate enriched PtdIns occurs before PtdIns is transported to the plasma membrane.

In vitro activities of many of the enzymes acting upon phosphoinositides are modulated by polyamines. The hypothesis that polyamines regulate phosphoinositide metabolism was investigated using cells labelled in culture with [^{32}P]Pi. Intracellular polyamines increased several-fold when cells were exposed to exogenous polyamines. [^{32}P]Phosphoinositide synthesis was increased in cells exposed to putrescine for 6 hours. *In vitro* experiments confirmed that polyamines stimulate PtdIns synthase. PtdIns(4,5)P_2 turnover was inhibited by spermine. However, neither agonist-stimulated PtdIns synthesis nor agonist-stimulated PtdIns(4,5)P_2 hydrolysis were affected by polyamines.

The biochemical basis of malignant hyperthermia (MH), a disorder leading to hypersensitivity to anaesthetics, was investigated using leukocytes and lymphoblasts from human patients susceptable to MH. Contrary to data from the pig model of the disease, human MH patients had normal levels of Ins(1,3,5)P_3 5-phosphatase.

List of Abbreviations

ATP	adenosine triphosphate
BK	bradykinin
BSA	bovine serum albumin
C6	rat glioma
CMP	cytidine monophosphate
CMP-PtdOH	cytidine monophosphate-phosphatidic acid
CoA	coenzyme A
CTAB	cetyltrimethylammonium bromide
CTP	cytidine triphosphate
DMEM	Dulbecco's modified Eagle's medium
dpm	disintegrations per minute
EDTA	ethylene diamine tetraacetate
EGF	epidermal growth factor
EGTA	ethyleneglycol diamine tetraacetate
FBS	fetal bovine serum
GABA	gamma-aminobutyric acid
GAP-43	growth associated protein 43
GH_3	rat pituitary tumour cells
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Ins	inositol
lod	likelyhood ratio
lysoPtdIns	lysophosphatidylinositol
MH	malignant hyperthermia
N1E-115	murine neuroblastoma
PBS	phosphate buffered saline
PDGF	platelet derived growth factor

Pi	inorganic phosphorus
PKC	protein kinase C
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdIns	phosphatidylinositol
PtdIns(3)P	phosphatidylinositol 3-phosphate
PtdIns(3,4)P_2	phosphatidylinositol 3,4-bisphosphate
PtdIns(3,4,5)P_3	phosphatidylinositol 3,4,5-trisphosphate
PtdIns(4)P	phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
PtdIns-PLC	phosphoinositide-specific phospholipase C
PtdOH	phosphatidic acid
SD	standard deviation
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
v/v	volume/volume

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Introduction

Both C6 glioma and N1E-115 neuroblastoma cells are capable of responding to extracellular agonists through activation of the phosphoinositide signal transduction pathway [7,293,115,459]. Agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) results in formation of at least two intracellular second messengers: diacylglycerol (DAG) which activates protein kinase C, and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) which elicits Ca^{2+} release from intracellular stores [269,35,373,374,411,267,390]. Since at least a portion of the hydrolysis of PtdIns(4,5)P_2 occurs at the plasma membrane [372,427], any factors which limit the amount or availability of PtdIns(4,5)P_2 at the plasma membrane are likely to have profound effects on signal transduction. Several aspects of phosphoinositide metabolism were addressed in this thesis using cultured cells of neural origin.

In 1987 Imai and Gershengorn suggested that phosphatidylinositol (PtdIns) synthesis can take place at the plasma membrane of GH_3 pituitary tumor cells [164]. Local recycling of agonist-sensitive phosphoinositide in the plasma membrane is an attractive hypothesis and is consistent with reports that agonist-generated DAG is preferentially used for PtdIns resynthesis [142,357,102,103]. However, only one of the enzymes necessary for complete phosphoinositide resynthesis (PtdIns synthase) was demonstrated at the plasma membrane of GH_3 cells. To rigorously test this hypothesis in neural cells, the subcellular distributions of all of the enzymes necessary to resynthesize and metabolize phosphoinositides within C6 glioma and N1E-115 neuroblastoma cells were investigated.

Phosphoinositides can also be degraded by phospholipase A_2 in agonist-stimulated cells [295,394]. The products of this reaction are lysoPtdIns and fatty acids. Thus, reacylation of lysoPtdIns at the plasma membrane could also influence the total quantity of phosphoinositides at the plasma membrane. PtdIns is enriched in arachidonate through a series of deacylation-reacylation reactions [149,151,4,82,83]. If this cycle were to exist at the plasma membrane it would impact on the quantity of phosphoinositides in that

membrane and the amount of arachidonate (a precusor of biologically active eicosanoids) present in PtdIns at the plasma membrane. As a test of this hypothesis, the ability of whole N1E-115 cells and isolated cell membranes to acylate 1-acyl-lysoPtdIns was investigated.

The availability of phosphoinositides for signal transduction may also be governed by sequestration of phosphoinositides within the plasma membrane by positively charged biological molecules. Phosphoinositides bind to the naturally occuring polyamines: putrescine, spermidine, and spermine [64,405,265,421]. Furthermore, extensive studies have demonstrated modulation of phosphoinositide metabolism *in vitro* by polyamines (reviewed in [364]). To test the hypothesis that polyamines regulate or modulate phosphoinositide metabolism in whole cells, the effects of increased intracellular polyamine content on phosphoinositide metabolism in N1E-115 cells were investigated under both basal and agonist stimulated conditions.

Malignant hyperthermia (MH) is a serious and often fatal complication of anaesthesia. In this disorder, halogenated anaesthetics trigger abnormal calcium fluxes within cells of susceptible individuals [235,272]. Inbred strains of swine that exhibit similar symptoms have been used as models of the human disorder. Swine susceptible to MH were reported to be deficient in Ins(1,4,5)P_3 5-phosphatase [108], an enzyme that was partially inhibited by halothane. This enzyme degrades Ins(1,4,5)P_3 and limits duration of Ca^{2+} fluxes in cells by this inositol phosphate. Ins(1,4,5)P_3 5-phosphatase levels and the effect of halothane on this activity was assessed in leukocytes and transformed lymphoblasts of humans with MH to determine if the swine and human disorders share the same defect.

Background

I. Structure and distribution of phosphoglycerides

Glycerophospholipids are major components of eukaryotic membranes. The simplest of the glycerophospholipids, phosphatidic acid (PtdOH), is composed of two fatty acids esterified to the sn-1 and sn-2 positions of L-glycerol 3-phosphate (Figure 1). The other glycerophospholipids are formed by esterification of an alcohol group to the phosphate moiety. Esterification of inositol, choline, ethanolamine, serine, and glycerol to the phosphate moiety results in the formation of phosphatidylinositol (PtdIns), phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), and phosphatidylglycerol (PtdGro), respectively. Diphosphatidylglycerol ($PtdGro_2$) is composed of two phosphatidic acid molecules bound to the sn-1 and sn-3 positions of glycerol. Glycerophospholipids are not the only lipids containing phosphorus in eukaryotic membranes. Sphingomyelin (SM), another important phospholipid, is composed of phosphocholine esterified to a long chain amino alcohol to which one fatty acid is covalently attached in amide linkage.

All classes of phosphoglycerides have a negative charge on the phosphate group at pH 7.0. In addition they may have charges due to the nature of the head group. Thus, at pH 7.0 PtdOH, PtdSer, PtdGro, $Ptd(Gro)_2$, and PtdIns are negatively charged whereas PtdCho, PtdEtn, and SM are zwitterions of neutral charge. Phosphoglycerides are amphipathic molecules with polar head groups and non-polar fatty-acyl tails. This property allows phosphoglycerides to form bilayers in which the polar head groups but not the non-polar fatty-acyl tails are exposed to the aqueous phase. As a result, phospholipids are key components of biological membranes.

Each of these different phospholipid classes is a heterogeneous group due to variations in the fatty acids esterified to the sn-1 and sn-2 positions of glycerol. Usually in mammalian cells, the fatty acid esterified to the sn-1 position is saturated whereas the fatty acid esterified to the sn-2 position is often unsaturated.

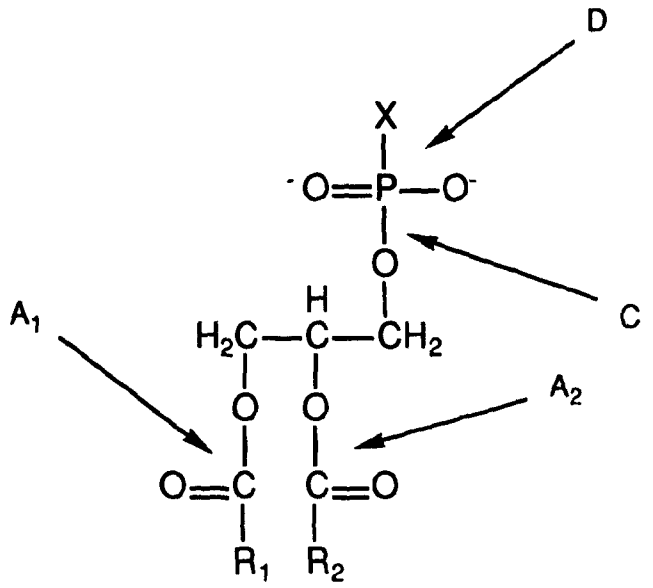


Figure 1. The general structure of phosphoglycerides and site of hydrolysis by phospholipases A_1, A_2, C, and D. R_1 and R_2 are $C_nH_{(2n+1)-2y}$ chains bound in ester linkages to the sn-1 and sn-2 positions of glycerol, respectively (where y is the number of double bonds). X represents a variety of alcohol head groups including inositol, choline, ethanolamine, serine, and glycerol.

Glycerophospholipids can be enzymatically hydrolyzed by various phospholipases. These phospholipases are characterized by which bonds in the phosphoglycerides they cleave. Phospholipases A_1 and A_2 remove the fatty acid residues from the sn-1 and sn-2 positions to generate lyso-phospholipids and free fatty acids. This is an important step in the production of arachidonic acid (a precursor of prostaglandins, thromboxanes, and leukotrienes). Hydrolysis of the bond between the phosphate and glycerol residues is catalysed by phospholipase C. The products of this reaction are diacylglyerol and the phosphorylated head group. Lastly, phospholipase D catalyzes hydrolysis of the bond between the phosphate and head group moieties producing phosphatidic acid and a free alcohol.

Phospholipids, particularly sphingomyelin, are unequally distributed among cellular membranes. The distribution of phospholipids between plasma membrane and microsomes in murine neuroblastoma cells (N1E-115) and rat glioma cells (C6) is shown in Table 1. Asymmetry of phospholipid distribution between inner and outer leaflets of membrane bilayers adds another level of complexity. PtdCho and SM are found mostly in the outer leaflet of plasma membrane whereas PtdEtn, PtdIns, and PtdSer are largely confined to the cytoplasmic side of the membrane [46,298].

Table 1. Distribution of phospholipids in plasma membrane and microsomes of N1E-115 and C6 cells.

	N1E-115 mouse neuroblastoma		C6 rat glioma	
Lipid Fraction	Plasma Membrane	Microsomes	Plasma membrane	Microsomes
Lyso-PtdCho	1.5	n.d.	n.d	3.4
SM	7.9	3.7	16.5	2.2
PtdCho	49.3	56.6	26.0	50.1
PtdSer	4.3	5.4	15.6	27.9
PtdIns	9.9	9.8	3.2	13.3
PtdEtn	23.5	20.1	34.9	19.7
$Ptd(Gro)_2$	3.4	5.2	3.9	1.2

Values are % of phospholipid recovered in subcellular fractions. Data are from Chakravarthy *et al.* [59] and Cook *et al.* [75].

II. Structure, physical and chemical properties of the phosphoinositides

The presence of inositol in biological materials has been known since the nineteenth century [418]. Myo-inositol is an isomer of hexahydroxycyclohexane. There are nine possible isomers; myo-inositol is by far the most common in biological materials. Inositol lipids are composed almost exclusively of myo-inositol. There is, however, evidence that PtdIns glycans, believed to be synthesized from PtdIns [238], contain chiro-inositol, another isomer of inositol [259,226].

When drawn in a Haworth projection, myo-inositol has 3 cis and 3 trans hydroxyl groups. A chair conformation more accurately represents the molecule. The most energetically favorable chair conformation has 1 axial and 5 equatorial hydroxyl groups (Figure 2). Phosphates may be covalently linked to any of the 6 hydroxyl groups. A great deal of confusion concerning the numbering of the carbons in the inositol ring has been caused by strict adherence to old IUPAC nomenclature rules. Under these rules addition or removal of phosphates could necessitate switching between the D- and L- numbering systems. IUPAC now recommends that the D- numbering system be used exclusively. Myo-inositol is numbered in the D- designation such that the axial hydroxyl group is on carbon 2 of the cyclohexane ring and carbon 3 is immediately counterclockwise (counting from above with the axial hydroxyl pointing up, out of the page) and so forth. Carbon number one of the inositol ring is immediately clockwise from the axial hydroxyl [2].

In 1942 Folch and Woolley demonstrated that inositol was present in an alcohol insoluble fraction of brain phospholipids [107]. It is now apparent that there are a variety of lipids that contain inositol moieties including phosphoglycerolipids (known collectively as phosphoinositides [106]), inositol-containing sphingolipids [228], inositolglycan lipids [345], and inositolglycan lipid protein anchors [237]. This thesis will consider the metabolism of phosphoinositides in cultured cells of glial and neuronal origin.

Like other phosphoglycerides, the two acyl chains of phosphatidylinositol are bound to the sn-1 and sn-2 positions of glycerol. Inositol is covalently linked via a

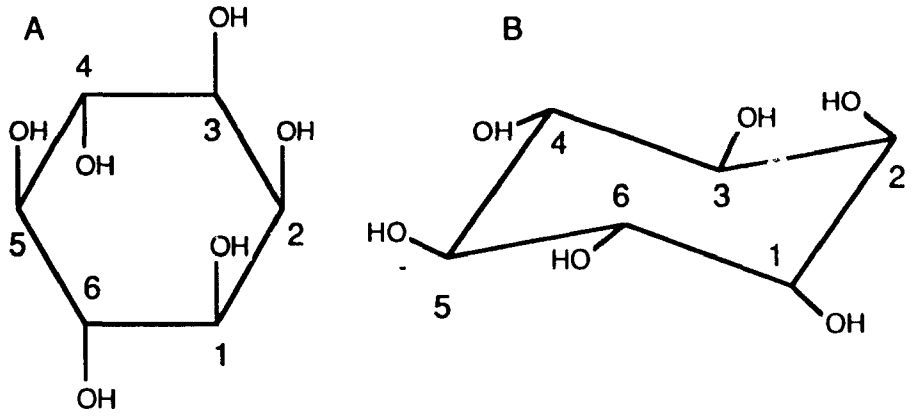


Figure 2. The structure of myo-inositol represented in a Haworth projection (A) and in chair conformation (B). There are 8 other isomers of inositol. They are: scyllo-, D-chiro-, L-chiro-, epi-, neo-, muco-, cis-, and allo-inositol

phosphate diester to the sn-3 position of the glycerol moiety. Inositol is bound to the phosphate via the oxygen on the D-1 position of the inositol ring [328,20]. Phosphatidylinositol may have additional phosphates bound in monoester linkages to the inositol ring. Inositol phospholipids have been found with phosphates bound to the 3, 4, and 5 positions of inositol [420,49,119,448]. The most common phosphoinositides are phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns(4)P), and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Figure 3). Small quantities of phosphoinositides phosphorylated on the D-3 position (PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3) have been found in a number of cell types [256]. These special phosphoinositides appear to be important in cellular responses to mitogens which activate tyrosine kinases [423,270].

Due to their phosphate moieties, the phosphoinositides are anionic at physiological pH. As a result they avidly bind to positively charged ions and molecules. The phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate, bind divalent cations strongly though without much specificity [313,311,312]. In addition, they strongly bind to positively charged organic molecules such as the antibiotics neomycin and gentamycin and the polyamines putrescine, spermidine, and spermine [359,234,64,265,276].

In many tissues and cell types a large proportion of the phosphoinositides possess stearate (18:0) esterified to the sn-1 carbon and arachidonate (20:4) esterified to the sn-2 carbon of glycerol. In rat liver and bovine brain the stearoyl-arachidonoyl species constitutes 73 % and 60 % respectively of total PtdIns molecular species [148,149].

The phosphoinositides (Figure 3) can be interconverted by specific phosphatases and kinases, even under resting conditions. This futile cycle may have important regulatory significance controlling the amounts and types of substrates available to the PtdIns-PLC.

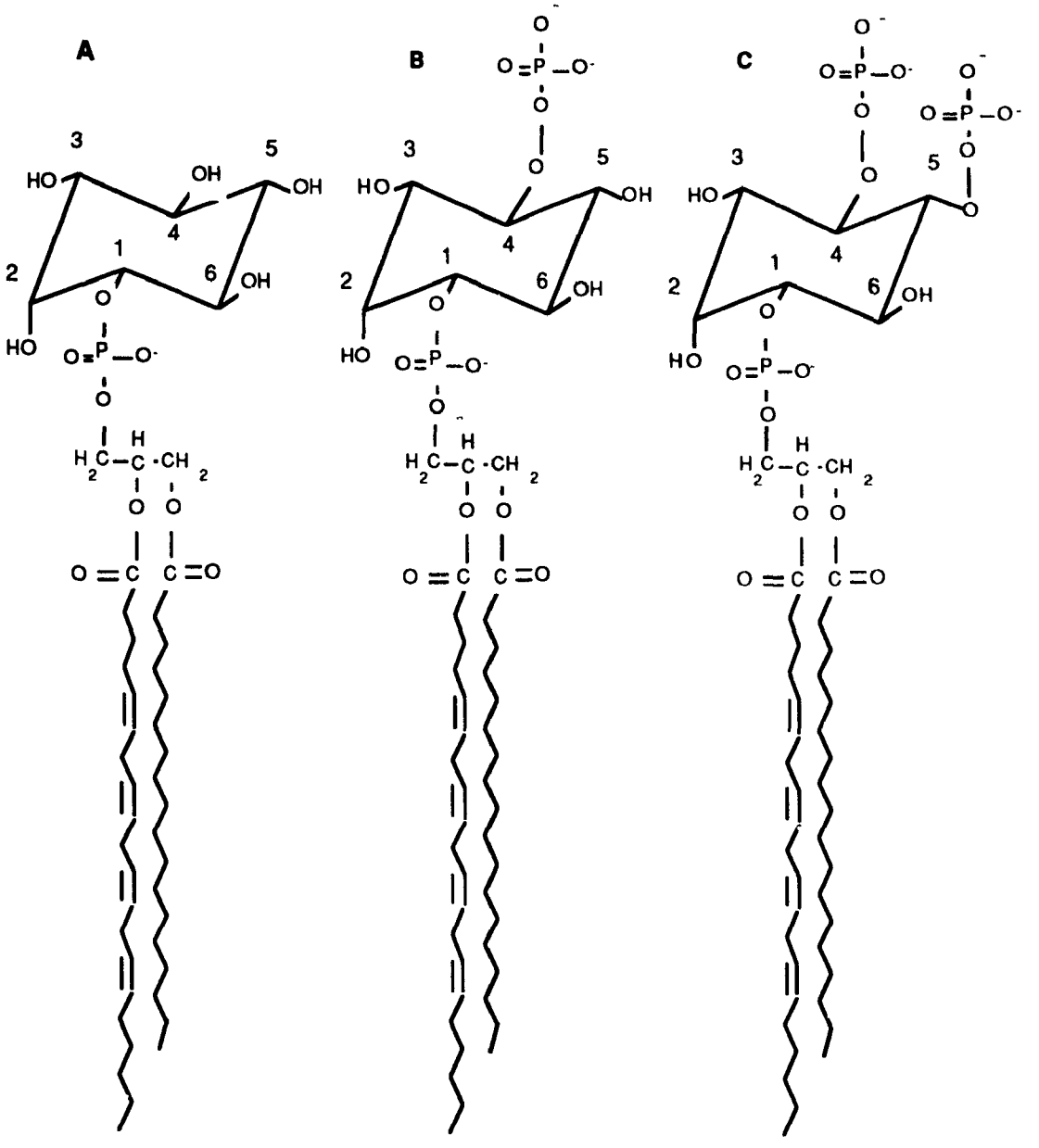


Figure 3. Structures of the phosphoinositides. Phosphatidylinositol (A), phosphatidylinositol 4-phosphate (B), and phosphatidylinositol 4,5-bisphosphate (C). Bond lengths are not drawn to scale.

Despite their importance for signal transduction (to be described below), the phosphoinositides are quantitatively minor components of cell membranes. PtdIns accounts for 7.5 mole % of total phospholipid in mouse N1E-115 neuroblastoma cells [115]. Polyphosphoinositide levels are much lower. Neuroblastoma cells contain 0.7 and 0.6 mole % of total phospholipid as PtdIns(4)P and PtdIns(4,5)P_2 respectively. C6 glioma cells have similar phosphoinositide levels. They contain 10.0, 1.5, and 0.7 mole % of total phospholipid as PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2, respectively [115].

III. Phosphoinositide metabolism

The biosynthetic pathway for the synthesis of phosphatidylinositol was established in the late 1950's by Agranoff and coworkers [3]and by Paulus and Kennedy [318]. In their 1960 paper Paulus and Kennedy [318] suggested that phosphatidylinositol is synthesized in liver according to the scheme:

1. L-α-glycerophosphate + 2 RCO-S-CoA → phosphatidic acid + 2 CoA-SH
2. phosphatidic acid + CTP ↔ CMP-phosphatidic acid + P-Pi
3. CMP-phosphatidic acid + inositol ↔ phosphatidylinositol + CMP.

The existence of this pathway is now well documented although there are alternate routes by which some of the steps occur.

A. Individual steps in PtdIns biosynthesis

1. Formation of phosphatidic acid

Phosphatidic acid (PtdOH) can be formed in many different ways. The first step in *de novo* PtdOH synthesis is formation of lysoPtdOH via acylation of sn-glycerol-3-phosphate to form 1-acyl-sn-glycerol-3-phosphate [211]. Different isozymes of this enzyme exist in microsomes, mitochondria and nuclei [30,14,17]. LysoPtdOH is also synthesized via acylation of dihydroxyacetone phosphate in microsomes and peroxisomes followed by reduction of acyl-dihydroxyacetone phosphate [30,43]. Finally, lysoPtdOH is acylated to form PtdOH using a second acyl-CoA [211,30]. 1-Acyl-sn-glycerol-3-phosphate acyltransferase in brain and liver prefers acyl-CoAs in the order: oleoyl-CoA >

palmitoyl-CoA > arachidonoyl-CoA [457,15,17]. Once formed PtdOH can be used for synthesis of CMP-PtdOH or dephosphorylated to form diacylglycerol for PtdCho, PtdEtn, and triacylglycerol synthesis [43]. Phosphorylation and acylation of monoacylglycerol is another way that PtdOH can be formed [326,377]. As a result, monacylglycerol derived from DAG can be incorporated into PtdIns. Swiss 3T3 cells preferentially incorporate sn-2-arachidonoyl monoacylglycerol into sn-1-stearoyl-2-arachidonoyl phosphatidylinositol [377]. The contribution that this reaction makes to *in vivo* lipid metabolism is not clear but potentially triacylglycerols and phospholipids could be sources of monoacylglycerol.

PtdOH can also be formed by the enzymatic phosphorylation of diacylglycerol by diacylglycerol kinase [141]. Thus, since DAG is a product of phospholipase C mediated phosphoglyceride hydrolysis, DAG can be reused for synthesis of phospholipids through PtdOH. Diacylglycerol kinase has wide-spread distribution within cells. In swiss 3T3 cells there are distinct cytosolic and membrane bound activities [249]. Plasma membranes of GH_3 pituitary tumour cells contain DAG kinase [167]. In rat brain, DAG kinase is present in cytosol, microsomes, and plasma membrane [400]. Furthermore, rat brain diacylglycerol kinase translocates from soluble to membrane-bound compartments in response to membrane diacylglycerol [39]. MacDonald *et al.* have demonstrated the existence of a platelet derived growth factor (PDGF) stimulated membrane-bound diacylglycerol kinase that specifically phosphorylates diacylglycerol containing arachidonate [248,249]. They suggest that this may participate in the formation of arachidonate enriched species of PtdIns. Since DAG is a product of phospholipase C mediated hydrolysis of phosphoinositides, DAG kinase specific for arachidonate rich DAG may serve to isolate phosphoinositide derived DAG from DAG derived from other lipids. DAG kinase is inhibited *in vitro* by spermine [380]. *In vivo* inhibition of DAG kinase could lead to increased DAG levels within cells resulting in potentiation of cellular responses to hormones. Alternately, PtdOH can be formed by the action of phospholipase D on phospholipids [21,61].

2. CTP:PtdOH cytidylyltransferase

The next step in PtdIns formation is the biosynthesis of CMP-phosphatidic acid (CMP-PtdOH). This activated form of PtdOH serves as a donor of PtdOH in the formation of PtdIns [318], PtdGro [204], and Ptd(Gro)$_2$ [156] in mammalian cells. CMP-PtdOH is present in bovine liver at a concentration of 5-17 μmol/kg, less than 1 % of the concentration of PtdOH in this tissue [416]. The low levels of CMP-PtdOH suggests that formation of this lipid may be the rate limiting step in phosphatidylinositol synthesis as well as the synthesis of PtdGro and Ptd(Gro)$_2$. CMP-phosphatidic acid is synthesized by CTP:PtdOH cytidylyltransferase (EC 2.7.7.41), a membrane bound enzyme which catalyzes the reaction;

$$\text{PtdOH} + \text{CTP} \leftrightarrow \text{CMP-PtdOH} + \text{PPi}.$$

Initially, it was reported that CDP-choline was used in the synthesis of CMP-PtdOH but later reports established that CTP was the preferred substrate [3,318].

Genetic studies in yeast have revealed regulation of CTP:PtdOH cytidylyltransferase by regulatory factors that also affect inositol biosynthesis [205,154]. Addition of either choline or ethanolamine to growth medium containing inositol caused yeast cells to have reduced levels of CTP:PtdOH cytidylyltransferase (measured *in vitro*). Choline and ethanolamine did not have any effect in the absence of inositol in the medium. A yeast strain mutant in inositol biosynthesis regulation also lacked regulation of CTP:PtdOH cytidylyltransferase activity by choline and ethanolamine. This indicates a very complex regulatory system involving multiple phospholipid precursors in yeast. The existence of such regulation at the gene level in mammalian cells is unknown.

CTP:PtdOH cytidylyltransferase is present in the endoplasmic reticulum, mitochondria, but not the golgi complex of rat liver [156,434]. Neuronal nuclei also synthesize CMP-PtdOH [414]. Generally, it has been believed that the cytidylyltransferase does not reside in the plasma membrane [139,43].

There is conflicting evidence concerning the role that CMP-PtdOH plays in

determining the final molecular species profile of PtdIns. Newly synthesized PtdOH, CMP-PtdOH, and PtdIns in alveolar macrophage microsomes contain very little 18:0-20:4 species [287]. Newly synthesized PtdIns in rat liver also contains very little 18:0-20:4 species [149,147]. This suggests that, in these tissues, the enzymes involved in *de novo* PtdIns synthesis have little specificity for arachidonate enriched species. Holub and Piekarski studied the specificity of CTP:PtdOH cytidylyltransferase towards different molecular species of PtdOH by using rat liver microsomes which had been endogenously labelled with [^{14}C]glycerol [153]. Newly formed CMP-PtdOH had a fatty acid profile similar to that of PtdOH. Thus CTP:PtdOH cytidylyltransferase of liver is not specific for particular molecular species of PtdOH. In contrast, CMP-PtdOH isolated from bovine brain is enriched in arachidonate [417]. PtdOH does not show similar enrichment [18]. Therefore in brain, either CTP:PtdOH cytidylyltransferase is selective for certain molecular species of PtdOH, CMP-PtdOH participates in deacylation/reacylation cycles, or significant phosphatidate exchange occurs between CMP-PtdOH and PtdIns that has been modified by deacylation/reacylation. Recently Lin *et al.* have reported a bovine brain CMP-PtdOH cytidylyltransferase that is specific for 1-stearoyl, 2-arachidonoyl PtdOH [230]. *De novo* synthesis of 1-stearoyl, 2-arachidonoyl enriched PtdIns may be characteristic of brain whereas in other tissues deacylation/reacylation plays a larger role in determining the final molecular species profile of PtdIns.

3. PtdIns synthase

PtdIns synthase or CMP-phosphatidate:inositol phosphatidyltransferase (EC 2.7.8.11) catalyzes the following reaction;

$$\text{CMP-PtdOH} + \text{Ins} \leftrightarrow \text{PtdIns} + \text{CMP}.$$

Paulus and Kennedy suggested that the reaction mechanism was:

1. CMP-phosphatidic acid + enzyme → enzyme-phosphatidic acid + CMP
2. enzyme-phosphatidic acid + inositol → phosphatidylinositol + enzyme.

The reaction, given the proper conditions, can also run in the reverse direction. This

results in the formation of CMP-PtdOH [143]. Reversal of PtdIns synthase has been demonstrated in mouse pancreas [143] and rabbit lung [45]. In fact during lung surfactant biosynthesis large amounts of CMP are formed (as a result of PtdCho synthesis) and PtdIns mass decreases resulting in more CMP-PtdOH available for surfactant PtdGro synthesis [45]. CMP-PtdOH formed by this route would be enriched in stearoyl-arachidonoyl species. This could also account for the *in vitro* CMP-dependent exchange reaction that has been observed between free and covalently bound inositol [318].

The enzyme has been purified from rat brain, rat liver and yeast [92,408,101]. The magnesium concentration required for maximal activity of the purified brain enzyme is 10 mM. Manganese was substantially less effective than magnesium at the same concentration [92]. PtdIns synthase from rat brain has an apparent K_m of 4.6 mM for Ins. The normal concentration of inositol within cells is 1-3 mM. This suggests that enzyme activity may be determined in part by the concentration of Ins. This may explain the decreased rate of PtdIns synthesis in peripheral nerve of diabetic animals (a condition leading to reduced levels of intracellular Ins due to glucose inhibition of Ins uptake) [120,447,460,92]. PtdIns synthase is sensitive to the acyl chains present in its substrate CMP-phosphatidate. Rat brain PtdIns synthase prefers dipalmitoyl CMP-PtdOH to either dioleoyl, didecanoyl or CMP-PtdOH synthesized from egg lecithin [229].

PtdIns synthase is present in the endoplasmic reticulum but not the mitochondria or golgi complex of rat liver [434]. Using sucrose density centrifugation, Imai and Gershengorn reported the presence of PtdIns synthase in plasma membrane of GH_3 pituitary tumour cells [164]. This is a important finding in terms of the supply of substrate for agonist stimulated phosphoinositide metabolism. In their experiments, they have found that endoplasmic reticulum and plasma membrane contain distinct PtdIns synthase activities, and that, compared to endoplasmic reticulum, the plasma membrane activity has significantly lower apparent K_m values for inositol, CMP-PtdOH, and manganese. Thyrotropin-releasing hormone could stimulate [^{3}H]PtdIns formation in both plasma

membrane and endoplasmic reticulum of cells which had been permeabilized and then resealed in the presence of [^{3}H]Ins. Imai and Gershengorn suggest that this reflects PtdIns biosynthesis in both membranes. Several important points must be emphasized. Plasma membrane incorporation of [^{3}H]Ins into PtdIns occurs in the absence of exogenous CMP-PtdOH and is greatly enhanced by Mn^{2+} [164]. Thus, Mn^{2+}-dependent CMP-PtdOH-independent [^{3}H]Ins exchange (discussed below) may have contributed to apparent PtdIns synthase activity in the plasma membrane of GH_3 cells. Also, Imai and Gershengorn do not report distribution of any nuclear membrane markers in the sucrose gradient that they used. Therefore, contamination of the plasma membrane fraction with nuclear PtdIns synthase is a possibility [16]. Furthermore, agonist dependent appearance of [^{3}H]PtdIns in the plasma membrane may be due (at least in part) to transport of [^{3}H]PtdIns from the endoplasmic reticulum. In view of the above points it is possible that the plasma membrane PtdIns synthase activity in GH_3 cells is overestimated.

Mammalian PtdIns synthase competes for CMP-PtdOH with phosphatidylglycerol phosphate synthase. A Chinese hamster ovary cell line which cannot synthesize Ins accumulates PtdGro in the absence of exogenous Ins [97]. This implies that PtdIns synthase and PtdGro synthase utilize a common pool of CMP-PtdOH.

In addition to *de novo* synthesis of PtdIns via PtdIns synthase, myo-inositol can be incorporated into PtdIns by Ins exchange catalyzed by either PtdIns synthase in the presence of CMP, or by a nucleotide-independent PtdIns:Ins exchange enzyme. Incorporation of radiolabelled Ins into PtdIns can occur when PtdIns synthase acts in reverse to transfer the PtdOH moiety of PtdIns to CMP to form CMP-PtdOH and free Ins. The PtdOH is transfered back to radiolabelled Ins in the forward PtdIns synthase reaction forming labeled PtdIns and CMP. This can be observed if [^{3}H]inositol is added to biological membranes in the presence of $MgCl_2$ and CMP. No net synthesis of PtdIns occurs but radiolabel is rapidly incorporated into PtdIns [37,36].

Another route of inositol incorporation into PtdIns is via PtdIns:Ins exchange operating in the absence of either CMP or CMP-PtdOH. A PtdIns:Ins exchange enzyme has been solubilized and partially purified from rat liver [410,409]. The enzyme has optimal activity with $MnCl_2$ although it has low but detectable activity (< 6 % of maximum) in the presence of $MgCl_2$ [409]. Free Ins stimulates enzyme catalyzed release of [^{3}H]Ins from prelabelled [^{3}H]PtdIns [409], suggesting that Ins exchange is not mediated by a phospholipase D which would hydrolyze PtdIns in the absence of Ins. The physiological role of this nucleotide-independent exchange is not clear since no net PtdIns synthesis occurs. When rat liver membranes are incubated with $MnCl_2$ and [^{3}H]Ins most of the [^{3}H]Ins incorporation was into highly unsaturated molecular species of PtdIns. In contrast, [^{3}H]Ins when administered *in vivo* radiolabels a different, less unsaturated pool of PtdIns [145]. Therefore, it appears unlikely that Mn^{2+}-dependent, nucleotide-independent inositol exchange is very active *in vivo*.

There is some confusion concerning the divalent cation requirements of PtdIns synthase and the Ins:PtdIns exchange enzyme. Early investigators disagreed about whether PtdIns synthase is most active with $MgCl_2$ [3] or $MnCl_2$ [318]. Purified PtdIns synthase from rat brain and rat liver is most active in the presence of $MgCl_2$ [408,92], and many investigators use $MgCl_2$ to assay PtdIns synthase in crude preparations [239,287,16,84]. However, some investigators use $MnCl_2$ [218,229] and some even use both $MgCl_2$ and $MnCl_2$ in PtdIns synthase assays [164,165,77]. Formation of [^{3}H]PtdIns through the CMP-dependent reverse reaction is most active in the presence of $MgCl_2$ but significant activity occurs in the presence of $MnCl_2$ [78,264]. The CMP-independent Ins:PtdIns exchange activity is most active in the presence of $MnCl_2$ [410,409,264].

4. Acyl chain remodelling of PtdIns

The physical and biological properties of membranes can be altered by changes in the fatty acid composition of phosphoglycerides. Furthermore, acylation-deacylation reactions influence the availability and concentration of physiologically active fatty acids. It

is apparent that phosphatidylinositol is extensively remodelled after synthesis. As synthesized from CMP-PtdOH, the fatty acid profile of PtdIns is characterized by the presence of a relatively short saturated fatty acid at the sn-1 position (ie. palmitate), and a longer monounsaturated fatty acid at the sn-2 position (ie. oleate) [147]. This pattern directly reflects the fatty acid composition of both the precursors PtdOH and CMP-PtdOH in liver [147]. However, at steady state the fatty acid profile is very different. Typically, 60 to 80 % of PtdIns has stearate and arachidonate at the one and two positions respectively [148,149,150,131]. This change in fatty acid profile, which may have very important effects on the second messenger function of PtdIns, is accomplished by a cycle of deacylation-reacylation reactions [149,151,4,82,83].

Acylation of lysolipids is an important step in fatty acid remodelling of phospholipids. Several different mechanisms for acylation of lysophospholipids have been proposed. 1. Fatty acyl-CoA:lysophospholipid acyltransferase following ATP-dependent acyl-CoA synthesis from free fatty acid [221,222,193,146,152,344]. 2. Acyl-CoAs can be synthesized by acyltransferase working in reverse [173,84]. This reaction requires CoA but not ATP. Lysophospholipid acyltransferase can then use the acyl-CoA to acylate a lysophospholipid. 3. CoA and ATP-independent transacylation from phospholipid to phospholipid [339].

Acylation of lysophosphatidylinositol (lysoPtdIns) with fatty acyl-CoA occurs in rat liver microsomes [152,173,146], rat brain microsomes [19], myelin [436], and pigeon pancreas microsomes [192,193]. Keenan and Hokin have shown that 1-acyl-lysoPtdIns can be acylated using either palmitoyl-CoA or oleoyl-CoA and that in pigeon pancreas there is a marked preference for acylation with oleate [193]. In rat liver microsomes acylation of 1-acyl-lysoPtdIns can occur by CoA mediated transfer of arachidonate from PtdCho to 1-acyl-lysoPtdIns [173]. The authors suggested that this could be due to reversible acyltransferase activity. However, this may not be the case in platelets. Kramer *et al.* have argued that such CoA-dependent transacylation of 1-acyl-lysoPtdIns with endogenous

arachidonate does not occur to any great extent in platelet membranes and that absolutely none occurs in the absence of CoA [212,213].

Acylation of 1-acyl-lysoPtdIns and 2-acyl-lysoPtdIns has been examined in rat liver microsomes by Holub and Piekarski [146,152]. Acylation of 1-acyl-lysoPtdIns with endogenous fatty acid requires both CoA and ATP (although some reaction may occur in the absence of ATP) [146] indicating that the primary pathway for lyso-PtdIns acylation requires formation of fatty acyl-CoA from free fatty acid. When the specificity for different fatty acyl-CoAs was examined it was found that the greatest reaction rate occurs with arachidonoyl-CoA. Thus, this could be the step which leads to the enrichment of PtdIns with arachidonate at the 2-position. Rat brain microsomes show similar specificity [19]. In studies with 2-acyl-lysoPtdIns, containing predominantly arachidonate, stearoyl-CoA is preferred over palmitoyl-CoA by a factor of almost two [152]. This accounts for enrichment with stearate in the sn-1 position.

1-acyl-lysoPtdIns acyltransferase (E.C. 2.3.1.23) has been purified from bovine heart muscle microsomes [356]. The isolated enzyme is specific for 1-acyl-lysoPtdIns. It is active with a broad range of acylCoAs, although it is most active with long chain unsaturated CoA esters in the order: arachidonoyl-CoA > oleoyl-CoA > eicosadienoyl-CoA > linoleoyl CoA. Little activity occurs with palmitoyl-CoA or stearoyl-CoA [356].

LysoPtdIns acyltransferase may be subject to regulation within cells. Phorbol ester (which activates protein kinase C) induces lysoPtdIns acyltransferase activity using arachidonoyl-CoA in smooth muscle cells [188]. Bradykinin increases phospholipid deacylation-reacylation in rat renal medulla slices [394]. In slices pre-labelled with [^{14}C]arachidonate, bradykinin stimulates a transient decrease in [^{14}C]PtdIns and a transient increase in lysoPtdIns. Phospholipid and lysophospholipid levels return to control values after 10 min [394]. Since phosphoinositides can be metabolized by phospholipase A_2 in agonist stimulated cells [295,394], the distribution of lysoPtdIns acyltransferase activity

within cells may influence the quantity of phosphoinositides present in the plasma membrane for signal transduction.

B. Polyphosphoinositide metabolism

5. PtdIns 4-kinase

In 1949, Folch isolated a phospholipid fraction having a phosphorus to lipid ratio of 2 [105]. This was the first demonstration of the existence of the polyphosphoinositides. We now know that this crude lipid fraction was composed of a mixture of PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2. The first demonstration of a kinase capable of phosphorylating PtdIns was by Colodzin and Kennendy in 1965 [72]. Subsequent work showed that the enzyme PtdIns 4-kinase (EC 2.7.1.67) is widely distributed within cells; it is found in plasma membrane [325], golgi membrane [71], nuclear membrane [66,65,88], lysosomal membrane [56], mitochondrial membrane [381], endoplasmic reticulum membrane [135], endocytic vesicles [56] and cytosol [185] depending on which tissue is examined. PtdIns 4-kinase from human neutrophils prefers ATP as a phosphate donor compared to GTP. Mg^{2+} is the preferred divalent cation although low activity occurs with Mn^{2+}. Ca^{2+} inhibits the enzyme [327].

PtdIns 4-kinase is subject to several types of regulation. Some studies show that the enzyme is feedback inhibited by its product PtdIns(4)P and by PtdIns(4,5)P_2 [327,329]. This suggests that hormone-stimulated breakdown of the polyphosphoinositides may allow PtdIns 4-kinase to be active, thus generating more substrate for the phospholipase C. Other studies show no such inhibition of the enzyme [247] or even increases in enzyme activity in the presence of PtdIns(4)P [166]. There is also evidence that PtdIns 4-kinase is under the control of a small G protein in rat liver membranes [428,429,399,431,430]. This suggests that extracellular agonists may directly stimulate the phosphorylation of PtdIns. Growth factor stimulation of PtdIns 4-kinase has been the subject of many studies. However, earlier studies were done before the existence of PtdIns 3-kinase was known and the product may have been mistakenly identified as

PtdIns(4)P [34,247]. It is now evident that PtdIns 3-kinase is activated by PDGF and EGF in some cell types [274,10,369].

Polyamines may play a role in PtdIns phosphorylation. PtdIns 4-kinase in membranes from A431 cells is activated eight fold by spermidine and spermine [438]. Half-maximal stimulation ocurred at 1.5 mM polyamine. Similar results have been observed in *Xenopus laevis* oocyte membranes [58], human neutrophils [327], rat mast cells [217], and human polymorphonuclear leukocytes [380], although, not in rat liver plasma membranes [241]. Polyamines activate PtdIns 4-kinase *in vitro* by removing the requirement for super-physiological requirements for magnesium [380]. Spermine shifts the Mg^{2+} dependency of PtdIns 4-kinase towards low Mg^{2+} levels so that maximal activity occurs in the physiological Mg^{2+} concentration range. Spermine enhances phosphorylation of PtdIns at low concentrations of PtdIns. Therefore, the spermine-PtdIns complex may be of higher affinity to the kinase than is the Mg^{2+}-PtdIns complex [380].

6. PtdIns(4)P 5-kinase

Phosphorylation of PtdIns(4)P to PtdIns(4,5)P_2 was demonstrated in 1966 by Kai *et al.* [182]. PtdIns(4)P 5-kinase (EC 2.7.1.68) exists in both soluble and membrane bound forms. In rat liver, PtdIns(4)P 5-kinase is localised predominantly at the plasma membrane [69] and is present in myelin [160]. This enzyme is membrane bound in erythrocytes [112]. Some cell types have significant cytosolic PtdIns(4)P 5-kinase activity [67,166]. Recently, PtdIns(4)P 5-kinase has been reported in the nuclear matrix [321].

Little is known about the regulation of this enzyme. PtdIns(4)P 5-kinase like the 4-kinase may be feedback regulated by its product PtdIns(4,5)P_2 [435,166,327]. PtdIns(4)P 5-kinase also prefers ATP over GTP, and Mg^{2+} over Mn^{2+}. It is inhibited by Ca^{2+} [327]. Association with the EGF receptor has been noted, suggesting that PtdIns(4)P 5-kinase may be regulated directly by this receptor [57]. *In vitro* activation of PtdIns(4)P 5-kinase by polyamines has been reported [240,241,380,327,294]. Partially purified PtdIns(4)P 5-kinase from rat brain is stimulated several fold by spermine and spermidine [240].

Spermine lowered the magnesium concentration required for optimal activity of the soluble enzyme to the physiological range. Rat liver plasma membrane PtdIns(4) 5-kinase is stimulated 3-fold by 1 mM spermine *in vitro*; however, there was no large shift in the Mg^{2+} dependency of the enzyme [241].

7. PtdIns(4,5)P_2 5-phosphatase

Shortly after the polyphosphoinositides were first demonstrated, Sloane-Stanley showed that there were enzymes, present in brain homogenates, which caused the release inorganic phosphate from these lipids [379]. PtdIns(5)P does not occur within cells. Therefore, dephosphorylation of PtdIns(4,5)P_2 proceeds first with removal of the phosphate bound to the 5 position of inositol and then removal of the 4 position phosphate [415]. It is apparent that the phosphoinositide phosphatases and kinases form a futile cycle. The phosphomonoester phosphates of PtdIns(4)P and PtdIns(4,5)P_2 turnover extremely rapidly [362,111]. The half-lives of PtdIns(4,5)P_2 and PtdIns(4)P are approximately 1 min in rabbit erythrocyte membranes [111].

Originally, it was thought that dephosphorylation of PtdIns(4,5)P_2 to PtdIns was catalyzed by a single enzyme. Experiments using crude enzyme preparations from rat kidney showed that PtdIns(4)P and PtdIns(4,5)P_2 competed for the same phosphatase [76]. It is now known that there are two different enzymes, one capable of removing the phosphate on position 5 of the inositol ring of PtdIns(4,5)P_2 and one capable of removing the 4-phosphate of PtdIns(4)P.

PtdIns(4,5)P_2 5-phosphatase (EC 3.1.3.36) has been partially purified from both protozoa and human erythrocytes and is clearly distinct from activities removing the 5-phosphate from soluble inositol phosphates. It has no activity towards PtdIns(4)P *in vitro* [343,305]. PtdIns(4,5)P_2 5-phosphatase is largely cytosolic in brain [355,308]. However, activity has also been demonstrated in myelin [355], and plasma membrane [380].

Very little is known about the regulation of the phosphoinositide monoesterases *in*

vivo. *In vitro* PtdIns(4,5)P_2 5-phosphatase activity is inhibited by the polyamine spermine. Activity of the enzyme acting on endogenous PtdIns(4,5)P_2 in plasma membrane of human polymorphonuclear cells is reduced by 50 % in the presence of 0.25 mM spermine [380]. However, PtdIns(4,5)P_2 5-phosphatase activity with either endogenous or exogenous substrate is not affected in rat liver plasma membranes by 1 mM spermine [241].

