CHARACTERISTICS AND REGULATION OF PHOSPHOLIPASES AND LYSOPHOSPHOLIPASES IN THE GUINEA-PIG HEART

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Ketan Badiani

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To my parents

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List of Abbreviations

AA	Arachidonic acid
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
ATP[S]	Adenosine-5'-*O*-(3-thiotriphosphate)
ANSA	1-Amino-2-naphthol-4-sulphonic acid
Bt_2cAMP	Dibutryl cyclic adenosine 3':5'-monophosphate
C	Carbon
°C	Degrees Celsius
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
Ci	Curie
Co.	Company
CoA	Coenzyme A
Corp.	Corporation
$cPLA_2$	Cytosolic phospholipase A_2
Da	Daltons
dpm	Disintegrations per minute
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
EC	Enzyme classification
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(ß-amino-ethyl ether)N,N'-tetraacetic acid
EGF	Epidermal growth factor

GDP	Guanosine-5'-diphosphate
GDPßS	Guanosine-5'-O-(2-thiodiphosphate)
GMPPNP	Guanylylimidodiphosphate
GTP	Guanosine-5'-triphosphate
GTP[S]	Guanosine 5'-*O*-(3-thiotriphosphate)
GPC	*sn*-glycero-3-phosphocholine
GPE	*sn*-glycero-3-phosphoethanolamine
GPL	*sn*-glycero-3-phospholipid
hr	Hour
HPLC	High-performance liquid chromatography
KCl	Potassium chloride
kDa	Kilodaltons
K_m	Michaelis-Menten constant
l	Litre
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
LPS	Lysophosphatidylserine
M	Molar
min	Minute
mg	Milligram
Mg^{2+}	Magnesium
ml	Millilitre
mM	Millimolar

mRNA	messenger RNA
NaF	Sodium Fluoride
N_2	Nitrogen
NaCl	Sodium chloride
nm	Nanometre
nM	Nanomolar
NEM	N-Ethylmaleimide
p	Statistical probability
PA	Phosphatidic acid
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PIP	Phosphatidylinositol-4-phosphate
PIP_2	Phosphatidylinositol-4,5 bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PS	Phosphatidylserine
Rf	Relative mobility
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
t.l.c	Thin-layer chromatography

TPA	Tetradecanoyl phorbol acetate
Tris	[*Tris*-(hydroxymethyl)aminomethane]
μ	Micron
μCi	Microcurie
μl	Microlitre
μM	Micromolar
μmol	Micromole
v	Volume
w	Weight

Abstract

Many cells and tissues respond to extracellular stimuli by mobilizing arachidonic and other polyunsaturated fatty acids from the sn-2 position of membrane phospholipids. It has been suggested that one possible route for the Ca^{2+}-independent release of sn-2 fatty acids could involve the sequential hydrolysis of the phospholipid by a phospholipase A_1 and lysophospholipase A_2. The major aim of this study has been to determine the characteristics and regulation of phospholipase A_1 and lysophospholipase A_2 activities in guinea-pig heart microsomes. This involved using radiolabelled phospholipid substrates for in vitro assays of enzyme activity. A Ca^{2+}-independent lysophospholipase A_2 was characterized that exhibited highest activity with 2-arachidonoyl and 2-linoleoyl GPE. This enzyme displayed characteristics that were different from a 1-acyl GPE and 2-acyl GPC lysophospholipase activities in guinea-pig heart microsomes. A Ca^{2+}-independent PC-hydrolyzing phospholipase A_1 activity was characterized that displayed highest activity with 1-palmitoyl-2-linoleoyl GPC. This enzyme displayed a preference for substrates which contain palmitic acid at the sn-1 position and the nature of the fatty acid at the sn-2 position affected the rate of the enzyme activity. The addition of GTP[S] inhibited the hydrolysis of 1-palmitoyl-2-linoleoyl GPC and 1-palmitoyl-2-arachidonoyl GPC. This inhibition was not duplicated by any other nucleotide tested suggesting that the enzyme activity was regulated by an inhibitory G protein. A Ca^{2+}-independent PE-hydrolyzing phospholipase A_1 activity was also characterized. This PE-hydrolyzing phospholipase A_1 displayed highest activity with 1-stearoyl-2-arachidonoyl GPE and preferred PE molecular species which contained arachidonate at the sn-2 position. Again, the rate of hydrolysis of the sn-1 position by the PE-hydrolyzing phospholipase A_1 was influenced by the nature of the

fatty acid at the sn-2 position. The hydrolysis of 1-16:0-2-18:2 GPE was enhanced by the addition of GTP[S] and GMPPNP but not by any other nucleotide. The phospholipase A_1 activity with 1-16:0-2-18:2 GPE was also stimulated by dl-isoproterenol and this activation could be specifically blocked by butoxamine, a $ß_2$-adrenergic receptor antagonist. Experiments suggest that G protein mediated activation of phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE occurs subsequent to activation of the $ß_2$-adrenergic receptor. Taken together, the results of this study indicate that phospholipases A_1 and lysophospholipases A_2 exist in guinea-pig heart microsomes that could work in concert to selectively release arachidonic, linoleic, or possibly other polyunsaturated fatty acids subsequent to cell stimulation.

1. Introduction

1.1. The Biological Membrane

The organization of metabolic activity in all higher cells depends in a large part on the compartmentalization of specific cellular processes. This compartmentalization is afforded by the biological membrane. The biological membrane, of which the plasma membrane is one example, acts as a barrier as it encloses every cell or particular components of the cell and so defines the limits or boundaries within the cell and of the cell itself. The nature of the membrane allows it to be semi-permeable. The membrane of the cell is a principal factor in determining the environment inside the cell.

1.1.1. The Structure of the Biological Membrane

The principal components of membranes are lipids, proteins, and carbohydrates (1,2,3,4). Although the composition of the membrane may vary greatly from one cell type to another, most of the basic structural and functional concepts are applicable to all cells. The amount of carbohydrate is usually small (10% or less), whereas the lipids of the membrane account for approximately 40% of the mass of the membrane, the balance being made up by protein. The structure of the membrane was elucidated as early as 1925 was determined to be a bilayer of lipids (5). A further modification of this bilayer model was made by Davson and Danielli in 1935 (6). The currently accepted model for the structure of the biological membrane was first proposed in 1972 by Singer and Nicholson (7).

The Singer and Nicholson fluid mosaic model of membrane structure encompassed and satisfied all the thermodynamic requirements for the structure of the

membrane. In this model, the biological membrane can be envisaged as being a sandwich of two layers of phospholipid. The matrix of the bilayer is the phospholipid. The proteins that are integral to the membrane are each arranged in a manner that satisfies the thermodynamic restrictions of the particular protein. This means that the ionic and highly polar portion of the protein protrudes from the surface of the bilayer into the aqueous phase of either the cytosol or the extracellular space while the apolar and hydrophobic portion of the protein is embedded within the lipid bilayer.

One consequence of such a membrane structure is that there is a lack of order to the organization of the components in the membrane. The reason for this randomness is that there is a lack of long-range interaction intrinsic to the membrane. There is very little interaction that enforces a distribution of a protein(s) in the membrane since the entire matrix of the bilayer is phospholipid. It is very likely that short-range order does exist (7). Direct interactions may exist between the protein and the lipid. A lipid dependence of many membrane enzyme activities has been inferred from the "re-activation" of purified membrane proteins by dispersion of the protein in model-membrane systems (1). Normal enzyme function can often be restored by lipids or even by detergents which simply provide an appropriate non-polar environment (1). Experiments have determined that for some enzymes a "boundary-layer" of lipid must exist to confer enzyme activity and hence micro-domains of specific lipids may exist within this sea of phospholipid (1).

Another consequence of the lipid nature of the membrane matrix is that the membrane would be fluid (7). There is good experimental evidence to show that the lipids of cell membranes are in a fluid state not unlike a viscous aqueous solution. Phospholipids in a bilayer may undergo lateral or rotational motion in the plane of the

bilayer (4). The rate at which lateral motion occurs was studied by Hubbell, Kornberg and McConnell (8,9) who determined that the rate of phospholipid neighbour exchange was approximately once every microsecond. However, phospholipids are thermodynamically unfavoured to undergo transverse motion or "flip-flop" across from one side of the bilayer to the other. McConnell and Kornberg found that the rate of flip-flop is once every 6 hours (3,4,9). The rate of flip-flop has been shown to be accelerated by the participation of putative phospholipid flippases (10). Proteins may also undergo lateral or rotational motion in the plane of the bilayer. Neither proteins nor phospholipids are thermodynamically favoured to undergo flip-flop. The rate of movement of the proteins in the plane of the bilayer is limited by the viscosity of the phospholipid. The fluid mosaic model of the biological membrane is a very dynamic one.

1.1.2. The Composition of the Biological Membrane

There are essentially three components to the biological membrane: lipids, proteins, and carbohydrate. Three classes of lipids found are: phospholipids, neutral lipids, and glycolipids. Phospholipids and glycolipids share one property and that is that they are both amphipathic. Amphipathic molecules have a dual nature to them. One portion of the lipid molecule will be hydrophilic while another portion will be hydrophobic.

The most common membrane lipids are phospholipids. Phospholipids all have the general structure of a glycerol carbon backbone with a polar head group attached at the sn-3 (stereospecific number) position and two long chain hydrocarbons linked to the sn-2 and sn-1 positions. The hydrophilic polar head group will associate with

the aqueous phase of the cell while the long chain fatty acids will extend away from the polar head group and preferentially interact with other hydrophobic groups in the centre of the lipid bilayer. Hence, the polar head groups of phospholipids form the surfaces of the bilayer while the centre of the bilayer is formed by the long chain hydrocarbon groups. Some common phospholipids are depicted in Figure 1. The hydrophilic head group can be either choline, ethanolamine, serine, or inositol. The hydrophobic fatty acid tails can differ in length, but generally they are 14-24 carbons in length. The sn-1 fatty acid is usually saturated while the sn-2 fatty acid may have one or more cis double bonds. It is this amphipathic nature of phospholipids that allows them to spontaneously form micelles or bilayers in solution. The major classes of phospholipids of mammalian cells are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM), and bis-phosphatidylglycerol also known as cardiolipin (CL). In general, phosphatidylcholine or phosphatidylethanolamine are the most abundant.

Phospholipids can also be of different subclasses. Phospholipids which differ in subclass differ with respect to the covalent linkage of the fatty acid at the sn-1 position. The majority of phospholipids in mammalian cell membranes are composed of diacyl phospholipids (eg. 1-acyl-2-acyl phosphatidylcholine), but in certain tissues or cell types, phospholipids exist in which the sn-1 fatty acid is attached with an O-alkenyl bond or as an O-alkyl group (11). Phospholipids can therefore be ether linked and have either an ether linked alkyl group or an α,ß-unsaturated hydrocarbon. Both the O-alkyl and the O-alkenyl phospholipids constitute ether-linked subclass of phospholipids. These can have either choline or ethanolamine headgroups.

Figure 1

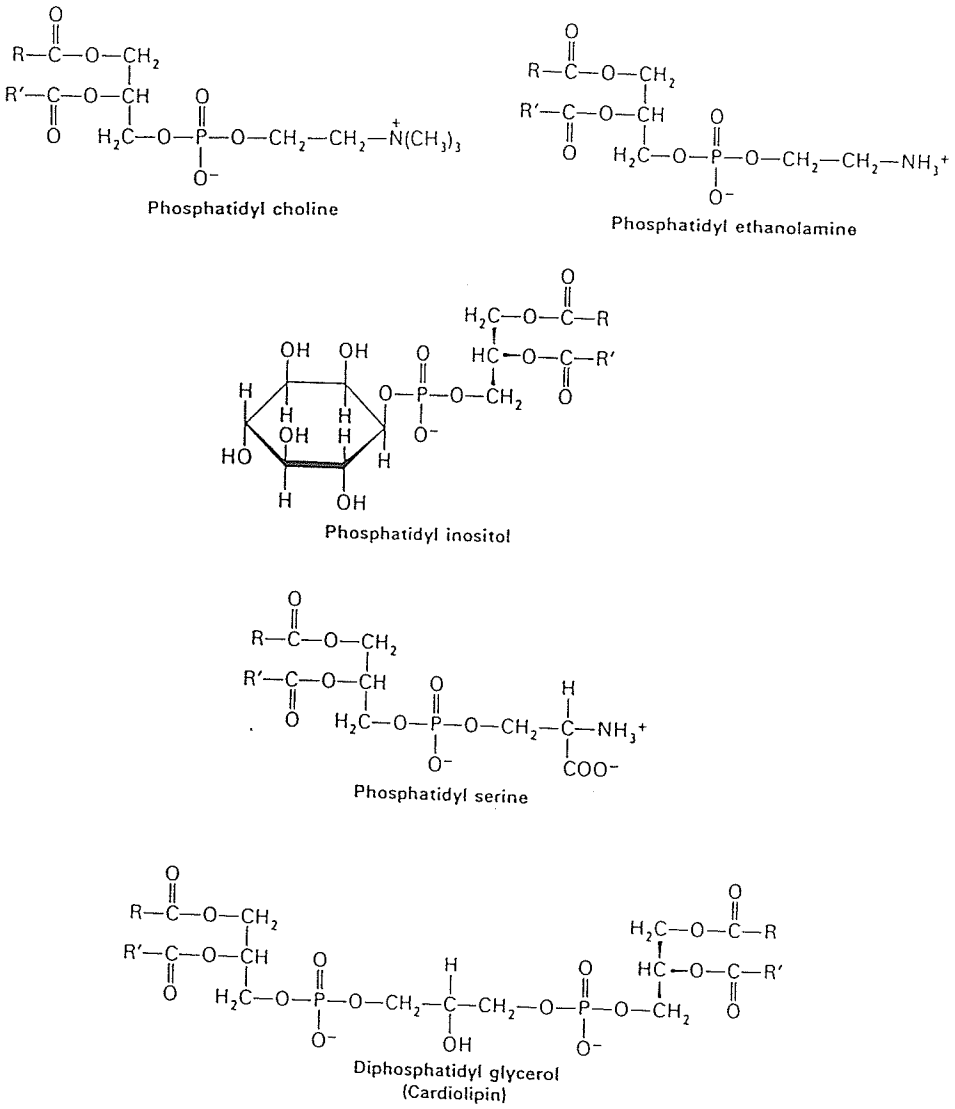

Fig. 1. Some common phospholipids found in mammalian cells. Adapted from ref. (1,2).

Ether linked phosphatidylcholine is termed plasmanylcholine while the vinyl ether linked phosphatidylcholine is termed plasmenylcholine to differentiate them from diacyl phosphatidylcholine. Vinyl ether linked phospholipids are termed plasmalogens.

Biological membranes are vectorial structures with an asymmetric distribution of the phospholipids in the inner and outer leaflet of the bilayer. Monolayers of the red cell membrane contain different proportions of the different phospholipids (12). The asymmetry in the red blood cell membrane is of a rather simple nature in which the choline phospholipids, phosphatidylcholine and sphingomyelin, constitute the outer half of the bilayer while the inner half (the cytosolic side) of the bilayer consists of the amino phospholipids, phosphatidylethanolamine and phosphatidylserine. Phosphatidylinositol is found on the cytoplasmic face of the bilayer, while cardiolipin is exclusively found in mitochondrial membranes. This asymmetry is not absolute as almost every type of phospholipid is present on both sides of the membrane bilayer but in different amounts. The low rate of flip-flop movement of phospholipids aids in the maintenance of this asymmetry. The function of phospholipid asymmetry is largely unknown.

A glycolipid molecule has a hydrophobic tail consisting of a ceramide molecule, similar to that of sphingomyelin. The hydrophilic portion of the amphipathic glycolipid is composed of a number of simple sugar residues linked together to form an oligosaccharide. Glycolipids are found exclusively on the outer or extracellular face of the membrane bilayer.

Cholesterol, fatty acids, diacylglycerol, and triacylglycerol comprise the neutral lipids. Cholesterol is the major neutral lipid found in the membrane. It has a steroid ring structure and like other neutral lipids is found in either monolayer of the bilayer.

Cholesterol will orient itself with its polar head group aligned with the polar head groups of the phospholipids.

Membrane proteins are also distributed asymmetrically in the phospholipid bilayer. Membrane proteins can span either both or one monolayer of the membrane. Protein asymmetry is maintained by the low rate at which the protein can change its orientation. For a large membrane protein to invert its orientation, many polar and charged groups would have to be forced through the hydrophobic core of the lipid bilayer (7,12). Membrane proteins can be classified as being either peripheral or integral proteins. The distinction between the two classes of proteins is that integral proteins are much more difficult to dissociate from the membrane than are peripheral proteins. Often the solubilization of integral proteins requires the use of detergents, acids, urea, or other chaotropic agents. Peripheral membrane proteins are more easily solubilized from the membrane with salt or the addition of a cation chelator (1,2,7). The inference from this empirical classification of membrane proteins is that peripheral membrane proteins are held to the membrane by rather weak, noncovalent, electrostatic interactions and are not tightly associated with the membrane lipid. In contrast, integral membrane proteins are quite the opposite as they are held to the membrane very tightly. The majority of proteins in most plasma membranes are integral membrane proteins (7).

The final component of the membrane is the carbohydrate. Carbohydrates are found as oligosaccharides on membrane glycolipids and glycoproteins. As mentioned above, membrane glycolipids are consistently found in the outer leaflet of the membrane bilayer. Glycolipids contain sugar residues linked to sphingosine. Glycoproteins contain sugar residues which are attached to the protein either through

an N-linkage to the nitrogen of asparagine or the sugar residues are O-linked through the amino acid serine. All glycoproteins are located in the outer leaflet of the membrane bilayer. The function of carbohydrates on the cell surface is not clearly defined, but, there is evidence that the carbohydrates play a role in cell-cell recognition (1,2).

1.1.3. The Function of the Biological Membrane

Some of the functions of the biological membrane have already been mentioned above. A great many of the functions of the cell are maintained by the enzymes and proteins of the cell membrane. But, the cell membrane itself also has several functions. The cell membrane defines the geographical limits of the cell. Other membranous compartments within the cell define the limits of particular cellular organelles.

The membrane bilayer plays a role in defining the environment within the cell. The membrane prevents some substances from entering the cell and others from leaking out. It regulates the traffic of materials moving between the interior and the exterior of the cell. All substances moving from the extracellular space to the interior of the cell must cross the cell membrane.

The cell membrane serves as a reservoir for a number of biologically active molecules which are derived from phospholipids. For example, when an appropriate ligand (eg. a hormone or growth factor) binds to a cell surface receptor, phosphatidylinositol 4,5-bisphosphate is hydrolyzed to diacylglycerol and inositol 1,4,5-trisphosphate (13). The inositol trisphosphate is involved in activation of intracellular Ca^{2+} release and diacylglycerol activates protein kinase C (14). Particular fatty acids

in the phospholipid are precursors of biologically active compounds called eicosanoids (eg. prostaglandins, leukotrienes, etc. 15,16,17). Degradation of phospholipids by enzymes will release these fatty acids which are metabolized to eicosanoids. More recently, it has been determined that sphingolipids and their breakdown products may inhibit protein kinase C (18).

1.2. Degradation of Membrane Lipids

Much of the initial interest in the study of degradation of membrane phospholipids was the discovery in the 1950's that membrane phospholipids were synthesized from precursors and could be broken down post-synthesis into their constituent parts within the cell (21,22,23,24,25). The existence of enzymes that degrade phospholipids was known of as early as 1932 when Belfanti and Arnaudi found that pancreatic juice could release fatty acids from phosphatidylcholine (26). Currently, the impetus for studying the degradation of phospholipids is the role of phospholipid degradation in the production of lipidic cellular second messengers such as diacylglycerol, platelet activating factor and arachidonic acid.

1.2.1. Pathways for the Degradation of Membrane Lipids

There are several metabolic routes by which membrane lipids are degraded. In general, the catabolism of lipids is via phospholipases and lipases. Several reviews have been published on this subject (19,20,21,22,23). All four ester bonds in a phospholipid are susceptible to enzymatic hydrolysis. Phospholipases are defined by the specific ester bond that they attack (Figure 2). Phospholipases A_1 hydrolyze the

Figure 2

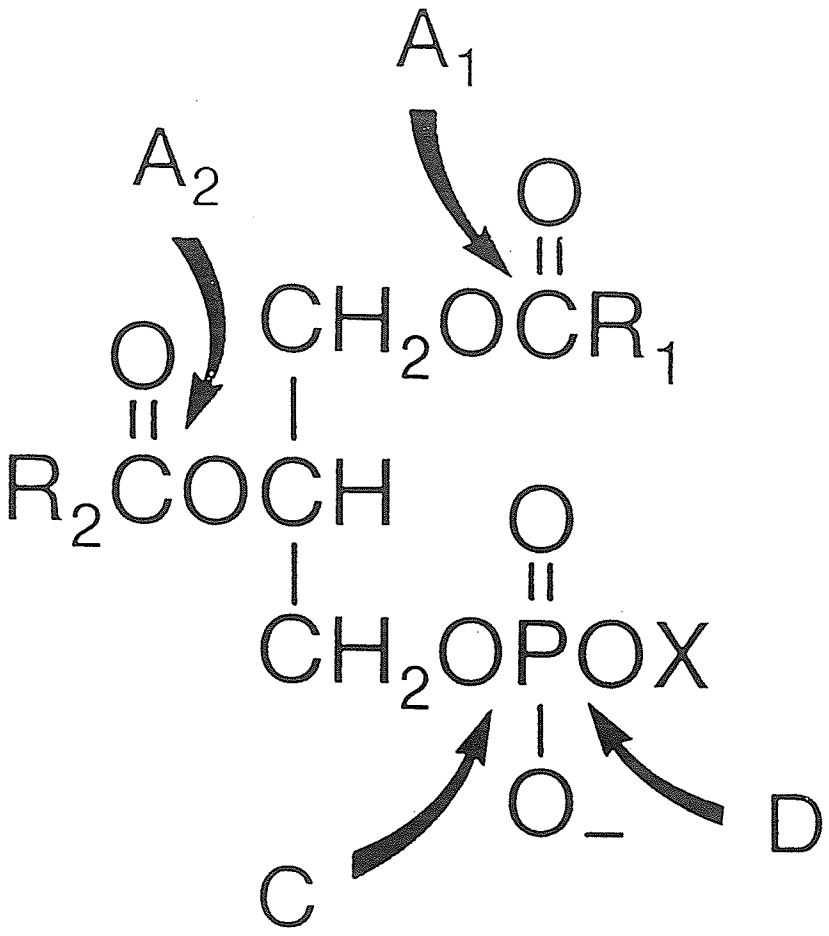

Fig. 2. Positional specificity of phospholipase attack on phospholipids. R_1COO and R_2COO refer to the fatty acids on the sn-1 and sn-2 positions, respectively, of the stereospecifically number phospholipid and X refers to the polar head group. Adapted from ref. (19,21).

sn-1 fatty acyl ester bond, while phospholipases A_2 hydrolyze the sn-2 ester bond. Phospholipases C cleave the phosphodiester bond proximal to the glycerol backbone and produce a diacylglycerol and a free phosphobase. The enzyme which degrades sphingomyelin called sphingomyelinase carries out an analogous reaction with sphingomyelin that the phospholipase C does with phospholipids. Those phospholipid hydrolyzing enzymes that attack the phosphodiester linkage on the side distal to the glycerol backbone are called phospholipases D.

Phospholipase A hydrolysis produces lysophospholipids[1] (either 1-acyl or 2-acyl lysophospholipids) and fatty acids. Lysophospholipids may be further degraded by lysophospholipases A_1 or A_2. The diglyceride product of phospholipase C action can also be further degraded to its constituent fatty acids by diglyceride and monoglyceride lipases. Phosphatidic acid the product of phospholipase D hydrolysis can be further degraded, in principle by either phospholipase A_1 or phospholipase A_2 activities. More likely, the phosphatidic acid will be converted to a diacylglycerol by the enzyme phosphatidate phosphohydrolase and then deacylated by the mono- and diglyceride lipases.

Defining whether a protein extract has a particular phospholipase activity is very difficult for a number of reasons. One reason is very obvious from the preceding paragraph, many of the products of phospholipase hydrolysis can arise via the action of one or a combination of phospholipase or lipase activities. It is not unusual to find that a phospholipase activity has other associated phospholipid metabolising enzyme

[1] The nomenclature for lysophospholipids in this thesis is to designate the fatty acid that is present. Hence, 1-acyl GPE refers to a lysophospholipid that can also be defined as 1-acyl-2-lyso GPE as the sn-2 fatty acid is missing.

activities. Many examples of such enzymes exist (section 1.3.). Phospholipases have been purified that can also catalyze transacylation reactions and transesterification reactions. Altogether, it seems that for enzymes that carry out a simple hydrolytic reaction, the classification of the phospholipases is difficult.

1.3. The Phospholipases

1.3.1. Phospholipase A_1

Phospholipase A_1 is defined as a hydrolytic activity that is capable of removing the sn-1 fatty acid from a phospholipid. Phospholipase A_1 activities have been found in both protein extracts from prokaryotes (27,28) and eukaryotes (29,30,31) in lysosomes, membranes, mitochondria, and cytosol subcellular fractions (32).

Scandella and Kornberg (27) were the first to purify a phospholipase A_1 activity from E. coli membranes. The enzyme was purified 5000-fold after solubilization from the membrane with sodium dodecyl sulfate. It had a molecular weight of 29 kDa and was active with an optimal pH of 8.4. As is the case with many purified phospholipases A_1, the E. coli enzyme did not have a precisely defined substrate. In the presence of 0.05% Triton X-100, the enzyme hydrolyzed 1-acyl glycerophosphocholine at a rate that was two-fold faster than observed with diacyl phosphatidylethanolamine under the same assay conditions. However, the enzyme did not hydrolyze 2-acyl lysophospholipids or triolein. The purified enzyme was defined by the products that were formed. On the basis of its substrate specificity, the E. coli phospholipase A_1 was described as a phospholipase A_1 with lysophospholipase activity.

It is interesting to note that detergent had an effect on the substrate specificity

of the E. coli phospholipase A_1 (27). This effect was also observed when the bovine pancreatic phospholipase A_1 was assayed in the presence of detergent (33). A phospholipase A_1 activity from bovine pancreas was purified to homogeneity using the dual radiolabelled substrate 1-[^{3}H]-palmitoyl-2-[^{14}C]-linoleoyl glycerophosphoethanolamine as the substrate for the enzyme assay (33). Using this substrate, it was possible to characterize unambiguously the lipase activity that hydrolyzed the substrate. The phospholipase A_1 was shown to be stimulated by the inclusion of 0.5 mM EDTA and 1 mg/ml sodium deoxycholate in the enzyme assay. The phospholipase A_1 activity was found to have an optimal pH of 7.5. The enzyme was purified 154-fold following chromatography on CM-cellulose, gel filtration, DEAE ion exchange, and hydroxylapatite resins. Surprisingly, in all fractions the phospholipase A_1 activity was also associated with a lysophospholipase activity. However, electrophoresis revealed one band of protein in the final pooled column fractions. The lysophospholipase activity of the pancreatic enzyme was completely inhibited by the presence of sodium deoxycholate. Hence, in the presence of the detergent, the enzyme only exhibits one lipolytic activity. Under assay conditions with intermediate amounts of sodium deoxycholate, complete deacylation of the phospholipid could be achieved. This 60 kDa phospholipase exhibits a high lysophospholipase activity with 1-acyl glycerophosphocholine and some activity with 2-acyl glycerophosphocholine.

Another pancreatic phospholipase A_1 activity was purified in 1981 by Fauvel et al. (34,35,36) from guinea-pig pancreas. The authors themselves have characterised this enzyme as being a cationic lipase displaying high phospholipase A_1 activity. Two lipases (Ia and Ib) were resolved after the last gel filtration step with a molecular mass

of 37 and 42 kDa respectively. The Ia and Ib lipases have indistinguishable isoelectric points of 9.3-9.4. The two lipases also cannot be distinguished on the basis of their substrate specificities. The enzymes hydrolyze dioleoylglycerol > monoleoylglycerol > trioleoylglycerol > phosphatidylcholine > 1-acyl glycerophosphocholine > phosphatidylethanolamine. The guinea-pig pancreatic enzyme differed from its bovine counterpart in having a high lipase activity with triolein as the substrate and the guinea-pig enzyme was insensitive to diisopropyl flurophosphate unlike the bovine enzyme. These pancreatic phospholipases A_1 described above probably function in the digestion of foods and are extracellular in their localization.

Intracellular phospholipases A_1 have also been described in heart (29,37,38), liver (30,32) and brain (39). Phospholipases A_1 in heart have been characterized by a number of groups. However, to date, only a partial purification of a rat heart sarcoplasmic reticulum phospholipase A_1 has been achieved (40). Hence, the study of phospholipases A_1 of the heart has been limited to defining the optimal conditions for assay of the enzymes from the various subcellular fractions (29,37,38). The partial purification of phospholipase A_1 from rat heart sarcoplasmic reticulum was achieved by the solubilization of the heart membranes by sodium taurodeoxycholate or octyl glucoside or with potassium chloride (40). The solubilised enzyme was purified 4-5 fold after HPLC gel filtration chromatography. Two peaks of activity were found when the column fractions were assayed with di[^{14}C]-oleoyl phosphatidylcholine. Peak I had an apparent molecular mass of 60 kDa while peak II eluted in the void volume of the column indicating a large molecular mass. There was some lysophospholipase activity in the column fractions with phospholipase A_1 activity. The authors did not state with what substrate the lysophospholipase activity was assayed. Each peak of

phospholipase A_1 activity also had an associated phospholipase C activity. The addition of 6 mM $CaCl_2$ did not cause any significant change in the peak I or peak II phospholipase A_1 activities. EGTA was inhibitory to the peak II enzyme activity up to a concentration of 3 mM EGTA after which there was no change in the activity with increasing concentration of the chelator. The addition of micromolar amounts of Ca^{2+} to a solution of 3 mM-EGTA pretreated solubilized peak II enzyme resulted in an increase and eventual restoration of the phospholipase A_1 activity up to the level of activity obtained with addition of 3 mM Ca^{2+}. The two peaks of phospholipase A_1 activity could be further distinguished on the basis of their differential sensitivity to heating and Hg^{2+} ions. The authors suggested that this peripheral membrane phospholipase A_1 activity might be the same phospholipase A_1 they had previously characterized as being cytosolic (37).

Phospholipases A_1 have been described and partially purified from rat liver cytosol and lysosomes (41,42,43), however, the best studied of all the liver phospholipases A_1 is that activity from liver plasma membranes that is heparin releasable (30,32,44,45,46,47). It has a molecular mass of 60-70 kDa but can form oligomers as it has also been purified with a molecular mass of 180 kDa (30). It has a broad substrate specificity and can be easily dissociated from rat liver plasma membranes by perfusion with heparin. The enzyme hydrolyzes triacylglycerol, diacylglycerol, monoacylglycerol, and phospholipids. It carries out transesterification and transacylation reactions in addition to the hydrolytic reaction. Due to its broad substrate specificity, the enzyme has a number of names. It has been called monoacylglycerol acyltransferase, hepatic triglyceride lipase, and phospholipase A_1 (47). It has been suggested that <u>in vivo</u> this enzyme acts as a phospholipase A_1 and is

implicated in lipoprotein metabolism. There is evidence that in vivo inactivation of this enzyme by specific antibodies induces a change in the subclasses of high density lipoproteins (48,49). The substrate specificity of this phospholipase A_1 was studied using mixed micelles of phospholipid and Triton X-100 (50). The phospholipase A_1 preferred phosphatidylethanolamine over phosphatidylcholine. When present together, phosphatidylcholine inhibited the hydrolysis of phosphatidylethanolamine, but phosphatidylethanolamine had no effect on the hydrolysis of phosphatidylcholine. The ether lipids choline plasmalogen and ethanolamine plasmalogen had no effect on the hydrolysis of phosphatidylethanolamine (32). It is curious that the enzyme prefers phosphatidylethanolamine as a substrate since lipoproteins (especially HDL) contain a larger proportion of phosphatidylcholine than phosphatidylethanolamine.

1.3.2. Phospholipase A_2

The existence of phospholipases A_2 (PLA_2) was shown more than a century ago. The interest in this enzyme revolves around its role in the release of unsaturated fatty acids especially arachidonic acid and thereby initiating the biosynthesis of the eicosanoids and platelet-activating factor (14,15,16,51). Phospholipases A_2 hydrolyze the sn-2 fatty acid of phospholipids. Phospholipases A_2 can be divided into two groups: the extracellular phospholipases and the intracellular phospholipases A_2.

1.3.2.1. Extracellular phospholipases A_2

Initial studies on phospholipases A_2 were performed with snake and bee venoms which are a rich source of very active phospholipases A_2 (19,20). The enzymes from venoms are the most completely studied of all the phospholipases. Some of the enzymes have been crystallized and the x-ray diffraction patterns have

been used to construct models of the structures of the enzymes. As well the catalytic mechanism has also been deduced for the phospholipases A_2. Studies have revealed that all snake and bee venom phospholipases A_2 have some common features (56). With few exceptions, it was found that all such phospholipases A_2 contained as many as 5-7 disulfide bonds. The molecular masses of the snakes and bee venom enzymes were remarkably similar, approximately 14 kDa. Other features that appear to be conserved in structure include a Ca^{2+}-binding loop involving the amino acids Tyr-28, Gly-30, and Asp-49, and an α-helical region at the N-terminus. These enzymes require Ca^{2+} and are optimally active in the acid or alkaline pH ranges depending upon the source of the enzyme. Henrikson et al. (52) were the first to classify the primary structures of the venom phospholipases A_2 into two major groups: Group I and Group II. The Group I phospholipases consist of venoms of the snakes of the species Elapidae, Hydrophidae, and other Old World snakes, while the Group II phospholipases A_2 are found in Crotalidae and Viperidae variety of snakes. A key difference between the structures of the two groups of enzymes is that the Group I enzymes have a disulfide bond between Cys-11 and Cys-77 whereas, the Group II enzymes are missing this bond but have instead a disulfide link at Cys-50 and Cys-132 and also contain an additional seven amino acid residues at the carboxyl-terminus (52,53). The two classes of enzymes tend to have different pharmacological properties (53). Snake venoms usually contain one or the other type depending upon their phylogenetic origin. Recent evidence indicates that lizards of the species Heloderma contain a PLA_2 enzyme that is different from the Group I and Group II enzymes described above and represent a third group. The Group III enzymes have the following characteristics: they are Ca^{2+} dependent, have an alkaline pH optimum

and are glycosylated (53). The venom from bees has a phospholipase A_2 that falls into the Group III as well.

