THE REGULATION OF PHOSPHOLIPID METABOLISM IN MAMMALIAN TISSUES

By

Jason T. Wong

A thesis submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements for the degree of

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of DOCTOR OF PHILOSOPHY

To my family

Mom, Dad, Sandra and Grandpa

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I would like to thank my supervisor, mentor and friend Dr. Patrick Choy. Pat, through your patient guidance over the years, you have given me valuable lessons in perseverance and enthusiasm for research and indeed for life. I shall always be in your debt.

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I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

Sir Isaac Newton

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LIST OF ABBREVIATIONS

$AACOCF_3$	arachidonoyltrifluoromethylketone
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cAMP	cyclic AMP
cDNA	complementary deoxyribonucleic acid
CDP-	cytidine diphospho-
CoA	coenzyme A
$cPLA_2$	cytosolic phospholipase A_2
CTP	cytosine triphosphate
DMSO	dimethylsulfoxide
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
ERK	extracellularly regulated kinase
GTP	guanosine triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
IFN	interferon
IL	interleukin
JNK	c-Jun N-terminal kinase

K_m	Michaelis-Menten coefficient
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAPK/ERK kinase
mRNA	messenger ribonucleic acid
PAP	phosphatidate phosphatase
pBPB	*para*-bromophenacyl bromide
PDGF	platelet-derived growth factor
PEMT	phosphatidylethanolamine *N*-methyltransferase
PG	prostaglandin
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
SAPK	stress-activated protein kinase
$sPLA_2$	secretory phospholipase A_2
Tris	tris(hydroxymethyl) aminomethane
Tx	thromboxane

ABSTRACT

The purpose of this study was to investigate the regulation of phospholipid biosynthesis and catabolism in mammalian tissues. Phospholipids form a fluid bilayer that provides both a selective permeability barrier and a matrix with which membrane proteins are associated. In addition to their structural role, phospholipids can be hydrolyzed to provide biological signalling molecules, such as arachidonic acid and its metabolites, known collectively as eicosanoids. The release of arachidonic acid is the rate-limiting step in eicosanoid synthesis.

In mammalian tissues, a considerable portion of phospholipid is synthesized *de novo* via phosphatidic acid. We hypothesize that there are at least two levels of control of phospholipid synthesis in the heart: (a) the activities of key biosynthetic enzymes; and (b) the energy status of the heart. Lidocaine was used as an agent to modulate phospholipid synthesis, while hearts were perfused under hypoxic conditions in order to alter the energy status of the heart. Perfusion of hearts in the presence of lidocaine enhanced the incorporation of [^{3}H]glycerol into lysophosphatidic acid, phosphatidic acid, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, and diacylglycerol. Phosphatidylcholine synthesis was reduced. Enzyme assays after heart perfusion showed that lidocaine stimulated phosphatidic acid phosphatase, which catalyzes the formation of 1,2-diacyl-*sn*-glycerol. In separate experiments, the addition of lidocaine to the assay mixtures did not directly affect this enzyme. In contrast, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase, which catalyzes the formation of lysophosphatidic acid, was

stimulated by the direct addition of lidocaine, whereas CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase, which catalyzes the final step in phosphatidylcholine synthesis, was inhibited.

Perfusion of hearts under hypoxic conditions caused a general decrease in the biosynthesis of phospholipids. Lidocaine partially restored the synthesis of most phospholipids examined, with the exception of phosphatidylcholine. ATP and CTP levels were reduced under hypoxia and were not restored by lidocaine. CTP:phosphatidic acid cytidylyltransferase, which catalyzes the rate-limiting step in phosphatidylinositol synthesis, was stimulated under hypoxia, and was further stimulated by lidocaine under hypoxia.

We conclude that lidocaine affects the regulation of phospholipid biosynthesis in the heart via both direct and indirect modulation of key enzymes. Furthermore, hypoxia lowered the energy status of the heart, which lead to depressed levels of the metabolites ATP and CTP, ultimately resulting in decreased phospholipid synthesis. The partial restoration of phospholipid synthesis by lidocaine appeared to be mediated at least in part by modulation of enzyme activities, indicating that perturbation of one level of control of phospholipid synthesis could be partially counteracted by modulation of another.

Lysophosphatidylcholine is a product of phosphatidylcholine hydrolysis by phospholipase A_2 (PLA_2), and is present in cell membranes, oxidized lipoproteins, and atherosclerotic tissues. It has the ability to alter endothelial functions and is regarded as a causal agent in atherogenesis. In this study, the modulation of arachidonic acid release by lysophosphatidylcholine in human umbilical vein endothelial cells was examined.

Incubation of endothelial cells with lysophosphatidylcholine resulted in an enhanced release of arachidonic acid in a time- and concentration- dependent manner. Maximum arachidonic acid release was observed at 10 min of incubation with 50 μM lysophosphatidylcholine. Lysophosphatidylcholine species containing palmitoyl ($C_{16:0}$) or stearoyl ($C_{18:0}$) groups elicited the enhancement of arachidonic acid release, while other lysolipids such as lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, or lysophosphatidic acid were relatively ineffective. Lysophosphatidylcholine-induced arachidonic acid release was decreased by treatment of cells with PLA_2 inhibitors such as *para*-bromophenacyl bromide and arachidonoyl trifluoromethyl ketone. Furthermore, arachidonic acid release was attenuated in cells grown in the presence of antisense oligodeoxynucleotides that specifically bind $cPLA_2$ mRNA. Treatment of cells with lysophosphatidylcholine resulted in a translocation of PLA_2 activity from the cytosolic to the membrane fractions of cells. Lysophosphatidylcholine induced a rapid influx of Ca^{2+} from the medium into the cells, with a simultaneous enhancement of protein kinase C (PKC) activity in the membrane fractions. The lysophosphatidylcholine-induced arachidonic acid release was attenuated when cells were preincubated with specific inhibitors of PKC (staurosporine and Ro31-8220) or a specific inhibitor of mitogen-activated protein kinase/extracellular regulated kinase kinase (PD98059). Taken together, the results of this study show that lysophosphatidylcholine caused the elevation of cellular Ca^{2+} and the activation of PKC, which stimulated $cPLA_2$ in an indirect manner and resulted in an enhanced release of arachidonic acid.

1 INTRODUCTION and LITERATURE REVIEW

1.1 The Biological Membrane

1.1.1 Introduction: structure and function of the biological membrane

The biological membranes of eukaryotic cells comprise plasma membranes and intracellular membranes. Biological membranes perform several functions that are essential to the life of eukaryotic cells. The plasma membrane delineates the boundaries of the cell and separates it from its environment, while the intracellular membrane system defines the intracellular organelles of eukaryotic cells. The organelles perform various functions essential to cell viability, and include the endoplasmic reticulum, Golgi apparatus, nucleus, mitochondria, lysosomes, and peroxisomes. In addition to its structural roles, biological membranes act as selective permeability barriers and regulate the flow of compounds that pass through those membranes. Finally, biological membranes participate in the transmission of inter- and intra-cellular signals. The hydrolysis of membrane lipids provides biologically active messenger molecules that mediate a wide variety of cellular responses (Liscovitch and Cantley 1994).

The general structure of biological membranes can be summarized in the Singer and Nicolson fluid mosaic model (Fig. 1) (Singer and Nicolson 1972). Lipids and proteins comprise the major structural components of biological membranes, while carbohydrates (associated with lipid or protein moieties) form a minor component of most membranes.

Most membrane lipids are amphipathic in nature, and assume a bilayer organization in the presence of an aqueous environment. The hydrophilic ("polar head group") regions of the lipids tend to orient themselves toward the aqueous phase, while the hydrophobic ("nonpolar tail") regions are oriented toward the centre of the bilayer where they are sequestered from water. Under physiological conditions, most and usually all lipid membrane bilayers are fluid in nature, which allows lipid and protein molecules to diffuse laterally within the plane of the membrane (Singer and Nicolson 1972; Cullis and Hope 1991). The bilayer organization of membrane lipids provide a matrix with which membrane proteins are associated.

In summary, the lipid moieties of biological membranes perform roles related to membrane structure, fluidity and permeability, and the association and function of the protein moieties. In the following sections, aspects of the structure and function of membrane lipids, proteins, and carbohydrates will be considered.

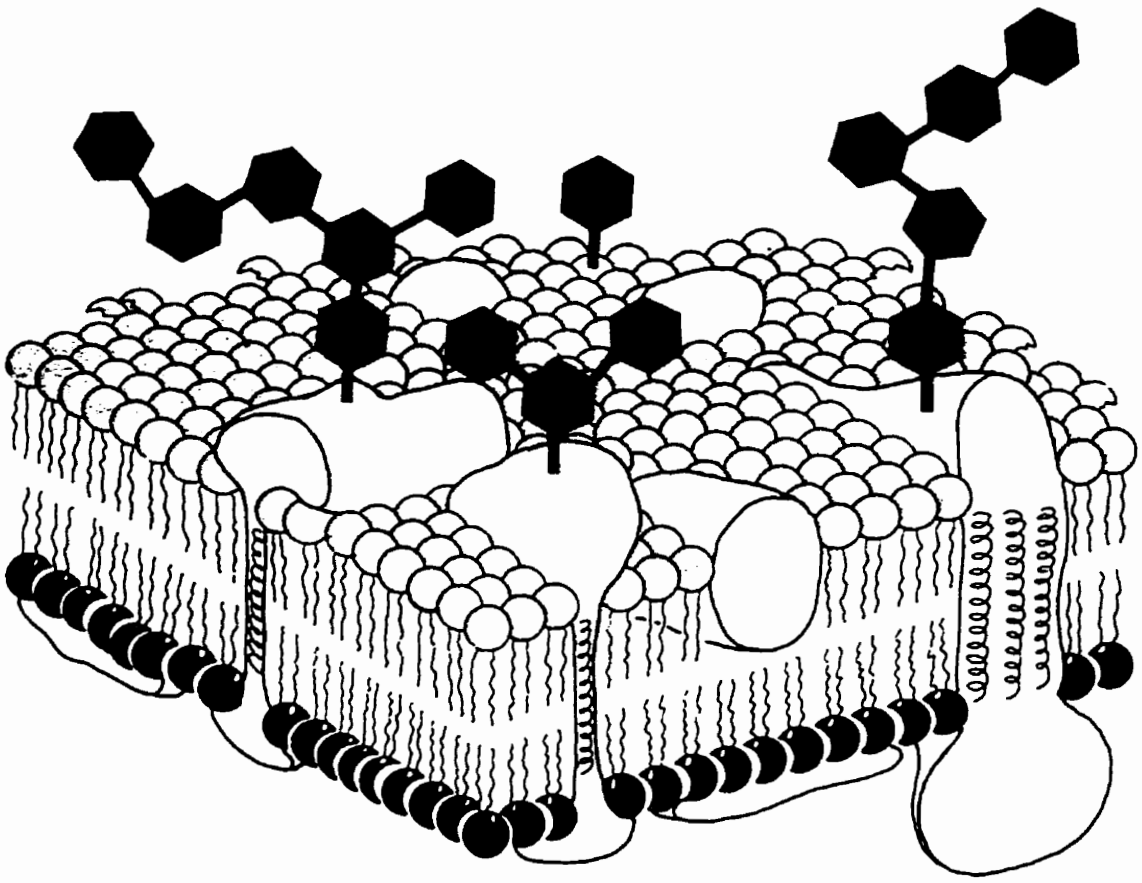


Figure 1. The fluid mosaic model of the eukaryotic plasma membrane depicting lipids proteins, and carbohydrate moieties.

1.1.2 Membrane lipids

The major classes of lipids found in eukaryotic membranes include glycerol-based phospholipids ("phosphoglycerides"), sphingosine-based lipids ("sphingolipids"), which also include the glycosphingolipids, and cholesterol. The terms "phospholipid" and "phosphoglyceride" will be used interchangeably in this work to refer to the glycerol-based phospholipids. The phosphoglycerides predominate in most eukaryotic membranes, and include phosphatidylcholine, phosphoatidylethanolamine, phosphatidylserine, phosphatidylinositol, and diphosphatidylglycerol (cardiolipin) (Fig. 2) (Cullis and Hope 1991). Cholesterol is another major component of mammalian plasma membranes and is a key modulator of membrane fluidity (Bloch 1991).

Fig. 2 depicts the structure of a phosphoglyceride molecule. The phosphoglyceride shown in Fig. 2 contains fatty acids linked, via their carboxyl groups, by ester bonds to the hydroxyl groups at the *sn*-1 and *sn*-2 positions of the glycerol moiety. These "1,2-diacyl-*sn*-glycerophospholipids" are the major form of phosphoglycerides in most mammalian tissues. Phosphoglycerides can be further subdivided according to the type of chemical linkage at the *sn*-1 position; the so-called "ether-linked phospholipids" include 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine and 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphocholine and their ethanolamine-containing analogues. Ether-linked phospholipids are a minor constituent of most mammalian tissues, but are found in significant quantities in some mammalian hearts and other electrically active tissues (Snyder 1991; Horrocks and Sharma 1982). Ether-linked phospholipids are present in very low quantities in rat and hamster heart. The fatty acid group at the *sn*-1 position is usually saturated, while an

unsaturated fatty acid is usually esterified at the *sn*-2 position (Holub and Kuksis 1978; Ansell and Spanner 1982; Cullis and Hope 1991). The hydroxyl group at the *sn*-3 position of the glycerol moiety is linked to a phosphate group, which may in turn be linked to the hydroxyl group of choline, ethanolamine, inositol, serine, glycerol or phosphatidylglycerol to form phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol or diphosphatidylglycerol (cardiolipin), respectively (Fig. 3). Phosphatidylcholine and phosphatidylethanolamine are the two most abundant phosphoglycerides in most mammalian tissues (White 1973; Ansell and Spanner 1982).

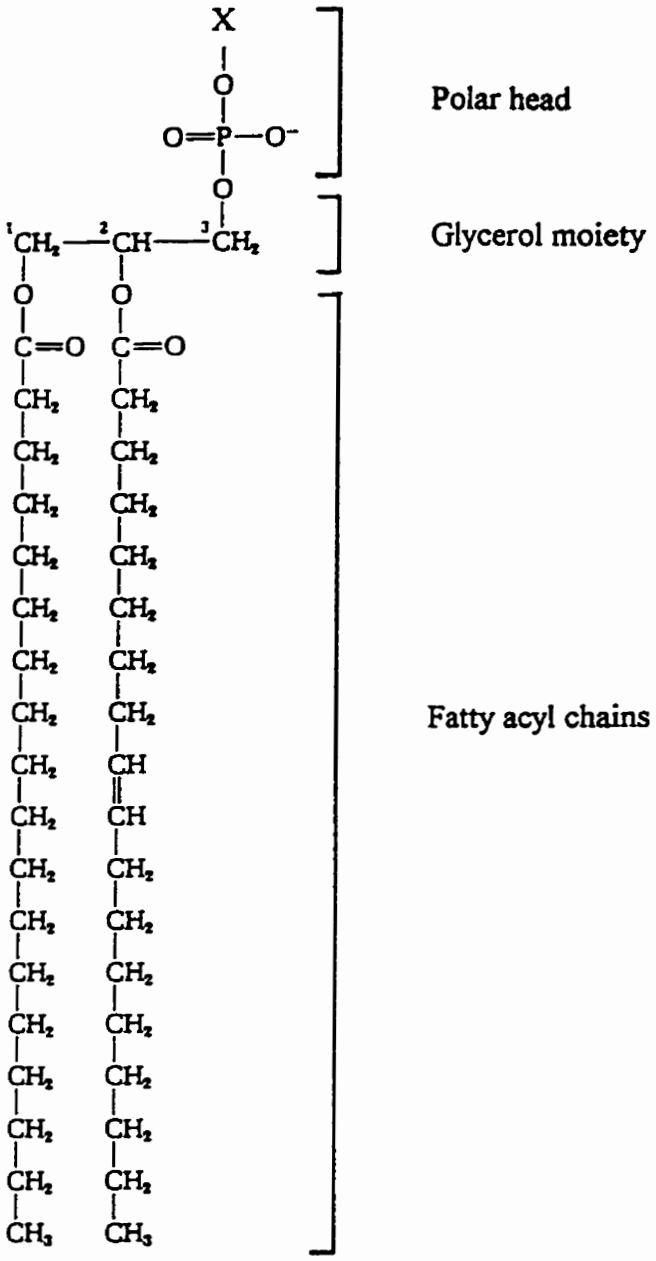


Figure 2. General structure of a phosphoglyceride molecule. X = Head group alcohol moiety, usually choline, ethanolamine, inositol or serine (see also Fig. 3).

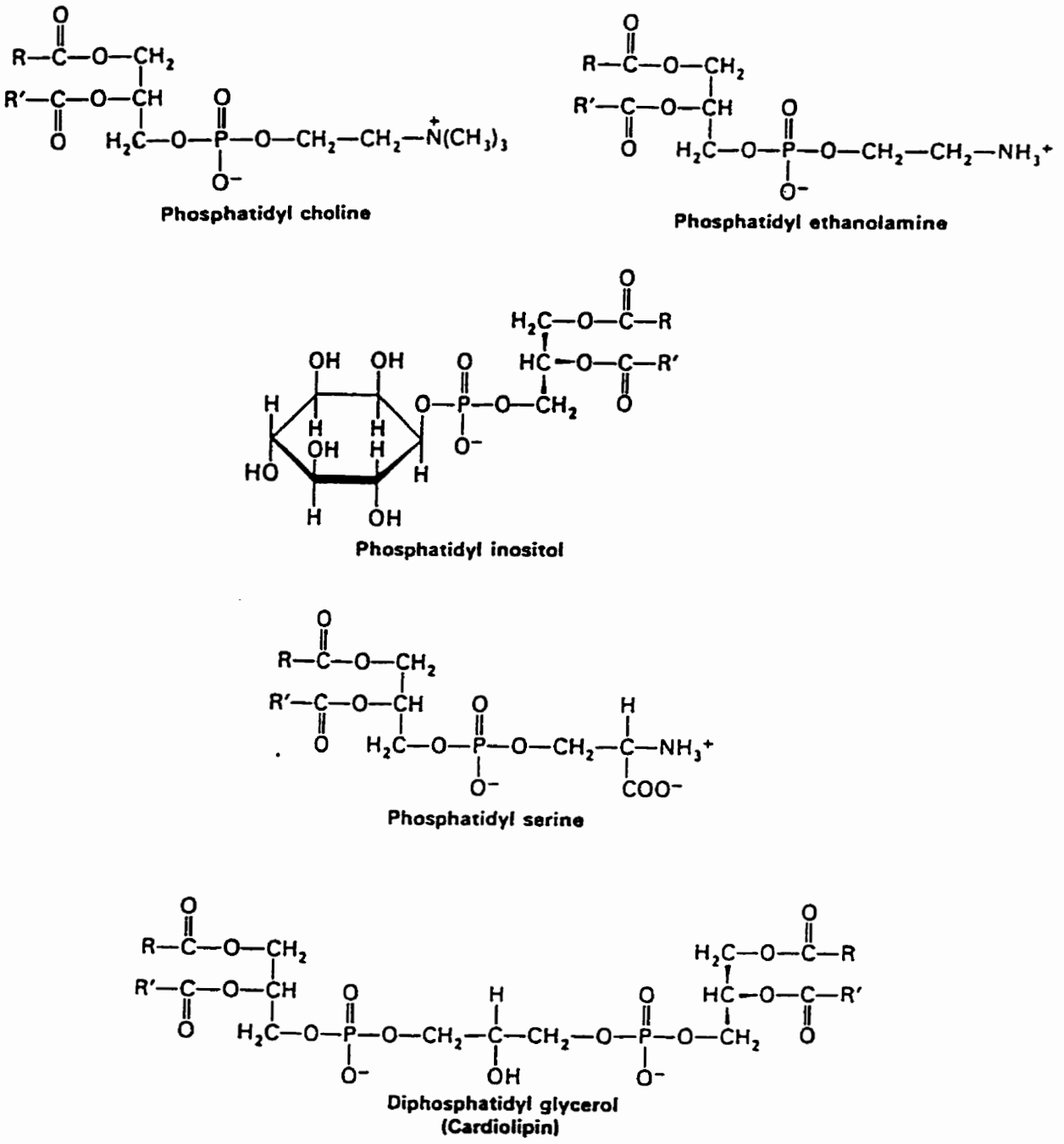


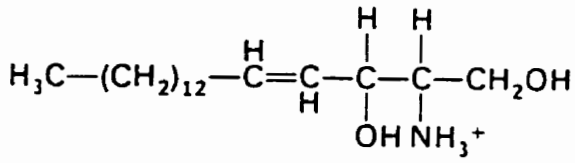
Figure 3. Five major types of phospholipids found in mammalian tissues.

As mentioned earlier, sphingosine-based lipids are known as sphingolipids (Fig. 4). Ceramide contains a fatty acid linked to the amino group of sphingosine via an amide bond. The major naturally occuring sphingolipid is sphingomyelin, which is formed by the transfer of a phosphocholine moiety to the terminal hydroxyl group of ceramide (Sweely 1991). Sphingomyelin occurs mainly in the plasma membrane, and is a major component of the myelin sheath that surrounds myelinated nerves. Glycosphingolipids consist of carbohydrate moieties linked to the terminal hydroxyl group of ceramide (Fig. 4). Cerebroside is the simplest glycosphingolipid, and consists of a single glucosyl or galactosyl residue linked to the terminal hydroxyl group of ceramide. More complex glycolipids known as gangliosides contain oligosaccharide chains with one or more residues of *N*-acetylneuraminic acid. Glycolipids occur on the extracellular leaflet of plasma membranes and function in cellular recognition and adhesion (Sweely 1991).

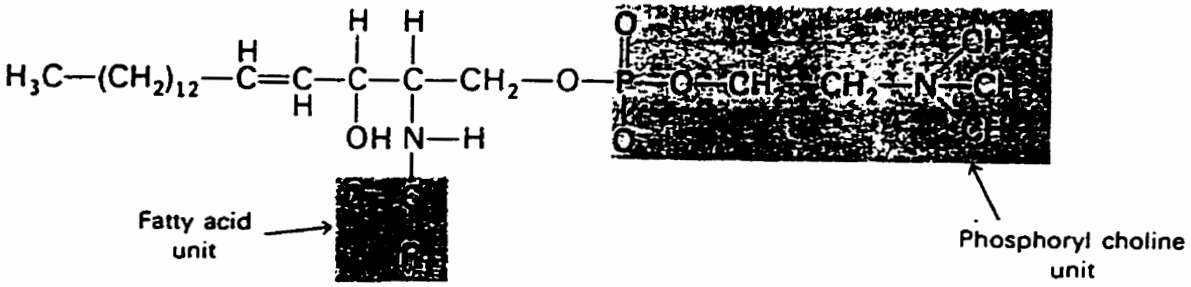
Choline-containing lipids such as phosphatidylcholine and sphingomyelin generally constitute 40 to 60% of membrane phospholipids, but there is considerable variation in their contribution to the membrane lipid composition of different tissues (Table 1) (White 1973). Those lipids that bear no net charge at neutral pH ("zwitterionic lipids"), which include phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, comprise a large portion of the membrane lipid pool of most tissues. Lipids that bear a negative charge at neutral pH ("acidic lipids") such as phosphatidylinositol, phosphatidylserine and cardiolipin comprise a somewhat lesser portion of the total lipid. The differences in membrane composition are usually greater between different tissues than between the

same tissue in different animals (White 1973).

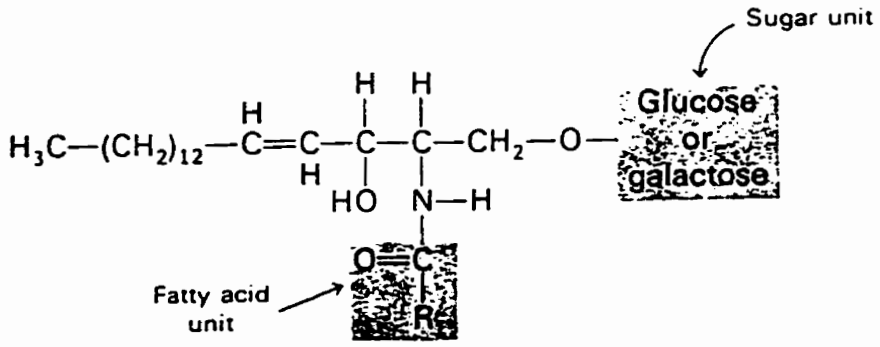
Cholesterol is an important component of some eukaryotic membranes, notably mammalian membranes, but is rarely found in prokaryotes (Bloch 1991). Cholesterol functions to modulate membrane fluidity. The intercalation of cholesterol into lipid bilayers restricts the motion of the fatty acyl moieties of membrane lipids, thereby lowering membrane fluidity (Bloch 1991). Cholesterol is also biologically important as a metabolic precursor of bile acids and the steroid hormones.



Sphingosine



Sphingomyelin



Cerebroside

Figure 4. Sphingosine, shingomyelin, and cerebroside (a glycolipid).

Table 1A. Phospholipid composition of some mammalian tissues.

Values for phospholipid compostion are expressed as percentage of total phospholipid. Adapted from White (White 1973). *n.d.*, not detected.

Tissue →	**Brain**			**Heart**			**Liver**		
Source →	Human	Rat	Cow	Human	Rat	Cow	Human	Rat	Cow
Phosphatidylcholine	30	37	30	40	36	42	44	51	56
Lysophosphatidylcholine	n.d.	nd	n.d.	4	0.6	n.d.	1	1	n.d.
Phosphatidylethanolamine	36	36	33	26	30	28	28	25	13
Phosphatidylinositol	3	3	6	6	4	4	9	7	8
Phosphatidylserine	18	12	17	3	3	2	3	3	4
Sphingomyelin	13	6	12	5	3	12	5	4	6
Cardiolipin	n.d.	2	0.7	9	11	9	4	5	4
Other	n.d.	nd	0.5	7	12	2	7	1	3

Table 1B. Lipid composition of some biological membranes.

From Cullis and Hope (Cullis and Hope 1991).

Lipid	Erythrocyte[a]	Myelin[a]	Mitochondria[b] (inner and outer membrane)	Endoplasmic reticulum[b]
Cholesterol	23	22	3	6
Phosphatidylethanolamine	18	15	35	17
Phosphatidylcholine	17	10	39	40
Sphingomyelin	18	8	-	5
Phosphatidylserine	7	9	2	5
Cardiolipin	-	-	21	-
Glycolipid	3	28	-	-
Others	13	8	-	27

The date are expressed as weight % of total lipid.
[a]Human sources.
[b]Rat liver

1.1.3 Membrane proteins

As mentioned earlier, the bilayer organization of membrane lipids provide a matrix with which membrane proteins are associated. The membrane proteins help to carry out many membrane functions including roles as molecular pumps, ion channels, energy transducers, receptors, signal transducers and enzymes. The protein content of membranes vary according to cell and membrane type. For example, myelin membrane has a relatively low protein content of 18%, while the inner membranes of energy-producing organelles such as mitochondria and chloroplast have a protein content of up to 75%.

Membrane proteins may be broadly classed according to their mode of interaction with the membrane (Alberts *et al.* 1994c). Peripheral proteins are associated with the membrane by noncovalent interactions such as electrostatic interaction or hydrogen bonding. Peripheral proteins may be dissociated from membranes by relatively mild treatments such as altering the ionic strength of the environment or changing the pH. Integral membrane proteins contain hydrophobic domains that interact extensively with the hydrophobic fatty acid regions of membrane lipids. Some integral proteins intercalate into one leaflet of the membrane bilayer, while others span the entire membrane one or several times, depending on the particular protein. The lipid microenvironment surrounding membrane proteins can influence protein function (Yeagle 1989). Dissociation of integral proteins from membranes requires the use of detergents or organic solvents to disrupt the membrane lipid bilayer. Most enzymes involved in phospholipid metabolism are integral membrane proteins (Esko and Raetz 1983; Vance 1985).

1.1.4 Membrane carbohydrates

Eukaryotic cell membranes usually have a carbohydrate content ranging from 2 to 10%, in the form of glycolipids and glycoproteins. The carbohydrate moieties of glycoproteins may be linked via oxygen in the side chains of threonine or serine residues ("*O*-linked" glycoproteins) or to the amide nitrogen in the side chain of asparagine ("*N*-linked"). The carbohydrate moieties of glycolipids and glycoproteins are located exclusively on the extracellular side of plasma membranes (Alberts *et al.* 1994c). Cell-surface carbohydrates function in cell-cell adhesion and recognition, modulation of receptor activity, antigenic specificity, extracellular matrix interactions, and growth regulation (Alberts *et al.* 1994a; Sweely 1991; Nathan and Sporn 1991).

1.2 The *de novo* Biosynthesis of Glycerolipids in Mammalian Tissues

1.2.1 Introduction

The biosynthesis of phospholipids occurs through the condensation of fatty acylthioesters with water-soluble precursors. These reactions are catalyzed by a system of more than 25 enzymes (Esko and Raetz 1983). Most lipid metabolic enzymes are integral membrane proteins (Vance 1985; Esko and Raetz 1983), but notable exceptions include phosphatidate phosphatase and CTP:phosphocholine cytidylyltransferase (discussed below). Furthermore, the lipid metabolic enzymes must often act simultaneously on both membrane-associated and water-soluble substrates. Given these characteristics, lipid enzymes have proven to be refractory to purification and kinetic analysis. However, the past decade has seen many strides in our understanding of these enzymes at the molecular (*ie.* amino acid and/or genetic) level. In the following sections the biosynthesis of glycerol-containing lipids will be discussed (Fig. 5), with an emphasis on phosphoglyceride synthesis.

1.2.2 *sn*-Glycerol-3-phosphate

The *de novo* biosynthesis of the phosphoglycerides occurs via the progressive acylation of *sn*-glycerol-3-phosphate, followed by the addition of the different polar head groups at the *sn*-3 position (Fig. 5). The plasma membrane is impermeable to *sn*-glycerol-3-phosphate but is freely permeable to glycerol (Lin 1977). As such, glycerol has been used as a metabolic precursor to study glycerolipid biosynthesis in hepatocytes (Sundler

and Akesson 1975), lymphocytes (Allan and Michell 1975), rat liver (Akesson *et al.* 1970; Brindley and Bowley 1975) and hamster heart (Tardi *et al.* 1992). After it has been taken up into the cell, glycerol is converted into *sn*-glycerol-3-phosphate by the action of glycerol kinase (EC 2.7.1.30) (Lin 1977; Esko and Raetz 1983). In rat liver, this reaction accounts for up to 20% of the total *sn*-glycerol-3-phosphate pool (Esko and Raetz 1983). Glycerol kinase has been identified and purified from many mammalian tissues and species (Lin 1977), and has since been cloned from human (Guo *et al.* 1993), mouse (Huq *et al.* 1996), yeast (Pavlik *et al.* 1993), and bacterial sources (Holmberg and Rutberg 1989; Pettigrew *et al.* 1988). Another source of *sn*-glycerol-3-phosphate is via the reduction of dihydroxyacetone phosphate by the action of *sn*-glycerol-3-phosphate dehydrogenase (Brindley 1991).

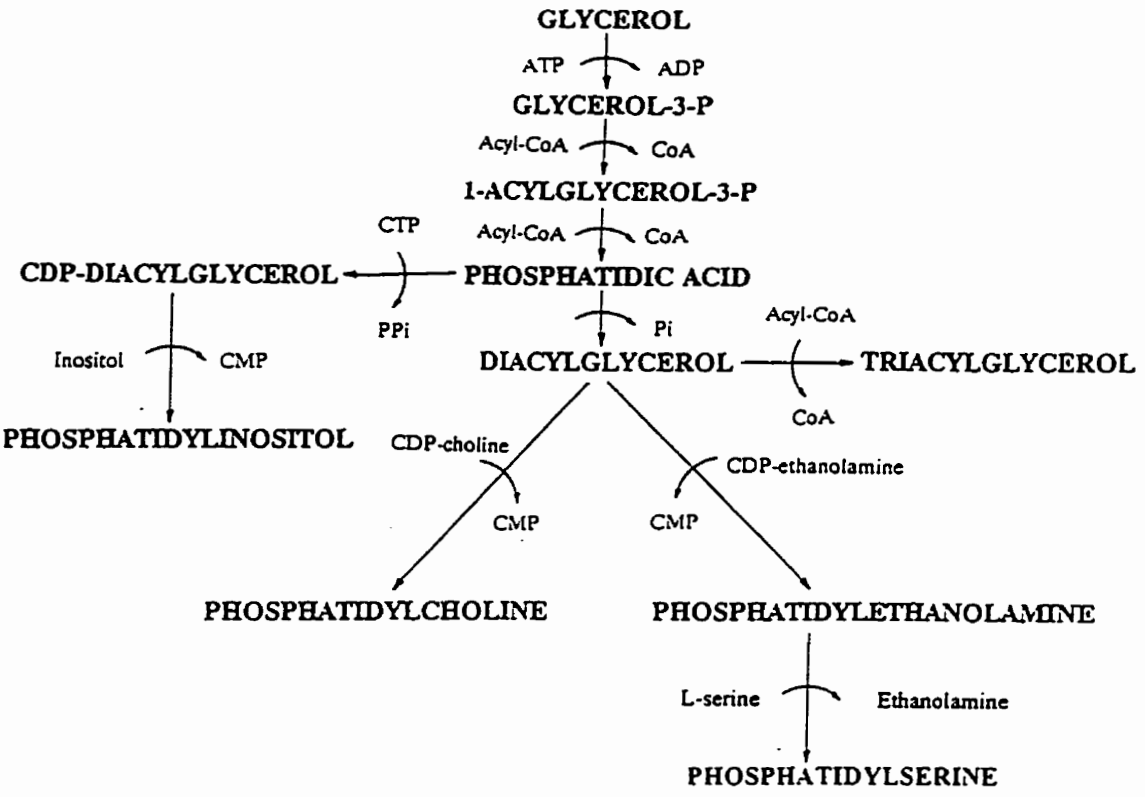


Figure 5. Pathways for the biosynthesis of phosphoglycerides.