8. PtdIns(4)P 4-phosphatase

Palmer and Mack demonstrated the existence of a phosphatase in erythrocyte membrane that was capable of hydrolyzing the monoester phosphates of PtdIns(4)P and lysoPtdIns(4)P [251]. This enzyme does not hydrolyze GroPtdIns(4)P, Ins(1,4)P_2, PtdIns(4,5)P_2, or lysoPtdIns(4,5)P_2. In contrast to PtdIns(4,5)P_2 5-phosphatase, this enzyme is not cation dependent. PtdIns(4)P 4-phosphatase (EC 3.1.3.36) is present in the membrane fractions of human, porcine, and lapine erythrocytes [250]. Oddly, ovine erythrocyte PtdIns(4)P 4-phosphatase activity is mainly cytosolic [250].

Little is known about the regulation of this enzyme. Spermine, inhibits PtdIns(4)P 4-phosphatase as measured by an *in vitro* detergent solubilized assay [382]. Inhibition of the enzyme occurs with as little as 0.05 mM spermine. Maximal inhibition (45 %) occurs when the *in vitro* spermine concentration is 5 mM. Spermidine also inhibits the enzyme at the same concentrations but to a lesser degree. On the other hand, 5 mM putrescine stimulates the activity of PtdIns(4)P 4-phosphatase *in vitro* by 30 % [382]. However, results of experiments using rat liver do not show any spermine effect. At 1 mM, spermine does not alter dephosphorylation of endogenous or exogenous PtdIns(4)P in rat liver plasma membrane [241].

9. Phosphoinositide specific PLC

The phosphoinositide specific phospholipases C (PtdIns-PLC) are phosphodiesterases which hydrolyze the glycerophosphate bond of phosphoinositides generating diacylglycerol and an inositol phosphate. These enzymes hydrolyze PtdIns,

PtdIns(4)P and PtdIns(4,5)P_2 to yield 6 water-soluble inositol phosphates corresponding to cyclic 1,2-phosphate and 1-phosphates of inositol mono-, bis-, and tris-phosphate [225]. PtdIns-PLC has wide spread distribution within cells. It has been found in plasma membrane, cytosol, endoplasmic reticulum and nuclei [225,90,183,239,322,88]. Production of cyclic 1,2-inositol phosphates may be a consequence of the mechanism by which PtdIns-PLC acts [35]. PtdIns-PLC catalyzes nucleophilic attack of the phosphodiester bond by water. The 2-position hydroxyl on the inositol ring also has a slight tendency to attack the phosphodiester bond resulting in formation of the observed cyclic products. There are several subtypes of PtdIns-PLC. Three classes (γ, β, δ) of isozymes have been identified on the basis of their primary structure. A fourth class (α) has been tentatively identified. However, PtdIns-PLCα cDNA has not yet been shown to encode a functional enzyme [31]. Comparisons of the corresponding cDNA sequences reveal that the γ, β, and δ subtypes share two regions of high sequence similarity while the rest of the structures varies more freely [190]. There is further variation within each subtype: ie. PtdIns-PLC γ-1 and PtdIns-PLC γ-2. The various PtdIns-PLC subtypes have different *in vitro* substrate specificities and calcium requirements. PtdIns-PLC β is specific for PtdIns(4,5)P_2 at high or low calcium concentrations [191]. In contrast, PtdIns-PLC δ prefers PtdIns(4,5)P_2 and PtdIns(4)P at low calcium concentrations but will readily hydrolyze PtdIns at high calcium concentrations [353]. PtdIns-PLC γ hydrolyzes PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 with equal efficiency [351]. The PtdIns-PLC isozymes also differ in the amount of cyclic products that they produce. The ratio of cyclic to non-cyclic inositol phosphates produced decreases in the order: PtdIns-PLC β > PtdIns-PLC δ > PtdIns-PLC γ [200]. Cyclic 1,2-inositol phosphate production is also dependent on the lipid substrate. The ratio of cyclic to non-cyclic inositol phosphates produced by PtdIns-PLC hydrolysis of phosphoinositides decreases in the order: PtdIns > PtdIns(4)P > PtdIns(4,5)P_2 [200]. Up to 80 % of the inositol phosphate produced by PtdIns-PLC β acting on PtdIns is inositol 1,2-cyclic phosphate (Ins(1:2,cyc)P) [200]. Some reports

suggest that cyclic inositol phosphates are minor products. Only 1-2 % of PtdIns(4,5)P_2 hydrolysis by PtdIns-PLC results in inositol 1:2cyclic,4,5-trisphosphate (Ins(1:2cyc,4,5)P_3) in stimulated rat parotid glands and WRK1 cells [129,454]. The cyclic inositol phosphates are acid labile and as a result may have been under-reported in many cases when cell extracts were prepared under acidic conditions.

The γ family of phospholipase Cs are regulated by tyrosine phosphorylation [290,168,201,199,315]. The other families are probably controlled by G proteins and/or Ca^{2+} (discussed below). When phospholipase γ associates with the PDGF receptor, three tyrosine residues and at least one serine residue in the phospholipase are phosphorylated [341]. Site directed mutagenesis of the tyrosine residues and expression of the resulting gene product in NIH 3T3 cells has revealed that phosphorylation of the tyrosine residues is necessary for optimal activation of the enzyme by the receptor. In one case, replacement of one of the tyrosines with phenylalanine completely blocked activation even though serine phosphorylation still ocurred [199]. Thus, tyrosine phosphorylation regulates phospholipase γ *in vivo*. Phosphorylation by protein kinase C , a serine kinase, also stimulates PtdIns-PLC γ *in vitro* [44].

It has been suggested that, under resting conditions, cellular PtdIns-PLC must be under inhibitory control. *In vitro* studies using purified PtdIns-PLC have shown that even in the absence of activators phosphoinositide supplies would be exhausted in a matter of seconds [6,340]. One could postulate that there is continuous synthesis of PtdIns(4,5)P_2, but on the scale required it would expend a great deal of ATP. Furthermore, without some sort of inhibitory control there would be high basal levels of Ins(1,4,5)P_3 and DAG within cells leading to continuous activation of second messenger systems. Meldrum and colleagues propose two possible scenarios for this suppression of activity [267]. Firstly, PtdIns-PLC could be bound to some endogenous inhibitors which dissociate or become inactive following receptor occupancy. Secondly, PtdIns-PLC could be in an active form within cells but cannot gain access to its substrate until some receptor mediated event

modifies either the enzyme or its substrate.

There is growing evidence of endogenous inhibitors of PtdIns-PLC. The γ class of PtdIns-PLC have src homology domains [320,96,340]. This region has been shown to interact with endogenous regulatory molecules inhibiting the tyrosine kinase activity of the src gene product [320]. Possibly the same or similar molecules interact with PtdIns-PLC affecting its activity [267]. Micro injection of PtdIns-PLC β or γ into quiescent NIH 3T3 cells results in dose dependent induction of DNA synthesis [383]. This ocurred even though total cellular PtdIns-PLC activity was not greatly increased. Thus, injected PtdIns-PLC is different from the endogenous enzyme. The endogenous phospholipase could be sequestered from its substrates or be inhibited by an endogenous inhibitor.

PtdIns-PLC δ is strongly inhibited by sphingomyelin [319]. Physiological concentrations of sphingomyelin in detergent micelle and liposome assays of PtdIns-PLC activity inhibit enzyme activity by 50 %. It has been suggested that sphingomyelin acts as a physiological inhibitor of PtdIns-PLC δ, which enables the enzyme to be regulated by activation [319].

PtdIns-PLC could also be regulated by interference with its ability to interact with its substrate. Although its substrate is membrane bound, most PtdIns-PLC is cytosolic [225,90,183]. PtdIns-PLC translocates from the cytosol to membranes of HER cells in response to epidermal growth factor and platelet derived growth factor where the active enzyme can begin hydrolyzing phosphoinositides [202].

Due to their negative charge the phosphoinositides bind strongly to positively charged molecules. The cytoskeletal protein profilin binds to PtdIns(4,5)P_2 *in vitro* and prevents its hydrolysis by PLC [117]. The polyamines putrescine, spermidine and spermine also bind phosphoinositides [64,265]. In experiments using permeabilized cells, cell lysates, membranes, and purified PtdIns-PLC, polyamines have been shown to positively or negatively modulate *in vitro* PtdIns-PLC activity depending on the concentration of polyamine used. One could hypothesize that polyamines may act as

endogenous modulators of PtdIns-PLC activity, which allow the enzyme to be regulated by receptor mediated activation.

Studies with permeabilized GH_3 cells indicate that polyamines inhibit guanine nucleotide-stimulated phosphoinositide synthesis [451]. Phosphoinositide turnover in polymorphonuclear leukocyte lysates is decreased by polyamines [380]. These two studies suggest that polyamines inhibit PtdIns-PLC activity although they do not rule out other effects such as modulation of G protein activity by polyamines. Soluble PtdIns-PLC from rat brain and mouse pancreatic islets is activated [94,134] whereas soluble PtdIns-PLC from platelets is strongly inhibited by polyamines [286]. PtdIns-PLC from human amnion is activated 3 to 8 fold by polyamines [354]. Purified PtdIns-PLC δ activity from rat liver is modulated by polyamines. Half maximal activation by spermine occurs at 0.15 mM, with optimal effects between 0.2 and 0.5 mM. Spermidine and putrescine stimulate half maximally at 0.9 and 6 mM respectively, with optimum effects at 2 mM and 10 mM respectively [123]. In general, low concentrations of polyamines activate and high (millimolar range) concentrations inhibit PtdIns-PLC.

C. Receptor activated phosphoinositide metabolism

Many cell types including rat glioma and mouse neuroblastoma cells exhibit enhanced phosphoinositide turnover in response to external stimuli [7,293,115,459]. This signalling system has been thoroughly reviewed elsewhere and is schematically represented in Figure 4 [269,35,373,374,411,267,390].

Faced with a wide variety of extracellular signals and having a plasma membrane that is impermeant to most molecules, cells often utilize second messengers to elicit an intracellular response to an extracellular signal. The phosphoinositide cascade is one of these second messenger systems. The cascade begins with binding of an agonist to a membrane bound receptor. Agonists that have their intracellular effect through the G protein linked-phosphoinositide cascade bind to a particular class of receptors. These receptors are similar to rhodopsin in that they are transmembrane proteins with seven

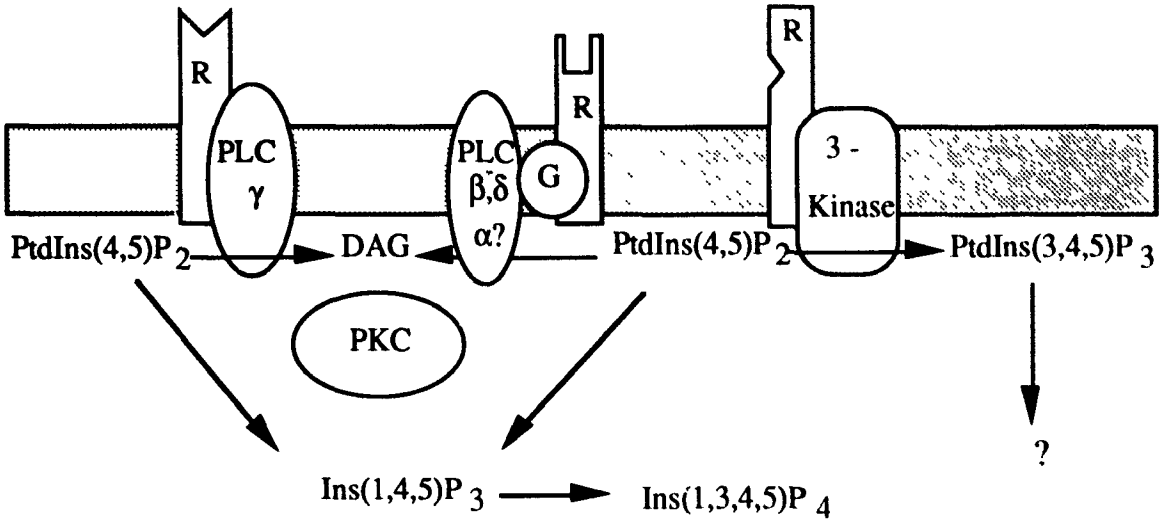


Figure 4. The phosphoinositide second messenger system. Agonist binding to receptors (R) activates PtdIns-PLC through G proteins (G) or by tyrosine phosphorylation of PtdIns-PLC by the activated receptor or an intermediary tyrosine kinase. Hydrolysis of PtdIns4,5)P_2 produces the second messengers DAG and Ins(1,4,5)P_3. DAG is a physiological activator of protein kinase C (PKC). Inositol phosphates mediate Ca^{2+} fluxes within cells. Phosphorylation of PtdIns(4,5)P_2 3-kinase on specific tyrosine residues by an activated receptor results in the formation of PtdIns(3,4,5)P_3 which has unknown cellular effects.

membrane-spanning domains [89,163]. This activated receptor then interacts with a hetero-trimeric ($\alpha\beta\gamma$) guanine nucleotide binding protein termed a G protein. G proteins which interact with these receptors are a large family of highly conserved membrane associated proteins [288,42]. G proteins exist in active and inactive states depending on whether GTP or GDP is bound. Interaction with a receptor which has bound an agonist stimulates exchange of GTP for GDP bound to the α subunit, thus dissociating the α subunit from the $\beta\gamma$ components. The activated GTP-α complex is then free to stimulate phosphoinositide specific phospholipase C until the α subunit's intrinsic GTPase activity hydrolyzes the GTP permitting reassociation of the α subunit with the $\beta\gamma$ components. There are at least 15 distinct genes encoding mammalian α subunits [389]. Alternative splicing of some α subunit transcripts adds even more diversity [38,426].

The first demonstration of G protein mediated activation of a phosphoinositide specific phospholipase C was in stimulated rat mast cells [118]. Histamine release and phosphoinositide hydrolysis is stimulated in permeabilized mast cells by the non-hydrolyzable GTP analogue GTP-γS [118,68]. This nucleotide binds to the G protein α subunit causing long-term stimulation of phosphoinositide hydrolysis. Another clue indicating the involvement of G proteins in phosphoinositide hydrolysis is the ability of pertussis toxin to inhibit the signalling pathway. Pertussis toxin catalyses the ribosylation of some types of α subunits thereby preventing their action. However, this inhibition does not occur in all cases. Bradykinin induced phosphoinositide hydrolysis is inhibited by pertussis toxin in NIH 3T3 cells [155] but not in neuroblastoma cells [109]. Clearly, a variety of G proteins are used by cells and not all of them are pertussis toxin sensitive. Direct activation of phosphoinositide specific phospholipase C by a purified G protein has recently been demonstrated [385]. This novel G protein (Gq, which contains αq) activates PtdIns-PLC β by increasing the affinity for calcium and V_{max}. The αq subunit is pertussis toxin insensitive [310,396]. Purified PtdIns-PLC β, but not PtdIns-PLC γ or PtdIns-PLC δ, is stimulated by the αq subunit [412]. In addition to normal, physiological receptor

activation, bradykinin activates G proteins in a receptor independent fashion [52,284] as do synthetic polyamines and a wasp venom peptide [8]. These compounds interact with a locus on the α subunit of the G protein which also interacts with receptors [137] and lead to inositol phosphate production in mast cells [52].

Recently, it has been reported that polyamines can directly stimulate G proteins [53,51]. *In vitro* GTPase activity of G proteins is stimulated by putrescine, spermidine, and spermine. Rat mast cells exhibit enhanced histamine release and intracellular inositol phosphate production in the presence of extracellular polyamines [51]. These effects can be blocked by pertussis toxin indicating that polyamines are acting at or before G proteins in the signal transduction pathway [51].

Recent evidence indicates that G protein coupled receptors are not the sole means of phosphoinositide specific phospholipase C activation. Receptors that contain tyrosine kinase activity can also stimulate phosphoinositide hydrolysis. The γ class of phosphoinositide specific phospholipase C can be phosphorylated by several tyrosine kinase receptors independently of trimeric G proteins [441,266]. Binding of PDGF to its receptor activates the receptor's intrinsic tyrosine kinase activity and leads to activation of PtdIns-PLC as measured by inositol phosphate production [34].

Phosphoinositide specific phospholipase C will hydrolyze the diester bond in phosphoinositides. The products of the hydrolysis of $PtdInsP_2$ are diacylglycerol and inositol 1,4,5-trisphosphate. These two molecules are the second messengers that propagate the signal within the cell. Diacylglycerol is a known activator of protein kinase C (discussed later). The diacylglycerol produced may serve as a substrate for lipases releasing arachidonic acid, a precursor of physiologically active eicosaniods. Otherwise, DAG may be reused for lipid synthesis. $Ins(1,4,5)P_3$ causes release of calcium from intracellular stores and directly or indirectly induces calcium entry from the exterior (discussed later).

Phosphorylation and dephosphorylation of inositol phosphates turns off the signal

from Ins(1,4,5)P_3 and generates at least one other molecule, inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) with possible effects on cellular calcium levels. Ultimately, inositol is regenerated for resynthesis of PtdIns.

Concomitant with the degradation of phosphoinositides is compensatory synthesis of PtdIns which may persist for many minutes. In some tissues this is due to *de novo* synthesis of complete PtdIns molecules [365,361]. In other systems, only the inositol phosphate moiety shows enhanced turnover suggesting that DAG is recycled back into PtdIns [189,357,102,425,103,242].

D. Phosphoinositide pools

Stimulation of PtdIns(4,5)P_2 specific phospholipase C mediated hydrolysis produces the second messengers diacylglycerol and inositol 1,4,5-trisphosphate. The availability of substrate for the phospholipase C may limit the quantities of diacylglycerol and inositol 1,4,5-trisphosphate produced. The amount of phosphoinositide available for hydrolysis depends on both the initial quantity of phosphoinositide present and the capacity of the metabolic machinery to replace it during the agonist response.

It is important then to determine whether all or only some of the total cellular phosphoinositide turns over in response to agonist (agonist-sensitive phosphoinositide). In 1964 Hokin and Hokin examined [^{32}P]Pi labelled avian salt glands that had been stimulated with acetylcholine and subsequently blocked with atropine, allowing them to study the changes in phospholipid metabolism that took place in response to a short defined stimulus [142]. When acetylcholine was added to the salt glands there was a dramatic increase in radiolabel incorporation into PA and to a lesser extent PtdIns. When the antagonist was added, thus blocking further stimulation of the glands, radiolabel that had appeared in PA decreased while simultaneously label increased in PtdIns. The kinetics suggested a precursor product relationship between these labelled lipids. The actual cellular content of PtdIns did not seem to change. Two implications of these results have received great attention since. Firstly, the phosphoinositide that turned over was just a small pool of the

cellular total. Secondly, diacylglycerol produced by phospholipase C action was phosphorylated and preferentially recycled back into PtdIns. It was soon demonstrated in several cell types that stimulated incorporation of radiolabelled glycerol, oleic acid or linoleic acid into PtdIns was much smaller than incorporation of [^{32}P]Pi or [^{3}H]inositol [189,357,102,425,103,242]. Furthermore, phosphoinositides, DAG, and PtdOH share the same diacylglycerol backbone in thrombin stimulated platelets [260]. These findings suggest that the diacylglycerol backbone of phosphoinositides may be recycled during the agonist response.

In 1973 L. E. Hokin's group [114] demonstrated that stimulation of guinea pig pancreas by secretagogues *in vivo* increased the incorporation of [^{3}H]inositol into lipid. Furthermore, the kinetics of this incorporation indicated that there was a pool of PtdIns that was synthesized in response to the secretagogues that did not mix with the bulk of the cellular PtdIns. Autoradiographic and subcellular fractionation studies in a number of tissues suggested no single discrete location for this increased incorporation of label [224,138,140,1,114], although label was always seen in the endoplasmic reticulum and may have been quickly distributed from there to other locations.

Fain and Berridge have studied compartmentation of phosphoinositide metablolism in blowfly salivary glands. Agonist-stimulation of glands prelabelled for a short time with either [^{32}P]Pi or [^{3}H]inositol resulted in loss of 80 % of the radiolabel in PtdIns with no change in total mass of PtdIns [98]. Also, glands which had been exhaustively pretreated with 5-hydroxytryptamine needed only to resynthesize not more than 1.3 % of the total PtdIns to restore half maximal response. This indicated that the hormone responsive pool of PtdIns was small. Furthermore, this pool was preferentially labelled by radioactive precursors at short incubation times, indicating that it was turning over faster than the bulk of the PtdIns within these glands. Evidence of functional compartmentalization of PtdIns has also been found in smooth muscle [23], erythrocytes [113], pancreatic islets [338], platelets [41,437], brain slices [363], and brain membranes [218].

Some of the strongest evidence in favor of compartmentation of PtdIns has come from the laboratory of Monaco, who labelled WRK1 mammary tumor cells with [^{32}P]Pi in the presence or absence of vasopressin. A second challenge with vasopressin revealed that the PtdIns synthesized in response to the first addition of vasopressin was much more sensitive to the second challenge than was PtdIns synthesized under resting conditions [277]. This pool of hormone sensitive PtdIns was estimated to represent 17% of the total cellular PtdIns. Furthermore, under resting conditions, this hormone-sensitive pool of PtdIns was more labile than the hormone-insensitive pool. It incorporated [^{32}P]Pi at a greater rate than the hormone-insensitive pool indicating that the hormone-sensitive pool turns over more rapidly even in the absence of hormone [279]. PtdIns was not transfered from the insensitive to the sensitive pool [279]. Koréh and Monaco extended these observations by investigating the relationship of hormone-sensitive and insensitive PtdIns to PtdIns(4,5)P_2 [210]. They demonstrated that hormone-sensitive PtdIns is the major precursor of PtdIns(4,5)P_2 within WRK-1 cells and that this PtdIns(4,5)P_2 is likewise hormone-sensitive. The small amount of PtdIns(4,5)P_2 synthesized from the hormone-insensitive pool of PtdIns remained hormone-insensitive.

In a 1987 study, using WRK-1 cells, Monaco demonstrated that incorporation of [^{3}H]inositol into hormone-sensitive and insensitive pools of phosphoinositides does not change with time, nor is incorporation of [^{3}H]inositol into the hormone-sensitive pool stimulated by agonist [278]. Taken with the [^{32}P]Pi results, this is an apparent contradiction. According to the accepted biosynthetic pathway for PtdIns biosynthesis, [^{32}P]Pi and [^{3}H]inositol should enter into PtdIns at the same rate. Monaco counters that CTP-dependent synthesis of PtdIns could show differential incorporation of the two radiolabels if the radiolabelled precursors equilibrate with their intracellular pools at different rates. No loss of prelabelled [^{3}H]phosphoinositide in response to agonist is seen unless lithium chloride is present in the incubation medium. Li^{+} is an uncompetitive inhibitor of inositol phosphate phosphatase and inositol polyphosphate 1-phosphatase

[125]. This suggests that [^{3}H]inositol derived from [^{3}H]inositol phosphates released following phospholipase C activation is preferentially and rapidly reincorporated into PtdIns unless lithium is present to inhibit inositol monophosphate phosphatase.

Label from inositol could enter PtdIns by three routes; CTP-dependent *de novo* synthesis, manganese dependent exchange and CMP-dependent exchange of inositol catalyzed by PtdIns synthase. The pool of agonist insensitive PtdIns is also the one which participates in the inositol exchange reaction. Addition of manganese to the medium greatly enhances incorporation of [^{3}H]inositol into PtdIns by exchange of free inositol with the head group of PtdIns; no net synthesis results. Manganese stimulates [^{3}H]inositol incorporation into a hormone-insensitive pool of PtdIns [278,218,363].

A cyclic system whereby diacylglycerol produced by phospholipase C action on the hormone-sensitive pool is recycled back into the agonist sensitive pool is central to most hypotheses of a metabolically distinct pool of phosphoinositides. The best evidence that PtdOH derived from phosphorylation of DAG is recycled back into PtdIns is based on differences observed in the rate of incorporation of glycerol, inositol, fatty acids and phosphate [142,357,102,103]. Interpretation of the data from those experiments is limited by the unknown kinetics of entry and equilibration of the precursors used. Importantly, it is clear that the total mass of PtdIns does not change significantly under conditions in which the rate of [^{32}P]Pi incorporation dramatically increases.

Results obtained by Monaco's group with WRK-1 cells have been questioned. Michell and co-workers have performed experiments with WRK-1 cells using [^{3}H] and [^{14}C]inositol to examine pooling of PtdIns. They labelled cells to isotopic equilibrium with [^{14}C]inositol and only briefly with [^{3}H]inositol to search for functional heterogeneity within these cells. After exposure to an agonist, the ratio of [^{3}H] to [^{14}C] in inositolphosphates produced in response to agonist reflected the radiolabelling of total cellular PtdIns [271,245,246]. This would seem to indicate that there is no functionally distinct, rapidly turning over pool of PtdIns in these cells. However, they have recently

determined that the difference between their results and those of Monaco's group may be due to an unexplained effect of insulin in the tissue culture medium [203]. Therefore, it is difficult to see exactly how their early results compare with those of Monaco's group. It seems that, as both groups have suggested, the likelihood of observing functional compartmentation of PtdIns depends on the choice of radioactive precursors used and their kinetics of entry and equilibration within cells [271,278].

E. Diacylglycerol as a second messenger

1. Protein kinase C activation

The second messenger action of diacylglycerol produced on hydrolysis of PtdIns(4.5)P_2 by phospholipase C is important to the understanding of signal transduction. Diacylglycerol is believed to be a physiological activator of a family of calcium- and phospholipid-dependent, serine/threonine specific protein kinases collectively known as protein kinase C. They are identified on the basis of these functional criteria or on the basis of structural similarity.

According to a model proposed by Bell, protein kinase C docks to a cluster in the membrane formed by PtdSer and Ca^{2+} [29]. Binding of diacylglycerol to this complex drastically reduces the calcium requirement for enzyme activation. This process occurs in several steps [26]. The first step is the formation of an non-productive enzyme-Ca^{2+}-phospholipid complex [27]. Several Ca^{2+} ions may be involved and the apparent affinity of the enzyme for Ca^{2+} is greatly enhanced by phospholipid [28]. The second step is binding of DAG (or phorbol ester) which leads to activation of PKC, presumably through a conformational change in the protein structure. There is, however, a component of cellular PKC activity which is only extractable with detergents and may be constitutively active PKC [26]. PKC can also associate with detergent insoluble cell fractions. This includes association with the cytoskeleton [198] and with the nucleus [257].

PKC was initially identified by Nishizuka's group in 1977 [169]. PKC can be reversibly stimulated by diacylglycerol in the presence of phosphatidylserine and calcium

[406,407]. It soon became apparent that multiple forms of PKC exist, sometimes within the same cells. Grouping the isozymes according to their amino acid sequence (predicted from cDNA sequence) reveals the presence of at least nine forms of PKC [157,390,291]. The functional significance of these different isozymes is currently an area of intense investigation. No large differences have been observed in the diacylglycerol requirements of the various PKC isozymes [390]. However, their calcium requirements vary greatly. The α, β and γ isoforms bind to membranes in a calcium dependent manner [453], while ϵ and δ isoforms bind independently of calcium [198,297].

Evidence is emerging that the different PKC isozymes have different substrate specificities. For example it is now known that the α, β and γ isoforms have different affinities for the EGF receptor and GAP-43 [161,375]. With respect to the inositol second messenger system, it has been shown that inositol 1,4,5-trisphosphate 3-kinase and possibly inositol 1,4,5-trisphosphate 5-phosphatase are regulated by protein kinase C. The V_{max} of inositol 1,4,5-trisphosphate 3-kinase decreases by 75 % following phosphorylation by PKC [376]. The data concerning activation of inositol 1,4,5-trisphosphate 5-phosphatase is contradictory. Connolly and coworkers have shown that protein kinase C phosphorylates the 5-phosphatase and that this increases its activity [73]. However, other studies show no effect on the 5-phosphatase [302,195].

Activation of protein kinase C with phorbol esters generally causes inhibition of the hydrolysis of phosphoinositides [194,352,158,316,458]. However, this may not be the case in all tissues [40]. Since diacylglycerol is produced by the action of phospholipase C, phosphorylation of phosphoinositide specific phospholipase C by PKC could represent a form of feedback regulation. Furthermore, this feedback regulation differentially affects the phospholipase C isoforms. It has been reported that treatment of cells with phorbol ester (which stimulates PKC) results in phosphorylation of PLC-β but not PLC-γ or PLC-δ. Phosphorylation of PLC-β did not change its *in vitro* activity but may alter its interaction with a putative guanine nucleotide binding protein thereby preventing activation of the

PLC-β [352].

Naturally occurring polyamines modulate *in vitro* PKC activity. *In vitro* protein kinase C activity is inhibited by polyamines non-competitively with respect to Ca^{2+} and PtdSer [336]. Polyamines greatly interfere with the formation of the active membrane-associated enzyme complex formed between inside-out erythrocyte membrane vesicles and purified protein kinase C [281].

2. PtdIns and PtdCho as sources of DAG

Phosphatidylcholine (PtdCho) is the major phospholipid in mammalian cells. At one time PtdCho was believed to be metabolically inert compared with the phosphoinositides. However, it is the largest potential source of DAG and arachidonate within the cell. There is now growing evidence that PtdCho turnover in response to agonists generates diacylglycerol over a much longer time period than does phosphoinositide turnover [433,432].

A PtdCho-specific phospholipase C has been partially purified from canine myocardium [452]. PtdCho-specific phospholipase C has been shown to be important in: cell growth [282], in the action of ras [219,333] and src oncogenes [86], and cellular responses to vasopressin [446,55], endothelin and bradykinin [433,432].

Several important caveats must be kept in mind when reviewing this literature. To date, the identification of PtdCho as a source of DAG has relied on comparisons of DAG molecular species with the molecular species present in PtdIns and PtdCho. As Kennerly [197] points out several important assumptions are made in these studies. First, they assume the molecular species of DAG is not significantly altered by fatty acid remodelling. Secondly, the molecular species of metabolically active PtdIns and PtdCho are similar to those of the whole cell mass of these lipids. Third, the molecular species of PtdIns(4)P and PtdIns(4,5)P_2 are similar to those of PtdIns. In fact there are small differences in the molecular species pattern of polyphosphoinositides in bovine brain. PtdIns(4,5)P_2 has significantly more 18:1-20:4 and 18:0-20:3 than PtdIns [148]. The issue of whether the

molecular species pattern of metabolically active PtdIns and PtdCho is similar to that of the whole cell mass of these lipids is important because phospholipids may be specifically enriched in certain fatty acids depending on their subcellular location. A point not addressed by Kennerly is that in the studies by van Blitterswijk *et al.* [433,432] and the studies by Kennerly [196,197], identification of the source of DAG is based on the observation that the ratio of saturated fatty acid to arachidonate is much higher in PtdCho than it is in PtdIns (van Blitterswijk *et al.* used [^{3}H] arachidonate and [^{14}C] palmitate labelling whereas Kennerly used mass determinations). Palmer *et al.* have shown that agonist stimulated phosphatidylinositol synthesis, presumably linked to turnover, continues in N1E-115 cells for at least 30 minutes [309]. If this turnover involves newly synthesized PtdIns, then the resulting DAG might have a fatty acid profile similar to that of PtdIns as it is synthesized *de novo*, rich in palmitate at the one position and oleate in the two position [147]. Therefore, at least some DAG produced could be derived from newly synthesized PtdIns instead of PtdCho.

Macara [244] has questioned the results of Lacal *et al.* [219] and Price *et al.* [333] in which they identified phospholipase C action on PtdCho on the basis of formation of phosphocholine and DAG. Elevated phosphocholine concentrations in ras-transformed NIH 3T3 cells arise from increased choline kinase activity, not PtdCho hydrolysis. It should be noted that detecting hydrolysis of PtdCho by measuring the loss of label from PtdCho is likely to be very insensitive given the large mass of PtdCho within cells and the comparatively small quantity of DAG produced.

van Blitterswijk *et al.* [432] have suggested that DAG production from PtdCho occurs mainly by phospholipase D and PtdOH phosphohydrolase, although initially it is produced by both phospholipase C and D. They conclude that the PtdIns-PLC and PtdCho-PLC are suppressed by activated protein kinase C and that the degradation of PtdCho by phospholipase D and PtdOH phosphohydrolase is stimulated by protein kinase C.

F. Inositol phosphates as second messengers

1. Metabolism of inositol phosphates

In 1983 Streb and coworkers demonstrated that inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) added to permeabilized acinar cells elicited release of calcium from intracellular stores [398]. Calcium fluxes occur within cells on a time scale consistent with the production and metabolism of $Ins(1,4,5)P_3$ [32,91,268]. Furthermore, there are specific $Ins(1,4,5)P_3$ receptors. The number of different $Ins(1,4,5)P_3$ receptors is uncertain but at least 2 forms can be derived from alternative splicing of mRNA [81]. Purified $Ins(1,4,5)P_3$ receptors have been reconstituted into lipid vesicles where they bind $Ins(1,4,5)P_3$ and as a result mediate calcium fluxes [100]. On the basis of these and other experiments it is now commonly accepted that $Ins(1,4,5)P_3$ formed as a product of the agonist stimulated hydrolysis of $PtdIns(4,5)P_2$ is a signal eliciting the release of calcium from intracellular stores into the cell cytosol.

The subsequent metabolism of $Ins(1,4,5)P_3$ is complex (Figure 5). It can be phosphorylated on the 3-position generating inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$), a compound which also has calcium mobilizing abilities. Alternately, it is dephosphorylated by $Ins(1,4,5)P_3$ 5-phosphatase forming inositol 1,4-bisphosphate ($Ins(1,4)P_2$). $Ins(1,4)P_2$ is inactive with regard to calcium mobilization. Many phosphorylation and dephosphorylation reactions have been observed producing a wide variety of inositol phosphates. In addition, as noted above 1:2-cyclic inositol phosphates are formed by PtdIns-PLC. Compared to $Ins(1,4,5)P_3$, $Ins(1:2cyc,4,5)P_3$ is at least 10 times less effective a promoter of calcium release [450,227]. Thus, the physiological role of cyclic inositol phosphates is not clear. One interesting observation is that transfected cells over-expressing inositol 1,2-cyclic phosphate 2-phosphohydrolase (the only enzyme known to hydrolyze the cyclic bond) grow to a lower density at confluence than control cells [348]. Thus, cyclic inositol phosphates may have a role in regulating cell growth. These compounds are metabolized only slowly and as a result they can accumulate in

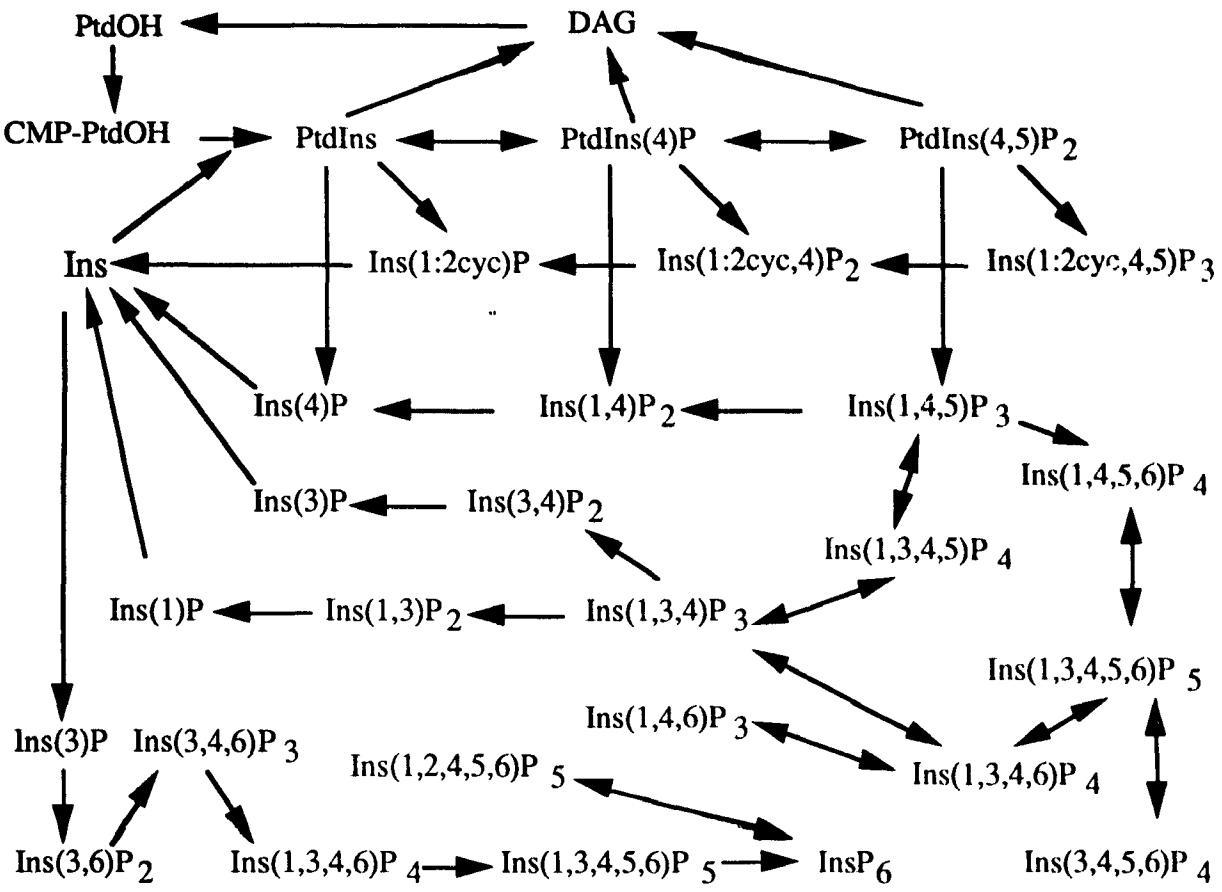


Figure 5. The phosphoinositide cascade and inositol phosphate metabolism in animal cells. All inositol phosphates shown are D-isomers. Not all of these pathways necessarily exist in all tissues or organisms. Figure is adapted from reviews by Rana and Shears [337,373,374].

stimulated cells even though their rate of production is low [159]. The recently discovered inositol lipids phosphorylated on the 3 position are very poor substrates for PtdIns-PLC and do not contribute to receptor mediated accumulation of inositol phosphates [370].

Evaluation of the role of Ins(1,3,4,5)P_4 has been complicated by experiments which did not account for contamination of Ins(1,3,4,5)P_4 with Ins(1,4,5)P_3, metabolism of Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3, or competition between Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 for the 5-phosphatase enzyme [411]. However, specific Ins(1,3,4,5)P_4 binding sites have been identified in a number of tissues and they are clearly distinct from Ins(1,4,5)P_3 receptors [95]. Most data from experiments using Ins(1,3,4,5)P_4, suggest that Ins(1,3,4,5)P_4 acts together with Ins(1,4,5)P_3 to regulate calcium entry into the cell [334,335,171,172].

The metabolism of Ins(1,4,5)P_3 is regulated in several ways. The 3-kinase is activated by calcium/calmodulin and inhibited by protein kinase C and A. Evidence for the regulation of the 5-phosphatase is scant or contradictory [374]. However, it is inhibited *in vitro* by polyamines [371].

2. Intracellular calcium stores

The nature of the intracellular organelles from which calcium is released in response to Ins(1,4,5)P_3 is a subject of great controversy. Originally, endoplasmic reticulum was identified as the source of calcium. More recently, it has been proposed that a specialized calcium storing organelle, termed the calciosome, is the Ins(1,4,5)P_3 sensitive calcium store in non-muscle cells [439]. This identification has now been questioned.

When Ins(1,4,5)P_3 was first shown by Streb *et al.* [398] to cause the release of calcium from intracellular organelles, it was believed that the only organelles capable of storing calcium in non-muscle cells were endoplasmic reticulum and mitochondria. Streb *et al.* showed that the Ins(1,4,5)P_3-sensitive pool was capable of storing and releasing calcium in the presence of inhibitors of mitochondrial function; hence, it was assumed that

the endoplasmic reticulum was the Ins(1,4,5)P_3-sensitive store of calcium. This was supported by subcellular fractionation results indicating that the Ins(1,4,5)P_3-sensitive pool co-purified with endoplasmic reticulum markers [330,331,180,332,25,397]. More recently, however, several groups have clearly separated calcium uptake, Ins(1,4,5)P_3 binding and Ins(1,4,5)P_3 induced calcium release from endoplasmic reticulum markers. This has been achieved in a wide variety of cell types including neutrophils [214], parotid acinar cells [136], HL60 cells [439], as well as adrenal cortex [349], brain [5] and liver cells [215]. In these studies the Ins(1,4,5)P_3 receptor is associated with a light membrane fraction, possibly plasma membrane or some unique smooth membrane compartment that is tightly associated with the plasma membrane. Immunocytochemical analysis has shown that the Ins(1,4,5)P_3 receptor is enriched in a special structure of stacked smooth-membrane cisterae associated with the rough endoplasmic reticulum of Purkinje cells. Neither calcium pump nor calcium-binding proteins are seen in the stacked cisternae and the function of the Ins(1,4,5)P_3 receptor in these sites remains a mystery [358,243]. The identity of the intracellular organelles from which Ca^{2+} is released by Ins(1,4,5)P_3 are unclear. Clearly, Ins(1,4,5)P_3 intracts with specific receptors which are also Ca^{2+} channels and allows Ca^{2+} to be released from intracellular calcium stores.

3. Calcium fluxes and waves

The calcium fluxes elicited by Ins(1,4,5)P_3 are temporally and spatially complex. Receptor mediated calcium fluxes have been observed to occur within cells as spikes [455] and waves [346]. Various models have been proposed to account for these observations. They fall into two basic types. One series of models propose that Ins(1,4,5)P_3 levels are regulated by feedback and vary with the local calcium concentration [411]. Indeed, calcium does regulate Ins(1,4,5)P_3 levels [128] and the agonist-specific shapes of the calcium spikes are best explained in this manner [456]. The second type of model suggests that sustained increases in Ins(1,4,5)P_3 concentration cause cyclical release of calcium. For example, complex calcium fluxes could occur if Ins(1,4,5)P_3 causes calcium mobilization

and then the Ins(1,4,5)P_3 receptor desensitizes. The calcium stores could then resequester calcium until the receptor resensitizes and the sustained high level of Ins(1,4,5)P_3 causes it to discharge again. These models have been further complicated by suggestions that the initial rise in calcium concentration causes calcium uptake into either Ins(1,4,5)P_3-sensitive or insensitive stores and that calcium-dependent calcium release occurs [33,275].

G. Novel polyphosphoinositides

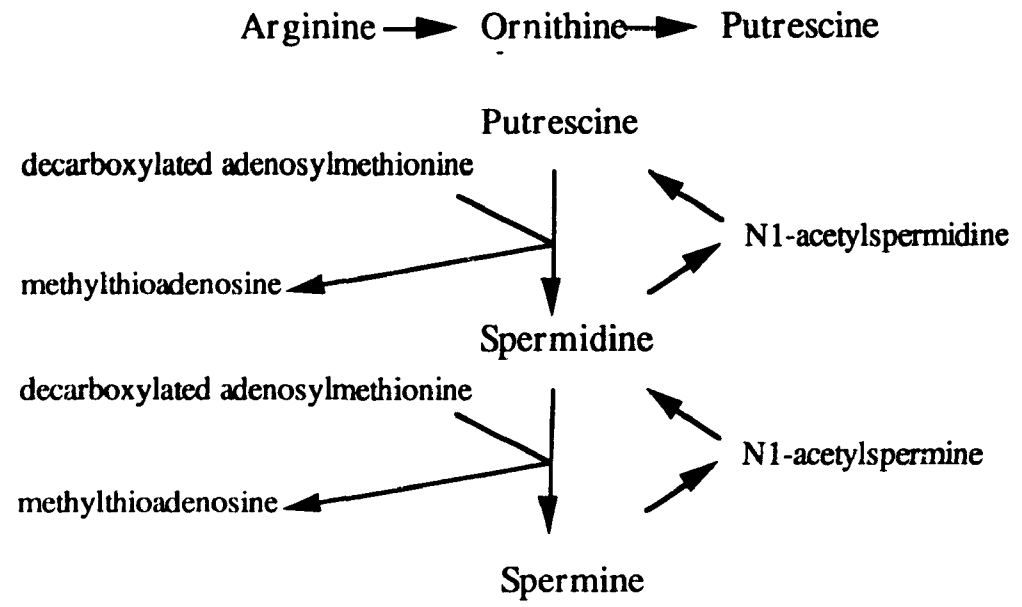
Recently, three novel polyphosphoinositides have been identified. All three have a phosphomonoester on the three position of the inositol ring. The combined action of phosphoinositide 3-kinase and the better characterized 4- and 5-kinases produces PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [423,448,424]. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are not found in unstimulated cells but are produced following cell stimulation by growth factors whose receptors are, or which activate, tyrosine kinases [423,10,369,216]. The 3-phosphate containing phosphoinositides are very minor cell components. Based on radiolabel incorporation they represent only 1 % of the levels of the other polyphosphoinositides [392,424,216]. All three of these phosphoinositides can be produced by the action PtdIns 3-kinase acting on the appropriate substrate *in vitro* [280]. However, the *in vivo* route for synthesis of PtdIns(3,4,5)P_3 is a matter of some controversy. One way to study this is to very briefly label cells with [^{32}P]Pi such that the phosphomonoester groups on inositol lipids are not yet in equilibrium with the γ phosphate of ATP. The last phosphate to be added to any phosphoinositide will have the highest specific activity. In thrombin stimulated platelets and PDGF stimulated NIH 3T3 cells the specific activities of the phosphomonoester groups suggest that the 3-kinase acts first followed by phosphorylation by 4- and 5-kinases producing PtdIns(3,4,5)P_3 [79,80]. Similar experiments with stimulated neutrophils suggest that PtdIns(3,4,5)P_3 is formed by 3-phosphorylation of PtdIns(4,5)P_2 [393]. The reason for this apparent contradiction is not clear. Perhaps there are tissue specific routes for PtdIns(3,4,5)P_3 synthesis. Significantly, phosphoinositides phosphorylated on the 3 position do not appear to be good

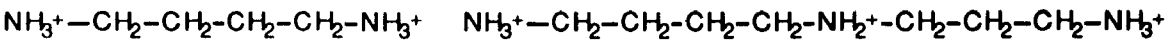
substrates for PtdIns-PLC [370]. The only known route for degradation of 3-phosphorylated inositol lipids is through a 3-phosphomonoesterase [231].