Mammals have retained both the Group I and Group II enzymes. The human pancreatic extracellular phospholipase A_2 is a Group I enzyme with a molecular mass of 13,800 daltons. It consists of a single polypeptide chain of 123 amino acids linked by six disulfide bonds (54,55). The Group II phospholipases A_2 are found in many tissues such as liver (56), spleen, lung (56), in a number of cell types such as platelets (57,58), vascular smooth muscle cells (59), synovial fluid (58), human seminal plasma (56) and other non-pancreatic sources (56). All these enzymes are extracellular and may be secreted in response to an appropriate stimulus.

Although all these enzymes are from different sources, some general characteristics were observed. First, the catalytic mechanism of all these enzymes is identical, as well as the geometry of the active site residues is similar. However, they have different substrate specificities. The generally accepted mechanism for the action of this enzyme is that a His-48 assisted by Asp-99 extracts a proton from a water molecule, the resulting hydroxide molecule acts as a general base to attack the carbonyl carbon at the sn-2 position of the phospholipid (60,61). The carbonyl may be co-ordinated to the catalytic Ca^{2+}. The Ca^{2+} is also held in place by Asp-49 and is co-ordinated to a phosphate group on the phospholipid. Tyr-69 determines the stereospecificity of the enzyme through its interaction with the phosphate group of the substrate. This mechanism is similar to that of the serine esterase chymotrypsin. Such a reaction mechanism is known as a proton relay system (62).

It seems reasonable that in the reducing environment inside the cell that the intracellular phospholipase A_2 should be quite different from its extracellular

counterpart. The constraints of seven disulfide bonds in a protein molecule will maintain a pressure for the molecule to remain quite similar to its original ancestor. This is exemplified quite well by the extracellular phospholipases A_2. From reptiles to mammals, the three dimensional structure of the enzyme has been well conserved over all these years of evolution. Without the constraints of the disulfide bonds, the intracellular phospholipase A_2 will probably differ from the Group I, II, and III enzymes.

1.3.2.2. Plasmalogen selective phospholipase A_2

Plasmalogens are found in every mammalian tissue, but they are especially enriched in tissues such as the brain and the heart (63,64,65). The plasmalogen content of the liver is low (66,67). One postulated function of plasmalogens is to serve as a reservoir of arachidonic acid. This may be very true considering that studies have shown that a large proportion of polyunsaturated fatty acids (in particular arachidonic acid) are esterified at the sn-2 position of plasmalogens (64,65). Plasmalogens may also be important in ion transport across membranes (65). The presence of these molecules in relatively large quantities in the heart and their presumed functions suggests that these compounds may be very important in the normal physiological and pathophysiological functioning of the heart. Further evidence for such a hypothesis is in part found in the "amphiphile hypothesis" (68) and the significance of myocardial lysophospholipid and eicosanoid generation in the heart in situations of ischemia and arrythmia (69,70,71).

In 1985, Gross and Wolf identified a phospholipase A_2 activity in canine myocardial subcellular fractions (72). This phospholipase A_2 activity was a Ca^{2+}-independent phospholipase A_2. This characteristic was dramatically different from the small molecular weight, extracellular enzymes described in section 1.3.2.1. The

extracellular phospholipases A_2 required millimolar concentrations of Ca^{2+} for activity (56). In fact, this myocardial phospholipase A_2 activity was actually inhibited by the addition of 1 mM $CaCl_2$ relative to a control with EGTA added or no additive (72). Activity was detected when the enzyme was assayed with radiolabelled phosphatidylcholine or plasmenylcholine. The activity was localized to both the microsomal and cytosolic compartments of the canine myocyte. The activity with plasmenylcholine was highest in the cytosol, while the phospholipase A_2 activity with phosphatidylcholine was highest in the microsomal fraction. Activity was found with both substrates in both the cytosolic and microsomal fractions. The cytosolic Ca^{2+}-independent myocardial phospholipase A_2 was purified to homogeneity in 1990 by Hazen et al. (73). The enzyme was purified 154,000 fold and had a molecular mass of 40 kDa which differs significantly from the extracellular phospholipases A_2 described in section 1.3.2.1. All the column fractions during the purification were assayed with radiolabelled 1-O-alkenyl-2-[^{3}H]-oleoyl glycerophosphocholine. The purification scheme involved ion-exchange, chromatofocusing, ATP-agarose, and HPLC on hydroxylapatite. The phospholipase A_2 was bound specifically to an ATP-agarose column and was eluted by ATP but not any other adenosine nucleotide. The substrate specificity of the enzyme revealed that it had highest activity with plasmenylcholine versus other choline glycerophospholipids. The enzyme also showed some specificity for a particular molecular species of plasmenylcholine. It displayed 150% more activity with 1-palmitoyl-2-arachidonoyl plasmenylcholine versus 1-palmitoyl-2-oleoyl plasmenylcholine. The enzyme had the following preference for the particular substrates assayed: 1-palmitoyl-2-arachidonoyl plasmenylcholine > 1-O-palmitoyl-2-arachidonoyl GPC > 1-palmitoyl-2-oleoyl plasmenylcholine > 1-palmitoyl-2-arachidonoyl GPC > 1-palmitoyl-

2-oleoyl GPC. This selectivity for plasmalogen substrate was exhibited in mixed micelles of both plasmenylcholine and phosphatidylcholine. The myocardial cytosolic phospholipase A_2 is specific for the hydrolysis of the sn-2 ester linkage in choline and ethanolamine diradylglycerophospholipids. The enzyme did demonstrate intrinsic lysophospholipase activity with 1-palmitoyl lysophosphatidylcholine and some palmitoyl CoA hydrolase activity. In the presence of mixed micelles of LPC and plasmenylcholine, there was no detectable release of fatty acid from LPC. Many properties of this plasmalogen-selective phospholipase A_2 distinguish it from the extracellular phospholipases A_2 previously described. Plasmalogen hydrolyzing phospholipase A_2 has been detected in a number of other tissues besides the heart. Intracellular phospholipase A_2 activities with plasmalogen substrates have been detected in bovine, human, and rat brain (74,75), PC12 pheochromocytoma, neuroblastoma, and glioblastoma cells (75), in sheep platelets (76), and vascular smooth muscle cells (77).

Initially, Gross and Wolf (72) described this phospholipase A_2 activity in both the cytosolic and microsomal subcellular fractions of canine myocardium. Further work by Hazen, Ford, and Gross (78) determined that the membrane-associated phospholipase A_2 from rabbit myocardium was activated by a period of ischemia. Using isolated-perfused rabbit hearts as their model system, it was found that under ischemic conditions, the activity of a microsomal phospholipase A_2 with plasmalogen substrate was increased relative to a non-ischemic control. The concentration of Ca^{2+} in the model system did not change the degree of activation of the rabbit myocardial microsomal phospholipase A_2 activity with plasmenylcholine as a substrate. A similar activation under ischemic conditions was not observed when diacyl

phosphatidylcholine was used as a substrate. Moreover, the addition of actinomycin D and cycloheximide did not alter the profile of activation suggesting that this enzyme is present at all times in the rabbit heart and is not newly synthesized under conditions of ischemia. Subcellular fractionation of the ischemic rabbit heart indicated that only the microsomal phospholipase A_2 activity and not any other plasmalogen selective phospholipase A_2 activity (eg. the cytosolic activity discussed above) was activated in this manner. In addition, an analysis of the molecular species of plasmenylcholine that was hydrolyzed by the ischemia-activated rabbit phospholipase A_2 revealed that it, like the canine myocardial cytosolic phospholipase A_2 (73), preferred to hydrolyze 1-O-palmitoyl-2-arachidonoyl plasmenylcholine versus 1-O-palmitoyl-2-oleoyl plasmenylcholine. However, the level of activation by ischemia of the hydrolysis of 1-O-palmitoyl-2-arachidonoyl plasmenylcholine was significantly greater than the level of activation of 1-O-palmitoyl-2-oleoyl plasmenylcholine. The difference in the ratio of the hydrolysis of the two molecular species of plasmenylcholine was dramatically different from the ratio of the specific activities for the hydrolysis of the two molecular species of plasmenylcholine in heart microsomes that were not prepared from ischemic hearts. It is possible to infer from this data that multiple microsomal plasmalogen selective phospholipases A_2 may exist in rabbit myocardium that are differentially activated under conditions of ischemic stress. There is evidence for multiple plasmalogen selective phospholipases A_2 in vascular smooth muscle (77). Unfortunately, more physical evidence on which to base this hypothesis is lacking as the membrane-bound form of the plasmalogen selective phospholipase A_2 has not been purified. The regulation of the plasmalogen selective phospholipases A_2, in particular by nucleotides and protein-protein interactions, will be further discussed in

section 1.5.

1.3.2.3. High molecular weight phospholipase A_2

Recently, attention in the field of phospholipases A_2 has focused on an enzyme which has properties that indicate that it may be responsible for the deacylation of phospholipids to release arachidonic acid in vivo. In 1988, Leslie et al. stirred up new interest in the field of phospholipases A_2 when they characterized and purified a phospholipase A_2 from the macrophage cell line RAW 264.7 (79). The enzyme was initially characterized with plasmalogen substrate 1-O-hexadecyl-2-[^{3}H]-arachidonoyl GPC. In determining the subcellular localization of the enzyme, they found that hydrolysis of the substrate releasing labelled arachidonate was found to only occur in the cytosol and that there was essentially no detectable phospholipid hydrolyzing activity in the membrane fraction under similar assay conditions. The cytosolic phospholipase A_2 activity hydrolyzed the plasmalogen substrate with an optimal pH of 8.0, but exhibited a rather broad pH optimum. The enzyme exhibited an absolute requirement for Ca^{2+} as there was no activity when it was assayed in the presence of EGTA. Concentrations of Ca^{2+} as low as 0.4 μM resulted in detectable phospholipase A_2 activity. The greatest increase in activity of the enzyme was found when it was assayed at millimolar concentrations of Ca^{2+}. When the substrate specificity of the phospholipase A_2 was examined, the enzyme preferred to hydrolyze 1-acyl-2-arachidonoyl GPC versus 1-O-hexadecyl-2-arachidonoyl GPC (79). Furthermore, the activity with the arachidonoyl containing substrates was 4-5 fold greater than those observed with linoleoyl containing substrate and 8 times greater than substrates with oleic acid. The enzyme displayed higher activity with 1-palmitoyl-2-arachidonoyl GPC than it did with 1-acyl-2-arachidonoyl GPE or with 1-acyl-2-arachidonoyl GPI. The

enzyme was subsequently purified 1000-fold from the cytosol of RAW 264.7 cells (79). It had a molecular mass of 70 kDa by SDS-PAGE under non-reducing conditions and 60 kDa under reducing conditions. The purified enzyme had characteristics identical to the crude cytosolic enzyme. The substrate specificity of the pure enzyme showed that it had maintained its preference for arachidonoyl containing substrates with a requirement for Ca^{2+}. The purified enzyme displayed a greater activity with 1-acyl-2-arachidonoyl GPE than with 1-acyl-2-arachidonoyl GPC. The pure enzyme was inhibited by all the detergents tested. Interestingly, this cytosolic phospholipase A_2 was inhibited 50% by bromophenylacylbromide. Bromophenylacylbromide is an alkylating agent that is known to alkylate the His-48 in the active site of the snake venom and other secretory or extracellular phospholipases A_2 thus completely inhibiting the activity. The fact that this alkylating agent does not completely inhibit the cytosolic PLA_2 ($cPLA_2$) implied that the composition of the amino acid residues at the active site of this intracellular phospholipase A_2 was different from that of the extracellular phospholipases A_2 discussed in section 1.3.2.1.

Subsequent to this study by Leslie et al. (79) several other investigators purified a similar enzyme from the U937 monocyte cell line (80,81,82). The purification of a 56 kDa enzyme with properties similar to that of the 70 kDa enzyme was reported by Diez and Mong (80). The authors claimed that the enzyme was purified to homogeneity after only a 2000-fold purification. Again the U937 cell enzyme was 56 kDa, Ca^{2+}-dependent and had a pH optimum of 8.0. The enzyme was purified by assaying all column fractions with 1-O-hexadecyl-2-[^{3}H]arachidonoyl GPC. The U937 cell enzyme was distributed differentially in the particulate and soluble fractions depending upon the amount of Ca^{2+} in the buffer mixture that was used to

homogenize the cells. In fact, Channon and Leslie have demonstrated that the RAW 264.7 cell phospholipase A_2 also exhibits a Ca^{2+}-dependent association with the membrane (83). The RAW 264.7 cell enzyme was found in the soluble fraction (100,000 x g) of the cell homogenate when the initial homogenization was performed in buffers containing cation chelators. Sixty to seventy percent of the activity was found to be associated with the membrane fraction when the cells were homogenized in a buffer containing 230 - 450 nM Ca^{2+}. This concentration of Ca^{2+} is comparable to the intracellular Ca^{2+} concentration in stimulated cells. Although the exact mechanism of translocation is not known, the enzyme has been shown to translocate to the membrane in vivo (84).

In the past few years, several other groups have purified this same phospholipase A_2 from U937 cells (81,82) by as much as 280,000-fold. These studies have shown that the cytosolic phospholipase A_2 is not a 56 - 70 kDa enzyme, rather it is a 100 - 110 kDa enzyme. The 60 kDa protein is a contaminating protein which can form dimers that tail into the active fraction in a size-exclusion chromatographic step. Hence, the purification procedures of Leslie (79) and Diez and Mong (80) resulted in an incorrect determination of the true size of the enzyme. However, all of the studies on the phospholipase A_2 corroborate the substrate specificity, Ca^{2+}-dependency and membrane translocation of the enzyme found previously (79,80). Moreover, in assays with mixed micelles the U937 $cPLA_2$ hydrolyzes 1-palmitoyl-2-arachidonoyl GPC at a rate that is at least 3-fold greater than any other molecular species of 1-palmitoyl-2-acyl phosphatidylcholine examined (80).

The cDNA of the $cPLA_2$ from U937 cells has been isolated, cloned, and expressed in cells by two groups independently (84,85). The sequence of the cDNA

predicts a protein of 85.2 kDa which is smaller than the 100 kDa protein purified by many investigators. The sequence of the cDNA has absolutely no identity to that of any other known phospholipase A_2 including the low molecular weight phospholipases A_2 from venoms. The protein had a calculated isoelectric point of 5.1 and was 749 amino acids in length. When the cDNA of the $cPLA_2$ was expressed in CHO cells, the recombinant cells had greatly enhanced PLA_2 activity relative to appropriate controls. Interestingly, the cDNA of the $cPLA_2$ did show some homology in a region with a Ca^{2+}-lipid binding domain that is also found in protein kinase C, and other proteins that are known to translocate to the cell membrane upon Ca^{2+} and phospholipid stimulation. Expression of the portion of the sequence for the Ca^{2+}-lipid binding domain indicated that it is responsible for the translocation of the enzyme in response to Ca^{2+}. The expressed protein was found to translocate to the membrane at concentrations of Ca^{2+} as low as 10 nM. The cDNA sequence also predicts several sites at which the protein can be phosphorylated by Ser/Thr kinases. The $cPLA_2$ has been shown to be phosphorylated in vitro by protein kinase C (86) and by the mitogen-activated protein kinase (MAP kinase,87). Finally and most importantly, the expressed protein hydrolyzed a mixture of endogenous U937 membranes such that selective release of arachidonate was noted. In U937 cell membranes, 20% of the fatty acids at the sn-2 position contain arachidonate, the recombinant enzyme hydrolyzed arachidonate containing phospholipids at a rate that was 4 times higher than the rate of hydrolysis of oleate containing phospholipids which are present in 3 times greater quantity. It is quite clear that the $cPLA_2$ might very well be the enzyme that is responsible for intracellular arachidonate release. Much is also known about the regulation of the $cPLA_2$ by Ca^{2+}, protein phosphorylation, and guanine-nucleotide

binding proteins. This is further discussed in section 1.5.

The $cPLA_2$ is a phospholipase A_2. However, it has also been found to exhibit some lysophospholipase A_1 and transacylase activities (88,89). When the $cPLA_2$ was assayed during purification by Leslie (88), the column fractions with phospholipase A_2 activity also exhibited activity that was able to hydrolyze 1-palmitoyl lysophosphatidylcholine. Moreover, an antibody to the $cPLA_2$ also cross reacts with fractions that contain the lysophospholipase A_1 activity. This suggested that a single protein had two distinct activities. The lysophospholipase A_1 activity did not require Ca^{2+}. The specific activity of the lysophospholipase A_1 was greater than the phospholipase A_2 activity. The effect of different pH on phospholipase A_2 activity was mirrored in the lysophospholipase A_1 activity. The substrate specificity of the lysophospholipase A_1 activity was not examined. Also, it is not known in great detail what regulates the lysophospholipase A_1 activity. However, when the enzyme is presented with a dual labelled substrate, there is release of products indicative of phospholipase A_2 activity at a rate that is 10 times greater than the lysophospholipase activity. The lysophospholipase activity is stimulated by Ca^{2+} if the activity is assayed in the presence of glycerol or Triton X-100 (89). The identification of the $cPLA_2$ as a transacylase (catalyzes the production of 1 mol of phosphatidylcholine from 2 mol of lysophosphatidylcholine) suggests that the $cPLA_2$ forms an acyl-enzyme intermediate in its catalysis of the hydrolytic reaction. This further implies that the $cPLA_2$ does not catalyze the hydrolysis of fatty acid through a proton relay system as described for the extracellular phospholipase A_2. Another enzyme with phospholipase A_2 activity has been identified, purified and cloned from sheep platelets that has an acyl-enzyme intermediate in its catalytic mechanism. This is a 30 kDa protein (90). It is not

inconceivable that there may be a family of high molecular weight phospholipases.

1.3.3. Phospholipase C

1.3.3.1. Phosphatidylinositol-specific phospholipase C

The phosphoinositides are a group of membrane phospholipids which are unique in that the headgroup, myo-inositol can be phosphorylated. The three predominant members of the phosphoinositides in mammalian tissues are phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2). The numbers refer to the position of the phosphates on the inositol ring. Altogether, these three lipids usually constitute less than 8% of total membrane phospholipids (2).

Hokin and Hokin in the 1950's spurred the initial interest in the study of the breakdown of phosphatidylinositol when they discovered an unusual phenomenon later termed the "PI response" (91,92,93). The Hokins' found that an increase in lipid radiolabelling, which accompanies secretion, in agonist stimulated tissue is confined to the lipids PI and phosphatidic acid (PA). The "PI response" is explained if the phosphoinositide is cleaved by a phospholipase (phospholipase C) producing diacylglycerol and an inositol phosphate. These results indicate that there is a degradation and renewal of the phosphobase of the PI molecule.

Attention therefore focused on the isolation of a phospholipase C which was active against phosphatidylinositol. The phospholipase C which catalyzes the hydrolysis of PI is present in most mammalian cells, plant cells, and micro-organisms (94,95,96). One of the first purified PI-specific phospholipase C reported from animal cells was a 70 kDa enzyme purified 4300-fold from rat liver cytosol (97). There is evidence for the presence of multiple isoenzymes of PI-specific phospholipase C (PI-

PLC) in various tissues. Multiple and distinct enzymes have been purified and characterized in sheep seminal vesicles (98), bovine platelets (99), and bovine brain (100,101,102).

The membrane localization of phospholipase C has always been a source of controversy. All the PI-specific PLCs that have been described are from the cytosolic fraction of cells or tissues. If the phospholipase C were to play a role in the production of the cellular second messengers inositol trisphosphate and diacylglycerol, the enzyme had to be located where phosphatidylinositol was located, that is in the plasma membrane. A counterpart to the bovine brain 150 kDa cytosolic enzyme was purified by two laboratories from the particulate fraction of bovine brain (103,104). Another approach that was used to try to determine the cellular localization of the phospholipase in living cells was to permeabilize the cell membrane to allow the cytosolic components to leak out. When streptolysin O, a bacterial cytolysin is added to cells, it was determined that the phospholipase C exits the cell much slower in comparison to enzymes which are truly cytosolic such as lactate dehydrogenase (105). This suggests that possibly the phospholipase C may be in a dynamic equilibrium between the membrane and the cytosol or that a mechanism exists whereby the cytosolic phospholipase C may become membrane associated. Such mechanisms have been postulated for a number of other enzymes such as protein kinase C, phosphatidate phosphohydrolase, and the cytosolic high molecular weight phospholipase A_2.

The bovine brain cytosolic enzymes (100,101,102) have become representative examples of the different classes of the PI-specific phospholipases C. cDNAs corresponding to the phospholipases C from bovine brain cytosol were initially isolated

and cloned (94,95,110,111). Since then several more isoenzymes have been purified and their amino acid sequences have been deduced from their cDNA sequences (93,94,95,106,107,108,109,110,111). From comparison of these predicted amino acid sequences and on the basis of results of experiments to determine the antigenic relatedness of these isoenzymes, it is suggested that all mammalian PI-specific phospholipases C (PLC) can be placed in either of three classes: PLC-ß, PLC-γ, and PLC-δ. Each type is a single polypeptide protein from a single specific gene. The PLC-ß class of enzymes have molecular masses of 150-154 kDa. PLC-γ enzymes have a molecular mass of 145-148 kDa and the PLC-δ have molecular masses of 85-88 kDa. In each class, there are subtypes, for example there are two enzymes, PLC-ß1 and PLC-ß2 within the PLC-ß class.

A total of 16 amino acid sequences (14 mammalian and 2 Drosophila PLC enzymes) and their corresponding cDNA sequences have been classified into one of the three categories (95). There is low sequence similarity between the classes but high sequence similarity between members of the same class. Significant similarity is demonstrated at two regions by all three classes of phospholipases C (94,110). The conservation of the sequences in these two regions implies that they may be domains which are important in the catalytic function of the phospholipase C. Unlike the PLC-ß and PLC-δ enzymes, PLC-γ can be thought of as consisting of three domains not two. PLC-γ has an additional domain termed the SH domain which is not present in PLC-ß or PLC-δ. The SH domain is similar in sequence to regions of members of the src-encoded family of tyrosine kinases (112,113). The SH-domain has a non-catalytic function in PLC-γ (114). As well, since only PLC-γ has the SH-domain, it is possible that the regulation of this class of phospholipase C is different from the other classes

of PI-PLC.

Finally, PI-specific phospholipase C activity has been purified that is distinct from any of the above discussed phospholipases C. These phospholipases C are the α-class of enzymes (93,111). They were first described in rat liver and guinea-pig uterus. These appeared to be immunologically distinct from the other three classes of PI-PLC isoenzymes. The amino acid sequence of PLC-α showed no identity to any other PLCs that had been isolated and cloned. The PLC-α cDNA was obtained from rat basophillic leukemic cell libraries using rabbit serum against the 62 kDa guinea-pig uterus PLC (115). The PLC-α clone displayed a considerable degree of homology to a thiol:protein disulfide oxidoreductase (115). Furthermore, since the PLC-α cDNA has not been expressed and shown to have intrinsic phospholipase C activity, the question of whether a PLC-α exists has been raised.

A great deal is now known about this very important class of phospholipase. Of all the phospholipases, our knowledge of the PI-specific phospholipases C is the most detailed at the molecular and cellular level and in terms of its regulation, function and structure.

1.3.3.2. Phosphatidylcholine-specific phospholipase C

The stimulation of hydrolysis of PIP_2 by PI-specific PLC is the initial response of many cells to various agonists, neurotransmitters and hormones. Hydrolysis of inositol phospholipids by phospholipase C results in the production of inositol trisphosphate and diacylglycerol. It was noticed by Bocckino et al. (116) that the production of diacylglycerol in response to some stimuli exceeds that attributable to PIP_2 hydrolysis. This production of diacylglycerol has been found in conjunction with an increase in the release of choline and phosphocholine (117,118 and the references

therein). Since the basal intracellular levels of choline and phosphocholine are high, 1.3 mM and 0.23 mM respectively in liver (119), it seemed likely that the production of choline and phosphocholine was not via a de novo pathway but was most likely produced by the breakdown of phosphatidylcholine. An analysis of the molecular species of diacylglycerol that were produced upon agonist stimulation confirmed that in stimulated mast cells, fibroblasts, and hepatocytes, the diacylglycerol that was produced was not from degradation of PI but from the breakdown of PC (120,121).

The production of choline and phosphocholine can occur through one of two mechanisms: either through phospholipase C hydrolysis of PC or phospholipase D hydrolysis of PC. Some products of phospholipase D or phospholipase C hydrolysis are interconvertible via the action of specific kinases and phosphatases. Phosphocholine can be produced via agonist stimulated breakdown of PC by phospholipase C. This phospholipase C that hydrolyzes PC would be expected to be different from the PI-specific phospholipases C described in section 1.3.3.1.

Cell free preparations from a number of rat tissues exhibit phospholipase C activities that metabolize phosphatidylcholine (72,122,123,124). Phospholipases C that hydrolyze PC have been purified from dog heart cytosol (72) and U937 monocytes (125). A neutral active phospholipase C was partially purified from canine myocardial cytosol by ion-exchange, chromatofocusing, and gel-filtration (72). The partially pure enzyme had a pI as determined by chromatofocusing of 7.4 and a molecular mass as determined by gel filtration chromatography of 29 kDa. The phospholipase C hydrolyzed substrates in the following order: diacylphosphatidylcholine > 1-alkenyl-2-acyl phosphatidylcholine (plasmenylcholine) > phosphatidylethanolamine. The enzyme displayed no activity towards sphingomyelin or phosphatidylinositol. The

myocardial phospholipase C had a pH optimum between 7-8. Its activity was inhibited by 70-80% in the presence of 2 mM EDTA or EGTA and completely inhibited by the detergents Triton X-100 and sodium deoxycholate. Nucleotides had no effect on the phospholipase C activity.

Another phosphatidylcholine-specific phospholipase C was identified and purified using a novel strategy from the human monocytic cell line U937 (125). It was hypothesized that phospholipase C hydrolysis of PC may have a role in the pathogenesis of rheumatoid arthritis. Therefore, an approach was taken to purify the U937 cell phospholipase C using the Bacillus cereus enzyme as a tool. The enzyme from B. cereus prefers PC as a substrate. The investigators made antibodies to the bacterial enzyme and immobilized the antibody on a column resin. The result was a 3000-fold one step purification procedure that bound a 40 kDa enzyme. The bacterial phospholipase C was only 23 kDa. In fact, this strategy worked so well, that the investigators were able to separate an activity that hydrolyzes 1-acyl-2-arachidonoyl phosphatidylcholine from an activity that hydrolyzes 1-acyl-2-arachidonoyl phosphatidylinositol. The enzyme is similar to the dog heart enzyme in a number of characteristics. The U937 cell phospholipase C had an optimal pH of 7-8 and was inhibited by EGTA.

Aside from these examples, there has been little work on the purification of PC-specific phospholipases C (PC-PLC). The majority of studies have characterized the mechanism of stimulation of the PC-PLC and its regulation. Such studies have involved labelling phospholipid precursors and then determining if there is any formation of radiolabelled products of phospholipase C hydrolysis such as [^{32}P]-phosphocholine or [^{3}H]-diacylglycerol or both. The production of diacylglycerol by PC

breakdown represents a second stage of diacylglycerol generation. This second phase is a sustained phase of diacylglycerol generation. Phosphatidylethanolamine is not considered to be a major contributor to this second phase of diacylglycerol generation. It has been shown that a sustained activation of diacylglycerol production occurs in response to mitogens, growth factors, phorbol esters, and transformation by oncogenic *Ras* (126,127,128,129). The function of such a sustained production of diacylglycerol and presumably a sustained activation of protein kinase C is thought to be important for the maintenance of signals necessary for long term responses such as cell proliferation, differentiation, and mitogenesis.

1.3.4. Phospholipase D

Many agonists cause rapid hydrolysis of phosphatidylcholine generating diradylglycerol (117,118). This generation of diradylglycerol may occur through either a direct or indirect mechanism. The direct mechanism involves the hydrolysis of phosphatidylcholine via a phosphatidylcholine-specific phospholipase C (discussed in section 1.3.3.2.). The indirect mechanism for the generation of diradylglycerol involves the hydrolysis of phosphatidylcholine initially by a phospholipase D to produce a molecule of phosphatidic acid (PA) and a molecule of free choline. The PA is further metabolised by a PA phosphatase to produce a diradylglycerol. This indirect pathway for the formation of various subclasses of diacylglycerol has been shown to operate in a number of cell types (130,131).

Phospholipases D hydrolyze phospholipids at the terminal phosphodiester bond generating PA and a polar head group. Phospholipases D also have a transphosphatidylation activity that removes the head group and transfers the phosphatidyl moiety to certain nucleophiles such as ethanol, ethanolamine, glycerol,

propanol, or other primary alcohols (132). This unique reaction has been exploited to determine if in vivo certain agonists produce diacylglycerol via phospholipase C or phospholipase D. Phospholipase C cannot catalyze a transphosphatidylation reaction. This transphosphatidylation reaction in addition to several rather ingenious protocols for radiolabelling phospholipids (128) have been used by many investigators to provide evidence for the formation of diacylglycerol in addition to that produced by PIP_2 hydrolysis. The diacylglycerol produced by phospholipase D is usually in the second, more sustained phase of formation of diacylglycerol. In fact, diacylglycerol production by phospholipase D can be activated subsequent to activation of protein kinase C by by the breakdown of PIP_2 which also produces diacylglycerol (130,131,133).

Phospholipase D activity was described as early as 1947 when Hanahan and Chaikoff invoked its existence to explain the abundance of choline and ethanolamine in carrots (134). Phospholipase D activities have been described in many plants and micro-organisms (135). Phospholipase D (PLD) activity was thought to be absent from mammalian tissues until 1973 when Saito and Kanfer described a PLD activity in a solubilized preparation of rat brain membranes (136). This enzyme was partially purified 240-fold and was determined to have a molecular mass of 200 kDa (137). A human eosinophil PLD activity of 50 kDa molecular mass was characterized in 1976 (138). The subcellular localization of the eosinophil enzyme is not known as the procedure involved using sonicates of eosinophils. Since then PLD activity has been detected in a number of mammalian systems including liver (116), lung (139), endothelial cells (140), and HL-60 cells (141). All of these PLD activities have been associated with the membrane subcellular fraction.

Interestingly, there is one report of the partial purification of a cytosolic PLD

and the comparison of this enzyme with its membrane counterpart (142). The authors concluded that assay conditions used by previous investigators inhibited the activity of the cytosolic PLD and so prevented its discovery. Moreover, the authors state that it is not impossible that this cytosolic PLD may be able to translocate to the membrane upon activation by an appropriate signal not unlike several other enzymes. This is the first evidence for multiple isoenzymes of PLD in a single tissue (142). There is still a great deal to be added to our knowledge of PLD. Further information will surely come with the complete purification of a phospholipase D and its cloning.

1.3.5. Lysophospholipases A_1

Lysophospholipases are enzymes that catalyze the hydrolysis of fatty acyl ester bonds in lysophospholipids. The activation of phospholipase A (either A_1 or A_2) results in the liberation of free fatty acid and the production of an equimolar amount of lysophospholipid. A 1-acyl lysophospholipid can be produced by the action of a phospholipase A_2 on a diacyl phospholipid. Similarly, the action of phospholipase A_1 on a diacyl phospholipid produces a 2-acyl lysophospholipid. Lysophospholipases A_1 hydrolyze 1-acyl lysophospholipids to release fatty acid.

Lysophospholipase activity has been detected in both eukaryotes and prokaryotes (21) and is present in most tissues examined (143). Studies on the subcellular distribution of lysophospholipases indicates that they are present in most subcellular fractions including cytosol, membranes, and mitochondria (144,145,146,147). Lysophospholipases A_1 are active in the absence of Ca^{2+} (21) and are inhibited by different detergents such as Tween-80, Triton X-100, cholate and deoxycholate (21,33,148). It has also been observed that the specific activity of lysophospholipases A_1 is greater than that of either phospholipase A_1 or phospholipases A_2. This results

in very little accumulation of lysophospholipid during degradation of diacylphospholipid (21). Lysophospholipases have been purified from a number of sources.

De Jong et al. (149) solubilised a lysophospholipase activity from bovine liver by treatment of homogenates with n-butanol. Passage of the solubilised protein extract over a DEAE-cellulose column resulted in the separation of the total activity into two peaks of lysophospholipase activity. Peak I was purified 3600-fold to homogeneity. The enzyme had a molecular mass of 25,000 Da and had a neutral pH with an isoelectric point of 5.2. The second enzyme, peak II, was purified 770-fold to obtain a pure protein with a molecular mass of 60,000 Da. It was active at a neutral pH and had an isoelectric point of 4.5. Long chain diacylphospholipids were not hydrolyzed to any extent by both enzymes. Both the beef liver lysophospholipases were inhibited by the detergents Triton X-100 and sodium deoxycholate. Both enzymes exhibited general esterolytic properties as they hydrolyzed tributyrin and *p*-nitrophenylacetate in addition to 1-acyl glycerophosphocholine.

Van den Bosch and his group determined the characteristics of a cytosolic rat-liver lysophospholipase (146). The enzyme showed highest activity with 1-myristoyl lysophosphatidylcholine. This cytosolic lysophospholipase was active at pH 7.0 and was also active in the presence of EDTA. The enzyme also exhibited some activity with diacylphosphatidylcholine. It hydrolyzed 1-acyl-2-linoleoyl phosphatidylcholine. The rat liver enzyme also displayed activity with 2-acyl glycerophosphocholines. The activity with 2-stearoyl or 2-oleoyl glycerophosphocholine was 50% that observed with the corresponding 1-acyl glycerophosphocholine. The enzyme did not hydrolyze triolein.

A lysophospholipase A_1 was purified 164-fold from homogenates of beef pancreas. The lysophospholipase had a molecular mass of 63 kDa as determined by gel filtration and an isoelectric point of 5.0-5.8. Again, the enzyme was active in the absence of any cations and in the presence of EDTA. No activity was observed when diacyl phosphatidylcholine was used as a substrate. The enzyme preferred 1-acyl glycerophosphocholine as a substrate.