1.2.3 1-Acyl-*sn*-glycerol-3-phosphate (lysophosphatidic acid)

The first committed step in the synthesis of the phosphoglycerides is the acylation of *sn*-glycerol-3-phosphate by fatty acyl-CoA thioesters in a reaction catalyzed by glycerophosphate acyltransferase (acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase) (EC 2.3.1.15), to yield 1-acyl-*sn*-glycerol-3-phosphate (Kent 1995). The acyltransferase exhibits the lowest specific activity among the enzymes in the pathway for phosphatidic acid synthesis in guinea pig parotid gland (Soling *et al.* 1989) and in an adipocyte cell line (Coleman *et al.* 1978), suggesting that the acyltransferase step may be rate-limiting. A cDNA for the mitochondrial glycerophosphate acyltransferase has been cloned from mouse liver (Shin *et al.* 1991; Yet *et al.* 1993). Its expression and activity increases upon adipocyte differentiation, and decreases upon starvation and is restored by refeeding (Coleman *et al.* 1978; Yet *et al.* 1993; Shin *et al.* 1991; Ericsson *et al.* 1997). Expression is induced by insulin (Yet *et al.* 1993; Shin *et al.* 1991). The promoter region of the mouse mitochondrial gene has been characterized (Jerkins *et al.* 1995), and gene expression has been found to be regulated by transcription factors involved in regulation of fat metabolism and adipocyte development (Ericsson *et al.* 1997).

In mammals, microsomal and mitochondrial isozymes have been identified based on differences in sensitivity to heat, sulfhydryl reagents and Mg^{2+} requirements (Saggerson *et al.* 1980; Bell and Coleman 1980). The two isozymes differ in their substrate preference; the mitochondrial enzyme exhibits greater activity toward saturated acyl-CoA species, while the microsomal enzyme exhibits little substrate preference (Monroy *et al.* 1972; Haldar *et al.* 1979; Yamada and Okuyama 1978; Jerkins *et al.* 1995). The two

enzyme activities also differ in their K_m values for the substrates (Nimmo 1979; Yamada and Okuyama 1978) and in their pH optima (Haldar 1978). The mitochondrial enzyme has been purified to apparent homogeneity from rat liver (Vancura and Haldar 1994). An alternative pathway for the formation of 1-acyl-*sn*-glycerol-3-phosphate is via the acylation of dihydroxyacetone phosphate followed by the reduction of acyldihydroxyacetone phosphate (Esko and Raetz 1983). The acylation of *sn*-glycerol-3-phosphate and dihydroxyacetone phosphate appears to be catalyzed by the same mitochondrial enzyme (Schlossman and Bell 1976).

1.2.4 Phosphatidic acid

Phosphatidic acid may be regarded as occupying a branch point in phosphoglyceride biosynthesis (Fig. 5) (Kent 1995). Phosphatidic acid can react with CTP to yield cytidine diphosphodiacylglycerol, which is the precursor for biosynthesis of the acidic phospholipids phosphatidylinositol, phosphatidylglycerol, and cardiolipin (see sections 1.2.5 and 1.2.6). Alternatively, phosphatidic acid may be dephosphorylated in a reaction catalyzed by phosphatidate phosphatase (EC 3.1.3.4) to yield diacylglycerol, which is a precursor for the biosynthesis of the zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine, and the neutral lipid triacylglycerol (see also sections 1.2.7, 1.2.8, and 1.2.9). In animals, phosphatidylserine is formed via base-exchange with other phospholipids (section 1.2.10).

Phosphatidic acid is formed *de novo* by acylation of 1-acyl-*sn*-glycerol-3-phosphate in a reaction catalyzed by 1-acylglycerophosphate acyltransferase (acyl-

CoA:lysophosphatidic acid acyltransferase) (EC 2.3.1.51). Like glycerophosphate acyltransferase, 1-acylglycerophosphate acyltransferase appears to exist as microsomal and mitochondrial isozymes, based on differential sensitivities to various inhibitors (Esko and Raetz 1983). 1-Acylglycerophosphate acyltransferase activity has been shown to be distinct from that of glycerophosphate acyltransferase by using a combination of detergent solubilization, chromatographic and centrifugation techniques (Esko and Raetz 1983). 1-Acylglycerophosphate acyltransferase is also distinct from 2-acylglycerophosphate acyltransferase, based on differences in substrate specificities; the former is specific for unsaturated fatty acyl donors, while the latter preferentially utilizes saturated acyl donors (Yamashita *et al.* 1973). 1-Acylglycerophosphate acyltransferase was activated via a mechanism involving protein kinase A (PKA) and calmodulin-dependent protein kinase in guinea pig parotid gland lobules stimulated by isoproterenol and carbachol (Soling *et al.* 1989). Activation of the acyltransferase via cAMP was also shown in hamster hearts perfused with methyl-lidocaine (Lee *et al.* 1995).

A cDNA for 1-acylglycerophosphate acyltransferase has recently been cloned from a human heart library (Eberhardt *et al.* 1997). The mRNA for the acyltransferase was detected in most tissues examined, with the highest expression in liver and pancreas. The expressed recombinant protein exhibited higher activity using arachidonoyl-CoA (C20:4) as the acyl donor than using stearoyl-CoA (C18:0) or palmitoyl-CoA (C16:0). The enzyme was unable to utilize 1-acyl-*sn*-glycerol-3-phosphorylcholine (lysophosphatidylcholine) as an acyl acceptor.

1.2.5 Cytidine diphosphate 1,2-diacyl-*sn*-glycerol (CDP-diacylglycerol)

CDP-diacylglycerol is formed via the transfer of a cytidine diphosphate moiety from CTP to phosphatidic acid in a reaction catalyzed by CTP:phosphatidate cytidylyltransferase (CDP-diacylglycerol synthase, EC 2.7.7.41). In mammals, the formation of CDP-diacylglycerol from phosphatidic acid is a committed step in the biosynthesis of the acidic phospholipids phosphatidylinositol, phosphatidylglycerol and cardiolipin (Kent 1995). The phosphatidate cytidylyltransferase requires Mg^{2+} for activity (Sturton and Brindley 1977), and has been found in microsomal and mitochondrial fractions of mammals (Esko and Raetz 1983; Mok *et al.* 1992). The microsomal and mitochondrial acitivities have distinct properties with respect to CTP and Mg^{2+} requirements, substrate specificities and sensitivity to sulfhydryl reagents (Mok *et al.* 1992). The cytidylyltransferase has been purified from yeast (Kelley and Carman 1987).

cDNA clones for mammalian cytidylyltransferases have been isolated from human (Weeks *et al.* 1997; Heacock *et al.* 1996) and rat (Saito *et al.* 1997) sources. The recombinant rat protein exhibited some preference for 1-stearoyl-2-arachidonoyl-*sn*-glycerol-3-phosphate (C18:0/C20:4-PA) as a substrate (Saito *et al.* 1997). Cytidylyltransferase activity was inhibited mildly by phosphatidylinositol, more strongly by phosphatidylinositol 4-monophosphate, and most strongly by phsophatidylinositol 4,5-bisphosphate (Saito *et al.* 1997). Phosphatidate cytidylyltransferase is thought to participate in the regulation of phosphoinositide levels for intracellular signalling, as overexpression of human and *Drosophila* forms of the enzyme caused amplification of phosphoinositide-mediated signalling responses in human endothelial cells (Weeks *et al.*

1997) and in *Drosophila* photoreceptor cells (Wu *et al.* 1995), respectively.

1.2.6 Phosphatidylinositol

Phosphatidylinositol is formed by the condensation of CDP-diacylglycerol and *myo*-inositol in a reaction catalyzed by phosphatidylinositol synthase (CDP-diacylglycerol:*myo*-inositol 3-phosphatidyltransferase, EC 2.7.8.11). Phosphatidylinositol synthase activities have been purified from human placenta (Antonsson 1994) and rat liver (Monaco *et al.* 1994). Both of these enzyme preparations required Mg^{2+} or Mn^{2+} for activity; however, the human enzyme was activated to a far greater degree by Mg^{2+} than by Mn^{2+}, whereas the rat enzyme was activated to a greater degree by Mn^{2+}. A cDNA for phosphatidylinositol synthase has been cloned from a rat brain library (Tanaka *et al.* 1996). mRNA for this enzyme was detected in all rat tissues examined, with the most abundant expression found in brain and kidney.

Phosphatidylinositol has received attention for its role in the transduction of biological signals within cells (Berridge 1987; Berridge and Irvine 1989). Successive phosphorylation of phosphatidylinositol produces phosphatidylinositol 4,5-bisphosphate. The hydrolysis of this molecule upon agonist stimulation yields diacylglycerol and inositol 1,4,5-trisphosphate, which act as intracellular second messengers (Berridge 1987; Berridge and Irvine 1989). Inositol 1,4,5-trisphosphate causes the release of Ca^{2+} from intracellular stores. Diacylglycerol and Ca^{2+} together activate protein kinase C, which in turn phosphorylates various intracellular proteins. The increased Ca^{2+} and protein phosphorylation lead to a wide variety of cellular responses.

1.2.7 1,2-Diacyl-*sn*-glycerol

1,2-Diacyl-*sn*-glycerol is derived from the hydrolysis of the phosphate moiety of phosphtidic acid by phosphatidate phosphatase (EC 3.1.3.4). Like phosphatidic acid, diacyl-*sn*-glycerol occupies a branch point in glycerolipid metabolism. It may condense with CDP-choline to form phosphatidylcholine in a reaction catalyzed by CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase (EC 2.7.8.2) (see section 1.2.8). In a parallel pathway, 1,2-diacyl-*sn*-glycerol can condense with CDP-ethanolamine to form phosphatidylethanolamine in a reaction catalyzed by CDP:ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase (EC 2.7.8.1) (see section 1.2.9). Diacylglycerol may be acylated in a reaction catalyzed by acyl-CoA:diacylglycerol acyltransferase (EC 2.3.1.20) to yield triacylglycerol.

There exist two distinct forms of phosphatidate phosphatase, designated PAP1 and PAP2 (Jamal *et al.* 1991; Day and Yeaman 1992; Gomez-Munoz *et al.* 1992). PAP1 exists in cytosol and microsomes, while PAP2 is bound to the plasma membrane. PAP1 requires Mg^{2+} for activity and is inhibited by the sulphydryl reagent *N*-ethylmaleimide, while PAP2 does not require Mg^{2+} and is insensitive to *N*-ethylmaleimide (Jamal *et al.* 1991). PAP1 is thought to participate in glycerolipid biosynthesis (Jamal *et al.* 1991). With the appropriate signals, for example in the presence of fatty acids, acyl-CoAs or phosphatidic acid, PAP1 translocates from cytosol to the membrane compartment, which is the site of glycerolipid metabolism (Hopewell *et al.* 1985). This isoform is also thought to be regulated by phosphorylation by PKA, with the phosphorylated form tending to dissociate from membranes and translocating to the cytosol (Gomez-Munoz *et al.* 1992;

Pittner *et al.* 1985; Butterwith *et al.* 1984)

PAP2 has received attention for its role in signal transduction. In the plasma membrane, the hydrolysis of phosphatidylcholine by phospholipase D yields phosphatidic acid, which is then hydrolyzed by PAP2 to yield 1,2-diacyl-*sn*-glycerol, an acitivator of protein kinase C (Exton 1990; Siddiqui and Exton 1992a). PAP2 is an integral membrane protein, and has been purified from rat liver (Waggoner *et al.* 1995; Fleming and Yeaman 1995). Two human cDNA clones encoding PAP2 isozymes have been identified (Kai *et al.* 1996). PAP2 is not activated by fatty acids or phospholipids, and there is no evidence that this isoform is regulated by phosphorylation (Fleming and Yeaman 1995).

Studies have indicated that there exist more than one intracellular pool of diacylglycerol, and that these pools do not readily mix (Binaglia *et al.* 1982; Rustow and Kunze 1985). Diacylglycerol involved in *de novo* glycerolipid synthesis is thought to represent a distinct pool. It has been proposed that the enzymes involved in phosphatidylcholine biosynthesis are organized in a multienzyme complex, in which metabolic intermediates are channelled from one enzyme active site to the next without mixing with the existing endogenous membrane lipids (Rustow and Kunze 1987; George *et al.* 1989).

1.2.8 Phosphatidylcholine

In mammalian tissues, several pathways are known for the formation of phosphatidylcholine (Fig. 6). The majority of this phospholipid is formed from choline via the cytidine diphosphocholine (CDP-choline) pathway (Kennedy 1962; Zelinski *et al.*

1980; Vance 1985). In this pathway, choline is taken up by the cell through a choline uptake system, after which choline kinase (EC 2.7.1.32) catalyzes the transfer of a phosphate moiety from ATP to choline to yield phosphocholine. Phosphocholine is then converted to CDP-choline in a reaction catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15). The condensation of CDP-choline and 1,2-diacyl-*sn*-glycerol to form phosphatidylcholine is catalyzed by CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase (EC 2.7.8.2). In the methylation pathway, phosphatidyl-ethanolamine is methylated by the transfer of methyl groups from *S*-adenosylmethionine, in a series of reactions catalyzed by phosphatidylethanolamine-*N*-methyltransferase (EC 2.1.1.17) (Bremer and Greenberg 1961; Ridgway 1989). This pathway contributes significantly to phosphatidylcholine formation in the liver but not in other organs (Ridgway 1989; Vance 1991). Another possible pathway is the Ca^{2+}-mediated exchange of choline for the head group of another phospholipid (Dils and Hubscher 1961; Kanfer 1989).

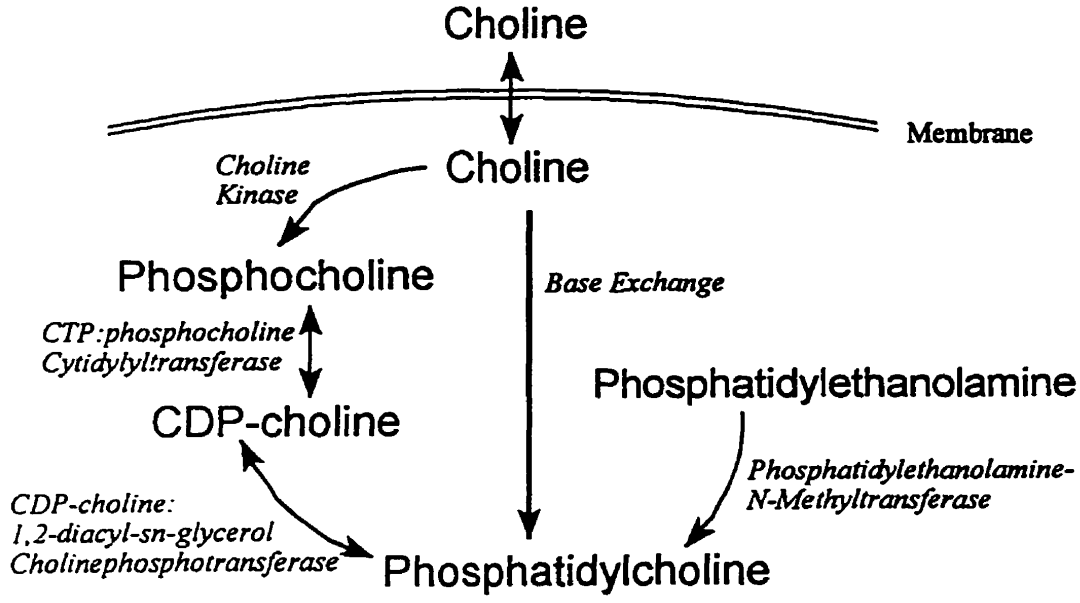


Figure 6. Pathways for the biosynthesis of phosphatidylcholine.

1.2.8.1 CDP-choline pathway

Choline is an essential nutrient in the diet of mammals (Ishidate 1989). In the heart, choline is taken up by a saturable mechanism with a K_m of 0.1 mM (Zelinski *et al.* 1980). Thus, it is possible that the plasma choline concentration (approximately 0.18 mM) may provide a mechanism for the regulation of choline uptake in the heart. Choline uptake has been found to be competetively inhibited by ethanolamine, while conversion of choline into phosphocholine or the biosynthesis of phosphatidylcholine was unaffected (Zelinski and Choy 1984; Zha *et al.* 1992). Choline did not inhibit ethanolamine uptake (Zelinski and Choy 1984). The possible rate-limiting role of the reaction catalyzed by choline kinase was suggested in a study in which rat hearts were perfused under ischemic conditions (Choy *et al.* 1992). Ischemia severely lowered the ATP and CTP levels in the hearts, with a concomitant decrease in the synthesis of phosphatidylcholine. The conversion of choline to phosphocholine was reduced, resulting in an accumulation of choline. This study demonstrated that the reaction catalyzed by choline kinase can become rate-limiting in the event of a greatly diminished ATP pool.

Choline kinase has been purified from a number of mammalian sources (Ulane *et al.* 1978; Ishidate *et al.* 1984; Uchida and Yamashita 1990; Porter and Kent 1990). Two forms of this enzyme have been described based on differences in immunological properties, chromatographic behaviour, and substrate specificities (Cao and Kanfer 1995; Uchida 1994a). Enzymatically, the two forms differed in their relative abilities to catalyze phosphorylation of choline, ethanolamine, *N*-monomethylethanolamine and *N,N*-dimethylethanolamine. cDNAs for choline kinase has been cloned from rat liver (Uchida

and Yamashita 1992) and a human glioblastoma library (Hosaka *et al.* 1992). There exist at least three alternatively spliced transcripts of the rat gene (Uchida 1994b).

The conversion of phosphocholine to CDP-choline, catalyzed by CTP:phosphocholine cytidylyltransferase, is widely regarded to be the rate-determining step in the biosynthesis of phosphatidylcholine via the CDP-choline pathway (Vance 1989; Vance 1991; Tronchere *et al.* 1994; Kent 1995). The cytidylyltransferase has been purified from rat liver (Choy *et al.* 1977; Weinhold *et al.* 1986; Feldman and Weinhold 1987), and has been cloned from a rat liver cDNA library (Kalmar *et al.* 1990). The cDNA predicts a protein of 367 amino acids, with a molecular weight of 42 kDa (Kalmar *et al.* 1990). The enzyme exists in both soluble and membrane-associated forms, and the translocation of the enzyme between the cytosol and membrane compartments is thought to be the primary posttranslational mechanism for regulating its activity (Vance 1989; Vance 1991; Tronchere *et al.* 1994; Kent 1995). The soluble form is relatively inactive, and its activity is enhanced upon translocation to intracellular membranes where it is activated by association with certain mambrane phospholipids (Vance 1989; Tronchere *et al.* 1994; Cornell 1991a; Cornell 1991b). Recently, cytidylyltransferase has been shown to exist in both the cytoplasm and the nucleus, and it can associate with the endoplasmic reticulum or the nuclear membrane (Vance 1989; Tronchere *et al.* 1994; Houweling *et al.* 1996; Watkins and Kent 1992; Wang *et al.* 1993b; Wang *et al.* 1993a). Translocation of the enzyme to membranes can also be induced by fatty acids, diacylglycerol, and acidic phospholipids (Vance 1989). The soluble form of the cytidylyltransferase is highly phosphorylated, while the membrane-associated form is in a dephosphorylated state

(Vance 1989; Wang *et al.* 1993a; Watkins and Kent 1991). It is thought that the lesser negative charge of the dephosphorylated state allows the enzyme to associate more readily with membranes (MacDonald and Kent 1994; Wang and Kent 1995; Houweling *et al.* 1994).

The final step in the biosynthesis of phosphatidylcholine via the CDP-choline pathway is catalyzed by CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase. Enzyme activity is found in microsomal and mitochondrial fractions, and requires divalent cations such as Mg^{2+} or Mn^{2+} for activity (Ghosh *et al.* 1990; O *et al.* 1989). Purification of the cholinephosphotransferase has proven difficult, due to its susceptibility to loss of activity upon extrication from its native membrane environment, though partial purification has been reported (O and Choy 1990; Ishidate *et al.* 1993; Bru *et al.* 1993). A gene for yeast cholinephosphotransferase, designated *CPT1*, has been cloned from the yeast *Saccharomyces cerevisiae* (Hjelmstad and Bell 1987). The *CPT1* gene predicts a 407 amino acid protein with seven transmembrane helices (Hjelmstad and Bell 1990).

While it is clear that CTP:phosphocholine cytidylyltransferase plays a major role in the regulation of phosphatidylcholine biosynthesis, the step catalyzed by the cholinephosphotransferase may offer an additional point of control. The coordination of phosphatidylcholine biosynthesis with other major metabolic pathways in the liver, via regulation at the cholinephosphotransferase-catalyzed step, was revealed in studies involving fasted hamsters (O and Choy 1993). In the livers of hamsters fasted for up to 48 h, phosphatidylcholine biosynthesis was reduced due to a number of factors, including a decreased rate of choline uptake; reductions in the pool sizes of ATP, CTP; reductions

in metabolites in the CDP-choline pathway such as phosphocholine, CDP-choline and diacylglycerol; and decreased activity of cholinephosphotransferase. The inhibition of cholinephosphotransferase was due to accumulation of arginosuccinate in the livers of fasted hamsters, which was found to be an endogenous inhibitor of cholinephosphotransferase. In the fasting animal, an increased utilization of amino acids for gluconeogenesis in the liver leads to activation of enzymes in the urea cycle (Sakami and Harrington 1963; Schimke 1962; Snodgrass 1981). The regulation of phosphatidylcholine synthesis via inhibition of cholinephosphotransferase by arginosuccinate may represent a novel mechanism for the coordination of phospholipid and protein metabolism in the liver during gluconeogenesis (O and Choy 1993).

1.2.8.2 Methylation of phosphatidylethanolamine

The methylation of phosphatidylethanolamine accounts for 20 to 40% of phosphatidylcholine synthesis in the liver, but does not contribute extensively in other tissues (Ridgway 1989; Vance 1991). The methylation pathway involves three successive transfers of methyl groups from *S*-adenosylmethionine. There appear to be at least two immunologically distinct forms of the methyltransferase in rat liver, designated PEMT1 and PEMT2 (Cui *et al.* 1993). PEMT1 is located on the cytosolic surface of the endoplasmic reticulum, and has been purified from rat liver microsomes (Ridgway and Vance 1987; Ridgway 1989). A cDNA for PEMT2 has been cloned and was found to be associated exclusively with the mitochondria-associated membrane, a unique membrane fraction in the liver (Cui *et al.* 1993). PEMT2 expression was increased in rats fed a

choline-deficient diet, and this was regarded as a mechanism whereby the choline-deficient rats could maintain the biosynthesis of phosphatidylcholine. Surprisingly, the expression of PEMT2 in hepatoma cells decreased the growth rate of the cells (Cui *et al.* 1994). This growth suppression was attributed at least in part to a down-regulation of phosphatidylcholine biosynthesis via the CDP-choline pathway due to decreased CTP:phosphocholine cytidylyltransferase (Cui *et al.* 1995). Similarly, PEMT2 expression was decreased during liver growth in perinatal rats and liver regeneration (Houweling *et al.* 1997; Cui *et al.* 1997).

1.2.8.3 Base exchange reaction

A minor pathway for phosphatidylcholine synthesis is the exchange of free choline with the head group of another phospholipid (Dils and Hubscher 1961; Kanfer 1989). This reaction does not result in a net synthesis of new phospholipid, but contributes to the remodelling of preexisting phospholipids (Kanfer 1989). This reaction is catalyzed by a base-exhange enzyme and requires Ca^{2+}. Substrates for the choline base exchange enzyme (*ie.* acceptor phospholipids) include phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (Kanfer 1989; Siddiqui and Exton 1992b). Enzyme activity was detected in subcellular membranes including plasma membranes, microsomes, Golgi, mitochondria, and nuclei. The plasma membrane activity is thought to be subject to regulation by a G-protein linked to purinergic receptors (Siddiqui and Exton 1992b). The nuclear activity is thought to contribute to the remodelling of phospholipids associated with chromatin (Albi and Viola-Magni 1997).

1.2.8.4 Influence of energy status of the heart on phosphatidylcholine biosynthesis

Both ATP and CTP are required cofactors in the CDP-choline pathway and the availability of these high energy compounds may affect the rate of phosphatidylcholine biosynthesis (Vance and Choy 1979; Choy *et al.* 1992; Choy 1982; Hatch and Choy 1990; Hatch and McClarty 1996). An increase in cytoplasmic CTP in polio-infected HeLa cells was shown to cause an enhancement of phosphatidylcholine biosynthesis in these cells, but an increase in ATP level did not affect the phosphorylation of choline (Choy *et al.* 1980). The effects of ATP and CTP levels on phosphatidylcholine biosynthesis in the heart was examined using a strain of cardiomyopathic hamsters as an experimental model (Choy 1982). Through autosomal recessive inheritance, cardiomyopathy develops spontaneously in a strain of inbred Syrian hamsters (BIO 14.6 strain) in which the myocardium exhibits degenerative lesions with 100% incidence (Bajusz 1971). Severe decreases in the ATP and CTP concentrations in the hearts of myopathic animals were observed (Choy 1982). Incorporation of radioactive choline into phosphocholine, and the pool size of phosphocholine, was unaltered despite the depressed ATP levels. In contrast, the labelling and pool size of CDP-choline was decreased. However, the net rate of phosphatidylcholine synthesis was maintained in the myopathic hearts, and this phenomenon was attributed to an increase in CTP:phosphocholine cytidylyltransferase activity. This activation of cytidylyltransferase was regarded as a compensatory mechanism to maintain a minimum CDP-choline level in order to prevent a net reduction of phosphatidylcholine biosynthesis. This study further substantiated the rate-limiting role of cytidylyltransferase in the CDP-choline pathway.

The effect on phosphatidylcholine synthesis of an acute reduction in the energy status of the heart was examined using hypoxic and ischemic isolated heart models (Choy *et al.* 1992; Hatch and Choy 1990). Hypoxic conditions were produced by perfusing the hearts with Krebs-Henseleit buffer saturated with N_2 rather than O_2, while ischemia was induced by perfusing with oxygenated buffer at 10% of the normal flow rate. Severe decreases were observed in the levels of ATP and CTP under hypoxia and ischemia, with resulting decreases in phosphatidylcholine biosynthesis. Examination of choline-containing metabolites revealed differences between the reaction of the hearts to hypoxia or ischemia. Under ischemia, a decrease in the conversion of choline to phosphocholine was observed, but CDP-choline labelling was unaffected (Choy *et al.* 1992). In contrast, hypoxia did not alter the conversion of choline to phosphocholine, but the conversion of phosphocholine to CDP-choline was decreased (Hatch and Choy 1990). As was the case in the myopathic hamster model, a concomitant increase in CTP:phosphocholine cytidylyltransferase activity was observed under hypoxia (Hatch and Choy 1990). This stimulation of cytidylyltransferase was attributed to an accumulation of fatty acids in the hypoxic heart. The importance of the CTP supply to phosphatidylcholine synthesis was corroborated in a study in which rat heart myoblastic (H9c2) cells were incubated with cyclopentenylcytosine, whose metabolite, cyclopentenylcytosinetriphosphate, is a potent and specific inhibitor of CTP synthetase (Hatch and McClarty 1996). The level of CTP in the cells was reduced to 10% of control levels, while other nucleotides were unaffected. The synthesis of phosphatidylcholine and other phospholipids was reduced by treatment of the H9c2 cells with cyclopentenylcytosine (Hatch and McClarty 1996). These studies

show that the intracellular ATP and CTP concentrations are important factors for the maintenance of phosphatidylcholine biosynthesis in the heart.

1.2.9 Phosphatidylethanolamine

Phosphatidylethanolamine is a major phospholipid in mammalian tissues (White 1973; Vance 1991). There are three pathways for phosphatidylethanolamine biosynthesis: a CDP-ethanolamine pathway that parallels the CDP-choline pathway for phosphatidylcholine synthesis; the decarboxylation of phosphatidylserine; and a Ca^{2+}-mediated base exchange pathway (Fig. 7) (Vance 1991). The relative contribution of each pathway varies between cell and tissue types (Kent 1995). For example, in Chinese hamster ovary cells, phosphatidylserine decarboxylation is the major pathway for phosphatidylethanolamine biosynthesis (Miller and Kent 1986), whereas the CDP-ethanolamine pathway is a major route in heart, kidney, and liver (Zelinski and Choy 1982b; Arthur and Page 1991).

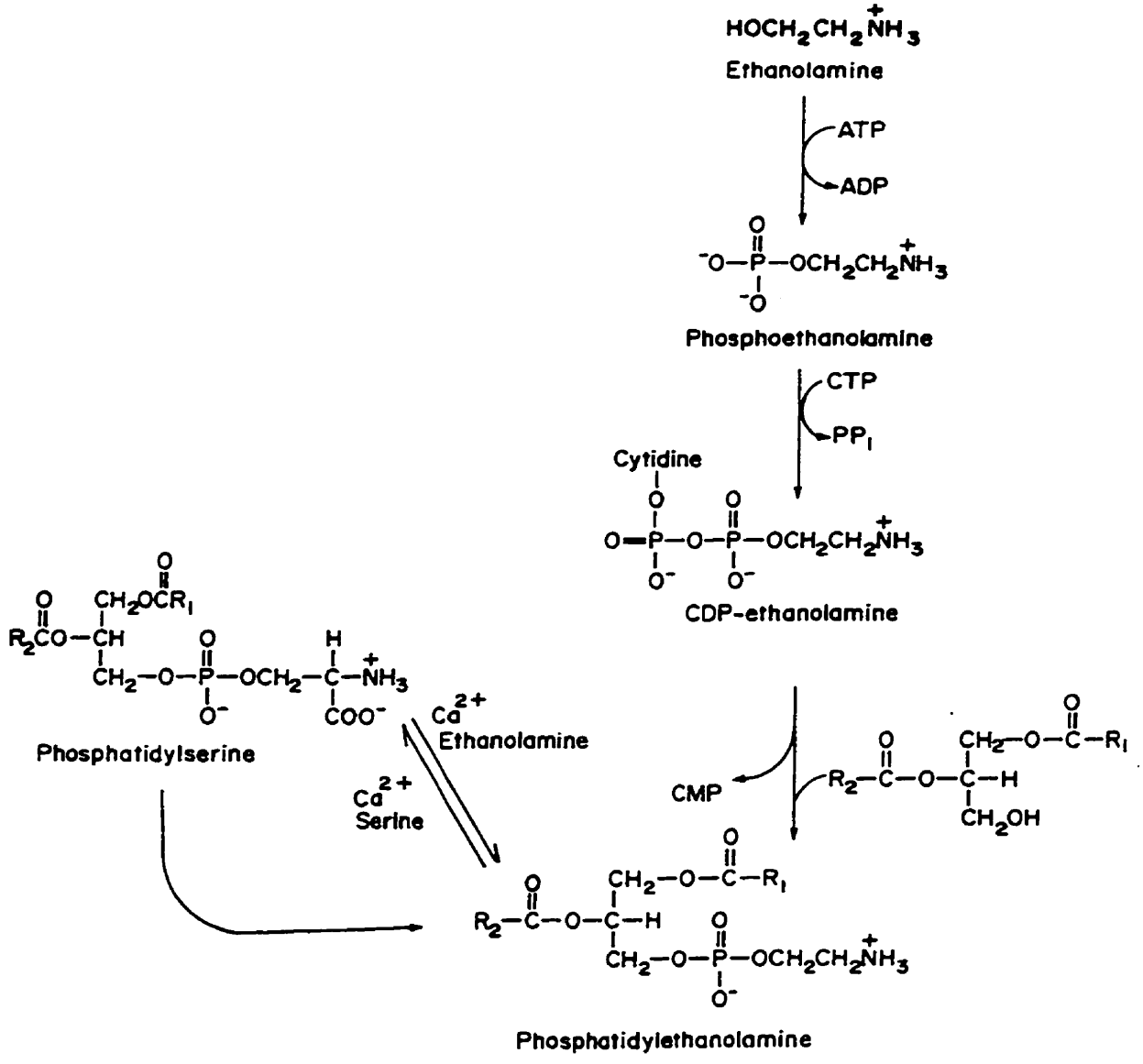


Figure 7. Pathways for the biosynthesis of phosphatidylethanolamine.