A possible clue as to the function of these lipids comes from the association of the 3-kinase with tyrosine kinases. PtdIns 3-kinase associates with immunoprecipitates of polyoma middle T/pp60c-src and ligand-activated PDGF receptor suggesting that it could be important in the transduction of signals by these two tyrosine kinases; perhaps the phospholipid serves as a signal itself [449]. Majerus and colleagues have suggested an interesting hypothesis concerning the function of 3-phosphate containing phosphoinositides [256]. PtdIns(4,5)P_2 binds to both profilin and gelsolin thus promoting actin polymerization [178,117]. However, the time course of thrombin stimulated actin polymerization is such that rapid actin polymerization takes place when PtdIns(4,5)P_2 levels are falling. On the other hand, PtdIns(3,4,5)P_3 levels do rise rapidly on thrombin stimulation. Thus, PtdIns(3,4,5)P_3 may serve to promote actin polymerization.

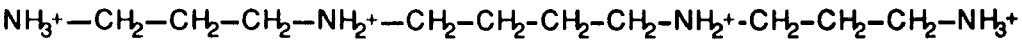
H. Polyamines and the inositol cycle

Three polyamines, putrescine, spermidine, and spermine, are ubiquitous components of eukaryotic cells. Their structures are shown in Figure 6. The polyamines are synthesized in mammalian cells by the pathway in figure 6; reviewed by Tabor and Tabor [401]. The first step is the formation of ornithine and urea from arginine by arginase. Decarboxylation of ornithine to putrescine (1,4-diaminobutane) is catalysed by ornithine decarboxylase, a pyridoxal phosphate-dependent enzyme. Spermidine is synthesized by addition of an aminopropyl group to putrescine. The aminopropyl donor is decarboxylated S-adenosylmethionine. Mammalian cells contain very low levels of decarboxylated S-adenosylmethionine; its synthesis is activated by putrescine and inhibited by spermidine [324]. Formation of decarboxylated S-adenosylmethionine is the rate limiting factor in spermidine synthesis [324]. Addition of another aminopropyl group results in spermine, the longest and most highly charged of the three polyamines. The polyamines can be interconverted by the action of specific N-acetyltransferases and an





Putrescine Spermidine



Spermine

Figure 6. Structure and metabolic relationships of the polyamines: putrescine, spermidine, and spermine.

FAD-dependent polyamine oxidase [324,367]. Acylation of spermine with acetyl-CoA yields N1-acetylspermine. Oxidative cleavage, whereby the aminopropyl residue which originated from decarboxylated S-adenosylmethionine is removed, results in the formation of spermidine. If the initial substrate was spermidine, the product of this pathway is putrescine. Acetylation of polyamines forming N8-acetyl-polyamine derivatives takes place in the nucleus [368]. Acetylation is a means whereby the net positive charge of polyamines is decreased thus liberating them from anionic binding sites such as DNA, RNA, negatively charged protein domains, and anionic lipids [367]. In addition, polyamines are degraded by the action of Cu^{2+}-dependent amine oxidases [324]. Putrescine can be oxidized by diamine oxidase yielding γ-aminobutyraldehyde which can be further oxidized to γ aminobutyrate (GABA). Brain has low levels of diamine oxidase; therefore the predominate route for polyamine degradation in brain is via oxidation of monoacetylated putrescine [366]. This pathway also results in GABA formation and therefore can influence neurotransmission. Lastly, intracellular polyamines can be transported out of cells. In humans, polyamines are excreted unchanged in urine [130].

Ornithine decarboxylase is the principle point at which polyamine synthesis is regulated. Ornithine decarboxylase levels are very low in quiescent cells and increase manyfold in response to a number of agonists including nerve growth factor [121], epidermal growth factor [391], acetylcholine [99], and insulin [122]. The induction of ornithine decarboxylase is regulated at the transcriptional level [350] and is stimulated by cyclic AMP in both glioma and neuroblastoma cells [12]. Treatment of cultured cells with phorbol esters, which activate protein kinase C, also induces ornithine decarboxylase synthesis [292] and hence leads to accumulation of polyamines. Intracellular polyamine concentrations change during the cell cycle [285]. Putrescine content is increased 5.5 fold in postischaemic rat cerebral cortex [317,85]. Concentrations also rise in cancer. Tissue putrescine and spermine increase 4 and 1.2 fold, respectively, in human esophageal tumors. Serum polyamine concentrations are doubled in these cancer patients [236].

Polyamines are actively transported into cells. Transport of polyamines is increased in response to a number of stimuli, such as adrenocorticotrophic hormone, acetylcholine and phorbol ester [99]. Transport is subject to feedback regulation. Mouse neuroblastoma NB-15 cells have a sodium-dependent transport system that is inhibited when the intracellular content of polyamines is high [342]. Transport of putrescine by NB-15 cells is repressed during the course of differentiation [63]. Transport of polyamines is under hormonal control in bovine adrenocortical cells [99]. ACTH, angiotensin, acetylcholine or phorbol ester stimulate uptake of putrescine and spermine by adrenocortical cells.

The naturally occurring polyamines, putrescine, spermidine and spermine, are attractive candidates as regulatory molecules for phosphoinositide metabolism. Their protonated amine groups are positively charged at physiological pH. Polyamines are present in high concentrations within mammalian cells (0.1-2mM) [347,403,401,445]. Furthermore, these concentrations can vary depending on cell growth, differentiation and hormonal stimulation [62,133,99]. Polyamines are actively taken up by cells [342] and are releasable from nerve terminals during neuronal excitement in a calcium-dependent manner [127]. It has been shown that polyamines bind tightly to negatively charged phospholipids such as PtdIns and PtdSer [64] as well as to polyphosphoinositides [405,265,421]. As noted above polyamines modulate *in vitro* activities of a number of enzymes involved in phosphoinositide mediated cell signalling including: PtdIns 4-kinase [438,58,327,380], PtdIns(4)P 5-kinase [240,241,380,327], PtdIns(4,5)P_2 specific phospholipase C [94,286,354,380,451,123], protein kinase C [336,281], PtdIns(4,5)P_2 5-phosphatase [380], PtdIns(4)P 4-phosphatase [382], and inositol 1,4,5-trisphosphate 5-phosphatase [371].

Due to their cationic nature the polyamines share many characteristics with inorganic cations such as magnesium and calcium. However, there are important differences. Polyamines have multiple charges distributed at fixed lengths along a flexible carbon chain. The amine groups on each end of spermine are separated by 1.6 nm [232].

The whole molecule has a net charge of approximately 3.5 at pH 7.4 [181]. As such, polyamines can interact with membranes in ways that inorganic cations do not. They can bridge distances between charged lipids and proteins in biological membranes and modify the electrostatic potential of membranes. It is conceivable that polyamines cluster, and possibly phase separate, negatively charged phospholipids within the membrane.

Once bound to biological membranes polyamines can change electrostatic potentials thereby affecting the activity of membrane bound enzymes and enzymes whose substrates are membrane bound. The surface potential of biological membranes containing 20 % negative phospholipids is approximately -40 mV in 100 mM NaCl [263]. Some of the aspects of cation-membrane interactions can be accounted for by the Gouy-Chapman-Stern theory of membrane electrostatics [262]. According to calculations based on this theory, non-adsorbed spermine at physiological pH is 30 times more effective than divalent cations at changing the surface potential of membranes. In fact, some spermine adsorbs to membranes and may lay flat across them thus changing the charge density of the membrane rather than simply screening the charge [64]. Thus, the effect on surface potential is even greater. These surface effects may explain the ease with which polyamines lower divalent cation requirements for some *in vitro* enzyme assays [380] in which ions serve dual functions of enzyme activation and moderation of surface charge.

A wide variety of abilities have been ascribed to the polyamines. They can interact with biological membranes thus stabilizing and protecting them. Added polyamines stabilize bacteria and protoplasts against osmotic shock [255,388]. Exogenous polyamines cause a dose-dependent immobilization of membrane phospholipids in *Escherichia coli* possibly by bridging between phospholipids or proteins [388]. Polyamines, through their phospholipid binding capabilities may constitute a passive cellular defence mechanism against lipid peroxidation [404]. Their effects on membrane-bound enzymes *in vitro* are many. In addition to those already described, other lipid metabolizing enzymes such as microsomal sn-glycerol-3-phosphate acyltransferase and 1,2-diacyl-sn-glycerol

acyltransferase are stimulated by spermine [174,175,24]. Polyamines stimulate the *in vitro* activity of phosphatidate phosphohydrolase and CTP:phosphocholine cytidylyltransferase [176,177]. Polyamines also inhibit some reactions. Prostaglandin E_2 synthesis is inhibited by spermine in a phospholipid-dependent manner [162].

In some systems intracellular calcium fluxes are mediated by polyamines. Submillimolar concentrations of spermine and spermidine in the presence of Mg^{2+} stimulate Ca^{2+} uptake by mitochondria [179]. A rapid and sustained increase in intracellular polyamines mediates testosterone induced calcium fluxes across plasma membranes and efflux from intracellular calcium stores in kidney cortex [209]. Polyamine levels rise because of activation of a latent membrane-bound form of ornithine decarboxylase. Similar results have been observed in K^+ depolarized synaptosomes and in response to β-adrenergic agonists [170,208]. These results suggest that polyamines serve in some circumstances as intracellular second messengers.

I. Malignant hyperthermia and the inositol cycle

Malignant hyperthermia (MH) is a rare but often fatal complication of general anesthesia with halogenated anesthetics such as halothane. It is characterized by rapidly rising body temperature, increased production of CO_2 and lactate, and ultimately by severe cardiovascular distress. It is one of the main causes of death due to anaesthesia [47]. This syndrome is found in both humans and in swine [124]. In humans the reported incidence of MH varies from 1 in 12 000 in children to 1 in 40 000 in adults [48,386]. In humans, the trait is inherited in an autosomal dominant fashion whereas in swine inheritance of the halothane sensitivity is autosomal recessive or co-dominant [47]. The swine and human disorders present very similar symptoms and it has been proposed that they reflect the same biochemical defect [296]. In this disorder, halogenated anaesthetics trigger abnormal calcium fluxes within cells of susceptible individuals [235,272].

Because of the serious nature of the disorder, much work has been done on diagnostic procedures for detection of MH before anaesthetics are used. This has been

hampered by confusion concerning the nature of the biochemical defect or defects leading to halothane sensitivity. A number of diagnostic tests for MH have been proposed; reviewed in [300]. Serum creatine kinase levels are sometimes increased in individuals susceptible to MH. However this is unreliable and non-specific. Tests linking susceptibility to MH with factors such as plasma cholinesterase levels, platelet nucleotide depletion, human leukocyte antigen type, electrophysiological measurements, muscle ATP depletion, adenylate kinase deficiency, adenylate deaminase deficiency, calcium uptake by sarcoplasmic reticulum, and heat production, have proved to lack either universality, sensitivity or specificity [300]. A successful diagnostic test based on contractures induced in muscle biopsies by caffeine and halothane has been developed and is widely used [299]. A better diagnostic procedure using material that is easier to obtain was described by Klip and coworkers. They observed that exposure of lymphocytes from MH susceptible individuals to halothane increased intracellular Ca^{2+} levels as compared to control [207]. They have also made the same observation with inbred pigs, thus supporting the argument that the human and pig disorders are the same [206].

The biochemical basis for this halothane dependent increase in intracellular Ca^{2+} in swine appears to be a deficiency in Ins(1,4,5)P_3 5-phosphatase [108]. Swine susceptible to MH have 10 fold higher Ins(1,4,5)P_3 levels compared to controls. Ins(1,4,5)P_3 5-phosphatase activity in the sarcoplasmic reticulum of MH susceptible swine is considerably lower than in control animals while myoplasmic activity (which is much lower than sarcoplasmic activity) is unaffected. Halothane inhibits the activity of myoplasmic Ins(1,4,5)P_3 5-phosphatase thus exacerbating the enzyme deficiency in the MH susceptible animals. Decreased ability to dephosphorylate Ins(1,4,5)P_3 would result in elevation of Ins(1,4,5)P_3 levels and prolongation of Ca^{2+} release from intracellular stores. This in turn could result in manifestation of the malignant hyperthermia phenotype. Presumably, in the absence of halothane myoplasmic Ins(1,4,5)P_3 5-phosphatase levels are sufficient to prevent excessive Ca^{2+} fluxes even though the sarcoplasmic reticulum Ins(1,4,5)P_3 5-

phosphatase levels are low in MH susceptible animals. It should be noted that the suggestion that Ins(1,4,5)P_3 is a physiological regulator of Ca^{2+} in skeletal muscle is contradicted by experiments in which injection of heparin (that blocks Ins(1,4,5)P_3 induced Ca^{2+} release) does not affect myoplasmic Ca^{2+} signals in skeletal muscle [314]. Furthermore, skinned muscle fibers respond weakly to added Ins(1,4,5)P_3 [442]. Thus, the significance of the Ins(1,4,5)P_3 5-phosphatase deficiency in MH swine is not clear.

Thesis Investigations

The phosphoinositides are quantitatively minor components of mammalian cell membranes. However, through the formation of second messengers they play a central role in cellular responses to a number of extracellular stimuli. As the preceding sections have demonstrated, the metabolic pathways for phosphoinositide synthesis and degradation are well established. The regulation of these pathways is less well understood. Particularly, the regulation of phosphoinositide substrate levels for agonist-stimulated phosphoinositide-specific phospholipase C is not well documented in whole cells. This is true from the point of view of phosphatidylinositol synthesis and control of the phosphoinositide kinases and phosphatases. This thesis investigates the subcellular distribution of the enzymes involved in polyphosphoinositide metabolism and their regulation by polyamines.

As agonist-stimulated phosphoinositide hydrolysis occurs in the plasma membrane, it is important to assess the capacity of the plasma membrane to resynthesize substrates for the phospholipase C. The degree to which plasma membrane can participate in PtdIns synthesis from DAG or from lysoPtdIns is of great interest. Acylation of lysoPtdIns also reflects part of the deacylation-reacylation pathway. The nature and subcellular localization lysoPtdIns acylation in N1E-115 cells was investigated.

The biochemical basis of malignant hyperthermia in humans was investigated with the aim of determining if cells from malignant hyperthermia-susceptible individuals could be used as a model to determine whether coupling of inositol phosphate production and calcium release from intracellular organelles is altered.

I. Objectives

The overall objective of this thesis has been to examine the control of phosphoinositide levels in cultured mammalian cells. In particular, the hypothesis that the small portion of cellular phosphoinositide participating in signal transduction might be preferentially recycled within the plasma membrane was tested in rat glioma (C6) and

mouse neuroblastoma (N1E-115) cells in culture. Also examined was the hypothesis that polyamines play a regulatory role in the phosphoinositide cycle. The following specific questions were asked:

1. Do isolated plasma membranes from C6 and N1E-115 cells contain the enzymes necessary for PtdIns synthesis (DAG kinase, CTP:PtdOH cytidylyltransferase, and PtdIns synthase)?
2. What is the subcellular distribution of enzymes involved in polyphosphoinositide synthesis and degradation?
3. Does the plasma membrane have the capacity to acylate lysophosphatidylinositol?
4. What are the characteristics of lysoPtdIns acylation in microsomes?
5. Does exposure to exogenous polyamines affect basal phospholipid metabolism, particularly phosphoinositide metabolism?
6. Does exposure to exogenous polyamines affect agonist stimulated phosphoinositide metabolism?
7. Do polyamines affect *in vitro* PtdIns synthesis?
8. Is inositol 1,4,5-trisphosphate 5-phosphatase deficient in patients susceptible to malignant hyperthermia?

II. Cultured cells as a model

Several different cell lines were used to investigate phosphoinositide metabolism. C6 glioma cells derived from a rat glial tumour and N1E-115 cells derived from a mouse neural tumour were used as a model system of phospholipid metabolism. Lymphoblast cell lines were used to examine Ins(1,4,5)P_3 5-phosphatase levels in cell lines derived from individuals susceptible to malignant hyperthermia. Cultured cells offer several advantages for investigation of phosphoinositide metabolism. They are considerably simpler than whole animals or even isolated tissues. Experiments can be performed under defined metabolic conditions which can be easily manipulated. There are, however, disadvantages to transformed cell models. These cell lines are derived from tumours or are transformed

with a virus, and great care must be exercised when results obtained in culture are extrapolated to normal non-transformed cells. Also, the relative homogeneity of these cultured cell lines, while offering several advantages, represents a reductionist model of whole tissues and cannot accurately model events where there is interaction between multiple cell types and tissues existing in different micro-enviroments.

III. Experimental approach

The subcellular distribution of enzymes involved in phosphoinositide metabolism was studied by fra onation of cell membranes using Percoll density gradient centrifugation. Enzyme assays were performed using isolated subcellular fractions under optimized conditions in which reaction rates were constant.

In vitro effects of polyamines on PtdIns synthase catalyzed inositol head group exchange and phosphatidylinositol synthesis, and on nucleotide-independent inositol exchange, were studied using [^{3}H]inositol incorporation into cell lysates.

Effects of polyamines on phospholipid metabolism were investigated by culturing cells with exogenous polyamines under conditions that were demonstrated to lead to increased intracellular polyamine levels. Addition of inorganic [^{32}P]Pi to the culture medium allowed phospholipid metabolism to be observed in pulse and pulse-chase conditions.

To assess the capacity of these cells to acylate lysophosphatidylinositol, in culture and *in vitro* experiments were performed. Acylation of [^{3}H]lysoPtdIns was followed both in culture by adding [^{3}H]lysoPtdIns to the culture medium under a variety of conditions and determining to nature and location of radioactivity incorporated into the cells. *In vitro* acylation was measured using subcellular fractions obtained by Percoll den.ity gradient centrifugation.

The possibility that human malignant hyperthermia is due to a deficiency in inositol 1,4,5-trisphosphate 5-phosphatase was tested in blood samples from individuals susceptible to malignant hyperthermia. *In vitro* assays of inositol 1,4,5-trisphosphate 5-

phosphatase activity were performed in leukocyte and lypmphoblast cell preparations. The effects of halothane and succinylcholine on enzyme activity were tested.

Materials and Methods

I. Materials

A list of the radioactive compounds, their specific radioactivities, and suppliers is given in Table 2. A list of non-radioactive chemicals and materials and their suppliers is given in Table 3. High specific activity γ-[^{32}P]ATP was prepared by Dr. Jason Hoffman, Dalhousie University [443]. $PtdInsP_2$ was prepared from bovine brain by Rita Breckon [304] and used for the enzymic preparation of PtdInsP [303].

Table 2. Radiolabelled compounds

Radioisotope (specific activity)	Source	Address
[2-^{3}H]AMP (15 Ci/mmol)	Dupont NEN products	Mississauga, ON
[2-^{3}H]inositol (16.2 Ci/mmol)	Dupont NEN products	Mississauga, ON
[^{3}H]putrescine (35.6 Ci/mmol)	Amersham	Oakville, ON
[5-^{3}H]CTP (20 Ci/mmol)	Amersham	Oakville, ON
[^{32}P]ATP	Dr. Jason Hoffman	Dalhousie
[^{32}P]Pi (9000 Ci/mmol)	Dupont NEN products	Mississauga, ON

Table 3. General chemicals and materials

Material	Source	Address
1-Butanol	Fisher Scientific Co.	Dartmouth, NS
1,10 diaminodecane	Sigma Chemical Co.	St. Louis, MO
Acetic Acid	BDH	Dartmouth, NS
Acetone	BDH	Dartmouth, NS
Acetonitrile	Fisher Scientific Co.	Dartmouth, NS
AMP	Sigma Chemical Co.	St. Louis, MO
ATP	Sigma Chemical Co.	St. Louis, MO
$Ba(OH)_2$	Fisher Scientific Co.	Dartmouth, NS
Bicinchoninic acid	Pierce Chemical Co.	Rockford, Ill
BSA	Pierce Chemical Co.	Rockford, Ill
$CaCl_2$	J.T. Baker	Phillipsburg, NJ
Chloroform	Fisher Scientific Co.	Dartmouth, NS
CMP	Sigma Chemical Co.	St. Louis, MO
CMP-PtdOH	Sigma Chemical Co.	St. Louis, MO
CTAB	BDH	Dartmouth, NS
CTP	Sigma Chemical Co.	St. Louis, MO
Cytochrome c	Sigma Chemical Co.	St. Louis, MO
sn-1,2-diacylglycerol	Serdary Research Labs	London, ON
Dansyl Chloride	Pierce Chemical Co.	Rockford, Ill
Diethyl ether	Anachemica	Rouses Pt., NY
Dithiothreitol	BDH	Dartmouth, NS
DMEM	Flow Laboratories	Mississauga, ON
EDTA	Sigma Chemical Co.	St. Louis, MO

EGTA	Sigma Chemical Co.	St. Louis, MO
Ethanol	Fisher Scientific Co.	Dartmouth, NS
Ethanolamine	Sigma Chemical Co.	St. Louis, MO
Fetal bovine serum	Flow Laboratories	Mississauga, ON
Fiske Subarow reagent	Fisher Scientific Co.	Dartmouth, NS
Fluorescamine	Sigma Chemical Co.	St. Louis, MO
LiCl	J.T. Baker	Phillipsburg, NJ
HCl	BDH	Dartmouth, NS
Hepes	Sigma Chemical Co.	St. Louis, MO
HPLC column RP-18	Beckman Instruments	Montreal, PQ
Inositol	Sigma Chemical Co.	St. Louis, MO
iso Butanol	Fisher Scientific Co.	Dartmouth, NS
KCl	Fisher Scientific Co.	Dartmouth, NS
KCN	J.T. Baker	Phillipsburg, NJ
KOH	Fisher Scientific Co.	Dartmouth, NS
Lactate dehydrogenase kit	Sigma Chemical Co.	St. Louis, MO
Methanol	Fisher Scientific Co.	Dartmouth, NS
$MgCl_2$	J.T. Baker	Phillipsburg, NJ
$MnCl_2$	Fisher Scientific Co.	Dartmouth, NS
NaCl	BDH	Dartmouth, NS
NADPH	Sigma Chemical Co.	St. Louis, MO
NaOH	Fisher Scientific Co.	Dartmouth, NS
Newborn calf serum	Flow Laboratories	Mississauga, ON
NH_4OH	Fisher Scientific Co.	Dartmouth, NS
Perchloric acid	Fisher Scientific Co.	Dartmouth, NS
Percoll	Pharmacia Chemicals	Montreal, PQ
Petroleum ether	Fisher Scientific Co.	Dartmouth, NS

Phospholipase A_2	Sigma Chemical Co.	St. Louis Mo
Potassium Oxalate	Fisher Scientific Co.	Dartmouth, NS
Proline	Sigma Chemical Co.	St. Louis, MO
$Ptd(Gro)_2$	Serdary Research Labs	London, ON
PtdCho	Serdary Research Labs	London, ON
PtdEtn	Serdary Research Labs	London, ON
PtdGro	Serdary Research Labs	London, ON
PtdIns	Serdary Research Labs	London, ON
PtdOH	Serdary Research Labs	London, ON
PtdSer	Serdary Research Labs	London, ON
Putrescine	Sigma Chemical Co.	St. Louis, MO
Ready Safe Liquid scintillation cocktail	Beckman Instruments	Montreal, PQ
Sodium borate	Fisher Scientific Co.	Dartmouth, NS
Sodium citrate	Sigma Chemical Co.	St. Louis, MO
Spermidine	Sigma Chemical Co.	St. Louis, MO
Spermine	Sigma Chemical Co.	St. Louis, MO
Sucrose	Sigma Chemical Co.	St. Louis, MO
TLC plates G	Fisher Scientific Co.	Dartmouth, NS
TLC plates HL	Analtech	Newark, DE
Tricine	Sigma Chemical Co.	St. Louis, MO
Triethylamine	BDH	Dartmouth, NS
Tris	Sigma Chemical Co.	St. Louis, MO
Triton X-100	Sigma Chemical Co.	St. Louis, MO
$ZnSO_4$	Fisher Scientific Co.	Dartmouth, NS

II. Methods

A. General methods

1. Cell culture

C6 rat glioma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). N1E-115 murine neuroblastoma cells were grown in DMEM containing 5% FBS and 5% newborn bovine serum. All cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells for use in subcellular fractionation studies were grown in 150 cm^2 flasks containing 45 ml of culture medium. Otherwise, cells were grown in 60 mm dishes containing 6.5 ml of culture medium. Cells were harvested at confluence. Lymphoblastoid cell lines were prepared by Robert Zwicker. Lymphocyte preparations were exposed to Epstein-Barr virus [289]. This virus transforms B lymphocytes. Subsequent treatment with cyclosporin A prevents suppression of the transformed cells by suppressor T cells and cytotoxic T cells [289]. This procedure results in establishment of permanent lymphoblastoid cell lines which can be kept in continuous culture.

2. TLC

Thin layer chromatography was performed in pre-equilibrated paper-lined tanks at room temperature. Several different solvent systems were used.

Separation of LysoPtdCho, SM, PtdCho, PtdSer, PtdIns, PtdEtn, PtdGro and $Ptd(Gro)_2$ was achieved by chromatography on silica gel G plates developed in chloroform/ethanol/triethylamine/water (4:5:4:1) for 100 min (system A) [422]. PtdOH comigrates with PtdEtn. Phospholipid bands were visualized by spraying the plate with molybdenum blue [87].

For the separation of PtdInsP and $PtdInsP_2$, samples were plated on activated silica gel HL plates. Before use plates were briefly immersed in 2.5% potassium oxalate and air dried. The dry plates were then activated in an oven at 110 °C for 30 min and allowed to cool to room temperature in a dry box. The plates were developed in

chloroform/ethanol/acetic acid/water (80:30:26:24:15) until the solvent reached the top of the plate (system B). Phospholipid bands were visualized by exposing the plate for 1-2 min to iodine vapour.

3. Protein assay

Protein in subcellular fractions was determined by the use of a fluorescamine based assay [378]. Freshly prepared aliquots of subcellular fractions containing 10-50 µg of protein were mixed with 3.5 ml of borate buffer (12.5 mM, pH 8.4). Fluorescamine in acetone (0.5 ml, 0.3 mg/ml) was added and the samples were left at room temperature for at least 10 min before fluorescence was measured in an Amico-Bowman fluorimeter at 390 nm excitation and 475 nm emission.

The bicinchoninic acid reagent was used for the determination of protein in methanol/water cell extracts [384]. This reagent is used to detect proteins via the biuret reaction. 10 - 50 µl aliquots of sample were mixed with 200 µl of BCA reagent. After several h, protein was detected by measuring absorbance at 562 nm in a Biorad microtitre plate reader. For all protein assays standard curves were constructed using BSA.

4. Phosphorus determination

When phospholipids were used as substrates in enzyme assays it was necessary to determine their concentration by measuring phosphorus content. Phosphorus was determined by measuring the formation of a coloured complex of inorganic phosphorus and ammonium molybdate following reduction with aminonapthosulfonic acid (ANSA) [104]. Aliquots of lipid extracts or gel bands from TLC separations were digested in 1 ml of 70 % perchloric acid at 180 °C for 30 min. After the samples were completely digested the following additions were made with mixing between each addition: 6 ml of distilled water, 0.4 ml of 5 % ammonium molybdate and 0.2 ml of 0.154 g/ml ANSA reagent. Samples were then placed in a boiling water bath for 10 min. Absorbance of the cooled samples at 820 nm was determined and compared to that obtained with phosphorus standards.

5. Radioactivity determination

a. Liquid scintillation counting

Samples scraped from TLC plates and aqueous radioactive samples were added directly to 5 ml of Beckman liquid scintillation cocktail (Ready Safe). Care was taken that the resulting sample-scintillation cocktail mixtures were always monophasic. Samples in organic solvents were placed in scintillation vials and organic solvents evaporated before addition of the scintillation cocktail. Radioactivity was determined in a Beckman LS 7800 liquid scintillation counter with quench correction.

b. Scanning of TLC plates

Distribution of radioactivity on TLC plates was determined using a computer controlled Bioscan imaging system 200 scanner. This system could not be used in circumstances in which the phospholipid bands were very close together or were irregularly shaped; these samples were scraped instead. The TLC scanner was used to determine the distribution of radioactivity on the plates and total radioactivity was calculated from liquid scintillation counting of an aliquot of the material spotted on the TLC plates.

c. Statistics

Where possible, data are presented as means ± standard deviation. Differences between measured quantities were assessed for statistical significance with Student's t-test, and p values are provided.

B. Subcellular fractionation studies

1. Subcellular procedure

Subcellular fractions of both glioma and neuroblastoma cells were prepared essentially as described for glioma cells [75] except that only a single gradient was used. Cells were removed from flasks by scraping (C6) or agitation (N1E-115) and washed twice with ice-cold 0.25 M sucrose buffered at pH 7.0 with 40 mM Hepes. The washed cell pellet (5-6 x 10^7 cells) was suspended in 5 ml of isotonic lysis buffer (25 mM Tricine, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, pH 9.6). Suspended cells were disrupted by

nitrogen cavitation (10 min at 600 lb/in^2 for glioma and 3 min at 100 lb/in^2 for neuroblastoma cells). An aliquot of the lysate was removed and the remainder was centrifuged at 900 g for 5 min. The supernatant was removed and the pellet resuspended in lysis buffer and sedimented as above. The resulting pellet (crude nuclear fraction) was resuspended in lysis buffer. A 2.5 ml aliquot of the combined supernatant was layered on top of 9.0 ml solution of 40 % Percoll (by volume), 200 mM KCl, 10 mM $MgCl_2$, and 200 mM Tricine adjusted to pH 9.3 with 0.1 M NaOH. Percoll is a suspension of polyvinylpyrrolidone coated silica particles. Fractions were separated in a self-forming density gradient during centrifugation at 100 000 x g for 12 min (8.4 x 10^9 ω^2t). Five fractions were collected from the top of the gradient. Volumes of fractions were 2.5, 1.9, 2.1, 2.9, and 1.5 ml for glioma cells and 3.0, 1.6, 3.0, 0.9, and 2.4 ml for neuroblastoma cells. All fractions were dialyzed against 40 mM Hepes (pH 7.2) for 6-12 h and stored at 4°C. Enzyme activities were assayed within 24 h.

2. 5'-nucleotidase

The plasma membrane marker 5'-nucleotidase (EC 3.2.1.30) was measured by the method of Avruch *et al.* [11]. In this procedure the unreacted substrate is separated from the product by precipitation. Reaction conditions were: 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 50 µM [^{3}H]AMP (880 dpm/nmol), and an aliquot of cell fraction in a total volume of 1 ml at 37 °C. Aliquots contained 20 and 2 µgm of protein for N1E-115 and C6 cells respectively. Reactions were terminated with 0.7 ml of 5 % $ZnSO_4$ after which samples were placed on ice. 10 µl of 1 mM AMP and 0.7 ml of 0.3 M $Ba(OH)_2$ were added and the samples were mixed thoroughly and centrifuged for 10 min at 500 x g. Aliquots (0.75 ml) of the supernatant were counted by liquid scintillation.

3. Lactate dehydrogenase

Lactate dehydrogenase (EC 1.1.1.27), a marker for the cytosol, was measured with the aid of a kit obtained from the Sigma Chemical Co. [440]. Production of NADH from NAD during the formation of pyruvate from lactic acid was measured

spectrophotometrically at room temperature. Using 10-100 µl of cell fractions the change in absorbance at 340 nm was measured and activity was calculated using the molar extinction coefficient of NADH.

4. NADPH:cytochrome c reductase

The endoplasmic reticulum marker NADPH:cytochrome c reductase (EC 1.6.99.1) was measured spectrophotometrically by following the reduction of cytochrome c by NADPH [387]. Reaction conditions were 50 mM KH_2PO_4 pH 7.7, 1 mM KCN, 50 µM cytochrome c, 100 µM NADPH, and 5-100 µl of cell fraction in a total volume of 500 µl at room temperature. All assay components except NADPH were mixed together and preincubated at room temperature for 3 min. NADPH was added and the change in absorbance at 550 nm was followed for 1.5 min. Activity was calculated based on the molar extinction coefficient of reduced cytochrome c.

5. Phosphatidylinositol synthase

PtdIns synthase (EC 2.7.8.11) was measured by incubating 10-50 µg protein of cell fraction in 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM [^{3}H]inositol (100 dpm/pmole), 0.1 mM CMP-PtdOH, and 0.1 % Triton X-100 at 37 °C in a total volume of 100 µL. Reactions were initiated by adding aliquots of cell fractions to the reaction mixture. Reactions were terminated by addition of 2 ml of chloroform/methanol/12 N HCl (100/100/1, v/v/v) and the phases separated by addition of 0.5 ml of 2 M KCl. The lower phase was washed three times with methanol/1 M KCl/chloroform (47/48/3, v/v/v) and radioactivity incorporated into lower phase lipid was measured by scintillation counting. No incorporation of [^{3}H]inositol occurred in the absence of CMP-PtdOH.

6. PtdIns 4-kinase and PtdIns(4)P 5-kinase

Activity measurements of PtdIns 4-kinase (EC 2.7.1.67) and PtdIns(4)P 5-kinase (EC 2.7.1.68) were as described by MacDonald *et al.*. [247] using purified PtdIns and PtdIns(4)P, respectively, as substrates. Reaction conditions were: 50 mM Hepes pH 7.25, 1 mM dithiotreitol, 18 mM $MgCl_2$, 450 µM [γ-^{32}P]ATP (500 dpm/pmole), 0.17 % Triton X-

100, 10-25 µl of cell fraction, and either 4 mM PtdIns or 0.5 mM PtdIns(4)P in a total volume of 75 µl. Reactions were started by addition of $MgCl_2$ to the mixture. After 2 min at 30 °C the reactions were stopped with 1 ml of $CHCl_3/CH_3OH$/12N HCl (66/33/1, by vol.) after which 1 ml of ideal upper phase ($CH_3OH/H_2O/CHCl_3$, 48/47/3) was added. After thorough mixing and partitioning into two phases (aided by centrifugation) the upper phase was discarded and the lower phase was washed once with ideal upper phase. Reaction products were isolated on oxalate-treated silica gel HL plates developed in TLC system B. Bands corresponding to PtdIns(4)P and PtdIns(4,5)P_2 were scraped off of the plates and radioactivity determined by scintillation counting.

7. PtdInsP_2 phospholipase C

Calcium-dependent PtdIns(4,5)P_2 specific phospholipase C (EC 3.1.4.11) was measured by the method of Palmer [306]. This assay is based on colourimetric detection of inorganic phosphorus released by alkaline phosphatase action on Ins(1,4,5)P_3 which is a product of PtdIns(4,5)P_2 specific phospholipase C. Reaction conditions were: 30 mM Hepes pH 7.2, 100 mM KCl, 3 mM PtdIns(4,5)P_2, 6 mM CTAB, 10-40 µl cell fraction and either 1 mM EDTA or 0.5 mM $CaCl_2$ in a total volume of 100 µl. Two tubes containing EDTA and two tubes containing $CaCl_2$ were used for each sample. Reactions were started by addition of cell fraction to the pre-equilibrated reaction mixture. After incubation at 37 °C for 10-60 min the reaction was stopped with 40 µl of 10 mM EDTA. Alkaline phosphatase (10 units) was added to half of the tubes and samples were incubated for a further 10 min after which time 0.35 ml of 5% SDS/50 mM EDTA was added to all tubes. The samples were then analysed with a Technicon autoanalyzer using a molybdate system to detect inorganic phosphorus. Interference by Percoll particles from the gradients was avoided by including a continuous flow dialyzer in the autoanalyzer system. Retrieval of inorganic phosphate from Percoll-containing samples and standards was 37 %. Activity was calculated as phosphorus specifically released in samples incubated in the presence of $CaCl_2$ and treated with alkaline phosphatase.

8. Digitonin extraction of cytosolic PtdIns-PLC

The distribution of PtdIns-PLC following stimulation of C6 with serotonin and N1E-115 cells with bradykinin was investigated by partitioning the cells into cytosolic and non-cytosolic fractions. Digitonin interacts specifically with β-hydroxy-sterols and perforates the plasma membrane and outer mitochondrial membranes [252]. Cytoplasmic enzymes rapidly diffuse out of digitonin permeabilized cells; the remainder of the cell components can be collected in Triton X-100 [252]. C6 cells were grown in 35 mm dishes. The culture medium was removed and cells incubated for 2 h in 1.25 ml of DMEM. 10 μM serotonin was added and the cells incubated for 0, 0.5,1,2,5,8,12, and 30 min. Incubations were terminated by replacing the DMEM with 1.25 ml of ice-cold 50 mM Hepes pH 7.4, 0.5 mg/ml digitonin. After 2 min the digitonin solution was removed and the remaining cell material was washed with a further 0.5 ml of buffered digitonin. Residual cell material was collected by scraping the dishes in 1.75 ml 0.5 % Triton X-100. Assays for PtdIns(4,5)P_2 specific phospholipase C were performed on aliquots of the digitonin and Triton X-100 extracts.

9. PtdIns(4,5)P_2 5-phosphatase

The magnesium-dependent PtdIns(4,5)P_2 5-phosphatase (E.C. 3.1.3.36) was measured by the method of Palmer [307]. Activity was detected by measuring the release of inorganic phosphorus. Reaction conditions were: 45 mM Hepes pH 7.2, 200 mM KCl, 2 mM EGTA, 5.6 mM CTAB, 5 mM dithiothreitol, 2.8 mM PtdIns(4,5)P_2, 10-30 μl of cell fraction and 1 mM $MgCl_2$ in a total volume of 150μl. 1 mM EDTA replaced $MgCl_2$ in the blanks. Incubations were for 15-45 min at 37 °C. Reactions were stopped by the addition of 0.35 ml of 50 mM EDTA in 5% SDS. Inorganic phosphorus was measured with an autoanalyzer as above. Values from blanks containing EDTA were subtracted from each reading. Activity was calculated as inorganic phosphorus specifically released in the presence of substrate and $MgCl_2$.

10. PtdIns(4)P 4-phosphatase

This assay was very similar to that for PtdIns(4,5)P_2 5-phosphatase except that PtdIns(4)P was used as a substrate and that the activity was measured in the presence of EDTA as the enzyme has no divalent cation requirement. Release of inorganic phosphorus was compared to controls which had no PtdIns(4)P added [307].

11. CTP:phosphatidate cytidylyltransferase

CTP:phosphatidate cytidylyltransferase (EC 2.7.7.41) activity was measured by a modification of the method of Liteplo and Sribney [233]. Aliquots of the cell fractions (10-50 µg protein) were pre-incubated for 5 min at 37 °C in the presence of 100 mM Tris-HCl pH 7.2, 4 mM dithiothreitol, 1 mM [^{3}H]CTP (2500 dpm/nmole), 0.1 % Triton X-100, and 1 mM PtdOH (total volume of 90 µl). Reactions were started by addition of 10 µL of 200 mM $MgCl_2$. Reactions were terminated by addition of 2 ml of chloroform/methanol/12 N HCl (100/100/1, v/v/v) and the phases separated by addition of 0.5 ml of 2 M KCl. The lower phase was washed three times with methanol/1 M KCl/chloroform (47/48/3, v/v/v) and radioactivity incorporated into lower phase lipid was measured by scintillation counting. Blank values were obtained by performing the incubation in the absence of cell fraction and adding the appropriate amount of cellular fraction after the reaction was stopped with organic solvents.

12. Diacylglycerol kinase

DAG kinase (EC 2.7.1.107) was measured by following the incorporation of radioactivity from [γ-^{32}P]ATP into the appropriate product in the presence of exogenous substrate according to the method of Besterman *et al.*. [39]. Assay conditions were 50 mM tris-HCl pH 7.6, 1 mM dithiotreitol, 10 mM $MgCl_2$, 130 µM PtdSer, 1.5 mM DAG, 1.5 mM [γ-^{32}P]ATP (666 dpm/nmole), and 10-20 µl of cell fraction in a total volume of 100 µl. Reactions were incubated at 30 °C for 10 min after which they were stopped with 50 µl 12 N HCl and 1.5 ml of water. The reaction mixtures were then extracted with 1 ml of 1-butanol. The butanol containing upper phases were then washed twice with 1 ml of

butanol-saturated water. Since the only radioactive lipid product formed was identified as PtdOH by thin layer chromatography, only the formation of radioactive lipid was routinely measured.

C. LysoPtdIns experiments

1. Preparation of LysoPtdIns

Preparation of 1-acyl[^{3}H]lysoPtdIns was performed in 4 steps. First, cell lysates were incubated with [^{3}H]inositol under conditions that promoted the incorporation of radiolabel into lipid. Newly formed [^{3}H]PtdIns was isolated from total lipid by chromatography on immobilized neomycin and, after conversion to the appropriate salt form, was deacylated in the sn-2 position with bee venom phospholipase A_2. Finally, lysoPtdIns was separated from [^{3}H]inositolglycerolphosphate (a product of minor lysophospholipase activity in the bee venom preparation) and residual [^{3}H]PtdIns by solvent extraction.

a. Formation of [^{3}H]PtdIns

Optimized conditions for the incorporation of [^{3}H]inositol into lipid were 0.1 ml N1E-115 cell lysate (containing 0.3 mg protein), 1 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM CMP, 50 mM Hepes pH 7.4, and 200 µCi of [^{3}H]inositol (16.2 Ci/mmol) in a total volume of 0.5 ml. The reaction was allowed to proceed for 2 h at 37 °C. The reaction was stopped and two phases created by the addition of 0.25 ml of 2 N HCl, 1 ml of methanol, and 2.5 ml of chloroform. The lower phase was dried under N_2 and resuspended in 2 ml of chloroform/methanol (1:1, v,v).

b. Purification of PtdIns

PtdIns was isolated by chromatography on immobilized neomycin [304]. Neomycin was reductively coupled to porous glass beads and packed into a 1 ml column [360] through which the lipid extract containing [^{3}H]PtdIns was passed. To remove other lipids, the column was then washed with 2 ml of chloroform/methanol (1/1, v/v), 2 ml of chloroform/methanol (1/2, v/v), and 6 ml of chloroform/methanol/88 % formic acid

(5/10/1, v/v/v). [^{3}H]PtdIns was eluted from the column with 6 ml of chloroform/methanol/40 mM ammonium formate (5/10/2, v/v/v).

PtdIns was then converted to a calcium salt. The eluate was evaporated under nitrogen and the residue was redissolved in 2 ml of $CHCl_3/CH_3OH$ (84/16, v/v). One ml methanol/ 6 N HCl/chloroform (47/48/3,v/v/v) was added and the resulting biphasic mixture mixed thoroughly and allowed to settle into 2 phases with the aid of centrifugation at 600 x g for 10 min. The resulting lower phase was washed twice with 2 ml of methanol/20 mM $CaCl_2$/chloroform (47/48/3, v/v/v) and kept for deacylation. All of the radioactivity in the resulting sample comigrated with PtdIns in TLC system B.

c. Formation of 1-acyl[^{3}H]lysoPtdIns

The lower phase containing [^{3}H]PtdIns was evaporated under nitrogen and then resuspended in 5 ml of diethylether. Bee venom phospholipase A_2 (300 units in 100 µl 50 mM HEPES pH 7.4) was added and the mixture sealed under nitrogen. The enzymatic digest was mixed vigorously at 37 °C for 30 min. The reaction was stopped by adding 1 ml of ethanol. [^{3}H]lysoPtdIns was separated from any unreacted [^{3}H]PtdIns by extraction [84]. The preparation was dried under nitrogen and resuspended in 2 ml of chloroform/methanol (1/1, v/v). Two ml of H_2O was added; the sample mixed, and two phases were formed. [^{3}H]PtdIns partitioned into the lower phase while [^{3}H]lysoPtdIns and [^{3}H]inositol partitioned into the upper phase. The aqueous phase containing lysoPtdIns was reduced under nitrogen and 1 ml of water-saturated butanol was added to form two phases. Following centrifugation the resulting butanol phase was dried under nitrogen to yield [^{3}H]lysoPtdIns. All of the radioactivity in this sample co-migrated with lysoPtdIns standard in TLC.

Final yield of [^{3}H]lysoPtdIns was 10 µCi and the specific activity based on the amount of PtdIns present in the N1E-115 lysate [59] was 17 µCi/nmol.

2. Incorporation of 1-acyl-[^{3}H]lysoPtdIns by cultured cells

The uptake and subsequent metabolism of 1-acyl-lysoPtdIns was investigated in N1E-115 cells in culture. N1E-115 cells grown in 60 mm dishes were incubated in 2 ml of DMEM containing 220 000 dpm of 1-acyl-[^{3}H]lysoPtdIns. To terminate the incubation the medium was removed and the cells in the dish were washed 3 times with ice-cold PBS. Cells were removed from the dish in 3.6 ml of methanol/water (5/4, v/v). Three 10 µl aliquots were removed for protein determination and 4 ml of chloroform with 50 µl of 12 N HCl was added to the remainder. Samples were thoroughly mixed and allowed to separate into two phases with the aid of centrifugation at 600 x g for 10 min. The lower phase was removed and kept for later use. The upper phase was extracted twice with $CHCl_3/CH_3OH$ (84/16, v/v) which was set aside. The lower phases were combined and were evaporated under N_2 and resuspended in 100 µl of chloroform/methanol/water (75/25/3, v/v/v), from which an aliquot was taken for liquid scintillation counting. Samples were spotted on oxalate impregnated silica gel HL plates. The chromatograms were developed in TLC system B. Phospholipid bands were identified by comparison with standards and radioactivity was determined by liquid scintillation counting of the scraped bands.

Subcellular fractionation studies were undertaken to determine the subcellular site of PtdIns formation from 1-acyl-lysoPtdIns. N1E-115 cells grown in 150 cm^2 tissue culture flasks were incubated in 12 ml of DMEM for 4 h after which time 40 µM 1-acyl-[^{3}H]lysoPtdIns (5 µCi) was added. Incubations were terminated at 1, 4, and 21 h by washing the cells in the flask 3 times with ice-cold PBS. The cells were then harvested and subcellular fractions obtained as above. Phospholipids were extracted from the subcellular fractions with iso-butanol/acetonitrile. 2 ml of iso-butanol/acetonitrile (2/1, v/v) was added to each Percoll-containing fraction (2-3 ml) and mixed thoroughly. Centrifugation at 400 x g for 10 min sedimented the Percoll at the bottom of an aqueous phase above which there was an iso-butanol/acetonitrile phase. The upper organic phase was removed and saved. More iso-butanol/acetonitrile (1 ml) was added to the Percoll containing aqueous phase. After mixing and centrifugation the upper phase was removed and saved. This was

repeated once more, and all 3 upper phases were combined. 2 ml of 3 M NaCl was added to the iso-butanol/acetonitrile extracts and after thorough mixing the mixture was centrifuged at 600 x g for 10 min. The upper, isobutanol/acetonitrile phase was removed and saved. 1 ml of iso-butanol/acetonitrile (2/1) was added to the salt phase and after mixing and centrifugation the upper organic phase was removed and combined with the original salt extracted organic phase. Radioactivity (taken to represent a mixture of [^{3}H]Ins and [^{3}H]GroPIns) in the residual aqueous phase was determined by liquid scintillation. No radiolabelled lipids were detected in the aqueous phase when that phase was evaporated, resuspended in chloroform/methanol/water (75/25/3), and analyzed with TLC system B. After evaporation of the iso-butanol and acetonitrile, the lipids were resuspended in 1 ml chloroform/methanol (84:16, v/v)) to which 1 ml of methanol/0.1 M HCl/chloroform (48:47:3) was added. After thorough mixing and centrifugation at 600 x g for 10 min, the lower phase was evaporated and resuspended in 100 µl of chloroform/methanol/water (75/25/3). Two 5 µl aliquots were taken to determine total radioactivity. Phospholipids in the remainder of the sample were separated by TLC system B.