The cDNA for this bovine pancreatic lysophospholipase has been isolated, cloned, and sequenced from a cDNA library of adult rat pancreas (150). While in the process of developing a strategy to improve the method of constructing cDNA libraries containing more full-length clones, Han et al. described the isolation of a cDNA clone which encodes for the full length lysophospholipase A_1 from rat pancreas which is similar to the bovine pancreatic lysophospholipase. The cDNA is 2067 bp in length. It has an open reading frame of 1839 nucleotides with 21 nucleotides at the 5' end and 207 nucleotides at the 3' end including the poly(A) tail. The cDNA sequence predicts for a protein of 612 amino acids that would have a molecular mass of 67,107 Da. The bovine pancreatic enzyme is 67,000 Da as determined by SDS-disc gel electrophoresis (148). The predicted size of the rat lysophospholipase and its predicted amino acid composition are comparable to that of the bovine lysophospholipase. In the absence of any peptide sequence for the bovine lysophospholipase A_1 to compare the sequence of this enzyme, Han et al. expressed the cDNA for the rat pancreatic lysophospholipase A_1 in Chinese Hamster Ovary cells (CHO cells) using an SV40 expression vector. They subsequently determined the activity of the expressed cDNA using cell extracts of the CHO cells as a source of enzyme. An assay with 1-[^{14}C]-palmitoyl lysophosphatidylcholine revealed that there

was a 12-fold difference in the lysophospholipase A_1 activity of control and transfected cells. Although the evidence presented by the authors is quite persuasive, comparison of the amino acid sequences or immunological evidence of similarity between the rat and bovine pancreatic lysophospholipases A_1 is still necessary for absolute confirmation.

A number of intracellular lysophospholipases have been purified. Lysophospholipases have been purified from HL-60 cells (151), several murine macrophage cell lines (152,153), rabbit myocardial cytosol (154) and human amnionic membranes (155). Many of these cells have more than one lysophospholipase A_1 activity. For example, anion exchange chromatography of WEHI 265.1 mouse macrophage cell homogenates revealed that there were three distinct lysophospholipase A_1 activities that will hydrolyze 1-pamitoyl LPC (153). Peaks 1 and 2 have molecular masses of 28 and 27 kDa while peak 3 was not homogeneous and analysis by SDS-PAGE revealed the presence of several bands including 57 and 110 kDa size proteins. The three peaks represent 30%, 17% and 53% of the total lysophospholipase activity respectively. Peak 3 had significant phospholipase A_2 activity when assayed using 1-stearoyl-2-[^{14}C]-arachidonoyl GPC. This peak 3 is believed to be the high molecular weight cytosolic phospholipase A_2 which exhibits lysophospholipase A_1 activity and was described in RAW 264.7 mouse macrophages (88). This phospholipase A_2 is described further in section 1.3.2.3. All three lysophospholipases in the WEHI 265.1 cell line preferred 1-acyl glycerophosphocholine as substrate, but they hydrolyzed 1-acyl glycerophosphoethanolamine at rate that was only 10% that with choline substrate. Even less activity was displayed with 1-acyl lysophosphatidylserine. None of the lysophospholipases hydrolyzed 1-acyl

lysophosphatidylinositol. In the absence of Ca^{2+}, none of the lysophospholipases displayed any phospholipase A_1, phospholipase A_2, nonspecific esterase, transacylase, or acyltransferase activity. The lysophospholipase A_1 activity of the Peaks 1 and 2 enzymes were unaffected by Ca^{2+}, Mg^{2+} and EDTA. The peak 3 lysophospholipase was inhibited 40% in the presence of 10 mM $CaCl_2$ or $MgCl_2$. In summary, the results from this paper (153) indicate that in WEHI 265.1 cell lines, there are three soluble lysophospholipases. Of these three, one is found in a number of other cell types including RAW 264.7 cells and U937 monocytes (80,88). The other two lysophospholipases are probably distinct enzymes as they have different amino acid compositions. The cellular localization and relationship of these enzymes remains to be determined.

In another study with $P388D_1$ cells, multiple lysophospholipases were again discovered (152). Two lysophospholipases A_1 were identified in the macrophage-like cell line $P388D_1$. The two activities were separated by Blue-Sepharose chromatography. The purified lysophospholipase I had a molecular mass of 27 kDa, while the partially purified lysophospholipase II had a molecular mass of 28 kDa. The size of these two enzymes correlates very well with the peak 1 and peak 2 lysophospholipases identified in WEHI 265.4 cells (153). The $P388D_1$ lysophospholipases had isoelectric points of 4.4 and 6.1 respectively. The lysophospholipase I peak had pH optimum of 7.5-9.0. The peak I enzyme was the only enzyme characterized in this study due to difficulties in purifying the peak II enzyme to homogeneity. No other phospholipase, transacylase, or acyltransferase activity was associated with the purified lysophospholipase A_1. The enzyme was active in the presence of EDTA, Ca^{2+}, Mg^{2+}, and Fe^{2+} cations, however, all the other cations

tested dramatically inhibited the enzyme. The two enzyme activities from $P388D_1$ cells were found to be immunologically distinct as antibody raised to the peak I enzyme failed to react with the peak II enzyme (157). Although some of the characteristics of the WEHI 265.1 enzymes are similar to the $P388D_1$ cell lysophospholipases, it is not known if these enzymes are related either genetically or immunologically. Lysophospholipase I from $P388D_1$ (152) exhibits little substrate specificity for chain length of fatty acid and hydrolyzes 1-acyl lysophosphatidylglycerol as well as 1-acyl glycerophosphocholine (156,157).

An examination of the lysophospholipases of butyric acid treated and untreated HL-60 cells reveals that both types of cells have three lysophospholipases A_1 (151). Interestingly, butyric acid treatment results in the increased expression of only one of these three enzymes. The lysophospholipase activity of HL-60 homogenates as assayed with 1-[^{14}C]-palmitoyl lysophosphatidylcholine was divisible into three distinct peaks of activity after hydrophobic interaction chromatography. The three peaks of activity had molecular masses of 69 kDa, 20 kDa, and 22 kDa respectively. The 20 kDa enzyme was increased in cells treated with butyric acid, while the activity of the other two enzymes was not changed. It should be stated that the small molecular weight enzymes were purified to homogeneity while the 60 kDa enzyme was not homogenous. Amino acid sequence analysis of the 20 and 22 kDa enzymes revealed that although these enzymes had similar molecular masses, they are not identical with respect to amino acid sequence, but they do share a good deal of homology. This suggests that these two enzymes may have evolved from the same ancestral gene, but that they developed to provide the cell with different functions during differentiation. The functions and subcellular localization of the

various lysophospholipases are not yet known. The amino acid composition of the HL-60 lysophospholipases peak 2 and 3 enzymes were found to be different from the amino acid compositions of the rat pancreatic lysophospholipase (150) or the WEHI 265.1 enzymes (153). No information concerning the substrate specificity of the lysophospholipases or whether the enzymes catalyze any other reactions was provided.

1.3.6. Lysophospholipase A_2

In contrast to the large amount of information on lysophospholipases A_1, there is a paucity of information on lysophospholipases A_2. The hydrolysis of a diradylglycerophospholipid by either a plasmalogenase or a phospholipase A_1 will generate a free fatty acid from the sn-1 position and a 2-acyl lysophospholipid. Lysophospholipases A_2 hydrolyze 2-acyl lysophospholipids. Lysophospholipases have traditionally been studied with 1-acyl glycerophospholipids as the substrate and most studies have focused on lysophospholipases A_1. 2-Acyl lysophospholipids unlike their 1-acyl lysophospholipid analogues are unavailable commercially and are thermodynamically unstable due to the phenomenon of acyl migration which results in the formation of a 1-acyl lysophospholipid from the 2-acyl lysophospholipid. The synthesis of 2-acyl GPL are therefore a prerequisite for the study of lysophospholipases A_2.

Evidence has been reported of an activity in both bacteria and mammalian tissues that can degrade 2-acyl lysophospholipids (146,158,159,160). Lysophospholipase A_2 activity was first shown by Van den Bosch and his group in their studies on the rat liver cytosolic lysophospholipase (146). The rat liver activity hydrolyzed 2-stearoyl and 2-oleoyl glycerophosphocholine. This enzyme activity was

never characterized any further.

A lysophospholipase A_2 activity was described in subcellular fractions of the guinea-pig heart by Arthur and his laboratory (158,159). This group investigated the hydrolysis of 2-acyl glycerophosphocholine in the guinea-pig heart. In particular, the studies involved the characterization of the activities in mitochondrial and microsomal fractions of the heart (158,159). There was no information as to whether the cytosolic fraction of the heart also had any detectable lysophospholipase A_2 activity. Lysophospholipase A_2 activity in the microsomal fraction of the guinea-pig heart was active in the absence of the cations Ca^{2+} and Mg^{2+} (158). The enzyme had an optimal pH of 8.0 using 2-arachidonoyl GPC as the substrate. The microsomal enzyme was inhibited by metal ions Cu^{2+} and Zn^{2+}. The lysophospholipase A_2 had a preference for 2-linoleoyl GPC as its substrate. This specificity for 2-linoleoyl GPC was not maintained in assays containing an equimolar mixture of 2-arachidonoyl GPC and 2-linoleoyl GPC. In order, to differentiate whether the lysophospholipase A_2 activity was similar to the lysophospholipase A_1 activity, the effect of pH, fatty acids, and various alkylating agents on the two lysophospholipase activities was examined. It was found that the effect of fatty acids and alkylating agents differed for the two lysophospholipases. This study documents that in guinea-pig heart microsomes, there are possibly two distinct lysophospholipases A, however, a definitive conclusion on this will have to await purification of the two activities.

Studies were carried out by Badiani et al. (159) which compared the lysophospholipase A_1 and A_2 activities in guinea-pig heart mitochondria. The mitochondrial lysophospholipase A_2 activity was shown to be Ca^{2+} and Mg^{2+}-independent. However, 5 mM Mg^{2+} activated the hydrolysis of 2-arachidonoyl GPC

relative to that of a control. The reagents NEM and DTNB inhibited the activity of the lysophospholipase A_2 completely. The lysophospholipase A_2 also demonstrated a specificity for 2-acyl GPC containing linoleic acid. Again, this specificity for 2-linoleoyl GPC was not maintained in mixtures of 2-linoleoyl GPC and 2-arachidonoyl GPC. The lysophospholipase A_2 activity was inhibited by unsaturated fatty acids. Cardiolipin, a mitochondrial phospholipid inhibited the mitochondrial lysophospholipase A_2 activity in a concentration dependent manner. The mitochondrial lysophospholipase A_1 does not share many of the above characteristics of the lysophospholipase A_2, especially with respect to its acyl specificity, its susceptibility to inhibition by unsaturated fatty acids and the effect of cardiolipin. Taken, together these results suggest that in guinea-pig heart mitochondria, there may be distinct lysophospholipases A_1 and lysophospholipase A_2 in different subcellular fractions (159).

Finally, only one lysophospholipase A_2 activity has been purified thus far. This lysophospholipase A_2 was purified and characterized in 1985 (160). This lysophospholipase A_2 is bound to the inner membrane of E. coli. The gene for this enzyme was isolated and cloned from mutants of E. coli K-12 that had an elevated level of this enzyme or were defective in this activity. The pldB gene coding for the lysophospholipase A_2 was cloned into a plasmid and then transformed into E. coli KL16-99 bacterial cells to aid in the purification of the enzyme. The enzyme was purified from the membrane fraction of these bacteria to homogeneity, a purification of 708-fold. The purified enzyme had a molecular mass of 38.5 kDa as determined by SDS-PAGE. The enzyme had an isoelectric point of 7.2. The substrate specificity of the enzyme indicated that it hydrolyzed 2-acyl GPC > 2-acyl GPE > 2-acyl glycerophosphoglycerol (GPG). Furthermore, it hydrolyzed 2-acyl GPC > 1-acyl GPC

> 1-acyl GPE >> 1-acyl GPG. The enzyme did not hydrolyze diacylphospholipids. The enzyme was heat-labile and was inactivated upon heating for 5 min at 55°C. Lastly, the characteristics of the E. coli K-12 lysophospholipase A_2 are different from the E. coli K-12 lysophospholipase A_1 (161). In all, there is good evidence to indicate that lysophospholipases A_2 are distinct from lysophospholipases A_1, however, more evidence is still required.

1.4. G proteins

Neurotransmitters, hormones, and growth factors transmit messages by binding to receptor molecules on the surface of the cell. Some of the receptors activate effector molecules via signal transducing molecules known as G proteins. G proteins are guanine nucleotide binding proteins. There have been a number of reviews published on the topic of G proteins (162,163,164,165,166). The G protein cycles between an inactive GDP-bound form and an active GTP-bound form. The unstimulated form of the G protein is the inactive GDP-bound form. The activation of the G protein from inactive to active form is initiated by the binding of the hormone (or other ligand) to its receptor. The actions of the G protein in going from an active to inactive form (or vice versa) is called the GTPase cycle or G protein cycle.

G proteins are heterotrimeric proteins consisting of three subunits designated α, ß, and γ in order of decreasing molecular mass. α Subunits are 39-46 kDa, ß subunits are 37 kDa, while γ subunits are 8 kDa. In mammals, G protein α, ß, and γ polypeptides are encoded by at least 16, 4 and 7 genes respectively (167). It is very probable that the number of these subunits that are known will increase in the near future. The activation of G protein coupled receptors results in the dissociation of heterotrimeric G proteins into Gα subunits and ßγ dimers. It is the Gα subunits that

bind GTP and GDP, have an intrinsic GTPase activity and interact with the effector molecule and receptor molecules. However, there is increasing evidence that indicates the ßɣ subunits may also interact with effector molecules (167). Furthermore, experiments have shown that different ß subunits of G proteins can determine the specific recognition of a G protein by its receptor (168). It is the binding of GTP to the G protein that decreases the affinity of the G protein for the agonist receptor complex and the subsequent dissociation of the heterotrimeric protein into its constituent subunits.

In the basal state, a receptor is unliganded and presumably does not associate with other components of the signalling pathway. The G protein(s) with which it can interact is inactive because of the high affinity of the G protein for GDP which is bound to the G protein. The affinity of the ligand-receptor complex for G protein-GDP is sufficient for their interaction. This complex formation facilitates the dissociation of GDP from the G protein. The complex of agonist, receptor, and nucleotide-free G protein is energetically stable but its lifetime is brief in the normal environment of the cell. In the cell, there is usually a high concentration of GTP. In such an environment, GTP replaces the position previously occupied by the GDP. The binding of GTP results in the disassociation of the G protein from the agonist-receptor complex. The G protein-GTP complex is the active form of the molecule. This complex is stable and can last for many seconds (163,164). During this time, the activated G protein interacts with the appropriate effector molecule. There is a great deal of specificity between a particular effector molecule and a particular G protein. The activated G protein alters the rate of function of the effector molecule. In the case of adenylyl cyclase, the first effector-G protein pair to be discovered, one G

protein can stimulate the production of many hundreds of molecules of cAMP in a single cycle of activation. The intrinsic GTPase activity of the G protein catalyzes the hydrolysis of the bound GTP to GDP; forming again the inactive form of the G protein and ending the cycle. This returns the system to its basal state. There is an aspect of amplification of signal in this system. The G protein is active for many seconds and so it can affect many effectors and a receptor can interact with and activate a number of G proteins in the period of one cycle.

Several criteria have been established to determine whether a particular effector is modulated by G protein(s) (170). This includes the following: (a) Aluminium tetrafluoride together with Mg^{2+} can interact with the Gα-GDP G protein to mimic the effect of GTP and activate the G protein. (b) Non-hydrolysable analogues of GTP keep the G protein in its active form and persistently activate the G protein. (c) In some cases, α subunits have specific amino acid residues that can be covalently modified by bacterial toxins. Cholera toxin will catalyze the ADP-ribosylation of a specific Arg residue in the Gα subunit. Pertussis toxin, another toxin can ADP-ribosylate a Cys residue in the carboxyl-terminus of the Gα subunit. Cholera toxin sensitive G proteins will be constitutively activated whereas, pertussis toxin G proteins will be inhibited as they will have an impaired ability to interact with receptors after ADP-ribosylation.

G proteins control a sophisticated signal processing network. Activation of effector molecules is now thought to depend on signals Gα subunits or βγ subunits. It is also not inconceivable that there is interaction of the messages from one receptor-G protein couple with that of another G protein receptor couple, thus resulting in "cross-talk" between the receptors in response to different extracellular signals (165,169). Also, effector molecules can control the rate of their deactivation and thus

also have a hand in the determination of the duration of the signals (171).

1.5. Regulation of the phospholipases

The products of phospholipid breakdown such as arachidonic acid, inositol trisphosphate, phosphatidic acid, and diacylglycerol have very important functions within the cell. Such products participate in intracellular events such as activation of protein kinases, cell growth, cell differentiation, repair of tissue or cell injury and inflammation. Given the importance of these products, it is not surprising that the mechanisms that result in the formation of these products should be regulated. The most direct mechanism which results in the formation of these compounds is the hydrolysis of phospholipids by phospholipases. In general, the methods for regulating the activity of the phospholipases includes: regulation by cations, protein phosphorylation, protein-protein interaction, regulation by guanine nucleotide binding proteins and regulation by coupling the activation of the phospholipase to stimulation of a cell surface receptor. Not all these mechanisms apply to all the phospholipases. In fact, very little is known about the specific mechanisms that regulate phospholipase A_1 and lysophospholipase activities. In contrast, a great deal is known about the regulation of intracellular phospholipase A_2 activity and PI-specific phospholipase C activity. Therefore their regulation will be described in this section.

1.5.1. Regulation of phospholipase A_2

Interest in the regulation of phospholipase A_2 activity has exploded within the last 5 years. The reason for the increased interest in this area of research has been two-fold: (1) There is evidence that phospholipase A_2 can be regulated by G proteins, and (2) the identification, purification and cloning of the intracellular $cPLA_2$ has lead to

the availability of many reagents (i.e. purified enzyme, recombinant cell lines, and anti-cPLA$_2$ antibodies, etc.) that can be used to dissect the pathways of its regulation.

The evidence that phospholipase A$_2$ may be regulated by G proteins has been accumulating for a number of years. There are two early studies which present good evidence for the stimulation of phospholipase A$_2$ activity via G proteins. When rat thyroid FRTL5 cells were pre-labelled with [^{3}H]-arachidonic acid, the addition of α_1-adrenergic receptor agonist norepinephrine resulted in arachidonic acid release (172,173,174). This could occur via activation of a receptor-coupled phospholipase A$_2$. In permeabilized cells, the addition of GTP[S], an activator of G proteins, and norepinephrine resulted in arachidonic acid release. This release of fatty acid could be inhibited completely by the addition of pertussis toxin indicating that the α_1-adrenergic receptor was coupled to a specific G protein and this was coupled to a phospholipase A$_2$. This experiment was also repeated successfully with cell membranes from the FRTL5 cell line with exogenous 1-acyl-2-arachidonoyl phosphatidylcholine as the substrate. Similar activation of the α_1-adrenergic coupled phospholipase A$_2$ and its G-protein was shown in MDCK cells (175).

The most direct evidence showing that phospholipase A$_2$ can be activated by G proteins was reported in studies on the activation of phospholipase A$_2$ by light in the rod outer segments of bovine retina. It was known previously that the light photoreceptor, rhodopsin is coupled to a cGMP phosphodiesterase via the G protein transducin or Gα_t (62). When the photoreceptor is stimulated by light, Gα_t is activated and inhibits the phosphodiesterase. In dark adapted rod outer segments, phospholipase A$_2$ was also activated in response to light (176). The addition of GTP[S] to crude rod outer segments stimulated this activity in the absence of any

light. Depletion of the transducin by hypotonic washing of the rod outer segments resulted in the reduction of both the light- and GTP[S] stimulated phospholipase A_2 activity. The addition of purified transducin in the dark to transducin depleted rod outer segments restored the GTP[S] stimulated activation of phospholipase A_2. This is clear evidence that transducin is coupled to phospholipase A_2 activation in rod outer segments. Interestingly, the addition of ßγ subunits of transducin stimulated phospholipase A_2 activity, while the addition of purified α-subunits of transducin inhibited the phospholipase A_2. This was the first example of evidence for the ßγ subunits playing a role in signal transduction (177). There is now considerable evidence in support of GTP-binding protein regulation of phospholipase A_2 activity in platelets, neutrophils, HL60 cells, Swiss 3T3 cells and many other cell types (178,179,180). In spite of this, the exact mechanisms of G protein activation of phospholipase A_2 are not known. Aside from transducin, it is also not known which G protein(s) activate phospholipase A_2.

By comparison, there is a great deal known about the regulation of the high molecular weight phospholipase A_2. One mechanism of regulation of this enzyme that has already been discussed is the translocation of the $cPLA_2$ to membrane vesicles in response to submicromolar concentrations of Ca^{2+} (sec. 1.3.2.3). The translocation of the enzyme brings it into contact with its substrate. Hence, this is the mechanism of activation of the $cPLA_2$ by Ca^{2+} ionophores such as A23187 (87). TPA and ATP treatment of CHO cells expressing the $cPLA_2$ was demonstrated to cause a 2-fold increase in the incorporation of ^{32}P into $cPLA_2$ (181). TPA had previously been shown to induce the phosphorylation of $cPLA_2$ in vitro (86). Phosphoamino acid analysis revealed that TPA and ATP treatment results in the phosphorylation of serine residues

in the $cPLA_2$ with no detectable tyrosine or threonine phosphorylation (181). PDGF and EGF treatment of Rat-2 cells transfected with $cPLA_2$ results in phosphorylation of serine residues (87). No other phosphorylation is detected. This indicates that the $cPLA_2$ may be phosphorylated in vivo by a Ser kinase. The phosphorylation of $cPLA_2$ increases the activity of the enzyme when assayed in an in vitro enzyme assay with exogenous substrate. The phosphorylation does not directly activate the enzyme but seems to "prime" the enzyme. Full activation is a Ca^{2+}-dependent process (87).

The identity of a Ser kinase that is responsible for activating $cPLA_2$ was recently reported to be MAP kinase (87). The $cPLA_2$ contains the consensus sequence for MAP kinase and it has been demonstrated in vitro that the MAP kinase phosphorylation of $cPLA_2$ at Ser-505 increases the activity of the enzyme towards PC micelle substrate (87). Mutants of $cPLA_2$, which have the Ser-505 changed to Ala by site-directed mutagenesis, cannot be phosphorylated in vitro by the MAP kinase nor can such mutants mediate agonist induced release of radiolabelled arachidonic acid from CHO cells that overexpress the mutant enzyme. It was further determined that TPA treatment results in the phosphorylation of a $cPLA_2$ identical to that produced by the in vitro phosphorylation of the enzyme by MAP kinase (87). The actual mechanistic step in the reaction mechanism that is affected by the phosphorylation of Ser-505 remains to be identified. In summary, it has been proposed that agents such as PDGF, which are G protein coupled to the PI-specific PLC, activate the $cPLA_2$ by stimulating protein kinase C and increasing the intracellular Ca^{2+} concentration. Protein kinase C phosphorylates MAP kinase. Activated (phosphorylated) MAP kinase will phosphorylate $cPLA_2$ and full activation of $cPLA_2$ will be due to activation by Ca^{2+}. Activation of the $cPLA_2$ can also occur in the presence of Ca^{2+} alone.

A recent study on the stimulation of arachidonic acid mobilization in Swiss 3T3 cells by PDGF has described that arachidonate mobilization occurs in a biphasic manner (182). The authors believe that $cPLA_2$ is responsible for the early phase of arachidonate release, but it is not responsible for a second phase of arachidonate mobilization. This second phase of arachidonate mobilization was dependent on de novo mRNA or protein synthesis as it was completely abolished by cyclohexamide or actinomycin D. The first phase of arachidonate release is not affected by these inhibitors. It has also been reported that macrophage colony stimulating factor induces the gene expression of the $cPLA_2$ (183). The actual mechanism in both cases is still unknown. These two reports indicate that there may be other proteins which are involved in the regulation of fatty acid (including arachidonic acid) release by phospholipases A_2. Additional regulatory mechanisms may be discovered that allow greater insight into the regulation of fatty acid release by phospholipase A_2.

Regulation of the activity of the $cPLA_2$ is dependent upon Ca^{2+}. Complete activation of the enzyme involves a Ca^{2+} dependent translocation of the enzyme. The $cPLA_2$ is a Ca^{2+} dependent enzyme, it is inactive in the absence of Ca^{2+} (79). The plasmalogen selective phospholipase A_2 is a Ca^{2+}-independent enzyme. Hence, there is a necessity for regulatory factors other than Ca^{2+} in the regulation of the plasmalogen selective phospholipase A_2. One mechanism that has been demonstrated to be involved in the regulation of this activity is ATP modulated protein-protein interactions. It was shown by Hazen and Gross in 1991 that there is ATP dependent regulation of this enzyme (184). ATP has the effect of augmenting the rate of phospholipid hydrolysis by this enzyme in a manner that does not require the participation of a kinase. When myocardial cytosol was incubated with ATP, there

was a discernable difference in the characteristics of the enzyme with respect to its rate of thermal denaturation, interaction with the substrate, or inactivation by a sulfhydryl reagent. However, when highly purified enzyme was incubated with ATP, these effects were not observed. This indicated that myocardial cytosol contained a component that was necessary for ATP to confer its effects. This regulatory factor was recently purified and identified to be an isoform of the glycolytic enzyme, phosphofructokinase (185). The phosphofructokinase forms a large molecular mass complex with the phospholipase A_2. It was determined that this regulatory factor of the phospholipase A_2 is a tetramer of phosphofructokinase of which a monomer is 85 kDa. The mechanism by which the ATP-phosphofructokinase complex can act to stimulate the plasmalogen selective phospholipase A_2 is not known. The binding of ATP to phosphofructokinase results in an allosteric alteration in the conformation of the phosphofructokinase which somehow influences the rate of phospholipid hydrolysis of the plasmalogen selective phospholipase A_2. The authors are quick to point out that this novel mechanism of regulation of this enzyme is just one possible mechanism for its regulation.

1.5.2. Regulation of phospatidylinositol specific phospholipase C

The hydrolysis of phosphatidylinositol by phospholipase C can be activated by the stimulation of numerous cell surface receptors. There are also a number of isoforms of PLC that are responsible for the hydrolysis of PI (sec. 1.3.3.1). Given these two facts, it is not surprising that there are multiple systems regulating the activity of PI-PLC. The regulation of PI-PLC is distinct for two of the known PLC families, PLC-ß and PLC-γ. PLC-ß is regulated by G-proteins while PLC-γ1 and PLC-γ 2 are regulated by receptor-tyrosine kinases.

The evidence in favour of G-protein regulation of PI-PLC is reviewed quite well by Fain et al. (186). The evidence for G-protein regulation of PLC can be summarized as follows: (a) Non-hydrolysable analogues of guanine nucleotides cause increased hydrolysis of endogenous PIP_2. (b) In some cell types, the addition of pertussis toxin blocked receptor stimulated inositol lipid hydrolysis. This occurred primarily in those cells and tissues of bone marrow origin. (c) PLC-linked receptors were shown to stimulate GTPase activity in membranes derived from a number of tissues. Investigations into the nature and identity of the G-protein regulating PI-PLC activity have shown there may be two distinct types of G-proteins that can be differentiated by their sensitivity to pertussis toxin. It is believed that there is a pertussis-toxin sensitive G protein and a pertussis toxin insensitive G-protein. The identity of the pertussis toxin insensitive G-protein (previously termed Gp) had eluded scientists until very recently when it was purified (187,188,189). Unfortunately, there has not been similar identification of the pertussis toxin sensitive G-protein.

Three laboratories have recently purified and identified G proteins that can stimulate PI-PLC (187,188,189). 42 and 43 kDa proteins were purified from liver membranes that activated PI-PLC. These proteins were not recognized by antiserum to any of the known G protein α-subunits. This indicated that these were novel G proteins. The 42 kDa protein was identified to be $G\alpha_q$ and the 43 kDa protein was identified as $G\alpha_{11}$ (189,190). Both of these are members of the Gq family of G proteins (190). The purified $G\alpha$ subunits had a very low GTPase activity and exchanged GTP for GDP very slowly. The affinity of these α-subunits for GTP or GTP analogues is very low. Reconstitution experiments demonstrated that these G proteins specifically activate PLC-ß but not PLC-δ or PLC-γ isoforms (191,192). The receptors

that activate PLC via these G proteins include thromboxane A2, bradykinin, angiotensin, histamine, vasopressin, and the muscarinic acetylcholine receptor (192). The mechanism of activation of PLC-ß by $G\alpha_q$ appears to be an increase in the apparent V_{max} of the enzyme with no change in the K_m for the substrate (192).

Although the regulation of effectors by G-proteins is generally mediated by the activated α-subunits, there are reports that the ßɣ-complex may also serve some regulatory functions (193). There is evidence that purified ßδ-subunits activate cardiac muscarinic gated K+ channels, type II and type IV adenylyl cyclases and inhibit the type I adenylyl cyclase (193). Recently, it has been shown that ßɣ-subunits can specifically activate PLC-ß2 but not the ɣ- or δ-forms of PLC from a number of sources (194,195,196).

PLC-ɣ appears to be activated by growth factor tyrosine kinases. Polypeptide growth factors such as PDGF, EGF, FGF, and insulin all mediate their actions by binding to and activating cell surface receptors that possess an intrinsic protein-tyrosine kinase activity. The binding of PDGF or EGF to their respective receptors stimulates the breakdown of PIP_2. Treatment of cells with PDGF or EGF not only promotes the hydrolysis of PIP_2 (197,198) and phosphorylation of PLC-ɣ1 (197,200) but also promotes the association of PLC-ɣ1 with the PDGF and EGF receptor (199,200). It is believed that the phosphorylation of Tyr-783 of PLC-ɣ1 is essential in the process of PLC-ɣ1 activation (110). To date none of the receptors or signal transducers that are coupled to PLC-δ are known.

It is also known that the PLC-ɣ can be activated by non-receptor protein tyrosine kinase activities especially in leukocytes (201). Phosphopeptide mapping revealed that the amino acids that are tyrosine phosphorylated by the growth factor

receptor tyrosine kinases and the non-receptor tyrosine kinases are identical. Finally, evidence also suggests that both protein kinase C and the cAMP-dependent protein kinase A (PKA) may attenuate the activity of PLC-γ via phosphorylation of Ser and Thr residues of the PLC (110,111).

Although, there have been some investigations into the regulation of phospholipase D and PC-specific PLC activities, the specific details are still very unclear. What is quite clear is that both of these enzymes can be regulated by receptor-stimulation, G proteins, activation by Ca^{2+}, and modulated by protein kinase C. The evidence in favour of such regulation is reviewed by Anthes and Billah (128).

There is some evidence in favour of receptor regulation of phospholipase A_1. In 1979, Franson et al. identified a Ca^{2+}-independent phospholipase A_1 activity in canine cardiac sarcolemma that could hydrolyze 1-acyl-2-linoleoyl GPE at pH 7.0 (202). This phospholipase A_1 activity could be stimulated 3-4 fold by the catecholamine analogue dl-isoproterenol. The mechanism by which this agonist mediated its effect was not investigated.

1.6. Adrenoreceptors

The catecholamines noradrenaline and adrenaline modulate a variety of diverse responses in mammals. The catecholamines are produced from nerve terminals and chromaffin tissue. Catecholamines produce their responses by binding to specific adrenergic receptors on the plasma membrane of cells. The binding of catecholamines or related drugs induce changes in these receptors that lead to a series of events that result in the characteristic observed physiologic effects of the drug (203). Classically, adrenoreceptors have been divided into α and ß types (204). Subtypes of these two classes of adrenergic receptors have now been discovered

(205,206). The differentiation of the receptors was made on the basis of the rank potency of a series of structurally related catecholamines. With the advent of molecular biology, a further definition of subtypes has been made. α-Adrenergic receptors can be either α_1 or α_2 type of which there are α_{1A}, α_{1B}, and α_{2A}, α_{2B}, α_{2C}, α_{2D} subtypes, as well as three subtypes of ß-receptor, $ß_1$, $ß_2$, and $ß_3$ (207).

All of the adrenoreceptors for which the amino acid sequence is known are members of the G protein linked family of receptors. All such G protein linked receptors are transmembrane spanning proteins which are in general between 402 and 560 amino acids in length. These proteins contain functional domains which mediate ligand binding and G-protein coupling (208,209). Adrenergic receptors are found in many cells and tissues. In the heart, catecholamines act to modulate the rate and force of the cardiac contraction (210).

1.7. Research Aims

Many cells and tissues respond to various extracellular stimuli by mobilizing arachidonic and other fatty acids. These bioactive fatty acids are metabolized to eicosanoids via the cyclooxygenase, lipoxygenase, and epoxygenase pathways (15,17). In most tissues, the synthesis of eicosanoids is limited by the availability of precursor fatty acids. The bulk of the eicosanoid precursor fatty acids in mammalian cells is esterified to the fatty acyl chains of glycerophospholipids (15,16,22). Such a localization of these bioactive fatty acids has led to the suggestion that the availability of free fatty acid is linked to the activation of the hydrolysis of phospholipids by phospholipases. It is generally believed that changes in the level of free fatty acid are the result of receptor stimulation of phospholipases. It is generally accepted that the main mechanism for the selective release of sn-2 fatty acids is via direct deacylation

of phospholipids by a phospholipase A_2 (16,22,211).

Most of the evidence cited to support the involvement of phospholipase A_2 in fatty acid release does not distinguish it from other possible routes for fatty acid release (212). Most experiments cited to support the involvement of phospholipase A_2 usually involve the pre-labelling of cell membrane phospholipids with radiolabelled fatty acid and then monitoring the release of radioactivity into the medium upon cell stimulation. Although it is true, that direct deacylation of phospholipids by phospholipase A_2 is a mechanism which explains the experimental findings, it is not the only mechanism. Other pathways may also explain such experimental findings. Figure 3 shows other pathways which can lead to an increase in the level of free fatty acid. The involvement of enzymes other than phospholipase A_2 such as phospholipase C, phospholipase D, and monoglyceride and diglyceride lipases have been shown to operate for fatty acid release (212,213,214,215).