1.2.9.1 CDP-ethanolamine pathway

The CDP-ethanolamine pathway is analogous to the CDP-choline pathway for phosphatidylcholine biosynthesis, and begins with the uptake of ethanolamine into the cell. Upon uptake, ethanolamine is phosphorylated in a reaction catalyzed by ethanolamine kinase (EC 2.7.1.82) to yield phosphoethanolamine. In the hamster heart, the uptake and phosphorylation of ethanolamine can become the rate limiting step in phosphatidylethanolamine biosynthesis under conditions of high exogenous ethanolamine (0.4 μM or higher) (McMaster *et al.* 1992). Uptake and phosphorylation of ethanolamine can also be inhibited by serine (McMaster and Choy 1992). Ethanolamine supply also seems to be important in controlling the rate of phosphatidylethanolamine biosynthesis in partially hepatectomized rat liver (Houweling *et al.* 1992).

It is thought that the same enzyme catalyzes the phosphorylation of both choline and ethanolamine, as the two enzyme activities copurify to homgeneity (Kent 1995; Porter and Kent 1990). However, choline and ethanolamine were shown to be phosphorylated independently in intact cultured rat lens (Ekambaram and Jernigan 1994). The independent kinetics were lost upon homogenization of the lens, and choline inhibited the phosphorylation of ethanolamine, and vice versa. Hence it is still unclear whether or not choline and ethanolamine are phosphorylated by a single enzyme.

The conversion of phosphoethanolamine to CDP-ethanolamine, catalyzed by CTP:phosphoethanolamine cytidylyltransferase (EC 2.7.7.14), is regarded as the rate-limiting step in phosphatidylethanolamine via the CDP-ethanolamine pathway (Sundler and Akesson 1975; McMaster *et al.* 1992). The phosphoethanolamine cytidylyltransferase

has been purified from rat liver and exists as a dimer with a molecular weight of 100 kDa (Sundler 1975; Vermeulen *et al.* 1993). The phosphoethanolamine cytidylyltransferase had been thought to be an exclusively cytosolic enzyme, and to not associate with intracellular membranes (Vermeulen *et al.* 1993). However, a subsequent study utilizing ultrastructural immunogold labelling indicated that this enzyme was located in both the cytosol and in association with the rough endoplasmic reticulum (van Hellemond *et al.* 1994). A human cDNA for this enzyme has recently been cloned by complementation of a mutant yeast strain (Nakashima *et al.* 1997).

The final step of the CDP-ethanolamine pathway is the condensation of CDP-ethanolamine and 1,2-diacyl-*sn*-glycerol to form phosphatidylethanolamine. This reaction is catalyzed by CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase (EC 2.7.8.1), which requires Mg^{2+} or Mn^{2+} for activity. This enzyme is an integral membrane protein and is located on the cytosolic face of the endoplasmic reticulum (Ballas and Bell 1980). Ethanolaminephosphotransferase activity was shown to be distinct from cholinephosphostransferase activity by chromatographic separation, and on the basis of different pH optima, heat lability and responses to divalent cations (O *et al.* 1989).

1.2.9.2 Decarboxylation of phosphatidylserine

The decarboxylation of phosphatidylserine to yield phosphatidylethanolamine is catalyzed by phosphatidylserine decarboxylase (EC 4.1.1.65). This enzyme is located on the inner mitochondrial membrane (Voelker 1989), though there has been a report of

phosphatidylserine decarboxylase activity associated with plasma membrane (Auchi *et al.* 1993). However, to date no further studies on the plasma membrane activity seem to have been published. The mitochondrial enzyme has been partially purified (Voelker and Golden 1992). A cDNA for the decarboxylase was cloned by complementation of a mutant Chinese hamster ovary cell line (Kuge *et al.* 1991). The decarboxylation of phosphatidylserine is regarded as the main route for phosphatidylethanolamine biosynthesis in certain cell lines (Miller and Kent 1986; Voelker 1984).

1.2.9.3 Base exchange reaction

Ethanolamine can also serve as a substrate for the phospholipid base exchange enzymes (Kanfer 1989). This pathway may account for up to 10% of phosphatidylethanolamine synthesis in hamster heart (Zelinski and Choy 1982b). An ethanolamine-serine base exchange activity was purified from rat brain microsomes that catalyzed the incorporation of ethanolamine and serine into phospholipids (Suzuki and Kanfer 1985). The enzyme exhibited a higher affinity for ethanolamine than serine. Kinetic studies suggested an identical binding site for ethanolamine and serine (Suzuki and Kanfer 1985).

1.2.10 Phosphatidylserine

Phosphatidylserine comprises 5 to 15% of the phospholipids in most mammalian tissues (White 1973). The sole route for phosphatidylserine biosynthesis in animals is via a base exchange reaction (Vance 1991). As metioned in the previous section, an

ethanolamine-serine base exchange enzyme has been purified (Suzuki and Kanfer 1985). There appear to be at least two enzyme activities catalyzing the incorporation of serine into phospholipids, designated serine-exchange enzymes I and II (Kuge *et al.* 1986a). Serine-exchange enzyme I utilized serine, ethanolamine and choline as substrates, while serine-exchange enzyme II did not use choline. In a related study, the authors proposed a sequential pathway for phosphatidylserine biosynthesis, in which serine-exchange enzyme I catalyzes the exchange of serine with phosphatidylcholine to yield phosphatidylserine; phosphatidylserine is decarboxylated to yield phosphatidylethanolamine; and phosphatidylethanolamine undergoes a base exchange catalyzed by serine-exchange enzyme II to regenerate phosphatidylserine (Kuge *et al.* 1986b).

1.3 The Catabolism of Phospholipids by Phospholipase A_2 and the Products of Phospholipid Catabolism

1.3.1 Introduction: the phospholipases A_2

Enzymes that catalyze the catabolism of phospholipids are referred to as phospholipases. The various phospholipase activities are named for the particular ester bond in the phospholipid molecule that they hydrolyze (Fig. 8). Phospholipase A_1 (EC 3.1.1.3.2) and phospholipase A_2 (EC 3.1.1.4) catalyze the hydrolysis of phospholipids at the *sn*-1 and *sn*-2 positions, respectively, to yield a lysophospholipid and a free fatty acid. Phospholipase C (EC 3.1.4.3) hydrolyzes phospholipids proximal to the phosphate group of phospholipids to yield diacylglycerol and a phosphorylated alcohol such as phosphocholine, while phospholipase D (EC 3.1.4.4) hydrolyzes phospholipids distal to the phosphate group to yield phosphatidic acid and an alcohol.

Enzymes with phospholipase A_2 activity seem to represent a large superfamily of enzymes, with a number of members having recently been recognized (Dennis 1997). At the time of this writing, phospholipase A_2 enzymes have been assigned to at least ten groups, based primarily on sequence homology (Dennis 1997; Mayer and Marshall 1993; Cupillard *et al.* 1997). The phospholipases A_2 are ubiquitous in nature and have been found in every human tissue examined (Dennis 1983). Groups designated I, II, and III are all secreted (*ie.* function in the extracellular environment); have relatively low molecular weights (around 14 kDa for Groups I and II, and 16 to 18 kDa for Group III); require millimolar concentrations of Ca^{2+} for catalytic activity; have high disulfide bond content;

and have histidine and aspartate in the active site (Dennis 1994; Dennis 1997). Group IA was initially identified in cobra venom; Group IB in porcine and human pancreas; Group IIA in rattlesnake and viper venom, and human synovial fluid and platelets; Group IIB in gaboon viper; and Group III from bees and lizards (Dennis 1994). The enzymes in these groups are often referred to as "secreted" phospholipase A_2 ($sPLA_2$).

Group IV phospholipase A_2 is often referred to as "cytosolic" phospholipase A_2 ($cPLA_2$). This group has a number of properties that distinguish it from Groups I to III (Clark *et al.* 1991; Dennis 1994; Dennis 1997; Mayer and Marshall 1993). Group IV enzymes are cytosolic; have a higher molecular weight (85 kDa); require micromolar concentrations of Ca^{2+} such as are found in the intracellular environment; and do not possess disulfide bonds. Whereas the $sPLA_2$s do not exhibit substrate specificity with respect to the fatty acid composition of phospholipid substrates, $cPLA_2$ preferentially hydrolyzes phospholipids with arachidonic acid at the *sn*-2 position. The regulation of this enzyme will be discussed in further detail in the next section.

A cytosolic Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) has been purified and cloned from Chinese hamster ovary cells and $P388D_1$ macrophages (Ackermann *et al.* 1994; Tang *et al.* 1997; Balboa *et al.* 1997). This enzyme has a high molecular weight (80 kDa), and has been designated as Group VI (Dennis 1997). This enzyme is thought to be involved in the remodelling of membrane phospholipids (Balsinde *et al.* 1995).

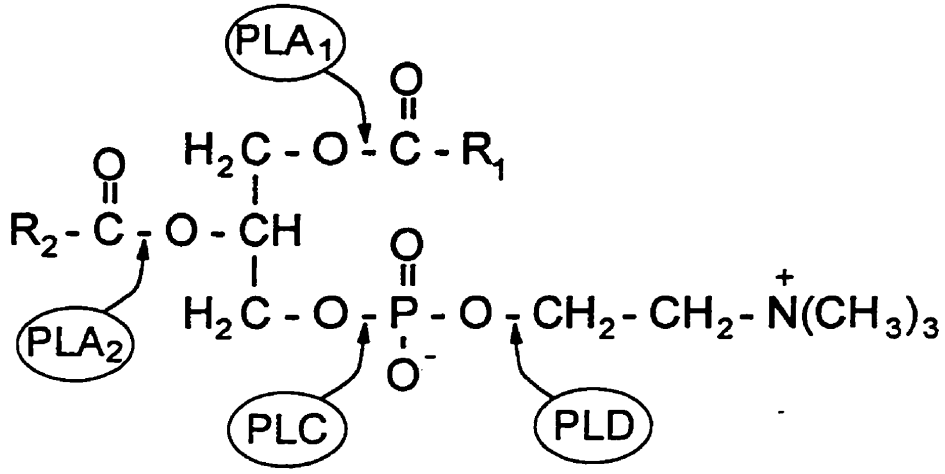


Figure 8. Sites of action of phospholipases.

1.3.2 Regulation of Group IV cytosolic phospholipase A_2

At least three levels of regulation of the Group IV cytosolic phospholipase A_2 (referred to herein as $cPLA_2$) have been identified: regulation of protein expression; phosphorylation of the enzyme protein; and Ca^{2+}-induced translocation to intracellular membranes (Clark *et al.* 1995).

A number of factors have been shown to increase the expression of $cPLA_2$, including interleukin (IL)-1α, IL-1β, tumour necrosis factor-α, macrophage colony-stimulating factor, epidermal growth factor (EGF), leukemia inhibitory factor (LIF), interferon (IFN)-γ, platelet-derived growth factor (PDGF), and lipopolysaccharide (Clark *et al.* 1995; Ikezono *et al.* 1997; Wu *et al.* 1994; Tay *et al.* 1994). The increases in $cPLA_2$ expression was shown to be due to increased transcription of $cPLA_2$ mRNA, to increased mRNA stability, or a combination of both. Factors demonstrated to increase the transcription of mRNA include EGF (Chepenik *et al.* 1994) and IFN-γ (Wu *et al.* 1994). Factors shown to increase the half-life of $cPLA_2$ mRNA include EGF and PDGF (Tay *et al.* 1994; Hack *et al.* 1996). cPLA2 expression has been shown to be decreased by IL-4 (Kuroda *et al.* 1997).

$cPLA_2$ possesses a consensus mitogen-activated protein kinase (MAPK; see section 1.4.3) phosphorylation site at serine 505 (Lin *et al.* 1993). Phosphorylation of the protein at this site by MAPK results in increased enzyme activity (Lin *et al.* 1993; Gordon *et al.* 1996). p42 MAPK has been implicated in the phosphorylation and activation of $cPLA_2$ in stimulated cells (Xing and Insel 1996; Xing *et al.* 1997; Kan *et al.* 1996). $cPLA_2$ can also be phosphorylated by the relatively novel p38 MAPK (Kramer *et al.* 1996; Waterman

et al. 1996). Although $cPLA_2$ has also been shown to be an *in vitro* substrate of protein kinase C (PKC; see section 1.4.2), phosphorylation of $cPLA_2$ by PKC does not result in enhanced enzyme activity (Lin *et al.* 1993). PKC is not thought to directly phosphorylate $cPLA_2$ in cellular systems; rather, PKC is thought to act as an upstream activator in certain cellular systems (Clark *et al.* 1995; Xing and Insel 1996; Xing *et al.* 1997).

$cPLA_2$ possesses an amino-terminal Ca^{2+}-dependent lipid-binding domain (Clark *et al.* 1991; Nalefski *et al.* 1994). This Ca^{2+}-dependent lipid-binding domain comprises amino acids 36 to 91 of $cPLA_2$, and shares homology with other membrane-associated enzymes such as "conventional" PKC isozymes and phospholipase C (Nalefski *et al.* 1994). This domain is responsible for the translocation of $cPLA_2$ to membranes. Translocation to membranes was induced by Ca^{2+} concentrations of $\geq$100 nM (Nalefski *et al.* 1994). In cellular systems, Ca^{2+} concentrations in this range (100 to 300 nM) were induced by agonists such as EGF, resulting in the Ca^{2+}-dependent translocation of $cPLA_2$ (Clark *et al.* 1995; Schalkwijk *et al.* 1995; Schalkwijk *et al.* 1996). The catalytic domain of $cPLA_2$ resides in the carboxy-terminal domain of the protein (Nalefski *et al.* 1994). Although Ca^{2+} is not required for catalytic activity, maximal activity is observed in the presence of Ca^{2+} and upon phosphorylation (Nalefski *et al.* 1994; Schalkwijk *et al.* 1996).

1.3.3 Arachidonic acid and its metabolites

Arachidonic acid (all-*cis*-5,8,11,14-eicosapentaenoic acid) is a 20-carbon fatty acid with *cis* double bonds at carbons 5, 8, 11, and 14 (Fig. 9). Arachidonic acid is stored within cellular phospholipids, and is esterified mainly at the *sn*-2 position of phospholipids

(Holub and Kuksis 1978). A primary route for release of arachidonic acid is via hydrolysis by phospholipase A_2. $cPLA_2$ has been implicated in the stimulation of arachidonic acid release by agonists including thrombin, ATP or lipopolysaccharide (Lin *et al.* 1992; Roshak *et al.* 1994; Bartoli *et al.* 1994). Group II $sPLA_2$ has also been implicated in the arachidonic acid release induced by certain agonists (Nakazato *et al.* 1991; Barbour and Dennis 1993b). Another route for arachidonic acid release is the initial hydrolysis of a phospholipid by phospholipase C, followed by release of arachidonic acid by diacylglycerol lipase and monoacylglycerol lipase (Exton 1990; Prescott and Majerus 1983; Martin and Wysolmerski 1987; Konrad *et al.* 1994). Alternatively, phospholipids may be sequentially hydrolyzed by phospholipase D, phosphatidate phosphatase, and diacylglycerol lipase (Exton 1990; Ishimoto *et al.* 1994; Wright and Malik 1996).

Once released, arachidonic acid may be converted into other biologically active metabolites, known collectively as eicosanoids (Smith 1989). Arachidonic acid and eicosanoids are not stored free within cells, but are released and synthesized upon stimulation by extracellular stimuli; thus, the release of arachidonic acid is an important control point in the regulation of its biological activity and of eicosanoid synthesis (Smith *et al.* 1991). Arachidonic acid may be metabolized via three major pathways (Fig. 10) (Smith *et al.* 1991). The "cyclooxygenase" pathway leads to the synthesis of the prostanoids, which include prostaglandins and thromboxanes. Lipoxygenase catalyzes the first step in the synthesis of the hydroxy- and hydroperoxy-eicosaenoic acids and the leukotrienes. The cytochrome p450 epoxygenases catalyze the first step in the synthesis of the epoxyeicosatrienoic acids.

The first enzyme in the cyclooxygenase pathway is also known as prostaglandin endoperoxide synthase or PGH synthase (EC 1.14.99.1) (Fig. 11) (Needleman *et al.* 1986; Smith *et al.* 1991). PGH synthase is an integral membrane protein found mainly in the endoplasmic reticulum, and exhibits two different enzyme activities that occur at two distinct sites within the protein (Smith 1989; Smith *et al.* 1991). The first activity is the cyclooxygenase activity, and catalyzes the insertion of two oxygen molecules into arachidonic acid to yield 15-hydroperoxy-9,11-endoperoxide (PGG_2), which contains a substituted cyclopentane ring (Smith 1989; Needleman *et al.* 1986). The second enzyme activity is a peroxidase activity, which catalyzes the reduction of the 15-hydroperoxyl group of PGG_2 to yield the 15-hydroxyl analogue, PGH_2 (Smith 1989; Needleman *et al.* 1986). PGH_2 may be metabolized to the various prostanoids, but the particular product that predominates depends on the particular enzymes that are present in a given cell, and is specific to different cells types (Smith *et al.* 1991; Needleman *et al.* 1986). For example, the major prostanoid formed in endothelial cells is prostacyclin (PGI_2), while platelets produce mainly thromboxane A_2 (TxA_2) (Smith *et al.* 1991; Needleman *et al.* 1986). Endothelial cells also produce some TxA_2 (Ingerman-Wojenski *et al.* 1981; Luscher *et al.* 1992). The formation of PGI_2 and TxA_2 from PGH_2 are catalyzed by PGI_2 synthase and TxA_2 synthase, respectively (Smith *et al.* 1991; Needleman *et al.* 1986).

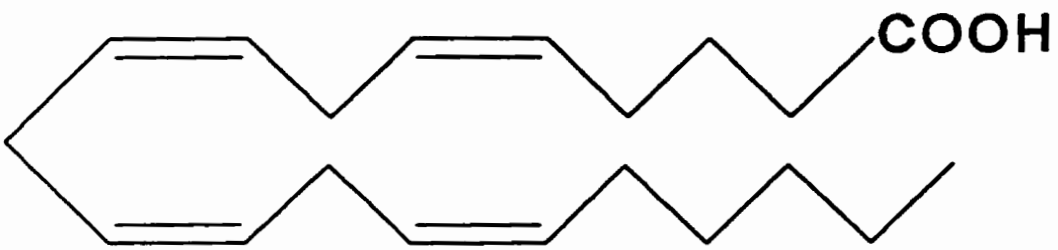


Figure 9. Structure of arachidonic acid (all-*cis*-5,8,11,14-eicosapentaenoic acid).

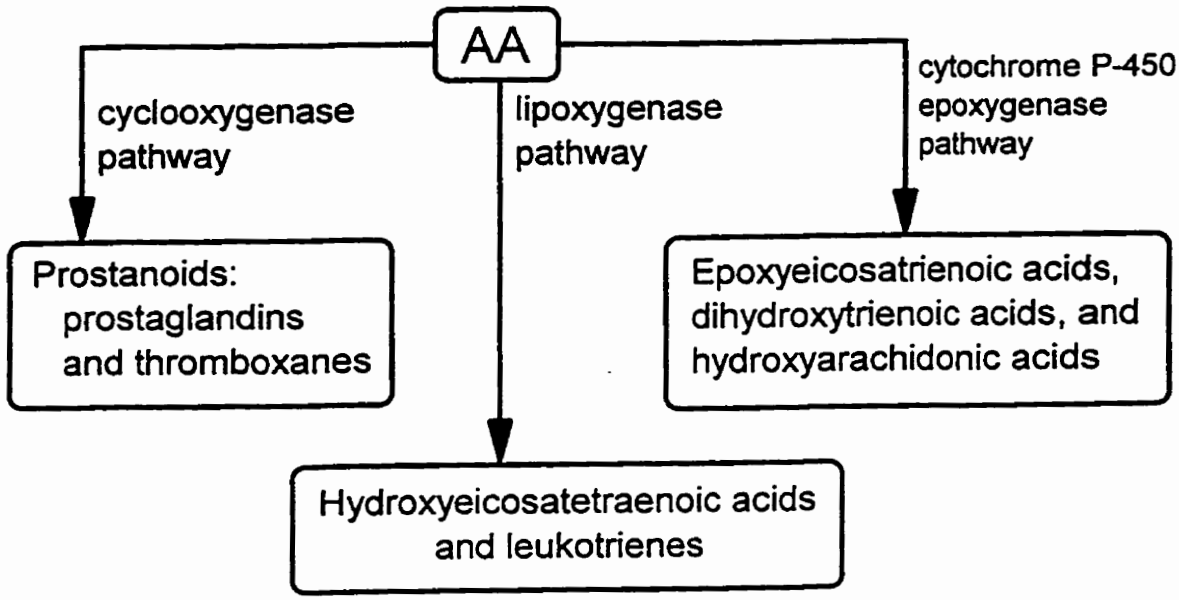


Figure 10. Pathways for the metabolism of arachidonic acid (AA).

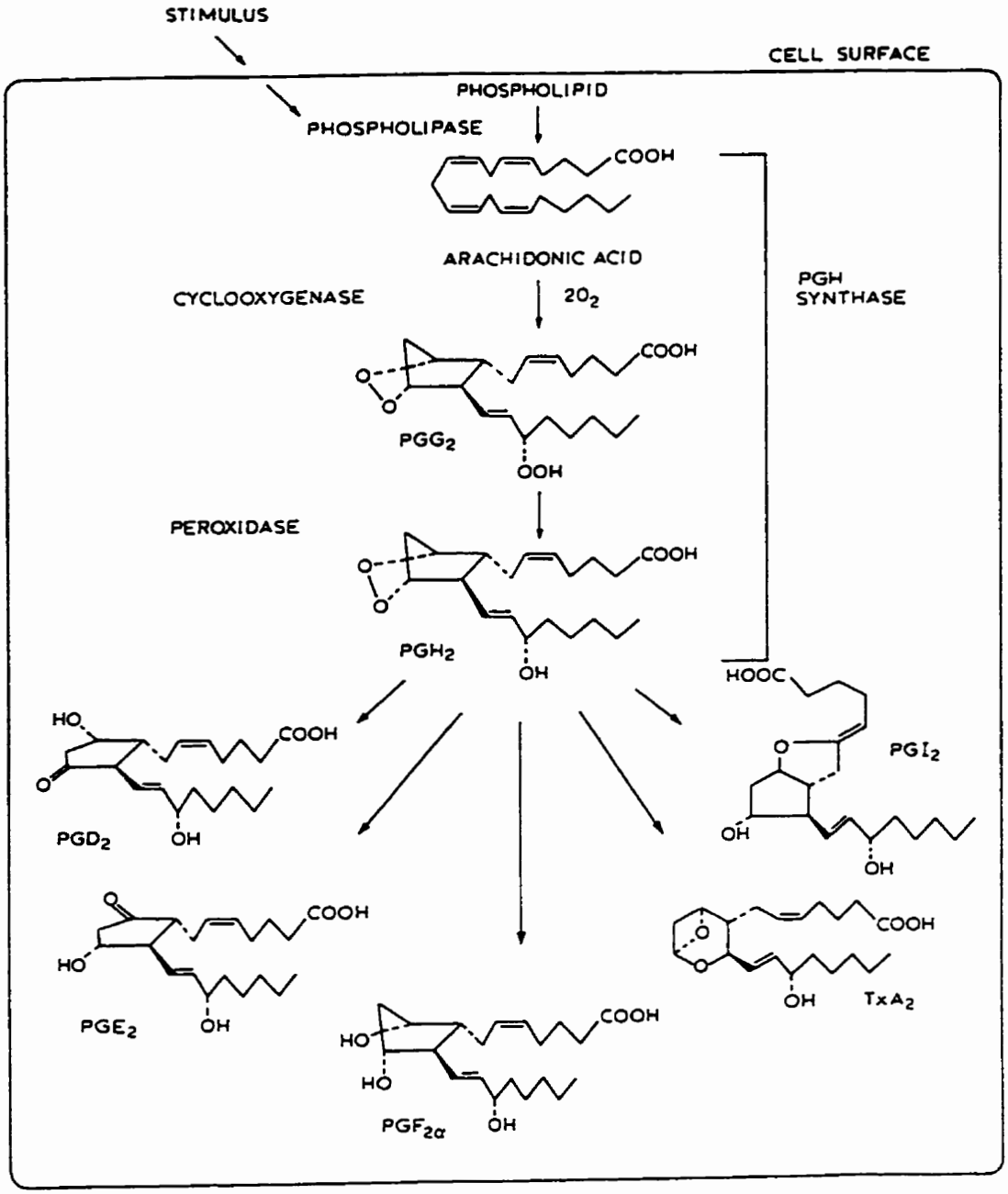


Figure 11. Pathways for prostanoid biosynthesis.

Cell types such as endothelial cells, platelets, vascular smooth muscle cells or leukocytes are able to utilize either endogenous or exogenous arachidonic acid for eicosanoid synthesis, and arachidonic acid released by cells such as endothelial cells can become substrates for eicosanoid synthesis by other cells (Hamberg and Samuelsson 1974; Salzman *et al.* 1980; Brotherton and Hoak 1983; Karim *et al.* 1996; Koll *et al.* 1997; Rao *et al.* 1994). The biological effects of the various eicosanoids depend on the particular compound and the target tissue (Smith 1989; Smith *et al.* 1991; Luscher *et al.* 1992; Needleman *et al.* 1986). TxA_2 induces platelet aggregation and vascular smooth muscle contraction, while PGI_2 functions as a platelet anti-aggregator and vasodilator (Smith 1989). It is thought that a balance of thrombotic/vasoconstricting factors such as TxA_2 and antithrombotic/vasorelaxing factors such as PGI_2 is important in maintaining normal endothelial function and preventing thrombosis, while an imbalance in these factors may be a form of endothelial dysfunction and may contribute to vascular diseases such as atherosclerosis (Gryglewski *et al.* 1976; Whittle and Moncada 1983; Bunting *et al.* 1983; Luscher *et al.* 1992; Rubanyi 1993).

Arachidonic acid itself can also function as a biological signalling molecule (Sumida *et al.* 1993). For example, arachidonic acid can activate PKC or modulate ion channels in many cell types (Sumida *et al.* 1993; Naor 1991). In endothelial cells, arachidonic acid suppressed the induction by IL-1 or tumour necrosis factor-α of gene products involved in inflammation (Stuhlmeier *et al.* 1997; Stuhlmeier *et al.* 1996). For example, arachidonic acid inhibited the expression of E-selectin, intercellular adhesion molecule-1 and IL-8 (Stuhlmeier *et al.* 1997; Stuhlmeier *et al.* 1996). It was shown

further that the suppression of these inflammatory mediators was due to the inhibition of activation of a transcription factor (nuclear factor-κB) that is involved in the expression of the mediators (Stuhlmeier *et al.* 1997).

1.3.4 Lysophosphatidylcholine

Another product of phosphatidylcholine hydrolysis by PLA_2 is lysophosphatidylcholine. This lysophospholipid possesses detergent properties at high concentrations (Weltzein 1979) but is quickly metabolized or reacylated (Tardi *et al.* 1990; Giffin *et al.* 1988). Lysophosphatidylcholine is a normal constituent of blood plasma (Phillips 1957), vascular tissue (Portman and Alexander 1969) and lipoproteins (Steinbrecher *et al.* 1984; Liu *et al.* 1994), but its levels are greatly elevated in hyperlipidemia (Rodriguez *et al.* 1987), atherosclerotic tissue (Portman and Alexander 1969), oxidized lipoproteins (Steinbrecher *et al.* 1984; Liu *et al.* 1994) and ischemic hearts (Kinnaird *et al.* 1988). Lysophosphatidylcholine has been shown to increase intracellular calcium levels in a number of cell types (Su *et al.* 1995; Chen *et al.* 1995; Inoue *et al.* 1992). It also perturbs the expression and/or activity of certain enzymes, such as PKC (stimulates) (Oishi *et al.* 1988; Sasaki *et al.* 1993; Kugiyama *et al.* 1992), and nitric oxide synthase (increases or decreases expression, depending on experimental conditions) (Hirata *et al.* 1995; Dudek *et al.* 1995; Zembowicz *et al.* 1995). A growing body of evidence has implicated lysophosphatidylcholine in the pathogenesis of cardiovascular diseases. Lysophosphatidylcholine in oxidized lipoproteins impairs vascular relaxation (Liu *et al.* 1994; Murohara *et al.* 1994; Chen *et al.* 1997).

Lysophosphatidylcholine is chemotactic for monocytes (Quinn *et al.* 1988) and T lymphocytes (McMurray *et al.* 1993). Lysophosphatidylcholine in oxidized low density lipoprotein induces mitogenesis of macrophages (Masakazu *et al.* 1994). In endothelial cells, lysophosphatidylcholine can induce the expression of genes for various growth factors (Kume and Gimbrone 1994; Nakano *et al.* 1994) and cellular adhesion molecules (Kume *et al.* 1992; Ochi *et al.* 1995). The perturbation of vascular endothelial function and recruitment of various cell types have been implicated as early events in atherogenesis (Ross 1993; Rubanyi 1993). Thus, given its many biological properties, lysophosphatidylcholine has been postulated to be an important causal agent in inflammation and atherosclerosis (Vadas and Pruzanski 1986; Steinberg *et al.* 1989).

1.4 Regulation of Cellular Functions by Protein Phosphorylation

1.4.1 Introduction: cell signalling

Cells within a multicellular organism must communicate in order to behave in a coordinated manner, and therefore possess many mechanisms for relaying extracellular and intracellular signals (Alberts *et al.* 1994b; Marks 1996). Extracellular signalling is effected by the release of messenger molecules, such as hormones, neurotransmitters or cytokines (Alberts *et al.* 1994b; Marks 1996). Types of signalling molecules include those that do not readily penetrate cell membranes, such as proteins, peptides, amino acids or nucleotides. These molecules typically interact with cell-surface receptors. Steroid hormones, thyroid hormones and retinoids are able to enter cells, and interact with receptors in the cytoplasm or nucleus of the target cell. The effector molecules controlled by the activated receptors include ion channels, G-proteins, enzymes such as adenylyl and guanylyl cyclases and protein kinases, and regulatory genomic sequences (Alberts *et al.* 1994b; Marks 1996). The phosphorylation of proteins by protein kinases is an ubiquitous mechanism for reversibly modifying protein function and transducing intracellular signals (Marks 1996). Two major types of protein kinases are protein kinase C (PKC) and the mitogen-activated protein kinases (MAPKs) (Alberts *et al.* 1994b; Marks 1996).

1.4.2 Protein kinase C

PKC was initially discovered as a serine/threonine-specific protein kinase that is activated by diacylglycerol and Ca^{2+}, and its name refers to its activation by Ca^{2+} (Marks

1996). Many diverse signalling molecules including hormones and neurotransmitters stimulate the hydrolysis of phospholipids such as phosphatidylinositol bisphosphate and phosphatidylcholine to yield diacylglycerol (Marks 1996; Newton 1995). In the case of phosphatidylinositol bisphosphate, its hydrolysis also yields inositol trisphosphate, which mobilizes intracelluar Ca^{2+}. Phospholipids may be hydrolyzed by specific phospholipases C to yield diacylglycerol, or by phospholipase D to yield phosphatidate and subsequently diacylglycerol (Newton 1995). It is thought that hydrolysis of phosphatidylinositol bisphosphate by a phosphatidylinositol-specific phospholipase C yields diacylglycerol for a transient activation of PKC, while hydrolysis of phosphatidylcholine by a phosphatidylcholine-specific phospholipase C or phospholipids by a phospholipase D give rise to a sustained PKC activation (Marks 1996; Nishizuka 1995). These phospholipases may be activated by the appropriate signalling molecules acting through mechanisms that include G-protein coupled receptors, receptor tyrosine kinases or non-receptor tyrosine kinases (Newton 1995). In general, prolonged activation of PKC results in a down-regulation of PKC (Marks 1996).