3. Assay of LysoPtdIns acylation *in vitro*

In vitro synthesis of [^{3}H]PtdIns from 1-acyl-[^{3}H]lysoPtdIns was assayed in plasma membrane, cytosolic and microsomal N1E-115 fractions. The following method was used to determine the conditions necessary for acylation of 1-acyl-[^{3}H]lysoPtdIns with endogenous fatty acids. Aliquots of subcellular fractions from N1E-115 cells (25 µg of protein) were incubated with 25 µM 1-acyl-[^{3}H]lysoPtdIns (3200 dpm/nmol), and 40 mM Hepes pH 7.4 at 37°C for 15 min in a total volume of 500 µl. Other reagents were added as indicated at the following concentrations: 10 mM EDTA, 10 mM $MgCl_2$, 5 mM ATP, 5 mM Coenzyme A. The reaction was initiated by addition of a subcellular fraction aliquot to the pre-equilibrated reaction mixture. After 15 min, reactions were terminated by adding 2 ml of chloroform/methanol (84:16) and 1 ml of methanol/0.1 M HCl/chloroform (48:47:3). After thorough mixing and centrifugation at 600 x g for 10 min the lower phase was

evaporated and resuspended in 100 µl of chloroform/methanol/water (75:25:3). Two 5 µl aliquots were taken to determine total radioactivity and the phospholipids in the remainder were separated in TLC system B.

D. Effect of polyamines on cultured cells

1. Polyamine incorporation

a. Incorporation of [^{3}H]putrescine

Uptake of [^{3}H]putrescine was measured in N1E cells. Cells were grown to confluence in 60 mm dishes and then 100 000 dpm of [^{3}H]putrescine was added to the medium. Various concentrations and times of incubation were investigated. Following incubation with [^{3}H]putrescine the cells were washed 4 times with 2 ml of DMEM containing 5mM putrescine. Cells were harvested and total radioactivity in the cells was determined by liquid scintillation counting.

Modulation of [^{3}H]putrescine uptake was characterized with N-ethylmaleimide, and bradykinin. Cells were pre-incubated with 5 mM N-ethylmaleimide for 5 min. The cells were rinsed twice and 1 µM [^{3}H]putrescine (200 000 dpm) was added in fresh DMEM. The effect of bradykinin on [^{3}H]putrescine uptake was measured by adding 10 µM bradykinin to the cells at the same time as the [^{3}H]putrescine.

b. HPLC of polyamines

The intracellular content of polyamines was determined by HPLC using a modification of the method of Chen *et al.* [62]. N1E-115 cells were exposed to 5 mM exogenous polyamine (putrescine, spermidine, or spermine) in 2 ml of DMEM. Incubations were terminated and cells were washed 4 times with ice cold PBS. Cells were harvested in 2 ml methanol/water (5/4, v/v) at which time 20 µl of 1 mM 1,10-diaminodecane was added as an internal standard. Aliquots for protein determinations were taken. 1 ml of 0.8 M trichloroacetic acid was added, and following 2 h at 4 °C, cell extracts were centrifuged at 600 x g for 10 min. A 600 µl aliquot of the supernatant was made alkaline by addition of 75 µl of saturated Na_2CO_3 and 450 µl of saturated $NaHCO_3$.

Amines were dansylated by addition of 500 µl of 20 mM dansylchloride in acetone. After 12 h at room temperature in the dark, 20 µl of 1.5 M proline was added to remove excess dansylchloride. After 20 min, acetone was evaporated under N_2 and the samples were dissolved in methanol. Polyamines were determined by HPLC using a reverse phase column (Beckman RP-18) and eluted with an acetonitrile-water gradient using 1,10-diaminodecane as an internal standard. Retention times were 3.5, 8.5, 9.5, 11.8 min for putrescine, spermidine, 1,10-diaminodecane, and spermine, respectively. Fluorescence of dansylated amines eluted from the column was detected with a flow through Amico spectrofluorimeter.

c. [^{32}P]Pi incorporation into phospholipids

In order to study the metabolism of the phosphoinositides, cells were incubated with [^{32}P]orthophosphoric acid ([^{32}P]Pi) and polyamines added to the cell culture medium. After exposure to [^{32}P]Pi and polyamines, incubations were stopped and lipids were extracted. With the TLC system used lysoPtdEtn migrates in the region of the polyphosphoinositides. C6 and N1E-115 cells contain large amounts of acid labile PtdEtn plasmalogen [413]. In order to efficiently extract polyphosphoinositides but avoid acid hydrolysis of PtdEtn plasmalogen a neutral extraction was performed and then the upper phase was extracted with organic solvents under acidic conditions. This procedure resulted in no detectable hydrolysis of plasmalogen in extracts from [^{14}C]Etn labelled cells. The procedure was as follows. [^{32}P]Pi Cell incubations were terminated by removing the culture medium and scraping the cells off of the dish in 3.6 ml of ice cold methanol/water (5/4, v/v). Three 10 µl aliquots were removed for protein determination and 4 ml of chloroform was added to the remainder. After centrifugation lower phases were removed and reserved (neutral lower phase). 100 µl of 12 N HCl was added to the remaining upper phase. The acidified upper phase was extracted twice with 2 ml of chloroform/methanol (84/16, v/v). The acidic lower phase extracts were combined and neutralized with 1 drop of 4 N NH_4OH before adding to the first neutral lower phase. The combined lower phases

were evaporated under N_2 and resuspended in 100 µl of chloroform/methanol/water (75/25/3, v/v/v), from which an aliquot was taken to determine total radioactivity in the sample. Portions of each sample was spotted on both silica gel G and oxalate impregnated silica gel HL plates. The chromatograms were developed in chloroform/ethanol/triethylamine/water (4/5/4/1, v/v/v/v) and chloroform/acetone/methanol/acetic acid/water (80/30/26/24/15, v/v/v/v/v), respectively. Phospholipid bands were identified by comparison with standards and radioactivity was determined by liquid scintillation counting of the scraped bands or by scanning for radioactivity with a Bioscan scanner 2000 radioactivity scanner.

Bradykinin stimulated synthesis of PtdIns was measured by incubation of the cells with 15 µCi [^{32}P]Pi per dish for 1 h in medium with and without added polyamines [116]. At 1 h, 10 µM bradykinin was added to some of the dishes and cells were further incubated in the same medium for 30 min after which time the incubations were stopped and lipids extracted as above (Figure 7).

Pulse-chase experiments were performed to measure bradykinin induced loss of polyphosphoinositides. Cells were prelabelled with 15 µCi [^{32}P]Pi for 1 h in medium, with and without added polyamines, after which time they were washed twice with DMEM. Fresh DMEM, with and without polyamines, was added. After 30 min of chase with non-radioactive phosphate from the medium, 10 µM bradykinin was added to some of the dishes for 45 seconds and incubations were terminated with ice-cold methanol/water (Figure 8) [116]. The resulting samples were analyzed as above.

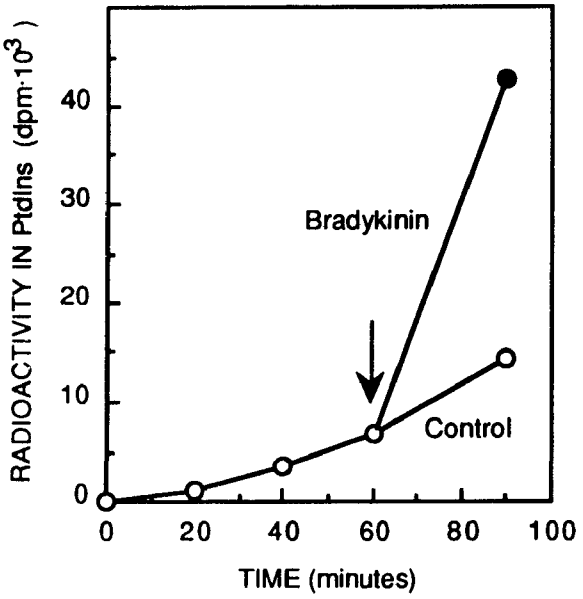


Figure 7. Bradykinin stimulated PtdIns turnover. Effect of 10 μM bradykinin on [^{32}P]Pi incorporation into PtdIns. N1E-115 cells grown in culture were incubated with 15 μCi of [^{32}P]Pi for 60 min in 2 ml DMEM. 10 μM bradykinin (in water) was added at the 60 min mark and cells incubated for a further 30 min. After a total of 90 min the cells were removed from the dishes in methanol/water and lipids were extracted with chloroform. PtdIns was isolated by TLC system A. Control dishes, (O); Bradykinin treated dishes,(●) [309].

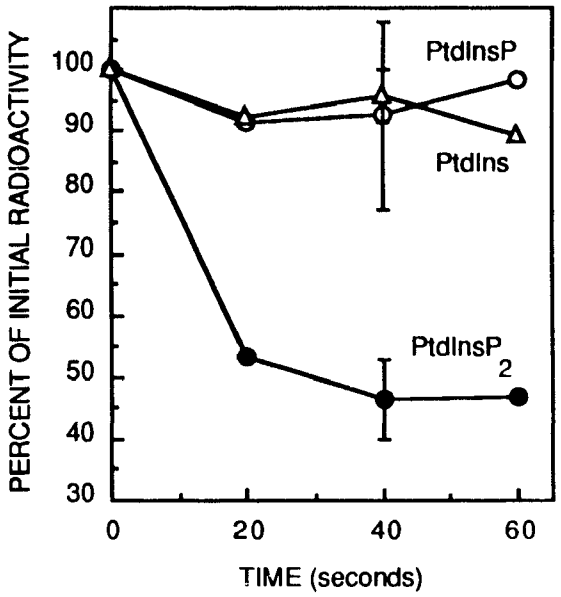


Figure 8. Bradykinin stimulated loss of PtdIns(4,5)P_2. Effect of 10 μM bradykinin on loss of [^{32}P]Pi incorporation from PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2. N1E-115 cells prelabelled with 15 μCi of [^{32}P]Pi for 1 h were washed twice with 2 ml of DMEM and incubated in DMEM (radioactiviity free) for a further 30 min. Bradykinin (10 μM) was added to some of the dishes and incubations were terminated after 20, 40, and 60 seconds. Lipids were analysed with TLC system B [309].

E. *In vitro* polyamine experiments

1. Ins exchange activity

In vitro incorporation of [^{3}H]inositol into PtdIns was measured in 100 µl of 100 mM HEPES buffer pH 7.4 containing polyamine, $MnCl_2$, $MgCl_2$ and CMP as noted in the figure legends of each experiment. The reaction mixtures containing 25 µg of N1E-115 lysate protein were incubated at 37 °C for 50 min. During this period incorporation of label into lipid was linear with time. Reactions were terminated by addition of 2 ml of chloroform/methanol/12 N HCl (100/100/1, v/v/v) and the phases separated by addition of 0.5 ml of 2 M KCl. The lower phase was washed three times with methanol/1 M KCl/chloroform (47/48/3, v/v/v). When analyzed by TLC (system B) all of the radioactivity in the lower phase was present as PtdIns. Routinely, only radioactivity in the lower phase was determined.

2. PtdIns synthase

The effects of polyamines on *in vitro* PtdIns synthase activity were determined under conditions described for the assay of PtdIns synthase in subcellular fractions. The effects of various concentrations of polyamines were tested. To determine the effect of 1 mM polyamines on the Mg^{2+} requirement of PtdIns synthase the optimal concentration of 10 mM $MgCl_2$ was varied.

F. Malignant hyperthermia experiments

1. Leukocyte preparation

Leukocytes were prepared from blood samples donated by healthy volunteers and by otherwise healthy volunteers with diagnosed malignant hyperthermia. 10 ml blood samples were collected in vacuum tubes containing heparin. The blood samples were layered on 8 ml of Dextran-Hypaque (4/10, g/g) [13]. Red blood cells were allowed to settle to the bottom of the Dextran-Hypaque mixture. Granulocytes form an intermediate layer. The upper layer of cells, containing leukocytes and platelets, were transfered to a clean test tube and centrifuged at 600 x g for 5 min. The platelet rich supernatant was

removed and discarded. The cell pellet was twice resuspended in saline and centrifuged at 600 x g for 5 min. The resulting washed cell pellet was resuspended in 3 ml of distilled water to lyse any remaining erythrocytes. After 90 seconds the cell suspension was restored to iso-osmotic conditions by the addition of 1 ml of 3.6 % NaCl (w/v). To remove residual platelets and disrupted erythrocytes the cell suspension was centrifuged at 800 x g for 5 min. The supernatant was removed and discarded. The resulting leukocyte enriched cell pellet was resuspended in 10 mM Hepes pH 7.4 and sonicated 3 times with a 60 watt probe sonicator for 10 seconds. The resultant cell lysate was brought to 20 % glycerol and could be frozen at -70 °C with no loss in Ins(1,4,5)P_3 5-phosphatase activity.

2. Ins(1,4,5)P_3 5-phosphatase assay

Ins(1,4,5)P_3 5-phosphatase activity of leukocytes and lymphoblasts was measured *in vitro* using HPLC to separate the substrate from the products of the reaction [108]. 10 µg of cell protein was incubated at 37 °C for 15 min in a total volume of 75 µl with 40 mM Hepes pH 7.4, 0.1 mM EGTA, 2 mM $MgCl_2$, and 10 µM [^{3}H]Ins(1,4,5)P_3 (300 dpm/pmol). The reactions were stopped with 0.925 ml of a solution containing 7.5 % trichloroacetic acid, 5 mM LiCl, and 0.5 mM $CdCl_2$. This terminated the reaction and caused proteins and lipids in the sample to precipitate. Precipitates were removed by centrifugation. The samples were then extracted 3 times with 1 ml of diethylether to remove trichloroacetic acid. Finally, the samples were filtered through a Millipore HV 0.45 µM filter and injected onto the HPLC column. Inositol and inositol phosphates were separated on a SAX anion exchange column using a sodium citrate-methanol buffer [258]. The procedure was as follows. The samples were injected onto a SAX column through which water/methanol (95/5) was flowing at a rate of 1.5 ml/min. After 4 min the solvent was changed to 1 M sodium citrate/methanol (95/5) and the run continued for a further 25 min after which time the column was re-equilibrated with water/methanol. Ins(1,4,5)P_3 was eluted after 15 min, Ins(1,4)P_2 was eluted at 9.5 min, a peak presumed to be InsP was eluted after 8.5 min, and Ins was eluted after 4 min. Radioactivity in the eluate was

detected with a Flo-1 liquid scintillation counter. Counting efficiency was calibrated with [^{3}H]Ins, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 standards.

Results

I. Subcellular Fractionation Studies; C6 rat glioma

A. Distribution of protein and marker enzymes

The subcellular distribution of enzymes involved in phosphatidylinositol synthesis and polyphosphoinositide metabolism was investigated in C6 rat glioma cells. A self-forming Percoll density gradient was used to separate plasma membrane, cytosol, and endoplasmic reticulum during ultracentrifugation.

Based on initial studies of the distribution of marker enzymes, the gradients were divided into 5 fractions (Figure 9, Table 4) that minimized cross contamination between the three major fractions representing cytosol (fraction 1), plasma membrane (fraction 3) and endoplasmic reticulum (fraction 5). The plasma membrane fraction had little cross-contamination: it had 6.0 ± 1.4 %, 3.2 ± 0.3 %, and 71.6 ± 4.9 % of total recovered lactate dehydrogenase, NADPH:cytochrome C reductase, and 5' nucleotidase, respectively. The greatest overlap of marker enzymes was found in intervening fractions (fractions 2 and 4). Fraction 1 exhibited characteristics similar to those observed before [75]. It contained 60 % of the protein and 72 % of the lactate dehydrogenase. A fraction roughly equivalent to fractions 2 and 3 had been taken previously as representative of plasma membrane and contained most (> 60 %) of the 5'-nucleotidase activity. Loss of some plasma membrane into fraction 2 was sacrificed to ensure minimal contamination of the plasma membrane fraction with cytosol (lactate dehydrogenase activity) (Table 4). Likewise, separation of fraction 4 minimized contamination of the plasma membrane fraction with endoplasmic reticulum (NADPH:cytochrome c reductase activity). The bottom microsomal fraction (fraction 5) contains 68 % of the total endoplasmic reticulum marker, NADPH:cytochrome c reductase. It is slightly contaminated with plasma membrane and cytosol, as it contains 8 % of the total 5'-nucleotidase, and 0.7 % of the total lactate dehydrogenase activities. This fraction was relatively crude as it also contained golgi, lysosomes, and mitochondria [75].

Table 4. Relative specific activity and recovery of enzyme activities in subcellular fractions of glioma (C6) cells.

Cells from 4 flasks were used for each fractionation (65.8±19.3 mg protein, mean ± SD, 13 experiments). After fractionation, 32.4±5.9 mg, 4.1±0.8 mg, 3.3±1.0 mg, 2.2±0.9 mg, and 4.4±1.9 mg were recovered in gradient fractions 1 to 5, respectively, and the remainder in the initial 900 x g crude "nuclear pellet". Enzyme activities in the total cell lysate are expressed as nmol/min/mg protein except for CTP:PtdOH cytidylyltransferase and PtdIns synthase which are pmol/min/mg protein. Relative specific activities were obtained by dividing the specific activity in each fraction by the specific activity of the initial lysate. Activity data are reported as the means ± SD for 3 experiments.

	Specific Activity (lysate)	Relative Specific Activity					Recovery (%)
		1	2	3	4	5	
Lactate dehydrogenase	50.7±7.4	1.14±0.04	2.03±0.14	0.93±0.18	1.06±0.61	0.1±0.02	94.3±7.6
5'-Nucleotidase	159±4.7	0.07±0	0.73±0.06	9.14±0.18	1.39±0.11	0.8±0.1	99.5±13.0
NADPH:cyt. c reductase	7.0±0.4	0.18±0.02	0.33±0.08	0.33±0.03	1.27±0.64	5.3±0.4	86.0±19.9
DAG kinase	0.5±0.1	0.24±0.02	1.91±0.12	2.95±0.31	2.19±0.3	1.8±0.2	101±7.5
CTP:PtdOH cytidylyltransferase	261±24	0.03±0.01	0.04±0.05	0.29±0.13	0.07±0.04	4.8±0.06	70.0±3.5
PtdIns synthase	79.8±2.7	0.05±0.03	0.32±0.21	0.40±0.13	0.68±0.21	4.5±0.4	110±10.9
PtdIns 4-kinase	31.5±1.4	0.48±0.02	0.85±0.04	0.79±0.07	1.37±0.02	3.9±0.03	81.7±2.5
PtdIns(4)P 5-kinase	11.7±0.7	0.08±0	0.52±0.01	2.84±0.01	1.40±0.02	3.7±0.03	104±9.7
PtdIns(4,5)P_2 phospholipase C	140±31.5	0.44±0.17	0.62±0.88	1.65±0.23	1.21±0.73	0.35±0.1	78.9±7.4
PtdIns(4,5)P_2 5-phosphatase	39.7±2.1	0.70±0.08	0.62±0.28	1.19±0.20	1.39±0.47	0.8±0.07	85.7±4.7
PtdIns(4)P 4-phosphatase	47.8±1.1	0.19±0.03	0.62±0.32	1.47±0.13	2.88±0.08	0.9±0.2	64.1±3.5

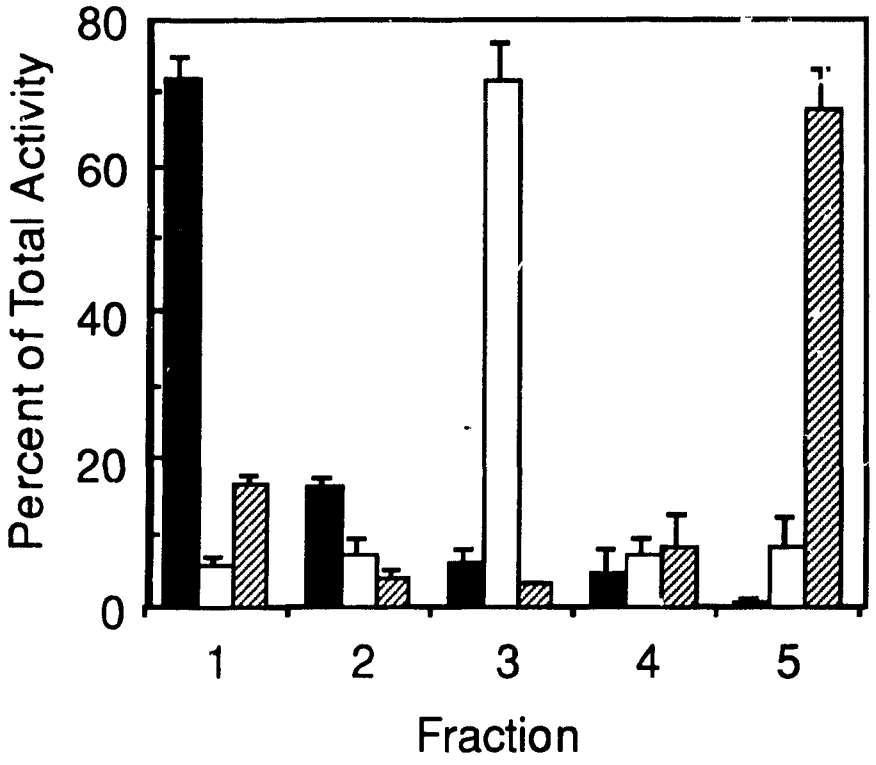


Figure 9. Subcellular distribution of marker enzymes for cytosol, plasma membrane and endoplasmic reticulum in rat glioma cells (C6). Enzyme markers are lactate dehydrogenase (solid bars), 5'-nucleotidase (open bars), and NADPH:cytochrome c reductase (hatched bars). Results are expressed as percent of the total activity recovered in the gradient. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient are shown in Table 4. Data are the means ± standard deviations for three independent experiments in which all assays were done in triplicate.

B. Distribution of enzymes that participate in PtdIns synthesis

The hypothesis that plasma membrane has the capacity to resynthesize PtdIns was tested in C6 glioma cells. PtdIns resynthesis following phospholipase C degradation of $PtdInsP_2$ requires three enzymes. The first of these, DAG kinase,was distributed throughout the gradient (Figure 10). The plasma membrane fraction had slightly more DAG kinase activity than the other fractions (25.5 %). CTP:PtdOH cytidylyltransferase and PtdIns synthase activities were recovered primarily in the microsomal fraction. However, small amounts of both activities (4 % and 7 %, respectively) were found in the plasma membrane fraction. The relative specific activities of these enzymes in each subcellular fraction is shown in Table 4.

Since agonist-sensitive phosphoinositide represents only a small portion of the total cellular phosphoinositide (variously estimated at 5-15 %), resynthesis of this pool of lipid in the plasma membrane might require only a small portion of the synthetic enzymes to be located there. To assess whether cytidylyltransferase and PtdIns synthase activities observed in the plasma membrane fraction represent contamination with endoplasmic reticulum or real but limited capacity of the plasma membrane for PtdIns synthesis, increasing amounts of the microsomal fraction were added to a constant amount of plasma membrane fraction . The synthetic activities were compared to the activity of the endoplasmic reticulum marker, NADPH: cytochrome c reductase (Figure 11). When extrapolated to zero reductase activity, regression lines for both cytidylyltransferase and PtdIns synthase activities passed the vertical axis at or slightly below zero. Assuming NADPH:cytochrome c reductase to be a valid endoplasmic reticulum marker that is absent from plasma membrane, the results indicate that glioma plasma membrane does not have even minor amounts of either of the two enzyme activities necessary for synthesis of PtdIns from PtdOH.

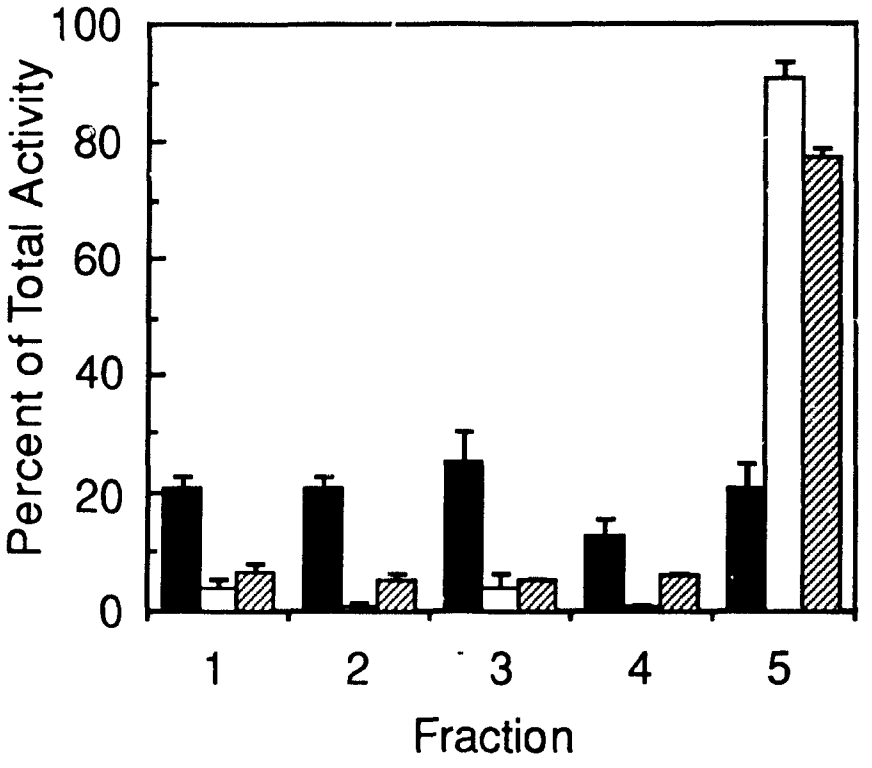


Figure 10. Subcellular distribution of enzymes of phosphatidylinositol synthesis in rat glioma cells (C6). Enzymes are DAG kinase (solid Bars), CTP:phosphatidic acid cytidylyltransferase, (open bars) and PtdIns synthase (hatched bars). Fraction 1, cytosol; Fraction 3, plasma membrane; and Fraction 5, endoplasmic reticulum. Results are expressed as percent of the total activity recovered in the gradient. Specific activities of enzymes in the post-nuclear supernatant and their total recovery from the gradient are shown in Table 4. Data are the means ± standard deviations for three independent experiments in which all assays were done in triplicate.

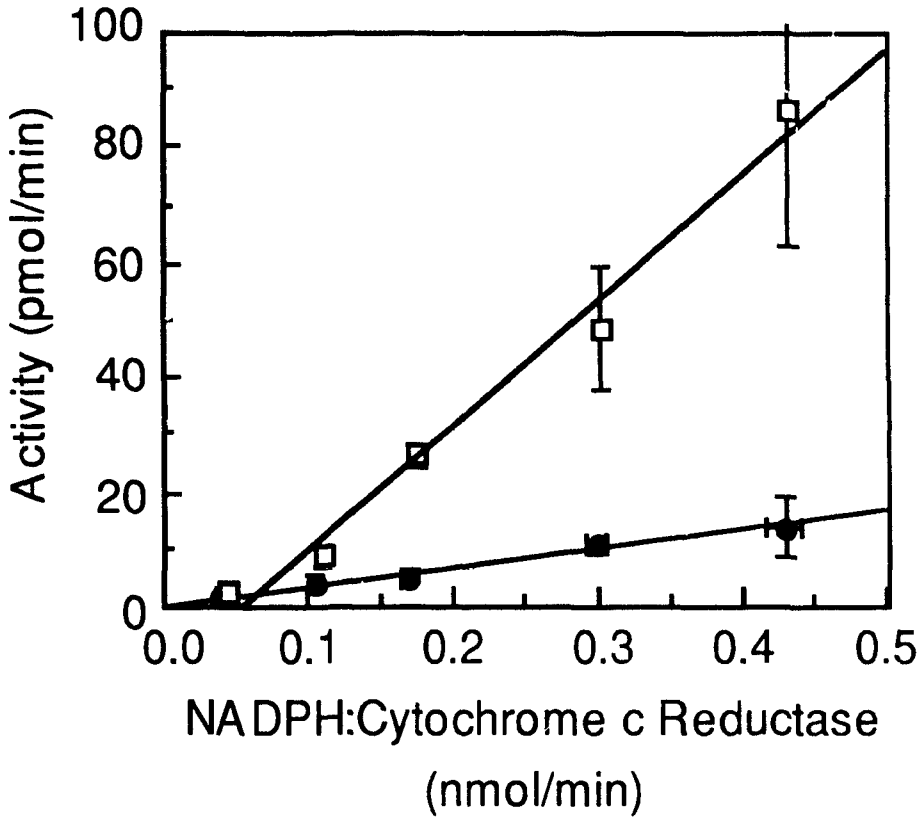


Figure 11. Estimation of contamination-dependent CTP:PtdOH cytidylyltransferase and PtdIns synthase activities in rat glioma plasma membranes. Increasing aliquots of microsomes (fraction 5) were added to a fixed aliquot of plasma membrane (fraction 3). Data are for one experiment in which each point represents the means ± standard deviations for three mixtures of the two fractions from a single gradient. CTP:PtdOH cytidylyltransferase, (□); PtdIns synthase, (●). Results are typical of 3 experiments in which only the slope of the regression line varied due to the gradient fractions having slightly different specific activities for the enzymes.

C. Distribution of enzymes involved in polyphosphoinositide synthesis

When assayed in the presence of exogenous substrate, the PtdIns and PtdIns(4)P kinases exhibited different bimodal distributions (Figure 12). PtdIns 4-kinase was recovered mainly in the cytosol (37 %) and microsomal (41 %) fractions but not in the plasma membrane. Knowing the distribution of marker enzymes in the gradient, it is possible determine whether low levels of some of the other enzymes measured in some fractions is due to contamination. PtdIns 4-kinase is found in both the cytosol and microsomes. A rough calculation can be performed to determine if the small amount present in the plasma membrane is due to contamination with cytosol and microsomes. Multiplying the ratio of lactate dehydrogenase in fractions 3 and 1 with the amount of PtdIns 4-kinase present in fraction 1 gives an estimate of how much PtdIns 4-kinase activity in the plasma membrane fraction is due to contamination with cytosol (fraction 1). Cytosolic contamination would result in 3.1 % of the total PtdIns 4-kinase activity to be found in the plasma membrane. Similar calculations to estimate microsomal contamination of the plasma membrane show that this would result in contamination of the plasma membrane fraction with another 2 % of the total PtdIns 4-kinase activity. Therefore, if PtdIns 4-kinase were found in only the cytosol and microsomes, contamination with these two fractions would cause a total of 5.1 % of the PtdIns 4-kinase activity to be found in the plasma membrane fraction. The result of this rough estimate is very close to the observed PtdIns 4-kinase activity in the plasma membrane fraction (6 %). Therefore, PtdIns 4-kinase is probably not present in the plasma membrane. Fractions 3 and 5 contain 29 % and 51 % of the PtdIns(4)P kinase activity respectively. Similar calculations show that PtdIns(4)P kinase in the cytosol (fraction 1) is attributable to contamination with the membranous fractions. The relative specific activities of these enzymes in each subcellular fraction is shown in Table 4. Total PtdIns 4-kinase activity (2.07 μmol/min) is 2.7 times higher than total cellular PtdIns(4)P 5-kinase activity (0.77 μmol/min).

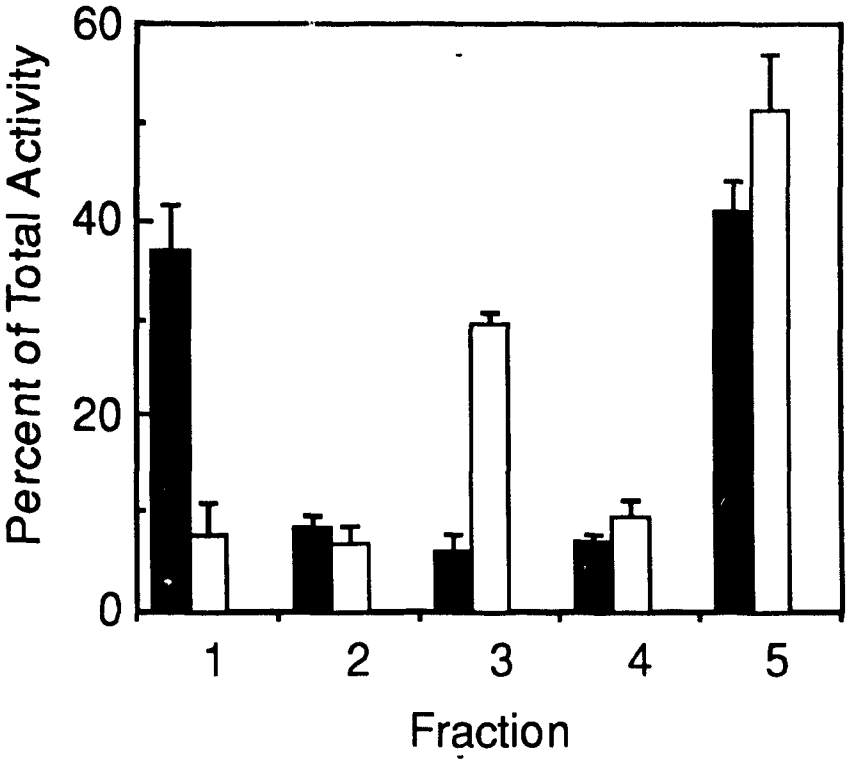


Figure 12. Subcellular distribution of phosphoinositide kinases in rat glioma cells (C6). Enzymes are PtdIns 4-kinase (solid bars), and PtdIns(4)P kinase (open bars). Fraction 1, cytosol; Fraction 3, plasma membrane; and Fraction 5, endoplasmic reticulum. Results are expressed as percent of the total activity recovered in the gradient. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient are shown in Table 4. Data are the means ± standard deviations for three independent experiments in which all assays were done in triplicate.

D. Distribution of enzymes involved in polyphosphoinositide catabolism

PtdIns(4,5)P_2 is sequentially dephosphorylated by two specific phosphatases. The first removes the phosphate on the 5-position of the inositol ring and the second removes the 4-position phosphate. Total *in vitro* PtdIns(4)P 4-phosphatase activity was similar (3.15 µmol/min) to total PtdIns(4,5)P_2 5-phosphatase activity (2.61 µmol/min). These two phosphatases showed different distributions in the Percoll gradient (Figure 13). PtdIns(4)P 4-phosphatase was distributed throughout the gradient whereas PtdIns(4,5)P_2 5-phosphatase was recovered mainly (63 %) in the cytosol. A small amount of PtdIns(4,5)P_2 5-phosphatase was detected in plasma membrane (11 %) and microsomes (9 %). On the basis of estimates like those described for PtdIns 4-kinase (described above), PtdIns(4,5)P_2 5-phosphatase activity in the microsomes is attributable to contamination with cytosol whereas plasma membrane activity was slightly higher than could be attributed to contamination alone . This suggests that the plasma membrane contains a low, but real, amount of PtdIns(4,5)P_2 5-phosphatase. The distribution of PtdIns(4,5)P_2 5-phosphatase was further investigated to determine if the activity measured in the plasma membrane fraction could be attributed to contamination with cytosol. Increasing volumes of cytosol (fraction 1) were added to a constant amount of fraction 3. PtdIns(4,5)P_2 5-phosphatase activity in the mixtures was plotted against lactate dehydrogenase activity (Figure 14). Extrapolation to zero lactate dehydrogenase activity indicated that, in the absence of cross-contamination with cytosol, there was little PtdIns(4,5)P_2 5-phosphatase activity in the plasma membrane fraction.

The PtdIns(4,5)P_2 specific phospholipase C was also recovered mostly in the cytosol (54 %), but fraction 3 contained 21 % of the total activity recovered in the gradient, indicating that a portion of the phospholipase C was associated with the plasma membrane. The plasma membrane contains only 6 % of the total lactate dehydrogenase activity, so PtdIns(4,5)P_2 specific phospholipase C activity in fraction 3 is clearly associated with the plasma membrane. PtdIns(4,5)P_2 specific phospholipase C activity in the microsomes

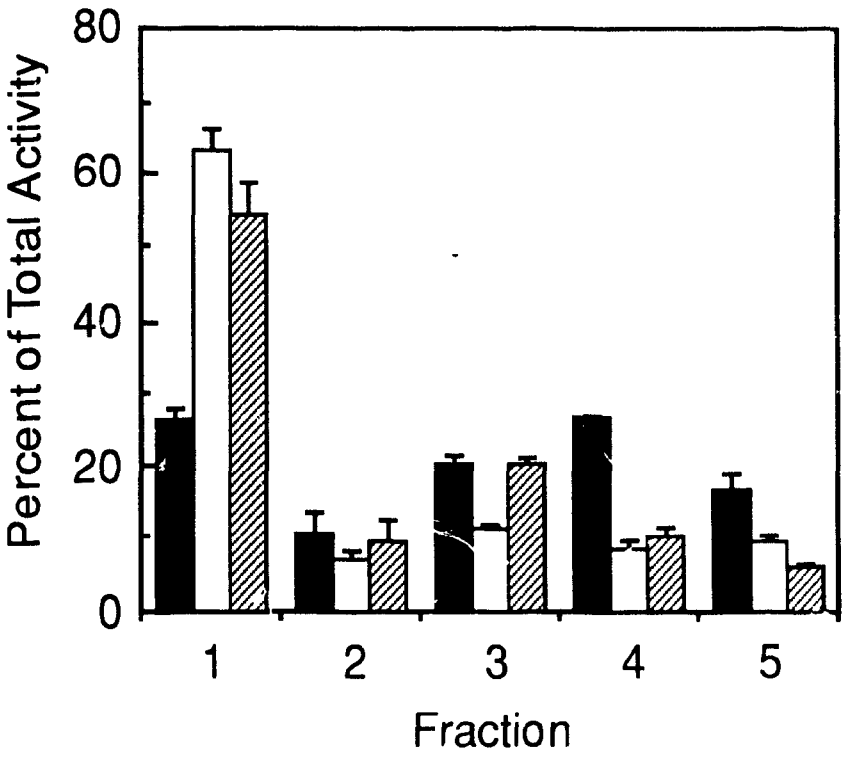


Figure 13. Subcellular distribution of enzymes of polyphosphoinositide catabolism in rat glioma cells (C6). Enzymes are PtdIns(4)P 4-phosphatase (solid bars), PtdIns(4,5)P_2 5-phosphatase (open bars) and PtdIns(4,5)P_2 specific phospholipase C (hatched bars). Fraction 1, cytosol; Fraction 3, plasma membrane; and Fraction 5, endoplasmic reticulum. Results are expressed as percent of the total activity recovered in the gradient. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient are shown in Table 4. Data are the means ± standard deviations for three independent experiments in which all assays were done in triplicate.

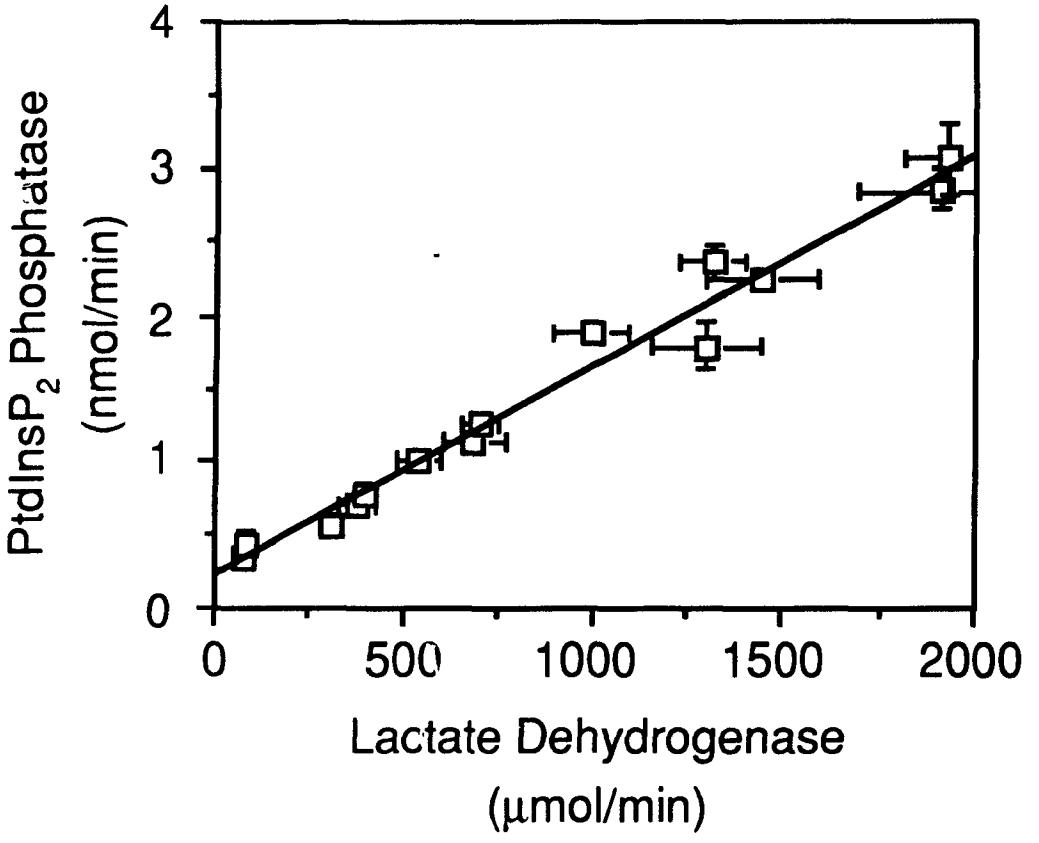


Figure 14. Estimation of contamination-dependent PtdIns(4,5)P_2 phosphatase activity in rat glioma plasma membranes. Increasing aliquots of cytosol (fraction 1) were added to a fixed aliquot of plasma membrane (fraction 3). Data is for three experiments in which each point represents the mean ± SD for three mixtures of the two fractions from a single gradient.

(6 %) is attributable to contamination of that fraction with plasma membrane and cytosol. Of the enzymes of polyphosphoinositide metabolism in C6 cells, PtdIns(4,5)P_2 specific phospholipase C is by far the most active *in vitro* (9.21 μmol/min). The relative specific activities of these enzymes in each subcellular fraction are shown in Table 4.

The large quantity of PtdIns(4,5)P_2 specific phospholipase C found in the cytosol raises the possibility of translocation from the cytosol to membranes. This was tested with digitonin permeabilized cells. Cytosolic enzymes rapidly diffuse out of digitonin permeabilized cells [252]. Permeabilization of C6 glioma results in release of 70-80 % of the total PtdIns(4,5)P_2 specific phospholipase C activity (Figure 15). This percentage is higher than the quantity found in fraction 1 (54 %) because the separation of the cell components is not absolute (Figure 13). PtdIns(4,5)P_2 specific phospholipase C in the digitonin extract includes soluble activity from fraction 1 (cytosol) and soluble activity contaminating the membrane fractions. Since 28 % of the lactate dehydrogenase activity was found in fractions other than the cytosol (fraction 1), the amount of PtdIns(4,5)P_2 specific phospholipase C measured in the cytosolic fraction is underestimated. Taking the loss of cytosolic PtdIns(4,5)P_2 specific phospholipase C into other fractions into account, and considering that contamination of the cytosol with plasma membrane is low, it is apparent that more than 70 % of the total PtdIns(4,5)P_2 specific phospholipase C activity in C6 cells is soluble. This agrees with a previous report of PtdIns(4,5)P_2 specific phospholipase C distribution between soluble and particulate fractions of these cells [115].

Phosphoinositide hydrolysis in C6 cells is stimulated by serotonin [7]. Treatment of C6 cells with 10 μM serotonin for various times did not alter the amount of PtdIns(4,5)P_2 specific phospholipase C that diffused out of the cells. Thus, no large scale translocation of the enzyme ocurred. This study was extended by examining the effects of bradykinin on PtdIns(4,5)P_2 specific phospholipase C distribution in N1E-115 cells (Figure 16). The distribution of this enzyme was not studied using Percoll gradients. However, previously published results show that 64 % of the enzyme activity in these cells is soluble [115].

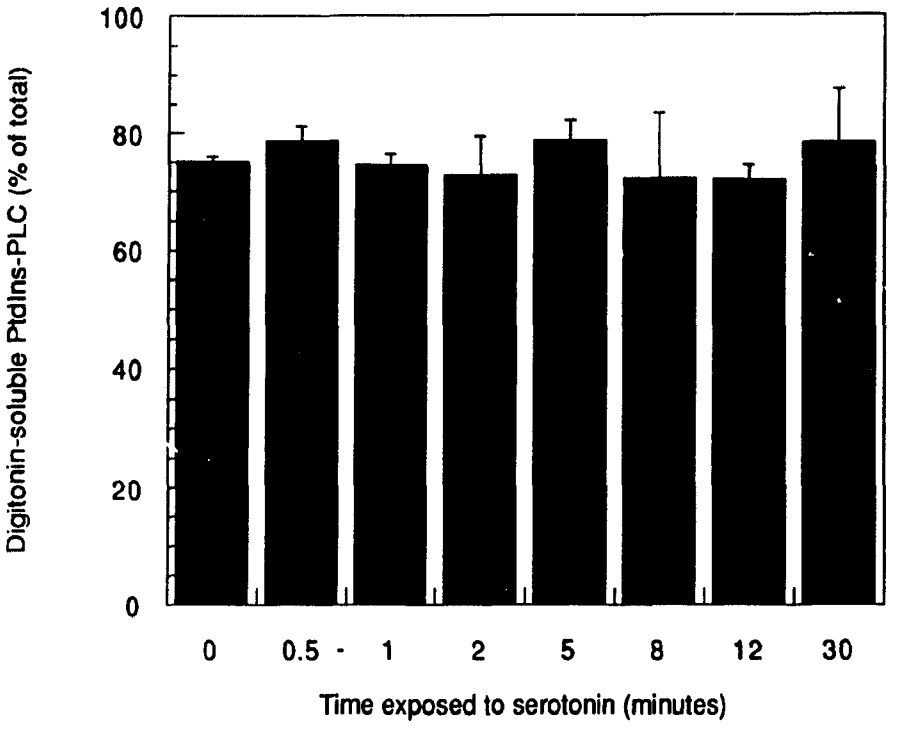


Figure 15. Effect of 10 μM serotonin on the distribution of PtdIns-PLC between digitonin and Triton X-100 soluble fractions of C6 cells grown in 35 mm dishes. The culture medium was removed and cells incubated for 2 h in 1.25 ml of DMEM. 10 μM serotonin was added and the cells incubated for the times indicated. Incubations were terminated by replacing the DMEM with 1.25 ml of ice-cold buffered digitonin. After 2 min the digitonin solution was removed and the remaining cell material was washed with a further 0.5 ml of buffered digitonin. Residual cell material was collected by scraping the dishes in 1.75 ml 0.5 % Triton X-100. Data are expressed as mean percent of the total recovered PtdIns-PLC activity which was found in the digitonin extract ± standard deviation for 3 separate dishes of cells..