Studies have shown that there can be Ca^{2+}-dependent and Ca^{2+}-independent pathways for the release of arachidonic acid (216,217,218,219,220). It is known that both the extracellular phospholipase A_2 and the $cPLA_2$ are both Ca^{2+}-dependent enzymes, their involvement in Ca^{2+}-independent pathways for fatty acid release can therefore be discounted. The Ca^{2+}-independent plasmalogen selective phospholipase A_2 is also most likely not involved in such selective fatty acid release as its involvement is restricted to releasing fatty acids from ether lipids. Therefore, it is likely that Ca^{2+}-independent pathways for the selective release of fatty acids from diacyl phospholipids do not involve of any of the currently known phospholipases A_2.

Figure 3

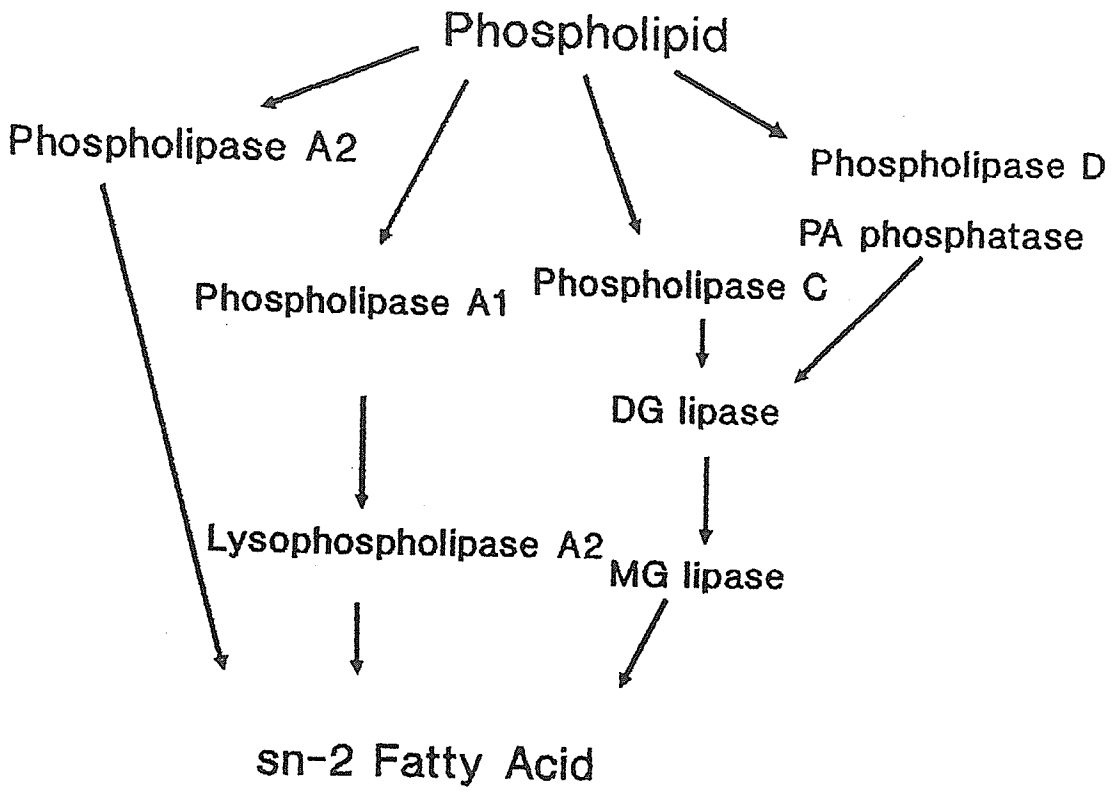

Fig. 3. Pathways which can lead to the release of the sn-2 fatty acid from a phospholipid.

Adapted from ref. (212).

One idea that has been suggested by many individuals is that selective release of fatty acids from diacyl phospholipids could occur via a phospholipase A_1/lysophospholipase A_2 pathway (16,22,212,220). In this pathway, the initial action of a phospholipase A_1 on the diacylphospholipid would result in the production of a 2-acyl lysophospholipid and a free fatty acid. The subsequent hydrolysis of the 2-acyl lysophospholipid by a lysophospholipase A_2 would result in the release of the eicosanoid precursor fatty acid.

Although this possibility of releasing sn-2 fatty acids by a phospholipase A_1/lysophospholipase A_2 pathway has been postulated by many investigators very little experimental evidence has been obtained to support it. The characteristics of the lysophospholipase A_2 or phospholipase A_1 that may be involved is not known. There is some evidence that this Ca^{2+}-independent pathway may operate for the release of fatty acids from phosphatidylinositol in endothelial cells (220). The major aim of this study has been to determine the characteristics and regulation of phospholipase A_1 and lysophospholipase A_2 activities in guinea-pig heart microsomes in an effort to ascertain whether these two enzymes may act together to selectively release fatty acids in the guinea-pig heart. The guinea-pig heart was chosen as our model system as previous studies had shown that a lysophospholipase A_2 activity was present in the microsomal fraction for the hydrolysis of 2-acyl GPC (158). Also, an analysis of the distribution of fatty acids in the guinea-pig heart revealed that there was a large amount of arachidonic acid esterified to the diacyl phosphatidylethanolamine fraction. The specific goals of this work were as follows:

1. To determine the existence and characteristics of a 2-acyl glycerophosphoethanolamine lysophospholipase activity in guinea-pig heart microsomes

especially with respect to its acyl specificity and Ca^{2+}-requirements. As there is very little information regarding the tissue distribution of this enzyme, this was also investigated.

2. To determine whether Ca^{2+}-independent phospholipase A_1 activities are present in the heart microsomal subcellular fraction and the acyl specificity of the enzymes with respect to different molecular species of PE and PC.

3. To determine if the heart microsomal phospholipase A_1 activity could be modulated by either guanine nucleotides and/or agonists that can couple to specific receptors.

2. Materials

2.1. Experimental Animals

Male guinea-pigs (250-300 g) and male Sprague-Dawley rats (250-300 g) were used during these studies. The animals were obtained from Charles River Canada (St. Constant, Quebec, Canada). The guinea-pigs were maintained on the Agway guinea-pig diet (Agway, Inc., Syracuse, New York) and tap water *ad libitum* in a light and temperature controlled room.

2.2. Chemicals and Reagents

1-Acyl-2-[1-^{14}C]-arachidonoyl GPE (50 mCi/mmol), 1,2-di[1-^{14}C]-palmitoyl GPE (52 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]-linoleoyl GPE (54 mCi/mmol), 1,2-di[1-^{14}C]-oleoyl GPC (52 mCi/mmol), [1-^{14}C]-oleoyl-CoA (52 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]-palmitoyl GPC (55 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]-linoleoyl GPC (54 mCi/mmol), 1-stearoyl-2-[1-^{14}C]-arachidonoyl GPC (56 mCi/mmol), and 1-palmitoyl-2-[1-^{14}C]-oleoyl GPC (54 mCi/mmol) were purchased from Amersham International (Oakville, Ontario, Canada). 1-Palmitoyl-2-[1-^{14}C]-arachidonoyl GPC (50 mCi/mmol) and [1-^{14}C]-arachidonoyl-CoA (47 mCi/mmol) were obtained from NEN DuPont (Mississauga, Ontario, Canada). Pig liver lysophosphatidylethanolamine, 1,2-dioleoyl GPE, pig liver lysophosphatidylcholine, and 1-palmitoyl-2-lyso GPE were the products of Serdary Research Laboratories (London, Ontario, Canada). 1-Stearoyl-2-arachidonoyl GPC, oleoyl-CoA, arachidonoyl-CoA, 1-palmitoyl-2-linoleoyl GPC, 1,2-dipalmitoyl GPC, 1-palmitoyl-2-oleoyl GPC, 1,2-dioleoyl GPE, 1,2-dipalmitoyl GPE, 1-palmitoyl-2-linoleoyl GPE, Triton X-100, Triton QS-15, sodium taurocholate, sodium deoxycholate, sodium taurodeoxycholate, Tween-20, hexadecyltrimethylammonium bromide, *dl*-isoproterenol, propranolol, clonidine, phenylephrine, dibutryl cAMP, butoxamine, atenolol, coenzyme A and trifluoperazine

were all purchased from the Sigma Chemical Company (St. Louis, Missouri, USA). Ion exchange resins CM-52 and DEAE-Sepharose were products of Whatman (Maidstone, Kent, England) and Pharmacia (Baie d'Urfe, Quebec, Canada) respectively. Linoleic acid, arachidonic acid, and oleic acid were obtained from NuChek Prep (Elysian, Minnesota, USA). 1-Palmitoyl-2-arachidonoyl GPC, 1,2-diarachidonoyl GPE, and 1,2-dilinoleoyl GPE were purchased from Avanti Polar Lipids (Pelham, Alabama, USA). Silicic acid was purchased from Bio-Rad (Montreal, Canada). All nucleotides were obtained from Boehringer Mannheim (St. Laval, Quebec, Canada). Folin-Cicolteau phenol reagent was obtained from BDH Chemicals Ltd. (Toronto, Ontario, Canada). Aquacide III flake polyethylene glycol was purchased from Calbiochem (San Diego, California, U.S.A). Ecolite scintillation fluid was a product of ICN Biomedicals (Toronto, Ontario, Canada). Anhydrous diethyl ether was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Frozen guinea-pig pancreas was purchased from Pel-Freez Biologicals (Rogers, Arkansas, U.S.A). All other solvents and chemicals were of reagent grade and obtained from Baxter-Canlab (Toronto, Ontario, Canada).

2.3. Enzymes

Triacylglycerol lipase from Rhizopus arrhizus and Savoy cabbage phospholipase D were purchased from Boehringer Mannheim. Phospholipase A_2 from Crotalus adamenteus was a product of the Sigma Chemical Co.

3. Methods

3.1. Preparation of subcellular fractions

Male rats or guinea-pigs (250-300 g) were killed by decapitation, the necessary organs were removed and placed on ice. The procedure used for subcellular fractionation of the tissues was a modification of that described by Arthur et al. (64)

to rapidly prepare microsomal material. Tissues were rinsed, minced and homogenized in an ice-cold buffer of 0.25 M sucrose, 10 mM Tris HCl, pH 7.4 and 2 mM EDTA. The homogenization of the tissue solution was carried out with two bursts of a Polytron homogenizer (Brinkmann) for approximately 30 sec each at a setting of 7. The resulting homogenate was then centrifuged at 20,000 x g for 10 minutes to sediment unbroken cells and mitochondrial material. The post-mitochondrial supernatant was re-centrifuged at 20,000 x g for 10 minutes to pellet mitochondrial material. The supernatants from the above centrifugations were combined and centrifuged at 100,000 x g for 1 hour. All centrifugations were carried out at 4 °C. The pellet from the ultracentrifugation is the microsomal material. This pellet was resuspended using a Dounce homogenizer ('A' pestle) in 0.25 M sucrose, 10 mM Tris HCl pH 7.4 buffer. The microsomes were stored at -80 °C until necessary. The protein content of the material was determined by the method of Lowry et al. (Sec. 3.5.2., 221).

3.2. Preparation of substrates

3.2.1. Preparation of 2-acyl glycerophosphoethanolamine

2-Acyl glycerophosphoethanolamine substrates with various radiolabelled fatty acids at the sn-2 position were prepared by the hydrolysis of the parent phosphatidylethanolamine with lipase from Rhizopus arrhizus. The reaction contained 4 μmol of phosphatidylethanolamine in a solution of 1.2 mM sodium deoxycholate, 30 mM Tris HCl pH 7.4 and 5 mM of $CaCl_2$ in a total volume of 6 ml. The hydrolytic reaction was initiated by the addition of 5 units of the lipase. The reaction was allowed to progress for 30 min at 37 °C in a shaking waterbath. The reaction was stopped by the addition of 12 ml of chloroform/methanol (2/1, v/v). The phases were

allowed to separate after mixing the tubes. The organic layer of the phase separation was kept and the upper layer was discarded. The solvent in the lower layer was evaporated and the lipid residue was redissolved in chloroform. The products of the hydrolysis were separated by CM-cellulose chromatography. The procedure for the separation of the lipids on CM-cellulose was essentially that described by Comfurius and Zwaal (222). The lysophospholipid produced by this method was certified to be 2-acyl GPE according to the methods outlined by Arthur (158,223). Briefly, by preparing 2-acyl lysophospholipids, then reacylating these lysophospholipids to diacyl phospholipids with a rat liver microsomal acyltransferase system and analysing the radiolabelled products produced upon phospholipase A_2 hydrolysis, it was determined that the radioactivity was associated predominantly with the fatty acid at the sn-2 position. The 2-acyl GPE was stored at -20 °C and used within 24 h of preparation.

3.2.2. Preparation of 1-acyl glycerophosphoethanolamine

1-Oleoyl GPE and 1-arachidonoyl GPE were prepared by a procedure similar to that described by Robertson and Lands (224). This method involves the acylation of a 2-acyl GPE by rat liver microsomal acyltransferases, followed by hydrolysis of the radiolabelled product via a snake venom phospholipase A_2 producing the necessary 1-acyl glycerophosphoethanolamine. In more detail, 2-acyl GPE was prepared by the procedures detailed in section 3.2.1. The 2-acyl LPE was acylated with radiolabelled fatty acyl-CoA using the rat liver microsomes as a source of LPE:acyl CoA acyltransferases to catalyze the acylation. The reaction conditions consisted of 0.02 M KH_2PO_4 buffer pH 7.1, 6 mM ATP, 0.01 M NaF, 5 mM $MgCl_2$, 0.92 mM radiolabelled acyl CoA (either oleoyl or arachidonoyl CoA) and 0.06 mM 2-acyl LPE. The incubation was carried out for 1 hour at 37 °C. The reaction was stopped using the appropriate

amount of chloroform/methanol (2/1, v/v) to yield a phase separation. The products of the acylation reaction in the lower layer were separated by CM-cellulose chromatography as described by Comfurius and Zwaal (222). The fractions containing phosphatidylethanolamine were pooled and subjected to Crotalus adamenteus phospholipase A_2 hydrolysis. The method for hydrolysis of phospholipids by this enzyme is described in section 3.2.7. The 1-acyl LPE product was recovered after another round of CM-cellulose chromatography.

3.2.3. Preparation of phosphatidylcholine substrates

The specific PC substrates used in studies on the guinea-pig heart phospholipase A_1 were prepared by the addition of radiolabelled and unlabelled phosphatidylcholine to a test tube to give the appropriate specific radioactivity (0.2-0.4 Ci/mol). Solvents were evaporated under a stream of nitrogen followed by the addition of 5% (w/v) solution of sodium taurodeoxycholate resulting in a solution with a detergent:lipid ratio of 1 mg detergent:0.5 μmol PC. The volume of the solution was adjusted to 1 ml by the addition of double-distilled water. The phospholipid was dispersed into solution by vortexed for 30-60 seconds.

3.2.4. Preparation of phosphatidylethanolamine substrates

The specific phosphatidylethanolamine substrates used in studies on the guinea-pig heart microsomal PE hydrolysing phospholipase A_1 were prepared in a manner similar to that described in section 3.2.3. Synthesized phosphatidylethanolamine (0.5 μmol was usually the starting amount. Synthesis is described in section 3.2.5.) was added to a test tube and the solvent was evaporated by nitrogen. Sodium taurodeoxycholate was added to the PE substrate such that a solution of 1 mg detergent : 0.5 μmol PE was produced. Double-distilled water was

added to bring the final volume of the solution to 1 ml and the mixture was dispersed by vortexed for 30-60 sec.

3.2.5. Transphosphatidylation of phosphatidylcholine to phosphatidylethanolamine

For the phospholipase A_1 studies with phosphatidylethanolamine substrates, all the substrates were synthesized. The phosphatidylethanolamine substrates were synthesized by phospholipase D catalyzed transphosphatidylation of the corresponding phosphatidylcholine (spec. radioactivity 0.2-0.3 Ci/mol). The procedure used was essentially that described by Comfurius and Zwaal (222).

3.2.6. Preparation of 1-[1-^{14}C]palmitoyl GPE from 1,2-di[1-^{14}C] palmitoyl GPE

The preparation of the 1-acyl lysophospholipid from a diacyl phospholipid is essentially done by performing a phospholipase A_2 catalyzed hydrolysis of the diacylphospholipid. Labelled 1,2-di[1-^{14}C]-palmitoyl GPE and unlabelled dipalmitoyl GPE was added to a screw capped test tube such that the final specific radioactivity of the solution was 0.2 Ci/mol. The total amount of phospholipid in the test tube was 10 μmol. The solvent was evaporated from the phospholipid by a stream of nitrogen. The phospholipid was resuspended in 2 ml of diethyl ether. The tube was capped and vortex mixed for 1-2 minutes to thoroughly dissolve the phospholipid. To this was added 50 μl of a 1 U/μl solution of Crotalus adamenteus venom phospholipase A_2 dissolved in solution of 0.1 M Tris HCl, 0.01 M $CaCl_2$, pH 7.4. The tube was capped, vortexed and incubated for at least three hours at room temperature with shaking. The hydrolytic reaction was stopped by removing the ether layer with nitrogen. The lipid residue was dried down and subsequently resuspended in an aliquot of chloroform. The lysophospholipid was separated from the

diacylphospholipid by silicic acid chromatography.

3.3. Enzyme Assays

3.3.1. Phospholipase A_2 assay

Phospholipase A_2 was measured by determination of the release of radiolabelled fatty acids from 1-acyl-2-[1-^{14}C]-arachidonoyl glycerophosphoethanolamine. The reaction mixture contained 200 μM of the radiolabelled substrate (spec. radioactivity 0.2-0.3 Ci/mol), 200 μg of guinea-pig heart microsomal protein, 100 mM Tris HCl pH 8.5, and double-distilled water to make a total volume of 500 μl. The reaction was started by the addition of the substrate and incubated for 10 min at 37 °C in a shaking waterbath. The reaction was stopped by the addition of 3 ml of 2/1 chloroform/methanol (v/v) and 1 ml 0.9% KCl (w/v) to the test tubes. The reaction products were isolated by t.l.c. and identified in a manner similar to that described for the assay of the lysophospholipase A_1.

3.3.2. Lysophospholipase A_1 assay

Lysophospholipase A_1 was measured by determining the release of fatty acid from 1-acyl GPE. The assay mixture consisted of 100 mM Tris HCl pH 8.0, 100 μg of guinea-pig heart microsomal protein and 125 μM of 1-[^{14}C or ^{3}H]-acyl GPE (spec. radioactivity 0.1-0.2 Ci/mol) in a total volume of 500 μl. The reaction was initiated by the addition of the substrate and incubated at 37 °C for 15 min in a shaking waterbath. After 15 min, the progress of the reaction was stopped by the addition of 3 ml of 2/1 chloroform/methanol (v/v) and 1 ml of 0.9% KCl (w/v). The phases were allowed to separate after vortex-mixing and centrifugation. Aliquots of the lower layer were removed for the determination of free fatty acid formed in the reaction. The free

fatty acid was isolated and quantitated after separation of the reaction products by t.l.c. in a solvent system of 60/40/4 heptane/isopropyl ether/acetic acid (by volume). The radioactivity associated with the fatty acid band was determined by scintillation counting.

3.3.3. Lysophospholipase A_2 assay

The assay of the lysophospholipase A_2 activity was essentially performed in a manner similar to that described for the lysophospholipase A_1 (Sec. 3.3.2.). The assay mixture contained 100 mM Tris HCl pH 8.0, 90 μg of guinea-pig heart microsomal protein and 200 μM of 2-[1-^{14}C]-acyl GPE (spec. radioactivity 0.15-0.2 Ci/mol) in a total volume of 500 μl. The assay was carried out at 37 °C for 10 min. The reaction was stopped by the addition of 3 ml of chloroform/methanol (2/1, v/v) and 1 ml of 0.9% KCl (w/v). The reaction products were identified as described in section 3.3.1. for the lysophospholipase A_1 assay. The radiolabel associated with the free fatty acid fraction was determined by scintillation counting.

3.3.4. Phospholipase A_1 assays with phosphatidylcholine substrate

Phospholipase A_1 activity was assayed by measuring the rate of formation of radiolabelled lysophosphatidylcholine from phosphatidylcholine labelled at the sn-2 fatty acid. The assay mixture consisted of 300 μM phosphatidylcholine (spec. radioactivity 0.2-0.4 Ci/mol), 100 μg of guinea-pig heart microsomal protein and 100 mM Tris HCl pH 8.0, in a total volume of 500 μl. The reaction was initiated by the addition of the substrate. The reaction was allowed to progress for 10 min at 37 °C until it was terminated by the addition of 3 ml chloroform/methanol (2/1, v/v) and 1 ml 0.9% KCl (w/v). Aliquots of the lower layer were taken after phase separation and used to determine the radioactivity in the lysophosphatidylcholine fraction. A fraction of the

lower layer was dried under nitrogen and the remaining lipid residue was redissolved in a small volume of chloroform and applied to a t.l.c. plate. The t.l.c. plate was developed in a solvent system of chloroform/methanol/water acetic acid (35/15/2/1, by volume). The bands were visualized after exposure of the t.l.c. plate to iodine vapour and the band corresponding to lysophosphatidylcholine was scrapped into a scintillation vial. The radioactivity associated with the LPC fraction was determined by scintillation counting.

3.3.5. Phospholipase A_1 assay with phosphatidylethanolamine substrate

This assay was performed in much the same way that for phospholipase A_1 activity with the phosphatidylcholine as the substrate (section 3.3.4.). The reaction conditions are 100 mM Tris HCl pH 9.0, 100 μg guinea-pig heart microsomal protein and 200 μM of the phosphatidylethanolamine substrate (spec. radioactivity 0.2 Ci/mol) in a total volume of 500 μl. Incubations were carried out for 10 min at 37 °C. The reaction was started by the addition of the substrate. After the 10 min time period, the reaction was quenched with chloroform/methanol (2/1, v/v) and 0.9% KCl (w/v) as described in section 3.3.4. The products of the hydrolysis were separated by t.l.c. on heat activated t.l.c. plates (110 °C for 1 hour) and subsequent development of the plate in a solvent of chloroform/methanol/water/acetic acid (100/75/4/7, by volume). The phospholipid band corresponding to LPE was scraped into scintillation vials and the radioactivity associated with the LPE fraction was determined by scintillation counting.

3.3.6. Pancreatic phospholipase A_1 assay

This assay is similar to that described by Fauvel et al. (34). The assay was carried out as follows: in a total volume of 500 μl, 0.2 M Tris HCl pH 8.0, 2.4 mM

sodium deoxycholate and an aliquot of the enzyme source to be assayed were combined. The reaction was initiated by the addition of 300 μM 1-palmitoyl-2-[1-^{14}C]-linoleoyl glycerophosphocholine (specific radioactivity 0.2 Ci/mol). The reaction was carried out at 37 °C for 15 min in a shaking waterbath. After 15 min, the reaction was halted by the addition of 3 ml of 2/1 chloroform/methanol (v/v). To further clarify the phase separation, 1 ml of 0.9% KCl (w/v) was added and the tubes were mixed and subsequently centrifuged in a bench centrifuge. After centrifugation, the upper layer was removed and the lower layer was kept. Carrier phospholipids were added to all the samples and the reaction products were separated by thin-layer chromatography. The t.l.c. plates were developed in two solvents but in only one direction. First, the t.l.c. plate was developed in a solvent of 70/30/4/2 chloroform/methanol/water/acetic acid (by volume) for approximately 8 cm from the bottom of the plate. The t.l.c. plate was then allowed to air dry and then developed in the same direction in a second solvent of 60/40/4 heptane/isopropyl ether/acetic acid (by volume). The products of the hydrolysis were visualized with iodine. The bands corresponding to fatty acid and lysophosphatidylcholine were scraped into scintillation vial and the radioactivity associated with these fractions was determined by scintillation counting.

3.4. Preparation of phospholipase A_1 from guinea-pig pancreas

The purification of phospholipase A_1 from guinea-pig pancreas cytosol was a modification of a procedure described by Fauvel et al. (34). The frozen guinea-pig pancreas was thawed and the fat was trimmed off. The pancreas was then washed in cold 0.1 M Tris HCl pH 9.0 buffer and cut into small pieces in this buffer. The minced pancreas was homogenized in approximately 5 volumes of the Tris buffer

using a Polytron homogenizer. The resulting homogenate was centrifuged at 200 x g for 10 min at 4 °C to remove unbroken cells. The supernatant was decanted and kept on ice while the pellet was resuspended in buffer and rehomogenized and centrifuged at 200 x g for 10 min. The pellets were discarded and the supernatants from both centrifugations were combined. This pooled supernatant was centrifuged at 100,000 x g for 1 hour to obtain pancreatic cytosol. The supernatant from the ultracentrifugation was combined with an equal volume of DEAE-Sepharose in 0.05 M Tris HCl pH 9.0 with 20 % glycerol (v/v) in a batch adsorption step. This mixture was stirred gently overnight at 4 °C. The DEAE-Sepharose was then centrifuged at 200 x g for 20 min at 4 °C to separate the non-adsorbed protein. All the supernatants were collected, pooled and this procedure was repeated for another 4 hours. The mixture was again centrifuged and the supernatant collected and concentrated with flake polyethylene glycol (Aquacide III). The concentrate was applied to a column of DEAE-Sepharose that had been previously equilibrated with 0.05 M Tris HCl pH 9.0 buffer. The column was eluted with the same 0.05 M Tris HCl pH 9.0 buffer. Column fractions were collected and assayed for phospholipase A_1 activity (see section 3.3.5. for details on the assay). Fractions containing the highest phospholipase A_1 activity were combined and concentrated with Aquacide III before the volume was adjusted to 50% (v/v) with glycerol. The enzyme was stored at -20 °C.

3.5. Analytical procedures

3.5.1. Determination of lipid phosphorous

The lipid phosphorous content of phospholipid samples was determined by the method of Bartlett (225). This procedure is based upon the release of inorganic phosphate by acid digestion of the sample, followed by the colorimetric determination

of this phosphate as a phosphomolybdate complex. An aliquot of the sample was dried down under nitrogen. Perchloric acid (1.1 ml) was added to all the tubes including those for the determination of a calibration curve. The tubes were incubated in a block heater at 160 °C for approximately 2 hours. The tubes were allowed to cool to room temperature. To the tubes for calibration curve was added an appropriate amount of inorganic phosphorous from a standard stock solution 10 μg/ml potassium phosphate to produce a range of Pi concentrations. The total volume of each tube was made up to 9 ml with double-distilled water. The tubes were vortexed and each tube was then added 800 μl of 5% ammonium molybdate (w/v) followed by mixing. ANSA solution (200 μL) was added to each tube. The tubes were subsequently mixed again and placed in a boiling water for 10 min. The tubes were allowed to cool to room temperature and the absorbance was measured at 830 nm. The ANSA reagent was prepared by dissolving 11.7 g of anhydrous sodium sulfite, 80 mg sodium metabisulfite and 0.2 g of ANSA in 100 ml of water. This solution is stable in the dark.

3.5.2. Determination of protein concentration

The protein concentrations of subcellular fractions were determined by the method of Lowry et al. (221) using bovine serum albumin (BSA) as a standard. Aliquots of the samples or BSA (0-100 μg) were added to test tubes. The volume in each tube was made up to 1 ml with the addition of 100 μl of 5% sodium deoxycholate (w/v) and double-distilled water. Subsequently 4 ml of a solution C (1/50 solution A/solution B, v/v) was added to each tube followed by mixing. Solution A was made by mixing 1 ml of 1% copper sulphate (w/v) and 1 ml of 2% sodium potassium tartrate (w/v). Solution B was 2% sodium carbonate (w/v) in 0.1 M sodium

hydroxide. The tubes were incubated at room temperature for 10 min and then 0.5 ml of 1 N Folin-Cicolteau phenol reagent was added to each tube. The tubes were vortexed and immediately incubated in a 60 °C waterbath for 10 min. The tubes were cooled to room temperature prior to determining the absorbance at 730 nm.

3.5.3. Determination of radioactivity

Radioactivity was determined by scintillation counting using a Beckman LS3801 counter with Ecolite (ICN Biochemicals Ltd.) as the scintillant.

3.5.4. Statistical Analysis

Statistical significance between groups was assessed by the unpaired "t" test as described by Huntsberger and Billingsley (226).

4. Results

4.1. Lysophospholipase A_2 activity with 2-acyl GPE in guinea-pig heart microsomes.

As an initial step in our study on a postulated phospholipase A_1/lysophospholipase A_2 pathway that functions for the selective release of fatty acids for eicosanoid biosynthesis in the guinea-pig heart, the characteristics of the lysophospholipase A_2, were investigated. The presence of a 2-acyl GPE hydrolyzing lysophospholipase has not been reported in the heart or any other tissue. We were interested in determining if this activity was present in guinea-pig heart microsomes. The incubation of 2-[^{14}C]-arachidonoyl GPE with heart microsomes resulted in the release of radiolabelled arachidonic acid. The activity that hydrolyzed 2-arachidonoyl GPE was further characterized with respect to conditions that optimized the release of the fatty acid. The activity had an optimal pH of 9.0 (Fig. 4) and an optimal substrate concentration of 150-300 μM (Fig. 5). The reaction rate was linear up to 15 minutes with 90 μg of guinea-pig heart microsomal protein. In contrast to these results, experiments with 2-linoleoyl GPE revealed that the lysophospholipase A_2 had an optimal substrate concentration of 50 μM. Increasing concentrations of 2-linoleoyl GPE greater than 50 μM greatly inhibited the enzyme activity. With 100 μM of 2-linoleoyl GPE, there was 40% less lysophospholipase A_2 activity than was obtained with 50 μM of 2-linoleoyl GPE. The enzyme activity did not display such kinetics with any of the other 2-acyl glycerophosphoethanolamine lipids used. The lysophospholipase A_2 activity was active in the presence of EDTA and EGTA. The heart microsomal lysophospholipase A_2 activity is therefore Ca^{2+}-independent. Moreover, the addition of

Figure 4

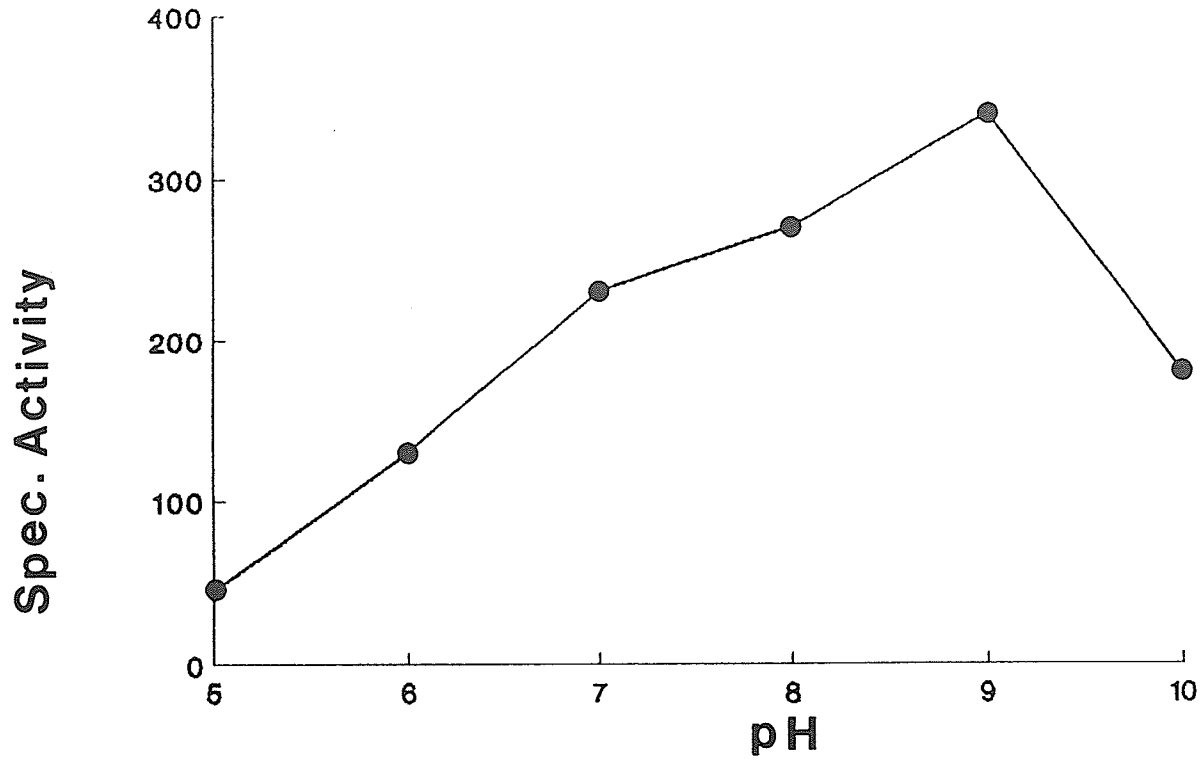

Fig. 4. The effect of pH on guinea-pig heart microsomal lysophospholipase A_2 activity.

The activity of the heart lysophospholipase A_2 was measured at different pH values with 100 μg microsomal protein, 100 nmol of 2-arachidonoyl GPE and buffer in a total volume of 500 μl. The buffers used were Tris-succinate (pH 5-6) and Tris-HCl (pH 7-10). Incubation and isolation of the reaction products are as described in the Materials and Methods section. The values represent the means of two experiments each done in triplicate. The standard deviation of all the values are less than 10% of the mean. The specific activity is expressed as nmol arachidonic acid released/hr/mg protein.