There exist at least 11 and possibly more PKC isoenzymes, and these have been grouped into three subfamilies based on the mechanisms of enzyme activation (Marks 1996; Newton 1995). The "conventional" PKCs (cPKC) require Ca^{2+} and diacylglycerol for activation, and include the α, βI, βII and γ subtypes; the "novel" PKCs (nPKC) require only diacylglycerol for activation, and include the ϵ, δ, η, θ and μ subtypes; and the "atypical" PKCs (aPKC) require neither diacylglycerol nor Ca^{2+} for activation, and include the ζ, λ and ι subtypes (Marks 1996). The subfamily names are historical designations

and are not to imply any physiological importance. All PKC isozymes require acidic phospholipids such as phosphatidylserine for catalytic activity, and thus are active in membranous environments (Marks and Gschwendt 1996; Newton 1995). PKCs exist in the cell cytoplasm and become associated with membranes upon activation (Marks 1996). In addition to diacylglycerol, other lipid or lipid-derived species are also able to activate the various PKC isozymes. These activators include *cis*-unsaturated fatty acids such as arachidonic acid and other *cis*-polyunsaturated fatty acids. Furthermore, lysophosphatidylcholine can activate cPKCs; phosphatidylinositol 3,4,5-trisphosphate can activate the nPKC isozymes ϵ and η and the aPKC isozyme ζ; and ceramide can activate PKCζ, (Marks and Gschwendt 1996; Nishizuka 1995; Sumida *et al.* 1993).

PKCs phosphorylate serine or threonine residues flanked by basic amino acid residues such as lysine or arginine (Marks and Gschwendt 1996; Newton 1995). Given this relatively low substrate specificity, a wide variety of proteins involved in a range of cellular functions serve as substrates for PKC. These functions include, among others, receptor desensitization, regulation of transcription, immune responses, cell proliferation, cell structure and motility, and learning and memory (Marks and Gschwendt 1996; Newton 1995). PKC can also participate in cross-talk with a MAPK pathway, as cPKC is able to phosphorylate Raf-1 (see next section) (Marks and Gschwendt 1996; Sozeri *et al.* 1992). As mentioned above, PKC can function as an upstream activator of $cPLA_2$ by such agents as epinephrine or ATP (Clark *et al.* 1995; Xing and Insel 1996; Xing *et al.* 1997).

1.4.3 Mitogen-activated protein kinases

The MAPKs are a group of homologous protein serine/threonine kinases that transduce signals in response to a wide variety of stimuli, including those involved in cell growth and differentiation, stress responses (*eg.* to ultraviolet radiation or osmotic shock), or inflammation (Naumann *et al.* 1996; Moriguchi *et al.* 1996; Cohen 1996; Cobb and Goldsmith 1995). There exist at least three parallel MAPK pathways, and each can be considered as a hierarchical kinase cascade consisting of three levels of kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (Fig. 12) (Naumann *et al.* 1996; Cobb and Goldsmith 1995). Each kinase in each pathway has also been given specific names. The first two closely related MAPKs to be identified are referred to as p44 and p42 MAPKs, or extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2), respectively (Naumann *et al.* 1996; Cobb and Goldsmith 1995). The MAPKKs that phosphorylate and activate the ERKs are often referred to as MAPK/ERK kinases 1 and 2 (MEK1 and MEK2), and the corresponding MAPKKK is referred to as MEK kinase (MEKK) (Cobb and Goldsmith 1995). A well-known MEKK in the ERK pathway is known as Raf-1, among others (Naumann *et al.* 1996; Cobb and Goldsmith 1995). The other two parallel MAPK pathways are referred to as the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, and the p38/HOG-1 pathway (Cobb and Goldsmith 1995). Among their many functions, the ERK pathway transduces signals related to cell growth or differentiation, while the JNK/SAPK and p38/HOG-1 pathways transduce signals related to stress responses or inflammation (Naumann *et al.* 1996; Cobb and Goldsmith 1995; Cohen 1996; Moriguchi *et al.* 1996).

Specific proteins upstream of the ERK pathway connect this pathway to events at the cell surface (Fig. 13) (Naumann *et al.* 1996; Cadena and Gill 1996). Many cytokine and growth factor receptors are receptor tyrosine kinases (Cadena and Gill 1996). Upon binding their ligands, these receptors dimerize and autophosphorylate. The phosphorylated receptors recruit the guanine nucleotide exchange protein Sos, with the aid of adaptor proteins such as Shc or Grb2 that recognize and bind only the phosphorylated receptors. Sos then activates the G-protein Ras by increasing the (active) GTP-bound form of Ras. The active Ras then activates Raf. As mentioned above, Raf can also be directly phosphorylated and activated by PKC.

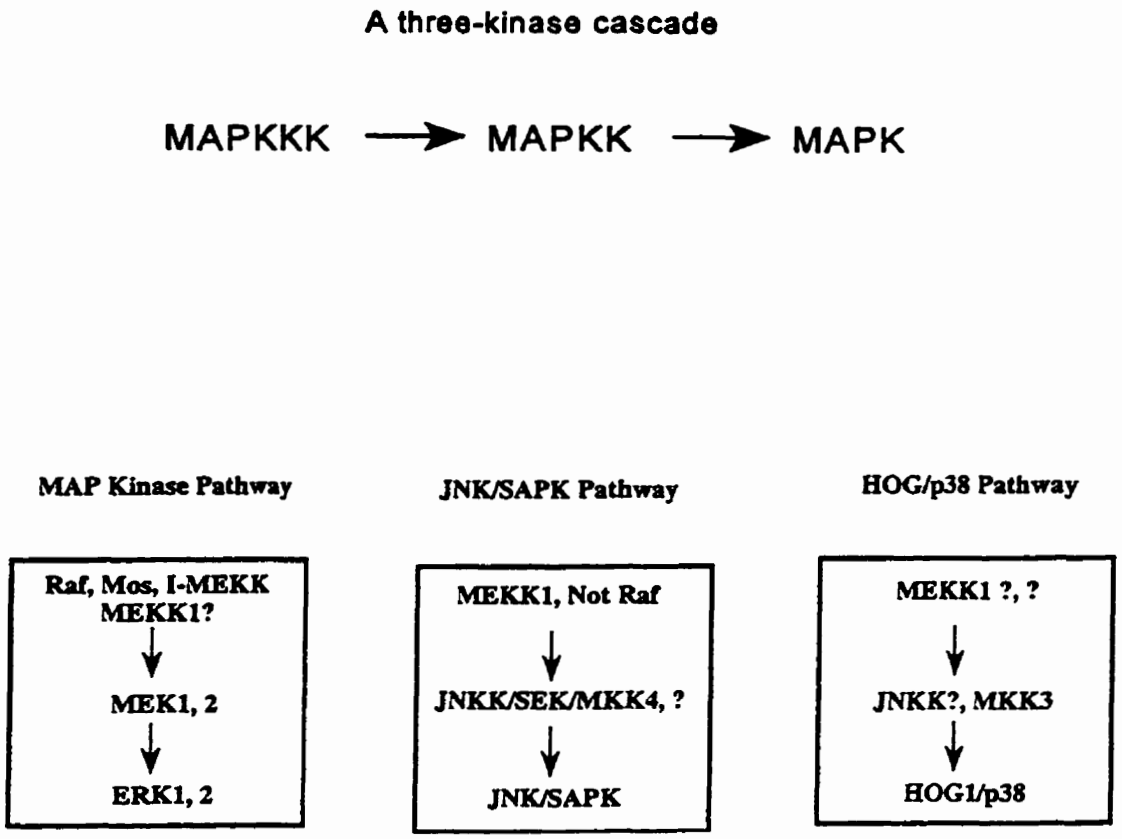


Figure 12. Parallel MAPK pathways in mammalian cells. (Adapted from Cobb and Goldsmith 1995.)

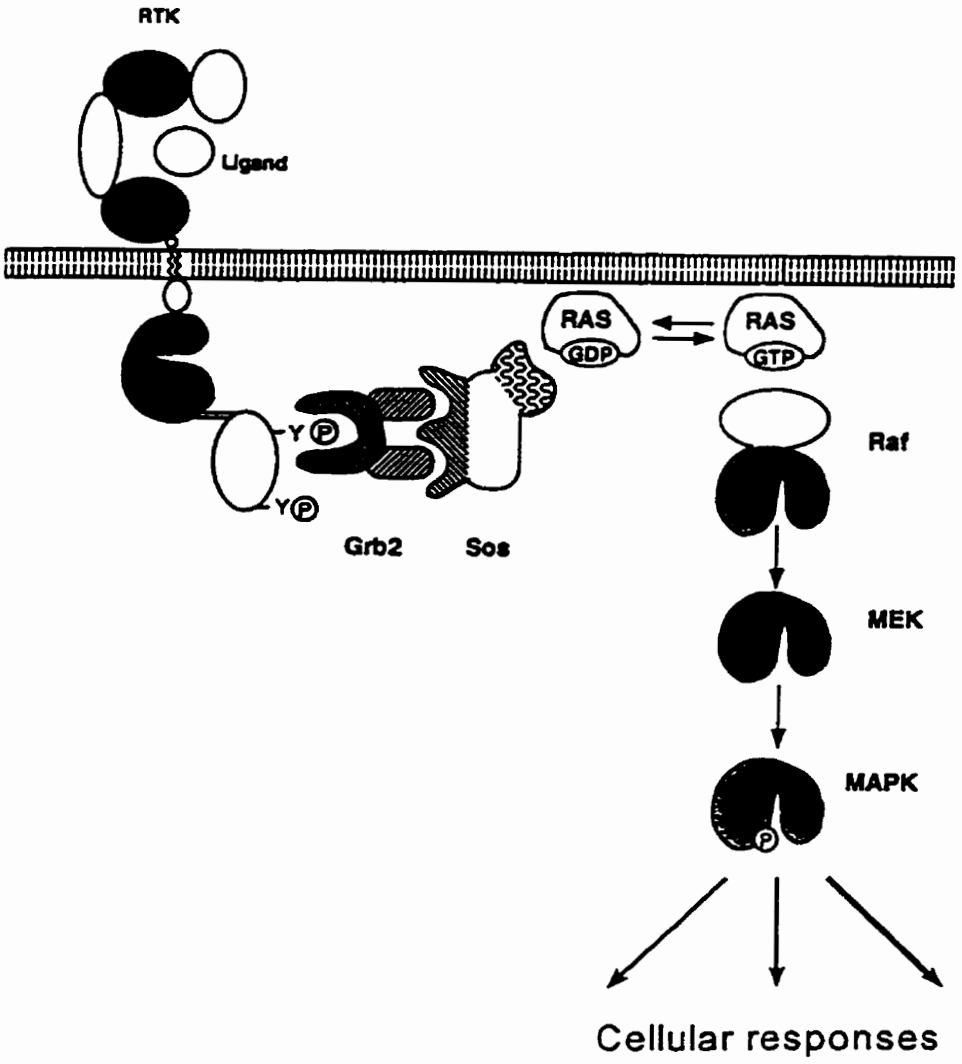


Figure 13. Signalling via the MAPK/ERK pathway. (Adapted from Cadena and Gill 1995.)

1.5 Lidocaine

Lidocaine is an amphiphilic compound, with a hydrophobic portion consisting of an aromatic ring and a hydrophilic portion consisting of a tertiary amine (Fig. 14). This compound has a pK_a of 7.65. Lidocaine has been used clinically as a local anaesthetic and in the treatment of cardiac arrhythmias, and is classified as a sodium channel blocking-agent (Hondeghem and Katzung 1984). Like other local anaesthetics, lidocaine interacts with both lipid and protein moieties of cellular membranes (Seeman 1975; Hondeghem and Katzung 1984).

In view of their ability to interact with the lipid and protein components of cellular membranes, lidocaine and other cationic amphiphilic compounds, such as the methylated lidocaine derivative methyl-lidocaine, have been used as probes to study the regulation of phospholipid metabolism (Tardi *et al.* 1992; Lee *et al.* 1995; Tardi *et al.* 1990; Chu and Lee 1994; Nishizawa *et al.* 1990; Horakova and Stolc 1988; Koul and Hauser 1987; Yada *et al.* 1986; Van Rooijen and Bazan 1986; Brindley and Bowley 1975; Allan and Michell 1975; Eichberg and Hauser 1974). For example, methyl-lidocaine was found to stimulate the biosynthesis of the acidic phospholipids phosphatidylinositol and phosphatidylserine (Tardi *et al.* 1992; Lee *et al.* 1995). This effect was attributed to a direct enhancement of CTP:phosphatidic acid cytidylyltransferase activity by methyl-lidocaine, and also to a stimulation of acyl-CoA:lysophosphatidic acid acyltransferase via a cyclic AMP- and protein kinase A-dependent mechanism (Tardi *et al.* 1992; Lee *et al.* 1995). In another study, lidocaine was found to decrease the incorporation of radiolabelled choline into

phosphatidylcholine in a monocyte-like cell line, and this phenomenon was thought to be due to an inhibition of choline uptake and to a decrease in CTP:phosphocholine cytidylyltransferase activity (Chu and Lee 1994). The purpose of the current study is not to investigate the antiarrhythmic or local anaesthetic properties of lidocaine; rather, this drug was used as a probe to study the regulation of of phospholipid biosynthesis in the heart (see also next section).

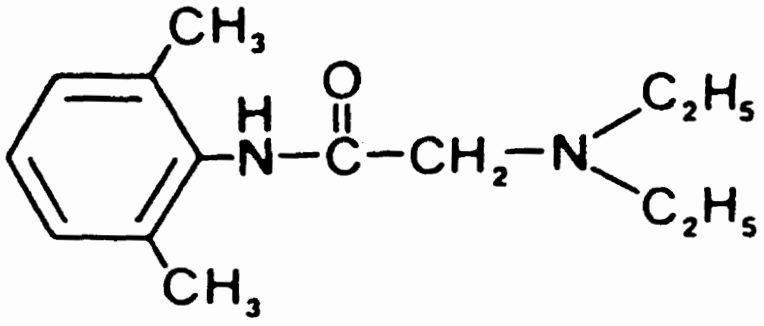


Figure 14. Structure of lidocaine.

1.6 Research Aims and Hypotheses

As mentioned above, phospholipids perform a structural role in membranes, and can also affect the function of various membrane proteins. Furthermore, the hydrolysis of phospholipids provides a source of biological signalling molecules. Thus, the biosynthesis and catabolism of phospholipids must be subject to certain forms of regulation. The purpose of this research is to study the regulation of biosynthesis and catabolism of phospholipids in mammalian tissues. Specifically, in the first part of this study, mechanisms involved in the regulation of phospholipid biosynthesis were studied in the hamster heart. We hypothesize that there exist at least two levels of control of phospholipid biosynthesis in the heart: (a) the activities of key biosynthetic enzymes; and (b) the energy status of the heart. Lidocaine was used as an agent to modulate phospholipid biosynthesis by potentially perturbing the activities of key biosynthetic enzymes, while hearts were perfused under hypoxic conditions in order to alter the energy status of the heart and to study the regulation of phospholipid biosynthesis under an altered energy state.

In the second part of this study, the regulation of catabolism of phospholipids in the form of arachidonic acid release was studied in human umbilical vein endothelial cells. As discussed in section 1.3.3, endothelial cells are a source of arachidonic acid and participate in the regulation of vascular homeostasis. The regulation of arachidonic acid release under the influence of lysophosphatidylcholine was studied. As discussed in section 1.3.4, lysophosphatidylcholine can cause elevated Ca^{2+} in certain cell types and

can modulate certain enzyme activities, including PKC. These events can potentially stimulate $cPLA_2$. Thus, we hypothesize that lysophophatidylcholine can modulate arachidonic acid release in endothelial cells.

The results of this study will help us to better understand the regulation of biosynthesis and catabolism of phospholipids in mammalian tissues.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Experimental animals

Male Syrian golden hamsters (120 ± 20 g) were used for studies of lipid biosynthesis. The hamsters were purchased from Charles River Canada (St. Constante, PQ). The hamsters were maintained on an Agway rodent diet RMH 3000 (Agway Inc., Syracuse, NY) and tap water *ad libitum*, in a light- and temperature-controlled room.

2.1.2 Culture of human umbilical vein endothelial cells

Endothelial cells were derived from human umbilical cord veins by the method of Jaffe (Jaffe 1984). Umbilical cords were obtained from Women's Hospital (Winnipeg). The umbilical vein was cannulated with tubing from butterfly needles and flushed with 50 mL of a buffer containing 140 mM NaCl, 4 mM KCl, 11 mM glucose, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, pH 7.4. The vein was then filled with 10 mL of 0.2% collagenase in HEPES-buffered saline (10 mM HEPES, 140 mM NaCl, 4 mM KCl, 11 mM glucose, pH 7.4) and incubated at 37°C for 15 min. The buffer and the dissociated cells were flushed with 40 mL HEPES-buffered saline into a 50 mL centrifuge tube containing 5 mL medium 199. The cells were sedimented by centrifugation at 180 x *g* for 10 min. The cell pellet was resuspended in 10 mL of fresh medium 199 and cultured on

a 75 cm^2 culture flask coated with 0.2% gelatin. The cells were incubated for 24 h at 37°C in an atmosphere of 95% air / 5% CO_2. The medium was then replaced with fresh medium 199 supplemented with endothelial cell growth supplement at a concentration of 30 μg/mL medium. Fresh medium was added on alternate days, and confluent cell monolayers were usually achieved within 7 to 9 days. For subculture, the cells were detached from the culture ware by trypsin digestion, and were subcultured in a 1:3 ratio. The cells used for experiments were in passage 2 or 3. The cells were verified to be endothelial cells by immunofluorescent microscopy for von Willebrand factor antigen (Jaffe 1984; Chan and Tran 1990).

2.1.3 Chemicals, biochemicals and other materials

Lidocaine hydrochloride was obtained from Astra Pharmaceutical Products (Mississauga, ON). [1,3-^{3}H]glycerol, [1-^{14}C]oleoyl-Coenzyme A (CoA), [1-^{14}C]palmitoyl-CoA, [5-^{3}H]CTP, *myo*-[2-^{3}H]inositol, [^{3}H]acetic anhydride, and 1-stearoyl-2-[1-^{14}C]arachidonoyl-L-3-phosphatidylcholine (55 mCi/mmol) were obtained from Amersham (Oakville, ON). CDP[*methyl*-^{14}C]choline, CDP[1,2-^{14}C]ethanolamine, and [5,6,8,11,12,14,15-^{3}H(N)]arachidonic acid (230.5 Ci/mmol) were obtained from the NEN Division of Dupont Co. (Dorval, PQ). BCl_3-methanol reagent was obtained from Supelco Canada (Mississauga, ON). Medium 199 with Hanks' salt and L-glutamine, heat-inactivated fetal bovine serum, trypsin, and other standard culture reagents were obtained from Gibco (Burlington, ON). Type I collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ, USA). Endothelial cell growth supplement was

obtained from Collaborative Biomedical Products (Bedford, MA, USA). Essentially fatty acid-free bovine serum albumin, ionophore A23187, phorbol 12-myristate 13-acetate, staurosporine, and *para*-bromophenacyl bromide were purchased from Sigma (St. Louis, MO, USA). PD098059 was a product of Calbiochem (La Jolla, CA, USA). Arachidonoyl trifluoromethyl ketone and H89 was obtained from Biomol Inc. (Plymouth Meeting, PA, USA). Ro31-8220 was a generous gift from Roche Research Centre (Welwyn Garden City, Hertfordshire, UK). Phospholipids, lysophospholipids and all other lipid standards were obtained from Serdary Research Laboratory (London, ON). Thin-layer chromatography plates (silica gel G) were products of Fisher Scientific Co. (Edmonton, AB). Anti-cPLA$_2$ polyclonal antibody was a generous gift from Drs. J.L. Knopf and L.-L. Lin of the Genetics Institute (Boston, MA, USA). Anti-human sPLA$_2$ monoclonal antibody was a product of Upstate Biotechnology Inc. (Lake Placid, NY, USA). All other chemicals, solvents and biochemicals were of reagent grade and were obtained from the Canlab Division of Baxter Company (Winnipeg) or Sigma Chemical Company (St. Louis, MO, USA).

2.2 Methods

2.2.1 Perfusion of the isolated hamster heart

Isolated hamster hearts were perfused with Krebs-Henseleit buffer (Krebs and Henseleit 1932) in the Langendorff mode, *ie.* in a retrograde manner by cannulation via the aorta (Langendorff 1895) (Fig. 15). The Krebs-Henseleit buffer was prepared fresh prior to perfusion by combining 100 mL solution A (70.1 g/L NaCl, 21.0 g/L $NaHCO_3$, 9.91 g/L dextrose), 10 mL solution B (3.55 g/100 mL KCl, 2.94 g/100 mL $MgSO_4 \cdot 7H_2O$, 1.63 g/100 mL $NaH_2PO_4 \cdot 7H_2O$), 5 mL solution C (3.73 g/100 mL $CaCl_2 \cdot 2H_2O$), and double distilled water to make a 1 L solution. (Final concentrations were 120 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM dextrose, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 1.25 mM $CaCl_2$.) For studies employing oxygenated buffer, the Krebs-Henseleit buffer was saturated with 95% O_2/5% CO_2 (Zelinski and Choy 1982a). For studies employing hypoxic buffer, the buffer was saturated with 95% N_2/5% CO_2 (Hatch and Choy 1990). Hamsters were sacrificed by decapitation, the hearts were removed, and the hearts were cannulated via the aorta. The isolated heart was stabilized with Krebs-Henseleit buffer for 10 min prior to perfusion with buffer containing 0 or 0.5 mg/mL lidocaine. The temperature of the buffer was maintained at 37°C during the perfusion. The perfusion flow rate was 4.0-4.5 mL/min. For studies of phospholipid biosynthesis, the buffer also contained 1.0 mM [1,3-^{3}H]glycerol (3 μCi/μmol). After perfusion, the heart was flushed with 10 mL Krebs-Henseleit buffer to remove residual radioactivity, cut into small pieces, blotted dry and weighed.

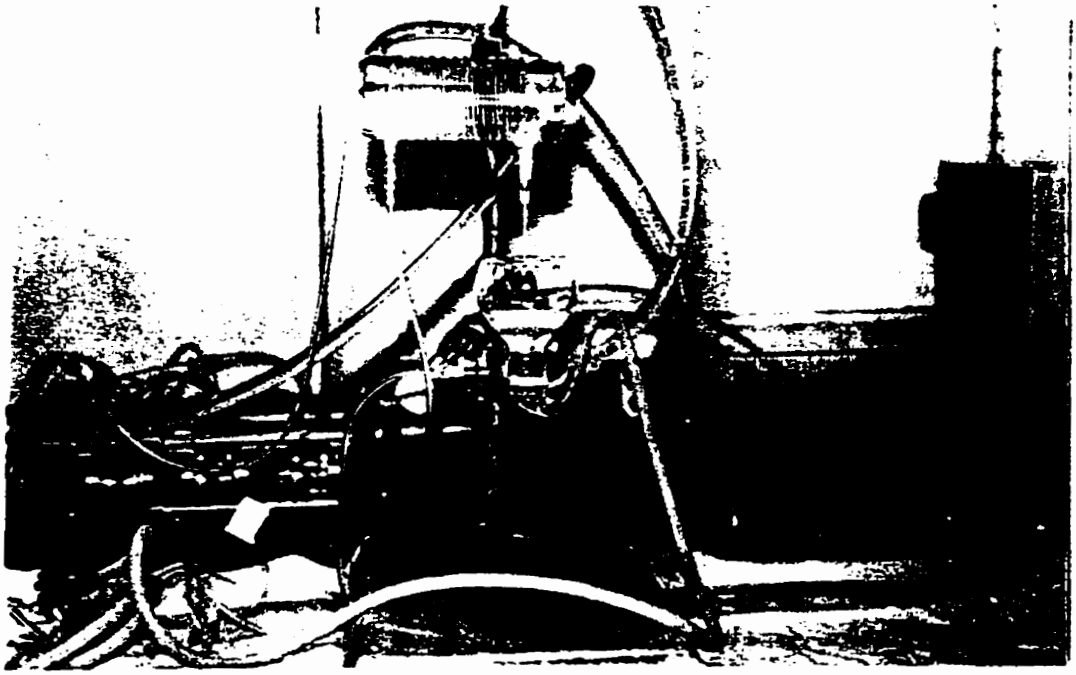


Figure 15. Perfusion of the isolated hamster heart in the Langendorff mode.

2.2.2 Determination of uptake and incorporation of radioactive glycerol

The hearts were homogenized in 10 mL chloroform/methanol (1:1, vol/vol), and the insoluble material was sedimented by centrifugation. The supernatant was removed and saved. The homogenized tissue was reextracted with 10 mL chloroform/methanol (1:1). The supernatants from the two extractions were pooled and an aliquot was taken for determination of radioactivity by scintillation counting. A 0.25 M KCl solution and chloroform were added to the supernatant to cause phase separation, and samples of the aqueous and organic phases were taken for radioactive determination. The extraction efficiency of labelled phospholipids by this method was 88-90%, and was not affected by lidocaine. The phospholipid and neutral lipid classes in the organic phase were separated by thin-layer chromatography in the following solvent systems. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine fractions were separated in a solvent containing chloroform/ methanol/ 15 M aqueous NH_3/ water (35:15:2:1, by vol). The solvent used for the separation of phosphatidic acid and lysophosphatidic acid contained chloroform/ methanol/ HCl (87:13:0.2, by vol) (McMurray and Jarvis 1978). Cytidine diphosphate (CDP-)diacylglycerol was separated in a solvent containing chloroform/ methanol/ acetic acid/ water (52:20:7:3, by vol) (Getz *et al.* 1970). The solvent for the separation of diacylglycerol contained light petroleum (b.p. 35-60° C)/ diethyl ether/ acetic acid (60:40:1, by vol). After visualization by iodine staining, the bands on the thin-layer chromatography plate corresponding to authentic standards were scraped into scintillation vials and their radioactivities were determined.

2.2.3 Lipid determinations

Phospholipid species were separated by thin-layer chromatography as described above. Phosphatidylcholine and phosphatidylethanolamine were quantified by lipid phosphorus determination according to the procedure of Bartlett (Bartlett 1959). For the determination of phosphatidylinositol and phosphatidylserine, the procedure of Rouser *et al.* was used (Rouser *et al.* 1966). For the determination of phosphatidic acid and lysophosphatidic acid, the procedure of Zhou and Arthur was used (Zhou and Arthur 1992). For the determination of diacylglycerol, the acyl groups were converted to the respective methyl esters by reaction with BCl_3-methanol (Metcalfe and Schmitz 1961). The fatty acid methyl esters were quantified by gas liquid chromatography using a Shimadzu Mini-2 gas -liquid chromatograph equipped with 15% DEGS (on 80/100 Chromosorb W/AW) columns. Heptadecanoic acid methyl ester was used as a standard for quantification.

2.2.4 Protein determination

Protein levels were determined by the method of Lowry *et al.* (Lowry *et al.* 1951) or by the bicinchoninic acid method (Smith *et al.* 1985).

2.2.5 Enzyme preparations

After perfusion, hearts were homogenized in a buffer containing 0.25 M sucrose and 10 mM Tris/HCl (pH 7.4). The postnuclear fraction was obtained by centrifugation at 2000 x *g* for 10 min. The postmitochondrial fraction was obtained by centrifugation of

the postnuclear fraction at 12,000 x *g* for 15 min. The cytosolic and the crude microsomal fractions were obtained by centrifugation of the postmitochondrial fraction at 100,000 x *g* for 60 min. The resulting supernatant was designated as the cytosolic fraction, while the microsomal pellet was suspended in 10 mM phosphate buffer, pH 7.4 (*e.g.*, 19 mL of 0.2 M KH_2PO_4, 81 mL of 0.2 M K_2HPO_4, adjust volume to 2 L; verify pH using a pH meter). Enzyme activities were assayed in the subcellular fractions as described in the following sections.

2.2.6 Acyl-CoA:*sn*-glycerol-3-phosphate and acyl-CoA:lysophosphatidic acid acyltransferases.

The activities of acyl-CoA:*sn*-glycerol-3-phosphate and acyl-CoA:lysophosphatidic acid acyltransferases were determined in the postnuclear fraction by the procedure of Batenburg *et al.* (Batenburg *et al.* 1986). For the acylation of *sn*-glycerol-3-phosphate, the assay mixture consisted of 100 mM Tris/HCl (pH 7.5), 1.5 mM *sn*-glycerol-3-phosphate, 90 mM sucrose, 0.5 mg of bovine serum albumin, 1 mM dithiothreitol, 40 μM [1-^{14}C]oleoyl-CoA (4000 dpm/nmol) and approximately 0.2 mg protein in a volume of 500 μL. The reaction was initiated by addition of the postnuclear fraction, incubated at 30°C for 30 min, and terminated by addition of 3 mL chloroform/ methanol (2:1, vol/vol). A solution containing 0.5 M NaCl was then added to the mixture to cause phase separation. The presence of a high salt concentration in the aqueous phase facilitated the recovery of lysophosphatidic acid in the organic phase. The labelled lysophosphatidic acid in the lower phase was resolved by thin-layer chromatography and the radioactivity in that

fraction was determined.

For the acylation of lysophosphatidic acid, the reaction was carried out under the same conditions, except that *sn*-glycerol-3-phosphate was replaced with 0.2 mM 1-palmitoyl-*sn*-glycerol-3-phosphate (Batenburg *et al.* 1986). The labelled phosphatidic acid formed in the reaction was separated by thin-layer chromatography and the radioactivity in that fraction was determined.

2.2.7 Posphatidate phosphatase assay

Phosphatidic acid phosphatase activity was assayed in the cytosolic and microsomal fractions by determining the production of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerol from the corresponding phosphatidic acid (Martin *et al.* 1987). The labelled phosphatidic acid was obtained from 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerophosphocholine by phospholipase D hydrolysis in a procedure adapted from Comfurius and Zwaal (Comfurius and Zwaal 1977). [^{14}C]Phosphatidylcholine (50 μmol, 2000 dpm/nmol) was dissolved in 2 mL diethyl ether. Phospholipase D (1.33 mg) was dissolved in 2 mL of a solution of 100 mM sodium acetate (pH 5.6) and 100 mM $CaCl_2$. The phosphatidylcholine substrate was mixed with the enzyme suspension in a screw-cap tube. The reaction mixture was incubated at 42°C for 30 min, then cooled on ice. Fresh enzyme (1.33 mg) was added to the mixture and incubated for an additional 30 min at 42°C. The reaction was terminated by removal of the diethyl ether under N_2. Phase separation was effected by addition of 6 mL chloroform/methanol (2:1, vol/vol). The lower phase was resolved by thin-layer chromatography in a solvent system containing chloroform/methanol/water/acetic acid

(50:37:2:3, by vol). The thin-layer chromatographic plate was sprayed with 0.25% 2'-7'-dichlorofluorescein in ethanol (weight/vol), and the phospholipid bands were visualized under UV light (Arvidson 1968). The band corresponding to phosphatidic acid was scraped from the plate and eluted by washing the silica gel with 4 mL chloroform/methanol/water/acetic acid (50:39:10:1, by vol). The samples were centrifuged at 1000 x *g* for 10 min and the supernatant was collected. The extraction was repeated twice more and 4 mL of 4 M NH_4OH was added to the combined supernatants to cause a phase separation. The upper phase was removed and the lower phase was washed with 2 mL methanol/water (1:1, by vol). The solvent in the lower phase was removed under a stream of N_2 and resuspended in 1 mL of chloroform/methanol (2:1, by vol).

The phosphatidate phosphatase assay mixture (total volume 100 µL) contained 100 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 0.2 mg bovine serum albumin, 0.6 mM 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerol-3-phosphate (2000 dpm/nmol), 0.4 mM phosphatidylcholine, 1 mM $MgCl_2$, 1 mM ethyleneglycol-*bis*(β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), 1 mM EDTA, 0.75 mM oleic acid and approximately 50 µg protein. The phosphatidic acid and phosphatidylcholine substrates were dispersed by sonication prior to their addition to the assay mixture. The cytosolic fraction was preincubated with oleic acid for 20 min at 37°C prior to the assay. The reaction was initiated by the addition of the appropriate subcellular fraction to the assay mixture and incubated at 37°C for 60 min. The reaction was terminated by the addition of 2 mL chloroform/ methanol (2:1, vol/vol). After phase separation, the radioactive diacylglycerol

in the organic phase was separated by thin-layer chromatography and the radioactivity in that fraction was determined.