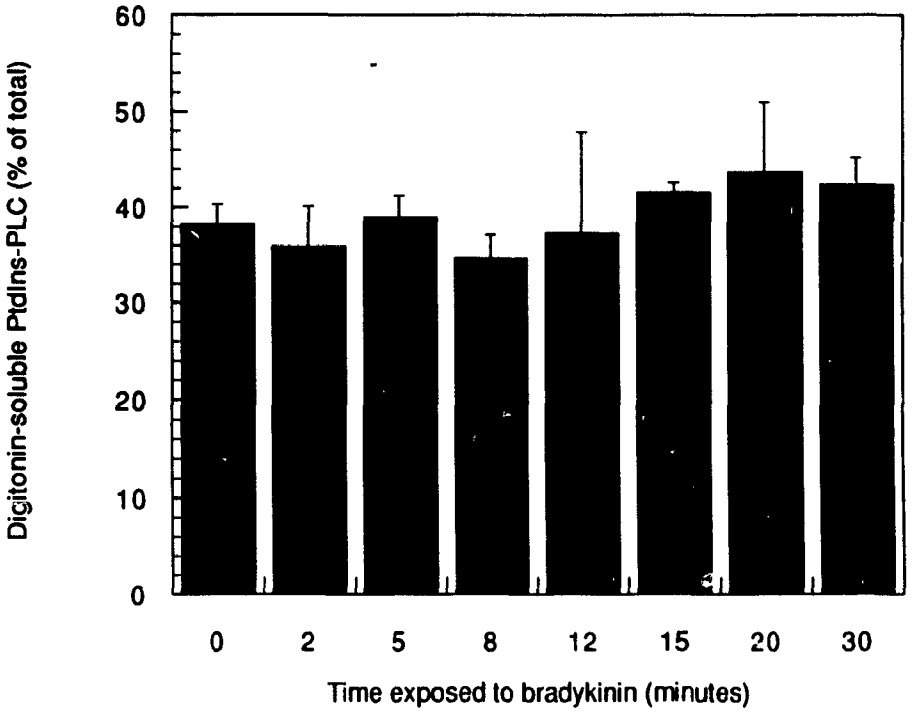


Figure 16. Effect of 10 μM bradykinin on the distribution of PtdIns-PLC between digitonin and Triton X-100 soluble fractions of N1E-115 cells grown in 35 mm dishes. The culture medium was removed and cells incubated for 2 h in 1.25 ml of DMEM. 10 μM serotonin was added and the cells incubated for the times indicated. Incubations were terminated by replacing the DMEM with 1.25 ml of ice-cold buffered digitonin. After 2 min the digitonin solution was removed and the remaining cell material was washed with a further 0.5 ml of buffered digitonin. Residual cell material was collected by scraping the dishes in 1.75 ml 0.5 % Triton X-100. Data are expressed as mean percent of the total recovered PtdIns-PLC activity which was found in the digitonin extract ± standard deviation for 3 separate dishes of cells.

Digitonin permeabilization of N1E-115 cells results in release of 40 % of the total PtdIns(4,5)P_2 specific phospholipase C in these cells. Incubation of the cells for various times after exposure to bradykinin, which is known to stimulate PtdIns(4,5)P_2 hydrolysis [116], does not result in redistribution of soluble and membrane bound PtdIns(4,5)P_2 specific phospholipase C in N1E-115 cells. Based on comparisons between how much PtdIns-PLC is released from the cells with published reports of PtdIns-PLC distribution within these cells, digitonin permeabilization of N1E-115 cells is less efficient than permeabilization of C6 cells. Considering that digitonin permeabilizes cells by interacting with sterols in the plasma membrane [252], reduced efficiency of permeabilization of N1E-115 cells may be because the ratio of cholesterol to phospholipid in the plasma membrane of these cells is only half of that in C6 glioma [59,75].

II. Subcellular Fractionation Studies; N1E-115 murine neuroblastoma cells

A. Distribution of protein and marker enzymes

Since observations for glioma cells conflicted with published reports of substantial PtdIns synthase activity in the plasma membrane of GH_3 cells [164,165], a second cell line (N1E-115 neuroblastoma) was investigated. Subcellular distribution of marker enzymes in the continuous gradient (Figure 17) was similar to that observed before for this cell line using a discontinuous Percoll gradient and dividing the gradient into five fractions of equal volume [59]. Fraction sizes were modified to optimize the purity of the three major fractions (Table 5). Distribution of marker enzyme activities was similar to that seen with glioma cells except that 5'-nucleotidase was distributed lower in the gradient. The cytosol fraction had no nucleotidase activity while fraction 5 at the bottom of the gradient (microsomes) was slightly contaminated with the plasma membrane marker (Figure 17). However, the plasma membrane fraction was almost devoid of contamination with either cytosol (lactate dehydrogenase) or endoplasmic reticulum (NADPH:cytochrome c reductase). Specific activities of the marker enzymes is shown in Table 5.

Table 5. Relative specific activity and recovery of enzyme activities in subcellular fractions of neuroblastoma (N1E-115) cells.

Data presented as described for Table 4.

Cell lysates used for each gradient contained 79.5 ± 6.5 mg protein (mean ± SD, 13 experiments). After fractionation, 43.4 ±4 .1 mg, 2.7 ± 0.1 mg, 2.1 ± 0.2 mg, 0.4 ± 0.004 mg, and 10.5 ± 0.4 mg was recovered in gradient fractions 1 to 5, respectively, with the remainder in the 900 x g crude "nuclear pellet".

	Specific Activity (lysate)	Relative Specific Activity					Recovery (%)
		1	2	3	4	5	
Lactate dehydrogenase	1.5±0.04	1.4±0.1	1.3±0.7	0.3±0.1	0.1±0.1	< 0.1	99.6±3.8
5'-Nucleotidase	2.4±0.6	< 0.1	3.5±0.2	8.7±0.8	8.9±3.2	0.5±0.1	79.4±19.6
NADPH:cyt.-c reductase	13.6±0.6	0.1±0.01	0.5±0.2	0.9±0.3	1.2±0.7	4.2±0.1	77.5±12.0
CTP:PtdOH cytidylyltransferase	22.0±0.2	< 0.1	0.1±0.1	0.2±0.1	0.3±0.1	3.3±0.1	75.8±8.1
PtdIns synthase	3.1±0.4	< 0.1	< 0.1	0.4±0.1	1.3±0.2	3.5±0.1	68.7±15.2

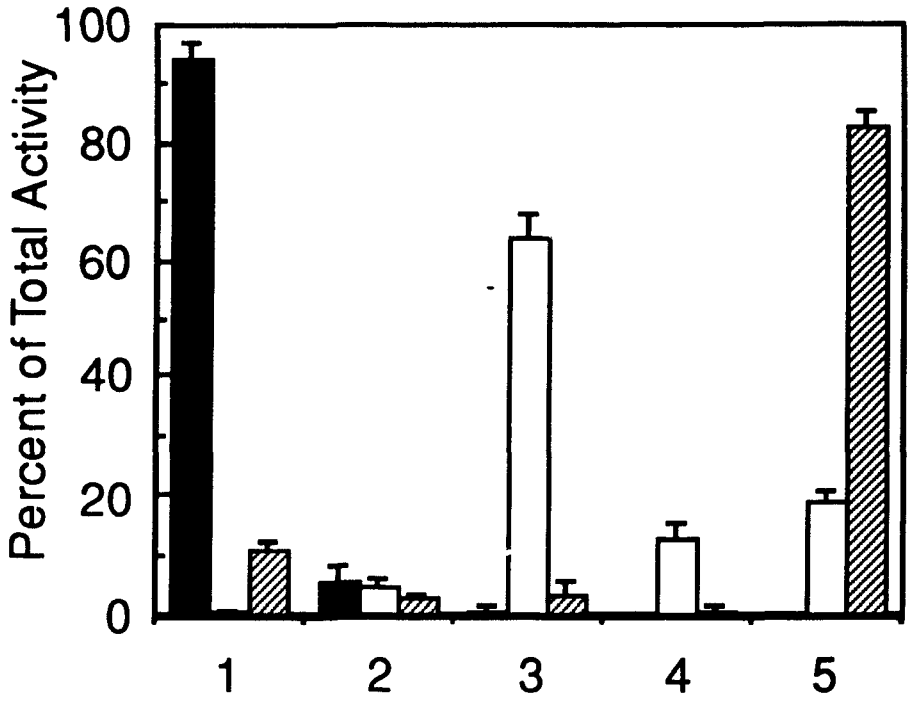


Figure 17. Subcellular distribution of marker enzymes for cytosol, plasma membrane and endoplasmic reticulum in murine neuroblastoma cells (N1E-115). Enzymes are lactate dehydrogenase (solid bars), 5'-nucleotidase (open bars), and NADPH: cytochrome c reductase (hatched bars). Results are expressed as percent of the total activity recovered in the gradient. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient are shown in Table 5. Data are the means ± standard deviations for three independent experiments in which all assays were done in triplicate.

B. Distribution of enzymes involved in PtdIns synthesis

Distribution in the gradient

Virtually all CTP:PtdOH cytidylyltransferase (96 %) and PtdIns synthase (93 %) activities were recovered in the microsomal fraction (Figure 18). The activities of these two enzymes in plasma membrane were lower in N1E-115 neuroblastoma than in C6 glioma. The plasma membrane fraction had 1 % and 2 % of the total CTP:PtdOH cytidylyltransferase and PtdIns synthase activities respectively. The low activities of CTP:PtdOH cytidylyltransferase and PtdIns synthase observed in the cytosol and plasma membrane are attributable of contamination of those fractions with microsomal fraction. The specific activities of these two enzymes are shown in Table 5. Total cellular CTP:PtdOH cytidylyltransferase activity (measured *in vitro*) is 7 times greater than PtdIns synthase activity (1.75 nmol/min and 0.25 nmol/min respectively).

A further experiment was performed to be certain that the low levels of PtdIns synthase and CTP:PtdOH cytidylyltransferase activity in the plasma membrane fraction of N1E-115 cells were indeed due to contamination with the microsomal fraction. Increasing amounts of the microsomal fraction were added to a constant amount of plasma membrane fraction and the synthetic activities compared to the activity of the endoplasmic reticulum marker, NADPH: cytochrome c reductase (Figure 19). When extrapolated to zero reductase activity, regression lines for both cytidylyltransferase and PtdIns synthase activities passed the vertical axis at or slightly below zero. Assuming NADPH:cytochrome c reductase to be a valid endoplasmic reticulum marker that is absent from plasma membrane, the results indicate that neuroblastoma N1E-115 plasma membrane does not have either of the two enzyme activities necessary to synthesis PtdIns from PtdOH.

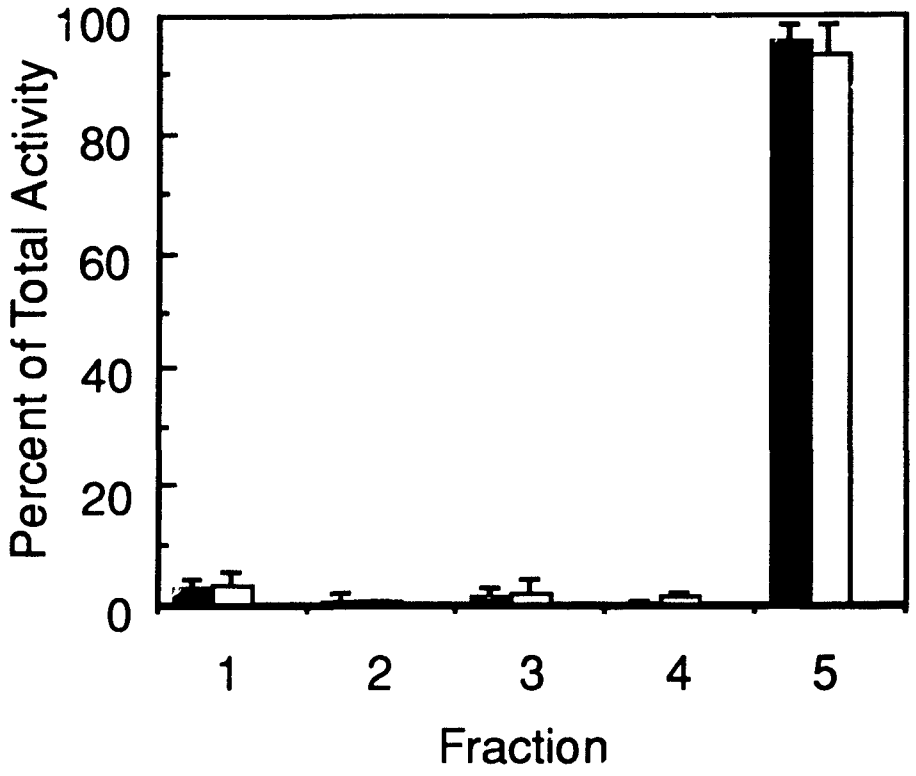


Figure 18. Subcellular distribution of enzymes of phosphatidylinositol synthesis in murine neuroblastoma cells (N1E-115). Enzymes are CTP:phosphatidic acid cytidylyltransferase, (solid bars) and PtdIns synthase (open bars). Fraction 1, cytosol; Fraction 3, plasma membrane; and Fraction 5, endoplasmic reticulum. Results are expressed as percent of the total activity recovered in the gradient. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient are shown in Table 5. Data are the means ± standard deviations for three independent experiments in which all assays were done in triplicate.

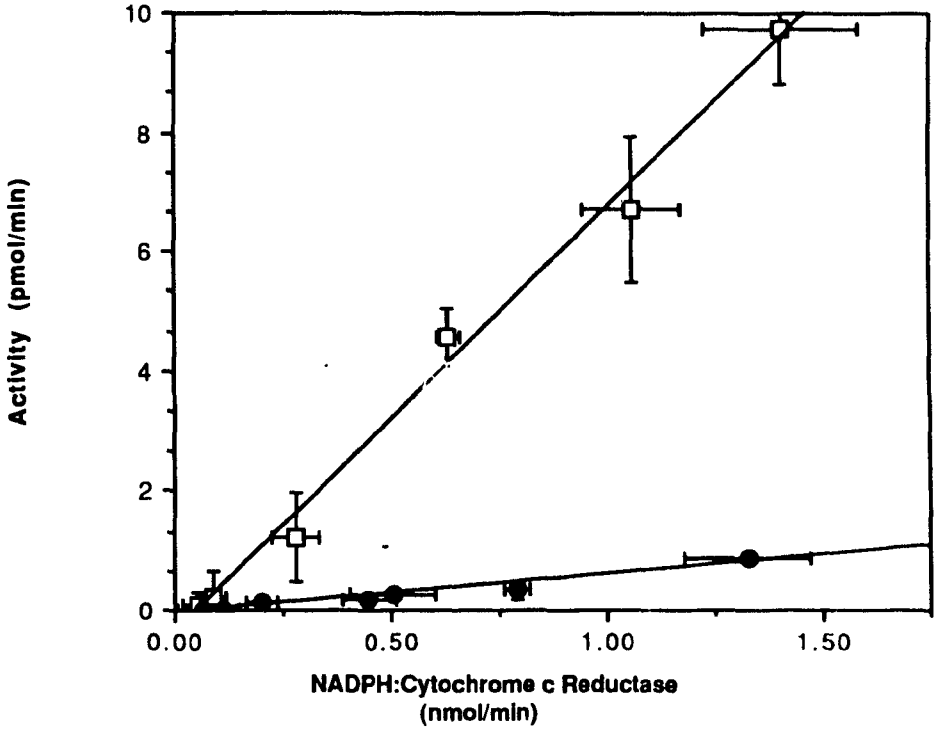


Figure 19. Estimation of contamination-dependent CTP:PtdOH cytidylyltransferase and PtdIns synthase activities in N1E-115 neuroblastoma plasma membranes. Increasing aliquots of microsomes (fraction 5) were added to a fixed aliquot of plasma membrane (fraction 3). Data are for one experiment in which each point represents the mean ± SD for three mixtures of the two fractions from a single gradient. CTP:PtdOH cytidylyltransferase, (□); PtdIns synthase, (●). Results are typical of 3 experiments in which only the slope of the regression line varied due to the gradient fractions having slightly different specific activities for the enzymes.

III LysoPtdIns metabolism

To investigate lysoPtdIns metabolism in cultured cells, 1-acyl-[^{3}H]lysoPtdIns was added to the culture medium and subsequent uptake and metabolism was monitored. The 1-acyl-lysoPtdIns used in this study was radiolabelled with [^{3}H]Ins. Figure 20 shows that N1E-115 cells did take up 2 μM 1-acyl-[^{3}H]lysoPtdIns and that they could either metabolize label into PtdIns or degrade [^{3}H]lysoPtdIns forming non-lipid, water soluble products (presumably [^{3}H]GroPIns and/or [^{3}H]Ins). Total radioactivity in the cells increased quickly for the first 2 h and continued to increase slightly thereafter. Cell associated [^{3}H]lysoPtdIns peaked at 2 h and then decreased slightly by 4 h whereas radioactivity continued to appear as PtdIns for at least 4 h (Figure 20). Using 2 μM lysoPtdIns no polyphosphoinositides were observed at any time point. When N1E-115 cells were exposed to various concentrations of [^{3}H]lysoPtdIns for 2 h, uptake into the cells increased in a concentration dependent manner (Figure 21). Uptake of [^{3}H]lysoPtdIns was strongly dependent on concentration up to 40 μM. Higher concentrations of [^{3}H]lysoPtdIns had little effect on uptake of [^{3}H]lysoPtdIns. The amount of [^{3}H]lysoPtdIns in the cells was, at all concentrations of [^{3}H]lysoPtdIns in the medium, greater than the amount of [^{3}H]PtdIns derived from [^{3}H]lysoPtdIns. [^{3}H]PtdIns increased with time and concentration of added exogneous lysoPtdIns but did not exceed 15 % of the total cell associated radioactivity at 2 h. The amount of water soluble hydrolysis products from [^{3}H]lysoPtdIns was low, < 10 % at low concentrations of exogenous lysoPtdIns but increased dramatically at concentrations greater than 10 μM and reached a plateau at 40 μM at which point hydrolysis products constitute 40 % of the total incorporated label. They too, remained less in quantity than cell associated [^{3}H]lysoPtdIns at all concentrations. At 40 and 100 μM [^{3}H]lysoPtdIns there was detectable, albeit low, synthesis of radiolabelled PtdIns(4)P and PtdIns(4,5)P_2 (Figure 22). The polyphosphoinositides constitute 2.5 % of total cell associated radioactivity in cells exposed to 100 μM 1-acyl-[^{3}H]lysoPtdIns for 2 h.

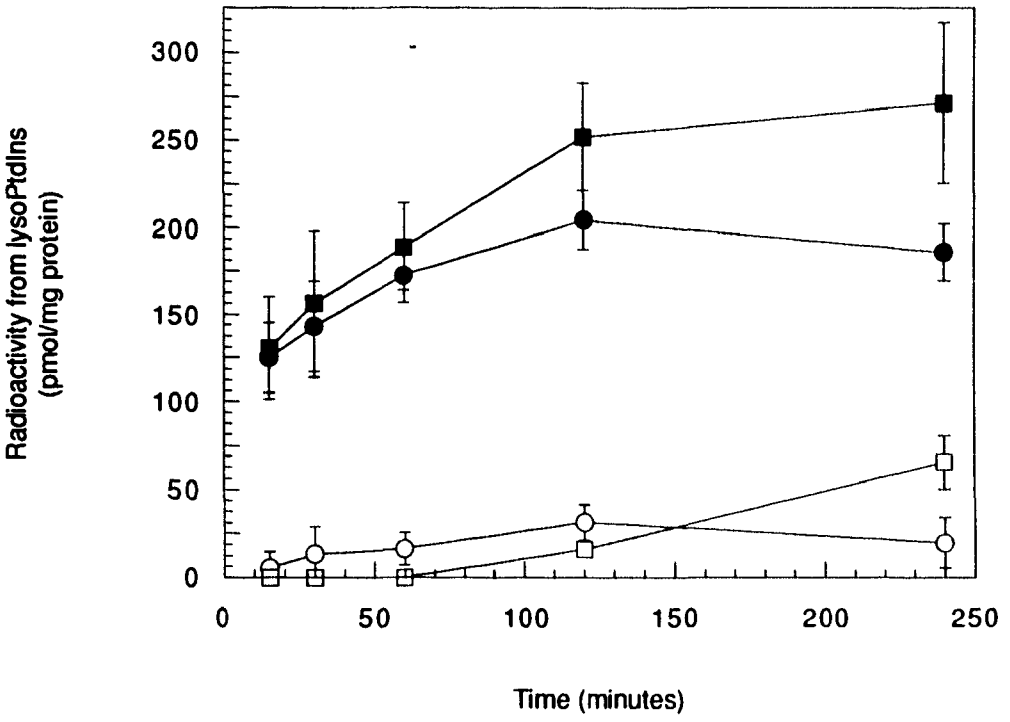


Figure 20. Time course of 1-acyl-lysoPtdIns uptake and metabolism by N1E-115 cells in culture. 2 μM 1-acyl-[^{3}H]lysoPtdIns (55 dpm/pmol) was added to 2 ml of DMEM and cells incubated for the times indicated. Cells were rinsed 3 times with cold PBS then phospholipids were exhaustively extracted under acidic conditions. Radioactivity in the organic phase was analysed with TLC system B. Total cell associated radioactivity, (■); LysoPtdIns, (●); PtdIns, (□); and water soluble products (O). Data are means ± standard deviation for 3 separate dishes of cells.

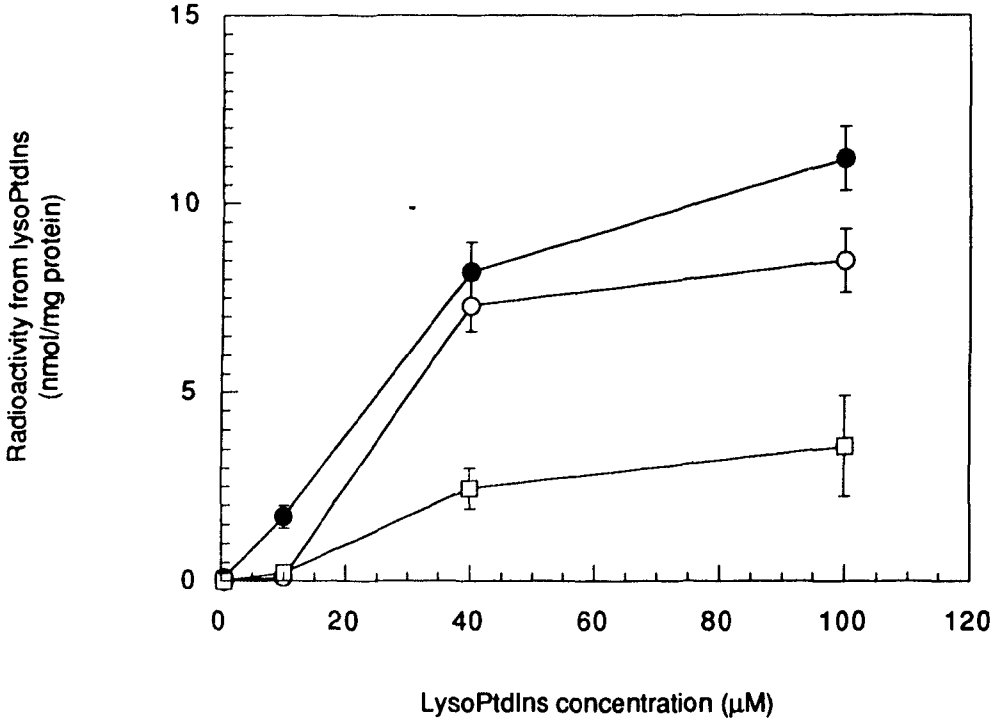


Figure 21. Concentration dependence of 1-acyl-lysoPtdIns uptake and metabolism by N1E-115 cells. Cells were incubated in 2 ml of DMEM containing 220,000 dpm of 1-acyl-[^{3}H]lysoPtdIns for 2 h. Cells were rinsed 3 times with cold PBS then phospholipids were exhaustively extracted under acidic conditions. Radioactivity in the organic phase was analysed with TLC system B. LysoPtdIns, (●); PtdIns, (□); and water soluble hydrolysis products (O). Data are means ± standard deviation for 3 separate dishes of cells.

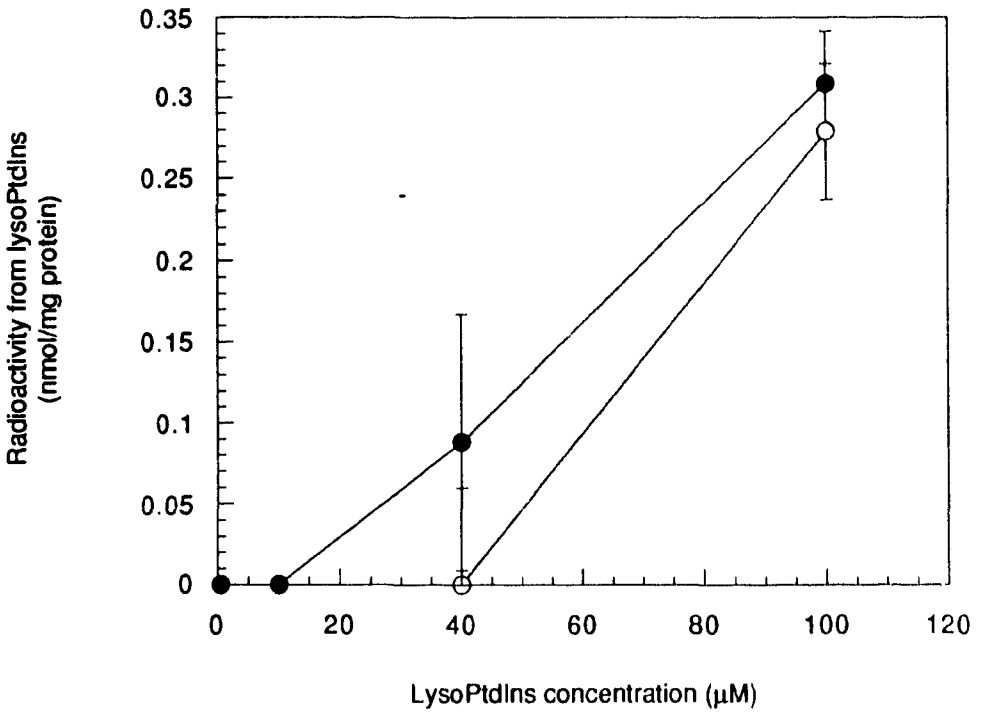


Figure 22. Concentration dependence of polyphosphoinositide synthesis from 1-acyl-lysoPtdIns N1E-115 cells. Cells were incubated in 2 ml of DMEM containing 220 000 dpm of 1-acyl-[^{3}H]lysoPtdIns for 2 h. Cells were rinsed 3 times with cold PBS then phospholipids were exhaustively extracted under acidic conditions. Radioactivity in the organic phase was analysed with TLC system B. PtdIns(4,5)P_2, (●); and PtdIns(4)P, (○). Data are means ± standard deviation for 3 separate dishes of cells.

Acylation of lysoPtdIns within the plasma membrane might affect the quantity of phosphoinositides and their fatty acid composition within the plasma membrane. Therefore, the subcellular distribution of 1-acyl-[^{3}H]lysoPtdIns metabolism was investigated using Percoll density gradients (Figure 23). The subcellular fractionation scheme used was the same as that used to determine the location of PtdIns synthesis within these cells. Subcellular fractions from N1E-115 cells prelabelled with 1-acyl-[^{3}H]lysoPtdIns for different periods of time were isolated and radioactivity derived from 1-acyl-[^{3}H]lysoPtdins was determined in the cytosolic, plasma membrane, and microsomal fractions.

Percoll is a suspension of polyvinylpyrrolidone coated silica particles. Significant amounts of this material partition into the lipid phase of chloroform/methanol/water extractions and, if Percoll is not completely removed it will interfere with separation of phospholipids on TLC. Furthermore, Percoll forms an intractable, solid mass when acid is added to a chloroform/methanol/water extraction . Therefore, it was necessary to devise a new extraction procedure to overcome these difficulties while retaining excellent recovery of phospholipids (particularly acidic phospholipids) and lysophospholipids (Methods and Apendix).

Incorporation of radiolabel from exogenous 1-acyl-[^{3}H]lysoPtdIns continued to 21 h. At 1, 4, and 21 h the cells had incorporated 266, 488, and 868 pmoles, respectively, of radioactivity from 1-acyl-[^{3}H]lysoPtdIns. The rate of radiolabel incorporation decreased with time. Water-soluble hydrolysis products of 1-acyl-[^{3}H]lysoPtdIns (likely [^{3}H]GroPIns and [^{3}H]Ins) were found in the cytosol only. Metabolism of 1-acyl-[^{3}H]lysoPtdIns within one h was limited. No PtdIns was detected in any of the fractions and little hydrolysis of 1-acyl-[^{3}H]lysoPtdIns ocurred. By one h [^{3}H]lysoPtdIns was widely distributed within the cells. The cytosolic fraction was enriched in [^{3}H]lysoPtdIns at 1 h. The possibility that [^{3}H]lysoPtdIns is redistributed during the fractionation procedure cannot be excluded. By 21 h the endoplasmic reticulum is enriched in

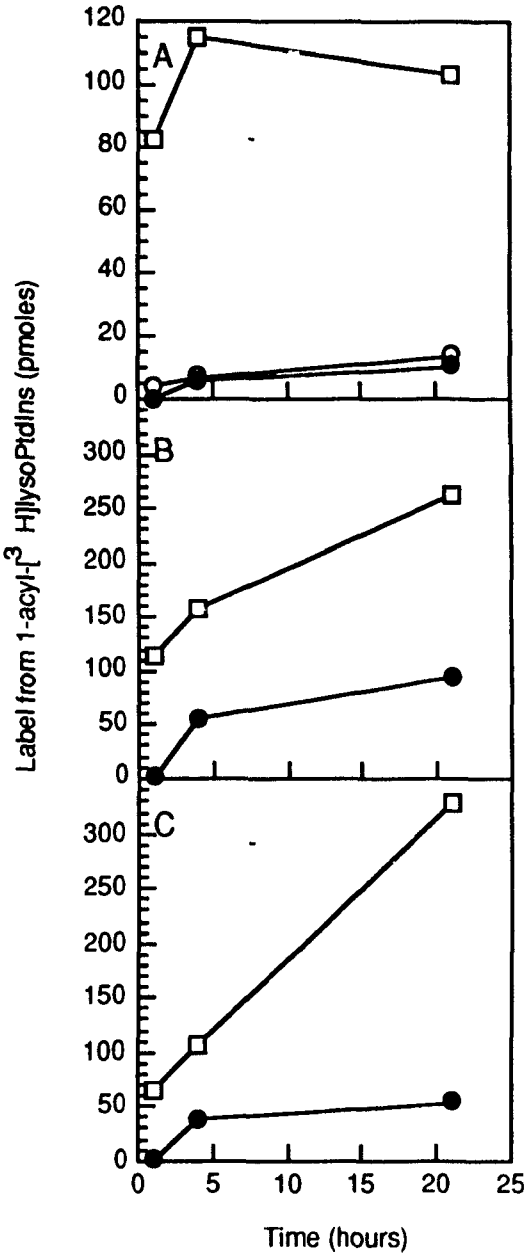


Figure 23. The subcellular distribution and metabolic fate of 1-acyl-[^{3}H]lysoPtdIns (5 μCi, 40 μM) added to the culture medium of N1E-115 cells grown in culture. Non-lipid hydrolysis products, (O); lysoPtdIns, (□); and PtdIns, (●). Panels are: A, Cytosol; B, Plasma membrane; C, Endoplasmic reticulum. Non-lipid hydrolysis products were present in the cytosol only. Data are total pmoles of [^{3}H]lysoPtdIns label appearing in each compound for each fraction.

[^{3}H]lysoPtdIns. By 4 h, [^{3}H]PtdIns was formed in the cells. [^{3}H]PtdIns was distributed in all cell fractions. The plasma membrane and microsomal fractions contained the most [^{3}H]PtdIns at 4 and 21 h. By 21 h the plasma membrane fraction contained almost twice as much [^{3}H]PtdIns as the microsomal fraction. The specific radioactivity of [^{3}H]PtdIns (moles of [^{3}H]PtdIns per mole of total PtdIns) can be calculated using the known quantities of PtdIns within the plasma membrane and microsomes [59]. The specific radioactivity of PtdIns within the plasma membrane was 3.8 fold higher than in the microsomal fraction at 4 h and is 4.6 fold higher at 21 h. This suggests that the plasma membrane may be the site of [^{3}H]PtdIns formation from 1-acyl-[^{3}H]lysoPtdIns, although synthesis of [^{3}H]PtdIns either by acylation of 1-acyl-[^{3}H]lysoPtdIns or by incorporation of [^{3}H]Ins within the microsomes and then transport to the plasma membrane is a strong possibility as this is where PtdIns synthesis from PtdOH occurs. [^{3}H]PtdIns recovered in the cytosol (10.4 pmoles) may be in the process of being transported between cellular membranes or may be due in part to contamination of that fraction with endoplasmic reticulum and plasma membrane (expected to result in approximately 6 pmoles based on contamination with enzyme markers Figure 17).

To test if the plasma membrane is capable of acylating 1-acyl-[^{3}H]lysoPtdIns and determine the contributions of the cytosolic and microsomal fractions to this process, *in vitro* assays of 1-acyl-[^{3}H]lysoPtdIns acylation were performed using subcellular fractions enriched in plasma membrane, cytosol, and microsomes. No acylation of 1-acyl-[^{3}H]lysoPtdIns was observed in the presence of aliquots of the plasma membrane using either endogenous fatty acids and cofactors or exogenous acyl-CoA. Acylation of 1-acyl-[^{3}H]lysoPtdIns with acyl-chains derived from endogenous sources was detected in aliquots of the microsomal fraction. Acylation ocurred in the presence of EDTA and CoA (Figure 24). Lower activity was observed in the presence of CoA, ATP, and $MgCl_2$. There was also some activity in the presence of EDTA alone. Acylation of 1-acyl-[^{3}H]lysoPtdIns in

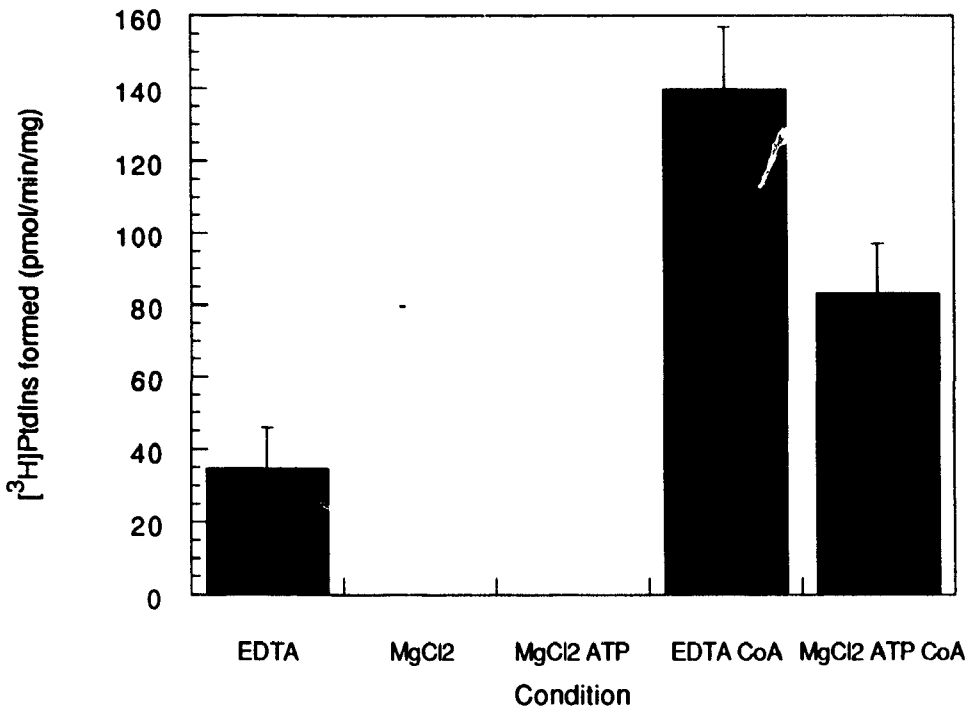


Figure 24. *In vitro* synthesis of [^{3}H]PtdIns from 1-acyl-[^{3}H]lysoPtdIns in N1E-115 microsomal fractions. Aliquots of the microsomal fraction from N1E-115 cells (enriched in NADPH cytochrome c reductase) were incubated with 25 μM 1-acyl-[^{3}H]lysoPtdIns (3200 dpm/nmol), and 40 mM HEPES pH 7.4. Other reagents were added as indicated at the following concentrations: 10 mM EDTA, 10 mM $MgCl_2$, 5 mM ATP, 5 mM Coenzyme A. The subcellular fractions contain $MgCl_2$. Data are for triplicate determinations ± standard deviation.

the presence of EDTA was highest if arachidonoyl-CoA was added (Figure 25). Oleoyl-CoA and palmitoyl-CoA were less effective substrates.

No acylation of 1-acyl-[^{3}H]lysoPtdIns was detected using cytosolic and plasma membrane fractions under any conditions. Contamination of those two fractions with microsomes should have resulted in low acylation activity (approximately 5 % of microsomal activity). The assay may not have been sensitive enough to detect low acylation activity in the cytosolic and plasma membrane fractions. Preliminary experiments using [^{14}C]acyl-CoA indicate that there was extensive hydrolysis of the fatty acyl CoA derivatives in the plasma membrane and cytosolic fractions. This may have prevented detection of acylation in those fractions.

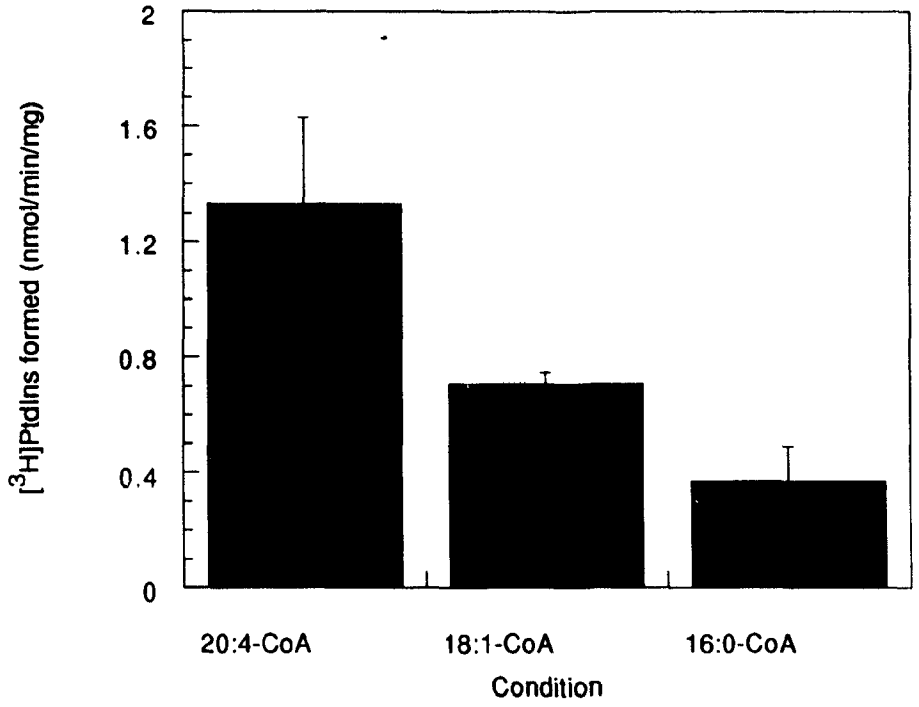


Figure 25. Acyl-Coenzyme A specificity of 1-acyl-[^{3}H]lysoPtdIns acylation. Aliquots from microsomal fractions were incubated with 25 μM 1-acyl-[^{3}H]lysoPtdIns, 40 mM HEPES pH 7.4, 10 mM EDTA and fatty acyl-CoAs as indicated at a concentration of 0.1 mM. Data are for triplicate determinations ± standard deviation.

IV. Effects of polyamines on cultured cells

Polyamines have many well documented effects on the *in vitro* activities of most of the enzymes that participate in phosphoinositide metabolism (discussed above)[438,58,327,380,240,241,380,327,94,286,354,380,451,123,336,281,380,382]. However, the relevance of these results to phosphoinositide metabolism in whole cells is not clear. The hypothesis that polyamines modulate phosphoinositide metabolism was tested by exposing N1E-115 cells grown in culture to exogenous polyamines. Phosphoinositide metabolism in whole cells was examined by radiolabelling the cells with [^{32}P]Pi and examining incorporation of this radiolabel into phospholipid. It was first necessary to determine whether N1E-115 cells were capable of incorporating exogenous polyamines and whether this resulted in an increase in total polyamine content of the cells.

A. Uptake of polyamines

1. Uptake of [^{3}H]putrescine

Uptake of [^{3}H]putrescine by N1E-115 cells was measured by incubating the cells in culture with [^{3}H]putrescine followed by extensive washing with DMEM, containing unlabelled putrescine, to reduce the quantity of polyamines adsorbed to the cell exterior. The dependence of putrescine uptake on concentration was measured by incubating the cells with [^{3}H]putrescine at various concentrations for 5 h. Uptake was linear for concentrations of putrescine from 0.05 mM to 10 mM (Figure 26) At 5 mM putrescine the N1E-115 cells had accumulated 69 nmoles of [^{3}H]putrescine per mg protein. This amounted to 1.6 % of the total putrescine in the medium.

The dependence of putrescine uptake on time was measured by incubating cells with 5 mM [^{3}H]putrescine for various times (Figure 27). N1E-115 cells quickly accumulated [^{3}H]putrescine. By 1 h they had accumulated 78 nmoles of [^{3}H]putrescine per mg protein. Maximal accumulation of [^{3}H]putrescine was 89 nmol/mg protein at 2 h. By 24 h the amount of [^{3}H]putrescine within the cells had decreased to 70 nmol/mg

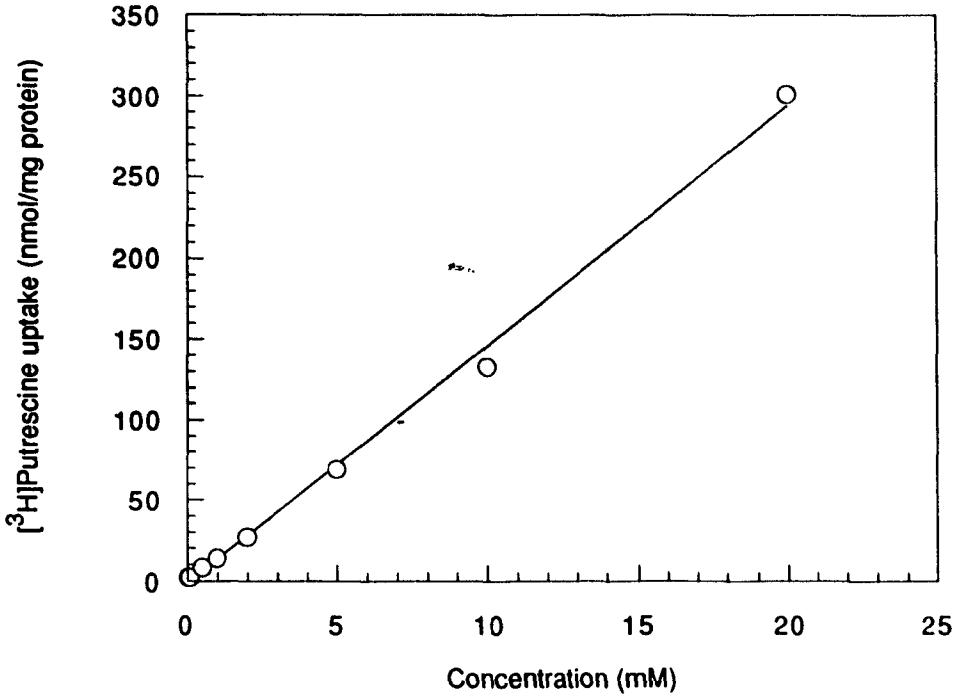


Figure 26. Dependence of [^{3}H]putrescine uptake on concentration by N1E-115 cells in culture. Cells were incubated with [3H]putresine at various concentrations for 5 h after which they were washed 3 times with cold PBS containing 5 mM putrescine. The washed cells were collected in 1 ml of water and radioactivity was determined by liquid scintillation counting. Data are for one experiment.