Figure 5

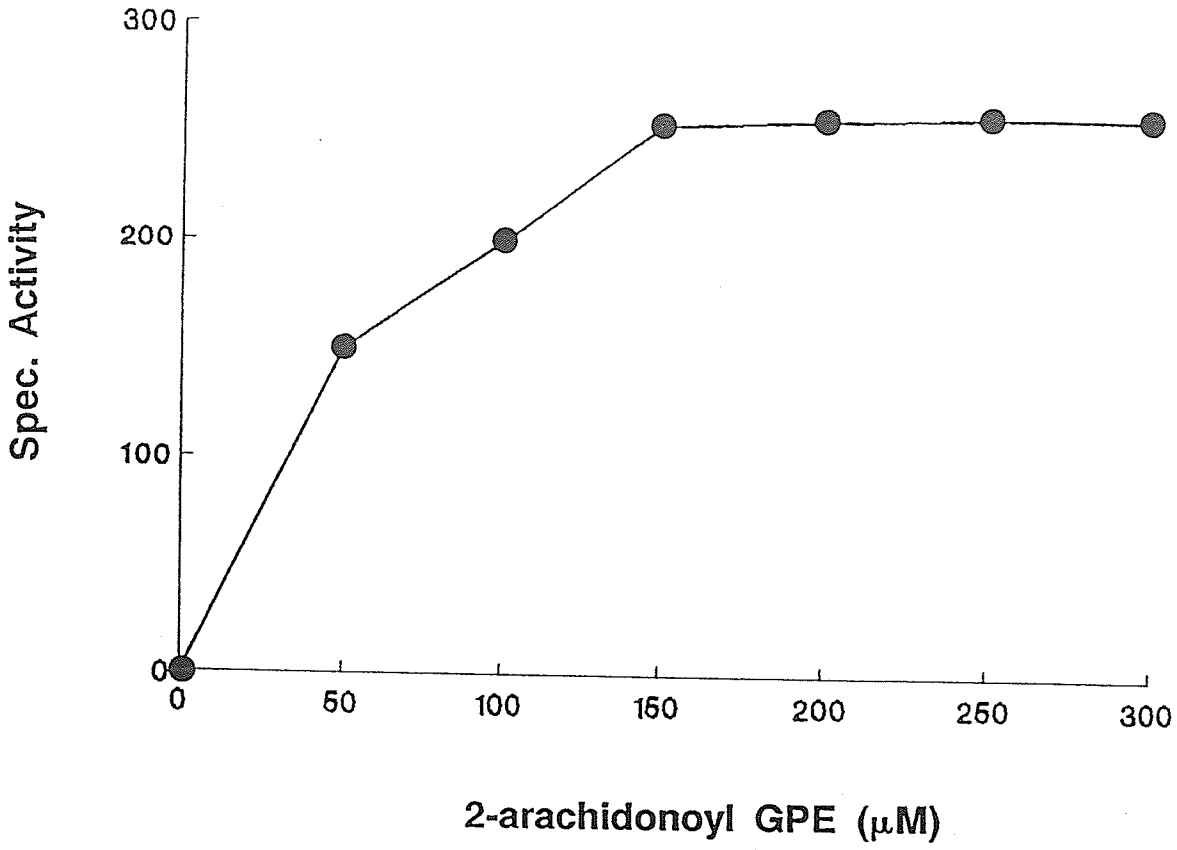

Fig. 5. The effect of 2-arachidonoyl GPE concentration on the guinea-pig heart lysophospholipase A_2 activity.

The activity of the guinea-pig heart microsomal lysophospholipase A_2 was determined as a function of the 2-arachidonoyl GPE concentration. The assay mixture contained 90 μg of microsomal protein with 100 mM Tris-HCl buffer pH 8.5 in a total volume of 500 μl. Incubation was for 10 min at 37 °C. The reaction products were isolated as described in the Materials and Methods section. The values represent the mean of two experiments each done in triplicate. The standard deviation of all the values is less than 10% of the mean. The specific activity is expressed as nmol arachidonic acid formed/hr/mg protein.

Figure 6

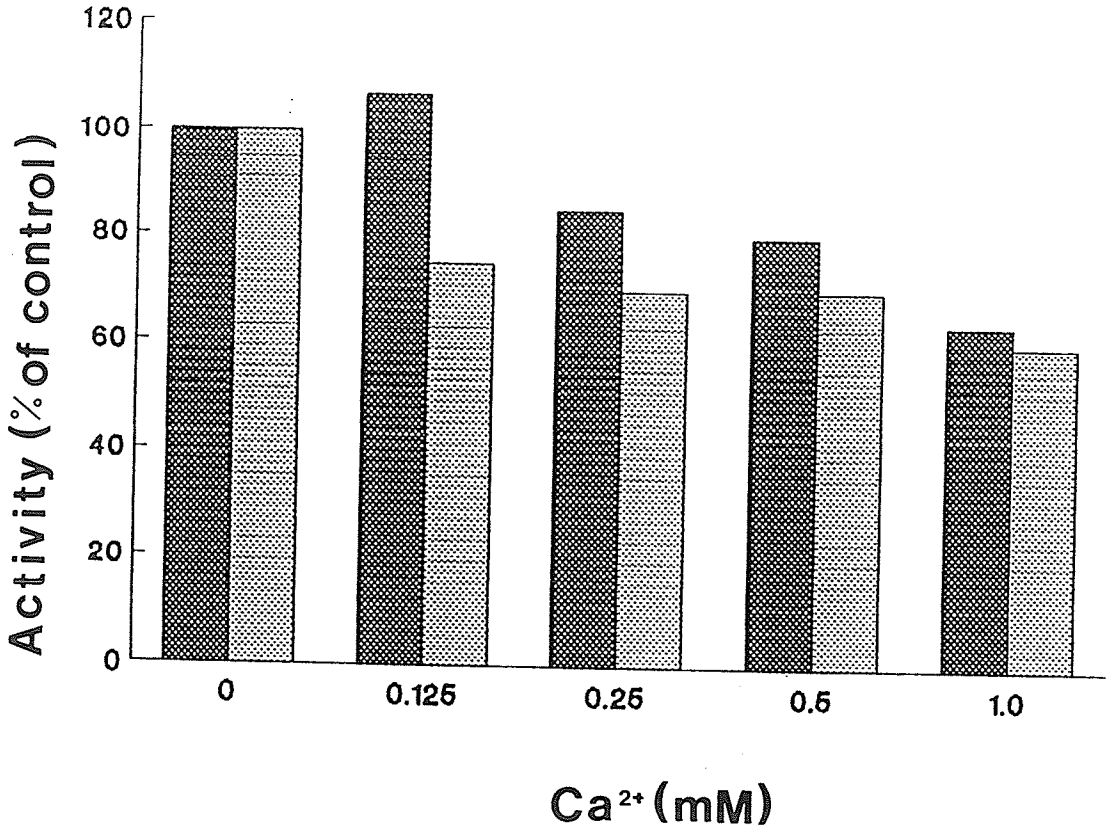

Fig. 6. Effect of Ca^{2+} on the guinea-pig heart 1-acyl GPE and 2-acyl GPE hydrolyzing activities in guinea-pig heart microsomes.

Lysophospholipase A_1 activity (dark bars) was assayed with 62.5 μM 1-palmitoyl GPE and lysophospholipase A_2 activity (light bars) was assayed with 2-arachidonoyl GPE (200 μM). The reaction conditions used for each activity are described in the Materials and Methods section. The results are expressed as a percentage of the activities obtained in the presence of 2 mM EGTA (control). The values are the means of two separate experiments each done in duplicate. In all cases, the standard deviation was less than 10% of the mean.

Ca^{2+} to the reaction mixture resulted in the inhibition of lysophospholipase A_2 activity (Fig. 6).

To determine if the guinea-pig heart microsomal lysophospholipase A_2 activity exhibited any selectivity for different molecular species of the 2-acyl GPE, the rate of hydrolysis of 2-arachidonoyl GPE, 2-oleoyl GPE and 2-palmitoyl GPE was compared at a substrate concentration of 200 μM. 2-Linoleoyl GPE was not compared in this set of experiments since with 200 μM of this substrate the activity of the lysophospholipase A_2 was greatly inhibited. The order of hydrolysis of the substrates relative to that of 2-palmitoyl GPE was as follows: 2-arachidonoyl GPE > 2-oleoyl GPE > 2-palmitoyl GPE, the activities with the substrates were respectively 10-fold greater and 2-fold greater than the hydrolysis of 2-palmitoyl GPE (Table 1). To be sure that these differences in activity were not due to differences in solubilities of the substrates, the effect of various detergents on the hydrolysis of 2-arachidonoyl GPE was investigated (Table 2). All of the detergents except Triton QS-15 (0.5%, w/v) inhibited the activity of the lysophospholipase A_2. Triton QS-15 at a concentration of 0.5% actually activated the activity of the lysophospholipase A_2 by 84%. Hence, the acyl specificity was determined in the presence of 0.5% Triton QS-15. The results of this experiment are shown in Table 3. Again, the enzyme displayed highest activity with 2-arachidonoyl GPE as the substrate. The lysophospholipase A_2 activity with 2-arachidonoyl GPE was 20-fold greater than that with 2-palmitoyl GPE and approximately 7-fold greater than that with 2-oleoyl GPE. The acyl specificity of the lysophospholipase A_2 was similar in the absence and presence of detergent. Identical experiments were performed using the substrates 2-arachidonoyl GPE, 2-linoleoyl GPE, and 2-palmitoyl GPE at concentrations of 50 μM in the presence and absence of the

Table 1. Acyl specificity of guinea-pig heart microsomal lysophospholipase A_2 in the absence of detergent.

Lysophospholipase A_2 activity in guinea-pig heart microsomes (90 μg) was assayed with different 2-acyl GPEs (200 μM; spec. radioactivity 0.15-0.2 Ci/mol) in 100 mM Tris-HCl, pH 8.5. In these experiments the assays were performed essentially as described in the Materials and Methods section. Values represent the means and standard deviations of three experiments each done in triplicate.

Substrate	Specific activity (nmol fatty acid/hr/mg protein)	Activity relative to 2-palmitoyl GPE hydrolysis
2-Palmitoyl GPE	56 ± 7	1
2-Oleoyl GPE	135 ± 8	2
2-Arachidonoyl GPE	566 ± 19	10

Table 2. Effect of detergents on guinea-pig heart lysophospholipase A_1 and A_2 activities.

Guinea-pig heart microsomal lysophospholipase A_1 and A_2 activities were assayed with 1-palmitoyl GPE and 2-arachidonoyl GPE respectively. Detergents were added to the incubation mixture to give a final concentration of 0.5%. Other reaction conditions are described in the Materials and Methods section. Identical incubations without detergent served as a control. The values represent the means and standard deviation of two experiments each of which was conducted in triplicate. The specific activity of the lysophospholipase A_1 in the absence of detergent was 82 nmol fatty acid released/hr/mg protein and the specific activity of the lysophospholipase A_2 in the absence of detergent was 452 nmol fatty released/hr/mg protein.

Detergent	Lysophospholipase activity (% of control)	
	A_1	A_2
None	100	100
Miranol DS	84 ± 21	26 ± 4
Triton X-100	33 ± 12	7 ± 0.1
Taurocholate	29 ± 6	18 ± 2
Triton QS-15	430 ± 62	184 ± 22

Table 3. Acyl specificity of guinea pig heart microsomal lysophospholipase A_2 in the presence of 0.5% Triton QS-15.

Lysophospholipase A_2 activity in guinea-pig heart microsomes was assayed with different 2-acyl GPEs (200 μM) in the presence of Triton QS-15 (0.5%) in 100 mM Tris-HCl pH 8.5 in a total volume of 500 μl. The substrate was prepared by sonication in the detergent solution. The assay was performed as detailed in the Materials and Methods section. The values are the means and standard deviation of three experiments done in triplicate.

Substrate	Specific activity (nmol fatty acid/hr/mg protein)	Activity relative to 2-palmitoyl GPE hydrolysis
2-Palmitoyl GPE	51 ± 5	1
2-Oleoyl GPE	151 ± 3	3
2-Arachidonoyl GPE	1039 ± 47	20

Table 4. Acyl specificity of guinea-heart microsomal lysophospholipase A_2 with 50 μM substrate in the absence of detergent.

Lysophospholipase A_2 activity was assayed with 50 μM of various 2-acyl GPE as substrates. The reaction conditions and procedure for the assay are described in the Materials and Methods section. The values are the means and standard deviation of three experiments each done in triplicate. The specific activity is expressed as nmol fatty acid released/hr/mg protein.

Substrate	Specific activity	Activity relative to 2-palmitoyl GPE hydrolysis
2-Palmitoyl GPE	65 ± 26	1
2-Linoleoyl GPE	370 ± 18	6
2-Arachidonoyl GPE	377 ± 42	6

Table 5. Acyl specificity of the guinea-pig heart microsomal lysophospholipase A_2 with 50 μM substrate in the presence of detergent.

The lysophospholipase A_2 activity in guinea-pig heart microsomes was assayed with various 2-acyl GPE in the presence of 0.5% Triton QS-15. The substrates were prepared by sonication in the detergent solution. The conditions for the assay and the method of quantification of the fatty acid released were that detailed in the Methods and Materials section. The values represent the means ± standard deviation of three experiments each of which was done in triplicate. The specific activity is expressed as nmol fatty acid released/hr/mg protein.

Substrate	Specific Activity	Activity relative to 2-palmitoyl GPE hydrolysis
2-Palmitoyl GPE	71 ± 13	1
2-Linoleoyl GPE	734 ± 38	10
2-Arachidonoyl GPE	694 ± 15	10

detergent Triton QS-15. The results of these experiments are shown in Tables 4 and 5. The inhibition of lysophospholipase A_2 activity with concentrations of 2-linoleyl GPE greater than 50 μM was also observed in the presence of detergent. When the acyl specificity was determined with 50 μM of substrate in the absence of detergent, the rates of hydrolysis of 2-arachidonoyl GPE and 2-linoleoyl GPE were very similar. The rates of hydrolysis of either substrate was still 6-fold greater than the rate hydrolysis of 2-palmitoyl GPE. In the presence of the detergent, this acyl specificity for 2-arachidonoyl GPE and 2-linoleoyl GPE was maintained as the rates of hydrolysis of both these substrates were 10-fold faster than the rate of hydrolysis of 2-palmitoyl GPE.

In order to determine if the specificity of the enzyme for 2-arachidonoyl GPE was still manifested in a mixture of substrates, the effect of presenting equimolar amounts of a pair of 2-acyl GPE was examined. Two pairs of substrates were used in these experiments: 2-arachidonoyl GPE/2-palmitoyl GPE and the pair of 2-linoleoyl GPE/2-arachidonoyl GPE. The results of this experiment are shown in Table 6. As can be seen from the results with the 2-palmitoyl/2-arachidonoyl GPE, the lysophospholipase A_2 maintained its specificity for 2-arachidonoyl GPE in the presence of 2-palmitoyl GPE. However, it was surprising that the enzyme activity with 2-arachidonoyl GPE was inhibited by 2-palmitoyl GPE. This result was unexpected given the high activity that the enzyme had demonstrated with 2-arachidonoyl GPE when it was presented singly. 2-Palmitoyl GPE hydrolysis was inhibited by the presence of 2-arachidonoyl GPE relative to a control with 2-palmitoyl GPE alone. The mutual inhibition observed with the pair of 2-palmitoyl GPE/2-arachidonoyl GPE was also observed with the 2-linoleoyl GPE/2-arachidonoyl GPE pair. The hydrolysis of both

Table 6. Effect of presenting mixtures of 2-acyl GPE on guinea-pig heart microsomal lysophospholipase A_2 activity.

Lysophospholipase A_2 activity was assayed with 90 μg of guinea-pig heart microsomal protein and the following pairs of 2-acyl GPE: 2-[^{14}C]-palmitoyl GPE/2-arachidonoyl GPE, 2-palmitoyl GPE/2-[^{14}C]-arachidonoyl GPE and 2-[^{14}C]-linoleoyl GPE/2-arachidonoyl GPE and 2-linoleoyl GPE/2-[^{14}C]-arachidonoyl GPE. Equimolar amounts of each substrate were sonicated together, and aliquots containing 50 μM of each substrate were used in the assays. Control tubes contained 50 μM of each individual substrate and their values are reported in Table 4. The assay was carried out as described in the Methods and Materials section. The values represent the means and standard deviation of two experiments each of which was done in triplicate. The specific activity is expressed as nmol fatty acid/hr/mg protein.

Pairs of substrates	Specific activity	Activity (% of control)
2-Palmitoyl GPE and	35 ± 6	54
2-Arachidonoyl GPE	165 ± 14	44
2-Linoleoyl GPE and	207 ± 9	56
2-Arachidonoyl GPE	219 ± 23	58

lysolipids was inhibited by approximately 40% by the presence of the other lysolipid. There was no enhancement of selective hydrolysis of either 2-acyl GPE when 2-linoleoyl GPE and 2-arachidonoyl GPE are presented together.

Preliminary results had indicated that the lysophospholipase A_2 activity was ubiquitously distributed in guinea-pig tissues. The acyl specificity of lysophospholipase A_2 activity in guinea-pig liver, lung, kidney, and brain microsomes was examined. These experiments were conducted using the same assay conditions that had been established for lysophospholipase A_2 activity in the heart microsomes. Triton QS-15 (0.5%) did not inhibit the activity of the lysophospholipase A_2 in any of these tissues (Table 7). The lysophospholipase A_2 activity was determined with 2-oleoyl GPE and 2-arachidonoyl GPE in the presence and absence of 0.5% Triton QS-15 (Table 8). In all tissues, the rate of hydrolysis of 2-arachidonoyl GPE was greater than the rate of hydrolysis of 2-oleoyl GPE whether in the presence or absence of detergent. The highest lysophospholipase A_2 activity was observed in brain microsomes. As with the heart microsomal lysophospholipase A_2, the Triton QS-15 activated the lysophospholipase A_2 activity in lung and brain microsomes but not the liver or kidney enzymes with 2-arachidonoyl GPE as the substrate. The reason for this is unclear.

Lysophospholipases, which hydrolyze 1-acyl lysophospholipids, are known to be present in the heart (see section 1.3.5, 154,158,159). It was therefore necessary to determine if the lysophospholipase A_2 that had been characterized was distinct from any lysophospholipase A_1 that hydrolyzed 1-acyl GPE in guinea-pig heart microsomes. Using 1-[^{14}C]-palmitoyl GPE as the substrate, we discovered that guinea-pig heart microsomes contained a lysophospholipase A_1 activity. This activity had an optimal pH of 8.0 (Fig. 7) and an optimal substrate concentration range of 45-150 μM (Fig. 8).

Table 7. The effect of 0.5% Triton QS-15 on guinea-pig microsomal lysophospholipase A_2 in various tissues.

The effect of the detergent Triton QS-15 (0.5%) on lung, liver, brain, and kidney microsomal lysophospholipase A_2 activity was assessed. The method for the assay is the same as that described in the Materials and Methods section. The substrate used was 2-arachidonoyl GPE (200 μM). The assay was initiated by the addition of the substrate. The results represent the means ± standard deviation of two separate experiments each assayed in duplicate. The results are expressed as a percentage of the activity of a control did not contain any detergent.

Tissue	Activity (% of control)
Liver	144 ± 14
Kidney	120 ± 25
Brain	216 ± 58
Lung	198 ± 17

Table 8. Acyl Specificity of lysophospholipase A_2 in different guinea-pig tissues.

Lysophospholipase A_2 activity in the microsomal fractions of various guinea-pig tissues was assayed with 2-oleoyl GPE and 2-arachidonoyl GPE (200 μM). The reaction conditions used were identical to those described for the assay of the heart microsomal lysophospholipase A_2 as outlined in the Methods and Materials section. The values represent the means and standard deviation of three experiments each done in duplicate. The substrate was prepared with and without Triton QS-15 (0.5%). The specific activity is expressed as nmol fatty acid released/hr/mg protein.

	Specific Activity			
Substrate	2-Oleoyl GPE		2-Arachidonoyl GPE	
Tissue	-QS-15	+QS-15	-QS-15	+QS-15
Lung	63 ± 14	61 ± 38	267 ± 78	487 ± 98
Liver	53 ± 28	55 ± 16	180 ± 28	219 ± 29
Brain	165 ± 12	220 ± 87	650 ± 15	1192 ± 236
Kidney	54 ± 16	50 ± 14	141 ± 32	155 ± 15

Figure 7

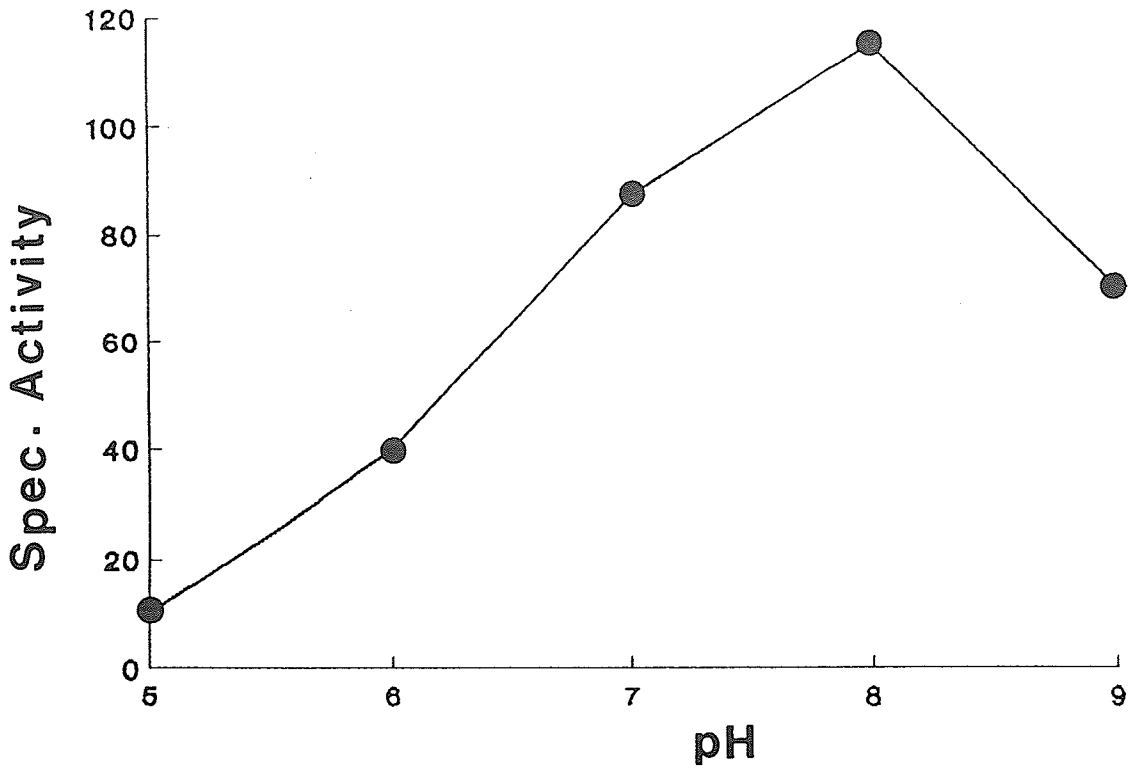

Fig. 7. The effect of pH on the guinea-pig heart microsomal lysophospholipase A_1 activity.

The activity of guinea-pig heart lysophospholipase A_1 was determined at different pH values using the buffers described in Figure 4. The reaction mixture contained 100 mM of buffer 100 μg guinea-pig heart microsomal protein and 100 nmol of 1-palmitoyl GPE in a total reaction volume of 500 μl. The isolation of the reaction products are as described in the Materials and Methods section. The activity is expressed as nmol palmitic acid produced/hr/mg protein. The values are the means of two experiments each of which was conducted in triplicate. The standard deviation of the values was less than 15% of the mean in all cases.

Figure 8

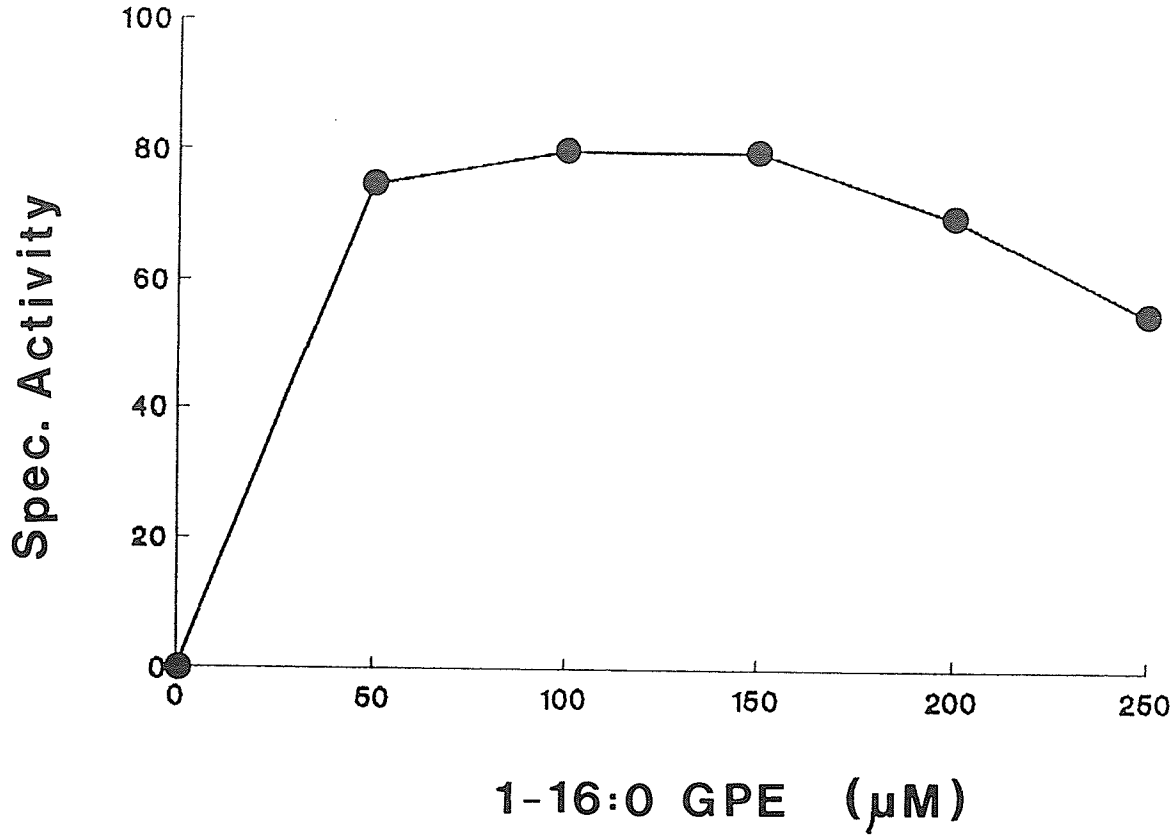

Fig. 8. The effect of 1-palmitoyl GPE concentration on the guinea-pig heart microsomal lysophospholipase A_1 activity.

The activity of the guinea-pig heart microsomal lysophospholipase A_1 was measured as a function of the substrate concentration. The assay mixture consisted of 100 mM Tris-HCl buffer, pH 8, 100 μg of heart microsomal protein and varying amounts of 1-palmitoyl GPE in a total volume of 500 μl. The assay was carried out as described in the Materials and Methods section. The specific activity is expressed as nmol palmitic acid produced/hr/mg protein. The values in each figure represent the means of two experiments each done in triplicate. In all cases, the standard deviation for each point was less than 15% of the mean.

Concentrations of substrate greater than 150 μM were found to inhibit the enzyme activity. The lysophospholipase A_1, like the lysophospholipase A_2, was active in the presence of cation chelators EDTA or EGTA (2 mM). The lysophospholipase A_1 was also inhibited by increasing concentration of Ca^{2+} less than 125 μM (Fig. 6). At a concentration of Ca^{2+} greater than 125 μM, the lysophospholipase A_1 is not affected by Ca^{2+}, whereas the lysophospholipase A_2 activity is inhibited. The effect of detergents on the lysophospholipase A_1 activity was also determined (Table 2). Like the lysophospholipase A_2 activity, the lysophospholipase A_1 activity was also inhibited by all the detergents to varying degrees except for Triton QS-15 (0.5%). Triton QS-15 stimulated the hydrolysis of 1-palmitoyl GPE by guinea-pig heart microsomes by approximately 4-fold. In addition, the inhibition of the lysophospholipase A_1 activity by Miranol DS was only slight (15% relative to a control), unlike the 75% inhibition observed on the lysophospholipase A_2 activity.

Next, the acyl specificity of the guinea-pig heart microsomal lysophospholipase A_1 was determined. The results of this experiment are shown in Table 9. The enzyme displayed highest activity with 1-palmitoyl GPE. The rate of hydrolysis of 1-palmitoyl GPE was 16 times faster than the rate of hydrolysis of 1-arachidonoyl GPE and approximately 9 times faster than the rate of hydrolysis of 1-oleoyl GPE. The rate of hydrolysis of 1-palmitoyl GPE by the lysophospholipase A_1 was 5-6-fold greater than the rate of hydrolysis of 2-palmitoyl GPE by the lysophospholipase A_2.

Finally, to be sure that the lysophospholipase A_2 activity that was characterized in the heart microsomes was not due to an unspecific activity of a Ca^{2+}-independent phospholipase A_2, the effects of Ca^{2+} and trifluoperazine on the lysophospholipase A_2 and phospholipase A_2 activity under assay conditions that were optimal for the

Table 9. Acyl Specificity of guinea-pig heart microsomal lysophospholipase A_1.

Guinea-pig heart microsomal lysophospholipase A_1 activity was measured with different species of 1-acyl GPE (125 μM; spec. radioactivity 0.1 Ci/mol) with Triton QS-15 (0.5%). All other reaction conditions are as described in the Methods and Materials section. The values are the means and standard deviation of three experiments, each done in triplicate. The specific activity is expressed as nmol fatty acid released/hr/mg protein.

Substrate	Specific activity	Activity relative to 2-arachidonoyl GPE hydrolysis
1-Palmitoyl GPE	394 ± 9	16
1-Oleoyl GPE	45 ± 6	2
1-Arachidonoyl GPE	24 ± 14	1

lysophospholipase A_2 were compared. The results of this experiment are shown in Table 10. At concentrations of 0.5 and 1 mM Ca^{2+} there was no significant effect on the phospholipase A_2 activity whereas, the activity of the lysophospholipase A_2 was inhibited by 40% under the same conditions. There was also a differential effect of trifluoperazine on the two enzyme activities. At a concentration of 0.5 mM, trifluoperazine inhibited the phospholipase A_2 activity but stimulated the lysophospholipase A_2 activity by 100%. The results of this experiment suggest that in guinea-pig heart microsomes, the phospholipase A_2 and lysophospholipase A_2 activities are distinct.

Table 10. Effect of Ca^{2+} and trifluoperazine on guinea-pig heart microsomal lysophospholipase A_2 and phospholipase A_2 activities.

Lysophospholipase A_2 and phospholipase A_2 activities in guinea-pig heart microsomes were determined as described in the Materials and methods section with 2-arachidonoyl GPE (200 μM) and 1-acyl-2-arachidonoyl GPE (200 μM) as the substrates. There was a period of preincubation in experiments with trifluoperazine (TFP), the microsomes were incubated with TFP at room temperature for 5 min before the initiation of the assay. The results are the means and standard deviations of two experiments performed in triplicate. The activities are expressed as percentage of a controls with either no TFP or with EGTA and EDTA (2 mM).

	Activity (% of control)	
Additive	Phospholipase A_2	Lysophospholipase A_2
None (control)	100	100
Ca^{2+} (0.5 mM)	88 ± 3	65 ± 7
Ca^{2+} (1.0 mM)	94 ± 4	60 ± 10
TFP (100 μM)	101 ± 13	135 ± 37
TFP (500 μM)	76 ± 11	228 ± 27

4.2. The regulation and characteristics of a PC-hydrolyzing phospholipase A_1 in guinea-pig heart microsomes.

It has been established previously that a lysophospholipase A_2 activity that hydrolyzes 2-acyl GPC exists in guinea-pig heart microsomal subcellular fractions (sec. 1.3.6., 158). This enzyme was Ca^{2+}-independent and demonstrated a preference for 2-linoleoyl GPC as its substrate. The characteristics of this enzyme led to the suggestion that this 2-acyl GPC lysophospholipase could work in concert with a phospholipase A_1 to release linoleic and arachidonic acids for further metabolism. However, the selective release of a single fatty acid would depend upon the concentration of the 2-acyl GPCs that were present. A large and very specific release of a particular fatty acid could occur if there was a large amount of one molecular species of 2-acyl GPC. 2-Acyl GPCs are generated by phospholipase A_1 hydrolysis of diacyl phosphatidylcholine. It was not known if phospholipase A_1 activity in the guinea-pig heart or any other tissue was selective for substrates with specific fatty acids at the sn-2 position. If the phospholipase A_1 functions in the selective release of fatty acids then the activity of the enzyme would be expected to be modulated by external stimuli acting on membrane receptors. While there is evidence for G protein modulation of phospholipases in the literature, there is no evidence of regulation of phospholipases A_1 by such proteins (227). In this next section of the study, the specificity of the phospholipase A_1 was investigated with respect to defined molecular species of PC and the possible modulation of the enzyme activity by guanine nucleotides.

Initial attempts to assay for phospholipase A_1 activity in guinea-pig heart microsomes revealed extremely low activity. When 1-palmitoyl-2-linoleoyl GPC was

incubated with guinea-pig heart microsomes, very little phospholipase A_1 activity was detected (0-3 nmol LPC produced/hr/mg protein). This low activity was observed regardless of the molecular species of PC used in the assay. Since other investigators had detected phospholipase A_1 activity when the detergent taurodeoxycholate was included in the assay (40), this approach was tried. Phospholipase A_1 activity in the heart microsomes could be detected when this detergent was used and the concentration of substrate was greater than 100 μM. The enzyme displayed an optimal pH of 8.0 (Fig. 9) and exhibited a substrate concentration profile that was characteristic of an enzyme that acts on micelles (Fig. 10). The enzyme activity increased with increasing substrate concentration until a concentration of 300 μM was reached. At concentrations of substrate greater than 300 μM, the enzyme activity was inhibited. The reaction rate was linear for 10 min at 37 °C with 100 μg of heart microsomal protein. The enzyme activity was enhanced significantly by the addition of taurodeoxycholate (1 mM) in the assay. This enhancement of activity was not observed with the detergents Triton X-100, Triton QS-15, and Tween-20, but increased phospholipase A_1 activity was demonstrated when deoxycholate was used in the assay (Table 11). The phospholipase A_1 activity when deoxycholate was used was 66% less than that with taurodeoxycholate. The effect of increasing substrate concentration on phospholipase A_1 activity was the same when either taurodeoxycholate or deoxycholate was used. In all subsequent experiments, the substrate was prepared with taurodeoxycholate. The final concentration of taurodeoxycholate in the enzyme assays was 1 mM. The phospholipase A_1 activity was not dependent on Ca^{2+} (100-500 μM) and was active in the presence of EDTA and EGTA (2 mM).

Table 11. The effect of detergents on the hydrolysis of PC by phospholipase A_1 in guinea-pig heart microsomes.

PC substrates were prepared with detergents in a ratio of 1 mg detergent per 0.5 μmol of phospholipid as described in the Materials and Methods section. The substrates were subsequently used in phospholipase A_1 assays with guinea-pig heart microsomes as the enzyme source. The substrate used in this assay was 1-16:0-2-18:2 GPC. The results represent the means and standard deviation of duplicate determinations from two separate preparations. The specific activity is expressed as nmol LPC produced per hour per mg protein.