2.2.8 CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase and CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase assays

CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase and CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase activities were assayed in the microsomal fraction by determining the transfer of CDP-[^{14}C]choline or CDP-[^{14}C]ethanolamine, respectively, to 1,2-diacyl-*sn*-glycerol (Zelinski *et al.* 1980; Zelinski and Choy 1982b). The reaction mixture for the cholinephosphotransferase assay (final volume 200 μL) contained 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.4 mM CDP-[^{14}C]choline (1.0 μCi/μmol), 0.2 mM 1,2-diacyl-*sn*-glycerol (prepared in 0.015% Tween 20), and approximately 50 μg protein. The final pH of the reaction mixture was 8.5. The reaction was initiated by the addition of the radioactive CDP-choline substrate, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 mL chloroform/methanol (2:1, vol/vol), and water was added to cause phase separation. The phosphatidylcholine in the organic phase was separated by thin-layer chromatography and the radioactivity in that fraction was determined.

The reaction mixture for ethanolaminephosphotransferase activity (final volume 200 μL) contained 10 mM Tris-HCl, 10 mM $MnCl_2$, 1 mM EDTA, 0.2 mM CDP-[^{14}C]ethanolamine (1.0 μCi/μmol), 0.2 mM 1,2-diacyl-*sn*-glycerol (prepared in 0.015% Tween 20), and approximately 50 μg protein. The final pH of the reaction mixture was

8.5. The reaction was initiated by the addition of the radioactive CDP-ethanolamine substrate, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 mL chloroform/ methanol (2:1, v/v), and water was added to the mixture to cause phase separation. The phosphatidylethanolamine in the organic phase was separated by thin-layer chromatography and the radioactivity in that fraction was determined.

2.2.9 CTP:phosphatidate cytidylyltransferase assay

CTP:phosphatidate cytidylyltransferase (CDP-diacylglycerol synthase) activity was assayed in the microsomal fraction using [5-^{3}H]CTP and phosphatidic acid as substrates (Kelley and Carman 1987). The reaction mixture contained 50 mM Tris/maleate (pH 6.5), 20 mM $MgCl_2$, 15 mM Triton X-100, 1.0 mM [5-^{3}H]CTP (10,000 dpm/nmol), 0.5 mM phosphatidic acid, and 50 μg protein. The reaction was initiated by addition of the microsomal fraction and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 2.5 mL 0.1 M HCl in methanol. After cooling, 5 mL chloroform was added to the mixture. The chloroform/methanol mixture was washed three times with 10 mL 2 M $MgCl_2$ (Belendiuk *et al.* 1978). The CDP-diacylglycerol in the organic phase was separated by thin-layer chromatography in a solvent system containing chloroform/methanol/acetic acid/water (25:14:2:4), and the radioactivity in that fraction was determined by scintillation counting.

2.2.10 Phosphatidylinositol synthase assay

Phosphatidylinositol synthase activity was assayed in the microsomal fraction by determining the transfer of *myo*-[^{3}H]inositol to CDP-dipalmitoylglycerol (Imai and Gershengorn 1987). The reaction mixture (total volume 100 µL) contained 100 mM Tris-HCl (pH 7.5), 1 mM EGTA, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 0.2% Triton X-100, 0.1 mM CDP-dipalmitoylglycerol, 0.1 mM *myo*-[^{3}H]inositol (5 x 10 ^{3}dpm/nmol), and 50 µg protein. The reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 1 mL chloroform/ methanol/ HCl (100:100:1, by vol) and 0.4 mL 0.9% KCl. After phase separation, the labelled phosphatidylinositol in the organic phase was separated by thin-layer chromatography and the radioactivity in that fraction was determined.

2.2.11 Serine base exchange enzyme assay

Serine base exchange enzyme activity was assayed in the microsomal fraction by determining the production of radioactive phosphatidylserine from [^{3}H]serine and phosphatidylethanolamine (Miura and Kanfer 1976). The reaction mixture (total volume 250 µL) contained 10 µM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (Hepes) (pH 7.3), 0.8 µmol phosphatidylethanolamine, 20 nmol [^{3}H]serine (78,000 dpm/nmol), 6 µmol $CaCl_2$, 50 µg bovine serum albumin, and 50 µg protein. The reaction was carried out at 37°C for 15 min, and terminated by the addition of 2 mL chloroform/methanol (2:1, v/v). After phase separation, the phosphatidylserine in the organic phase was separated by thin-layer chromatography and the radioactivity in that fraction was determined.

2.2.12 Determination of CDP-choline levels

CDP-choline was quantitated by a modification of the procedure of Vance *et al.* (Vance *et al.* 1980). After perfusion, hearts were stored temporarily in liquid nitrogen, and then homogenized in 6 mL chloroform/ methanol (2:1, v/v). Water (3 mL) was added to the homogenate to cause phase separation. The aqueous phase was removed and the organic phase was reextracted twice with 3 mL of water. The pooled aqueous phase extract was concentrated by evaporation under reduced pressure. The recovery of CDP-choline was calculated from a small amount of CDP-[*Me*-^{14}C]choline added to the tissue homogenate. The concentrated aqueous sample was dissolved in 10 mL water and applied to a Dowex AG1-X8 (OH^- form) column (1 cm x 30 cm). Choline was removed from the column by 60 mL methanol/water (1:1, v/v) followed by 10 mL water, and CDP-choline and phosphocholine were co-eluted with 200 mL 0.4 M NH_4HCO_3. The fraction containing the CDP-choline and phosphocholine was evaporated under reduced pressure and redissolved in 5 mL water. The sample was applied to a Norite A/celite (1:1, v/v) column (1 cm x 5 cm). Phosphocholine was eluted from the column with 20 mL water and 15 mL 2% ethanol, and CDP-choline was eluted with 30 mL 40% ethanol containing 1% NH_4OH. The CDP-choline fraction was treated with 10 units of alkaline phosphatase and 10 units of *Crotalus adamanteus* phosphodiesterase (type III) to yield choline. The choline in this sample was purified by extraction into 3 mL heptan-3-one containing tetraphenylboron (10 mg/mL). The choline in the heptan-3-one was back-extracted with 0.5 mL 0.4 M HCl followed by a second extraction with 3 mL 1.0 M HCl. The HCl extracts were combined and lyophilized, and the choline was determined by the

quantitative conversion of choline into [γ-^{32}P]phosphocholine. The reaction was catalyzed by choline kinase in the presence of [γ-^{32}P]ATP by a modification of the method of McCaman and Stetzler (McCaman and Stetzler 1977).

2.2.13 Studies on arachidonic acid release in endothelial cells

Cells were radiolabelled as described by Tran and Chan (Tran and Chan 1988). Cell monolayers grown to near-confluence in 35 mm culture dishes were incubated for 20 h with 1 μCi/mL [^{3}H]arachidonic acid in Medium 199 containing 10% fetal calf serum. The cells were washed three times with Hepes-buffered saline (140 mM NaCl, 4 mM KCl, 5.5 mM glucose, 10 mM Hepes, 1.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$; pH 7.4) containing various concentrations of essentially fatty acid-free bovine serum albumin. Aliquots of lysophospholipids, dissolved in chloroform/methanol (2:1, v/v), were dried under N_2 gas and then resuspended in Hepes-buffered saline containing bovine serum albumin, then used to challenge the cells.

After challenge of the cells, the buffer was removed and acidified with 50 μL glacial acetic acid. The mixture was centrifuged for 5 min at 800 x *g* and a 0.8 mL aliquot was used for lipid extraction in a solvent mixture consisting of chloroform/methanol/water (4:3:2, by vol). Oleic acid was added as an internal fatty acid standard. Free fatty acid in the organic phase was resolved by thin-layer chromatography in a solvent system consisting of hexane/diethyl ether/acetic acid (70:30:1, by vol). The fatty acid band was visualized by iodine vapour and its radioactivity was determined by liquid scintillation counting.

2.2.14 Treatment of cells with antisense oligonucleotides for $cPLA_2$ and $sPLA_2$

Antisense oligonucleotides for group II PLA_2 ($ASsA_2$: 5'-GAT CCT CTG CCA CCC ACA CC-3') (Barbour and Dennis 1993a) and for $cPLA_2$ ($AScA_2$: 5'-GTA AGG ATC TAT AAA TGA CAT-3') (Roshak *et al.* 1994) with phosphorothioate linkages were synthesized by the University Core DNA Services, University of Calgary (Calgary, AB). These oligonucleotides were designed to hybridize with the translation start site of the respective mRNAs. Complementary sense oligomers were used as controls. 72 h prior to challenge with lysophosphatidylcholine, cells were supplied with medium containing 10 μM oligonucleotides, and were supplied with fresh medium containing 10 μM oligonucleotides at 24 h intervals thereafter. The concentrations of oligonucleotides did not affect cell viability or arachidonic acid labelling.

2.2.15 Immunoblotting analysis of phospholipases A_2

Cell lysates containing approximately 50 μg protein were subjected to sodium dodecylsulfate/7.5% polyacrylamide gel electrophoresis. The protein fractions from the gels were transferred to nitrocellulose membranes, then allowed to react with a polyclonal anti-$cPLA_2$ antibody, or with an anti-$sPLA_2$ antibody. The membranes were then exposed to a goat anti-rabbit antibody (for detection of anti-$cPLA_2$ antibodies) or an anti-mouse antibody (for detection of anti-$sPLA_2$ antibodies) that were coupled to horseradish peroxidase. The $cPLA_2$ or $sPLA_2$ bands were detected on film using a Western blotting detection reagent kit (from Amersham) which yields a fluorescent compound via a reaction catalyzed by the peroxidase. The bands corresponding to $cPLA_2$ or $sPLA_2$ were

identified by comparison with authentic $cPLA_2$ or $sPLA_2$ standard proteins. In order to compare the relative intensities of the bands, the images on the photographic film were scanned, and the intensties of the scanned images were compared using a software product designed for this purpose (Band Leader, Shareware).

2.2.16 Determination of phospholipase A_2 activity

Cells were lysed by sonication in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 μM leupeptin, 10 μM aprotinin, 20 mM NaF, and 10 mM Na_2HPO_4. Cell lysates were centrifuged at 100000 x *g* for 60 min. The supernatant was designated as the cytosolic fraction, while the pellet was designated as the membrane fraction and resuspended in the buffer described above. PLA_2 activity in the subcellular fractions was determined by the hydrolysis of 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine to yield free radiolabelled arachidonic acid. After removal of organic solvent, the substrate was resuspended in DMSO (less than 0.5% of the final volume) by vigourous vortexing and sonication in a water bath sonicator. The assay mixture contained 50 mM Tris-HCl (pH 8.0), 1.5 mM $CaCl_2$, 0.9 nmol of 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (100000 dpm/assay) and approximately 10 μg protein in a final volume of 100 μL. The reaction mixtures were incubated at 37°C for 30 min, and were then quenched by the addition of 1.5 mL of chloroform/methanol (2:1, by volume). Total lipid was extracted and the radioactivity of the arachidonic acid released was determined as described above.

2.2.17 Monitoring of intracellular Ca^{2+}

Changes in cytosolic free Ca^{2+} were monitored using the fluorescent Ca^{2+} indicator fura-2 as described by Liu *et al.* (Liu *et al.* 1993). Briefly, monolayers grown on microscope coverslips were incubated in medium with 5 μM fura-2-AM for 30 min. Fura-2-AM is permeable to cells, and once inside the cells the compound is hydrolyzed by endogenous esterases to yield the cell-impermeable fura-2. The cells on the coverslip were transferred into a cuvette, rinsed with HEPES-buffered saline containing 0.025% bovine serum albumin, and immersed in the same buffer. Lysophosphatidylcholine was added to some of the buffer baths to determine its effect on intracellular Ca^{2+}. The cells containing fura-2 were sequentially excited with light at wavelengths of 340 and 380 nm generated by a SPEX dual wavelength spectrophotometer. The fluorescence emitted at 505 nm was recorded and quantitated with photomultiplier tubes coupled to a computer. The ratio of the intensities of fluorscent emission due to absorption at 340 and 380 nm was monitored as an indicator of changes in cytosolic Ca^{2+} levels. The isobestic (crossover) point of fura-2 remained constant during lysophosphatidylcholine treatment.

2.2.18 Determination of PKC Activity

Cells were sonicated in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 0.25 M sucrose, 0.3% β-mercaptoethanol, 10 μM benzamidine, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. The cell mixture was centrifuged at 1500 x *g* for 10 min. The supernatants were subjected to ultracentrifugation at 100,000 x *g* for 60 min to obtain the soluble and membrane fractions. Approximately

15-30 μg of protein from these fractions were used to determine PKC activity using a PKC assay kit (Amersham), which is based on the incorporation of ^{32}P from [γ-^{32}P]ATP into a PKC-specific substrate peptide.

2.2.19 Statistical analysis

Most data were analyzed using a two-tailed independent Student's *t* test. Where appropriate, one-tailed or paired *t* tests were used. For comparison of three or more groups of data, statistical significance was determined by analysis of variance. In all cases, the level of statistical significance was defined as $p < 0.05$.

3. EXPERIMENTAL RESULTS

3.1 Studies on *de novo* Phospholipid Biosynthesis

3.1.1 Effect of lidocaine on *de novo* phospholipid biosynthesis

The purpose of this portion of the study is to investigate the regulation of phospholipid biosynthesis. Amphiphilic compounds have been used to alter lipid biosynthetic enzyme activities and phospholipid biosynthesis in order to study their regulation (Brindley and Bowley 1975; Allan and Michell 1975; Yada *et al.* 1986; Koul and Hauser 1987; Eichberg and Hauser 1974; Tardi *et al.* 1992; Lee *et al.* 1995; Chu and Lee 1994). As glycerol forms the backbone for the glycerophospholipids, radiolabelled glycerol was used as a metabolic precursor to monitor glycerophospholipid biosynthesis in the perfused hamster heart, under the influence of lidocaine and hypoxia. Glycerol has been used previously as a precursor to study lipid biosynthesis in various mammalian tissues (Akesson *et al.* 1970; Allan and Michell 1975; Brindley and Bowley 1975; Tardi *et al.* 1992). In this portion of the study, the effect of lidocaine on phospholipid biosynthesis in the isolated hamster heart was investigated.

3.1.1.1 Perfusion of hamster hearts and uptake of [^{3}H]glycerol

Isolated hamster hearts were perfused with Krebs-Henseleit buffer containing 0.1 to 2.0 mM [^{3}H]glycerol (4.5 μCi/μmol) for 60 min. After perfusion, the total uptake of

radioactivity in the hearts were determined. The uptake of glycerol was found to increase in a concentration-dependent manner between 0.1-1.0 mM. Thus, a glycerol concentration of 1.0 mM was used for subsequent experiments on phospholipid biosynthesis. To determine the uptake of glycerol over time, hearts were perfused with 1.0 mM [^{3}H]glycerol (3 μCi/μmol) for 30 to 90 min. The glycerol uptake was found to be linear up to 60 min of perfusion (Fig. 16). When lidocaine was present at a final concentration of 0.5 mg/mL in the perfusate, an enhancement of uptake (88-128%) at all time points was observed (Fig. 16). Workers in our laboratory had shown previously that a lidocaine concentration of 0.5 mg/mL elicited a maximal enhancement of glycerol uptake without stopping the heart from beating (lidocaine at this concentration slows the heart rate, while higher concentrations completely stop the heart). The vast majority of the increase in radioactivity uptake was located in the organic phase of the tissue extracts (Fig. 16).

3.1.1.2 Incorporation of [^{3}H]glycerol into phospholipid fractions

The labelling of phospholipids and neutral lipids in the heart after perfusion was determined. Phospholipid and diacylglycerol fractions in the organic phase of the tissue extracts were separated by thin-layer chromatography and the radioactivity in each fraction was determined. As depicted in Fig. 17, perfusion of the heart with lidocaine for 60 min caused an increase in the incorporation of radioactivity into lysophosphatidic acid (181%), phosphatidic acid (135%), phosphatidylethanolamine (157%), CDP-diacylglycerol (152%), phosphatidylinositol (75%), and phosphatidylserine (90%). The level of labelling

in diacylglycerol was also increased by lidocaine perfusion (250%). In contrast, the labelling of the phosphatidylcholine fraction was decreased by 40%.

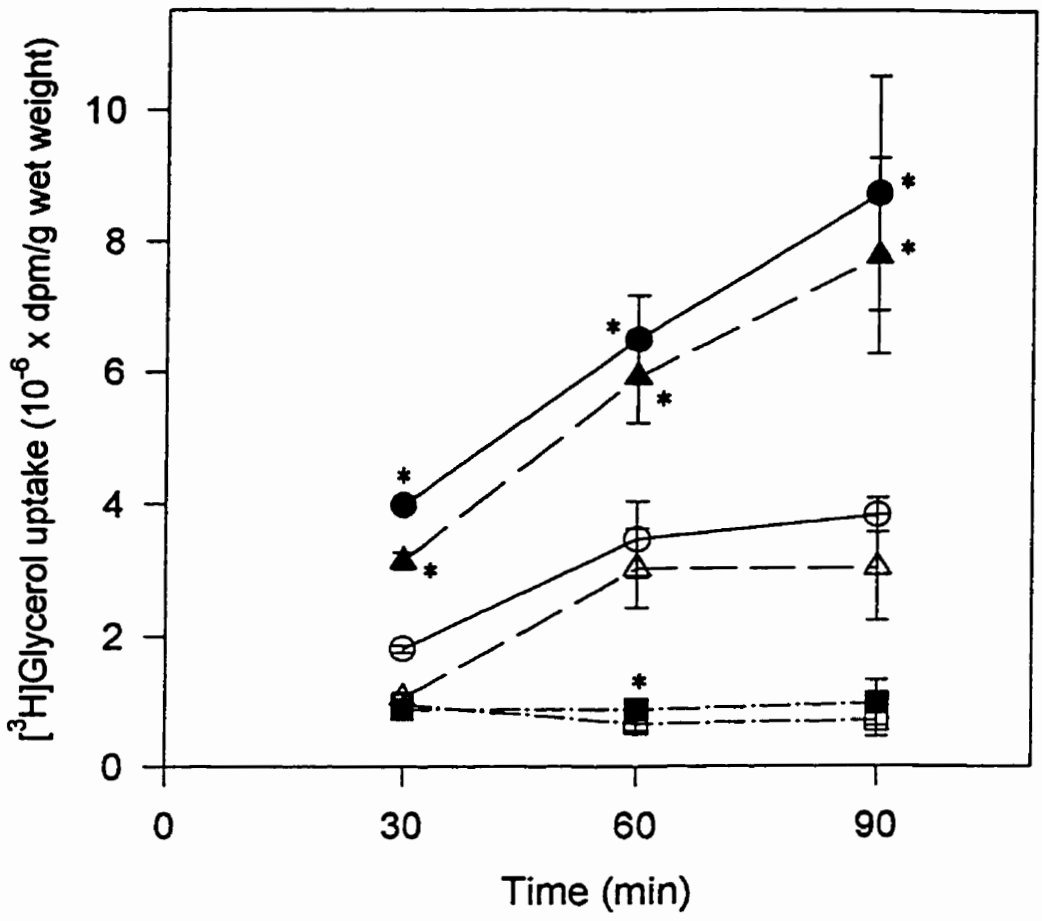


Figure 16. Uptake of glycerol in isolated perfused hamster hearts. Hamster hearts were perfused for the indicated times with Krebs-Henseleit buffer containing 1.0 mM [^{3}H]glycerol (3 μCi/μmol) in the absence or presence of 0.5 mg/mL lidocaine. After perfusion, the hearts were homogenized in chloroform/methanol (1:1, vol/vol) to obtain tissue extracts. The tissue extracts were separated into aqueous and organic phases. Aliquots of the tissue extracts (○,●) and the aqueous (□,■) and organic (△,▲) phases were used for radioactivity determination. *Open symbols*, control hearts, *ie.* perfused without lidocaine; *closed symbols*, hearts perfused with 0.5 mg/mL lidocaine. The results are expressed as means ± standard deviations. Values for 30 min of perfusion represent results from 4 separate experiments; those for 60 min, 11 experiments; and those for 90 min, 3 experiments. $^*p < 0.05$.

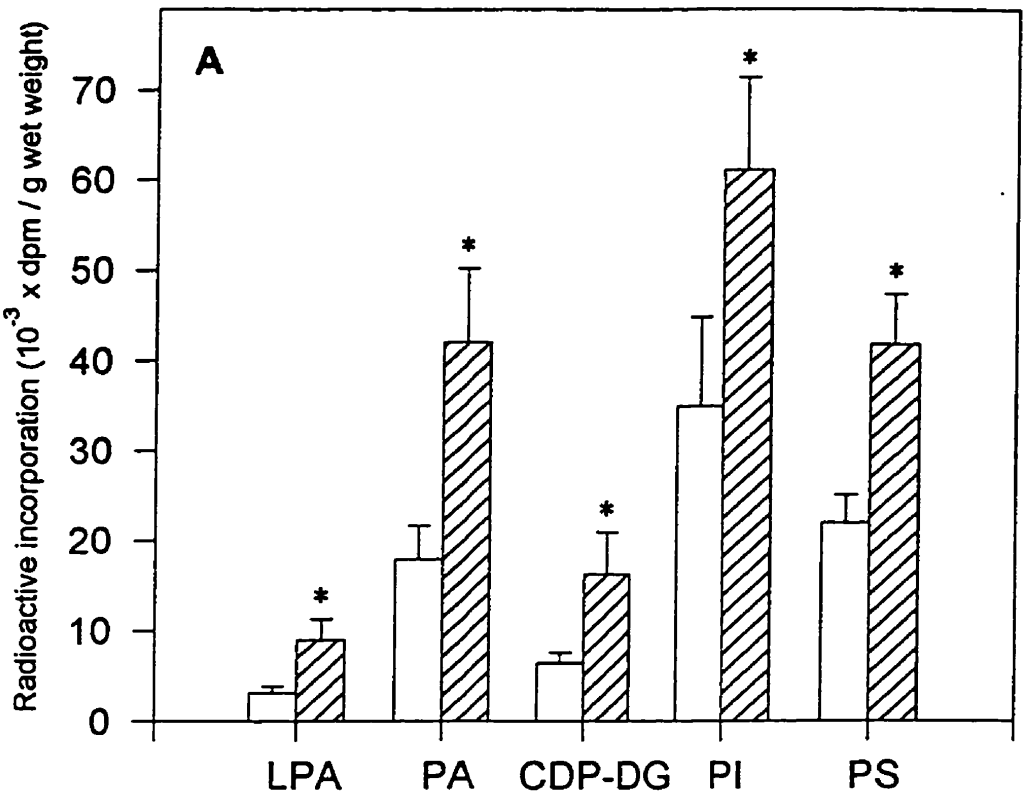


Figure 17. Incorporation of [^{3}H]glycerol into lipid fractions in the hamster heart. Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1.0 mM [^{3}H]glycerol (3 μCi/μmol) in the absence or presence of 0.5 mg/mL lidocaine. After perfusion, the lipid fractions were separated by thin-layer chromatography and the radioactivity in each fraction was determined. *Open bars*, control hearts, *ie.* perfused without lidocaine; *hatched bars*, hearts perfused with 0.5 mg/mL lidocaine. The results are expressed as means ± standard deviations of *n* separate experiments. *A*, incorporation of [^{3}H]glycerol into lysophosphatidic acid (LPA, $n = 12$); phosphatidic acid (PA, $n = 12$); CDP-diacylglycerol (CDP-DG, $n = 4$); phosphatidylinositol (PI, $n = 12$); and phosphatidylserine (PS, $n = 11$). (Continued on next page)

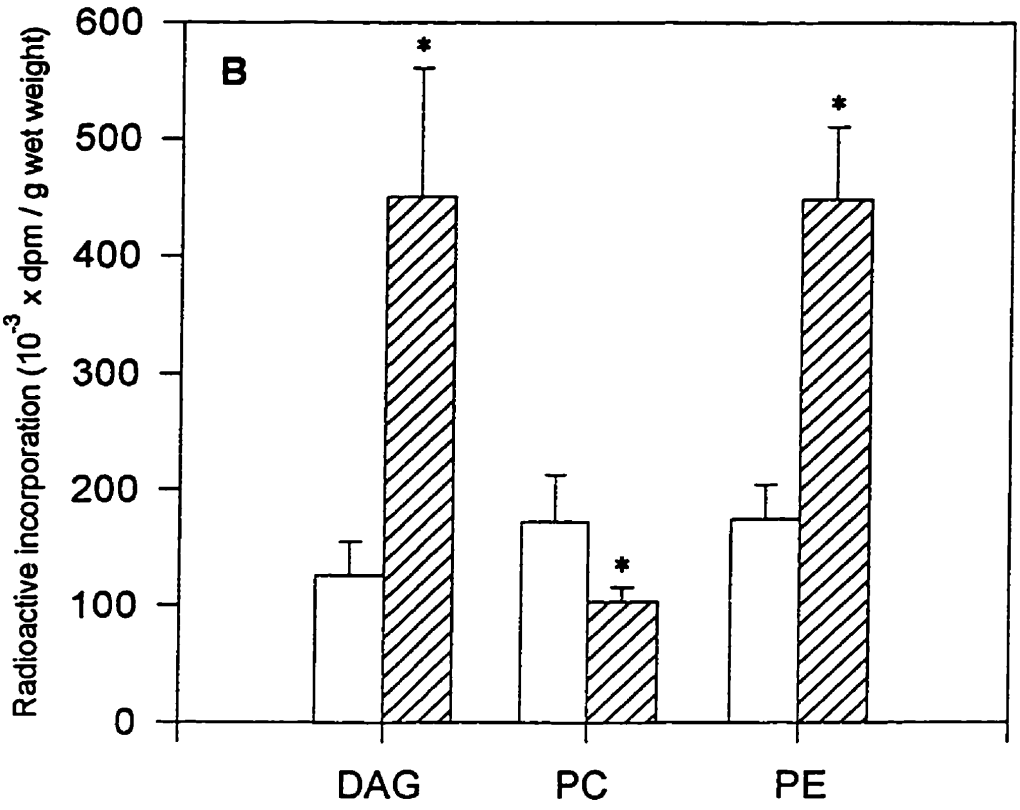


Figure 17 (continued). *B*, incorporation of [^{3}H]glycerol into diacylglycerol (DAG, n = 8); phosphatidylcholine (PC, n = 10); and phosphatidylethanolamine (PE, n = 9). *p < 0.05.

3.1.1.3 Phospholipid contents of hearts following perfusion

Conceivably, changes in the pool sizes of the lipid fractions in hearts perfused with lidocaine could cause the observed changes in the radioactive labelling of these fractions. Analysis of pool sizes of the various lipid fractions showed that lidocaine treatment did not cause any significant changes in the total amounts of phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol in the hamster heart (Table 2). In addition, the pool size of CDP-diacylglycerol, the immediate precursor of phosphatidylinositol, was examined and found to be 12.1 ± 0.1 nmol/g wet weight. The amount of this metabolite was not significantly affected in hearts perfused with lidocaine. However, lidocaine perfusion caused a significant (50%) increase in the lysophosphatidic acid levels in the hearts (Table 2).

Table 2. Phospholipid and diacylglycerol contents in perfused hamster hearts.

Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1.0 mM glycerol, in the absence (*Control*) or presence of 0.5 mg/mL lidocaine. After perfusion, the lipid fractions were separated by thin-layer chromatography and the amount of lipid in each fraction was determined. The results are expressed as mean ± standard deviation; the number of experiments is indicated in parentheses. $*p < 0.05$.

	Content (μmol lipid P/g wet weight of heart)	
Fraction	Control	Lidocaine
Lysophosphatidic acid	0.06 ± 0.01 (6)	0.09 ● 0.01* (3)
Phosphatidic acid	0.34 ± 0.08 (6)	0.28 ± 0.07 (6)
Phosphatidylinositol	1.23 ± 0.66 (6)	1.07 ± 0.43 (6)
Phosphatidylserine	1.80 ± 0.32 (6)	1.55 ● 0.25 (6)
Phosphatidylcholine	11.14 ± 1.39 (6)	11.85 ± 1.12 (6)
Phosphatidylethanolamine	10.41 ± 1.18 (6)	9.61 ± 0.55 (6)
Diacylglycerol[a]	6.54 ± 0.48 (9)	7.40 ± 1.24 (9)

[a]Diacylglycerol content is expressed as μmol/g wet weight of heart.

3.1.2 Effect of hypoxia on *de novo* phospholipid biosynthesis

The purpose of this part of the study is to investigate the hypothesis that phospholipid biosynthesis in the heart is subject to regulation by the energy status of the heart. Oxidative phosphorylation provides most of the energy requirements of the cardiac tissue under normal physiological conditions (Taegtmeyer 1984). Perfusion of the isolated heart under hypoxic conditions has been established as an experimental model to reduce the oxygen available to the myocardium, thereby greatly decreasing the amount of ATP that the tissue is able to generate (de Leiris *et al.* 1984). Since ATP is a precursor of CTP biosynthesis, a decrease in ATP synthesis would also result in a decrease in CTP levels (Swain and Holmes 1986; Hatch and Choy 1990). In this portion of the study, the effect of hypoxia on phospholipid biosynthesis in the isolated hamster heart was investigated.

3.1.2.1 Perfusion of hamster hearts under hypoxia

Hamster hearts were perfused with Krebs-Henseleit buffer containing 1 mM [1,3-^{3}H]glycerol. The buffer was saturated with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 prior to and during perfusion. Analysis of oxygen content in the perfusate showed that the partial pressure of O_2 in the oxygen-saturated buffer (for controls) was over 500 mm Hg, while the partial pressure of O_2 in the N_2-saturated buffer (for hypoxic perfusion) was less than 40 mm Hg. The presence of lidocaine in the buffers did not alter the partial pressure of oxygen. The partial pressure of CO_2 in all buffers was 35-40 mm Hg, which produced a pH value of 7.40 $\pm$ 0.05. The pH of the buffer was not altered in the hypoxic buffers. Hearts perfused under hypoxic conditions for 60 min displayed an abnormal

electrocardiogram which was reversible upon re-oxygenation (Hatch and Choy 1990). Some degree of mitochondrial swelling at 60 min of hypoxic perfusion was observed by electron microscopy, and the swelling was found to be reversible upon re-oxygenation (Hatch and Choy 1990). Hearts were perfused under hypoxic conditions for 60 min or less in subsequent experiments.

3.1.2.2 Uptake of [^{3}H]glycerol and incorporation into phospholipid fractions under hypoxia and effect of lidocaine

Hamster hearts were perfused in the presence of 1.0 mM glycerol (3 μCi/μmol) under normoxic or hypoxic conditions. Since lidocaine was found to increase phospholipid biosynthesis, we also perfused hearts under hypoxia in the presence of 0.5 mg/mL lidocaine. After perfusion the hearts were homogenized and the total uptake of labelled glycerol was determined. When the heart was perfused under hypoxic conditions, the overall uptake of radioactive glycerol was reduced by 47% (Fig. 18). The majority of the reduction in radioactivity was confined to the organic phase of the heart extracts, in which the radioactivity was decreased by 58% (Fig. 18). Analysis of the organic phase by thin-layer chromatography showed general reductions in radioactivity associated with all lipid fractions (Fig. 19).

When 0.5 mg/mL lidocaine was present in the hypoxic perfusion buffer, the reduction in total glycerol uptake became less severe (24% of control) than in hearts perfused under hypoxic conditions in the absence of lidocaine. This partial restoration of labelled glycerol uptake was also reflected in radioactivity found in the organic phase, in

which the radioactivity was 67% of that found in the control hearts. The organic phase of the heart extracts were analyzed by thin-layer chromatography, and it was observed that the radioactivity associated with most phospholipid fractions was similar to their control values in hearts perfused with lidocaine (Fig. 19). The sole exception to the restoration of radioactive labelling of phospholipids in lidocaine-perfused hearts was phosphatidylcholine, whose labelling remained depressed.