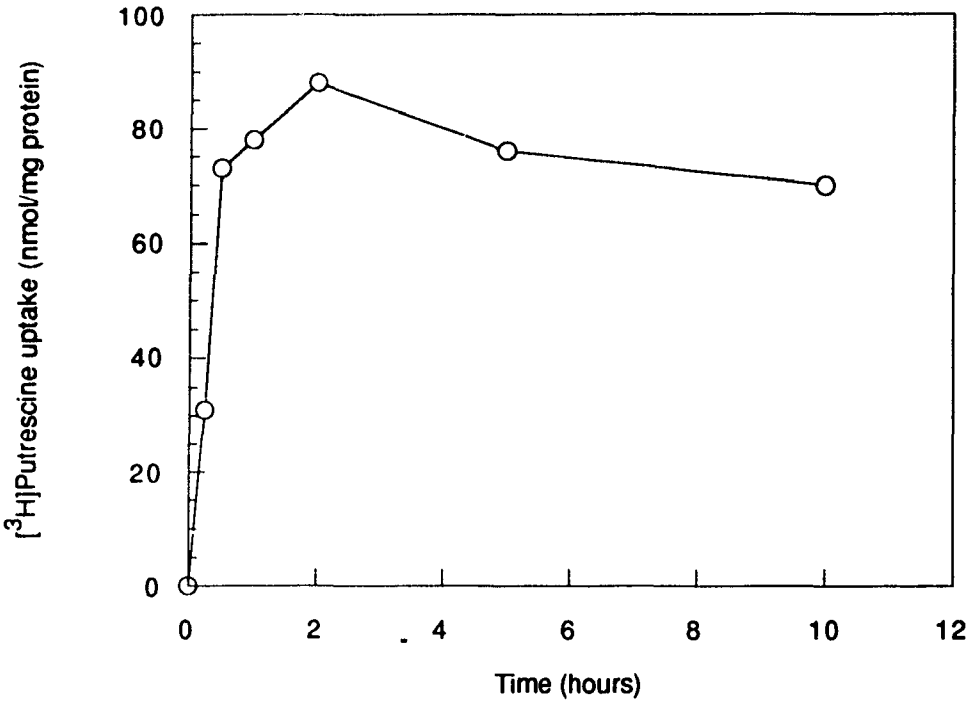


Figure 27. Dependence of [^{3}H]putrescine uptake on time by N1E-115 cells in culture. Cells were incubated with 5 mM [^{3}H]putrescine for various times after which they were washed 3 times with cold PBS containing 5 mM putrescine. The washed cells were collected in 1 ml of water and radioactivity was determined by liquid scintillation counting. Data are for one experiment.

protein. At all times tested cells were normal with regards to appearance, adherence to the dish, and total mg of cell protein recovered per dish. Therefore, long term exposure of the cells to 5 mM putrescine was not toxic. Also, putrescine was taken up by N1E-115 cells in a concentration dependent manner and equilibrium was reached by 2 h.

Putrescine uptake is mediated by a sulfhydryl containing protein and is subject to regulation by hormones and neurotransmitters [187,342]. Modulation of putrescine transport was examined in cells which were pre-incubated with N-ethylmaleimide, which covalently modifies sulfhydryl groups. Pre-incubation of N1E-115 cells with 5 mM N-ethylmaleimide for 5 min decreased [^{3}H]putrescine uptake by more than 93 % (Figure 28). Adherence of the cells to the dishes and total protein per dish was not altered by treatment of the cells with N-ethylmaleimide. The effect of bradykinin, which stimulates phosphoinositide turnover in neuroblastoma cells, on putrescine transport was examined. Bradykinin (10 μM) added at the same time as putrescine stimulated the transport of putrescine by greater than 65 % (Figure 28).

2. Measurement of polyamine mass

Incorporation of [^{3}H]putrescine demonstrated that N1E-115 cells were capable of transporting polyamines from the extracellular medium into the cell interior. However, this did not prove that polyamine content was increased or that polyamines were taken up into the cells in a biologically relevant manner. To address these points it was necessary to measure the mass of putrescine, spermidine, and spermine within the cells exposed to exogenous polyamines and in control cells.

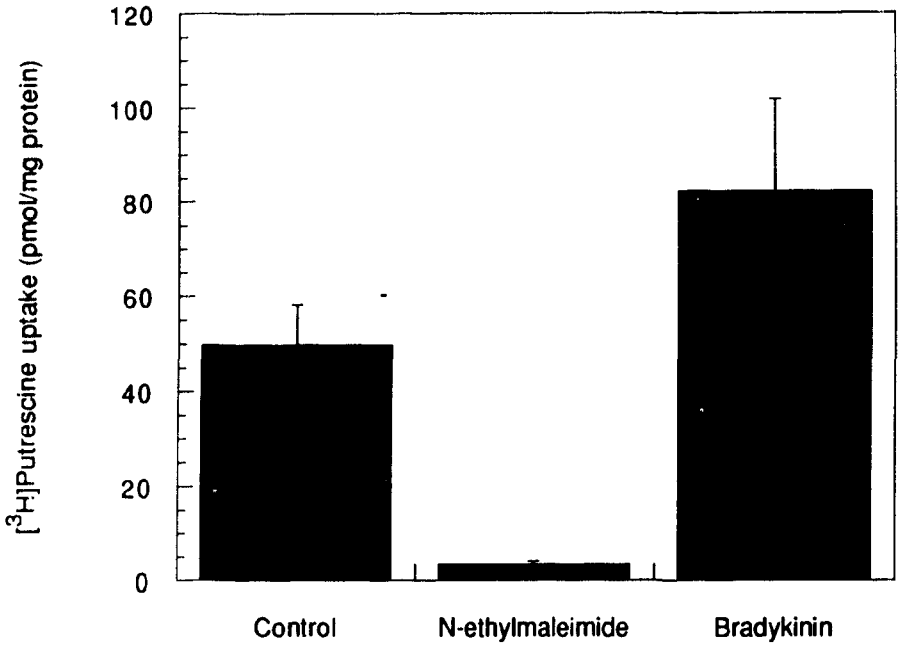


Figure 28. Effect of 5 mM N-ethylmaleimide and 10 μM bradykinin on uptake of [^{3}H]putrescine. Cells were incubated with 1 μM [^{3}H]putrescine for 20 min. Cells were pre-treated with N-ethylmaleimide for 5 min. Putrescine in DMEM was added to the cells after they had been rinsed twice. Bradykinin was added to the cultures at the same time as the putrescine. [^{3}H]Putrescine uptake by N-ethylmaleimide and bradykinin treated cells were significantly different from control ($p < 0.005$ and $p < 0.05$ respectively). Data are the means ± standard deviation for 3 separate dishes of cells.

Polyamine mass in N1E-115 cells was measured by HPLC (Figure 29). Under normal culture conditions N1E-115 cells had 2.2 ± 0.4 nmoles of putrescine per mg protein, 6.0 ± 1.0 nmoles of spermidine per mg protein, and 14.4 ± 0.6 nmoles of spermine per mg protein. Cells were incubated in culture with exogenous polyamines to raise the intracellular polyamine concentration. The exogenous concentration of polyamines was set at 5 mM for several reasons. Figures 26 and 27 show that 5 mM putrescine is rapidly incorporated into the cells. Also, this concentration was higher than the estimated concentration of polyamines within cells (0.1-2 mM) [347,403,401,445]. Finally, *in vitro* polyamine effects on enzymes of phosphoinositide metabolism occur between 1 and 10 mM polyamine [438,58,327,380,240,241,380,327,94,286] [354,380,451,123,336,281,380,382]. Exogenous polyamine concentrations above 5 mM were avoided to reduce the risk of toxicity [70,50].

Incubation of the cells for 1 h with 5 mM putrescine caused a 3 fold increase in the putrescine content of the cells. The intracellular spermine content was slightly, but significantly, increased following incubation with putrescine, presumably due to addition of aminopropyl groups to putrescine after incorporation into the cells. Incubation of the cells with 5 mM spermidine caused the intracellular content of spermidine to increase by more than a factor of 2. The intracellular content of putrescine and spermine were also increased. Putrescine content more than doubled due to degradation of spermidine after incorporation into the cells. When cells were exposed to 5 mM spermine the intracellular spermine content increased almost 4 fold and the putrescine level doubled due to degradation of spermine within the cells. Spermidine content increased by a small but statistically significant amount following incubation of the cells with spermine. Total polyamine content increased from 22 ± 2 in control cells to 31 ± 5, 37 ± 8, and 46 ± 5 nmol/mg protein in putrescine, spermidine, and spermine treated cells. Thus, treatment of the cells with exogenous polyamines results in up to a 2 fold increase in total polyamine content of the cells.

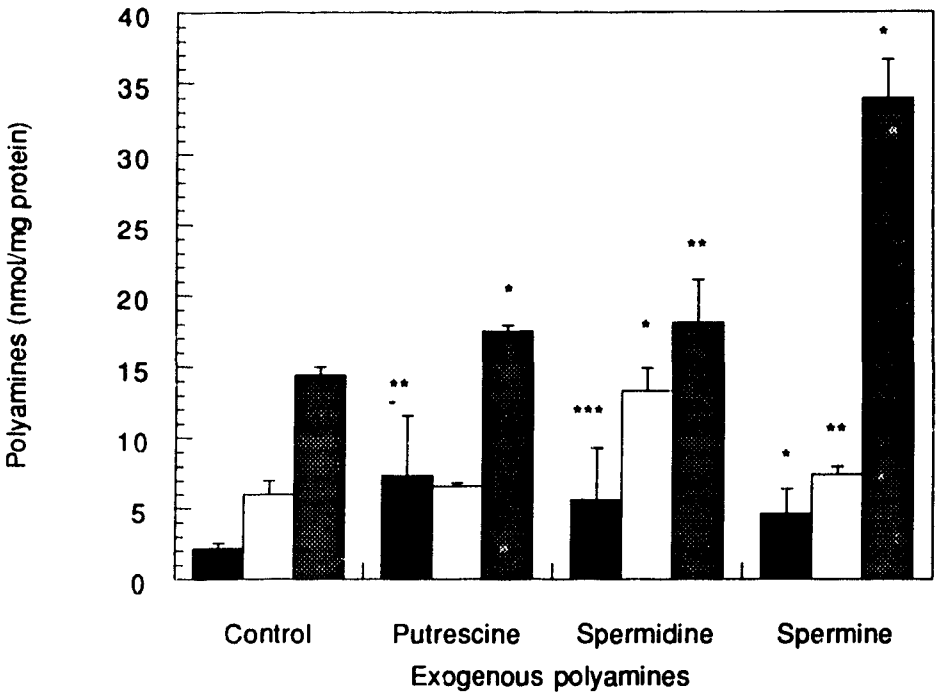


Figure 29. Intracellular content of polyamines in N1E-115 cells. Cells were incubated in the presence of 5 mM polyamines, as indicated on the X-axis for 1 h. Data are means ± standard deviation for 6 separate dishes of cells. Solid bars, Putrescine; Open bars, Spermidine; Grey bars, Spermine. Values of polyamine mass were significantly different from control values as indicated, $p < 0.005$, *; $p < 0.01$,**; $p < 0.025$,***.

B. Effects of polyamines on phosphoinositide metabolism in cultured cells

1. Basal phosphoinositide metabolism

To determine what effects increased intracellular polyamines content have on phosphoinositide metabolism, cells in culture were incubated with [^{32}P]Pi and exogenous polyamines. Incubation with [^{32}P]Pi allows determination of synthesis and, if mass remains constant, the metabolic turnover of phospholipids. For lipids synthesized from CMP-PtdOH, such as PtdIns and PtdGro, [^{32}P]Pi incorporation results from *de novo* PtdOH synthesis or phosphorylation of DAG. Radiolabel incorporation into PtdCho and PtdEtn is via phosphorylation of choline and ethanolamine. Radiolabel that has appeared in one class of phospholipid can appear in another following base exchange (ie. [^{32}P]Pi is incorporated into PtdEtn before appearing in PtdSer). Thus, during pulse conditions the rate of [^{32}P]Pi incorporation into a phospholipid class represents synthesis and this radiolabel incorporation occurs at well defined metabolic steps.

As demonstrated above (Figur · 29), N1E-115 cells accumulated significant amounts of polyamines when exposed to polyamines in the culture medium. To investigate the effects of changing polyamine levels on phosphoinositide metabolism, cells were incubated with [^{32}P]Pi and polyamines together for various times. At 1 h, cells incubated with putrescine, spermidine, and spermine showed no difference in their incorporation of [^{32}P]Pi into most phospholipid classes (Table 6). Putrescine did not alter [^{32}P]Pi incorporation into the phosphoinositides. However, incorporation of radiolabel into PtdIns(4,5)P_2 was selectively reduced to 60 % ($p < 0.025$) and 30 % ($p < 0.005$) of control by spermidine and spermine, respectively.

Cells incubated for one h with putrescine, spermidine, or spermine adhered to the surface of the culture dishes and total mg of protein recovered per dish was not affected. Incubation with spermine and spermidine, but not putrescine, for 4 h or longer caused the cells to lift off the dish and to be lost when the medium was removed resulting in lower

Table 6. Effect of polyamines on [^{32}P]Pi incorporation into phospholipids after 1 h in the presence of [^{32}P]Pi and 5 mM polyamine. Radiolabel incorporation into individual phospholipid classes is expressed as percentage of control values (from cells that were not exposed to polyamines). PtdIns(4,5)P_2 levels were significantly decreased by spermine ($p<0.005$) and spermidine ($p<0.025$). Data are the means of 3 experiments ± standard deviation. Each experiment was performed in triplicate.

Phospholipid	Exogenous Polyamine		
	Putrescine	Spermidine	Spermine
PtdCho	107 ± 6	84 ± 25	106 ± 22
PtdSer	136 ± 60	78 ± 34	114 ± 17
PtdIns	106 ± 30	90 ± 9	93 ± 18
PtdEtn/PtdOH	93 ± 18	88 ± 21	103 ± 39
PtdGro	98 ± 29	106 ± 4	103 ± 7
Ptd(Gro)$_2$	104 ± 7	99 ± 6	100 ± 1
PtdInsP$_2$	100 ± 42	60 ± 11	30 ± 6
PtdInsP	93 ± 22	91 ± 5	88 ± 21

recovery of protein per dish. Therefore, only putrescine was used in experiments designed to examine long term effects of polyamines on N1E-115 cells in culture. Cells exposed to 5 mM putrescine for up to 8 h were normal with regard to appearance, adherence, and no difference was found in the amount of cell protein per dish. [^{32}P]Pi incorporation into total phospholipid was not altered by 5 mM putrescine until 4 h of incubation (Figure 30). Radiolabel incorporation into total phospholipid from 1 to 4 h was not noticeably affected by exogenous putrescine. By 6 h, cells treated with exogenous putrescine had incorporated significantly more [^{32}P]Pi into phospholipid ($p < 0.025$) compared to cells not exposed to putrescine. Putrescine-treated cells had 1.2 fold more [^{32}P]Pi incorporated into total phospholipid at 6 h than control cells.

Putrescine-enhanced [^{32}P]Pi incorporation occured in specific phospholipid classes (Figure 31). Total radioactivity in PtdCho at 6 h was the same for both putrescine treated and control cells. In contrast, [^{32}P]Pi incorporation into PtdIns, PtdIns(4)P, PtdIns(4,5)P_2, and PtdEtn/PtdOH increase 1.6, 1.4, 1.3, and 1.2 fold, respectively, over control values. Radioactivity in the other phospholipid classes was not significantly affected by putrescine. Therefore, putrescine increased phosphoinositide synthesis at long time periods. Spermine and spermine have a short term effect, resulting principally in a decrease in [^{32}P]PtdIns(4,5)P_2 synthesis.

Polyamine effects on phosphoinositide metabolism were further characterized in [^{32}P]Pi pulse-chase experiments. Cells pre-labelled with [^{32}P]Pi in the presence of polyamines were incubated in non-radioactive medium (but with polyamines) for various times and [^{32}P]Pi incorporation into individual phospholipid classes was examined (Figure 32 and Figure 33). Controls had no exogenous polyamines at any time. Even though the radioactive medium had been removed, [^{32}P]Pi incorporation into lipid continued to increase for some time. The rate of incorporation of [^{32}P]Pi into total phospholipid after the medium was changed to fresh medium that did not contain [^{32}P]Pi, exceeded the rate of [^{32}P]Pi incorporation in the continuous presence of [^{32}P]Pi. Between 1 h and 2 h of

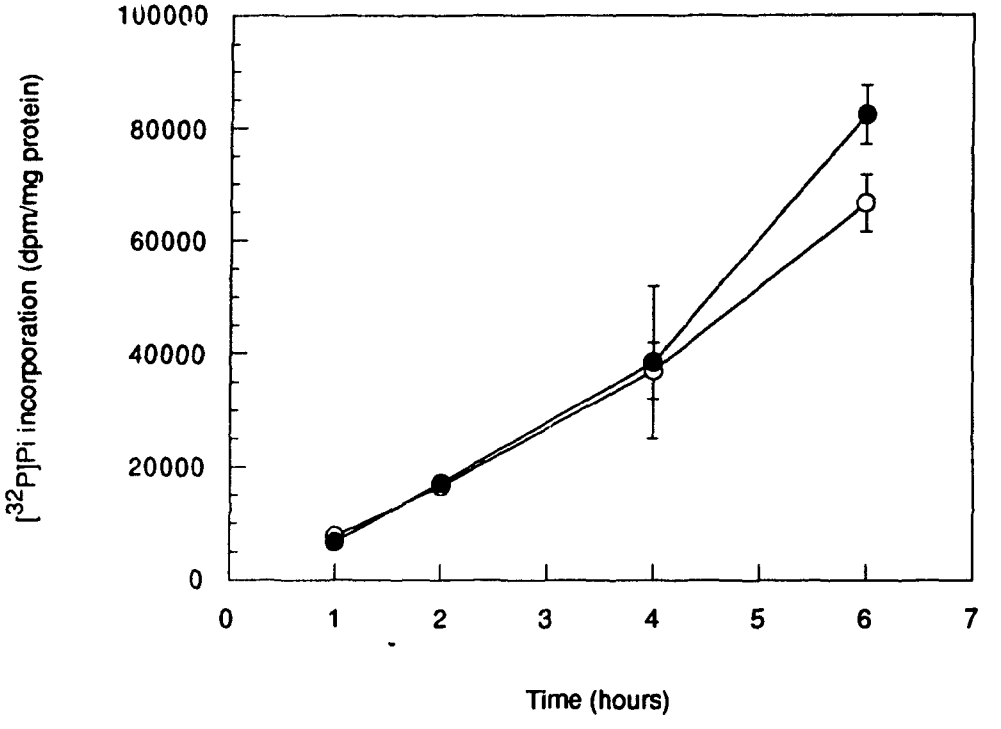


Figure 30. Effect of exogenous putrescine on incorporation of [^{32}P]Pi into total phospholipid. N1E-115 cells in culture were incubated with 5 mM putrescine and 15 μCi / dish of [^{32}P]Pi for 1 to 6 h. Data are total radioactivity soluble in chloroform/methanol expressed as dpm/mg protein ± standard deviation for 3 separate dishes of cells. Control cells, O; Putrescine treated cells, ●.

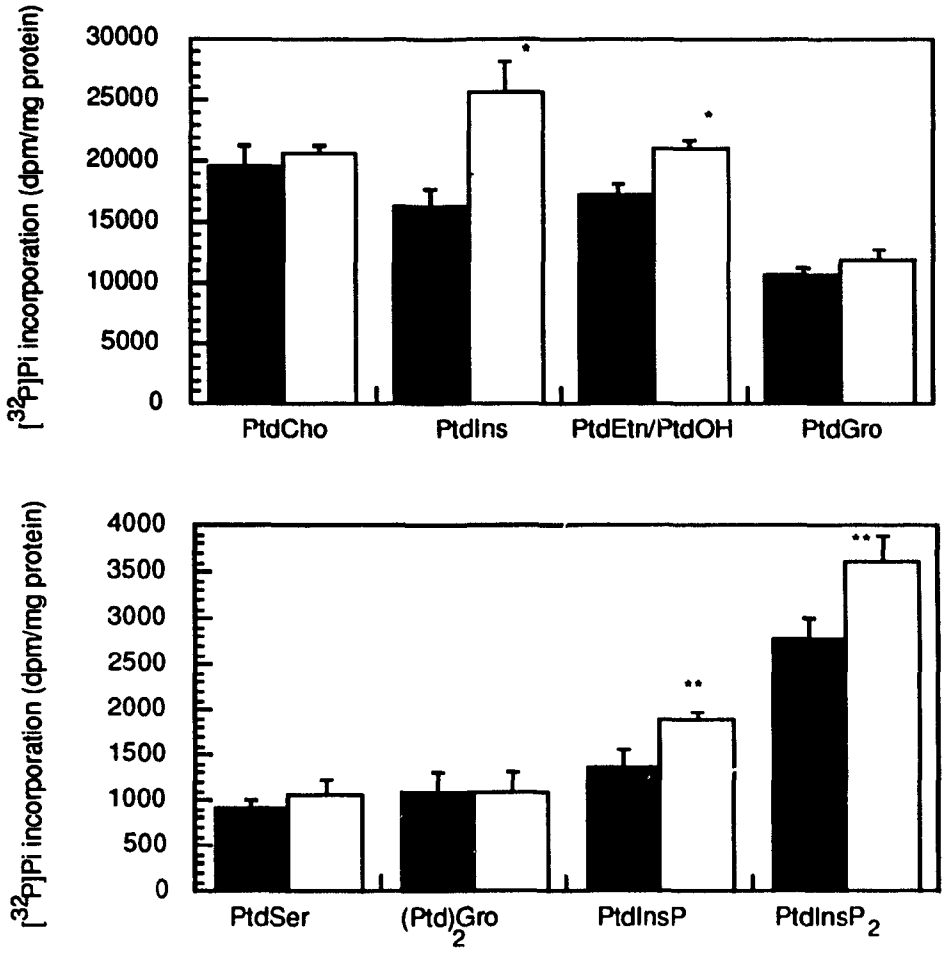


Figure 31. Effect of putrescine on [^{32}P]Pi incorporation into individual phospholipid classes. N1E-115 cells were incubated with [^{32}P]Pi and putrescine for 6 h. Cells were harvested and lipids extracted. Individual phospholipid classes were separated using TLC. Control cells, solid bars; Putrescine treated cells, open bars. Values of [^{32}P]Pi incorporation were significantly different from control values as indicated, $p<0.005$, *; $p<0.01$,**. Data are means ± standard deviation 3 separate dishes of cells.

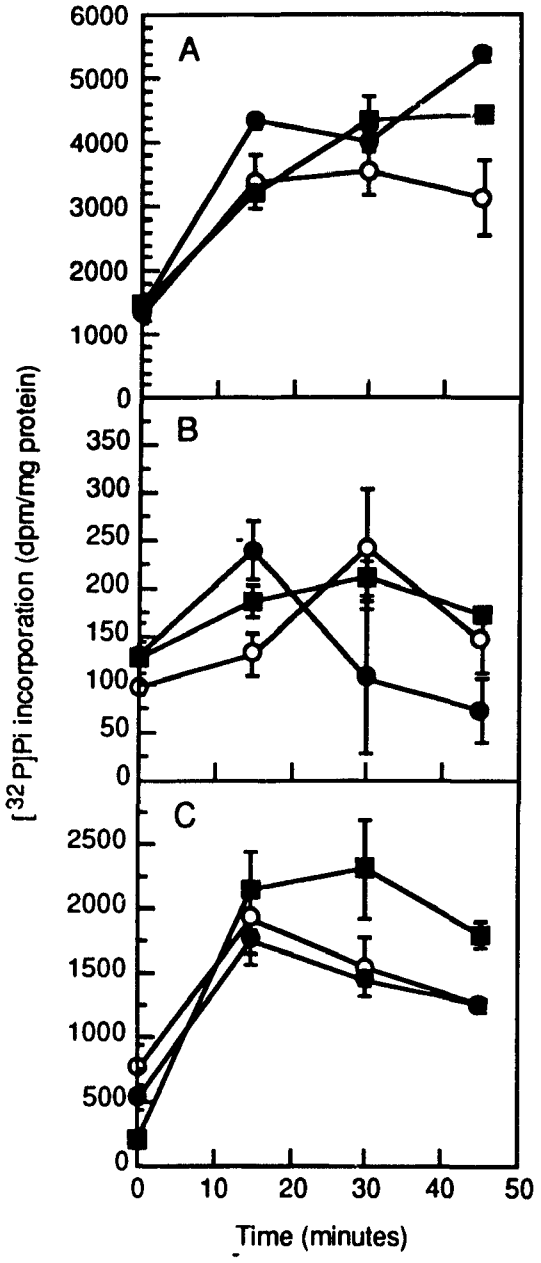


Figure 32. Effect of putrescine and spermine on chase of [^{32}P]Pi from phosphoinositides. Cells were pre-labelled with 15 μCi [^{32}P]Pi in the presence of 5 mM putrescine or spermine for 1 h. The cells were rinsed and further incubated in non-radioactive medium for the indicated times. Control cells, (O); Putrescine treated cells, (●); Spermine treated cells, (■). Panel A, PtdIns; Panel B, PtdIns(4)P; Panel C, PtdIns(4,5)P_2. Controls had no exogenous polyamines in the medium at any time. Data are means ± standard deviation for 3 separate dishes of cells.

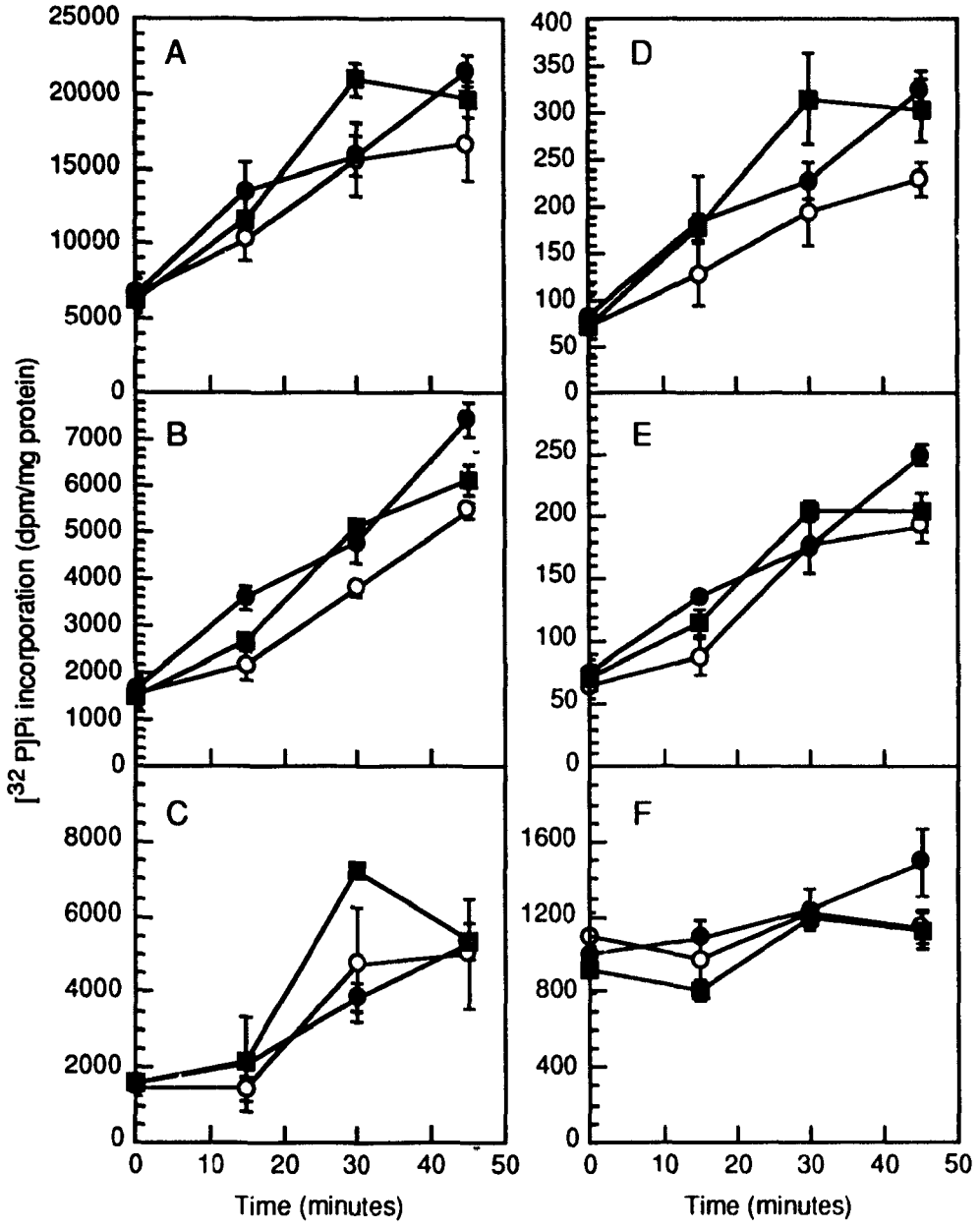


Figure 33. Effect of putrescine and spermine on chase of [^{32}P]Pi from phospholipids. Experiments were performed as in figure 32. Control cells, (O); Putrescine treated cells, (●); Spermine treated cells, (■). Panel A, Total lipid; Panel B, PtdCho; Panel C, PtdEtn/PtdOH; Panel D, PtdSer; Panel E, PtdGro; Panel F, $(Ptd)_2Gro$. Controls had no exogenous polyamines in the medium at any time. Data are means ± standard deviation for 3 separate dishes of cells.

pulse with [^{32}P]Pi, total [^{32}P]phospholipid within cells in the presence of [^{32}P]Pi increased from 7776 ± 834 to 16599 ± 1387 dpm/mg protein, a 2.1 fold increase in one h (Figure 30). In a chase experiment, total [^{32}P]phospholipid within cells exposed to [^{32}P]Pi for one h and then placed in non-radioactive medium for 0.5 h increases from 6632 ± 1029 to 15628 ± 2478 dpm/mg protein, a 2.4 fold increase in total [^{32}P]phospholipid in just 0.5 h (Figure 33).

During chase of [^{32}P]Pi with non-radioactive phosphate from the medium, radioactivity in PtdIns(4,5)P_2 continued to increase for 15 min in control and putrescine treated cells (Figure 32). After 30 min, [^{32}P]Pi PtdIns(4,5)P_2 had decreased in control and putrescine treated cells. In contrast, [^{32}P]Pi PtdIns(4,5)P_2 in spermine treated cells increased for a longer time and to a greater extent than in control cells despite the fact that it started out lower at the end of the [^{32}P]Pi pulse (as expected from Table 6). [^{32}P]Pi PtdIns(4,5)P_2 in spermine treated cells increased more than 10 fold in the 30 min after [^{32}P]Pi was removed from the medium. After 30 min, radioactivity in PtdIns(4,5)P_2 of control cells was only twice what it was when the radioactivity was removed from the medium (zero time). Despite the fact that at the end of the pulse period (zero time) [^{32}P]PtdIns(4,5)P_2 was low in spermine treated cells, after 30 min of chase spermine treated cells contained 1.5 fold higher [^{32}P]PtdIns(4,5)P_2 levels than control cells. This was due to the high rate of [^{32}P]Pi incorporation into PtdIns(4,5)P_2 of spermine treated cells during the chase period. Total radioactivity incorporation into PtdIns(4)P was low and there were no consistent differences between control, putrescine, and spermine treated cells (Figure 32). In control cells [^{32}P]PtdIns increased for the first 15 min of chase and then leveled off (Figure 32). [^{32}P]PtdIns increased for a longer time in both putrescine and spermine treated cells. Compared to control, [^{32}P]Pi incorporation into PtdCho and PtdSer was stimulated during the phosphate chase by putrescine and spermine. In contrast, no consistent effect of polyamines on [^{32}P]Pi incorporation into PtdGro, (Ptd)$_2$Gro or PtdEtn/PtdOH was seen during the phosphate chase (Figure 33).

2. Effect of polyamines on agonist stimulated phosphoinositide metabolism

To examine agonist stimulated loss of PtdIns(4,5)P_2, it was necessary to first radiolabel the cells for 1 h with [^{32}P]Pi and then submit the cells to a 30 min chase with medium containing non-radioactive phosphate. This chase period was sufficient to allow radioactivity in PtdIns(4,5)P_2 to decline (Figure 32). This allowed detection of the loss of labelled PtdIns(4,5)P_2 while [^{32}P]Pi incorporation due to resynthesis was reduced. Under control conditions, a 45 second treatment of the cells with bradykinin which activates PtdIns-PLC resulted in loss of 76 % of the prelabelled PtdIns(4,5)P_2 (Figure 34). A slightly lower proportion of the PtdIns(4,5)P_2 label (64 %) was lost in cells that had been treated with spermine. This bradykinin effect was specific for PtdIns(4,5)P_2 loss; PtdIns and PtdIns(4)P labelling were unaffected by short term exposure to bradykinin.

Agonist-stimulated synthesis of PtdIns occurs in N1E-115 cells (Figure 35). [^{32}P]PtdIns synthesis was enhanced more than 3 fold in cells pre-incubated with [^{32}P]Pi for 1 h and then incubated with [^{32}P]Pi and bradykinin together for a further 30 min. Exposure of N1E-115 cells to exogenous putrescine or spermine for 90 min in the absence of bradykinin did not alter [^{32}P]Pi incorporation into PtdIns. Cells exposed to putrescine or spermine were sensitive to bradykinin and agonist-stimulated [^{32}P]PtdIns synthesis was comparable to cells that had not been treated with exogenous polyamines (Figure 35). Thus, bradykinin stimulated PtdIns synthesis was not affected by polyamines in the extracellular medium or by increased intracellular polyamine levels.

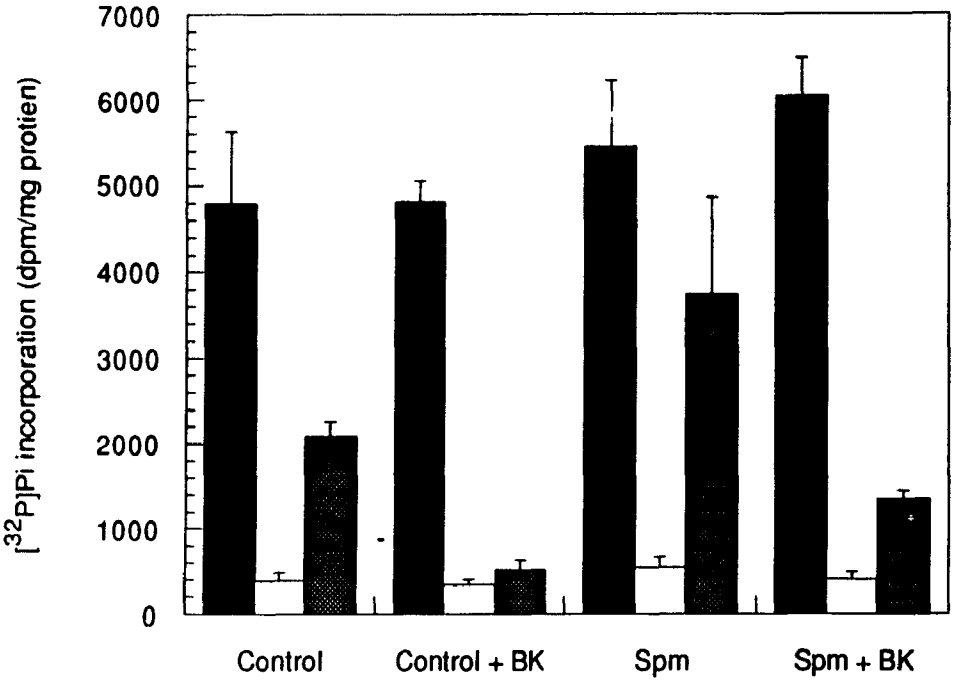


Figure 1. Effect of spermine on bradykinin stimulated PtdIns(4,5)P_2 hydrolysis. Cells were incubated with [^{32}P]Pi and the indicated polyamine for one h. The medium was removed and the cells were rinsed. The cells were incubated for a further 30 min in the continued presence of polyamine but without [^{32}]Pi. Bradykinin (BK, 10 μM) was then added and the incubations were terminated after a further 45 seconds. Solid bars, PtdIns; Open bars, PtdIns(4)P; Grey bars, PtdIns(4,5)P_2. Data are the means ± standard deviations for one experiment performed with 3 separate dishes of cells in each group and are representative of 2 experiments.

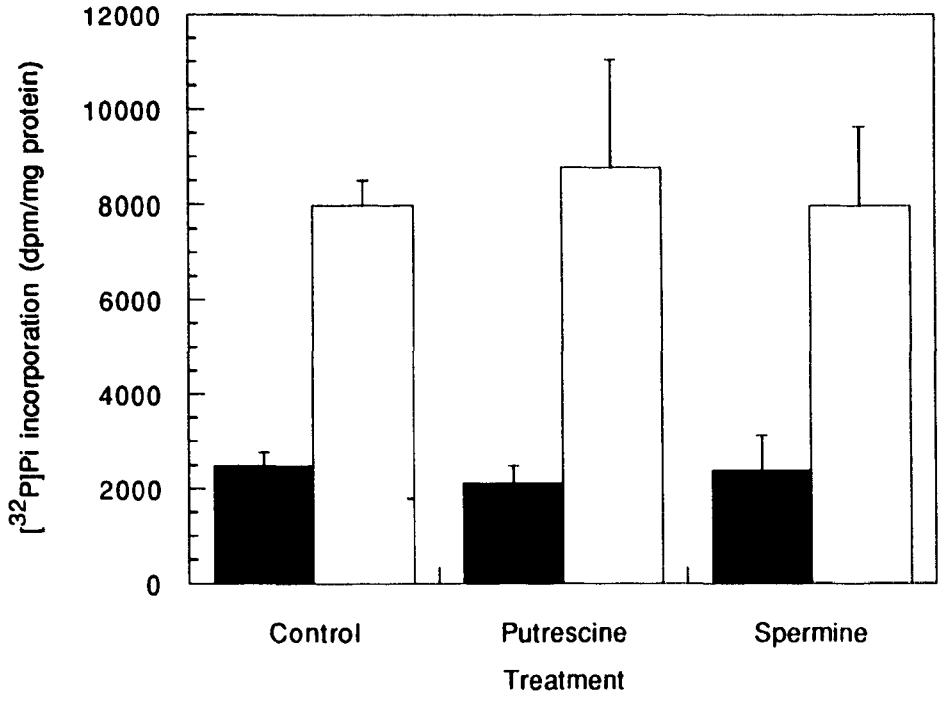


Figure 35. Effect of polyamines on bradykinin stimulated PtdIns synthesis in N1E-115 cells. Cells were incubated with [^{32}P]Pi and polyamines as indicated for one h at which time 10 μM bradykinin was added and the cells incubated in the same medium for a further 30 min. Solid bars, unstimulated cells; Open bars, bradykinin stimulated cells. Data are the means ± standard deviations for one experiment performed with 3 separate dishes of cells in each group and are representative of 2 experiments.

V. *In vitro* effects of polyamines on PtdIns synthesis

In vitro experiments with many of the enzymes involved with phosphoinositide metabolism have revealed modulation of enzyme activity by polyamines. This generally occurs in what is presumed to be the whole cell concentration range of the polyamines (1-3 mM). While the effects of polyamines on phosphoinositide specific kinases, phosphatases, and phospholipases have been examined, no data have been published concerning polyamine effects on PtdIns synthase or on the PtdIns:inositol exchange enzyme. N1E-115 cells exposed to exogenous putrescine and [^{32}P]Pi for 6 h had higher amounts of [^{32}P]PtdIns than cells labelled in the absence of putrescine (Figure 31). Since labelling of some phospholipids (i.e. PtdCho) was not altered, this suggested that PtdIns synthase activity was stimulated in these cells. *In vitro* assays of CMP-PtdOH-dependent PtdIns synthase, CMP-dependent cation-dependent Ins exchange, and CMP-independent Mn^{2+}-dependent Ins exchange were performed to assess this hypothesis.

Polyamine effects were first investigated using a detergent solubilized assay for CMP-PtdOH-dependent PtdIns synthase. The activity of PtdIns synthase increases with increasing concentrations of $MgCl_2$. Over a wide range of $MgCl_2$ concentrations, polyamines did not greatly affect PtdIns synthase activity. At 15 mM $MgCl_2$, 1 mM spermine and 1 mM spermidine marginally stimulated activity over control values (Figure 36). At a constant $MgCl_2$ concentration, various concentrations of polyamines did not substantially affect detergent solublized PtdIns synthase activity (Figure 37).

These studies were extended by examining the effect of polyamines on [^{3}H]Ins exchange activities. The effects of polyamines on membrane bound enzymes with lipid substrates are likely to be due, at least in part, to modification of membrane surface charge by ionic interaction with negatively charged phospholipids and proteins. Detergent present in the *in vitro* PtdIns synthase assay, which solubilized the membranes in large mixed micelles, may obscur any effect that polyamines would have on enzyme function in intact membranes. Therefore, polyamine effects on the CMP-dependent transient reversal of

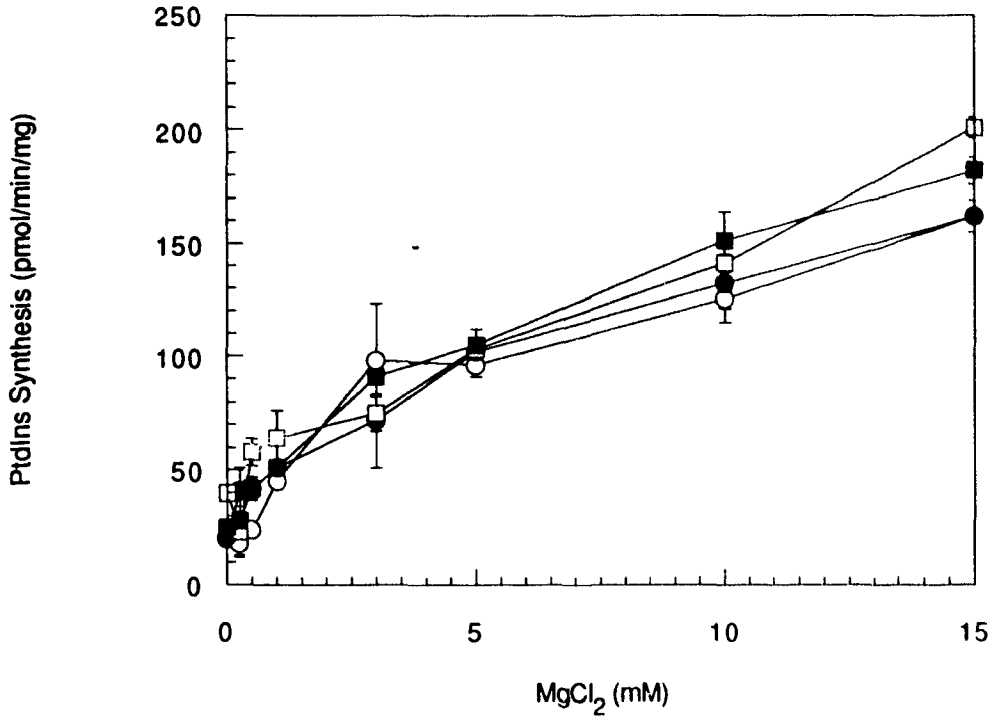


Figure 36. Effect of polyamines on the Mg^{2+} requirement of PtdIns synthase. *In vitro* assays of PtdIns synthase in N1E-115 lysates were performed in the presence of 1 mM polyamines and various $MgCl_2$ concentrations. Control, (O); Putrescine, (●); Spermidine, (□); and Spermine, (■). Data are the means ± standard deviation for 3 determinations.

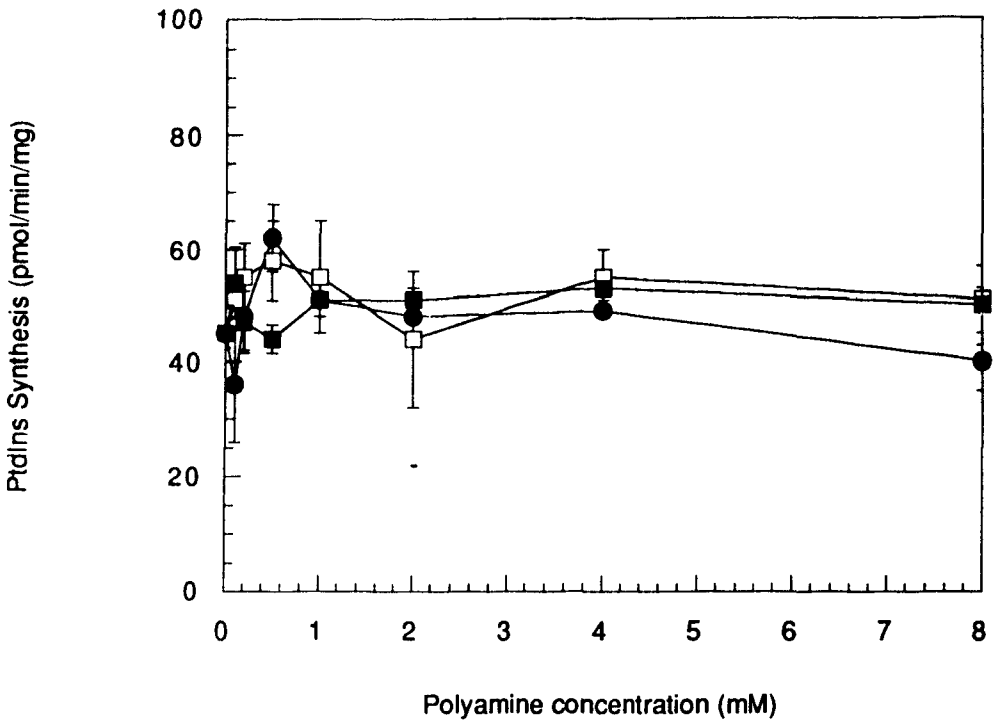


Figure 37. Effect of polyamine concentration on PtdIns synthase activity. *In vitro* assays of PtdIns synthase activity in N1E-115 lysates were performed in the presence of 1 mM $MgCl_2$ and various concentrations of polyamines. Putrescine, (●); Spermidine, (□); and Spermine, (■). Data are the means ± standard deviation for 3 determinations.

PtdIns synthase were investigated. This activity can be measured in intact membranes in the absence of detergents. As noted above, PtdIns synthase is reversible in the presence of CMP [37,36]. Hence, incubations of CMP and endogenous PtdIns with PtdIns synthase can result in formation of CMP-PtdOH and free inositol. In the presence of [^{3}H]Ins, PtdIns synthase can then catalyze formation of [^{3}H]PtdIns although no net synthesis occurs. Furthermore, nucleotide-independent incorporation of [^{3}H]Ins into PtdIns also occurs [409]. It is therefore necessary to distinguish between nucleotide-dependent and nucleotide-independent Ins exchange.