Detergent	Specific activity
None	0
Sodium taurodeoxycholate	74 ± 3
Sodium deoxycholate	21 ± 1
Triton X-100	0
Triton QS-15	0
Hexadecyltrimethylammonium bromide	0

Figure 9

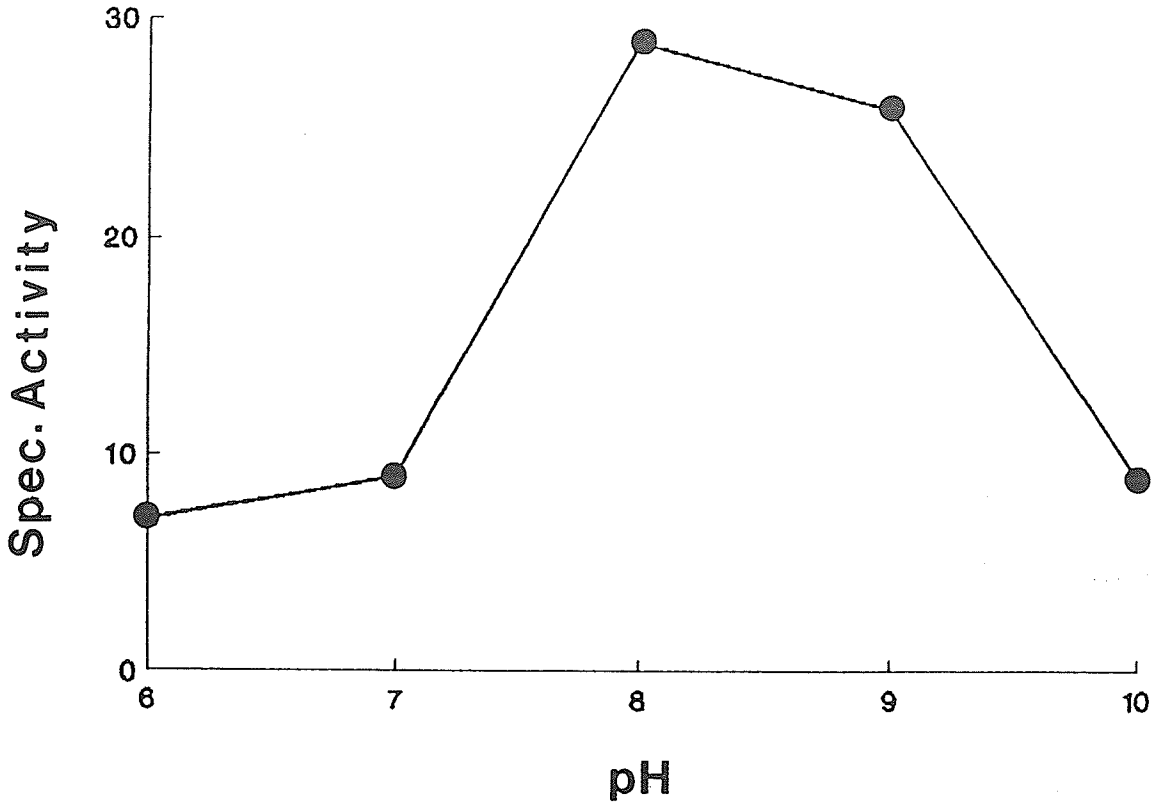

Fig. 9. The effect of pH on guinea-pig heart microsomal phospholipase A_1 activity.

The effect of pH on the guinea-pig heart microsomal phospholipase A_1 was examined with different buffers (Fig. 4). The phospholipase A_1 activity was assayed using 1-16:0-2-18:2 GPC as the substrate. The reaction mixture contained 100 mM of various buffers, 100 μM of PC substrate and 100 μg of heart microsomal protein in a total volume of 500 μl. The assay was performed as described in the Materials and Methods section. The values represent the means of two experiments each done in triplicate. The specific activity is expressed as nmol LPC formed/hr/mg protein. The standard deviation of all values was less than 15% of the mean.

Figure 10

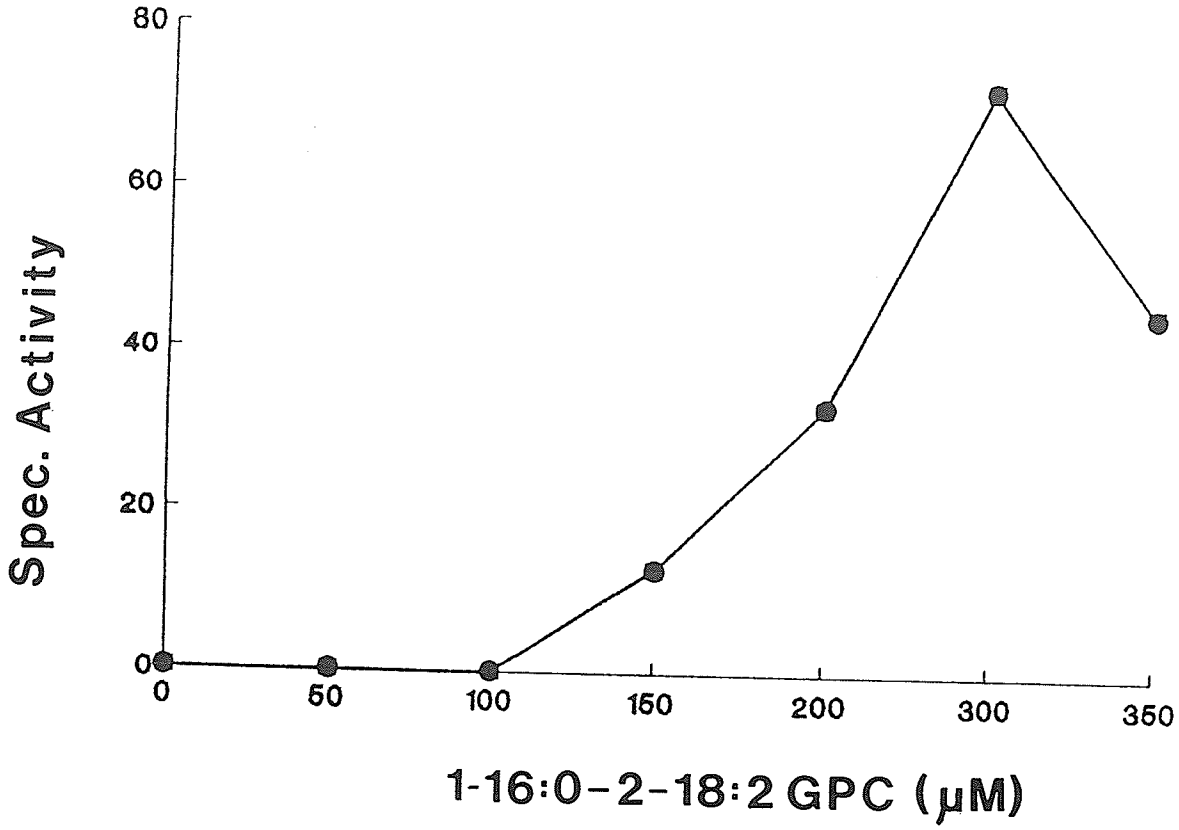

Fig. 10. The effect of concentration of 1-16:0-2-18:2 GPC on the guinea-pig heart microsomal phospholipase A_1 activity.

The effect of varying the concentration of PC substrate on phospholipase A_1 activity was determined. The reaction mixture contained varying concentrations of 1-16:0-2-18:2 GPC which was prepared with taurodeoxycholate (1 mM), 100 mM Tris HCl pH 8.0, and 100 μg heart microsomal protein in a total volume of 500 μl. The reaction products were isolated as described in the Methods and Materials section. The values represent the means of two experiments each performed in triplicate. The specific activity is expressed as nmol LPC produced/hr/mg protein. The standard deviation for all the values was less than 15% of the mean.

Having established the optimal conditions for the assay of phospholipase A_1 activity, its acyl specificity with respect to the molecular species of PC it hydrolyzed was next examined. To determine the acyl specificity of the phospholipase A_1, the activity of the enzyme was assayed with different molecular species of PC. The results of this experiment are shown in Table 12. The phospholipase A_1 displayed highest activity with 1-16:0-2-18:2 GPC (Appendix 1) as the substrate, while lowest activity was observed with 1-18:0-2-20:4 GPC. The order of increasing rates of hydrolysis of substrates was as follows: 1-18:0-2-20:4 GPC = 1-16:0-2-16:0 GPC < 1-16:0-2-18:1 GPC = 1-16:0-2-20:4 GPC < 1-16:0-2-18:2 GPC. Comparison of the hydrolysis of 1-18:0-2-20:4 GPC and 1-16:0-2-20:4 GPC, revealed that the 1-16:0-2-20:4 GPC species was hydrolyzed approximately 1.5 times faster than the 1-18:0-2-20:4 species. This suggests that the guinea-pig heart phospholipase A_1 prefers PC with palmitate rather than stearate at the sn-1 position. Studies with more molecular species are necessary to reach a firm conclusion. The palmitic acid at the sn-1 position was hydrolyzed at different rates depending on the fatty acid esterified at the sn-2 position. The order of decreasing hydrolysis of PC containing palmitic acid at the sn-1 position was the following with respect to the sn-2 fatty acids: linoleic > arachidonic = oleic > palmitic.

The possibility of receptor activation of the phospholipase A_1 was next investigated. Since other phospholipases were modulated by G proteins following receptor activation (227), we examined the effects of non-hydrolysable analogues of GTP on phospholipase A_1 activity. GTP analogues such as GTP[S] and GMPPNP are persistent activators of G proteins, hence a modulation of an activity by these nucleotides is considered evidence in favour of G protein modulation of the effector.

Table 12. Acyl specificity of the guinea-pig heart microsomal phospholipase A_1 with phosphatidylcholine as the substrate.

The acyl specificity of PLA_1 was determined with different molecular species of phosphatidylcholine. The substrates were presented as mixed micelles with taurodeoxycholate. The values represent the mean ± standard deviation of four experiments each done in triplicate. The specific activity is expressed as nmol LPC produced/hr/mg protein.

PC substrate	Specific activity	Activity relative to hydrolysis of 1-18:0-20:4 GPC
1-18:0-2-20:4 GPC	24 ± 4	1.0
1-16:0-2-16:0 GPC	26 ± 5	1.1
1-16:0-2-18:1 GPC	32 ± 2 [a]	1.3
1-16:0-2-20:4 GPC	33 ± 5 [a]	1.4
1-16:0-2-18:2 GPC	50 ± 5 [a]	2.1

[a]Significantly different from a sample assayed using 1-18:0-2-20:4 GPC as the substrate at a level of $p < 0.005$ by t test.

The hydrolysis of two molecular species of PC, 1-16:0-2-18:2 GPC and 1-16:0-2-20:4 GPC was assayed in the presence of GTP[S] (0-1 mM). The results are displayed in Table 13. Surprisingly, the effect of GTP[S] was to inhibit the phospholipase A_1 rather than to stimulate the activity. The hydrolysis of 1-16:0-2-20:4 GPC was more sensitive to inhibition by GTP[S] as it was decreased by 50% with as little as 1 μM GTP[S] while the hydrolysis of 1-16:0-2-18:2 GPC was not inhibited at all until a concentration of 1 mM GTP[S] was used.

The specificity of the inhibition by GTP[S] was next determined. As can be seen from the results in Table 14, a 1 mM concentration of ATP, GTP, GDPβS, or ATP[S] did not inhibit of the phospholipase A_1 as seen with 1 mM GTP[S] when either 1-16:0-2-20:4 GPC or 1-16:0-2-18:2 GPC was used as the substrate. In order to ascertain if this inhibitory effect could be due to the presence of cations in the GTP[S] preparation, we examined the effect of GTP[S] and cation chelators EDTA or EGTA on phospholipase A_1 hydrolysis with 1-16:0-2-18:2 GPC as the substrate. The outcome of this experiment is tabulated in Table 15. In the presence of 1 mM GTP[S] and either EDTA or EGTA (2 mM), the inhibition of phospholipase A_1 activity was still observed relative to control experiments which only had EDTA or EGTA as additives.

GDPβS is a persistent inactivator of G proteins as it promotes the maintenance of a G protein in its inactive heterotrimeric state. The ability of GDPβS to prevent the inhibition of phospholipase A_1 activity by GTP[S] was therefore determined. The results of this experiment are shown in Table 16. Preincubation of microsomes with GDPβS, followed by the addition of GTP[S] did not result in the inhibition observed with GTP[S] alone. This strongly suggested that GTP[S] activated a G protein which

Table 13. Effect of GTP[S] on guinea-pig heart microsomal phospholipase A_1 activity.

Phospholipase A_1 activity was assayed with GTP[S] (0-10^{-3} M) and the molecular species of phosphatidylcholine indicated. The assays were performed as described in the Materials and Methods section. The values represent the means and standard deviation of three experiments each done in triplicate. The specific activity is expressed as nmol LPE produced/hr/mg protein.

	Activity (% of control)	
Conc.	1-16:0-2-18:2 GPC	1-16:0-2-20:4 GPC
0	100	100
1 μM	103 ± 2	41 ± 8
0.1 mM	95 ± 2	58 ± 9
1 mM	68 ± 6	51 ± 15

Table 14. Effect of nucleotides on guinea-pig heart microsomal phospholipase A_1 activity.

Phospholipase A_1 activity was assayed in the presence of the various nucleotides. Controls assays were conducted in the absence of any nucleotides. The assay was performed as described in the Materials and Methods section. The values represent the means of three experiments each performed in triplicate. The values are expressed as a percentage of the activity of a control. The specific activities of phospholipase A_1 with no additives were 55 ± 7 and 34 ± 5 nmol LPC formed/hr/mg protein with 1-16:0-2-18:2 GPC and 1-16:0-2-20:4 GPC respectively.

	Activity (% of control)	
Addition	1-16:0-2-18:2 GPC	1-16:0-2-20:4 GPC
None	100	100
1 mM GTP	99 ± 8	113 ± 18
1 mM GDPβS	97 ± 14	134 ± 16
1 mM ATP[S]	100 ± 1	115 ± 5
1 mM ATP	95 ± 9	103 ± 6
1 mM GTP[S]	68 ± 6	51 ± 5

Table 15. Effect of cation chelators on GTP[S]-mediated inhibition of phospholipase A_1 hydrolysis.

Phospholipase A_1 activity was assayed in the presence of GTP[S] (1 mM), EDTA (2 mM), and EGTA (2 mM) together or individually using 1-16:0-2-18:2 GPC. The assay was performed essentially as described in the Methods and Materials section. The values represent the means ± standard deviation of three experiments each performed in triplicate. The specific activity of phospholipase A_1 with no additives was 47 ± 5 nmol of LPC formed/hr/mg protein. The values are expressed as a percentage of a control experiment with no additives.

Addition	Activity (% of control)
None	100
EGTA (2 mM)	93 ± 4
EDTA (2 mM)	84 ± 5
GTP[S] (1 mM)	62 ± 5
EDTA (2 mM) + GTP[S] (1 mM)	73 ± 7 [a]
EGTA (2 mM) + GTP[S] (1 mM)	68 ± 2

[a]Significantly different from a sample with EDTA only added at a level of $p < 0.005$ by t test.

modulated, either indirectly or directly, the phospholipase A_1.

The differential sensitivity to GTP[S] that was observed, when the effect of GTP[S] on the hydrolysis of two molecular species of phosphatidylcholine was examined was intriguing (Table 13). It could be that this sensitivity reflects the presence of distinct phospholipase A_1 enzymes which are affected to different extent by the molecules that modulate the phospholipase A_1 activity in response to GTP[S]. To investigate the possible existence of different phospholipases A_1 in guinea-pig heart microsomes, the effect of heating the heart microsomes prior to assaying for phospholipase A_1 activity was investigated. The results of this experiment are given in Table 17. The phospholipase A_1 activity that hydrolyzes 1-16:0-2-18:2 GPC is much more heat sensitive than the same phospholipase A_1 activity that hydrolyzes 1-16:0-2-20:4 GPC. After 1 minute of heating at 60 °C the phospholipase A_1 activity with 1-16:0-2-18:2 GPC is almost inhibited completely, meanwhile, in the same period of time, the activity that hydrolyzes 1-16:0-2-20:4 GPC is only decreased by 15%.

Table 16. Effect of GDPßS on GTP[S]-mediated inhibition of phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPC.

Guinea pig heart microsomes were incubated with GDPßS (1 mM) for 5 minutes at room temperature. Subsequently GTP[S] (1 mM) was added and the reaction was initiated by the addition of the radiolabelled substrate. The remainder of the assay was carried out exactly as described in the Materials and Methods section. The values represent the means ± standard deviation of three experiments each done in triplicate. The values are presented as a percentage of the activity observed with a control of no addition.

Addition	Activity (% Control)
None	100
GTP[S]	72 ± 10 [a]
GDPßS	96 ± 6
GDPßS + GTP[S]	96 ± 15

[a]Significantly different from a control with no additives at a level of $p < 0.005$ by t test.

Table 17. Effect of heating on phospholipase A_1 activity in guinea-pig heart microsomes.

Phospholipase A_1 activity was assayed after heating the heart microsomes at 60 °C for the indicated times followed by cooling to 4 °C in an ice bath for 5 min. The assays were performed as described in the Materials and Methods section. The results represent the means and standard deviation of two experiments each performed in triplicate. The activities are expressed as a percentage of the activity observed with controls that were not heat treated.

	Activity (% of control)	
Time	1-16:0-2-18:2 GPC	1-16:0-2-20:4 GPC
none	100	100
1 min	7 ± 1	83 ± 13
2 min	10 ± 1	84 ± 3
3 min	15 ± 5	83 ± 12
4 min	13 ± 8	64 ± 4
8 min	7 ± 2	20 ± 4

4.3. Regulation and characteristics of a phosphatidylethanolamine-hydrolyzing phospholipase A_1 activity in guinea-pig heart microsomes.

The identification of a 2-acyl GPE lysophospholipase activity in guinea-pig heart microsomes led to the hypothesis that fatty acids may be selectively released from PE by a phospholipase A_1/lysophospholipase A_2 pathway. The existence of such a pathway would require the presence of a PE-hydrolyzing phospholipase A_1 activity in the microsomes that exhibited selectivity for specific molecular species of PE. Although there are reports of PE-hydrolyzing PLA_1 activity in hamster, rat and canine heart microsomes (29,202), the studies were limited to optimising the assay conditions and subcellular localization of the enzymes. In this section of this study, the presence of a PE-hydrolyzing phospholipase A_1 activity in guinea-pig heart microsomes was investigated and characterized with respect to acyl specificity and modulation of its hydrolytic activity by guanine nucleotides and cations

The rate of hydrolysis of 1-16:0-2-18:2 GPE by phospholipase A_1 activity in guinea-pig heart microsomes ranged from 13-21 nmol LPE produced/hr/mg protein with different heart microsomal preparations. To eliminate possible differences in the solubility of different molecular species of PE in aqueous solution, the assays were conducted with mixed micelles of detergent and PE. The effects of a variety of detergents on the hydrolysis of 1-16:0-2-18:2 GPE by the phospholipase A_1 were examined (Table 18). Phospholipase A_1 activity was observed in the absence or presence of the bile salt detergents taurodeoxycholate and deoxycholate, however, only 0-20% of this activity was observed when this enzyme activity was assayed with mixed micelles of substrate and Triton X-100, Triton QS-15, or hexadecyl-

Table 18. Effects of detergents on hydrolysis PE by phospholipase A_1 in guinea-pig heart microsomes.

PE substrate (1-16:0-2-18:2 GPE) was prepared with detergents in a ratio of 0.001 g detergent/0.5 μmol of phospholipid as described in the Methods and Materials section. The substrates were subsequently used in PLA_1 assays with guinea-pig heart microsomes as the enzyme source. The results represent the means ± standard deviation of duplicate determinations from two separate preparations. The specific activity is expressed as nmol LPE formed/hr/mg protein.

Detergent	Specific Activity
None	17 ± 4
Sodium taurodeoxycholate	17 ± 3
Sodium deoxycholate	16 ± 4
Triton X-100	3 ± 2
Triton QS15	0
Hexadecyltrimethylammonium bromide	3 ± 1

trimethylammonium bromide (Table 18). At a concentration of 1 mM in the assay, taurodeoxycholate did not affect the hydrolysis of any of the molecular species of PE used in our studies. In all subsequent assays, a mixed micelle system of PE and taurodeoxycholate (1 mM in the assay) was used. The optimal conditions required for phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE were established. The optimal pH was 9.0 (Fig. 11) and the reaction rate was linear up to 15 minutes at 37 °C with 100 μg of heart microsomal protein. All the phosphatidylethanolamine substrates used in this study yielded maximum activities at concentrations between 200-300 μM (Fig. 12). A substrate concentration of 200 μM was selected for all further experiments.

To determine if the guinea-pig heart microsomal phospholipase A_1 displayed a selectivity for the hydrolysis of the sn-1 fatty acid, the relative rates of hydrolysis of different molecular species of PE were compared. The results of this experiment are shown in Table 19. From the data, in this table, it can be observed that the phospholipase A_1 displayed different rates of hydrolysis with different molecular species of PE. The order of decreasing rates of hydrolysis was 1-18:0-2-20:4 GPE > 1-16:0-2-20:4 GPE > 1-16:0-2-18:1 GPE = 1-16:0-2-18:2 GPE > 1-16:0-2-16:0 GPE. Phospholipase A_1 activity in the microsomes displayed a clear preference for 1-18:0-2-20:4 GPE compared to other molecular species. With respect to a preference for a particular type of sn-1 fatty acid, even though both 1-16:0-2-20:4 GPE and 1-18:0-2-20:4 GPE had arachidonate esterified at the sn-2 position, the former was hydrolyzed at almost twice the rate of the latter, suggesting a possible preference of the enzyme for stearate over palmitate. The unavailability of other radiolabelled molecular species with stearate at the sn-1 position prevented further exploration of the sn-1 preference

Figure 11

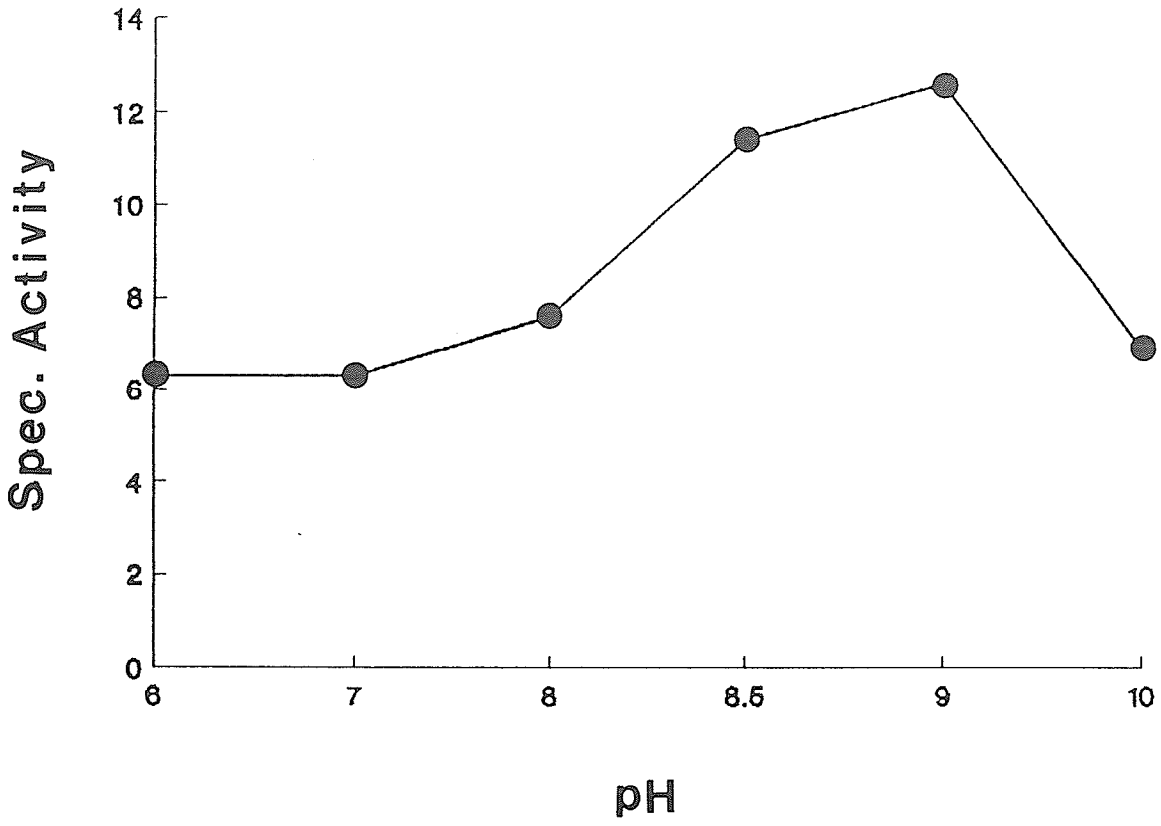

Fig. 11. The effect of pH on guinea-pig heart microsomal PE-hydrolyzing phospholipase A_1 activity.

The effect of pH on the guinea-pig heart microsomal phospholipase A_1 activity was examined with various buffers (Fig. 4). The phospholipase A_1 activity was assayed using 1-16:0-2-18:2 GPE as the substrate. The reaction mixture contained 100 mM of the various buffers, 100 μM of PE substrate and 100 μg of heart microsomal protein in a total volume of 500 μl. The assay was performed as described in the Materials and Methods section. The values represent the means of two experiments each performed with three separate preparations. The specific activity is expressed as nmol LPE formed/hr/mg protein. The standard deviation of all the values was less than 15% of the mean.

Figure 12

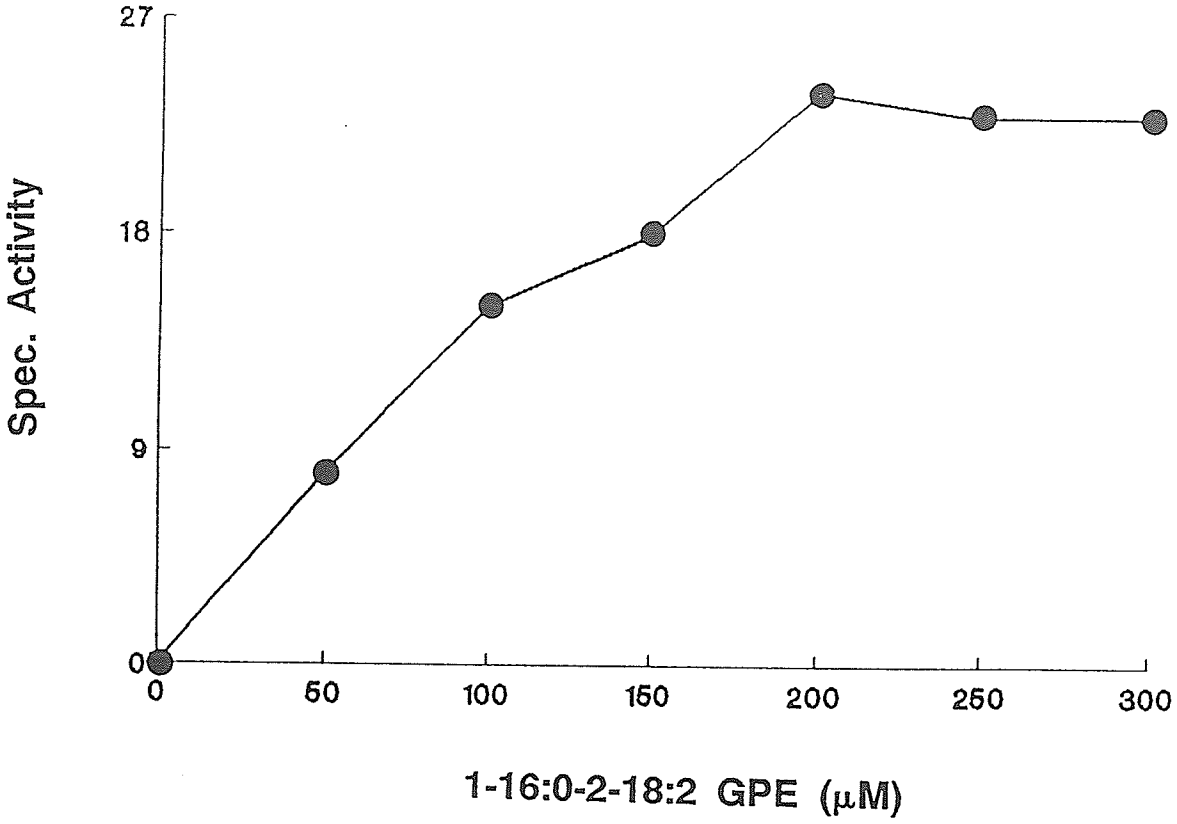

Fig. 12. The effect of different concentrations of 1-16:0-2-18:2 GPE the guinea-pig heart microsomal phospholipase A_1 activity.

The effect of varying the concentration of PE substrate on phospholipase A_1 activity was determined. The reaction mixture contained varying concentrations of 1-16:0-2-18:2 GPE which was prepared with the detergent taurodeoxycholate, 100 mM Tris HCl pH 9.0, and 100 μg heart microsomal protein in a total volume of 500 μl. The reaction products were isolated as described in the Materials and Methods section. The values represent the means of two experiments each done in triplicate. The specific activity is expressed as nmol LPE produced/hr/mg protein. The standard deviation of each value was less than 15% of the mean.

Table 19. Acyl specificity of guinea-pig heart microsomal phospholipase A_1 with PE as a substrate.

The acyl specificity of guinea-pig heart microsomal PLA_1 was determined with different molecular species of PE. The assay was conducted as described under Materials and Methods. Substrates were presented as mixed micelles of PE and taurodeoxycholate. The values represent the means ± standard deviation of four experiments each done in triplicate. The specific activity is expressed as nmol LPE produced/hr/mg protein.

PE Substrate	Specific Activity	Relative to the hydrolysis of 1-16:0-2-16:0 GPE
1-16:0-2-16:0 GPE	5 ± 1	1.0
1-16:0-2-18:2 GPE	15 ± 1	3.0
1-16:0-2-18:1 GPE	16 ± 7	3.2
1-16:0-2-20:4 GPE	43 ± 13	8.6
1-18:0-2-20:4 GPE	74 ± 15	15.0

of the activity. A comparison of the rate of hydrolysis of the other substrates, all of which had palmitate at the sn-1 position, revealed the following order of decreasing hydrolysis with respect to the sn-2 fatty acid: arachidonate > oleate = linoleate > palmitate. Thus, the rate of hydrolysis of the sn-1 position clearly depended on the nature of the fatty acid at the sn-2 position. The phospholipase A_1 has a preference for arachidonate containing substrates.

Modulation of phospholipase activities by cations is well documented (Section 1.3.). Therefore, the effect of Ca^{2+} and Mg^{2+} on the hydrolysis of 1-18:0-2-20:4 GPE and 1-16:0-2-18:2 GPE by the guinea-pig heart microsomal phospholipase A_1 was investigated. The results of these experiments are shown in Tables 20 and 21. The addition of EGTA (2 mM) slightly inhibited the activity of the phospholipase A_1 by about 10% relative to assays conducted with no additives, while an equivalent concentration of EDTA had no effect on the phospholipase A_1 activity (Table 21). As can be seen from the data in Table 20, the addition of Ca^{2+} (100-300 μM) stimulated the hydrolysis of 1-16:0-2-18:2 GPE by 30-60% while the hydrolysis of 1-18:0-2-20:4 GPE was stimulated only 30-50%. Thus the hydrolysis of both substrates was affected by Ca^{2+}. Similar studies with Mg^{2+} (100-300 μM) revealed a differential effect of this cation on the hydrolysis of the same two molecular species of phosphatidylethanolamine. From the results in Table 21, only the phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE was affected by Mg^{2+}. There was no effect of Mg^{2+} on phospholipase A_1 hydrolysis of 1-18:0-2-20:4 GPE. This differential effect of Mg^{2+} prompted us to investigate other differences between the hydrolysis of 1-18:0-2-20:4 GPE and 1-16:0-2-18:2 GPE by phospholipase A_1.

The effect of heating on the hydrolysis of 1-18:0-2-20:4 GPE and 1-16:0-2-18:2

Table 20. The effect of Ca^{2+} on guinea-pig heart microsomal phospholipase A_1.

The assay of phospholipase A_1 activity was performed as described in the Materials and Methods section. The results are expressed as a percentage of the activity observed in the presence of 2 mM EGTA. The specific activity of the phospholipase A_1 with 1-16:0-2-18:2 GPE was 30 ± 6 nmol LPE produced/hr/mg protein and 25 ± 7 nmol LPE produced/hr/mg protein in the absence and presence of 2 mM EGTA respectively. The activity with 1-18:0-2-20:4 GPE was 68 ± 2 nmol LPE with no additive while the specific activity was 59 ± 1 nmol LPE produced/hr/mg protein with 2 mM EGTA. The results represent the means and standard deviation of two experiments each performed in triplicate.

	Activity (% of control)	
Additive	1-16:0-2-18:2 GPE	1-18:0-2-20:4 GPE
2 mM EGTA	100	100
100 μM Ca^{2+}	130 ± 15[b]	130 ± 18[a]
200 μM Ca^{2+}	149 ± 17[c]	156 ± 10[c]
300 μM Ca^{2+}	160 ± 15[c]	150 ± 7[c]

[a]Significantly different from control at $p < 0.05$ level by t test.
[b]Significantly different from control at $p < 0.01$ level by t test.
[c]Significantly different from control at $p < 0.005$ level by t test.

Table 21. The effect of Mg^{2+} on guinea-pig heart microsomal phospholipase A_1.

Phospholipase A_1 activity was assayed as described in the Materials and Methods section with the indicated additives. The results are expressed as a percentage of the activity observed in control experiments which contained 2 mM EDTA. The values represent the means and standard deviation of two experiments each performed in triplicate. The specific activity of the phospholipase A_1 with 1-16:0-2-18:2 GPE was 24 ± 4 nmol LPE produced/hr/mg protein while the specific activity with 1-18:0-2-20:4 GPE was 68 ± 6 nmol LPE formed/hr/mg protein.