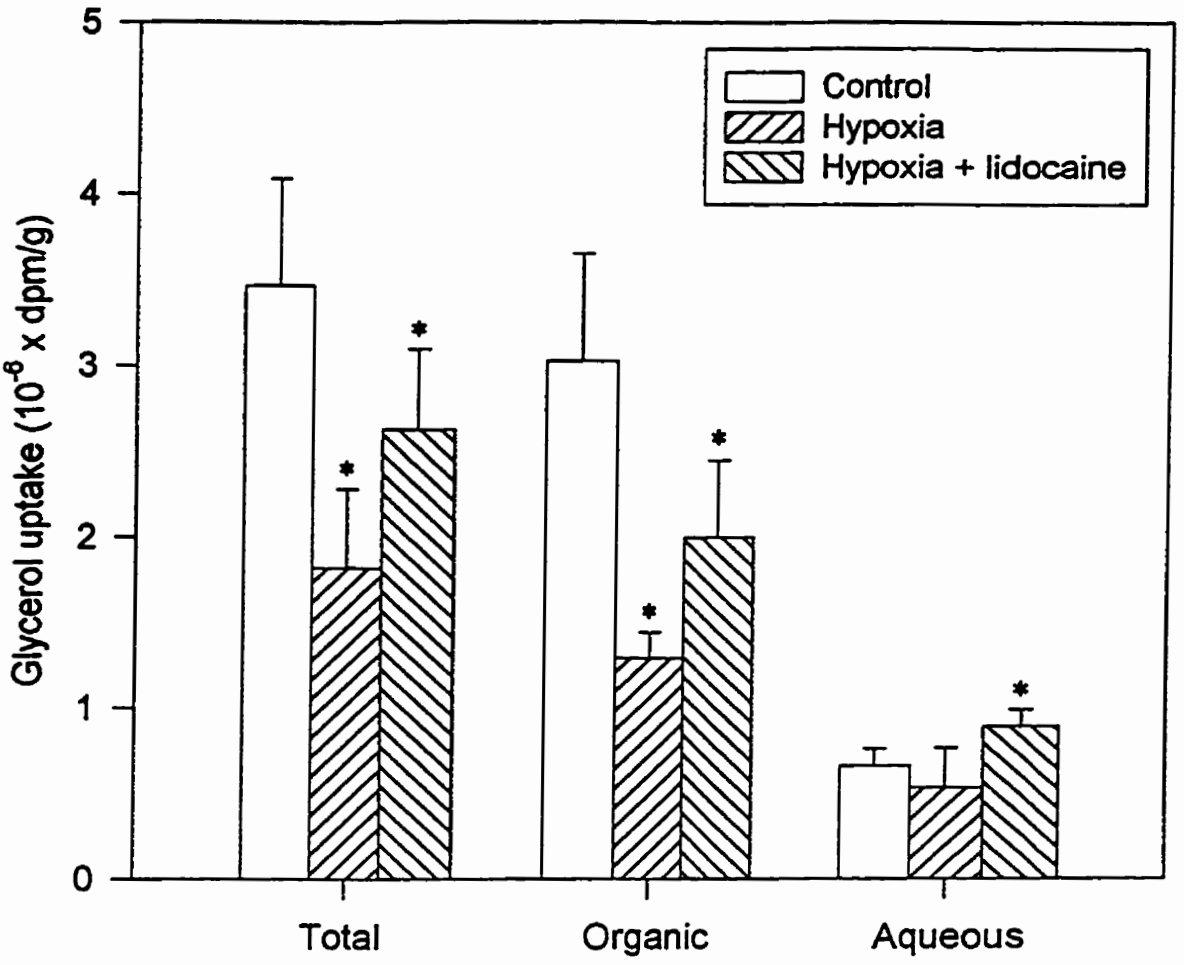


Figure 18. Effects of lidocaine and hypoxia on the uptake of [^{3}H]glycerol in perfused hamster hearts. Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1.0 mM [1,3-^{3}H]glycerol (3 μCi/μmol). *Control*, hearts were perfused with buffer saturated with 95% O_2; *hypoxia*, hearts perfused with buffer saturated with 95% N_2; *hypoxia + lidocaine*, hearts perfused with hypoxic buffer containing 0.5 mg/mL lidocaine. After perfusion, the hearts were homogenized in chloroform/methanol (1:1, vol/vol) to obtain tissue extracts. The tissue extracts were separated into aqueous and organic phases. Aliquots of the total tissue extracts and the organic and aqueous phases were used for radioactivity determination. The results are expressed as means ± standard deviation. Values for control hearts represent results from 13 separate experiments; those for hypoxic hearts, 4 experiments; and those for hypoxia + lidocaine, 3 experiments. *$p < 0.05$ compared with control.

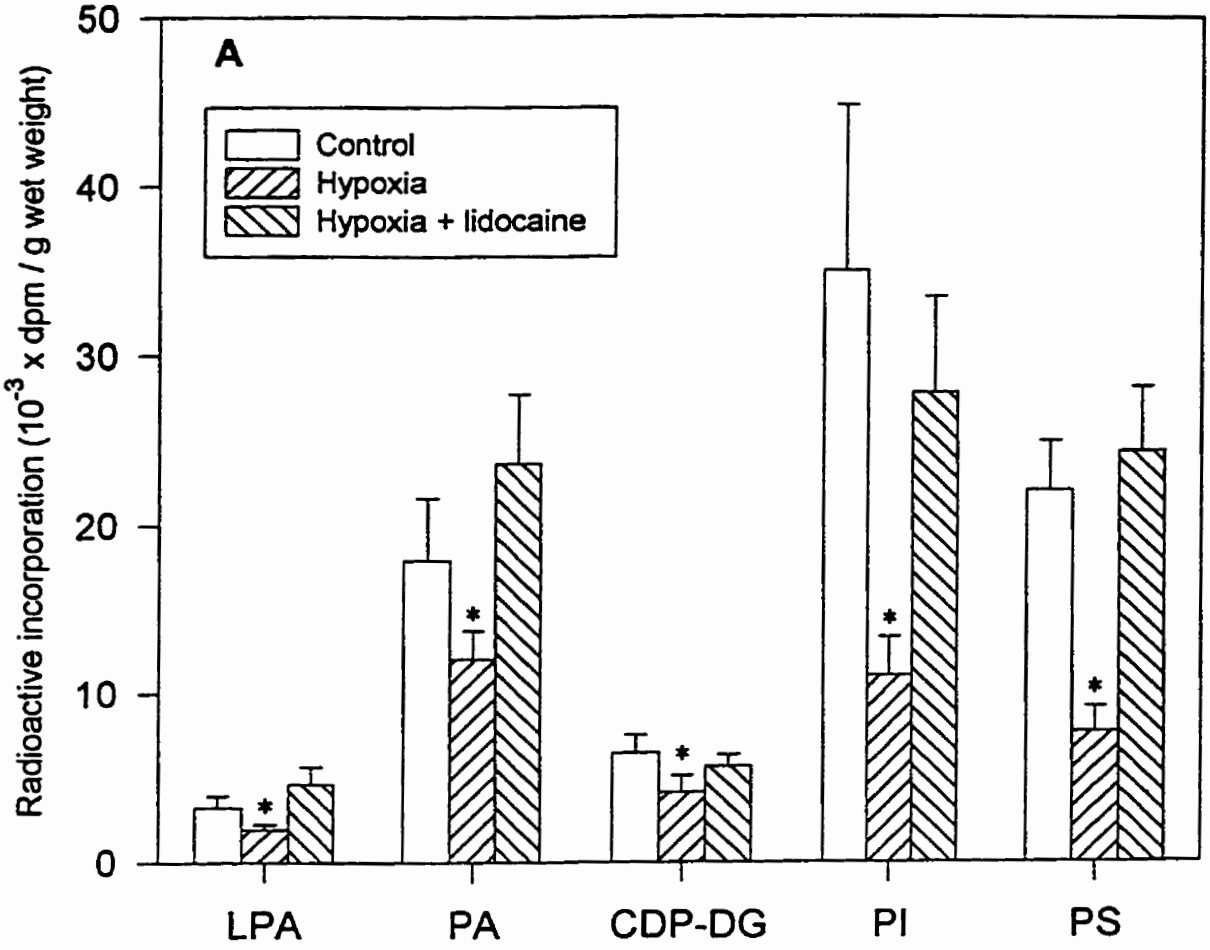


Figure 19. Effects of lidocaine and hypoxia on incorporation of [^{3}H]glycerol into phospholipids and diacylglycerol in the perfused hamster heart. Hearts were perfused as described in Fig. 18. After perfusion, lipid fractions were separated by thin-layer chromatography and the radioactivity associated with each fraction was determined. The results are expressed as means ± standard deviations. Values for control hearts represent results from 13 separate experiments; those for hypoxic hearts, 4 experiments; and those for hypoxia + lidocaine, 3 experiments. *A*, incorporation of [^{3}H]glycerol into lysophosphatidic acid (LPA); phosphatidic acid (PA); CDP-diacylglycerol (CDP-DG); phosphatidylinositol (PI); and phosphatidylserine (PS). (Continued on next page)

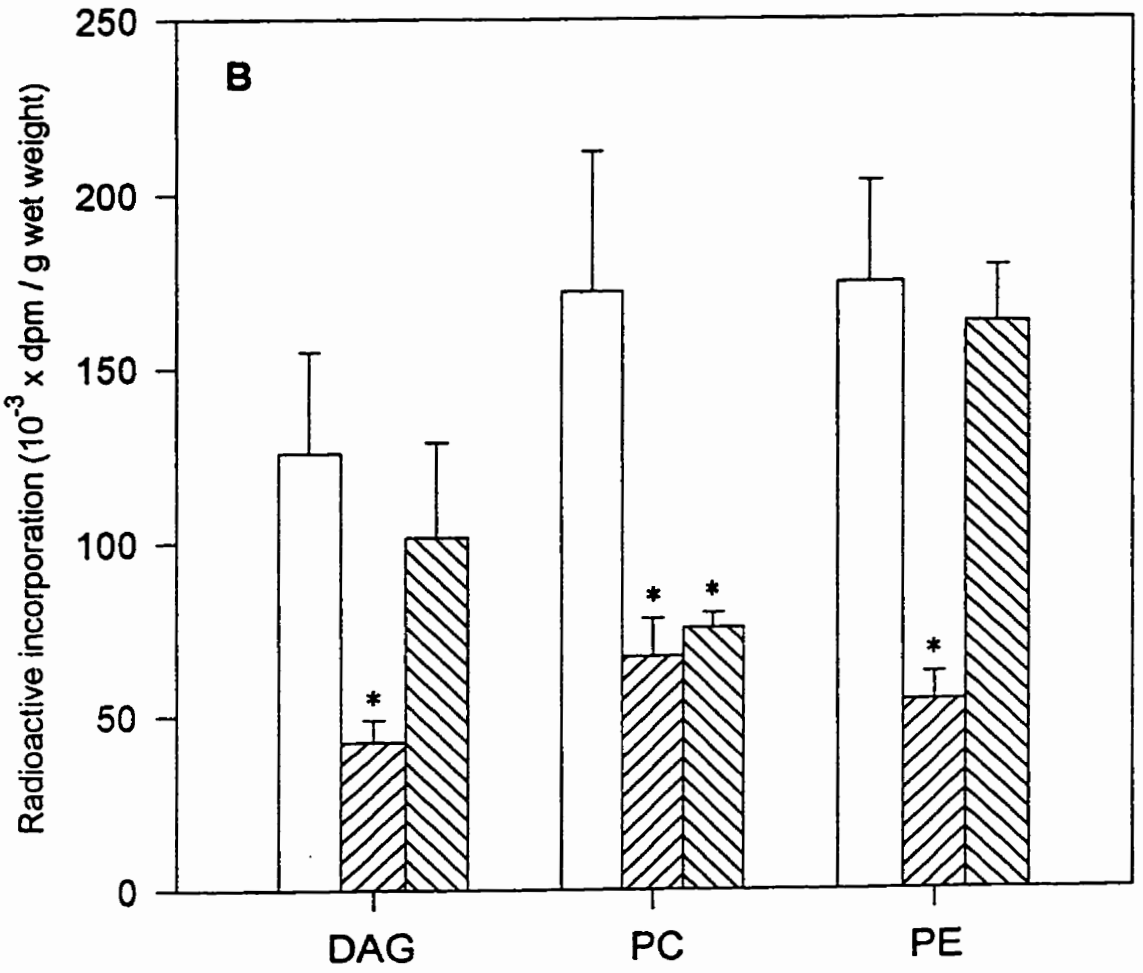


Figure 19 (continued). *B*, incorporation of [^{3}H]glycerol into diacylglycerol (DAG); phosphatidylcholine (PC); and phosphatidylethanolamine (PE). $*p < 0.05$.

3.1.2.3 Phospholipid contents of hearts following perfusion under hypoxia

The pool sizes of phospholipid and diacylglycerol fractions in the hearts perfused under hypoxic conditions or in the presence of lidocaine were determined. As depicted in Table 3, phospholipid and diacylglycerol contents in the heart were not affected by hypoxic perfusion or lidocaine treatment. Thus, the observed alterations in the labelling of the cardiac phospholipids did not appear to arise from changes in their pool sizes or those of their precursors.

3.1.2.4 CDP-choline contents in hamster hearts under hypoxia

CDP-choline is a key metabolite for the synthesis of phosphatidylcholine. Since phosphatidylcholine was the only phospholipid whose synthesis was decreased by lidocaine perfusion, the level of this metablolite in the hearts perfused under hypoxic conditions or in the presence of lidocaine was determined. Our results show that the CDP-choline content in the hamster hearts was not significantly changed by hypoxic perfusion or lidocaine perfusion, or by perfusion with lidocaine under hypoxic conditions (Table 4).

3.1.2.5 ATP and CTP levels in hamster hearts

The reduction of ATP and CTP levels in hypoxic hearts is well documented (de Leiris *et al.* 1984). The ATP and CTP contents in hamster hearts (2.54 ± 0.16 µmol/ g heart and 11.49 ± 2.24 nmol/g heart, respectively) have been determined in our laboratory (Hatch and Choy 1990). We have confirmed that the levels of ATP and CTP were reduced (23% and 48%, respectively) under hypoxia. Perfusion of hearts in the presence of lidocaine did not affect further the levels of these nucleotides.

Table 3. Phospholipid and diacylglycerol contents in control and hypoxic hamster hearts.

Hamster hearts were perfused with Krebs-Henseleit buffer containing 1.0 mM glycerol for 60 min. *Control*, hearts were perfused with buffer saturated with 95% O_2; *hypoxia*, hearts perfused with buffer saturated with 95% N_2; *hypoxia + lidocaine*, hearts perfused with hypoxic buffer containing 0.5 mg/mL lidocaine. After perfusion, the lipid fractions were separated by thin-layer chromatography and the amount of lipid in each fraction was determined. The results are expressed as mean ± standard deviation. Values for control hearts represent results from 9 separate experiments; those for hypoxic hearts, 3 experiments; and those for hypoxia + lidocaine, 3 experiments.

	Content (μmol lipid P/g wet weight of heart)		
Fraction	Control	Hypoxia	Hypoxia + Lidocaine
Lysophosphatidic acid	0.06 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
Phosphatidic acid	0.34 ± 0.08	0.34 ± 0.03	0.39 ± 0.13
Phosphatidylinositol	1.23 ± 0.48	1.14 ± 0.06	1.32 ● 0.13
Phosphatidylserine	1.80 ± 0.31	1.75 ± 0.03	1.89 ± 0.28
Phosphatidylcholine	11.74 ± 1.31	11.48 ● 1.00	10.46 ± 0.24
Phosphatidylethanolamine	10.41 ± 1.00	8.99 ± 1.08	9.66 ± 1.27
Diacylglycerol[a]	6.54 ± 0.48	7.30 ± 1.28	5.94 ± 1.04

[a]Diacylglycerol content is expressed as μmol/g wet weight of heart.

Table 4. CDP-choline levels in hamster hearts.

CDP-choline levels in the hearts were determined either before perfusion or after perfusion for 60 min with Krebs-Henseleit buffer, containing either 0 (control) or 0.5 mg/mL lidocaine. The results are shown as mean ± standard deviation for 4 experiments.

	CDP-choline content (nmol/g heart)
Unperfused hearts	104 ± 10
Perfused control	94 ± 6
Perfused with 0.5 mg/mL lidocaine	98 ± 8
Hypoxic	99 ± 8
Hypoxic + lidocaine	90 ± 10

3.1.3 Effects of lidocaine and hypoxia on phospholipid biosynthetic enzymes

3.1.3.1 Activities of enzymes following perfusion

Changes in the labelling of the cardiac phospholipids might arise from changes in activities of the enzymes responsible for their biosynthesis. Hence, the activities of the phospholipid biosynthetic enzymes in the hamster heart were determined subsequent to perfusion under hypoxia or with lidocaine. Hearts were perfused under the defined conditions for 60 min with Krebs-Henseleit buffer containing 1.0 mM glycerol. After perfusion, subcellular fractions were prepared and enzyme activities were determined as described in Materials and Methods. The activity of phosphatidic acid phosphatase was observed to be elevated (26%) in the microsomal fractions of hearts that had been perfused with lidocaine (Fig. 20). This effect was abolished when the microsomal fraction was incubated with 4.0 mM *N*-ethylmaleimide prior to the assay of enzyme activity, indicating that the increase in phosphatidic acid phosphatase activity was due to a *N*-ethylmaleimide-sensitive phosphatase activity. The increase in the microsomal enzyme activity was accompanied by a 30% decrease in the cytosolic enzyme activity. Perfusion under hypoxia decreased the activity of this enzyme in both microsomal and cytosolic fractions. The presence of lidocaine during hypoxic perfusion did not result in any significant increase in the phosphatidic acid phosphatase activity (Fig. 20).

The activity of CTP:phosphatidic acid cytidylyltransferase (for the formation of CDP-diacylglycerol) was not affected by lidocaine under normoxic conditions (Fig. 20). However, its activity was observed to be increased by 52% when assayed following hypoxic perfusion, and the presence of lidocaine in the hypoxic buffer resulted in a further

increase (170%) above the control activity.

The activities of the other phospholipid biosynthetic enzymes examined were not significantly affected by various experimental treatments (Fig. 20). Although the labelling of phosphatidylcholine was decreased by lidocaine, no change in the activity of CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase was detected when assayed after perfusion of hearts with lidocaine (Fig. 20).

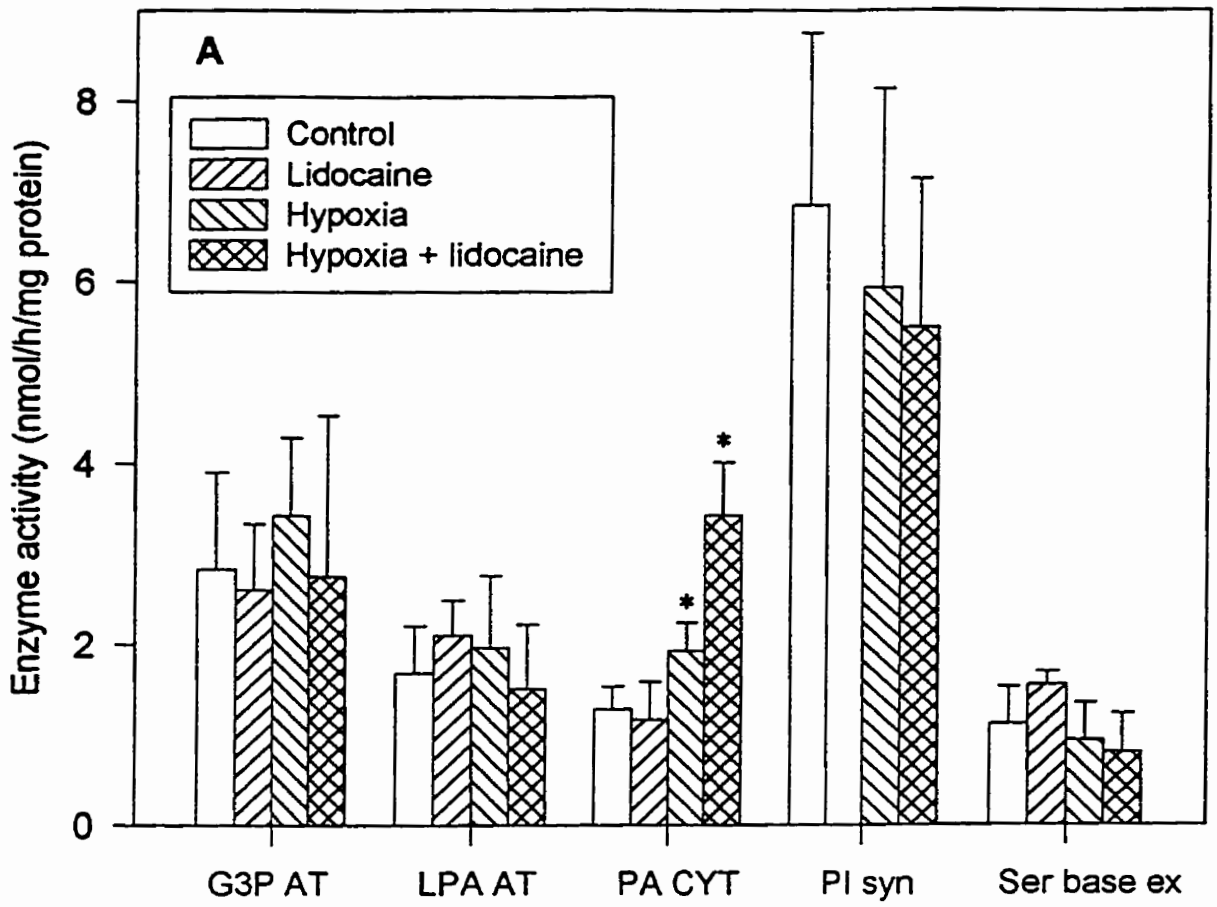


Figure 20. Activities of lipid biosynthetic enzymes in perfused hamster hearts. Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1.0 mM glycerol. *Control*, hearts were perfused with buffer saturated with 95% O_2; *lidocaine*, hearts perfused with oxygenated buffer containing 0.5 mg/mL lidocaine; *hypoxia*, hearts perfused with buffer saturated with 95% N_2; *hypoxia + lidocaine*, hearts perfused with hypoxic buffer containing 0.5 mg/mL lidocaine. After perfusion, subcellular fractions were prepared and enzyme activities were determined as described in Materials and Methods. Results are expressed as means ± standard deviations for 6 separate experiments. *A*, activities of enzymes for biosynthesis of acidic phospholipids. *G3P AT*, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; *LPA AT*, acyl-CoA:lysophosphatidic acid acyltransferase; *PA CYT*, CTP:phosphatidic acid cytidylyltransferase; *PI syn*, phosphatidylinositol synthase; *Ser base ex*, serine base exchange enzyme. (Continued on following page)

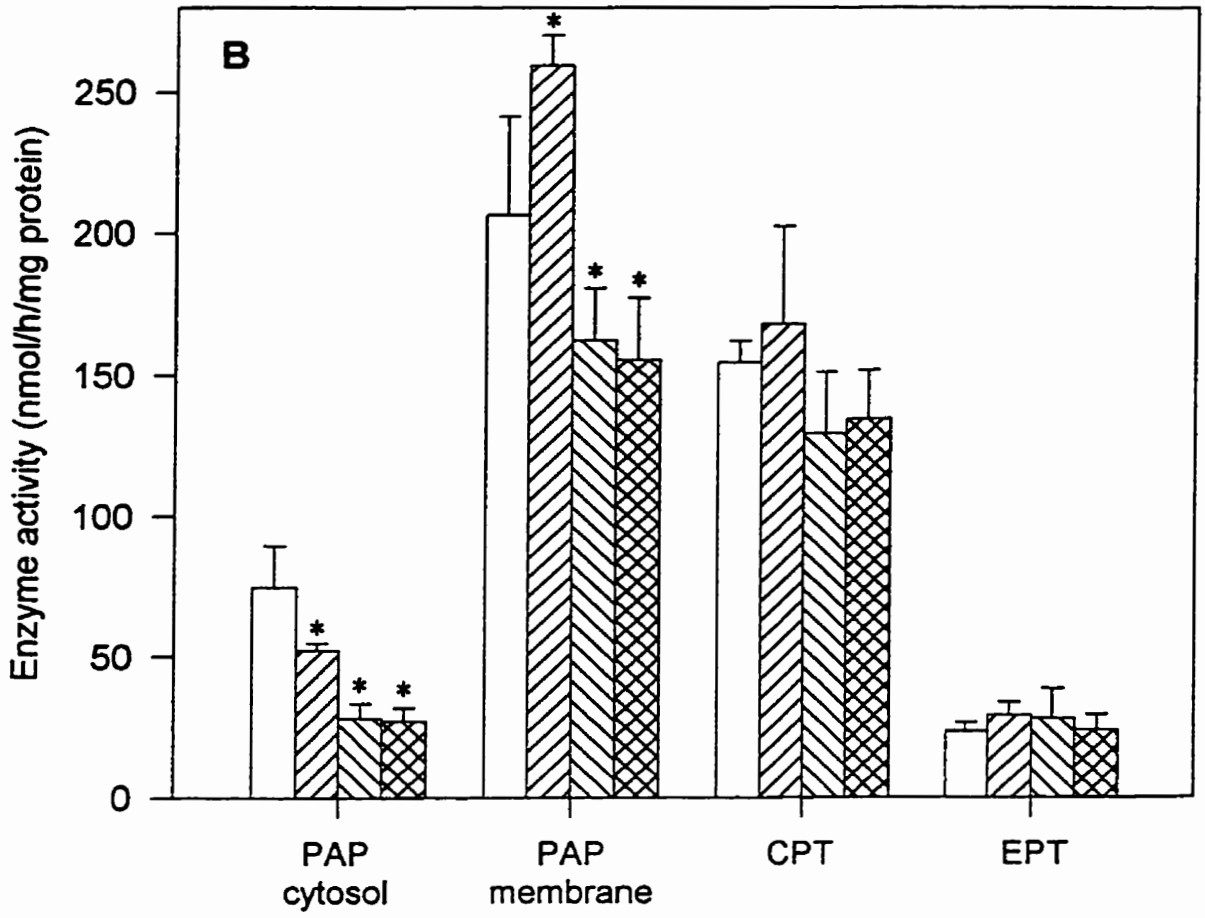


Figure 20 (continued). *B*, activities of enzymes for biosynthesis of zwitterionic phospholipids. *PAP cytosol*, phosphatidic acid phosphatase assayed in cytosolic fractions; *PAP membrane*, phosphatidic acid phosphatase assayed in membrane fractions; *CPT*, CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphtransferase; *EPT*, CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase. $^{*}p < 0.05$.

3.1.3.2 Effects of lidocaine on enzyme activities *in vitro*

The observed changes in the labelling of cardiac lipids might be caused by perturbations in the activities of the phospholipid biosynthetic enzymes. Hence, the activities of these enzymes in subcellular fractions of the hearts were assayed *in vitro*, in the absence or presence of lidocaine (Tables 5-7). The activity of acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase was stimulated in the presence of lidocaine (Table 5). This effect may account for the increased labelling of lysophosphatidic acid observed in lidocaine-perfused hearts.

As described above, the phosphatidic acid phosphatase and CTP:phosphatidic acid cytidylyltransferase activities were found to be altered following perfusion under various experimental conditions. However, the addition of lidocaine to the *in vitro* enzyme assays for these enzymes did not cause any significant change in their activities (Tables 5 and 6). The activities of other phospholipid biosynthetic enzymes were not significantly changed by the presence of lidocaine in the *in vitro* assays (Tables 5 and 6).

The activity of CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase was examined *in vitro*, in the absence or presence of lidocaine (Table 7). Lidocaine caused a decrease in the cholinephosphotransferase activity in microsomal fractions obtained from both normoxic and hypoxic hearts. This inhibition of cholinephosphotransferse activity by lidocaine was observed regardless of whether lidocaine was present in the perfusate during perfusion of the hearts. The inhibition of cholinephosphotransferase activity might explain the decrease in the labelling of phosphatidylcholine observed in hearts perfused with lidocaine.

Table 5. Effect of lidocaine on *in vitro* activities of enzymes for biosynthesis of acidic phospholipids.

Subcelluar fractions were obtained from hamster hearts and enzyme activities were assayed in the presence of 0 (control) or 0.5 mg/mL lidocaine. The results are expressed as mean ● standard deviation for three sets of experiments, each of which was performed in duplicate. Results were analyzed using a paired *t* test. $*p < 0.05$

	Enzyme activity (pmol/min/mg protein)	
Enzyme	Control	0.5 mg/mL lidocaine
Acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase	49.5 ● 18.8	110.7 ± 20.5*
Acyl-CoA:lysophosphatidic acid acyltransferase	17.5 ● 5.2	18.2 ± 5.2
CTP:phosphatidic acid cytidylyltransferase	5.5 ± 1.2	6.7 ± 1.7
Phosphatidylinositol synthase	8.7 ± 1.7	6.7 ± 0.5
Serine base exchange enzyme	2.2 ± 0.7	0.8 ± 0.5

Table 6. Effects of lidocaine on *in vitro* activities of phosphatidic acid phosphatase and CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase.

Subcelluar fractions were obtained from hamster hearts and enzyme activities were assayed in the presence of 0 (control) or 0.5 mg/mL lidocaine. The results are expressed as mean ± standard deviation for three sets of experiments, each of which was performed in duplicate. Results were analyzed using a paired *t* test.

	Enzyme activity (nmol/min/mg protein)	
Enzyme	Control	0.5 mg/mL lidocaine
Phosphatidate phosphatase	1.10 ± 0.32	1.25 ± 0.26
CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase	0.33 ± 0.05	0.26 ± 0.02

Table 7. Effect of lidocaine on the *in vitro* activity of CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase.

Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 0 (control) or 0.5 mg/mL lidocaine under nomoxic or hypoxic conditions. After perfusion, cholinephosphotransferase activities in the microsomal fractions were determined in the absence or presence of 0.5 mg/mL lidocaine. Results are expressed as mean ± standard deviation for 4 separate experiments. $^*p < 0.05$.

Enzyme source	CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase activity (nmol/h/mg protein)
Unperfused hearts	164 ± 20
Perfused control	159 ± 26
Perfused control + 0.5 mg/mL lidocaine	90 ± 14*
Perfused with lidocaine	165 ± 36
Perfused with lidocaine + 0.5 mg/mL lidocaine	94 ± 33*
Hypoxic perfusion	138 ± 26
Hypoxic perfusion + 0.5 mg/mL lidocaine	88 ± 23*

3.2 Studies on Arachidonic Acid Metabolism

In this portion of this study, we have investigated the catabolism of phospholipids. In addition to their structural function in cellular membranes, phospholipids may be regarded as a potential source of biological signalling molecules. The catabolism of phospholipids by phospholipases liberates lipoidal species which can then function as signalling molecules or be metabolized further to provide signalling molecules. As outlined in the Introduction and Literature Review, one such molecule is arachidonic acid. Thus, in this section we have investigated the regulation of arachidonic acid metabolism. Lysophosphatidylcholine is present in cell membranes, oxidized lipoproteins and atherosclerotic tissues. Since it can alter some endothelial functions and is regarded as a causal agent in atherogenesis (see also Introduction and Literature Review), we have investigated the regulation of arachidonic acid release in endothelial cells under the influence of lysophosphatidylcholine.

3.2.1 Effect of lysophosphatidylcholine on arachidonic acid release in human umbilical vein endothelial cells

3.2.1.1 Uptake and incorporation of [^{3}H]arachidonic acid into phospholipids in human umbilical vein endothelial cells

To determine the incorporation of [^{3}H]arachidonic acid into phospholipids in human umbilical vein endothelial cells, the cells were incubated for up to 24 h with 1.0μCi/mL [^{3}H]arachidonic acid in Medium 199 containing 10% fetal calf serum. After

the appropriate incubation period, the cells were collected and free arachidonic acid and phospholipid fractions were isolated as described in Materials and Methods. Results are shown in Fig. 21. Free arachidonic acid was detected in the cells within the first 2 h of incubation, and decreased to a steady state level within 8-10 h. The incorporation of [^{3}H]arachidonic acid into major phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine fractions were monitored (Fig. 21). Radioactive labelling of these phospholipids reached an equilibrium level within 8-24 h of incubation with [^{3}H]arachidonic acid. Hence, the cells were radiolabelled for approximately 20 h in subsequent experiments.