No [^{3}H]PtdIns was formed in the absence of divalent cations by the CMP-independent exchange (Figure 38 and 39). Maximal [^{3}H]PtdIns formation occured in the presence of 0.2 mM $MnCl_2$. Higher $MnCl_2$ concentrations did not further stimulate [^{3}H]PtdIns formation. 2 mM putrescine, spermidine, and spermine did not affect nucleotide-independent, Mn^{2+}-dependent [^{3}H]PtdIns formation (Figure 38). Nucleotide-independent [^{3}H]Ins incorporation into PtdIns also occured in the presence of $MgCl_2$ (Figure 39). However, in the absence of CMP, [^{3}H]PtdIns formation in the presence of $MgCl_2$ was only 2 % of that in the presence of $MnCl_2$. This low level of activity was not significantly modulated by either putrescine, spermidine, or spermine. Therefore, CMP-independent [^{3}H]Ins exchange was most active in the presence of low concentrations of $MnCl_2$ and was not affected by polyamines.

Mn^{2+}-dependent [^{3}H]Ins exchange activity was not stimulated by CMP (Figure 38 and 40). However polyamines stimulated [^{3}H]Ins incorporation into PtdIns by 40 % in the presence of CMP and Mn^{2+}, even though polyamines had no effect in the presence of Mn^{2+} alone (Figure 38). In the presence of low concentrations of $MnCl_2$ spermidine is the most potent activator. However, at higher concentrations of $MnCl_2$ all the polyamines stimulated the reaction equally. CMP-dependent activation of [^{3}H]Ins exchange is maximal at low (0.2 mM) $MnCl_2$ concentration and is not affected by higher $MnCl_2$ concentrations.

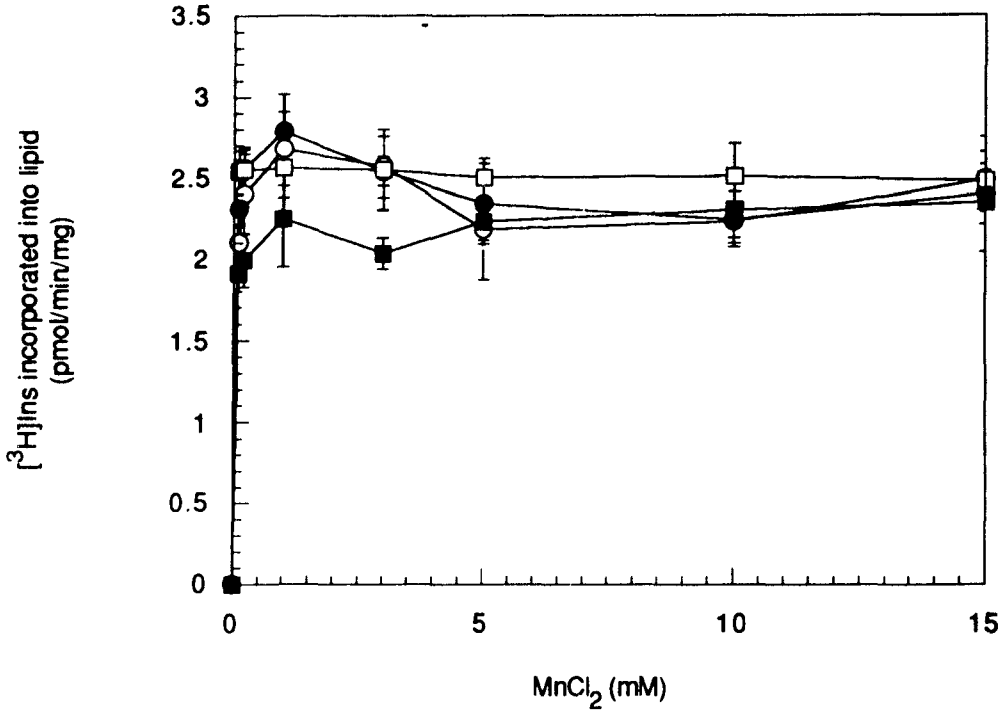


Figure 38. Effect of polyamines on CMP-independent [^{3}H]Ins incorporation into lipid in the presence of $MnCl_2$. *In vitro* incorporation of [^{3}H]Ins was measured in the presence of 2 mM polyamines as indicated and various concentrations of $MnCl_2$. Control, (O); Putrescine, (●); Spermidine, (□); Spermine, (■). Data are the means ± standard deviation for 3 determinations.

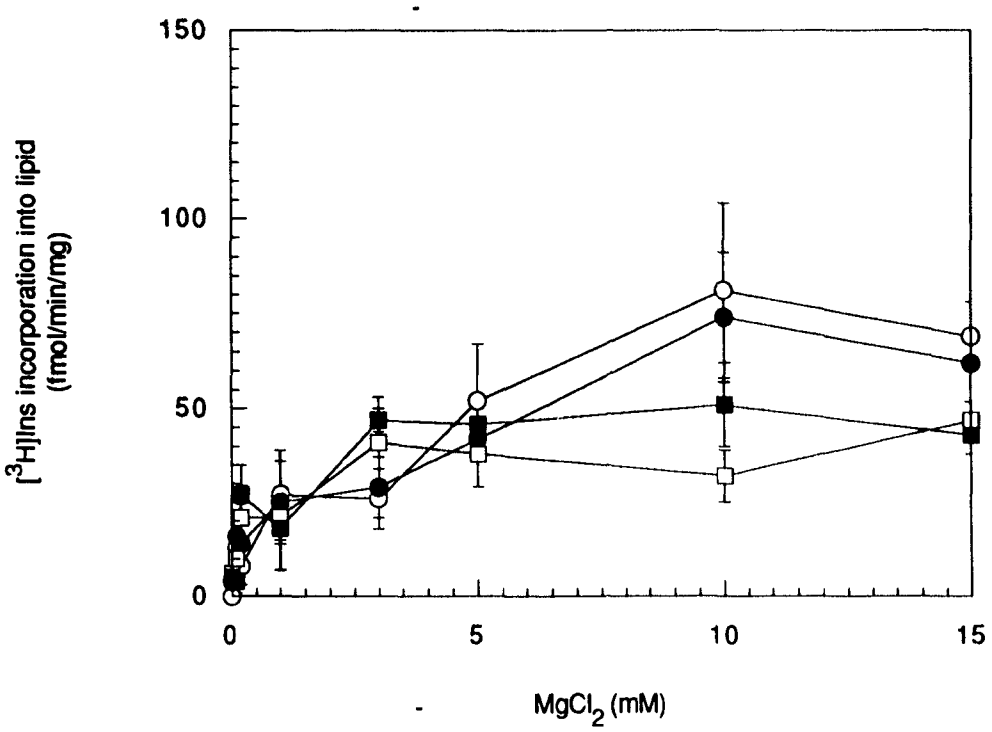


Figure 39. Effect of polyamines on CMP-independent [^{3}H]Ins incorporation into lipid in the presence of $MgCl_2$. *In vitro* incorporation of [^{3}H]Ins was measured in the presence of 2 mM polyamines as indicated and various concentrations of $MgCl_2$. Control, (O); Putrescine, (●); Spermidine, (□); Spermine, (■). Data are the me ns ± standard deviation for 3 determinations.

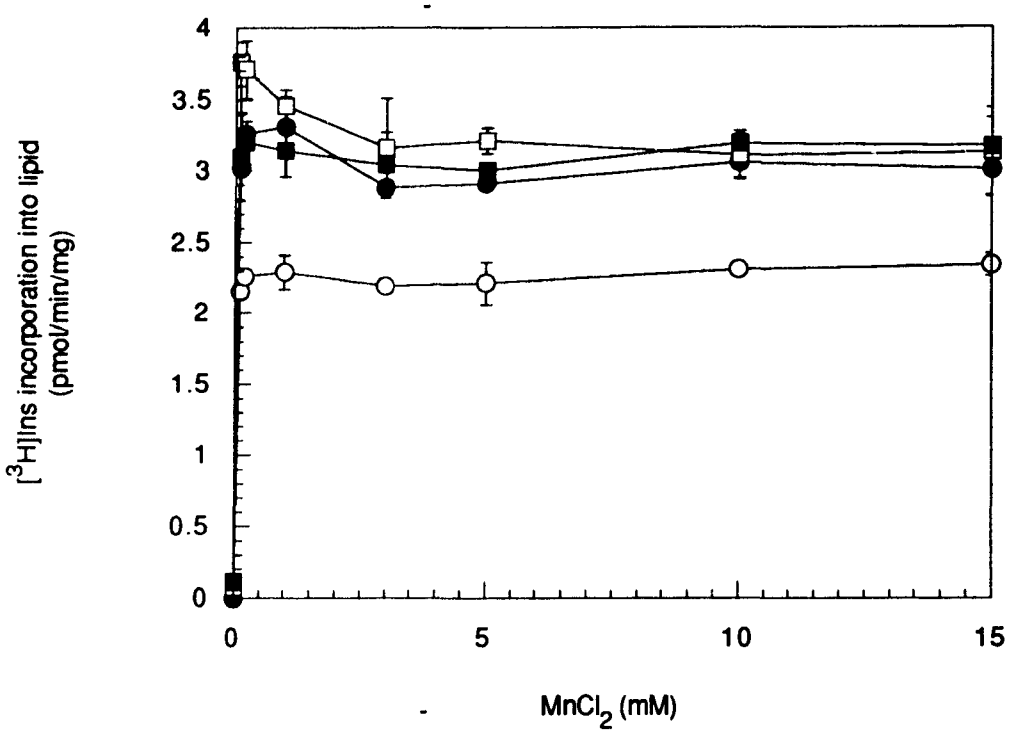


Figure 40. Effect of polyamines on [^{3}H]Ins incorporation in the presence of CMP and $MnCl_2$. *In vitro* [^{3}H]Ins incorporation into lipid was measured in the presence of 2 mM polyamines as indicated with various concentrations of $MnCl_2$ and 0.5 mM CMP. Control, (O); Putrescine, (●); Spermidine, (□); Spermine, (■). Data are the means ± standard deviation for 3 determinations.

In the presence of 10 mM $MgCl_2$, CMP caused a 20 fold stimulation of [^{3}H]Ins incorporation into PtdIns (Figures 39 and 41). CMP-dependent [^{3}H]Ins incorporation into PtdIns increased with increasing $MgCl_2$ concentrations. Putrescine, spermidine, and spermine (2 mM) caused up to 2 fold stimulation of CMP-dependent [^{3}H]Ins incorporation into PtdIns in the presence of Mg^{2+}; spermine was the most potent activator. Therefore, CMP-dependent [^{3}H]Ins exchange is clearly Mg^{2+}-dependent and is stimulated by polyamines. In the presence of $MnCl_2$, polyamine-stimulated, CMP-dependent exchange occured over and above the baseline Mn^{2+}-dependent, nucleotide-independent activity.

VI. Malignant hyperthermia

Swine susceptible to malignant hyperthermia (MH) have a deficiency of membrane bound Ins(1,4,5)P_3 5-phosphatase [108]. Halothane inhibits the soluble enzyme in muscle preparations of both normal and afflicted pigs. Thus, the decreased levels of membrane bound Ins(1,4,5)P_3 5-phosphatase exacerbate the halothane-induced reduction of Ins(1,4,5)P_3 5-phosphatase activity leading to manifestation of the disorder. To investigate whether Ins(1,4,5)P_3 5-phosphatase was deficient in humans exhibiting MH, blood samples were obtained from volunteers susceptible to the disorder and from individuals who had never suffered halothane related problems. Leukocyte enriched cell preparations were lysed and membranes were separated from the soluble fraction by centrifugation. Permanent cell lines were also established and used for further studies. Activity of Ins(1,4,5)P_3 5-phosphatase was determined in cell lysates and the soluble and insoluble fractions. Also, enzyme assays were performed in the presence of halothane and succinylcholine to determine if inhibition of enzyme activity occurred, and if there was any difference between MH and control samples.

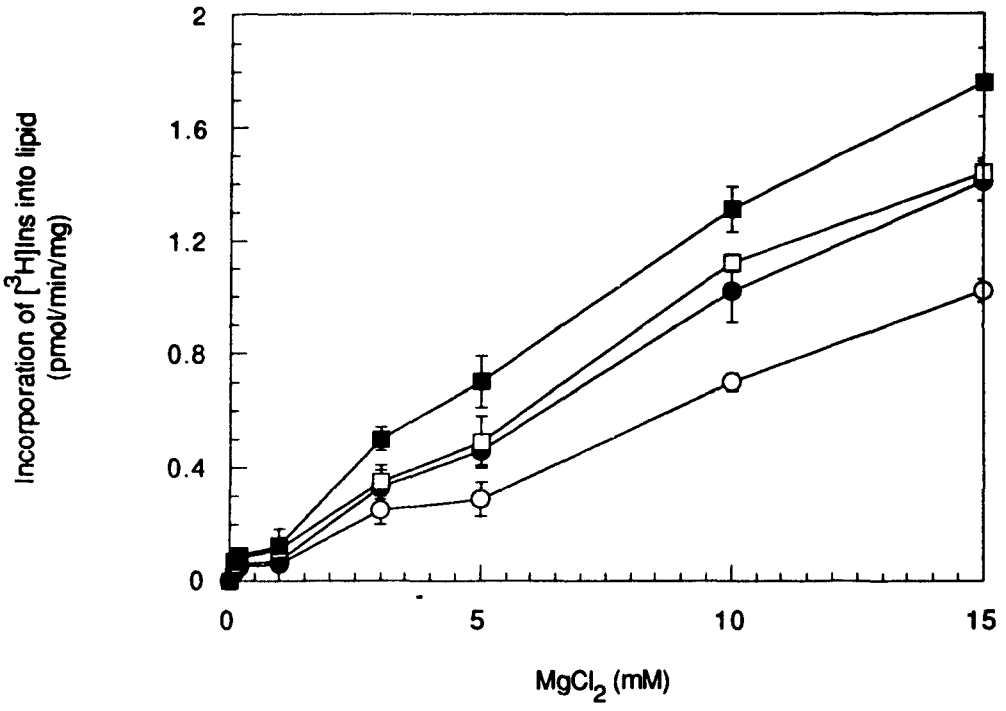


Figure 41. Effect of polyamines on [^{3}H]Ins incorporation in the presence of CMP and $MgCl_2$. *In vitro* [^{3}H]Ins incorporation into lipid was measured in the presence of 2 mM polyamines as indicated with various concentrations of $MgCl_2$ and 0.5 mM CMP. Control, (O); Putrescine, (●); Spermidine, (□); Spermine, (■). Data are the means ± standard deviation for 3 determinations.

Ins(1,4,5)P_3 5-phosphatase activity in leukocyte preparations of MH susceptible individuals was not different from activity in preparations from control individuals (Figure 42). Membrane bound Ins(1,4,5)P_3 5-phosphatase activity was sedimented by centrifugation (insoluble fraction), and was found to constitute 17 % of the total Ins(1,4,5)P_3 5-phosphatase activity in leukocytes. Neither soluble (Figure 43), nor insoluble activity (Figure 44) was lower in MH samples compared to control. Figures 43 and 44 show that 1 mM halothane slightly inhibited Ins(1,4,5)P_3 5-phosphatase activity in soluble and insoluble fractions of leukocyte preparations but did not differentially affect enzyme activity in samples from MH susceptible or control individuals.

Transformed lymphoblast cell lines were prepared from blood samples from malignant hyperthermia susceptible individuals. These cell lines (SW2515 and TB2516) and two control cell lines (AD0001 and MW0001) had similar levels of Ins(1,4,5)P_3 5-phosphatase activity when cell lysates were assayed *in vitro* (Figure 45). Furthermore, succinylcholine, a known cause of MH in susceptible individuals [283], did not affect Ins(1,4,5)P_3 5-phosphatase in lymphoblast lysates (Figure 46).

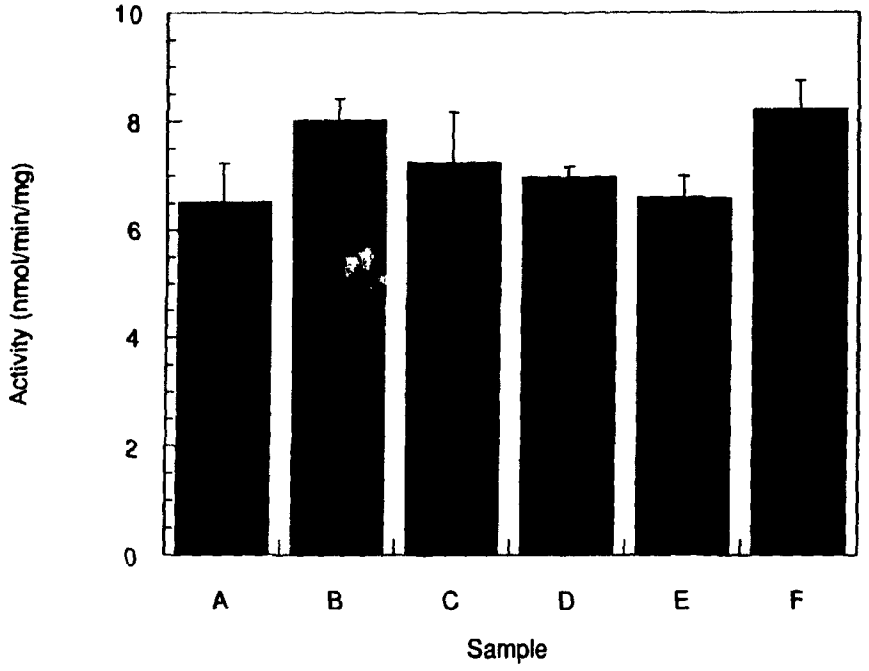


Figure 42. *In vitro* Ins(1,4,5)P_3 5-phosphatase activity in leukocyte preparations of malignant hyperthermia susceptible and control individuals. A and B are samples from volunteers susceptible to malignant hyperthermia. Samples C through F are from volunteers with no history of malignant hyperthermia. Data are for three determinations ± standard deviation.

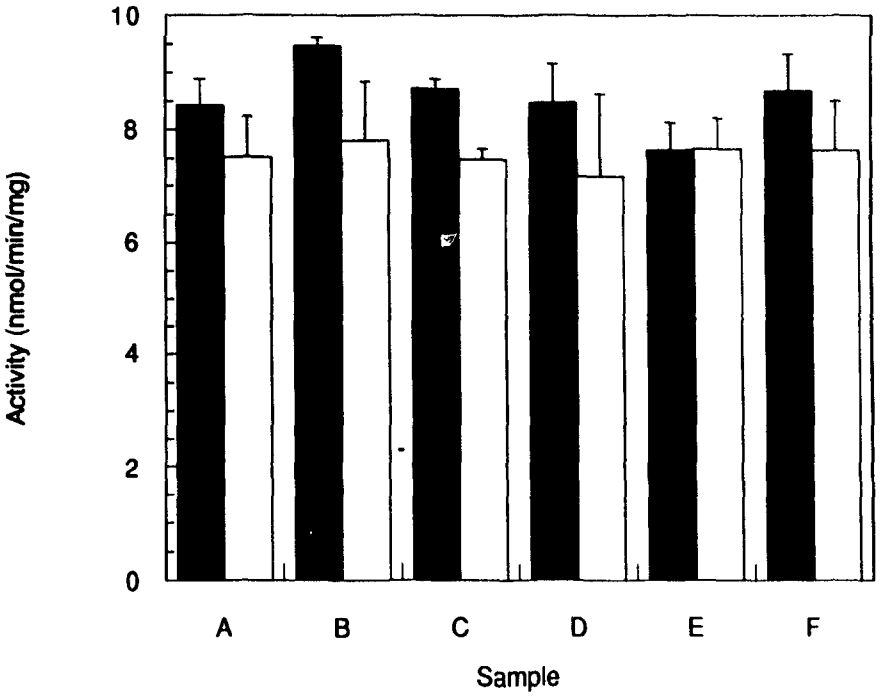


Figure 43. Effect of 1 mM halothane on *in vitro* Ins(1,4,5)P_3 5-phosphatase activity in the soluble fraction of leukocyte preparations. A and B are samples from volunteers susceptible to malignant hyperthermia. Samples C through F are from volunteers with no history of malignant hyperthermia. Solid bars, without halothane; Open bars, with halothane. Data are for three determinations ± standard deviation.

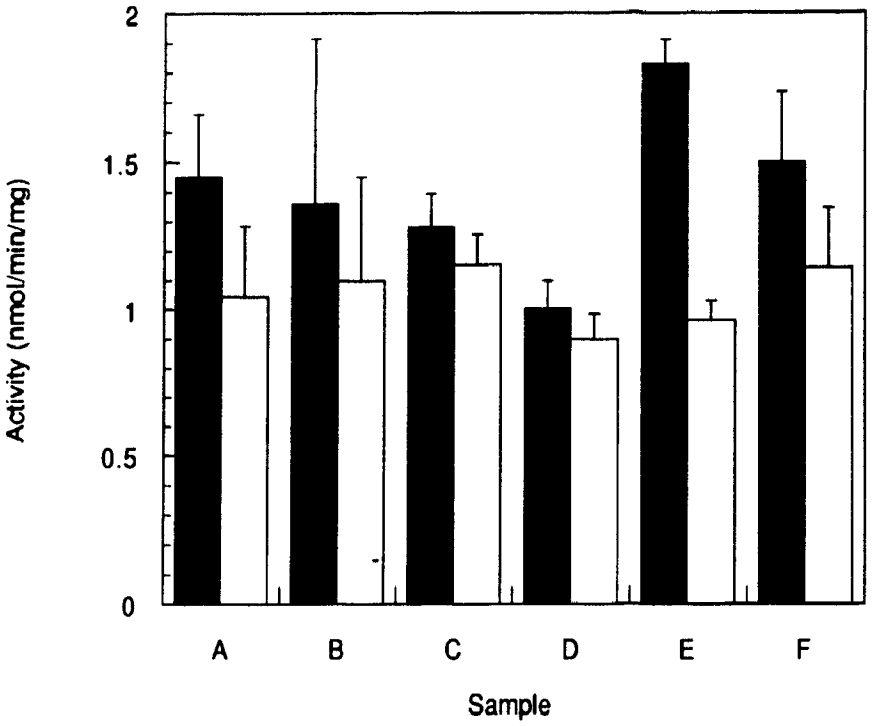


Figure 44. Effect of 1 mM halothane on *in vitro* Ins(1,4,5)P_3 5-phosphatase activity in the insoluble fraction of leukocyte preparations. A and B are samples from volunteers susceptible to malignant hyperthermia. Samples C through F are from volunteers with no history of malignant hyperthermia. Solid bars, without halothane; Open bars, with halothane. Data are for three determinations ± standard deviation.

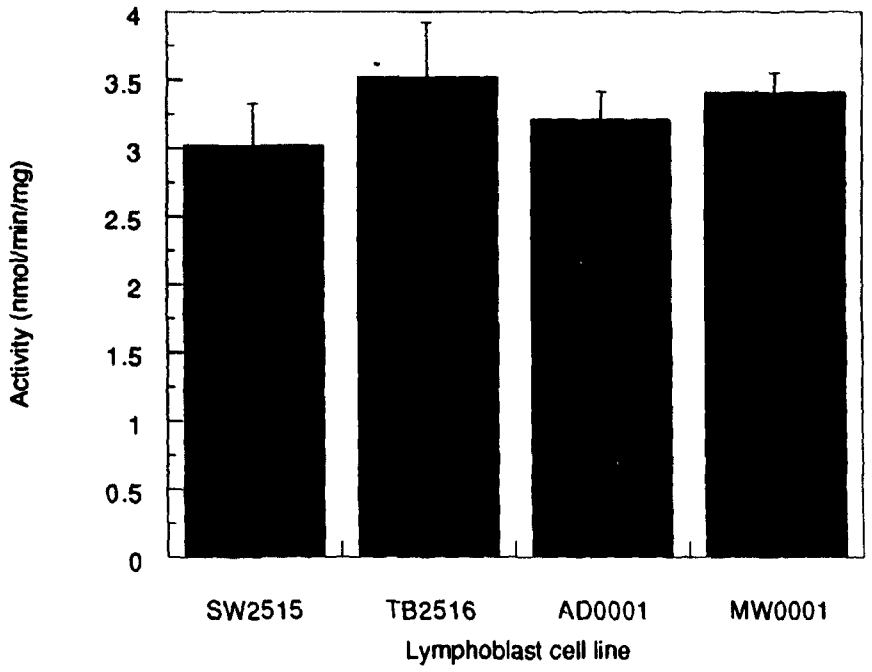


Figure 45. *In vitro* Ins(1,4,5)P_3 5-phosphatase activity in lymphoblast cell lysates. Cell lines SW2515 and TB2516 are derived from cell samples from malignant hyperthermia susceptible individuals. AD0001 and MW0001 are cell lines derived from adrenoleukodystrophy and control individuals. Data are for three determinations ± standard deviation.

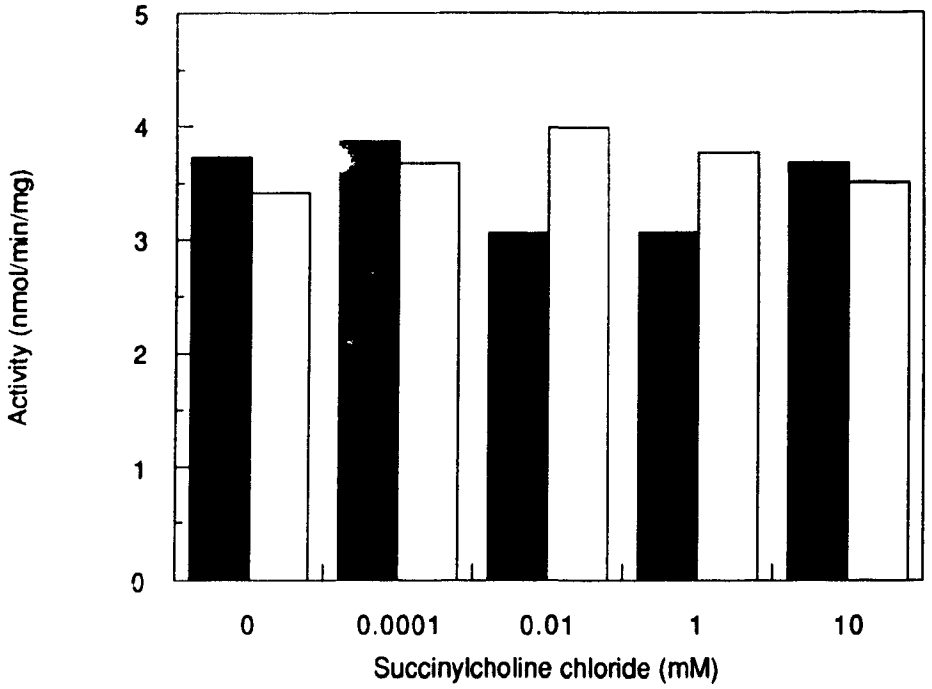


Figure 46. Effect of succinylcholine chloride on *in vitro* $Ins(1,4,5)P_3$ activity in lymphoblast lysates. Black bars correspond to activity in lysates of a cell line from a malignant hyperthermia sample (TB2515). Open bars are data from a cell line derived from an individual with no history of malignant hyperthermia (MW0001). Data are for single determinations.

Discussion

I. Subcellular fractionation of C6 glioma and N1E-115 neuroblastoma

A. Subcellular location of phosphatidylinositol synthesis

Although turnover of inositol phospholipids following stimulation by a variety of extracellular agonists can be demonstrated at the plasma membrane [372,427], it is not clear which of the complete sequence of metabolic events take place in the plasma membrane. In view of evidence in a variety of tissues for a metabolically distinct pool of agonist-sensitive phosphoinositide, it seemed possible that recycling of this fraction of the total cellular phosphoinositide complement might occur at the site of agonist-induced polyphosphoinositide hydrolysis in the plasma membrane. This hypothesis was tested by determining the subcellular distribution of all participating enzymes in cultured neural cells using a fractionation procedure originally developed for the isolation of high purity plasma membranes [75]. Three distinct fractions were isolated: cytosol, plasma membrane, and endoplasmic reticulum-enriched microsomes. These three fractions had minimal cross contamination as determined by the marker enzymes: lactate dehydrogenase, 5' nucleotidase, and NADPH:cytochrome C reductase.

The metabolic pathway for resynthesis of PtdIns from DAG generated by phospholipase C action involves three enzymes: DAG kinase, CTP:PtdOH cytidylyltransferase and CMP-PtdOH:inositol cytidylyltransferase. DAG kinase has been found in the cytosol, plasma membrane, and endoplasmic reticulum fractions of brain and other tissues [223,184,186,400,239]. Results presented here extend these observations to cultured cells of neural origin and show DAG kinase activity distributed in approximately equal amounts throughout the cytosol, plasma membrane and microsomal membranes of glioma cells. A similar assessment of the localization of PtdIns synthesis in hepatocytes was coincident with this work [239]. That study used Nycodenz gradients which did not yield a highly purified plasma membrane fraction but did afford a greater separation of endoplasmic reticulum and golgi. Fractionation of hepatocytes in Nycodenz gradients

showed the membrane-bound DAG kinase activity to be associated with the Golgi marker (galactosyltransferase) rather than with the endoplasmic reticulum [239]. In Percoll gradients, galactosyl transferase is localized in the heterogeneous microsomal membrane fraction at the bottom of the gradients [75] so endoplasmic reticulum and Golgi-associated membranes could not be distinguished. While Lundberg and Jergil [239] found no DAG kinase associated with the plasma membrane of hepatocytes, results with glioma cells clearly support a plasma membrane location for a significant amount of cellular DAG kinase activity. Since enhanced phosphatidic acid synthesis in the plasma membrane follows agonist-stimulated hydrolysis of $PtdInsP_2$ [372], it seems likely that DAG can be phosphorylated at or near its site of formation in the plasma membrane. A membrane-bound DAG kinase in 3T3 cells selectively phosphorylates arachidonoyl-DAG that would be produced by phosphoinositide hydrolysis [249]. This activity is distinct from the cytosolic activity which exhibits no acyl chain specificity, and has been implicated in the platelet-derived growth factor-stimulated formation of phosphatidic acid in these cells [248]. The DAG used to assay the kinase in the present study was derived from egg lecithin and therefore contains little arachidonic acid. Perhaps more plasma membrane associated DAG kinase activity would have been observed in glioma cells if DAG enriched in arachidonate had been used in the DAG kinase assays. Furthermore, DAG kinase activity may translocate to the plasma membrane in response to the appearance of DAG [39] and be part of a mechanism for limiting the DAG-mediated signal that activates protein kinase C.

In summary, DAG kinase was found in all subcellular fractions where it may play a role in basal metabolic functions and where it may serve to modulate activation of PKC by DAG. Plasma membrane PtdOH formation is the first step necessary for PtdIns resynthesis from agonist-generated DAG.

Following formation of PtdOH, the next two steps in PtdIns resynthesis are formation of CMP-PtdOH and transfer of the phosphatidyl group to inositol resulting in PtdIns. It has been believed that the CTP:PtdOH cytidylyltransferase and PtdIns synthase

required for PtdIns synthesis from PtdOH via CMP-PtdOH occur only in the endoplasmic reticulum although most studies could not exclude the possibility that a small portion of the total cellular activity might reside in the plasma membrane [43]. Since only a small portion of total cellular phosphoinositide is agonist-sensitive [98,279], low activity in the plasma membrane may be sufficient to recycle PtdIns within that membrane.

There is no cytidylyltransferase or PtdIns synthase activity in purified plasma membranes of either glioma or neuroblastoma cells that was not due to the very low level of endoplasmic reticulum contamination. That these low levels were due to contamination is confirmed by experiments in which increasing amounts of microsomal fraction were added to fixed quantities of plasma membrane fraction. When extrapolated to zero contamination with NADPH:cytochrome C reductase, regression lines for both PtdIns synthase and CTP:PtdOH cytidylyltransferase in glioma and neuroblastoma cells passed through or below the origin. Thus, all of the activity of these two enzymes in the plasma membrane fraction was due to cross-contamination.

In contrast, PtdIns synthase activity has been reported in liver plasma membrane [408]. More recently, a substantial portion of the cellular PtdIns synthase was found in the plasma membrane of rat pituitary (GH_3) cells [164]. Both CMP-independent Ins exchange and PtdIns synthase are membrane bound in turkey erythrocytes [264] although mammalian erythrocytes do not incorporate [^{3}H]Ins into PtdIns [126]. PtdIns synthase has also been found in the brush-border and basolateral membranes of rabbit proximal tubule cells [110]; two membranes that are biochemically distinct domains of the plasma membrane. In this case, the PtdIns synthase activity could not be attributed to contamination of the plasma membrane fractions with endoplasmic reticulum. It should be noted that these assays measured incorporation of [^{3}H]Ins into PtdIns in the presence of $MnCl_2$ and did not specifically exclude Mn^{2+}-dependent, CMP-PtdOH-independent Ins exchange. Thus, identification of the activity as CMP-PtdOH-dependent synthesis was not unambiguous. Nevertheless, it was suggested that PtdIns synthesis could occur in the

plasma membranes of these cells although no source of CMP-PtdOH in the plasma membrane was identified. PtdIns synthase can be partially solubilized from GH_3 cell membranes (both plasma membrane and endoplasmic reticulum) by salt extraction [77]. The high ionic strength in the Percoll gradients raises the possibility that PtdIns synthase and possibly CTP:PtdOH cytidylyltransferase might have been extracted from the membranes during the fractionation. This is not the explanation for the results presented here since (i) the salt concentration in the Percoll gradients was well below the optimal concentration (3 M) for solubilization of PtdIns synthase, and (ii) all the cytidylyltransferase and PtdIns synthase activities were in the microsomal fraction at the bottom of the gradient and no solubilized activities were present in the gradient. These results agree with those of Monaco [278] who detected no PtdIns synthase activity in the plasma membrane fraction of WRK-1 cells and Lundberg and Jergil [239] who obtained similar results with hepatocytes. In the latter study, the fractionation method did not clearly separate plasma membrane from endoplasmic reticulum. However, profiles of both CTP:PtdOH cytidylyltransferase and PtdIns synthase activities throughout the gradient followed the profile of the endoplasmic reticulum markers closely and they concluded that neither synthetic enzyme was present in plasma membrane.

Results from C6 and N1E-115 cells indicate that synthesis of PtdIns from DAG within the plasma membrane is not possible. The distributions of DAG kinase, CTP:PtdOH cytidylyltransferase, and PtdIns synthase indicate that although PtdOH can be formed in the plasma membrane from agonist generated DAG, complete PtdIns synthesis does not occur in that subcellular compartment. DAG or PtdOH must be transported to the endoplasmic reticulum for synthesis of PtdIns. Once formed, PtdIns must then be transported back to the plasma membrane.

Work presented in this thesis did not address the role of nuclei in PtdIns synthesis. However, PtdIns synthase has been found in neuronal nuclei isolated from rabbit cerebral cortex [16]. Microsomal and nuclear PtdIns synthase activities are clearly distinct. Nuclear

PtdIns synthase requires more CMP-PtdOH but less $MgCl_2$ and has a significantly lower apparent K_m for inositol than does the microsomal enzyme [16]. Given the existence of DAG kinase [381], CTP:PtdOH cytidylyltransferase, [414], PtdIns synthase [16], PtdIns 4-kinase [66], and PtdIns(4)P 5-kinase [321] in the nuclei, the possibility exists that DAG generated by PtdIns-PLC in the nuclei [88] is recycled back into phosphoinositides within that organelle. This may, in part, explain the phenomena of phosphoinositide pooling within some cells.

B. Subcellular location of enzymes involved in polyphosphoinositide synthesis

Since the step-wise phosphorylation of PtdIns to PtdIns(4,5)P_2 was shown to be catalyzed by two kinases located on the cytoplasmic side of human erythrocyte membranes [112], it has been presumed, with some experimental support, that PtdIns synthesized in the endoplasmic reticulum is phosphorylated after translocation to the plasma membrane. This is consistent with the view that at least a portion of plasma membrane PtdIns acts as a reservoir to replenish PtdIns(4,5)P_2 used in signal transduction. Prior studies of the distribution of these two kinases in other mammalian tissues have produced conflicting results. PtdIns 4-kinase has been found in the cytosol and in virtually every intracellular membrane from the nuclear envelope to the plasma membrane, depending on the tissue studied [69,166,400]. In glioma cells this enzyme activity is localized in the cytosol and the microsomal fraction. It is possible that PtdIns 4-kinase is loosely associated with the plasma membrane of whole cells and is removed from the membrane during subcellular fractionation. The absence of PtdIns 4-kinase from glioma plasma membrane, though surprising, is consistent with recent studies of hepatocytes [239] in which no appreciable activity was found in either the plasma membrane or the endoplasmic reticulum. Membrane-bound PtdIns 4-kinase originally assigned to Golgi in hepatocytes, was associated with a slightly lighter particle (possibly a lipid transport vesicle). As noted earlier, Golgi membranes migrate to the bottom of the Percoll gradient used in this study

and the PtdIns 4-kinase activity in the microsomal fraction of C6 glioma cells may represent Golgi-associated particles rather than a true endoplasmic reticulum location for this enzyme. In agreement with results presented here, PtdIns 4-kinase activity has recently been demonstrated in the microsomal fraction of chinese hamster ovary (CHO) cells [135]. Results from the present study suggest that either the cytosolic PtdIns 4-kinase acts on PtdIns on the surface of the plasma membrane *in vivo* or, alternatively, PtdIns may be phosphorylated in transit to the plasma membrane as proposed by Lundberg & Jergil [239].

While substantial PtdIns(4)P 5-kinase activity is present in the plasma membrane as well as the microsomal membranes of glioma cells, all activity found in the cytosol could be attributed to cross-contamination. The microsomal fraction of C6 glioma cells contains 1.7 times as much PtdIns(4)P 5-kinase activity as the plasma membrane suggesting that PtdIns(4,5)P_2 may serve some function within the microsomes. The plasma membrane associated activity can produce PtdIns(4,5)P_2 at the plasma membrane. In contrast with these results, PtdIns(4)P 5-kinase is almost exclusively localized in the plasma membrane of hepatocytes [69,239]. It is more widely distributed among cell membranes and the cytosol in whole brain [400] and is present in the microsomal membranes of CHO cells [135].

In summary, PtdIns 4-kinase is present in the cytosol and microsomes, while PtdIns(4)P 5-kinase is in the plasma membrane and microsomes of C6 cells. Synthesis of PtdIns(4,5)P_2 from PtdIns in the plasma membrane can only occur in these cells if PtdIns 4-kinase measured in the cytosolic fraction acts at the surface of the plasma membrane. Alternately, PtdIns(4)P may be formed in C6 cells before or during transport to the plasma membrane as suggested for hepatocytes. Once formed, PtdIns(4)P can be phosphorylated by PtdIns(4)P 5-kinase in the plasma membrane and microsomes.

C. Subcellular location of enzymes involved in phosphoinositide catabolism

Steady-state levels of the polyphosphoinositides are maintained in erythrocyte membranes and other tissues by an energy-dependent cycle in which the actions of PtdIns 4-kinase and PtdIns(4)P 5-kinase are reversed by the sequential action of a specific Mg^{2+}-dependent PtdIns(4,5)P_2 5-phosphatase [343,308] and a cation-independent PtdIns(4)P 4-phosphatase [251]. Consistent with earlier studies of whole tissues [308], PtdIns(4,5)P_2 5-phosphatase is almost entirely a soluble enzyme in glioma cells although there was very low activity in plasma membrane which was not completely attributable to cross-contamination . The possibility that the cytosolic activity may be derived from PtdIns(4,5)P_2 5-phosphatase loosely bound to the membranes and removed during subcellular fractionation cannot be entirely excluded. On the other hand, PtdIns(4)P 4-phosphatase is predominantly a membrane-bound activity present in the plasma membrane as well as intracellular membranes (60 70%) (Figure 13). Therefore, PtdIns(4)P 4-phosphatase and PtdIns(4,5)P_2 5-phosphatase (either the low plasma membrane activity or the cytosolic activity acting at the membrane surface) can both participate in phosphoinositide metabolism at the plasma membrane of glioma cells.

Results presented here for the PtdIns(4,5)P_2 specific phospholipase C agree with other studies [225,90,183,239,322] showing most of this activity to be localized in the cytosol with a lesser amount in the plasma membrane. In contrast to one report [183], no PtdIns(4,5)P_2 specific phospholipase C was detected in the microsomal fraction that could not be attributed to contamination with the plasma membrane fraction. It is presumed that the phospholipase C in the plasma membrane is responsible for agonist-dependent hydrolysis of PtdIns(4,5)P_2. The relationship between soluble and plasma membrane forms of the enzyme is not clear. They may be the same since highly purified preparations of each have similar kinetic properties and at least one of the soluble diesterase isozymes has been detected immunologically in membranes [444,22]. This raises the possibility of

translocation between the two compartments. In this thesis, digitonin permeablization of agonist-stimulated cells was used to examine translocation of total PtdIns-PLC activity. No agonist-dependent translocation of the PtdIns-PLC to membranes of glioma or neuroblastoma cells was observed. In these cells, serotonin and bradykinin stimulate PtdIns-PLC through G proteins [267], although the activated PtdIns-PLC isozymes have not been identified.

Translocation has been clearly demonstrated only for PtdIns-PLC γ. Agonist stimulated translocation of PtdIns-PLC has been demonstrated in rat basophilic leukemia cells [9] and in A431 cells [419]. In both cases tyrosine phosphorylation is involved. Aggregation of the IgE receptor on the surface of the leukemia cells activates hydrolysis of phosphoinositides and translocation of PtdIns-PLC γ from cytosol to membranes. Enzyme activity was down regulated by a tyrosine phosphatase [9]. Similarly, stimulation of the tyrosine kinase activity of the EGF receptor of A431 cells stimulates translocation of PtdIns-PLC γ.

Microsomes of glioma cells do not contain PtdIns-PLC. However, they contain PtdIns(4)P 5-kinase. If PtdIns(4,5)P_2 formed at the microsomes is hydrolyzed at those membranes, PtdIns-PLC must translocate to the microsomes within glioma cells.

In summary, PtdIns(4,5)P_2 5-phosphatase is largely a soluble enzyme whereas PtdIns(4)P 4-phosphatase is predominantly a membrane-bound activity present in the plasma membrane as well as microsomes of glioma cells. Assuming that cytosolic PtdIns(4,5)P_2 5-phosphatase can interact with membranes, both of the monoester phosphates of PtdIns(4,5)P_2 can be removed returning this lipid to the cellular pool of PtdIns.

Synthesis of PtdIns from DAG within the plasma membrane of C6 glioma and N1E-115 neuroblastoma cells is not possible. The subcellular locations of enzymes participating in phosphoinositide metabolism in C6 glioma cells is schematically represented in figure 47.

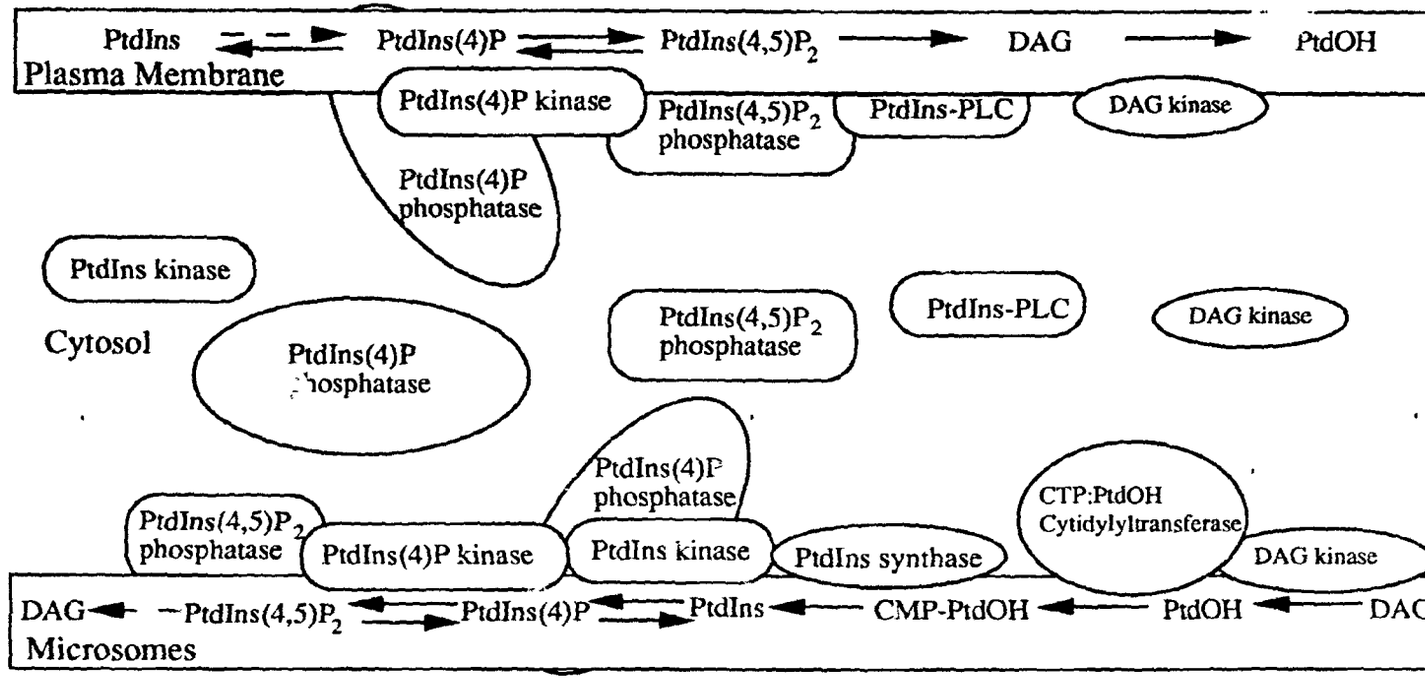


Figure 47. The distribution of enzymes involved in phosphoinositide metabolism in C6 glioma cells. Enzymes are shown as either plasma membrane bound, cytosolic, or microsomal. The pathways that can occur in plasma membranes and microsomes are shown. Arrows denote reactions that can be catalyzed by enzymes found in the membrane. Dashed arrows represent reactions that may occur in these membranes if cytosolic enzymes have access. The microsomes are composed of several different membranes, hence the complete pathway shown here might not occur.

The distributions of DAG kinase, CTP:PtdOH cytidylyltransferase, and PtdIns synthase indicate that although PtdOH can be formed in the plasma membrane from agonist generated DAG, complete PtdIns synthesis does not occur in that membrane. DAG or PtdOH must be transported to the endoplasmic reticulum for synthesis of PtdIns. Once formed, PtdIns could then be transported back to the plasma membrane. The mechanism by which preferential recyling of the DAG back into PtdIns might occur is not clear. Since PtdIns 4-kinase is found in the cytosolic fraction, it is difficult to determine where PtdIns(4)P is formed. It may be formed at a membrane or during transport of PtdIns. PtdIns(4)P 5-kinase is present in the plasma membrane, thus, PtdIns(4,5)P_2 can be formed at that membrane. PtdIns-PLC in the plasma membrane is well placed to interact with G proteins resulting in phosphoinositide hydrolysis at the plasma membrane. Although PtdIns synthesis may occur at the plasma membrane of some cell types (if CMP-PtdOH is availible at that site), this does not occur in neuroblastoma or glioma cells and therefore is not a general feature of cells that are capable of agonist-stimulated phosphoinositide metabolism.