	Activity (% of control)	
Addition	1-16:0-2-18:2 GPE	1-18:0-2-20:4 GPE
2 mM EGTA	100	100
100 μM Mg^{2+}	123 ± 10[a]	99 ± 3
200 μM Mg^{2+}	123 ± 7[a]	89 ± 10
300 μM Mg^{2+}	127 ± 12[b]	97 ± 5

[a]Significantly different from control at $p < 0.01$ level by t test.
[b]Significantly different from control at $p < 0.005$ level by t test.

GPE was determined. The results of these experiments are shown in Table 22. Microsomal membranes in buffer were incubated at 60 °C for varying times, cooled on ice for an additional 5 min and then assayed for phospholipase A_1 activity with either 1-18:0-2-20:4 GPE or 1-16:0-2-18:2 GPE. As can be observed from the data in Table 22, phospholipase A_1 activity with 1-18:0-2-20:4 GPE was reduced to 66% of that of a control after 1 min of heating at 60 °C and was undetectable after 2 min of heating the heart microsomes. The hydrolysis of 1-16:0-2-18:2 GPE was undetectable after only 1 minute of heating at 60 °C. Hence, the hydrolysis of 1-16:0-2-18:2 GPE was more sensitive to heating than the hydrolysis of 1-18:0-2-20:4 GPE.

The effect of 1-18:0-2-20:4 GPE on the hydrolysis of 1-16:0-2-18:2 GPE was investigated to determine whether the two substrates compete for the same active site. The results of these experiments are shown in Table 23. These results show quite definitely that the hydrolysis of 1-16:0-2-18:2 GPE was not affected by an equimolar concentration of 1-18:0-2-20:4 GPE. The effect of varying concentrations of 1-16:0-2-18:2 GPC on the hydrolysis of 1-16:0-2-18:2 GPE was investigated. The results of this experiment are shown in Figure 13. From this data, it is obvious that there is no inhibition of the hydrolysis of 1-16:0-2-18:2 GPE by the presence of the same molecular species of PC. Moreover, the addition of 1-16:0-2-18:2 GPC actually activates the hydrolysis of 1-16:0-2-18:2 GPE by approximately 2-fold.

The effect of guanine nucleotides on the hydrolysis of PE by the phospholipase A_1 was investigated to determine if G proteins may also modulate the hydrolysis of PE by phospholipase A_1. GTP[S] (0 - 1 mM) did not have any effect on the hydrolysis of 1-18:0-2-20:4 GPE (Table 24), but the rate of hydrolysis of 1-16:0-2-18:2 GPE was

Table 22. Effect of heating on phospholipase A_1 activity in guinea-pig heart microsomes.

Phospholipase A_1 activity was assayed after heating guinea-pig heart microsomes at 60 °C for varying times, followed by cooling to 4 °C in an ice-bath for 5 min. The assays were performed as described in the Materials and Methods section. The values are expressed as a percentage of the activity of an untreated controls. The results are the means and standard deviation of two experiments each done in triplicate. The specific activity for the hydrolysis of 1-18:0-2-20:4 GPE was 52 ± 6 nmol LPE produced/hr/mg protein while the specific activity for the hydrolysis of 1-16:0-2-18:2 GPE was 24 ± 1 nmol LPE produced/hr/mg protein.

	Activity (% of control)	
Heating period (min)	1-16:0-2-18:2 GPE	1-18:0-2-20:4 GPE
0	100	100
1	0	66 ± 12
2	0	0
5	0	0

Table 23. Effect of 1-18:0-2-20:4 GPE on the hydrolysis of 1-16:0-2-18:2 GPE by the guinea-pig heart microsomal phospholipase A_1.

Substrates were prepared with aliquots of 1-16:0-2-[^{14}C]-18:2 GPE with either 1-16:0-1-18:2 GPE alone or with 1-18:0-2-20:4 GPE in tubes followed by drying with nitrogen and the addition of taurodeoxycholate to give a detergent/lipid ratio of 1 mg detergent/0.5 μmol PE. The assays were performed as described in the Materials and Methods section. The values represent the mean and standard deviation of three experiments each done in duplicate. The specific activity is expressed as nmol of 2-[^{14}C]-18:2 GPE produced/hr/mg protein.

Substrates	Specific activity
1-16:0-2-[^{14}C]-18:2 GPE (150 μM)	22 ± 2
1-16:0-2-[^{14}C]-18:2 GPE (150 μM) + 1-18:0-2-20:4 GPE (150 μM)	22 ± 7

Table 24. Effect of GTP[S] on the hydrolysis of 1-18:0-2-20:4-GPE by phospholipase A_1.

PLA_1 activity was assayed with 1-18:0-2-20:4-GPE in the presence of GTP[S] (0-1 mM). The assays were conducted as described under Materials and Methods section with the modification that the GTP[S] solution was added prior to the addition of the substrate. The values are presented as a percentage of the activity of the samples with no GTP[S] added. The values represent the means ± standard deviation of two experiments each performed in triplicate. The specific activity for the hydrolysis of 1-18:0-2-20:4-GPE was 60 ± 7 nmol LPE formed/hr/mg protein.

Conc. GTP[S] (M)	Activity (% of control)
0	100
10^{-6}	97 ± 7
10^{-5}	92 ± 6
10^{-4}	95 ± 4
10^{-3}	90 ± 9

Figure 13

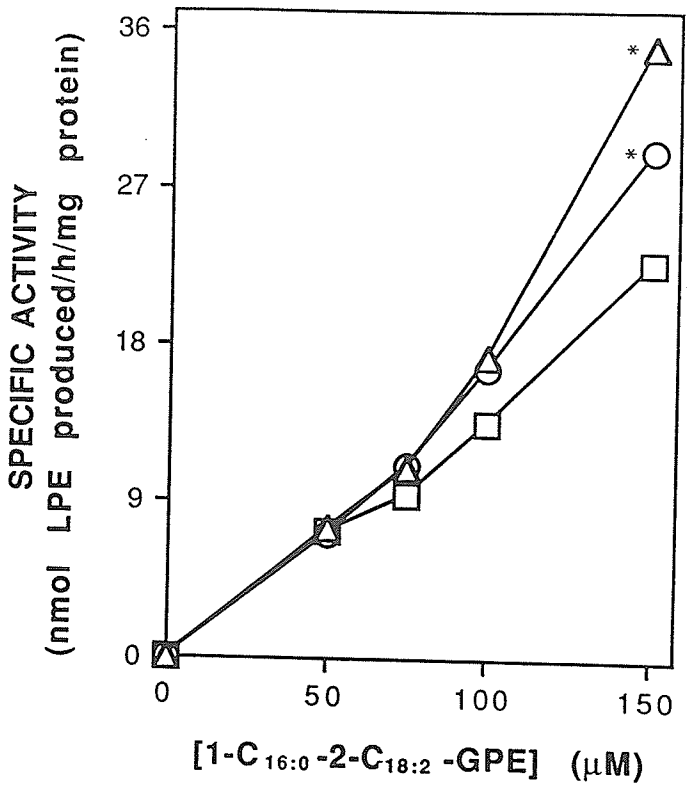

Fig. 13. Effect of 1-16:0-2-18:2 GPC on the hydrolysis of 1-16:0-2-18:2 GPE by guinea-pig heart microsomes.

The rate of phospholipase A_1 hydrolysis of 1-16:0-2-[^{14}C]18:2 GPE (50-250 μM) was measured in the presence of 0 μM (□), 150 μM (○) or 180 μM (△) 1-16:0-2-18:2 GPC. 1-16:0-2-18:2 GPE and 1-16:0-2-18:2 GPC were added separately to the reaction mixture. The assay was performed as described in the Methods and Materials section with the exception that the reaction was initiated by the addition of microsomal protein. The values represent the means of two independent experiments each performed in duplicate. The standard deviation for each point was less than 15% of the mean.
* $p < 0.05$ relative to corresponding value with 0 μM 1-16:0-2-18:2 GPE as determined by Student's t test.

increased by approximately 25-40% with 1 μM to 0.1 mM GTP[S] (Table 25). The hydrolysis of this molecular species of PE was inhibited by 15% by the addition of 1 mM GTP[S]. The nucleotide specificity of this activation of phospholipase A_1 was investigated with other nucleotides (Table 25). Of all the nucleotides tested, only the nonhydrolyzable analogue of GTP, GMPPNP and GTP itself were able to increase the rate of phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE. GMPPNP (1 μM - 10 μM) activated the hydrolysis of the substrate by 40%. Unlike 0.1 mM GTP[S], 0.1 mM GMPPNP did not result in an activation of phospholipase A_1. A slight activation of phospholipase A_1 hydrolysis of this substrate was seen with 1 μM GTP. No stimulation of the phospholipase A_1 activity was observed with GDPβS or ATP (1 μM). The inhibition seen with 1 mM GTP[S] was also seen with a similar concentration of GMPPNP, and ATP (Table 25).

To gain some insight into the modulation of the phospholipase A_1 activity by guanine nucleotides, the effect of cations and their chelators on the GTP[S] induced activation of 1-16:0-2-18:2 GPE was investigated. The results of these experiments are displayed in Table 26 and Table 27. The incubation of 1 μM GTP[S] with either 2 mM EDTA or 2 mM EGTA abolished the previously observed activation of the phospholipase A_1. The combined presence of 100 μM Mg^{2+} or Ca^{2+} and 1 μM GTP[S] resulted in a level of activation of phospholipase A_1 activity that was equal to that observed with either GTP[S] or cations alone. There was no additive effect of the divalent cations and GTP[S] on phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE.

Table 25. Effect of nucleotides on the hydrolysis of 1-16:0-2-18:2-GPE by guinea-pig heart microsomal phospholipase A_1.

Enzyme activity was assayed in the presence of different nucleotides. The assay was performed as indicated in Materials and Methods section with the addition of the nucleotides listed. In all cases the assay was initiated by the addition of the substrate. The values are expressed as a percentage of the activity of a control without any nucleotides. The values represent the means and standard deviation of three experiments each performed in triplicate.

	Activity (% of Control)				
Conc. (M)	GTP[S]	GMPPNP	GDPßS	GTP	ATP
0	100	100	100	100	100
10^{-6}	131 ± 10[c]	143 ± 9[c]	100 ± 12	114 ± 6[c]	95 ± 8
10^{-5}	123 ± 11[c]	140 ± 7[c]	ND	ND	ND
10^{-4}	142 ± 15[c]	102 ± 18	ND	ND	ND
10^{-3}	87 ± 6[c]	87 ± 5[c]	91 ± 10	94 ± 9	85 ± 5[b]

ND : not determined.
[a]Significantly different from control at $p < 0.025$ by Student's t-test.
[b]Significantly different from control at $p < 0.01$ by Student's t-test.
[c]Significantly different from control at $p < 0.005$ by Student's t-test.

Table 26. Effect of cation chelators on the GTP[S]-mediated activation of phospholipase A_1 activity.

Phospholipase A_1 hydrolysis of 1-16:0-2-18:2-GPE was assayed in the presence of cation chelators. The enzyme activity was determined as described under Materials and Methods section. The specific activity of the PLA_1 activity in the absence of any additives was 26 ± 4 nmol LPE produced/hr/mg protein. The values represent the means ± standard deviations of three experiments each done in triplicate. The activity is represented as a percentage of the activity of a control sample with no addition.

Addition	Activity (% of control)
None	100
2 mM EGTA	93 ± 7
2 mM EDTA	94 ± 5
1 μM GTP[S]	129 ± 13[a]
EGTA + GTP[S]	102 ± 9
EDTA + GTP[S]	93 ± 3

[a]Significantly different from control at $p < 0.005$ by Student's t test.

Table 27. Effect of cations on the GTP[S]-mediated activation of phospholipase A_1 activity.

PLA_1 activity was assayed in the presence of 100 μM Mg^{2+} or 100 μM Ca^{2+} or 1 μM GTP[S] or a combination of these additives. The enzyme activity was assayed with 1-16:0-2-18:2-GPE as substrate with the procedures described in the Materials and Methods. The values are expressed as a percentage of controls. Control samples did not contain any additives. The values are the means ± standard deviations of three experiments each performed in triplicate.

Addition	Activity (% of control)
None	100
GTP[S]	143 ± 15[a]
Mg^{2+}	140 ± 6[a]
Ca^{2+}	146 ± 17[a]
Mg^{2+} + GTP[S]	144 ± 17[a]
Ca^{2+} + GTP[S]	144 ± 21[a]

[a]Significantly different from control at $p < 0.005$ by Student's t test.

4.4. Regulation of the phosphatidylethanolamine hydrolyzing phospholipase A_1 by dl-isoproterenol.

In view of the report of Franson et al. of modulation of phospholipase A_1 activity in canine heart microsomes by dl-isoproterenol (202), the possible modulation of the PE-hydrolyzing phospholipase A_1 in guinea-pig heart microsomes by dl-isoproterenol was investigated. In these studies, a mixed micelle system of taurodeoxycholate (1 mM) and 1-16:0-2-18:2 GPE was used as substrate for the phospholipase A_1 activity. DL-Isoproterenol (10^{-3} to 10^{-13} M) could activate the guinea-pig heart microsomal phospholipase A_1 activity by approximately 30-50% (Table 28). At concentrations lower than 10^{-13} M, the phospholipase A_1 activity was inhibited by the dl-isoproterenol. Hereafter all references to isoproterenol will refer to the racemic dl-form.

To be sure that the 2-[^{14}C]-linoleoyl GPE that was detected was produced by phospholipase A_1 activity, triglyceride and diglyceride lipase activities in guinea-pig heart microsomes were assayed under the exact conditions used for the phospholipase A_1 assay except with the use of [^{3}H]-triolein and 1-acyl-2-[^{3}H]-acyl glycerol as the substrates. No diglyceride lipase activity was found under these assay conditions. However, there was appreciable triglyceride lipase activity under these assay conditions (Table 29). But the triglyceride lipase activity was not responsive to activation by GTP[S] or isoproterenol at similar concentrations that stimulated the phospholipase A_1 activity (Table 29). Therefore, the lysophosphatidylethanolamine that was produced upon stimulation of guinea-pig heart microsomes with isoproterenol was

Table 28. Effect of dl-isoproterenol on guinea-pig heart microsomal phospholipase A_1 activity.

Phospholipase A_1 activity in guinea-pig heart microsomes was assayed using 1-16:0-2-18:2 GPE as the substrate. The enzyme assay was performed as described in the Materials and Methods section with the exception that heart microsomes were pre-incubated with varying concentrations of dl-isoproterenol for 10 min at room temperature prior to the start of the assay. The values are presented as a percent of the activity of samples with no isoproterenol. The values represent the means and standard deviation of two experiments each performed in triplicate. The substrate was presented as a mixed micelle of taurodeoxycholate and PE. The specific activity for the hydrolysis of 1-16:0-2-18:2 GPE with no additives was 15 ± 1 nmol LPE formed/hr/mg protein.

Addition	Activity (% of control)
None	100
10^{-3} M Isoproterenol	139 ± 7
10^{-5} M Isoproterenol	146 ± 24
10^{-7} M Isoproterenol	140 ± 13
10^{-9} M Isoproterenol	139 ± 15
10^{-11} M Isoproterenol	139 ± 14
10^{-13} M Isoproterenol	127 ± 12
10^{-15} M Isoproterenol	53 ± 11

Table 29. Triglyceride lipase activity in guinea-pig heart microsomes.

Triglyceride lipase activity was assayed using [^{3}H]-triolein under the identical conditions used to assay for phospholipase A_1 activity with PE substrate. The assay was performed exactly as described for the PE-hydrolyzing phospholipase A_1 activity in the Materials and Methods section. There was a 10 min period of preincubation when isoproterenol was added. The reaction products were identified after t.l.c. was performed using a solvent system of 98/2/1 chloroform/methanol/acetic acid (by volume). The values represent the mean and standard deviation of two experiments each performed in triplicate. The specific activity is expressed as nmol fatty acid released/hr/mg protein.

Addition	Specific Activity
None	35 ± 4
1 nM dl-isoproterenol	35 ± 6
1 μM GTP[S]	37 ± 10

due to activation of a PE-hydrolyzing phospholipase A_1 activity.

To ensure that this activation of phospholipase A_1 activity was specifically due to the addition of isoproterenol to the heart membranes, studies were conducted with propranolol, a non-specific ß-adrenergic receptor antagonist (210,227). The results of this experiment are shown in Table 30. The addition of 1 µM propranolol has no effect on the guinea-pig heart microsomal phospholipase A_1 activity. In the presence of 1 µM dl-propranolol and various concentrations of isoproterenol, there was no observed activation of the phospholipase A_1 activity.

Isoproterenol is a non-selective ß-adrenergic receptor agonist. It can bind to and activate either the $ß_1$ or $ß_2$ receptor subtypes as well as α-adrenergic receptors although with a much lower affinity (227). To determine exactly which receptor type was being activated by isoproterenol and subsequently activating the phospholipase A_1, studies were carried out using specific receptor agonists and antagonists. Initial studies were aimed at determining whether activation of an α-adrenergic receptor was involved. Assays were conducted using the α_1-receptor agonist phenyleprine and the α_2-adrenergic receptor agonist clonidine at a concentration at which there is activation of phospholipase A_1 by isoproterenol. As can be seen from the results in Table 31, neither α-adrenergic receptor agonist had any effect on the rate of hydrolysis of 1-16:0-2-18:2 GPE by the phospholipase A_1. Next, using specific antagonists for $ß_1$- and $ß_2$-adrenergic receptors, experiments were performed to see if the isoproterenol mediated activation could be inhibited by one of these antagonists. Experiments were conducted using the $ß_1$-receptor specific antagonist atenolol and the $ß_2$-receptor specific antagonist butoxamine (227). The addition of 1 µM butoxamine by itself had no effect on the hydrolysis of 1-16:0-2-18:2 GPE by the phospholipase A_1, whereas,

Table 30. Effect of dl-propranolol on the dl-isoproterenol mediated activation of phospholipase A_1 activity.

Phospholipase A_1 activity was assayed in the presence of isoproterenol and propranolol. The assays are performed as indicated in the Materials and Methods section with the exception that the guinea-pig heart microsomes were preincubated for 10 min at room temperature with propranolol and isoproterenol. The values are presented as a percentage of the activity with no additive present. The values represent the means and standard deviations of two experiments each done in triplicate. The specific activity of the phospholipase A_1 for the hydrolysis of 1-16:0-2-18:2 GPE with no additive was 17 ± 3 nmol LPE produced/hr/mg protein.

Addition	Activity (% of control)
None	100
10^{-6} M Propranolol	104 ± 10
10^{-6} M Propranolol + 10^{-7} M Isoproterenol	92 ± 18
10^{-6} M Propranolol + 10^{-9} M Isoproterenol	90 ± 15
10^{-6} M Propranolol + 10^{-15} M Isoproterenol	91 ± 13

Table 31. Effect of α-adrenergic agonists on phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE.

Phospholipase A_1 activity was assayed with 1-16:0-2-18:2 GPE in the presence of the α_1-adrenergic agonist phenyleprine and the α_2-adrenergic agonist clonidine. The assays were carried out as described in the Materials and Methods section. Guinea-pig heart microsomes were preincubated with the agonist for 10 min at room temperature prior to the start of the assay. The values are presented as a percentage of the activity of a control with no agonist added. The values are the means and standard deviation of two experiments each performed in triplicate. The specific activity of the phospholipase A_1 without any additives was 13 ± 2 nmol LPE produced/hr/mg protein.

Addition	Activity (% of control)
None	100
10^{-4} M Isoproterenol	135 ± 6
10^{-4} M Phenyleprine	98 ± 10
10^{-4} M Clonidine	108 ± 14

Table 32. Effect of ß-adrenergic receptor antagonists on the dl-isoproterenol mediated activation of phospholipase A_1 activity in guinea-pig heart microsomes.

Phospholipase A_1 activity was assayed in the presence of the ß-adrenergic receptor agonist isoproterenol and the $ß_1$-adrenergic receptor antagonist atenolol and the $ß_2$-adrenoreceptor antagonist butoxamine. The microsomes were preincubated with the indicated amounts of agonist and antagonist for 10 min at room temperature. The assay was conducted as described in the Materials and Methods section with 1-16:0-2-18:2 GPE as the substrate. The values are presented as a percentage of controls with either atenolol or butoxamine. The values are the means and standard deviation of two experiments each done in triplicate. The specific activity of the phospholipase A_1 activity without any additions was 14 ± 4 nmol LPE/hr/mg protein. The specific activity was 7 ± 2 and 14 ± 2 with 1 µM atenolol and 1 µM butoxamine added respectively.

	Activity	
Addition	(% of Atenolol)	(% of Butoxamine)
10^{-6} M Atenolol	100	-
Atenolol + 10^{-7} M Isoproterenol	160 ± 6	-
Atenolol + 10^{-9} M Isoproterenol	140 ± 10	-
Atenolol + 10^{-11} M Isoproterenol	202 ± 12	-
10^{-6} M Butoxamine	-	100
Butoxamine + 10^{-7} M Isoproterenol	-	66 ± 11
Butoxamine + 10^{-9} M Isoproterenol	-	84 ± 9
Butoxamine + 10^{-11} M Isoproterenol	-	90 ± 4

addition of 1 μM atenolol inhibited this PE-hydrolyzing phospholipase A_1 activity by 50% relative to a control with no atenolol added (Table 32). Furthermore, the results shown in Table 32 indicated that the addition of atenolol did not inhibit the isoproterenol mediated stimulation of phospholipase A_1 activity. The addition of butoxamine (1 μM) to heart microsomes with various concentrations of isoproterenol did result in an inhibition of the isoproterenol activation of phospholipase A_1 activity. These results suggest that isoproterenol activates the $ß_2$-class of adrenergic receptor and this leads to activation of the guinea-pig heart microsomal phospholipase A_1.

In order to determine more specifically how activation of the $ß_2$-adrenergic receptor results in subsequent activation of phospholipase A_1 activity, experiments were performed to determine if the mechanism of activation involved Ca^{2+}. The results of such experiments are shown in Table 33. The isoproterenol mediated increase in activity was not observed in the presence of 2 mM EGTA. However, there was no additive or synergistic effect of Ca^{2+} and isoproterenol as the combined presence of these two activators did not result in an increase in phospholipase A_1 activity greater than that observed with either 100 μM Ca^{2+} or 1 nM isoproterenol alone. Since it was known that ß-adrenergic receptors are G protein coupled and that the PE-hydrolyzing phospholipase A_1 was also possibly G protein regulated (Sec. 4.3.), it was possible that isoproterenol could be activating the hydrolysis of 1-16:0-2-18:2 GPE via a G protein that is coupled to the ß-adrenergic receptor. Experiments were therefore carried out to test this possibility. The results of this experiment are shown in Table 34. The addition of 1 μM GTP[S] in the presence of 1 nM isoproterenol did not result

Table 33. Effect of Ca^{2+} and EGTA on the isoproterenol mediated activation of the guinea-pig heart microsomal phospholipase A_1.

Phospholipase A_1 activity was assayed in the presence of Ca^{2+}, EGTA, and isoproterenol either alone or combined. The phospholipase A_1 assay was performed as described in the Materials and Methods section with 1-16:0-2-18:2 GPE as the substrate. The guinea-pig heart microsomes were preincubated with the additives indicated for 10 min at room temperature prior to initiation of the assay. The values are presented as a percentage of the phospholipase A_1 activity observed without any additives. The values represent the means and standard deviation of three experiments each done in triplicate. The specific activity of the phospholipase A_1 in the absence of any additives was 13 ± 3 nmol LPE formed/hr/mg protein.

Addition	Activity (% of control)
None	100
1 nM Isoproterenol	157 ± 13
100 μM Ca^{2+}	165 ± 18
2 mM EGTA	83 ± 10
100 μM Ca^{2+} + 1 nM Isoproterenol	140 ± 9
2 mM EGTA + 1 nM Isoproterenol	93 ± 10

in an enhancement of the rate of hydrolysis of 1-16:0-2-18:2 GPE by the phospholipase A_1. Similar results were obtained when GTP was added with isoproterenol. However, evidence that a G protein may be involved was shown when experiments were performed involving the simultaneous addition of 1 μM GDPßS and 1 nM isoproterenol. The inclusion of GDPßS inhibits the isoproterenol mediated activation of phospholipase A_1. To test if GTP[S] and isoproterenol could activate phospholipase A_1 activity in a additive fashion, phospholipase A_1 activity was assayed in the presence of suboptimal concentrations of these compounds. The results of this experiment are shown in Table 35. At a concentration of 1 fM isoproterenol or 10 nM GTP[S], there is no activation of the phospholipase A_1. But the addition of 1 fM isoproterenol and 10 nM GTP[S] to the reaction mixture resulted in a 73% activation of the PE-hydrolyzing phospholipase A_1 activity. These results provide evidence of a link between the ß-adrenergic receptor activation and the phospholipase A_1 through a G protein. There was no effect of cAMP (1 nM) at a concentration that is greater than the basal level of cAMP in the heart (228) on the phospholipase A_1 (Table 36).

Table 34. Effect of nucleotides on the isoproterenol mediated activation of phospholipase A_1.

Phospholipase A_1 activity was assayed in the presence of 1 nM dl-isoproterenol and 1 μM of various nucleotides using 1-16:0-2-18:2 GPE as the substrate. Prior to initiation of the assay the guinea-pig heart microsomes were pre-incubated in buffer and water with the additives indicated for 10 minutes at room temperature. The assay was performed as described in the Materials and Methods section. The values represent the means and standard deviation of three experiments each performed in triplicate. The values are presented as a percentage of the activity of a control with no additives. The specific activity of the phospholipase A_1 without any additives was 12 ± 4 nmol LPE/hr/mg protein.

Addition	Activity (% of control)
None	100
1 μM GTP[S]	169 ± 15
1 nM Isoproterenol	152 ± 22
1 μM GTP[S] + 1 nM Isoproterenol	121 ± 15
1 μM GTP + 1 nM Isoproterenol	136 ± 17
1 μM GDPβS + 1 nM Isoproterenol	77 ± 15

Table 35. Effect of suboptimal concentrations of GTP[S] and dl-isoproterenol on the phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE.

Phospholipase A_1 was assayed in the presence of GTP[S] and isoproterenol at concentrations of these activators that did not activate the enzyme. The guinea-pig heart microsomes were pre-incubated for 10 min prior to the start of the assay with the various additives. The phospholipase A_1 activity was assayed using 1-16:0-2-18:2 GPE as the substrate according to the procedure given in the Materials and Methods section. The values represent the means and standard deviation of three experiments each performed in triplicate. The values are presented as a percentage of the specific activity observed with a control with no additives. The specific activity of this control was 12 ± 2 nmol LPE formed/hr/mg protein.

Addition	Activity (% of control)
None	100
10^{-9} M Isoproterenol	151 ± 8
10^{-15} M Isoproterenol	66 ± 14
10^{-8} M GTP[S]	111 ± 17
10^{-8} M GTP[S] + 10^{-15} M Isoproterenol	173 ± 21

Table 36. Effect of dibutryl-cAMP on the hydrolysis of 1-16:0-2-18:2 GPE by the guinea-pig heart microsomal phospholipase A_1.

Phospholipase A_1 activity was assayed in the presence of dibutyrl-cAMP. The dibutryl-cAMP was preincubated for 10 min with the heart microsomes prior to the start of the assay. The conditions and procedure for the assay of phospholipase A_1 activity with 1-16:0-2-18:2 GPE are as described in the Materials and Methods section. The values below represent the means and standard deviation of two experiments each performed in triplicate. The values are presented as a percentage of the activity found with a control which contained no additives. The specific activity of the phospholipase A_1 activity of this control was 17 ± 2 nmol LPE formed/hr/mg protein.

Addition	Activity (% of control)
None	100
1 nM dibutryl-cAMP	65 ± 4

5. Discussion

This study documents experiments which were performed in order to establish if fatty acids could be selectively released from membrane phospholipids in the mammalian heart by a phospholipase A_1/lysophospholipase A_2 pathway. In order to determine if indeed such a pathway exists, the approach of characterizing the two enzymes in the pathway was taken. The phospholipase A_1 and lysophospholipase A_2 activities as they exist in guinea-pig heart microsomes were studied and characterized with respect to acyl specificity and regulation of activity.

5.1. 2-Acyl GPE lysophospholipase A_2 activity in guinea-pig heart microsomes

The results of this study demonstrated the existence of a lysophospholipase A_2 activity in guinea-pig heart microsomes that hydrolyzes 2-acyl GPE. This lysophospholipase A_2 exhibited highest activity with 2-acyl GPE substrates containing arachidonic and linoleic acid. Hence, the lysophospholipase A_2 had a preference for specific molecular species of 2-acyl GPE. The lysophospholipase A_2 activity was observed in guinea-pig heart, brain, kidney, lung and liver. In all these tissues, the lysophospholipase A_2 displayed a preference for arachidonic containing substrates over other 2-acyl GPE substrates.

The lysophospholipase A_2 activity in the heart microsomal subcellular fraction appeared to be distinct from a lysophospholipase A_1 activity in the same subcellular fraction. The enzyme activities differed with respect to their sensitivity to various detergents, the effect of Ca^{2+}, and most importantly in their acyl specificity. The 2-acyl GPE lysophospholipase A_2 displayed highest activity with 2-arachidonoyl and 2-linoleoyl GPE while the 1-acyl GPE lysophospholipase exhibited lowest activity with 1-arachidonoyl GPE. In fact, the lysophospholipase A_1 exhibited highest activity with 1-

palmitoyl GPE; a substrate with which the lysophospholipase A_2 had lowest activity.

The different acyl specificities cannot be attributed to the different solubilities of the various substrates in solution as all the assays were conducted in the presence of the detergent Triton QS-15. This detergent itself had no inhibitory effect on the lysophospholipases. Such an acyl preference of lysophospholipase A_1 or lysophospholipase A_2 has not previously been shown. Previous studies have used only 1-palmitoyl GPC or 1-myristoyl GPC as substrates. Another study which assayed for lysophospholipase A_2 activity in rat liver cytosol used only 2-stearoyl GPC and 2-oleoyl GPC as the substrates (146). The rat liver cytosolic lysophospholipase A activity was not assayed with any other polyunsaturated fatty acid containing 2-acyl GPC or any 2-acyl GPE substrate (146). This study did demonstrate that the rat liver lysophospholipase activity had a slight preference for 2-oleoyl GPC over 2-stearoyl GPC (146). The acyl specificities of both the lysophospholipase A_1 and A_2 activities in this study are consistent with the expected molecular composition of membrane phospholipids.

The characteristics of the 2-acyl GPE lysophospholipase A_2 described in this study were different from those previously described for a 2-acyl GPC lysophospholipase A_2 in guinea-pig heart microsomes (158). These two lysophospholipases differed with respect to the pH required for optimal activity. The 2-acyl GPC lysophospholipase had an optimal pH of 8 while the 2-acyl GPE lysophospholipase has an optimal pH of 9. The 2-acyl GPE lysophospholipase was inhibited by Ca^{2+} whereas the lysophospholipase A_2 characterized in this study was not affected by Ca^{2+}. Even though both lysophospholipases A_2 exhibited a preference for unsaturated substrates, the acyl specificity of the two enzymes were different. The 2-

acyl GPC lysophospholipase A_2 exhibited highest activity with 2-linoleoyl GPC. The 2-acyl GPC displayed approximately 2-fold greater activity with 2-linoleoyl GPC than with 2-arachidonoyl GPC (161). The 2-acyl GPE lysophospholipase A_2 did not display a preference between 2-arachidonoyl GPE and 2-linoleoyl GPE when these substrates are presented at a concentration of 50 μM. At a concentration of 200 μM, the lysophospholipase A_2 displayed highest activity with 2-arachidonoyl GPE relative to the other 2-acyl GPEs.

There were also differences in the response of the 2-acyl GPE and 2-acyl GPC lysophospholipases to mixtures of different molecular species of their respective substrates. When 2-acyl GPC such as 2-palmitoyl GPC and 2-arachidonoyl GPC are presented as an equimolar mixture to guinea-pig heart microsomes, the rate of hydrolysis of 2-arachidonoyl GPC was decreased 87% relative to an experiment where 2-arachidonoyl GPC was presented singly (161). The rate of hydrolysis of 2-palmitoyl GPC by the 2-acyl GPC lysophospholipase A_2 was only decreased by 20% (161). If these observations were compared with those from a similar experiment with mixtures of 2-palmitoyl GPE and 2-arachidonoyl GPE, the rate of hydrolysis of 2-arachidonoyl GPE was only inhibited 44% while the rate of hydrolysis of 2-palmitoyl GPE was inhibited 54%. The end result of this experiment with mixtures of 2-acyl GPC was that there was a loss of selectivity of the lysophospholipase A_2 for 2-arachidonoyl GPC whereas in experiments with mixtures of 2-acyl GPE, there was still a 5-fold greater hydrolysis of 2-arachidonoyl GPE relative to that of 2-palmitoyl GPE.

With mixtures of 2-linoleoyl GPE and 2-arachidonoyl GPE, there was mutual inhibition of the 2-acyl GPE lysophospholipase A_2 activity. The end result was that there was no difference between the rate of hydrolysis of 2-arachidonoyl GPE and 2-

linoleoyl GPE when these substrates were presented together. The rates of hydrolysis of these substrates were however lower than those observed when the substrates were presented singly. In similar experiments performed with 2-linoleoyl GPC and 2-arachidonoyl GPC, a loss of selectivity of 2-linoleoyl GPC over 2-arachidonoyl GPC was observed to be the end result (158). This was due to greater inhibition of 2-linoleoyl GPC hydrolysis by the presence of 2-arachidonoyl GPC than vice-versa. The outcome of experiments with mixed substrates of 2-acyl GPC are certainly different from those with 2-acyl GPE substrates.

In all, this study has presented several lines of evidence that indicate that there are distinct activities responsible for the hydrolysis of the lysophospholipids: 1-acyl GPE, 2-acyl GPE, and 2-acyl GPC in guinea-pig heart microsomes. The 2-acyl GPE lysophospholipase A_2 appears to be distinct from the 1-acyl GPC, 1-acyl GPE and 2-acyl GPC lysophospholipases and as well from a Ca^{2+}-independent phospholipase A_2 in the heart microsomal fraction. Unambiguous proof will require the purification and characterization of the various enzyme activities.