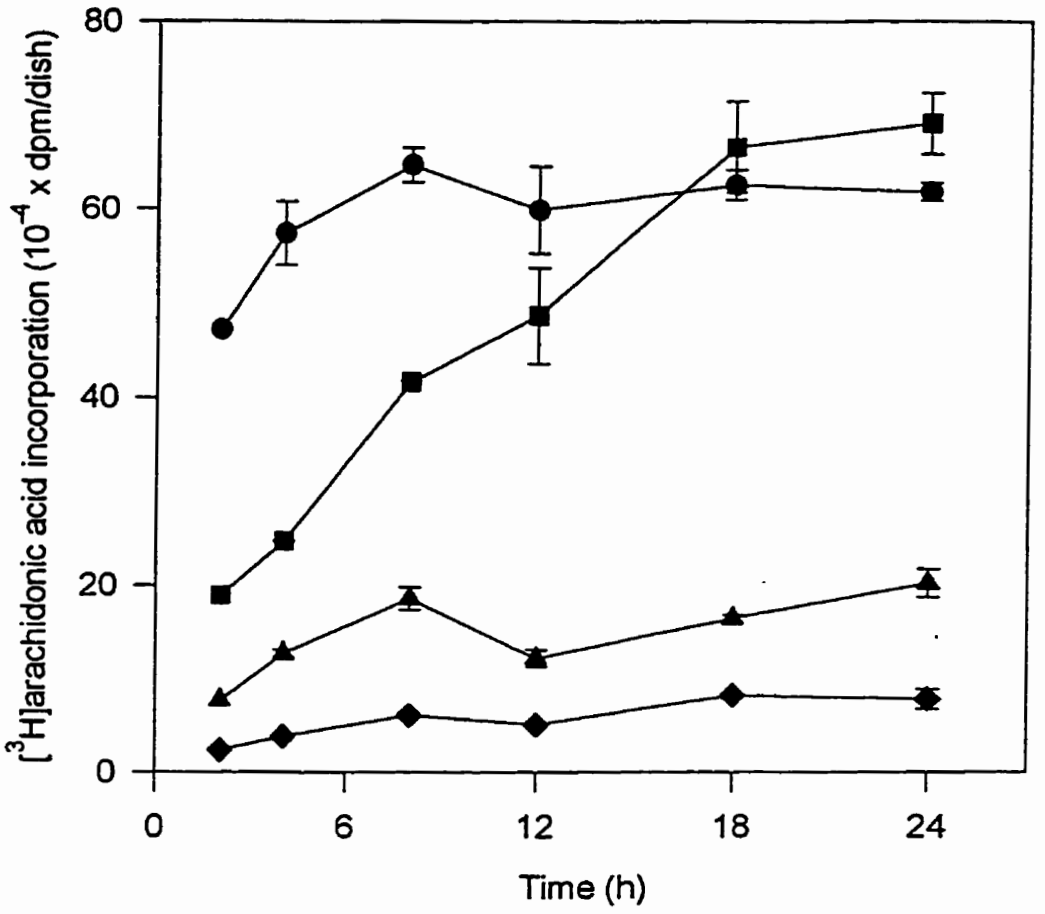


Figure 21. Incorporation of [^{3}H]arachidonic acid into phospholipids in endothelial cells. Near-confluent monolayers of endothelial cells were incubated for the indicated times in medium-199/10% fetal bovine serum containing 1.0 μCi/mL [^{3}H]arachidonic acid. At the end of each incubation period, the cells were harvested, phospholipid fractions were separated by thin-layer chromatography, and the radioactivity associated with each fraction was determined. Results shown for phosphatidylcholine (●), phosphatidylethanolamine (■), phosphatidylinositol (▲), and phosphatidylserine (◆). Results shown as means ± standard deviations of three separate determinations.

3.2.1.2 Modulation of arachidonic acid release by lysophosphatidylcholine

To determine the effect of lysophosphatidylcholine on arachidonic acid release, human umbilical vein endothelial cells were labelled with [^{3}H]arachidonic acid in Medium 199 containing 10% fetal calf serum for 20 h, as described in Materials and Methods. The cells were rinsed, and then incubated with Hepes-buffered saline containing 0.025% bovine serum albumin and 0 or 50 μM lysophosphatidylcholine for various time periods (Fig. 22). Lysophosphatidylcholine elicited a time-dependent arachidonic acid release which reached maximum at 10 min of incubation, after which time arachidonic acid release was slightly diminished. A nominal amount of bovine serum albumin was required in order to bind any arachidonic acid that was released into the buffer. The effects of various concentrations of lysophosphatidylcholine on arachidonic acid release was determined at bovine serum albumin concentrations ranging from 0.025 to 0.1% (w/v) (4 to 16 μM albumin) (Fig. 23). The effect of lysophosphatidylcholine on arachidonic acid release was affected by the albumin concentration (Fig. 23). Higher concentrations of lysophosphatidylcholine was required to elicit a stimulation of arachidonic acid release at higher albumin concentrations. For example, at 0.025% albumin (w/v), little arachidonic acid was detected below 25 μM lysophosphatidylcholine; however, at 50 μM lysophosphatidylcholine there was a dramatic increase in arachidonic acid released, peaking at more than 10-fold above the basal level. Lysophosphatidylcholine at this concentration has been shown to be non-lethal to endothelial cells during incubation times used in this study (Su *et al.* 1995), and we have confirmed cell viability under the incubation conditions by the exclusion of trypan blue dye. Concentrations of 50 μM lysophosphatidylcholine and 0.025% bovine serum

albumin were routinely used in subsequent experiments. Except for experiments in which the contribution of extracellular Ca^{2+} was explored (section 3.2.3), all buffers used for incubating the cells contained 1.5 mM Ca^{2+}.

Initial experiments on the effect of lysophosphatidylcholine on arachidonic acid release were performed using lysophosphatidylcholine derived from egg lecithin. Since egg lysolecithin contains mainly saturated acyl species, we tested the ability of palmitoyl ($C_{16:0}$)- and stearoyl ($C_{18:0}$)-lysophosphatidylcholine to stimulate arachidonic acid release. Fig. 24 shows that lysophosphatidylcholine containing palmitoyl and stearoyl chains induced a high release of arachidonic acid. To determine if the stimulation of arachidonic acid release is specific to lysophosphatidylcholine or if it is a property common to lysolipids, we tested the effect of other lysophospholipids such as lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, and lysophosphatidate on arachidonic acid release. As shown in Fig. 24, lysolipids with head groups other than choline were relatively ineffective in stimulating arachidonic acid release. Based on these results, lysophosphatidylcholine containing a palmitoyl ($C_{16:0}$) chain was routinely used in subsequent experiments.

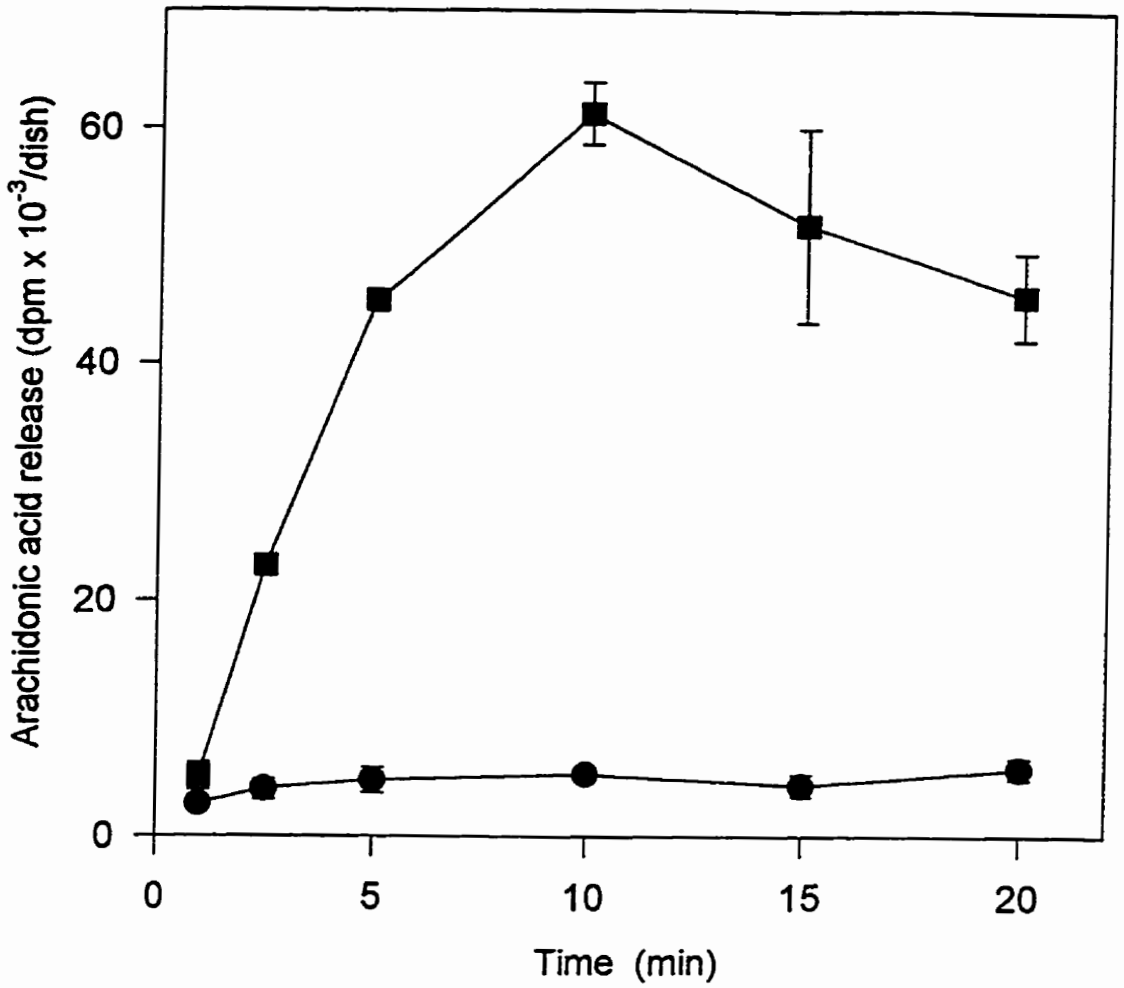


Figure 22. Time course of arachidonic acid release in endothelial cells. Near-confluent monolayers of endothelial cells were incubated for 20 h in medium-199/10% fetal bovine serum containing 1.0 μCi/mL [^{3}H]arachidonic acid. Cells were then washed three times with HEPES-buffered saline containing 0.025% (w/v) bovine serum albumin. The cells were incubated for the indicated times with HEPES-buffered saline/0.025% bovine serum albumin, containing 0 μM (●) or 50 μM (■) lysophosphatidylcholine. At the end of each incubation period, the buffer was collected, the arachidonic acid fraction was separated by thin-layer chromatography, and the radioactivity of the arachidonic acid fraction was determined. Values represent means ± standard deviations of three separate experiments.

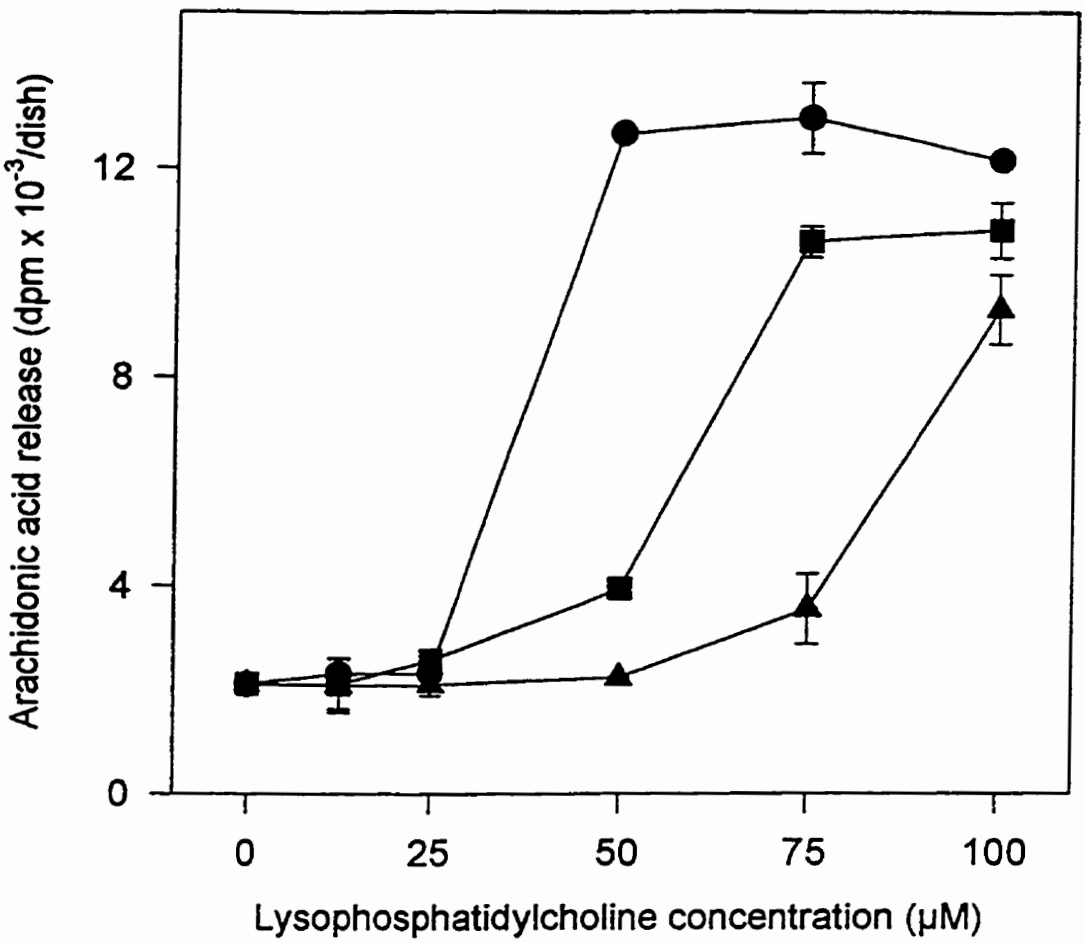


Figure 23. Effect of lysophosphatidylcholine concentration on arachidonic acid release in endothelial cells. Near-confluent monolayers of endothelial cells were incubated for 20 h in medium-199/10% fetal bovine serum containing 1.0 μCi/mL [^{3}H]arachidonic acid. Cells were then washed three times with HEPES-buffered saline containing 0.025% (w/v) bovine serum albumin. The cells were incubated for 10 min with the indicated concentrations of lysophosphatidylcholine in HEPES-buffered saline containing 0.025% (●), 0.05% (■), or 0.10% (▲) bovine serum albumin (w/v). At the end of the incubation period, the buffer was collected, the arachidonic acid fraction was separated by thin-layer chromatography, and the radioactivity of the arachidonic acid fraction was determined. Values represent means ± standard deviations of three separate experiments.

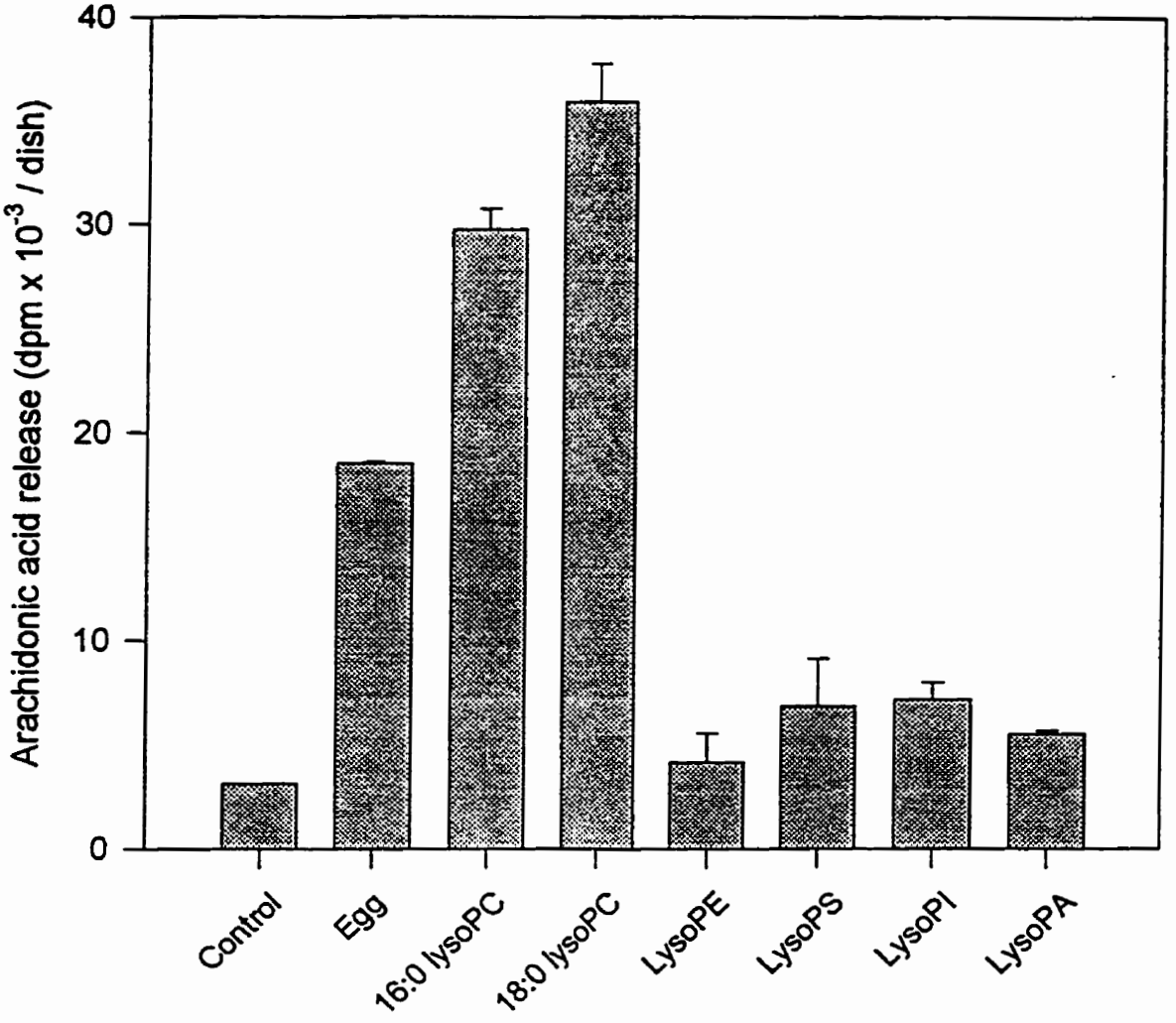


Figure 24. Effects of lysolipids on arachidonic acid release. Endothelial cells prelabelled with [^{3}H]arachidonic acid were incubated for 10 min with 0 μM (*Control*) or 50 μM of the indicated lysophosphatidylcholines or other lysolipids in HEPES-buffered saline. *Egg*, lysolecithin from egg; *16:0*, palmitoyl-lysophosphatidylcholine; *18:0*, stearoyl-lysophosphatidylcholine; *LysoPE*, lysophosphatidylethanolamine; *LysoPS*, lysophosphatidylserine; *LysoPI*, lysophosphatidylinositol; *LysoPA*, lysophosphatidic acid. Arachidonic acid released into the buffer was then determined as described above. Values represent means ± standard deviations of three separate experiments.

3.2.2 Role of phospholipases A_2 in arachidonic acid release induced by lysophosphatidylcholine

To determine whether the release of arachidonic acid was mediated by PLA_2, we examined the effects of the PLA_2 inhibitors *para*-bromophenacyl bromide (*p*BPB) and arachidonoyl trifluoromethylketone ($AACOCF_3$), the latter of which specifically inhibits the $cPLA_2$ (Street *et al.* 1993). As shown in Table 8, arachidonic acid release was significantly inhibited in those cells which were preincubated with these inhibitors prior to challenge with lysophosphatidylcholine. The inhibition of arachidonic acid release by up to 62% by $AACOCF_3$ indicates that the $cPLA_2$ may be involved in the arachidonic acid release induced by lysophosphatidylcholine. However, both $sPLA_2$ and $cPLA_2$ isoforms have been found to be present in endothelial cells, and may both participate in arachidonic acid release and prostacyclin synthesis (Murakami *et al.* 1993).

Table 8. Effect of PLA_2 inhibitors on lysophosphatidylcholine-induced arachidonic acid release.

Cells prelabelled with [^{3}H]arachidonic acid were incubated with the indicated concentrations of $AACOCF_3$ for 2 min or pBPB for 15 min prior to incubation for 10 min with 50 μM lysophosphatidylcholine in HEPES-buffered saline. Arachidonic acid released into the buffer was then determined as described above. Results are expressed as mean ± standard deviation of three separate experiments. $^*p < 0.05$ compared with cells incubated with 50 μM lysophosphatidylcholine alone.

Treatment	Arachidonic acid release (dpm x 10^{-3}/dish)	Inhibition (%)
Control (no lysophosphatidylcholine)	5.98 ± 0.40	
50 μM lysophosphatidylcholine	31.08 ± 2.35	
$AACOCF_3$ + lysophosphatidylcholine		
1 μM	19.11 ± 0.60*	39
25 μM	11.93 ± 1.55*	62
pBPB + lysophosphatidylcholine		
5 μM	22.09 ± 1.79*	29
25 μM	16.44 ± 2.61*	47

To further delineate which PLA_2 isoform is involved in the lysophosphatidylcholine-induced arachidonic acid release, we used antisense oligonucleotides toward $cPLA_2$ and $sPLA_2$ (see also Materials and Methods). These oligonucleotides were designed to bind specifically to the respective mRNAs and prevent the translation and synthesis of enzyme protein (Barbour and Dennis 1993b; Roshak *et al.* 1994). Complementary sense oligonucleotides were used as negative controls. Cells were cultured in the presence of sense or antisense oligonucleotides to either PLA_2 isoform for three days prior to challenge with lysophosphatidylcholine. Treatment of the cells with either sense or antisense oligonucleotides did not alter the total incorporation of [^{3}H]arachidonic acid. Lysophosphatidylcholine-induced arachidonic acid release was significantly attenuated in cells grown in the presence of antisense oligonucleotides for $cPLA_2$, compared with cells grown without oligonucleotides or with sense oligonucleotides (Fig. 25). The level of $cPLA_2$ protein after the treatment with antisense $cPLA_2$ oligonucleotides was determined by immunoblotting analysis with a polyclonal antibody for $cPLA_2$. The level of $cPLA_2$ protein was decreased (40 % reduction) by the antisense oligonucleotide treatment (Fig. 26). The immunoblotting analysis for $cPLA_2$ was conducted once. The reduction of $cPLA_2$ enzyme levels was corroborated by an *in vitro* assay for PLA_2 activity, in which we observed a decrease in PLA_2 activity in cells treated with antisense oligonucleotides toward $cPLA_2$, with a corresponding (30%) decrease in the activity associated with the membrane fractions. In cells treated with antisense $sPLA_2$ oligonucleotides, the lysophosphatidylcholine-induced arachidonic acid release was not significantly affected (Fig. 25), despite a 35 % decrease in the $sPLA_2$ protein level in those cells (Fig. 26). The immunoblotting analysis for $sPLA_2$ was carried

out three times, with similar results.

Taken together, the inhibition of arachidonic acid release by antisense oligonucleotides for $cPLA_2$ and by $AACOCF_3$ indicate that the $cPLA_2$ is involved in the lysophosphatidylcholine-induced arachidonic acid release. Therefore, we assayed *in vitro* the PLA_2 activity in subcellular fractions from control cells and from cells treated with lysophosphatidylcholine. As shown in Table 9, specific PLA_2 activity was decreased by 52% in the cytosolic fractions of lysophosphatidylcholine-treated cells, while activity in the membrane fractions was increased by 33%. The increase in the membrane PLA_2 activity is consistent with the notion that the enzyme translocated to cell membranes. The membrane-bound enzyme is regarded as the active form, as the cell membrane contains the endogenous phospholipid substrates of $cPLA_2$ (Clark *et al.* 1995). This notion was supported by an *in vitro* study, in which PLA_2 activity was determined after the treatment of cells with the antisense $cPLA_2$ oligonucleotides. The total PLA_2 activity was reduced in those cells, with a corresponding (30%) decrease in the activity associated with the membrane fractions.

In separate experiments, the direct effect of lysophosphatidylcholine on PLA_2 activity was examined by adding lysophosphatidylcholine to the PLA_2 assays. Lysophosphatidylcholine had no effect on PLA_2 activity when added directly to the enzyme assays (results not shown). Thus, the enhancement of PLA_2 activity observed in the membrane fraction of lysophosphatidylcholine-treated cells was likely due to an indirect mechanism occurring in the intact cells. Therefore, we investigated the potential roles of Ca^{2+} and protein kinases on the induction of arachidonic acid release by lysophosphatidylcholine.

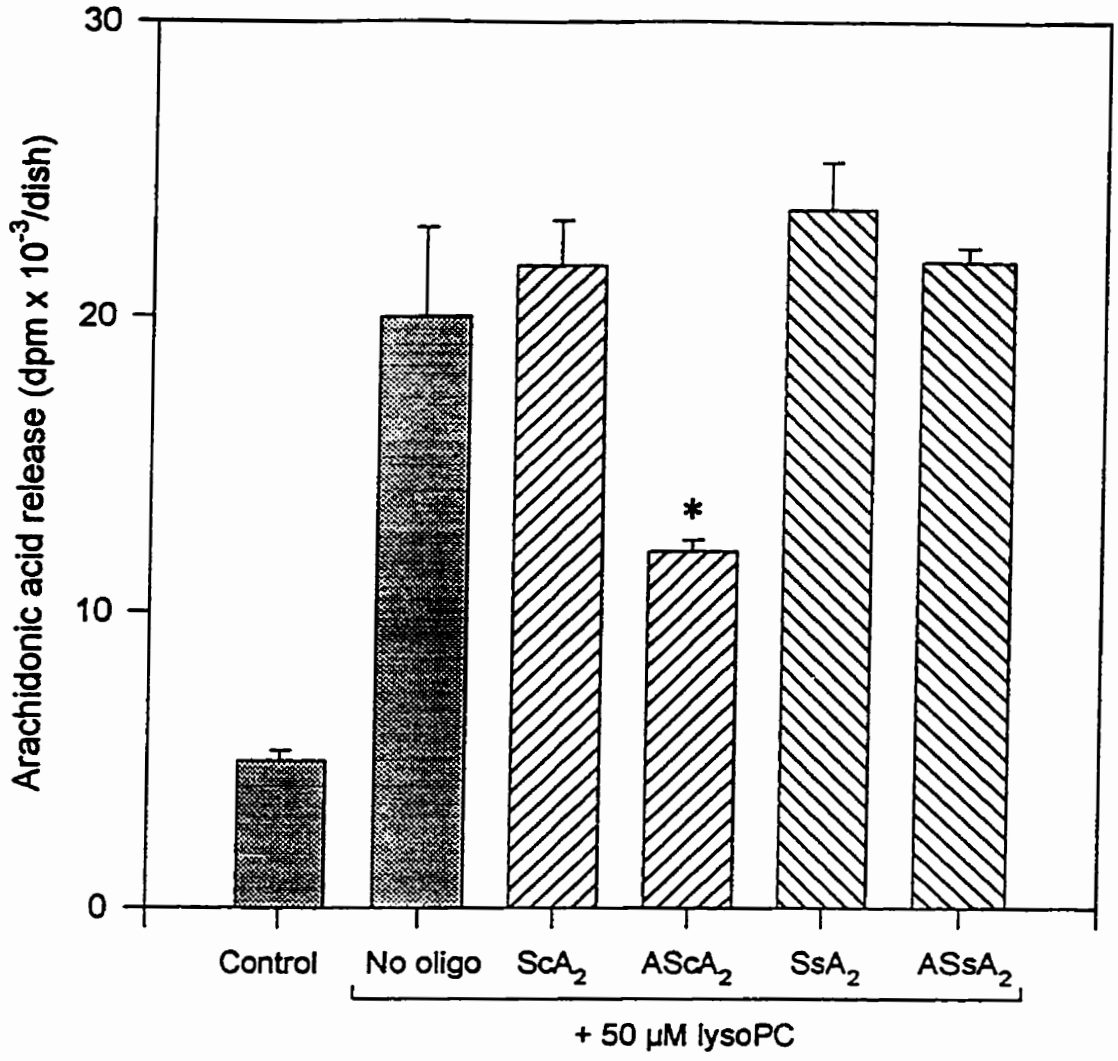
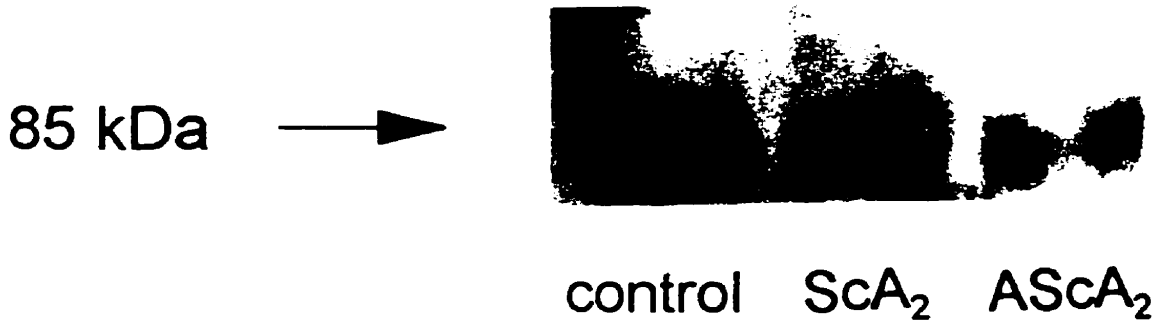


Figure 25. Effect of antisense PLA_2 oligonucleotides on arachidonic acid release. Endothelial cells were cultured in the presence of oligonucleotides for 72 h as described in Materials and Methods. Cells were labelled with [^{3}H]arachidonic acid for 20 h prior to incubation for 10 min with 50 μM lysophosphatidylcholine in HEPES-buffered saline. Arachidonic acid released into the buffer was then determined as described above. *Control*, cells cultured without oligonucleotides and not incubated with lysophosphatidylcholine; *No oligo*, cells cultured without oligonucleotide treatment prior to incubation with lysophosphatidylcholine; ScA_2, cells cultured in the presence of sense oligonucleotide for $cPLA_2$; $AScA_2$, cells cultured in the presence of antisense oligonucleotide for $cPLA_2$; SsA_2, cells cultured in the presence of sense oligonucleotide for $sPLA_2$; $ASsA_2$, cells cultured in the presence of antisense oligonucleotide for $sPLA_2$. Values represent means ± S.D. of three separate experiments. $^*p < 0.05$, compared with cells cultured in the absence of oligonucleotides prior to exposure to lysophosphatidylcholine.

Figure 26. Immunoblots of $cPLA_2$ and $sPLA_2$ in cells cultured in the presence of PLA_2 oligonucleotides. Cells were cultured for 72 h in the absence or presence of 10 μM sense or antisense oligonucleotides to $cPLA_2$ or $sPLA_2$. The levels of $cPLA_2$ (***Top***) or $sPLA_2$ (***Bottom***) in the cell lysates were quantitated by immunoblotting as described in Materials and Methods. ***Top, Control***, cells cultured without oligonucleotides; ScA_2, cells cultured in the presence of sense oligonucleotides for $cPLA_2$; $AScA_2$, cells cultured in the presence of antisense oligonucleotides for $cPLA_2$. ***Bottom, Control***, cells cultured without oligonucleotides; SsA_2, cells cultured in the presence of sense oligonucleotides for $sPLA_2$; $ASsA_2$, cells cultured in the presence of antisense oligonucleotides for $sPLA_2$.


85 kDa
control
ScA2
AScA2

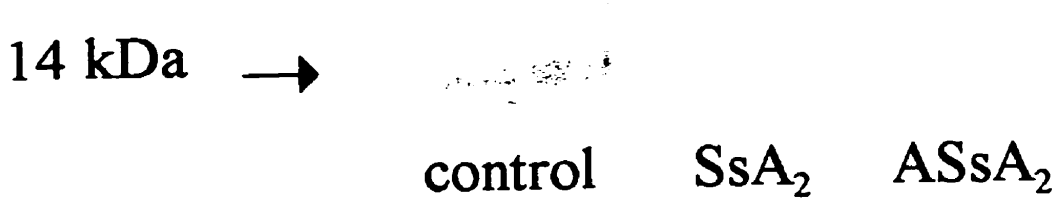

14 kDa
control
SsA2
ASsA2

Table 9. Effect of lysophosphatidylcholine on PLA_2 activity in endothelial cells.