II. Acylation of lysophosphatidylinositol

Acylation of 1-acyl-[^{3}H]PtdIns by N1E-115 neuroblastoma cells was assayed to determine whether N1E-115 cells have the capability to acylate 1-acyl-lysoPtdIns, where in the cell that activity resides and what are the *in vitro* characteristics of the reaction. The fatty acid profile of PtdIns is remodelled by a series of deacylation-reacylation reactions [4,149,151,82,83]. Thus, acylation of 1-acyl-lysoPtdIns is an important part of intracellular PtdIns metabolism. There are precedents for plasma membrane associated acylation; myelin (a modified plasma membrane) is capable of acylating 1-acyl-lysoPtdIns [436] and N1E-115 neuroblastoma plasma membranes contain 1-acyl-lysoPtdCho acyltransferase [60]. Furthermore, agonist stimulated phospholipase A_2 acts on phosphoinositides in some cells, producing lysoPtdIns [295,394]. Since acylation of lysoPtdIns may impact on the quantity and molecular species profile of PtdIns within the

plasma membrane, the ability of plasma membrane from N1E-115 cells to acylate 1-acyl-lysoPtdIns was assessed.

N1E-115 cells in culture will incorporate 1-acyl-[^{3}H]lysoPtdIns from the culture medium. Incorporation is time and concentration dependent. LysoPtdIns first encounters the plasma membrane. Consistant with this point of entry, lysoPtdIns was highest in this fraction at short times but was quickly distributed throughout the cell. This precluded preferential labelling of the plasma membrane (a secondary objective of the experiments). Hydrolysis of 1-acyl-[^{3}H]lysoPtdIns occurs before any [^{3}H]PtdIns is formed within the cells. Since the radiolabel was in the inositol moiety, it is possible that radiolabel could have been incorporated into PtdIns by PtdIns synthase or by Ins:PtdIns exchange. However, nucleotide-independent Ins:PtdIns exchange is a minor pathway in intact cells [145].

Subcellular fractionation of 1-acyl-[^{3}H]lysoPtdIns labelled cells showed that [^{3}H]PtdIns formed from 1-acyl-[^{3}H]lysoPtdIns is enriched in the plasma membrane. The specific activity (moles of [^{3}H]PtdIns per mole of total PtdIns) is 3.8 fold higher in the plasma membrane than the microsomes. Because of the complex nature of the microsomal fraction, this need not directly reflect a precursor-product relationship between [^{3}H]PtdIns in these two fractions. However, it is clear that at all times there is more [^{3}H]PtdIns in the plasma membrane than in the microsomal fraction. This suggests that either [^{3}H]PtdIns is formed at the plasma membrane, or it is formed in the microsomes and quickly transported to the plasma membrane. If [^{3}H]PtdIns is formed at the plasma membrane, it is not through the action of CTP:PtdOH cytidylyltransferase and PtdIns synthase because those two enzymes are not present in the plasma membrane of N1E-115 cells. The alternative is that plasma membranes of N1E-115 cells possess 1-acyl-lysoPtdIns acyltransferase activity. However, *in vitro* assays using subcellular fractions did not reveal any 1-acyl-[^{3}H]lysoPtdIns acyltransferase in either the cytosol or plasma membrane fractions. The possibility that activity may have been masked by hydrolysis of acyl-CoA derivatives by

these fractions cannot be excluded but it is likely that acylation of lysoPtdIns does not occur in the plasma membrane or cytosol.

The microsomal fraction (enriched in endoplasmic reticulum) had the ability to acylate 1-acyl-[^{3}H]lysoPtdIns. In the absence of added acyl-CoA derivatives, microsomes were capable of acylating 1-acyl-[^{3}H]lysoPtdIns with endogenous acyl-chains through CoA-dependent and CoA-independent transacylation. One might expect that 1-acyl-[^{3}H]lysoPtdIns would be degraded and the resulting [^{3}H]Ins incorporated into PtdIns through PtdIns synthase or Ins:PtdIns exchange. However, this does not account for these results because no exogenous CMP or CMP-PtdOH were present in the assay. Furthermore, Mg^{2+} (or Mn^{2+}) is required for PtdIns synthase and Ins:PtdIns exchange activity. Since formation of [^{3}H]PtdIns from 1-acyl-[^{3}H]lysoPtdIns was inhibited by magnesium (the subcellular fractions contain $MgCl_2$) and activated by EDTA, PtdIns synthase and Ins:PtdIns exchange activity do not account for the formation of [^{3}H]PtdIns. Therefore, [^{3}H]PtdIns was formed in these assays through the action of a 1-acyl-lysoPtdIns acyltransferase.

1-Acyl-[^{3}H]lysoPtdIns was acylated in microsomes of N1E-115 neuroblastoma cells using acyl-CoAs in the order arachidonoyl-CoA > oleoyl-CoA > palmitoyl-CoA, in agreement with previous results obtained in pancreas, brain, liver, and heart [193,19,146,356]. Thus, reacylation of lysoPtdIns within the microsomes of N1E-115 cells could contribute to enrichment of PtdIns with arachidonate.

In summary, N1E-115 neuroblastoma cells in culture incorporate 1-acyl-lysoPtdIns from the culture medium which is both degraded and acylated within the intact cells. No plasma membrane-associated 1-acyl-lysoPtdIns acylation activity was detected *in vitro*. Therefore, [^{3}H]PtdIns derived from [^{3}H]lysoPtdIns in the plasma membrane is most likely synthesized in the microsomes by 1-acyl-lysoPtdIns acyltransferase and then quickly transported to the plasma membrane. Preference of 1-acyl-lysoPtdIns acyltransferase for arachidonoyl-CoA is consistent with enrichment of PtdIns with arachidonate [150,131].

III. Effect of polyamines on phospholipid metabolism

Considering the large body of evidence demonstrating *in vitro* effects of polyamines on enzymes participating in the phosphoinositide cycle it is important to address the question of whether any of these *in vitro* effects can be detected in whole cultured cells. There are circumstantial associations between phosphoinositides and polyamines in whole cells. Polyamines and phosphoinositides both increase within cells during transformation [93,323]. Also, phorbol esters elevate phosphoinositide levels within cells and induce polyamine biosynthetic enzymes [350]and transport systems [99].

Polyamines, when added to the culture medium, are taken up by N1E-115 neuroblastoma cells. Prior treatment of the cells with the sulfhydryl reagent, N-ethylmaleimide, blocks putrescine transport as had been previously described [342]. A number of hormones and neurotransmitters stimulate polyamine transport [402]. A novel finding of the present study is that bradykinin stimulates putrescine transport in N1E-115 cells. Bradykinin, which is known to stimulate phosphoinositide turnover in these cells [115], stimulates putrescine transport by over 65 %. This modulation shows that [^{3}H]putrescine is transported into the cells rather than simply binding to the cell surface.

Under control conditions, N1E-115 neuroblastoma cells have 6.0 and 14.4 nmol/mg protein of spermidine and spermine, respectively. This is of the same order as bovine lymphocytes which have 7.3 and 8.6 nmol/mg protein of spermidine and spermine, respectively [445]. Raised spermine levels may be characteristic of transformed cells [236]. Incubation of N1E-115 cells with exogenous polyamines results in a 1.4 to 2 fold increase in total polyamine content. This closely matches increases seen in postischaemic brain and in tumours (2 -5 fold)[317,236] . Once taken up, polyamines can be metabolized to form other polyamines. Both shorter and longer chain polyamines were synthesized indicating the N1E-115 cells have active elongation and acetylation/oxidation pathways. Spermidine levels appear to be the most resistant to change. Treatment of the cells with exogenous putrescine caused increases in both putrescine and spermine but not spermidine.

However, spermine is synthesized from spermidine so the exogenous putrescine must be made into spermidine which is rapidly used for spermine synthesis.

These observations suggest that the polyamines which are taken up be N1E-115 cells participate in polyamine metabolism within these cells. Incubating cells with exogenous polyamines results in real, biologically significant (metabolizable) increases in polyamine content. Therefore, N1E-115 cells treated with exogenous polyamines are a good model for studying the effects of polyamines on phosphoinositide metabolism.

Specific, polyamine dependent changes in phospholipid metabolism have been observed in this study. One h incubations with [^{32}P]Pi and exogenous polyamines reveal a spermidine and spermine specific decrease in [^{32}P]Pi incorporation into PtdIns(4,5)P_2. This is not due to a polyamine mediated decrease in [^{32}P]ATP specific activity because [^{32}P]Pi incorporation into other phospholipids, such as PtdCho, is not affected. Therefore, in the presence of exogenous spermidine and spermine, either the mass or the turnover rate of PtdIns(4,5)P_2 in N1E-115 cells is reduced. This could be due to effects on: basal hydrolysis of PtdIns(4,5)P_2 by phospholipase C, activity of PtdIns(4)P 5-kinase, and/or PtdIns(4,5)P_2 5-phosphatase activity. If PtdIns(4,5)P_2 5-phosphatase activity was increased while PtdIns(4)P 5-kinase activity remained the same, or if PtdIns(4)P 5-kinase were inhibited, while PtdIns(4,5)P_2 5-phosphatase activity remained the same, [^{32}P]Pi incorporation into PtdIns(4)P would be higher than control. If basal phospholipase C activity were stimulated by spermidine and spermine, an increase in [^{32}P]Pi incorporation into PtdIns due to compensatory synthesis would be likely. Similarly, if basal PtdIns-PLC activity were decreased, PtdIns labelling would likely decrease. As neither PtdIns nor PtdIns(4)P [^{32}P]Pi incorporation increase, the most likely explanation is that spermine and spermidine (or perhaps spermine synthesized from spermidine) bind to PtdIns(4,5)P_2 within N1E-115 cells and slow the rate of [^{32}P]Pi incorporation into PtdIns(4,5)P_2 by inhibiting the futile turnover mediated by the enzymes PtdIns(4)P 5-kinase and PtdIns(4,5)P_2 5-phosphatase. The apparent specificity for PtdIns(4,5)P_2 may be due to

the fact that it is the most highly negatively charged of the phosphoinositides, and thus is the most likely to bind polyamines.

It is well established that long term exposure of cells in culture to exogenous spermine can result in cell death [70,50]. Therefore, the effects of long term exposure of the cells to polyamines were examined with putrescine, but not with spermine or spermidine which had toxic effects on these cells after 4 h. Exposure of the cells to 5 mM putrescine for up to 10 h had no effect on cell appearance or adherance to the dishes. At one h exogenous putrescine had no effect on [^{32}P]Pi incorporation into phospholipid. Longer incubation times revealed stimulation by putrescine of [^{32}P]Pi incorporation into specific phospholipid classes. Total radioactivity in PtdCho at 6 h is the same for both putrescine treated and control cells. In contrast, [^{32}P]Pi incorporation into PtdIns increases 1.6 fold in putrescine-treated cells. Putrescine stimulates radiolabel incorporation into the polyphosphoinositides to a lesser degree. [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns(4)P levels are 1.4 and 1.3 times higher in putrescine-treated cells than in control cells. Since putrescine does not affect all phospholipid classes, an effect though altered [^{32}P]ATP specific activity is unlikely. [^{32}P]PtdIns is increased by a 6 h treatment with putrescine to a greater extent than is either [^{32}P]PtdIns(4)P or [^{32}P]PtdIns(4,5)P_2 suggesting that long term treatment with putrescine may stimulate accumulation of PtdIns mass. Once formed, [^{3}H]PtdIns is used in polyphosphoinositide synthesis. Alternately, the specific radioactivity of PtdIns may be increasing without an increase in mass. If the specific radioactivity of the diester phosphate is less than either of the two monoester phosphates in polyphosphoinositides at 6 h, then an increase in diester phosphate specific activity may have been partly masked by radioactivity in the other two phosphates even though the polyphosphoinositides are derived ultimately from PtdIns. Stimulation of [^{32}P]PtdIns accumulation within N1E-115 cells may involve activation of PtdIns synthase. Therefore, polyamine effects on *in vitro* PtdIns synthase activity were investigated.

There was no detectable change in the average mg of protein per culture dish during the 6 h incubations either in the absence or presence of putrescine. Therefore, no large scale growth has ocurred during this time. It is important to note that the mass data presented here is for 1 h incubations with exogenous polyamines. Therefore, the intracellular content of the individual polyamines after 6 h of exposure to exogenous putrescine is not known,. It seems likely that the total polyamine content of the cells at 6 h is high and that perhaps a great deal of interconversion between putrescine, spermidine, and spermine has taken place.

Bradykinin stimulates PtdIns turnover in N1E-115 neuroblastoma cells [116]. In the present study neither putrescine nor spermine have any effect on agonist stimulated PtdIns turnover. By this measure, events upstream of stimulated PtdIns synthesis are not affected by polyamines. This is so, even though bradykinin stimulates putrescine transport, and hence, intracellular polyamine content may be even higher in bradykinin treated cells. The ability of bradykinin to bind to its receptor is unaltered by polyamines in the medium. Likewise, the ability of the agonist-receptor complex to activate a G protein is not prevented nor is subsequent activation of the phosphoinositide specific phospholipase C prevented. This suggests that polyamines are not binding to G proteins in N1E-115 neuroblastoma cells as had been suggested for mast cells [53,51]. Polyamines do not affect PtdIns synthesis under basal or agonist stimulated conditions during the 90 min time period of this experiment.

Comparing [^{32}P]Pi time course experiments with chase experiments in which [^{32}P]Pi is chased with non-radioactive phosphate reveals that changing the culture medium to initiate the chase stimulates incorporation of [^{32}P]Pi into phospholipid. Over the same time period the rate of [^{32}P]Pi incorporation in the absence of radioactivity in the medium is double that in the spent medium with [^{32}P]Pi. This is due to either accumulation of phospholipid mass within the cells or increased phospholipid turnover. Nutrients in fresh DMEM may stimulate cell growth and hence phospholipid synthesis. This complicates

interpretation of the results obtained in the presence of polyamines. However, it is clear that at the beginning of the chase period spermine treated cells have much less [^{32}P]PtdIns(4,5)P_2 than control cells, and that control and polyamine treated cells show enhanced [^{32}P]Pi incorporation due to the medium change. Radiolabel in PtdIns(4,5)P_2 of spermine treated cells is much more resitant to chase with non-radioactive phosphate than is [^{32}P]PtdIns(4,5)P_2 in control and putrescine treated cells. This is consistent with the notion that [^{32}P]PtdIns(4,5)P_2 levels are low in spermine treated cells at zero chase time (1 h pulse) because basal turnover, probably through the futile kinase-phosphatase cycle, is reduced.

Spermine treated cells show greater enhancement of [^{32}P]PtdIns(4,5)P_2 synthesis due to the medium change than do control cells. Increased polyamine levels are associated with cell growth [133,285]. While increased spermine levels within N1E-115 cells are not sufficient to stimulate cell growth, they may potentiate the effect of increased nutrients in the medium on cell growth. Cell growth may not have resulted in detectable changes in the amount of cellular protein per culture dish because of small variations in the number of cells in each dish at the begining of the experiment and because some of the cells are lost each time the medium is changed or the cells are washed.

Radiolabel continues to increase in PtdCho, PtdEtn, PtdGro, and Ptd(Gro)$_2$ for the full duration of the chase experiment while radiolabel in PtdIns starts to decline after 30 min of chase. This suggests that after a 1 h pulse with [^{32}P]Pi, PtdIns is much closer to equilibrium with the γ-phosphate of [^{32}P]ATP than are the other phospholipids. This is probably due to the fact that the total mass of PtdIns is low [59] and its rate of turnover is high.

[^{32}P]Pi was chased from the cells as a prelude to examining bradykinin stimulated loss of PtdIns(4,5)P_2. Bradykinin stimulates PtdIns-PLC in N1E-115 cells and this results in loss of 50-75 % of the prelabelled PtdIns(4,5)P_2 within 45 seconds. This agrees with previously published results [116]. Both control and spermine treated cells show

bradykinin stimulated hydrolysis of PtdIns(4,5)P_2. Much more radiolabelled PtdIns(4,5)P_2 is lost in the spermine treated cells, although the proportion of labelled polyphosphoinositide hydrolyzed due to bradykinin is similar to control. Therefore, as experiments with bradykinin stimulated PtdIns synthesis had suggested, spermine does not affect bradykinin binding to its receptor, G protein function, or PtdIns-PLC activation and function. Furthermore, because the proportion of labelled PtdIns(4,5)P_2 hydrolyzed is the same in control and spermine treated cells, the high levels of [^{32}P]PtdIns(4,5)P_2 in spermine treated cells are not due increased PtdIns(4,5)P_2 mass, but rather, due to slow chase of radiolabel as a result of decreased PtdIns(4,5)P_2 turnover.

Lack of any polyamine effect on agonist-stimulated phosphoinositide hydrolysis and synthesis suggests that *in vivo* agonist-sensitive phosphoinositide metabolism is not directly modulated by polyamines, even in those disease cases where polyamine content is elevated such as in postischaemic brain and in tumours [317,236]. However, polyamines may indirectly affect phosphoinositide signal transduction by increasing the synthesis of PtdIns. If this results in an increase in the total mass of PtdIns, the quantities of phosphoinositides available for signal transduction would be increased.

IV. Effect of polyamines on *in vitro* phosphatidylinositol synthesis

Polyamines modulate *in vitro* activities of a number of enzymes involved in phosphoinositide metabolism. Since time course data suggested that long term exposure of cells to putrescine stimulated [^{32}P]Pi incorporation into PtdIns, polyamine effects on PtdIns synthase and the Ins:PtdIns exchange enzyme were investigated. PtdIns synthase catalyzes Mg^{2+}-dependent transfer of PtdOH from CMP-PtdOH to inositol. It can be assayed using [^{3}H]Ins in the presence of CMP-PtdOH. Alternately, PtdIns synthase can be measured in the presence of [^{3}H]Ins and CMP by the exchange reaction. Reversal of the normal reaction results in transient formation of CMP-PtdOH and then incorporation of [^{3}H]Ins forming [^{3}H]PtdIns [37,36]. Assays using CMP and [^{3}H]Ins have the advantage that they require neither exogenous lipids nor detergents and thus are more suited to

examining the effects of polyamines which are likely to have effects through modification of electrostatic properties of the membranes. The disadvantage is that CMP-independent Ins:PtdIns exchange can take place as well. Many investigators have used either $MnCl_2$ or $MgCl_2$ to measure these activities. Some have used both divalent cations together. Therefore, the effects of $MnCl_2$ and $MgCl_2$ were examined in these studies.

Under all conditions tested, incorporation of [^{3}H]Ins into lipid absolutely requires either Mn^{2+} or Mg^{2+}. Polyamines did not stimulate detergent-solubilized CMP-PtdOH dependent activity, nor did they alter the Mg^{2+} requirement of the detergent solubilized assay as reported for Mg^{2+} requirement of phosphoinositide kinases [241,380]. Absence of polyamine effect may be due to the presence of detergent.

CMP-independent Ins:PtdIns exchange activity is more active with Mn^{2+} than Mg^{2+}. Low concentrations of Mn^{2+} are sufficient to fully activate the exchange. Therefore, the enzyme that catalyzes this exchange has high affinity for Mn^{2+}. In contrast to data from erythrocytes [264], CMP-independent exchange in N1E-115 lysates was not inhibited by high concentrations of Mn^{2+}. Also, CMP-independent exchange in these lysates was much less active in the presence of Mg^{2+} than it is in erythrocytes. Polyamines did not affect CMP-independent Ins:PtdIns exchange in the presence of Mn^{2+} or Mg^{2+}.

CMP dramatically increased Ins:PtdIns exchange in the presence of Mg^{2+} but not Mn^{2+}. This occurs through transient reversal of PtdIns synthase. This agrees with previous results [78]. Thus, the divalent cation requirements of CMP-independent exchange and CMP-dependent exchange (catalyzed by PtdIns synthase) are different; the former requires Mn^{2+} and the later requires Mg^{2+}. Incorporation of [^{3}H]Ins through reversal of PtdIns synthase in the presence of CMP and Mg^{2+} was stimulated by polyamines. Spermidine stimulated activity 2 fold over a wide range of Mg^{2+} concentrations. Polyamines also stimulated an otherwise undetectable, CMP-dependent exchange activity in the presence of Mn^{2+}. CMP-dependent, Mn^{2+}-dependent Ins:PtdIns exchange activity was very low and could not be detected in the absence of polyamines due

to the much higher activity of CMP-independent, Mn^{2+}-dependent exchange. However, polyamines stimulate this otherwise low CMP-dependent activity, even in the presence of low concentrations of Mn^{2+}.

In summary, long term stimulation of [^{32}P]PtdIns accumulation in cultured cells by putrescine suggested that PtdIns sythase was stimulated by polyamines. *In vitro* CMP-PtdOH dependent PtdIns synthase is not modulated by polyamines. This reaction is performed in the presence of detergent and exogenous lipid. Therefore, it does not accurately reflect the synthesis of PtdIns in native membranes. CMP-dependent Ins:PtdIns exchange was used as a measure of PtdIns synthase activity in intact membranes since this exchange is catalyzed by transient reversal of PtdIns synthase without exogenous lipids or detergents. PtdIns synthase is, by this measure, stimulated by polyamines. CMP-independent exchange activity is not affected. Hence, polyamine stimulation of PtdIns synthase can account for increased [^{32}P]PtdIns synthesis in whole cells treated with putrescine. The *in vitro* reaction is most strongly stimulated by elongated polyamines. Therefore, synthesis of spermidine and spermine from putrescine may have been been the factor responsible for the long term stimulation of PtdIns synthase.

V. Malignant hyperthermia

The inherited disorder malignant hyperthermia (MH) was investigated to determine the nature of the biological defect in humans and whether malignant hyperthermia cells could be used as a model system in which inositol phosphate dephosphorylation and hence calcium fluxes are altered. Some inbred strains of swine have a similar, or identical, disorder that has been used as a model of human MH. Two different mechanisms had been proposed for MH in swine. MH susceptible swine are somewhat deficient in membrane-bound Ins(1,4,5)P_3 5-phosphatase from muscle [108]. However, it had also been reported that the sarcoplasmic reticulum ryanodine receptor was altered in swine susceptible to MH [273]. The ryanodine receptor is a calcium channel found in the sarcoplasmic reticulum which binds the plant alkaloid ryanodine with high affinity [220,144]. Both of these

defects could lead to altered Ca^{2+} levels within cells. Published reports indicated that halothane triggered abberant Ca^{2+} fluxes in leukocytes of human MH patients [206, 207]. To test the hypothesis that Ins(1,4,5)P_3 5-phosphatase is deficient in humans with MH, *in vitro* activity of the enzyme was measured in lymphocytes and transformed lymphoblasts from patients with MH. Immortalized cells would provide a useful model of altered inositol phosphate metabolism if they were deficient in Ins(1,4,5)P_3 5-phosphatase.

Human leukocytes contain both cytosolic and membrane bound Ins(1,4,5)P_3 5-phosphatase activity. Membrane bound Ins(1,4,5)P_3 5-phosphatase activity constitutes 17 % of the total Ins(1,4,5)P_3 5-phosphatase activity in leukocytes. This is similar to membrane bound Ins(1,4,5)P_3 5-phosphatase levels in platelets (16 %) [74], but very different from levels reported in liver (over 70 %) [395,371].

The results in this study indicate that Ins(1,4,5)P_3 5-phosphatase activity is not deficient in leukocyte preparations of MH susceptible individuals compared to control. No differences were observed in Ins(1,4,5)P_3 5-phosphatase activity in lysates of leukocyte preparations. Neither cytosolic nor membrane bound activity was lower in MH samples compared to control. Foster and coworker reported that halothane inhibits cytosolic Ins(1,4,5)P_3 5-phosphatase [108]; the present study confirmed inhibition of soluble activity and extends this observation to membrane bound activity as well, although the extent of inhibition was low.

Results using transformed lymphoblast cell lines prepared from blood samples from malignant hyperthermia susceptible individuals confirmed the results obtained with leukocyte preparations. There were no detectable differences in Ins(1,4,5)P_3 5-phosphatase activity in malignant hyperthermia cell lines and controls. Exposing cell lysates of normal and MH lymphoblasts to succinylcholine did not reveal any difference in Ins(1,4,5)P_3 metabolism although succinylcholine is known to trigger MH [283].

The initial conclusion drawn from these studies was that human MH is not associated with deficient Ins(1,4,5)P_3 5-phosphatase and that swine are not suitable in this

respect as a model for the human disease. After these studies were completed, it was reported that halothane-induced intracellular calcium release in lymphocytes of MH susceptible and control humans are the same [301]. This contradicted the previously published reports, on which our study was based, that lymphocytes from MH susceptible individuals have increased levels of intracellular calcium after exposure to halothane but that control samples do not [206, 207]. Thus, lymphocytes may not have been an appropriate choice of tissue in which to attempt to detect inositol 1,4,5-trisphosphate 5-phosphate deficiencies.

Genetic linkage data from extended human pedigrees indicate that the malignant hyperthermia susceptibility locus is found at the q12-13.2 region of chromosome 19 in humans [132,261]. The locus controlling both human and porcine malignant hyperthermia is genetically linked to the glucose phosphate isomerase locus [261]. This is the region to which the ryanodine receptor has also been localized [253]. Co-segregation of malignant hyperthermia with ryanodine markers, resulting in a lod score of 4.20 at a linkage distance of zero centimorgans, indicates that mutations in the ryanodine receptor gene are the most likely cause of malignant hyperthermia [254]. Clearly, a defect in a sarcoplasmic reticulum calcium channel could lead to elevated Ca^{2+} intracellular levels observed in malignant hyperthermia [272]. Thus, it appears that in humans, and perhaps in some swine, the defect responsible for malignant hyperthermia results from a mutation in the ryanodine receptor gene. The deficiency in inositol 1,4,5-trisphosphate 5-phosphatase and its potential role if any in swine is in doubt.

Summary of major findings and conclusions

A. Major findings

1. Neither C6 glioma nor N1E-115 neuroblastoma cells have either CTP:PtdOH cytidylyltransferase or PtdIns synthase in their plasma membranes. These two enzymes were found exclusively in the endoplasmic reticulum enriched microsomal fraction. Thus, these two cultured cell lines are incapable of recycling agonist

generated DAG back into PtdIns within the plasma membrane and must transport PtdIns from the endoplasmic reticulum to the plasma membrane to replace phosphoinositides hydrolysed in the plasma membrane.

2. C6 glioma cells have plasma membrane bound PtdIns(4,5)P_2 phospholipase C. This activity can be activated by extracellular agonists. Most of the PtdIns(4,5)P_2 phospholipase C activity is cytosolic and it does not translocate to membranes of C6 or N1E-115 cells following stimulation with serotonin and bradykinin.
3. DAG kinase is distributed throughout the cell. Its presence in the plasma membrane suggests that the cells are able to modulate protein kinase C activation at the plasma membrane by phosphorylating DAG.
4. PtdIns 4-kinase is found in the cytosol and microsomal fractions. Plasma membrane PtdIns(4)P may be formed during or before transport to the plasma membrane. Cytosolic PtdIns 4-kinase activity may have access to PtdIns in the plasma membrane and form PtdIns(4)P at that membrane.
5. PtdIns(4)P 5-kinase is membrane bound and is found in both the plasma membrane and the microsomes. This suggests that PtdIns(4,5)P_2 may have important functions at a number of sites within glioma cells.
6. PtdIns(4)P 4-phosphatase is widely distributed throughout the cell and hence, can participate in phosphoinositide metabolism at the plasma membrane and other intracellular sites.
7. PtdIns(4,5)P_2 5-phosphatase is largely cytosolic. A small quanitity is membrane bound. Hence, it may participate in phosphoinositide metabolism at the plasma membrane and other sites.
8. N1E-115 cells in culture incorporate exogenous 1-acyl-[^{3}H]lysoPtdIns. The rapid formation of deacylated products suggests that [^{3}H]PtdIns which is formed arises not only from acylation of 1-acyl-[^{3}H]lysoPtdIns, but also from incorporation of [^{3}H]Ins into PtdIns via PtdIns synthase.

9. The microsomal fraction was the only subcellular fraction capable of acylating 1-acyl-[^{3}H]lysoPtdIns *in vitro*. The plasma membrane cannot acylate agonist-generated 1-acyl-lysoPtdIns or remodel the fatty acid profile of PtdIns.
10. Microsomal acylation of 1-acyl-[^{3}H]lysoPtdIns is most active with arachidonoyl-CoA. This accounts for the enrichment of PtdIns with arachidonate.
11. In addition to acyl-CoA dependent acylation, some CoA-dependent and CoA-independent acylation of 1-acyl-[^{3}H]lysoPtdIns transacylase activity with endogenous acyl chains is detected in the microsomal fraction.
12. N1E-115 cells rapidly incorporate exogenous putrescine, spermidine, and spermine. The intracellular polyamine content increased 1.5 - 2 fold.
13. Putrescine transport in N1E-115 cells is mediated by a sulfhydryl containing protein and is stimulated by bradykinin.
14. Once exogenous polyamines are transported into N1E-115 cells they are interconverted by addition and removal of aminopropyl groups.
15. Increased intracellular putrescine (or its metabolites) stimulates PtdIns synthase in cultured N1E-115 cells in the long term.
16. Changing the medium in which N1E-115 cells are growing to fresh medium stimulates phospholipid synthesis.
17. Cells pulsed with exogenous spermine and [^{32}P]Pi have much less [^{32}P]PtdIns(4,5)P_2 than control. That this is due to decreased turnover of PtdIns(4,5)P_2 is confirmed by [^{32}]Pi chase experiments showing that radiolabel is chased from [^{32}P]PtdIns(4,5)P_2 less efficiently than in control cells.
18. High intracellular polyamine levels do not affect agonist stimulated loss of PtdIns(4,5)P_2.
19. PtdIns synthesis in N1E-115 cells is stimulated by bradykinin and this process is not affected by polyamines.

20. Human leukocytes contain both soluble and membrane bound Ins(1,4,5)P_3 5-phosphatase activity. Halothane slightly inhibits both activities.

21. Human malignant hyperthermia is not due to deficient Ins(1,4,5)P_3 5-phosphatase activity. Neither leukocytes nor transformed lymphoblasts from malignant hyperthermia patients are deficient in Ins(1,4,5)P_3 5-phosphatase activity.

B. Conclusions

In conclusion, plasma membranes of N1E-115 and C6 cells have limited capacity for phosphoinositide metabolism. Resynthesis of PtdIns from agonist-generated DAG does not occur in the plasma membrane of these cells. Since these cells are capable of agonist-stimulated phosphoinositide metabolism, local resynthesis of PtdIns in the plasma membrane is not an obligatory requirement of the phosphoinositide signal transduction pathway. PtdIns must be transported from the endoplasmic reticulum to the plasma membrane to replace phosphoinositides hydrolyzed at the plasma membrane. Complete synthesis of PtdIns(4,5)P_2 from PtdIns is not mediated by the plasma membrane alone. Likewise, acylation of 1-acyl-lysoPtdIns does not occur in the plasma membrane. Agonist generated lysoPtdIns cannot be recycled back to PtdIns within the plasma membrane. Also, enrichment of PtdIns with arachidonate must occur in the microsomes and not in the plasma membrane, although, biologically active fatty acids may be important to the function of PtdIns in the plasma membrane. Thus, the plasma membrane is not an autonomous cellular compartment with regard to phosphoinositide metabolism. The phosphoinositide signal transduction pathway in the plasma membrane requires the combined actions of enzymes in the plasma membrane, cytosol, and microsomes.

High intracellular polyamine content does not interfere directly with agonist stimulated phosphoinositide metabolism. However, the futile cycle of phosphorylation and dephosphorylation of the 5-position monoester phosphate of PtdIns(4,5)P_2 is inhibited by spermine and possibly spermidine. Also, long term exposure of the cells to polyamines results in increased PtdIns synthesis. Thus, increased polyamine content in postischaemic

brain and in cancer probably does not directly affect signal transduction [317,236]. However, polyamines may indirectly affect phosphoinositide signal transduction by increasing the synthesis of PtdIns.

Human malignant hyperthermia is not caused by a defect in Ins(1,4,5)P_3 5-phosphatase. Therefore, pigs which are deficient in this enzyme are not a suitable model for malignant hyperthermia in humans.

Future experiments

The work described in this thesis details some of the aspects of phosphoinositide metabolism in C6 glioma and N1E-115 neuroblastoma cells. In particular, the results presented here shed light on factors that determine of the quantity and availability of phosphoinositides for agonist stimulated hydrolysis. However, many questions remain unanswered.

Future work in this area should be devoted to discovering the fate of agonist stimulated DAG at the plasma membrane. Clearly, DAG kinase is present in that membrane. DAG is probably phosphorylated thus as limiting its role as a second messenger. Data from many cell types suggests that this diacylglycerol is preferentially recycled back into phosphoinositides. N1E-115 neuroblastoma and C6 glioma are clearly incapable of PtdIns resynthesis in the plasma membrane. It would be instructive to determine whether DAG is preferentially recycled back into PtdIns in these cells. Perhaps preferential recycling of DAG is a feature of only those cell types that have plasma membrane associated PtdIns synthase.

PtdIns(4,5)P_2 specific PLC activity is mostly cytosolic although there was some activity in the plasma membrane. It would be of interest to determine the quantities and distributions of the various PtdIns-PLC isozymes.

PtdIns 4-kinase is not found in the plasma membrane. The site of PtdIns phosphorylation should be determined because this may strongly control plasma membrane phosphoinositide levels.

The effects of exogenous polyamines on phosphoinositide metabolism warrant further investigation. Polyamine effects on phosphoinositide mass should be further investigated. It would be useful to examine phosphoinositide metabolism in cells with reduced polyamine content. N1E-115 neuroblastoma and C6 glioma cells are transformed and may already have slightly increased levels of polyamines. It would be useful to find how transformed and non-transformed cells compare with respect to the effects of polyamines on phosphoinositide metabolism.

Some general approaches which could be used to investigate these issues are listed below:

1. The rate of loss of radiolabel from [^{32}P]Pi and [^{14}C]arachidonate labelled PtdIns could be measured in pre-labelled neuroblastoma and glioma cells under control and agonist-stimulated conditions. If there is preferential recycling much more phosphate label than arachidonate label would be lost.
2. PtdIns-PLC activity assays on subcellular fractions could be performed using PtdIns, PtdIns(4)P as well as PtdIns(4,5)P_2 to investigate the distribution of different isozymes of PtdIns-PLC.
3. Antibodies to various PtdIns-PLC isoforms could be obtained and used to study the presence and intracellular distribution of the various isozymes.
4. Time course studies using [^{32}P]Pi labelled cells in culture followed by subcellular fractionation could be performed to examine the kinetics of phosphoinositide synthesis and transport within different subcellular fractions of the cells.
5. It may be possible to reduce polyamine levels in N1E-115 cells by using specific inhibitors of polyamine synthesis. Preliminary evidence indicates that the ornithine decarboxylase inhibitors α-methylornithine and α-hydrazinoornithine did not reduce polyamine levels in N1E-115 cells below control values. However, many inhibitors of either ornithine decarboxylase or S-adenosylmethionine decarboxylase have been described including: (difluoromethylornithine, 1,3-diaminopropane, (E)-

2-(fluoromethyl)dehydroornithine, (2R,5R)-6-heptyne-2,5-diamine, S-adenosyl-1,8-diamino-3-thiooctane, and methylglyoxyal gis(guanylhydrazone). Thus, further studies using these inhibitors could be undertaken.

6. The effects of polyamines on phosphoinositide metabolism in non-transformed primary cultures of neural cells could be examined.

7. Mutant CHO cell lines which lack ornithine decarboxylase have been described [54]. These cells are dependent on exogenous polyamines. Phosphoinositide metabolism could be investigated in this cell line in the presence of different amounts and kinds of exogenous polyamines.

Appendix

I. Lipid extraction from subcellular fractions

Efficient extraction of lipids from Percoll density gradients is complicated by Percoll. In a chloroform/methanol extraction Percoll forms a large interface which extends down into the lipid containing lower phase. It is imperative to remove Percoll from the lower phase because Percoll in the lower phase will form an intractable gum if the sample is evaporated. Furthermore, even very small quantities of Percoll interfere with TLC separation of phospholipids. Passing lower phase lipid extract through silicic acid columns removes Percoll from the lipid fractions but is time consuming and results in some loss of phospholipids. To circumvent these problems a method was devised for extracting lipids from Percoll containing aqueous subcellular fractions using a 2:1 mixture of iso-butanol and acetonitrile. There are several advantages to biphasic lipid extraction with iso-butanol, acetonitrile, and water compared to the more traditional chloroform, methancl, water system. With iso-butanol/acetonitrile the lipid containing phase is the upper phase, thus, Percoll will settle down to the bottom, away from the lipid rich fraction. 40 % Percoll in gradient buffer could be extracted with iso-butanol/acetonitrile and when the upper phase was evaporated no Percoll gum was observed. As demonstrated with subcellular fractions from cells that had been pre-labelled with [^{3}H]oleic acid (18:1) (Figure 48), this extraction

procedure was quite efficient. With two extractions 99.7 % of the total radiolabel was recovered in the iso-butanol/acetonitrile extracts. This procedure also gives > 95 % recovery of acidic, zwiterionic and neutral lipids (Figure 49). Recovery of lysoPtdIns was quantitative (Figure 49). Unfortunately, significant amounts of cellular protein partition into the iso-butanol/acetonitrile phase. Protein interferes with resolublization of phospholipids when the organic solvents are evaporated. Proteins can be removed from the organic phase by back extraction with 3 M NaCl (Figure 49). This step also reduces the water content of the iso-butanol/acetonitrile phase, thus, decreasing the time necessary to evaporate the lipid containing organic phase. Finally, the organic phase is evaporated and lipids resuspended in a normal chloroform/methanol/water biphasic system (this is necessary because of the salt present in the iso-butanol/acetonitrile phase (Figure 50). During the iso-butanol/acetonitrile extractions any Percoll pellets which are difficult to resuspend can be more easily suspended after adding 1 ml of distilled water. The overall efficiency of the procedure measured with [^{3}H]18:1 labelled subcellular fractions is 95-99 % and, compared to chloroform methanol extractions coupled with silicic acid columns, takes half the time.

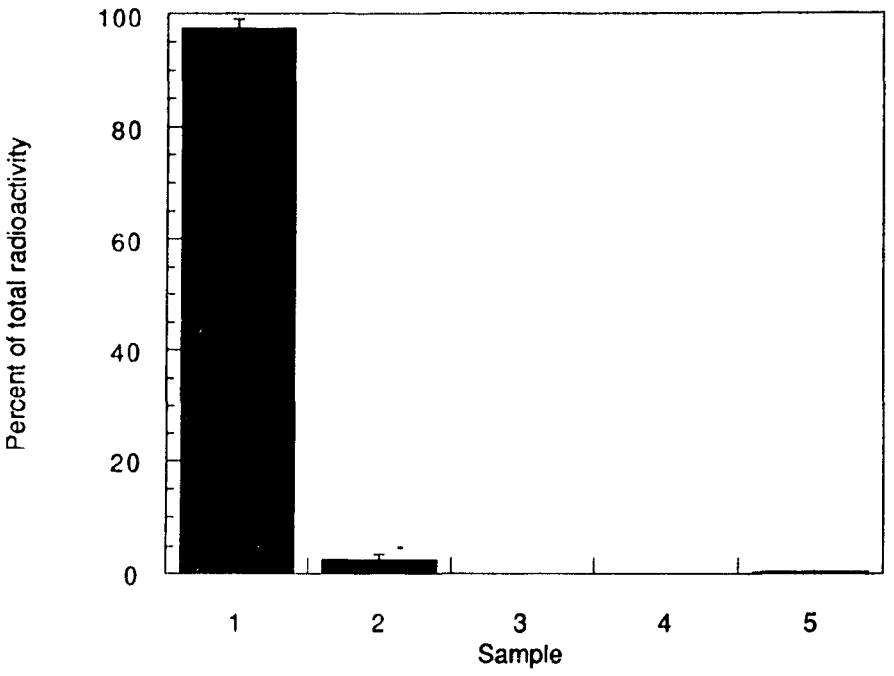


Figure 48. Extraction of lipids from a subcellular fraction with iso-butanol/acetonitrile. C6 glioma cells were incubated with 1 μCi [^{3}H]18:1 for 4 h. Subcellular fractions were obtained and the Percoll-containing membrane-rich fractions were combined. 1 ml aliquots of the fraction were extracted with 1 ml of iso-butanol/acetonitrile (2/1) 4 times. Sample 1 is radioactivity recovered in the first iso-butanol/acetonitrile extraction. Samples 2, 3, and 4 show the radioactivity recovered in subsequent extractions. Sample 5 is the radioactivity remaining in the Percoll pellet after 4 extractions. Data are for 3 determinations ± standard deviation.

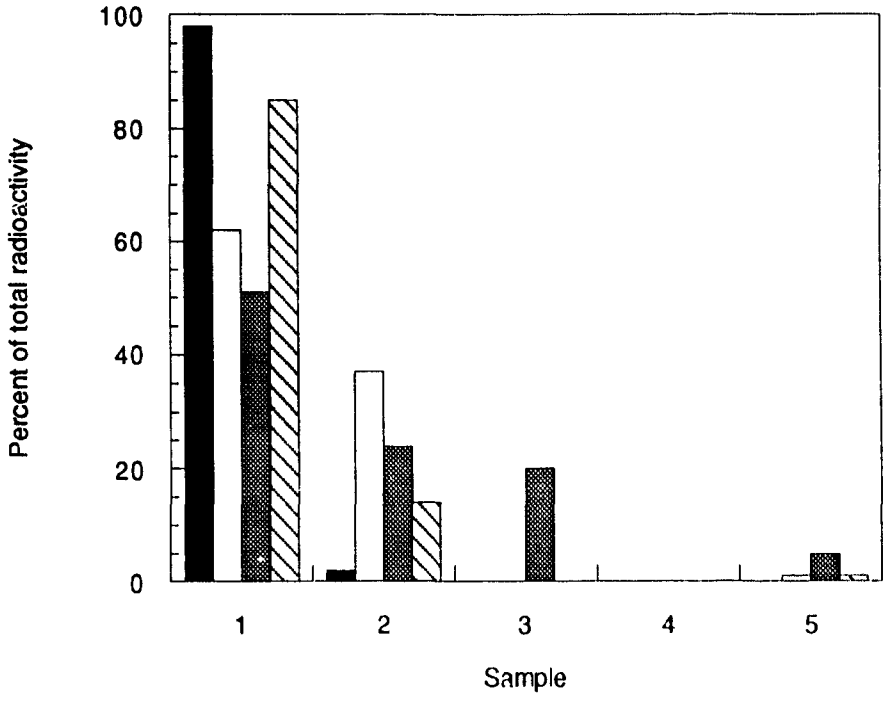


Figure 49. Extraction of selected lipids from 40 % Percoll. A suspension of 40 % Percoll in subcellular fractionation gradient buffer was made. 50,000 dpm of [^{3}H]lysoPtdIns (black bars), [^{3}H]PtdIns (open bars), [^{14}C]PtdCho (grey bars), and [^{14}C]triacylglycerol (hatched bars) was added to separate 2 ml Percoll suspensions and extracted with 2 ml of iso-butanol/acetonitrile (2/1) 4 times. Samples 1 through 4 show the radioactivity recovered in each of the 4 extractions. Sample 5 is radioactivity recovered in the Percoll pellets. Data are for single determinations.

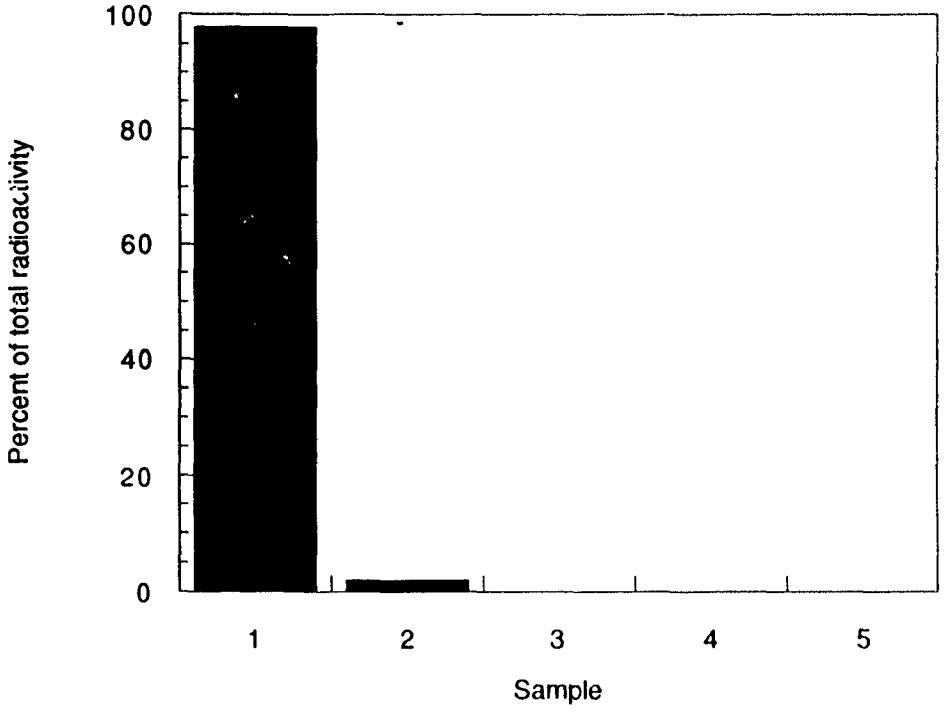


Figure 50. Loss of lipid into the salt wash. Subcellular fractions labelled with [^{3}H]18:1 were prepared as in figure X and extracted with iso-butanol/acetonitrile (2/1). The organic phase was extracted once with 2 ml of 3 M NaCl. The salt phase was back extracted 3 times with 1 ml of iso-butanol/acetonitrile. Sample 1 is percent of the total radioactivity that is left in the organic phase after 1 extraction with salt. Samples 2, 3, and 4 are percent of the total radioactivity recovered in iso-butanol/acetonitrile back washes of the salt phase. Sample 5 is radioactivity remaining in the salt phase after the back extractions. Data are for one experiment.

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