The characteristics of the guinea-pig heart microsomal lysophospholipase A_2 with particular reference to its acyl specificity and selectivity for 2-arachidonoyl GPE and 2-linoleoyl GPE are consistent with a role for this enzyme in the selective release of fatty acids from ethanolamine glycerophospholipids in the absence of any increase in the intracellular Ca^{2+}. The characteristics of this enzyme suggest that it may act to release polyunsaturated fatty acids and not just one specific fatty acid at all times. In view of the results of experiments with mixed 2-acyl GPE substrates, the operation of this enzyme in a pathway for the selective release of a particular fatty acid (eg. arachidonate) would require the presence of a phospholipase A_1 that is specific for the

hydrolysis of a specific molecular species of diacyl PE, leading to the generation of a large amount of one molecular species of 2-acyl GPE.

Accumulation of lysophosphatidylcholine in membranes is cytolytic and has been implicated in the pathogenesis of irreversible myocardial injury (68,229). In light of this, it is not surprising that the cell has a total of five different enzymes which can act to metabolize further lysophospholipids and hence prevent their accumulation. One of these five enzymes is the lysophospholipase A_2. It is still unclear what the exact role of this enzyme is in cell function. However, given the characteristics of the lysophospholipase A_2 shown in this study, the lysophospholipase A_2 may contribute to cellular phospholipid metabolism in a manner that is more specific than just general catabolism of lysophospholipids.

5.2. Phosphatidylcholine-hydrolyzing phospholipase A_1 activity in guinea-pig heart microsomes

The characteristics of the lysophospholipase A_2 with 2-acyl GPC indicated that this lysophospholipase A_2 could work in concert with a phospholipase A_1 to release fatty acids from phosphatidylcholine. The specificity of release of a given fatty acid would be dependent on the selectivity of the phospholipase A_1, that catalyzes the initial reaction, for a specific molecular species of phosphatidylcholine. The existence of a phospholipase A_1 with such specificity is not known. A phospholipase A_2 activity was purified from hamster heart cytosol with phospholipase A_1 activity against molecular species of PC containing arachidonate at the sn-2 position (230). There is no report of a membrane bound phospholipase A_1 displaying a preference for specific molecular species of any phospholipid.

This study has shown that a phospholipase A_1 activity is present in guinea-pig heart microsomes and that this activity catalyzes the hydrolysis of various molecular species of PC. The enzyme displayed a preference for substrates presented as micelles, it was Ca^{2+} independent and active at neutral pH. The guinea-pig heart microsomal phospholipase A_1 shares some characteristics of the partially purified phospholipase A_1 from rat heart sarcoplasmic reticulum (40). Both the guinea-pig heart and the rat heart phospholipase A_1 have optimal pHs for activity that are in the neutral pH range. The rat heart phospholipase A_1 was partially purified after solubilization of membranes with sodium taurodeoxycholate. This detergent activated the guinea-pig heart microsomal phospholipase A_1.

The PC-hydrolysing phospholipase A_1 showed highest activity with substrates containing a palmitic acid at the sn-1 position. The relative rates of hydrolysis of PC substrates containing palmitic acid at the sn-1 position but with various fatty acids at the sn-2 position were compared and it was observed that the nature of the fatty acid at the sn-2 position significantly affected the rate of hydrolysis of the sn-1 fatty acid. The order of decreasing hydrolysis was linoleate > arachidonate = oleate > palmitate. There are suggestions that the selectivity of phospholipases, especially phospholipase A_2, for arachidonate containing substrates may reflect the ease with which the enzyme penetrates different phospholipid micelles (231). This was not the case with the phospholipase A_1 in this study for several reasons. All the assays were conducted with substrate presented as mixed micelles of taurodeoxycholate which should render all the substrates equally soluble in aqueous solutions. The hydrolysis of arachidonate containing species of PE which would be expected to be the most favoured on the basis of the ease of penetration in the micelle (231) was not hydrolyzed at a rate that

was different from that of 1-palmitoyl-2-oleoyl GPC. Rather the enzyme displayed highest activity with 1-palmitoyl-2-linoleoyl GPC. Finally, the rate of hydrolysis of the other arachidonate containing species, 1-stearoyl-2-arachidonoyl GPC was similar to that of dipalmitoyl GPC. Therefore, the acyl specificity presented in Table 12 is believed to properly reflect the acyl specificity of the phospholipase A_1.

This study documents for the first time modulation of phospholipase A_1 activity by guanine nucleotides. Both the hydrolysis of 1-16:0-2-18:2 GPC and 1-18:0-2-20:4 GPC were inhibited by the addition of the non-hydrolysable analogue of GTP: GTP[S]. Hydrolysis of 1-16:0-2-20:4 GPC was inhibited by 50-60% by 1 μM GTP[S] while a 30% inhibition of 1-16:0-2-18:2 GPC hydrolysis was only observed with 1 mM GTP[S]. This effect of GTP[S] on the phospholipase A_1 activity was specific as it could not be reproduced by the addition of GTP, GDPβS, ATP[S] or ATP. The inhibitory effect of GTP[S] on phospholipase A_1 activity was observed in the presence and absence of the cation chelators EDTA and EGTA. Therefore, this inhibition was independent of the presence of cations. This inhibitory effect could be blocked by the presence of GDPβS. Taken together, the above results provide evidence for the involvement of a G protein in the inhibition of PC-hydrolysing phospholipase A_1 activity in guinea-pig heart microsomes. Definite proof would require the purification and reconstitution of the components. It should be mentioned that G protein mediated inhibition of phospholipase A_2 activity has been previously observed in experiments using retinal rod outer segments (177).

The results of experiments on the effect of GTP[S] and the effect of heating on the guinea-pig heart microsomal phospholipases A_1 that hydrolyze 1-16:0-2-18:2 GPC and 1-18:0-2-20:4 GPC suggest that there may be at least two distinct phospholipase

A_1 activities in the guinea-pig heart. Although these results do not provide conclusive evidence of distinct enzymes which would require purification of these activities, the evidence is consistent with such a postulate. Gel filtration chromatography of a taurodeoxycholate solubilised supernatant of rat heart sarcoplasmic reticulum identified two phospholipase A_1 enzymes which exhibited different susceptibilities to heat (40). Unfortunately, all the phospholipase A_1 assays performed by Trotz et al. (40) were done using 1-16:0-2-18:1 GPC as a substrate, hence it is not known whether the two phospholipases A_1 in the rat heart exhibit any acyl specificity.

The acyl specificities of the PC-hydrolyzing phospholipase A_1, the 2-acyl GPC lysophospholipase A_2, and the abundance of linoleic acid esterified at the sn-2 position of diacyl phosphatidylcholine in the guinea-pig heart (64), it is obvious that the phospholipase A_1 and the lysophospholipase A_2 may act in concert to selectively release fatty acids (especially linoleic acid) from diacyl PC. The results of this study would suggest that the activation of receptors coupled to G protein(s) activated in this study would result in the inhibition of fatty acid release by the phospholipase/lysophospholipase pathway rather than the stimulation of fatty acid release. Hence, the PC-hydrolyzing phospholipase A_1 may be coupled to an inhibitory G protein. This observed inhibition of phospholipase A_1 activity by GTP[S] does not, however, preclude the activation of PC-hydrolyzing phospholipase A_1 by a G protein subsequent to receptor activation. In our studies, the addition of GTP[S] to the membranes would promote the formation of free $G\alpha$ and free $\beta\delta$ subunits from multiple G proteins whose net effect might be the inhibition of the PC-hydrolyzing phospholipase A_1 activity. In intact cells, stimulation by an agonist will be expected to activate only one particular G protein. It is not therefore inconceivable that G

proteins are present in heart membranes which might activate the PC-hydrolyzing phospholipase A_1 but whose effects were not detectable under conditions of activation of multiple G proteins. Several enzymes are known whose activities are modulated by $\beta\gamma$ subunits and not the Gα subunits of G proteins (193). The experiments which were performed in this study do not distinguish whether the observed effects are due to the α or $\beta\gamma$ subunits of G proteins. The physiological significance of GTP[S] mediated inhibition is not known at this time.

5.3. Phosphatidylethanolamine-hydrolyzing phospholipase A_1 activity in guinea-pig heart microsomes.

The results of this study has revealed that guinea-pig heart microsomes contain a phospholipase A1 that hydrolyzes PE with an apparent preference for arachidonoyl-containing substrates. Previous studies by other investigators have reported the existence of PE-hydrolyzing phospholipase A_1 activities of myocardial origin (29,37,38). In all previous cases, the microsomal phospholipase A_1 activity was assayed with either 1-palmitoyl-2-linoleoyl GPE or 1-acyl-2-linoleoyl GPE. The microsomal PE-phospholipase A_1 activities in hamster, rat, and dog heart had optimal pHs between 7.5 and 8.0. In this study, the guinea-pig heart microsomal phospholipase A_1 had a more alkaline pH optimum of 9.0. The hamster and rat heart phospholipase A_1 activities were activated by high concentrations of Ca^{2+} (5 mM) and inhibited to some extent by EDTA while the canine heart phospholipase A_1 was activated by EDTA (5 mM) and inhibited by Ca^{2+} (5 mM). In this study, the phospholipase A_1 activity when assayed with 1-16:0-2-18:2 GPE was activated by micromolar concentrations of Ca^{2+}. EGTA (2 mM) but not EDTA (2 mM) slightly inhibited the enzyme activity. This is an indication that the PE-hydrolyzing phospholipase A_1 in this study is most likely different

from those characterized in dog, rat, and hamster heart.

The phospholipase A_1 activity in guinea-pig heart microsomes displayed a clear preference for 1-18:0-2-20:4 GPE as substrate compared with other molecular species of PE. The nature of the fatty acid at the sn-2 position influenced the rate of hydrolysis of the sn-1 fatty acid. A similar conclusion was reached in studies with the PC-hydrolyzing phospholipase A_1 described above. The differences in the rates of hydrolysis of the different molecular species of PE by the phospholipase A_1 cannot be attributed to differential solubilities of the various substrates used as all the assays were carried out in the presence of the detergent taurodeoxycholate. This preference for arachidonate-containing substrates is similar to that of the 2-acyl GPE lysophospholipase A_2 (Sec. 5.1.). Incidentally, the majority of PE species in guinea-pig heart microsomes contain arachidonate esterified at the sn-2 position (64). The sequential activity of this phospholipase A_1 and the 2-acyl GPE lysophospholipase A_2 on PE in the guinea-pig heart would be expected to result in the selective release of arachidonate. As other myocardial phospholipases A_1 have only been assayed with one molecular species of PE, a comparison between the acyl specificity obtained in this study cannot be made to other studies.

Evidence has also been obtained for the possible existence of distinct enzymes catalyzing the hydrolysis of different PE molecular species. This would suggest that the differences in the rates of hydrolysis of the different molecular species of PE may reflect differences in the rates of discrete enzymes. Both the hydrolysis of 1-16:0-2-18:2 GPE and 1-18:0-2-20:4 GPE were stimulated by Ca^{2+}, while Mg^{2+} only activated the hydrolysis of 1-16:0-2-18:2 GPE. The heat inactivation studies revealed that the activity hydrolyzing 1-16:0-2-18:2 GPE was very heat sensitive whereas the activity

hydrolyzing 1-18:0-2-20:4 GPE was still active after 1 min of heating at 60 °C. The results of assays with mixtures of substrates also indicate that these two molecular species of PE may be hydrolyzed by distinct enzymes. The hydrolysis of 1-16:0-2-18:2 GPE was not inhibited by the presence of 1-18:0-2-20:4 GPE. This lack of inhibition of hydrolysis of 1-16:0-2-18:2 GPE by equimolar amounts of 1-18:0-2-20:4 GPE suggests that the two substrates do not bind to a single substrate binding site. The non-identity of the activities hydrolyzing 1-16:0-2-18:2 GPE and 1-18:0-2-20:4 GPE was also indicated by experiments on the effect of GTP[S] on the PE-hydrolyzing activity. In all, the above findings suggest the presence of at least two phospholipase A_1 activities in the guinea-pig heart microsomes that are responsible for the hydrolysis of different molecular species of PE. Definitive proof for this conclusion would require the chromatographic separation or partial purification of two phospholipase A_1 activities from the heart microsomes each active with different molecular species of PE as substrate.

As mentioned above, a differential effect of non-hydrolysable analogues of GTP, GTP[S] and GMPPNP, was observed on the hydrolysis of 1-18:0-2-20:4 GPE and 1-16:0-2-18:2 GPE. The rate of hydrolysis of 1-18:0-2-20:4 GPE was unaffected by the addition of GTP[S] or GMPPNP. In contrast, the hydrolysis of 1-16:0-2-18:2 GPE was activated by the above nucleotides. This activation was not duplicated by the addition of GDPβS or ATP. Direct activation of G proteins by non-hydrolysable analogues of GTP is a well established procedure that implicates the involvement of G proteins in cellular processes. Therefore, the specific effect of GTP[S] and GMPPNP on the phospholipase A_1 activity with 1-16:-2-18:2 GPE suggests that the hydrolysis of this particular substrate may be modulated by a G protein. An activation of the

phospholipase A_1 activity was obtained with GTP but the level of activation was much less than that observed with GTP[S] and GMPPNP. This is not surprising as the active state of a G protein will be maintained for a much shorter period of time by GTP compared to GTP[S] or GMPPNP. Inhibition of phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE by 1 mM GTP[S], GMPPNP, and ATP was observed. The reason for this inhibition is not apparent, but an analogous observation was reported in studies on the effect of GTP[S] on phospholipase D activity in neutrophil membranes (232). The hydrolysis of phosphatidylethanolamine by phospholipase A_1 does not depend on the addition of Mg^{2+} or Ca^{2+}, but guanine nucleotide mediated activation of this phospholipase A_1 activity appears to be cation-dependent. In the presence of EGTA or EDTA (2 mM), the stimulatory effect of GTP[S] was abolished. A Ca^{2+} requirement for the stimulation of phospholipase A_2 activity by GTP analogues has been observed in neutrophils (180). In our studies, the addition of cations Ca^{2+} or Mg^{2+} did not increase the rate of phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE beyond that of hydrolysis of GTP[S] alone suggesting that the enzyme activity was maximally stimulated by either cations or G proteins.

A possible hypothesis to explain the above <u>in vitro</u> findings on the PE-hydrolyzing phospholipase A_1 activity is that the activity of the enzyme may be activated under resting and stimulated conditions. Under conditions of low Ca^{2+} concentration, stimulation by an agonist that does not mobilize intracellular Ca^{2+} could increase the activity of the enzyme by activating the postulated G protein. The activation of the G protein would lead to enhanced hydrolysis of 1-16:0-2-18:2 GPE by the phospholipase A_1. This would produce 1-lyso-2-18:2 GPE a substrate favoured by the 2-acyl GPE lysophospholipase A_2. The end result would be the specific release

of linoleic acid from PE. On the other hand, stimulation by a Ca^{2+}-releasing agonist may also activate the phospholipase A_1 independent of the G protein. An increase in the level of intracellular Ca^{2+} would, on the basis of the results of this study, lead to the activation of more than one PE-hydrolyzing phospholipase A_1 resulting in the hydrolysis of more than one molecular species of PE. However, the selective release of arachidonate from diacyl PE could still occur by this mechanism despite the apparent hydrolysis of more than one molecular species of PE considering: (a) the greater rate of hydrolysis of arachidonate containing PE substrates by the guinea-pig heart microsomal phospholipase A_1; (b) the large proportion (65%) diacyl PE in guinea-pig heart microsomes with arachidonate esterified at the sn-2 position (64); and (c) the high rate of hydrolysis of 2-arachidonoyl GPE by the guinea-pig heart microsomal lysophospholipase A_2 (Sec. 5.1.). The differential modulation of hydrolysis of molecular species of PE observed in this study by guanine nucleotides and cations may provide a means for the selective release of fatty acids from PE via the postulated phospholipase A_1/lysophospholipase A_2 pathway.

The characteristics of the PE-hydrolyzing phospholipase A_1 activities differ significantly from the PC-hydrolyzing phospholipase A_1 (Sec. 5.2.) in the same tissue subcellular fraction. The stimulation of phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE by guanine nucleotides contrasts with the effect of GTP[S] on the PC-phospholipase A_1. The acyl specificities of the PE- and PC-hydrolyzing phospholipases A_1 were different. In fact, while 1-18:0-2-20:4 GPC was the substrate with which the PC-hydrolyzing phospholipase A_1 displayed lowest activity, the corresponding molecular species of PE was the substrate with which the PE-hydrolyzing phospholipase A_1 displayed highest activity. The hydrolysis of PE by phospholipase A_1 was stimulated

by Ca^{2+} while the hydrolysis of PC by phospholipase A_1 was unaffected by Ca^{2+}. The phospholipase A_1 hydrolysis of PC and PE had completely different pH profiles. The hydrolysis of PE by the phospholipase A_1 was not affected by the detergent taurodeoxycholate or deoxycholate, meanwhile, these detergents had an activatory effect on the phospholipase A_1 hydrolysis of PC. The hydrolysis of 1-16:0-2-18:2 GPE was stimulated by the addition of 1-16:0-2-18:2 GPC. This indicates that the added PC may interact with the PE-hydrolyzing phospholipase A_1. The inability of PC to inhibit PE hydrolysis is an indication that the two substrates are hydrolyzed by distinct active sites. Taken together the data suggest that the PC and PE hydrolyzing phospholipases A_1 are discrete enzymes. It is intriguing that the hydrolysis of the PE molecular species containing linoleate is stimulated by GTP[S] while the hydrolysis of the corresponding molecular species of PC is inhibited by GTP[S]. Linoleic acid is a fatty acid that is found in large quantities in both the PE and PC fractions of guinea-pig heart phospholipids (64). We have yet to establish whether the postulated G protein responsible for modulating the activity of the PE-hydrolyzing phospholipase A_1 is the same as that postulated for modulating the activity of the PC-hydrolyzing phospholipase A_1 activity. As well, although we have suggested a role for G proteins in the activation and inhibition of PE and PC hydrolyzing phospholipases A_1 respectively, we cannot say whether the effect is directly on the enzyme or via an intermediary protein.

5.4. Isoproterenol mediated activation of phosphatidylethanolamine-hydrolyzing phospholipase A_1

The availability of a specific agonist for this phospholipase A_1 would allow

us to test the possibility that PE-hydrolyzing phospholipase A_1 is a receptor coupled G protein regulated phospholipase. This is a distinct possibility as microsomal subcellular fractions contain plasma membranes as well as other membraneous organellar compartments. Many such phospholipases have been discovered and have been classified as signal activated phospholipases (229). Such a role has not been extended to the phospholipase A_1. The possible modulation of the PE-hydrolyzing phospholipase A_1 by dl-isoproterenol was examined and it was found that isoproterenol (10^{-3} to 10^{-13} M) could activate the phospholipase A_1 hydrolysis of 1-16:0-2-18:2 GPE by 30-50%. There was a 50% inhibition of the phospholipase A_1 activity with a concentration of 1 fM isoproterenol. The reason for this inhibition is not known. The activation of phospholipase A_1 activity by isoproterenol was blocked by the addition of the ß-adrenergic receptor antagonist propranolol. In fact, even the inhibition observed with 1 fM isoproterenol was not seen in the presence of 1 µM of propranolol. This implies that the inhibition and more importantly, the activation of phospholipase A_1 was directly due to ligand induced activation of the ß-adrenergic receptors by isoproterenol. α-Adrenergic agonists did not affect the phospholipase A_1 activity. Isoproterenol is a non-selective ß-adrenergic receptor agonist (210,242) which activates both the $ß_1$- and $ß_2$-adrenergic receptor subtypes. Propranolol is a non-specific ß-adrenergic receptor antagonist (210,242). Hence, it was thought that the activation of the phospholipase A_1 is through a ß-adrenergic receptor. It was further demonstrated that activation of the $ß_2$-adrenergic receptor but not the $ß_1$-adrenergic receptor was necessary for stimulation of phospholipase A_1 activity. The addition of the $ß_2$-receptor specific antagonist butoxamine but not the $ß_1$-receptor antagonist atenolol inhibited the isoproterenol mediated enhancement of phospholipase A_1 activity.

The activation of the phospholipase A_1 by isoproterenol was dependent upon the presence of Ca^{2+}. The combination of EGTA (2 mM) and isoproterenol (1 nM) did not result in an activation of the phospholipase A_1 activity. Moreover, there was no synergistic effect of Ca^{2+} (100 μM) and isoproterenol (1 nM) on the phospholipase A_1 activity. An additive or synergistic effect might be expected if the mechanism of activation by these two compounds was independent of each other. The activation of enzyme activity by Ca^{2+} alone was not significantly different from the level of activation observed in the presence of isoproterenol. Similar observations had been made in studies on the activation of PE-hydrolyzing phospholipase A_1 activity by GTP[S] (Sec. 5.3.). It was hypothesized that the activation of the PE-hydrolyzing phospholipase A1 by isoproterenol may be via a G protein. Experiments involving the addition of 1 μM GTP[S] and 1 nM isoproterenol to guinea-pig heart microsomes did not result in the activation of phospholipase A_1 activity greater than that with 1 μM GTP[S] or 1 nM isoproterenol alone. In fact the phospholipase A_1 activity observed when these two compounds were added together was actually less than that observed when either activator was added separately. Isoproterenol will act to activate a subset of G proteins that can activate the phospholipase A_1, GTP[S] alone will activate multiple G proteins present in the heart microsomes, which would presumably include G proteins that are activated by isoproterenol. However, the lack of a synergistic or additive effect may be due to a number of reasons. One explanation is that the enzyme activity was maximally activated by the presence of one activator and the addition of the other activator would therefore not be expected to further increase the enzyme activity. Another explanation is that since the addition of GTP[S] activates multiple G proteins non selectively, G proteins could be activated that act to inhibit the

stimulation of phospholipase A_1 activity and hence a synergistic effect is not seen. This could also be responsible in part for the slightly decreased level of activation observed when both GTP[S] and isoproterenol are added together.

Evidence for the involvement of a G protein in mediating the isoproterenol activation of phospholipase A_1 activity is implied by the experiments involving the addition of GDPßS (1 nM) and isoproterenol (1 nM). GDPßS (1 μM) by itself does not inhibit the phospholipase A_1 activity with 1-16:0-2-18:2 GPE (Table 26). GDPßS will act to inhibit all receptor G protein coupled responses. To determine if the reason for not observing a synergistic effect with GTP[S] and isoproterenol could be due to the fact that the enzyme was maximally activated, experiments were performed at concentrations of GTP[S] and isoproterenol that did not result in activation of the phospholipase A_1 activity. Under these conditions, if a synergistic effect of GTP[S] and isoproterenol is possible, it should be observed as an activation of the phospholipase A_1 activity. The results of this experiment demonstrate quite well that GTP[S] (0.01 μM) and isoproterenol (1 fM) together can act to stimulate the activity of the phospholipase A_1 in a synergistic fashion. From these results, it is believed that the hydrolysis of 1-16:0-2-18:2 GPE by the phospholipase A_1 is modulated by a G protein that is coupled to the heart $ß_2$-adrenergic receptor. The characteristics of the G protein in this section of the study are very similar to those described in section 5.3. Although final proof would require purification of the particular G proteins and reconstitution of the receptor, G protein, and phospholipase A_1, we believe that there is a G protein that is coupled to this receptor and this enzyme. The results of the experiments with isoproterenol and GTP[S] indicate that the phospholipase A_1 may be a signal activated phospholipases. More importantly, the results suggest a

mechanism by which linoleic acid can be selectively released in the heart. The activation of the $ß_2$-adrenergic receptor by binding of a ligand will result in the activation of a G protein that can stimulate a PE-hydrolyzing phospholipase A_1 that can hydrolyze 1-16:-2-18:2 GPE. The subsequent action of the lysophospholipase A_2 that hydrolyzes the phospholipase A_1 product 2-18:2 GPE will result in the release of linoleic acid. The $ß_2$-adrenergic receptor is known to be coupled via a G protein to adenylyl cyclase (207). Hence, receptor occupation will induce the activation of adenylyl cyclase which will produce cAMP from ATP. To be sure that activation of the phospholipase A_1 was not due to cAMP or cAMP dependent protein kinase, we investigated the effect of dibutyrl-cAMP on the hydrolysis of 1-16:0-2-18:2 GPE. There was no activation of phospholipase A_1 hydrolysis of this phospholipid by cAMP. Although cAMP does not seem to be involved in the mechanism of activation of the phospholipase A_1, it is not impossible that the activation of the $ß_2$-adrenergic receptor activates a G protein whose $G\alpha$ subunits stimulate the adenylyl cyclase while the $ß\gamma$ subunits activate the PE-hydrolyzing phospholipase A_1. This situation would be analogous to that reported in retinal rod cells where $G\alpha_t$ of transducin stimulates the cGMP phosphodiesterase and $ß\gamma$ subunits stimulate phospholipase A_2 (177).

6. Conclusions and Speculation

The question arises as to the function of this pathway in the heart. The function of a pathway that results in the selective release of arachidonic acid is immediately obvious. Such a pathway for the selective release of arachidonic acid will act to provide the heart with a precursor for the formation of the eicosanoids. A pathway which results in the release of linoleic acid is not so obvious. Recently,

several reports have been published that suggest that unsaturated fatty acids may activate protein kinase C (233,234,235,236). A potential role for unsaturated fatty acids such as oleic and linoleic acid as second messengers was proposed as early as 1984. McPhail et al. demonstrated that a 100 μM concentration of arachidonate was able to stimulate a Ca^{2+}-dependent protein kinase C activity from detergent extracts of isolated neutrophils by approximately 2-fold relative to controls (233). Similar experiments were performed in the same study with various concentrations of other fatty acids. Linoleic acid, oleic acid and γ-linolenic acid all demonstrated the ability to activate protein kinase C 2-3-fold. Further work by a number of investigators has shown that unsaturated fatty acids such as oleic, linoleic, and arachidonic acid can potentiate the activity of Ca^{2+}-independent protein kinase C in vitro (234,235,236). The concentration of unsaturated fatty acid required to elevate protein kinase C activity differed among published reports, however, concentrations as low as 12 μM have been shown to activate protein kinase C activity 2-5 fold (234). The protein kinase C activity was stimulated the most by arachidonic acid > linoleic acid > oleic acid (233,234,236). The potency of the unsaturated fatty acids to enhance protein kinase C activity paralleled to some degree the number of cis-unsaturated double bonds in the fatty acid. In contrast, experiments have shown that neither palmitic acid, stearic acid, or trans-fatty acids were able to duplicate this activation (233,235). Thus, a role for unsaturated fatty acids as activators of protein kinase C has been postulated (237,238). Mechanisms that result in the liberation of such unsaturated fatty acids are mechanisms that could be involved in the regulation of protein kinase C (237,238). Cis-unsaturated fatty acids may activate protein kinase C that is not membrane-associated (239) in either a Ca^{2+}-dependent or independent manner (238). The

manner of activation would depend a great deal upon the mechanism by which the fatty acid was generated. Generation of fatty acids by the phospholipase A_1/lysophospholipase A_2 pathway could occur in a Ca^{2+}-independent manner. As stated above, the fatty acids released from PE may differ depending on the presence or absence of Ca^{2+}. For the moment this is speculation as in vivo activation of protein kinase C by a particular fatty acid or a particular pathway which generates a single species of fatty acid is not known.

Another function of the phospholipase A_1/lysophospholipase A_2 pathway may be to provide cells of the vasculature with linoleic acid from which 13-HODE, a lipoxygenase derived hydroxy fatty acid can be produced. This hydroxy fatty acid is produced by vascular endothelial cells and functions as a chemorepellant to maintain the thromboresistance of blood vessel walls (240). This compound is synthesized by endothelial cells from linoleic acid under resting conditions but not under stimulated conditions. Thus under stimulated conditions, the synthesis of 13-HODE is inhibited. One mechanism for inhibiting the production of this compound would be to prevent the release of precursor linoleic acid. This could be the function of the inhibitory G protein postulated in this study. It could inhibit the release of linoleic acid from 1-16:0-2-18:2 GPC by the phospholipase A_1/lysophospholipase A_2 pathway. The precursor for linoleic acid for 13-HODE synthesis has been suggested to be triacylglycerols (241), however, the study did not rule out the release of linoleic acid from phospholipids by the postulated pathway followed by replenishment of the fatty acid in the phospholipids by transfer of fatty acids from triacylglycerols.

Since it has not been established whether the proposed phospholipase A_1/lysophospholipase A_2 pathway is functional in cells, the physiological significance

has yet to be determined. Catecholamine-induced stimulation of fatty acid release or eicosanoid production in the heart has been reported (243,244). The proposed link between catecholamines and induction of phospholipase A_1 activity may therefore have physiological and pathophysiological implications. The activation of phospholipase A_1 under normal circumstances may serve to produce compounds required to modulate cellular processes (ie. through eicosanoids or protein kinase C). If catecholamines (eg. adrenalin and noradrenaline) do activate phospholipase A_1 in cardiac cells as the results of this study lead us to suggest, then the high concentrations of catecholamines that accumulate in ischemia coupled with a loss of normal receptor desensitization mechanisms (245,246) would result in the generation of high levels of fatty acids and lysophospholipids from the activation of the phospholipase A_1. Lysophospholipids and other amphiphiles have been implicated as causative agents in the development of arrhythmias (68,247). It is therefore possible that activation of cardiac phospholipase A_1 by catecholamines may play a role in the pathogenesis of ischemia-induced arrhythmias.

In conclusion, the results of this study provide a description of phospholipases A_1 and lysophospholipases A_2 in guinea-pig heart microsomes. The characteristics and regulation of these enzymes allows us to postulate that they could work in concert to selectively release either arachidonic or linoleic or possibly other polyunsaturated fatty acids upon stimulation of cells. The particular fatty acid that is produced upon cell stimulation would depend greatly upon the type of stimulus. It is not inconceivable that this pathway could operate in conjunction with a phospholipase A_2 pathway, but it is quite probable that this pathway will operate under conditions in which the phospholipase A_2 pathway would not operate. This study has presented a

number of novel findings with respect to the regulation of the phospholipase A_1 (Figure 14). The phospholipases A_1 in this study have been shown to be modulated by GTP and its analogues. This is indicative of G protein regulation of the enzyme activities. Also, this study documents evidence for the classification of a PE-hydrolyzing phospholipase A_1 to be a receptor-G protein coupled protein. This is a novel finding as far as we are aware and extends the family of signal activated phospholipases to include the phospholipase A_1 as a member. The studies therefore provide a firm basis for investigating the release of fatty acids from PC or PE in intact cells via a phospholipase A_1/lysophospholipase A_2 pathway.

Figure 14

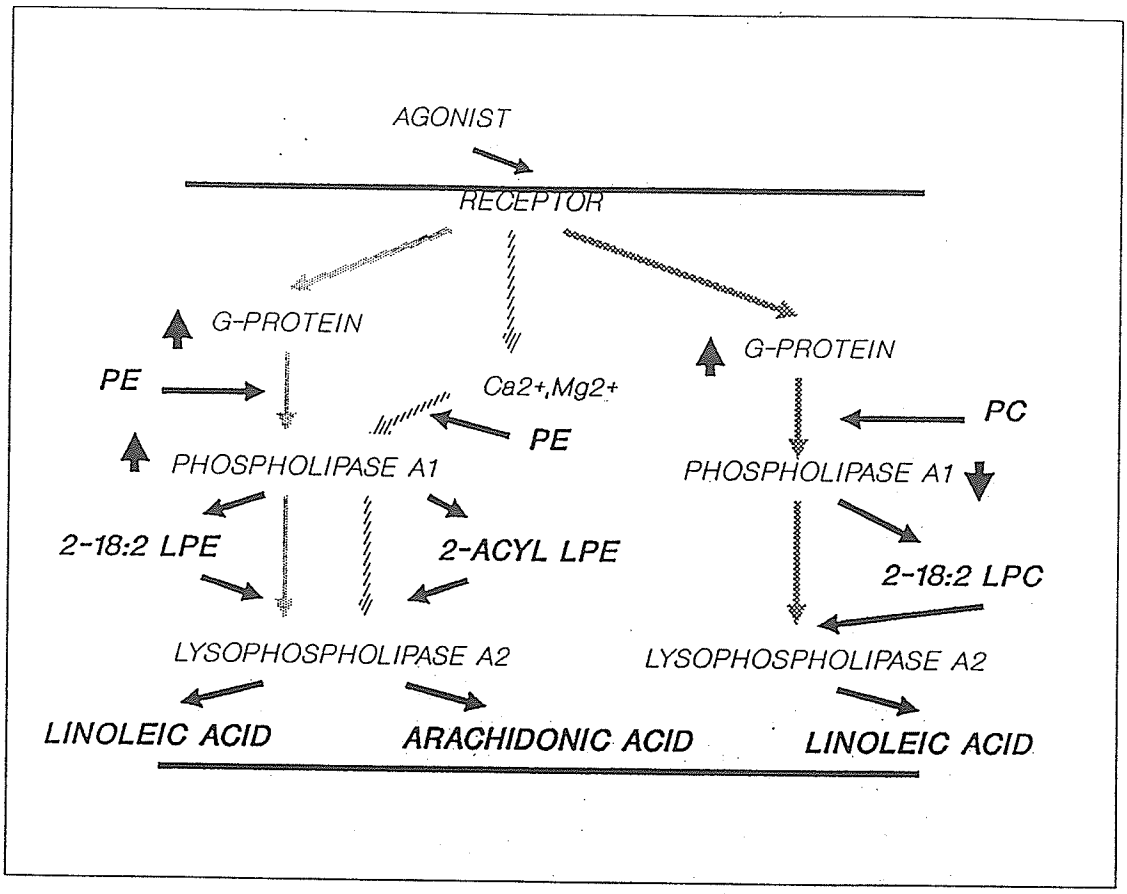

Fig. 14. Proposed model.

This model is derived from conclusions based upon the results of this study. It indicates how different fatty acids may be released under different forms of cell stimulation and cellular environments by a phospholipase A_1/lysophospholipase A_2 pathway in guinea-pig heart microsomes.

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Appendix 1. Nomenclature of some major long chain fatty acids.

Common Name	Systematic Name	Abbreviation
Palmitic acid	hexadecanoic acid	16:0
Stearic acid	octadecanoic acid	18:0
Oleic acid	9-octadecanoic acid	18:1
Linoleic acid	9,12-octadecanoic acid	18:2
Arachidonic acid	5,8,11,14-eicosatetraenoic acid	20:4