Cells were incubated for 10 min with 0 μM or 50 μM lysophosphatidylcholine in HEPES-buffered saline containing 0.025% bovine serum albumin. Cells were lysed, and PLA_2 activity was assayed in the cytosolic and membrane fractions as described in Materials and Methods. Results are expressed as mean ± standard deviation of three separate experiments. $^*p < 0.05$

Subcellular fraction	LysoPC treatment (μM)	PLA_2 activity (pmol/min/mg protein)
Cytosol	0	29.4 ± 0.2
	50	13.9 ± 2.6 *
Membrane	0	10.5 ± 0.8
	50	13.5 ± 0.3 *

3.2.3 Involvement of Ca^{2+} in arachidonic acid release induced by lysophosphatidylcholine

Since both $sPLA_2$ and $cPLA_2$ are regulated by Ca^{2+}, we investigated the role of Ca^{2+} in the arachidonic acid release induced by lysophosphatidylcholine. Cells were challenged with lysophosphatidylcholine in the presence of 0 to 1.5 mM Ca^{2+}. As shown in Fig. 27, the induction of arachidonic acid release by lysophosphatidylcholine was progressively suppressed with decreasing Ca^{2+} concentrations. Arachidonic acid release was completely abolished when Ca^{2+} was absent from the buffer (the calcium-fee buffer also contained 1 mM EDTA and 1 mM EGTA). Thus, the lysophosphatidylcholine-induced arachidonic acid release appeared to be dependent on the Ca^{2+} concentration in the buffer. Lysophosphatidylcholine has been shown to cause increases in intracellular Ca^{2+} concentrations (Su *et al.* 1995). In the current study, treatment of the cells with 50 μM lysophosphatidylcholine in the presence of 1.5 mM Ca^{2+} in the buffer caused an approximately three-fold increase in the intracellular Ca^{2+} level (Fig. 27, inset). In the absence of extracellular Ca^{2+}, neither lysophosphatidylcholine nor the calcium ionophore bromoA23187 was able to cause any change in cell Ca^{2+}. It appears that an influx of Ca^{2+} from an extracellular source was a prerequisite for the induction of arachidonic acid release by lysophosphatidylcholine, and that the rise of cellular Ca^{2+} was not derived from an intracellular pool.

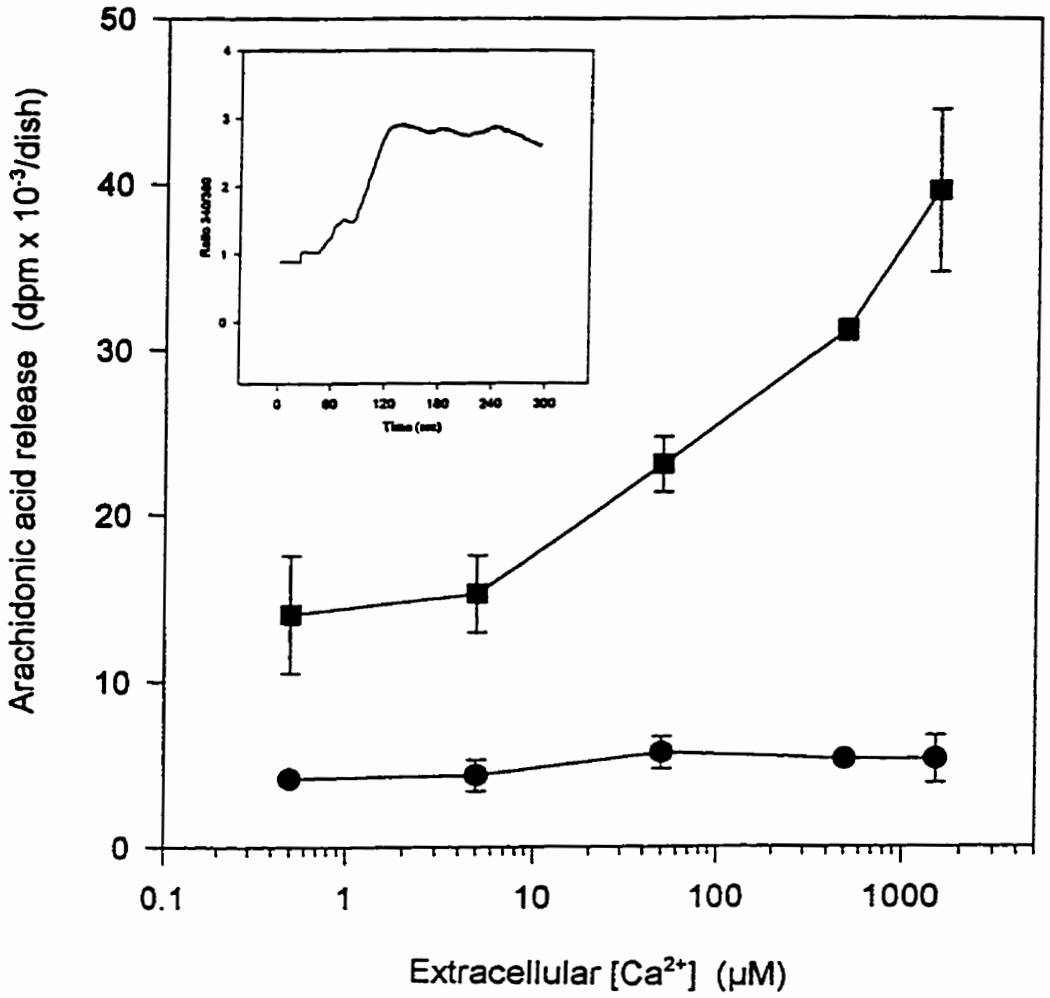


Figure 27. Effect of Ca^{2+} concentration on lysophosphatidylcholine-induced arachidonic acid release. Cells were labelled with [^{3}H]arachidonic acid for 20 h, and then incubated for 10 min with 0 μM (●) or 50 μM (■) lysophosphatidylcholine in HEPES-buffered saline containing the indicated Ca^{2+} concentrations. Arachidonic acid released into the buffer was determined as described above. Values represent means ± standard deviation of three separate experiments. ***Inset***, cells were challenged with 50 μM lysophosphatidylcholine in HEPES-buffered saline containing 0.025% bovine serum albumin, and the intracellular Ca^{2+} was monitored using fura-2 as described in Materials and Methods. A typical trace is shown.

3.2.4 Involvement of protein kinases in arachidonic acid release induced by lysophosphatidylcholine

3.2.4.1 Involvement of protein kinase C or protein kinase A

In addition to Ca^{2+}, phosphorylation events have also been shown to play a role in the regulation of $cPLA_2$ activity in a number of cell types (Clark *et al.* 1995; Qiu and Leslie 1994; Xing and Insel 1996). Thus, we investigated whether PKC or MAPK were involved in the lysophosphatidylcholine-induced arachidonic acid release. Cells were pretreated with the PKC inhibitors staurosporine (Tamaoki *et al.* 1986) or Ro31-8220 (Nixon *et al.* 1992) prior to challenge with lysophosphatidylcholine. For comparison, we also investigated the involvement of the cAMP-dependent protein kinase (PKA) by using the PKA inhibitor H89 (Chijiwa *et al.* 1990). As shown in Table 10, staurosporine and Ro31-8220 inhibited the arachidonic acid release induced by lysophosphatidylcholine by up to almost 70%. In contrast, H89 did not cause any significant inhibition, up to a dose (1.0 μM) far exceeding its K_i (0.05 μM) for PKA (Chijiwa *et al.* 1990). Lysophosphatidylcholine has previously been shown to modulate PKC activity in both cell-free and cell-based assays (Oishi *et al.* 1988; Sasaki *et al.* 1993; Kugiyama *et al.* 1992). Consistent with these findings, we observed that treatment of the cells with lysophosphatidylcholine for 5 min caused a 58% increase in PKC activity in the membrane fraction of the cells (Table 11).

To further investigate the role of PKC in the arachidonic acid release in endothelial cells, we incubated cells for 10 min with 200 nM phorbol 12-myristate 13-acetate, a known activator of PKC. Incubation of the cells with the phorbol ester resulted in a two-

fold stimulation of PKC activity (Table 11), and resulted in a two-fold increase in arachidonic acid release (Fig 28). In comparison, cells incubated with lysophosphatidylcholine exhibited a 5.7-fold increase in arachidonic acid release. When cells were incubated with both the phorbol ester lysophosphatidylcholine, a 7.6-fold increase in arachidonic acid release was observed (Fig 28). Thus, while it is clear that PKC activation can contribute to arachidonic acid release in endothelial cells, the stimulation of PKC activity alone cannot account for the arachidonic acid release induced by lysophosphatidylcholine.

Table 10. Effect of PKC and PKA inhibitors on lysophosphatidylcholine-induced arachidonic acid release.

Cells prelabelled with [^{3}H]arachidonic acid were incubated with the indicated concentrations of staurosporine, Ro31-8220 or H89 for 10 min. The cells were then incubated for an additional 10 min with 50 μM lysophosphatidylcholine. Arachidonic acid release was determined as described above. Results are expressed as mean ± standard deviation of three separate experiments. *$p < 0.05$ compared with cells incubated with 50 μM lysophosphatidylcholine alone.

Treatment	Arachidonic acid release (dpm x 10^{-3}/dish)	Inhibition (%)
Control (no lysophosphatidylcholine)	3.13 ± 0.63	
50 μM lysophosphatidylcholine	32.66 ● 2.19	
Staurosporine + lysophosphatidylcholine		
0.1 μM	18.28 ± 0.06*	44
1 μM	10.31 ± 0.16*	68
Ro31-8220 + lysophosphatidylcholine		
5 μM	15.63 ± 0.20*	52
10 μM	11.25 ± 0.05*	66
H89 + lysophosphatidylcholine		
0.1 μM	29.70 ± 1.25	9
1 μM	29.40 ± 2.19	10

Table 11. Effects of lysophosphatidylcholine and phorbol 12-myristate 13-acetate on PKC activity in endothelial cells.

Near-confluent monolayers of endothelial cells were incubated for 5 min with 50 μM lysophosphatidylcholine or 200 nM phorbol 12-myristate 13-acetate (PMA). PKC activity in the membrane fractions of the cells were determined as described in Materials and Methods. Results are expressed as mean ± standard deviation of three separate experiments. $*p < 0.05$

Treatment	PKC activity (pmol/min/mg protein)	Increase (%)
Control	80 ± 7	
Lysophosphatidylcholine	126 ± 5*	57.5
PMA	242 ± 22*	202.5

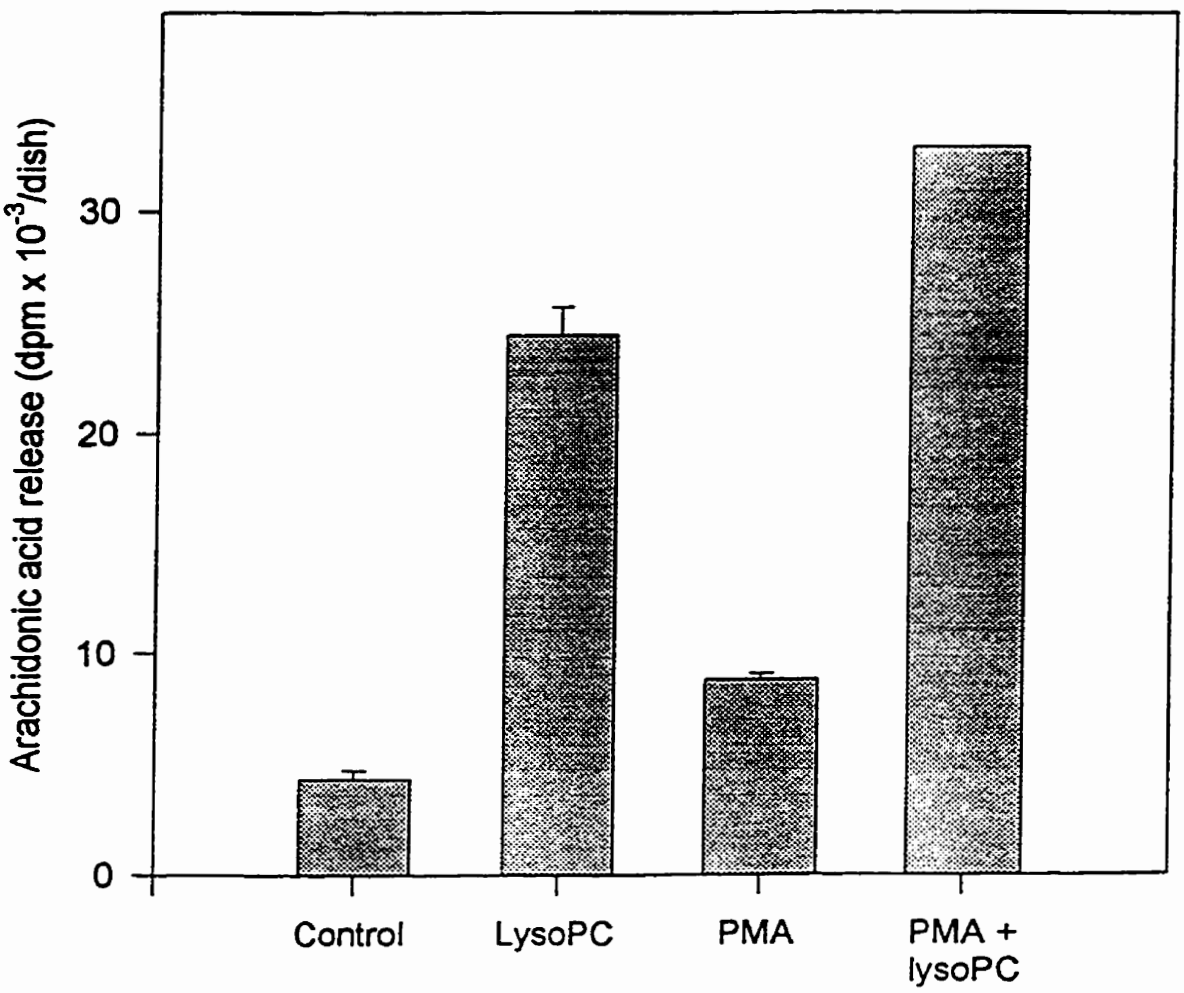


Figure 28. Effect of phorbol 12-myristate 13-acetate on arachidonic acid release in endothelial cells. Endothelial cells prelabelled with [^{3}H]arachidonic acid were incubated for 10 min with 50 μM lysophosphatidylcholine, 200 nM PMA, or a combination of both. Arachidonic acid released into the buffer was then determined as described above. Results are expressed as mean ± standard deviation of three separate experiments.

3.2.4.2 Involvement of a mitogen-activated protein kinase pathway

PKC has not been shown to directly phosphorylate $cPLA_2$ in any cellular system; rather, PKC is thought to be an upstream activator of $cPLA_2$ (Clark *et al.* 1995; Leslie 1997). The phosphorylation of $cPLA_2$ by MAPK results in enhanced phospholipase activity, and MAPK is thought to be responsible for $cPLA_2$ phosphorylation *in vivo* (Lin *et al.* 1993; Leslie 1997). Thus, we investigated the involvement of the MAPK pathway by determining the release of arachidonic acid in the presence of PD098059, an inhibitor of MAPK/ERK kinase (MEK) (Dudley *et al.* 1995) (Table 12). Cells were preincubated with the indicated concentrations of PD098059 for 30 minutes prior to challenge with lysophosphatidylcholine. Pretreatment of cells with PD098059 decreased the arachidonic acid released by lysophosphatidylcholine by up to 38%, relative to the arachidonic acid release caused by lysophosphatidylcholine alone.

Table 12. Effect of the MEK inhibitor PD098059 on lysophosphatidylcholine-induced arachidonic acid release.

Cells prelabelled with [^{3}H]arachidonic acid were incubated for 30 min with the indicated concentrations of PD098059 prior to incubation for 10 min with 50 μM lysophosphatidylcholine. Arachidonic acid released into the buffer was then determined as described above. Results are expressed as mean ± standard deviation of three separate experiments. *p < 0.05 compared with cells incubated with 50 μM lysophosphatidylcholine alone.

Treatment	Arachidonic acid release (dpm /dish)	Inhibition (%)
Control (no lysophosphatidylcholine)	2813 ± 149	
50 μM lysophosphatidylcholine	11689 ± 83	
10 μM PD098059 + lysophosphatidylcholine	8018 ± 184*	31.4
30 μM PD098059 + lysophosphatidylcholine	7270 ± 461*	37.8

4. DISCUSSION

4.1 Studies on *de novo* Phospholipid Biosynthesis

The *de novo* synthesis of phospholipids in mammalian tissues occurs via the progressive acylation of *sn*-glycerol-3-phosphate. Because *sn*-glycerol-3-phosphate does not readily permeate the plasma membrane, radiolabelled glycerol is usually used as a general precursor for studying phospholipid biosynthesis (Akesson *et al.* 1970; Allan and Michell 1975; Brindley and Bowley 1975; Arthur and Choy 1984; Tardi *et al.* 1992). In the hamster heart, glycerol is taken up and phosphorylated to yield *sn*-glycerol-3-phosphate in a reaction catalyzed by glycerol kinase. The *sn*-glycerol-3-phosphate is then acylated to form lysophosphatidic acid and phosphatidic acid, which provides a precursor for the synthesis of other phospholipids. The present study has revealed novel insights into the regulation of phospholipid biosynthesis in the heart. (1) Previous studies in our lab have shown a selective stimulation of acidic phospholipid biosynthesis in the hamster heart by methyl-lidocaine (Tardi *et al.* 1992; Lee *et al.* 1995); the current studies have demonstrated the capacity of the heart to increase phospholipid biosynthesis in general, under stimulation by lidocaine. This increase was correlated with stimulations in the activity of certain key enzymes. (2) Prior studies had shown a dependence of phosphatidylcholine and cardiolipin biosynthesis on the energy status of the heart (Choy 1982; Hatch and Choy 1990; Cheng and Hatch 1995); the current studies have

demonstrated that phospholipid biosynthesis in general is sensitive to the energy levels in the heart. Thus two levels of control of phospholipid biosynthesis were demonstrated in this study. (3) The partial restoration of phospholipid synthesis by lidocaine appeared to be mediated at least in part by modulation of enzyme activities; thus, perturbation of one level of control of phospholipid synthesis could be (partially) counteracted by modulation of another level of control. (4) Our studies have provided evidence for the possibility of regulation of phosphatidylcholine biosynthesis at the level of the cholinephosphotransferase-catalyzed step. This concept complements the well-established principle of regulation of phosphatidylcholine biosynthesis by modulation of phosphocholine cytidylyltransferase activity.

The notion that energy status and CTP supply could regulate the biosynthesis of phosphatidylcholine and other phospolipids was suggested by Vance and Choy in 1979 (Vance and Choy 1979), and has since been supported experimentally. In the hearts of cardiomyopathic hamsters, an increase in CTP:phosphocholine cytidylyltransferase activity helped to maintain phosphatidylcholine biosynthesis despite a decreased CTP supply (Choy 1982). The effects of acute reductions in the energy status of the heart was examined in hypoxic (Hatch and Choy 1990) or ischemic (Choy *et al.* 1992) hearts. In either case, decreases in ATP and CTP levels were observed, with concomitant decreases in phosphatidylcholine synthesis. Cardiolipin synthesis was also decreased under hypoxia (Cheng and Hatch 1995). ATP and CTP levels were reduced in the livers of hamsters fasted for up to 48 h, with an accompanying decrease in the synthesis of phosphatidylcholine in the livers of these animals (O and Choy 1993). That CTP levels could regulate phosphatidylcholine synthesis was supported in studies using poliovirus-

infected HeLa cells, which exhibited a two- to three-fold increase in CTP level and a two-fold increase in phosphatidylcholine synthesis (Choy *et al.* 1980). Direct evidence to support the importance of the CTP supply to phospholipid biosynthesis was provided in a study by Hatch and McClarty, in which H9c2 cells treated with cyclopentenylcytosine exhibited reduced CTP levels and a concomitant decrease in the synthesis of all phospholipids examined (Hatch and McClarty 1996). Phospholipid biosynthesis was restored when the cyclopentenylcytosine-treated cells were supplemented with exogenous cytidine. In the current studies the reduced energy level of the hamster heart under hypoxia compromised the synthesis of phospholipids (Fig. 19). The results of the current study support the hypothesis that the energy status of the heart can serve to regulate phospholipid biosynthesis in general. Our results further indicate that lidocaine has the ability to partially restore phospholipid synthesis during hypoxia (Fig. 19) without any significant effect on the reduced ATP and CTP levels. Thus it appears that the heart has a reserve capacity to modulate the rate of phospholipid synthesis in spite of decreased ATP and CTP levels.

CTP:phosphatidic acid cytidylyltransferase is thought to be rate-limiting for the synthesis of CDP-diacylglycerol, a precursor of phosphatidylinositol (Brindley and Sturton 1982; Sturton and Brindley 1977). The K_m for the purified cytidylyltransferase from *S. cerevisiae* was 1.0 mM (Kelley and Carman 1987), while an apparent K_m for the rat liver enzyme was 170 μM (Liteplo and Sribney 1980). The concentrations of CTP in the hamster hearts were below these values (Hatch and Choy 1990); thus, the decrease in CTP levels in the hypoxic hearts might be expected to affect the synthesis of CDP-diacylglycerol. Interestingly, an increase in CTP:phosphatidic acid cytidylyltransferase

activity was observed in the *in vitro* assays of subcellular fractions from the hypoxic hearts (Fig. 20). The apparent enhancement of CDP-diacylglycerol synthase activity during hypoxia could be a compensatory mechanism to maintain the proper level of CDP-diacylglycerol at low CTP concentrations. A similar compensatory increase in the activity of CTP:phosphocholine cytidylyltransferase was previously observed in hamster hearts perfused under hypoxia, and this increase was thought to help maintain a level of phosphatidylcholine synthesis during hypoxia (Hatch and Choy 1990).

Phosphatidate phosphatase catalyzes the conversion of phosphatidic acid to diacylglycerol and is regarded as a key enzyme in regulating the supply of diacylglycerol for the synthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol. The increase in the labelling of diacylglycerol under perfusion with lidocaine may be due to an increase in microsomal phosphatidate phosphatase (*ie.* PAP1) activity (Figs. 17, 20). This notion is supported by the observed increase in *N*-ethylmaliemide-sensitive phosphatidate phosphatase activity in microsomal fractions as assayed *in vitro* following lidocaine perfusion (Fig. 20). The increase in the microsomal enzyme activity might arise from the translocation of the enzyme from the soluble to the membrane compartment during lidocaine perfusion. Intriguingly, hypoxic perfusion caused a decrease in phosphatidate phosphatase activity in both cytosolic and microsomal fractions, which was not reversible by lidocaine (Fig. 20). Although no changes in the activities of acyl-CoA:glycerol-3-phosphate acyltransferase and acyl-CoA:lysophosphatidic acid acyltransferase were detected when these enzymes were assayed after perfusion (Fig. 20), the activity of the former was stimulated by the direct addition of lidocaine to the assay mixtures (Table 5). Thus, the restoration of diacylglycerol labelling by lidocaine under

hypoxia might be caused by the enhanced synthesis of its metabolic precursors. The direct action of lidocaine on the acyltransferases might not be detectable when assayed after perfusion of the heart in the presence of lidocaine, due to extensive dilution of the drug during the homogenization process.

The synthesis of phosphatidylcholine was distinct from the other phospholipids, as it was the only phospholipid whose synthesis appeared to be inhibited in hearts perfused in the presence of lidocaine (Figs. 17, 19). Another study, in which phosphatidylcholine biosynthesis was examined by monitoring the uptake of [^{3}H]choline into a monocyte-like cell line, also showed that lidocaine inhibited phosphatidylcholine biosynthesis (Chu and Lee 1994). This inhibition was attributed to a decrease in choline uptake and inhibition of CTP:phosphocholine cytidylyltransferase. The current study is consistent with that of Chu and Lee (1994) in that they both demonstrate an inhibition of phosphatidylcholine synthesis by lidocaine. Our study provides further insight into the control of phosphatidylcholine biosynthesis. In the current study, the incorporation of [^{3}H]glycerol into phosphatidylcholine was decreased by lidocaine under normoxic perfusion, in spite of the lidocaine-induced increase in the specific radioactivity of diacylglycerol (Fig. 17). Similarly, the incorporation of [^{3}H]glycerol into phosphatidylcholine remained depressed in the presence of lidocaine under hypoxia, despite the restoration in the specific radioactivity of diacylglycerol by lidocaine (Fig. 19). Furthermore, the CDP-choline pool was not changed by lidocaine or hypoxic perfusion (Table 4). Thus, the *in situ* activity of the cholinephosphotransferase (taken as the radioactivity incorporated into phosphatidylcholine over time, divided by the specific radioactivity of the precursor diacylglycerol) appears to be inhibited by lidocaine under

both normoxic and hypoxic perfusion. This notion is supported by the observation that cholinephosphotransferase activity was inhibited by the direct addition of lidocaine to the *in vitro* assay of cholinephosphotransferase activity (Table 7). The decrease in the radioactive labelling of phosphatidylcholine in the hamster hearts was unlikely to be due to an increase in catabolism of phosphatidylcholine, as the amount of phosphatidylcholine in the hearts remained constant during lidocaine perfusion (Tables 2, 3). As mentioned in the Introduction (section 1.2.8.1), phosphatidylcholine biosynthesis was depressed in the livers of fasted hamsters, and this inhibition was attributable at least in part to inhibition of the cholinephosphotransferase (O and Choy 1993). Thus, the results of the current study provide further evidence that regulation of the cholinephosphotransferase-catalyzed step can offer a point of control of phosphatidycholine biosynthesis, in addition to the well-documented regulation of the cytidylyltransferase-catalyzed step.

In contrast to the pattern of phosphatidylcholine labelling, the labelling of phosphatidylethanolamine correlated proportionately with that of diacylglycerol under all perfusion conditions (Figs. 17, 19). Although diacyl-*sn*-glycerol is the common precursor of phosphatidylcholine and phosphatidylethanolamine, the biosynthesis of these two phospholipids may be regulated independently of one another in the hamster heart.

4.2 Studies on Phospholipid Catabolism

The present study was conducted to investigate the regulation of phospholipid catabolism, specifically the control of arachidonic acid release in endothelial cells, under the influence of lysophosphatidylcholine. We found that exposure of the cells to lysophosphatidylcholine containing long saturated acyl chains induced a dose-dependent increase in the release of arachidonic acid, and that the effect was mediated through $cPLA_2$. Our findings support a model in which the induction of arachidonic acid release by lysophosphatidylcholine is dependent on Ca^{2+} influx and the activation of PKC. These processes result in the stimulation of $cPLA_2$ activity to give rise to an enhancement of arachidonic acid release. This model is summarized in Figure 29.

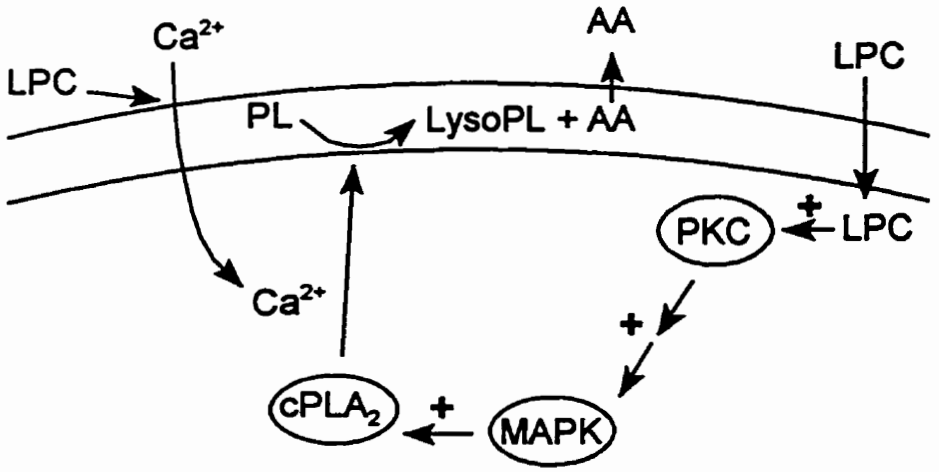


Figure 29. Proposed model for regulation of arachidonic acid release in endothelial cells by lysophosphatidylcholine.

A major pathway for arachidonic acid release from agonist-stimulated cells is via hydrolysis of phospholipids by PLA_2 (Mayer and Marshall 1993). Among the PLA_2 subtypes, the preference for arachidonic acid-containing substrates and the low (intracellular concentrations) requirement for Ca^{2+} of $cPLA_2$ have led many investigators to believe that this isoform is the main enzyme responsible for arachidonic acid release (Clark *et al.* 1995). The arachidonic acid release induced by lysophosphatidylcholine (Figs. 22, 23) was inhibited in a dose-dependent manner by the PLA_2 inhibitors pBPB and the $cPLA_2$-specific $AACOCF_3$ (Table 8). Furthermore, the arachidonic acid release by lysophosphatidylcholine was also attenuated in cells grown in the presence of antisense oligonucleotides for the $cPLA_2$ (Fig. 25). The observed enhancement of membrane-associated PLA_2 activity is consistent with an activation and translocation of $cPLA_2$ to membranes (Table 9). The decrease in soluble PLA_2 activity was not quantitatively recovered in the membrane fraction of the cell lysates. This result is not entirely surprising, as it was previously documented that the membrane component may interfere with the $cPLA_2$ activity when assayed *in vitro* (Channon and Leslie 1990). This phenomenon was also observed with other enzymes upon their association with membranes, and was attributed to a reduced accessibility of exogenous radioactive substrate to the enzyme, and/or to a "dilution" effect on the exogenous radioactive substrate, *ie.* the presence of endogenous membrane phospholipids lowered the effective specific activity of the radioactive substrate (Channon and Leslie 1990; Dagher and Hultin 1975; Nitisewojo and Hultin 1976). The normal Ca^{2+} concentration in endothelial cells is approximately 70 nM (Yang *et al.* 1994; Ehringer *et al.* 1996), and in the current study lysophosphatidylcholine was found to cause a three-fold increase in the intracellular Ca^{2+}

level (Fig. 27). The enhanced Ca^{2+} level caused by lysophosphatidylcholine is similar to those Ca^{2+} concentrations that were shown to cause the association of $cPLA_2$ with membranes (Nalefski *et al.* 1994; Channon and Leslie 1990). We conclude that the $cPLA_2$ is involved in the lysophosphatidylcholine-stimulated arachidonic acid release.

The contribution of $sPLA_2$ was also considered. Although antisense oligonucleotides for this isoform caused a reduction in $sPLA_2$ protein (Fig. 26), a corresponding attenuation of the lysophosphatidylcholine-induced arachidonic acid release was not detected in those cells (Fig. 25). This result suggests that the $sPLA_2$ does not contribute significantly to the arachidonic acid release stimulated by lysophosphatidylcholine. This finding is in accord with studies in which hormone-stimulated arachidonic acid release and eicosanoid production was attributed to $cPLA_2$ but not $sPLA_2$ (Lin *et al.* 1992; Bartoli *et al.* 1994; Roshak *et al.* 1994). It is interesting to note that the involvement of both the cytosolic and secretory PLA_2 subtypes in the release of arachidonic acid for PGI_2 synthesis has also been reported (Murakami *et al.* 1993).

Lysophosphatidylcholine, with the participation of diacylglycerol, phosphatidylserine and Ca^{2+}, has been shown to modulate PKC activity both *in vitro* and *in vivo* (Oishi *et al.* 1988; Sasaki *et al.* 1993; Kugiyama *et al.* 1992). The increase in intracellular Ca^{2+} caused by lysophosphatidylcholine (Fig. 27) may contribute to the enhancement of membrane-associated PKC activity (Table 11). Incubation of endothelial cells with phorbol ester caused a doubling of PKC activity and arachidonic acid release (Table 11, Fig. 28); however, this stimulation of arachidonic acid release is somewhat less than that observed with lysophosphatidylcholine. The contribution of PKC to the arachidonic acid release induced by lysophosphatidylcholine was further supported by the

observation that the PKC inhibitors staurosporine and Ro31-8220 attenuated the arachidonic acid release (Table 10). Thus, while it is clear that PKC is involved, PKC activation by itself cannot account for the stimulation of arachidonic acid release by lysophosphatidylcholine. A source of extracellular Ca^{2+} is also required (Fig. 27). It appears that lysophosphatidylcholine is able to stimulate arachidonic acid release by utilizing a PKC-dependent pathway and by increasing intracellular Ca^{2+} levels.

Although $cPLA_2$ is an *in vitro* substrate for PKC, the direct phosphorylation of $cPLA_2$ by PKC does not result in enhanced phospholipase activity (Lin *et al.* 1993), nor has PKC been shown to directly phosphorylate $cPLA_2$ *in vivo*. However, PKC is a known activator of the p42/p44 MAPK signalling cascade via phosphorylation of Raf-1 (Seger and Krebs 1995). The observation that the MEK1 inhibitor PD098059 attenuated arachidonic acid release (Table 12) implicates the involvement of the p42/p44 MAPK cascade in the lysophosphatidylcholine-induced arachidonic acid release. The concentrations of PD098059 used in this study (up to 30 μM) was similar to those used to almost completely inhibit the activation of MEK1, and to inhibit the activation of p42 MAPK by up to 80% (Dudley *et al.* 1995; Alessi *et al.* 1995). The partial inhibition of arachidonic acid release by PD098059 leaves open the possibility that lysophosphatidylcholine can also act through pathways that do not require the recruitment of p42/p44 MAPK. For example, the p38 MAPK is thought to participate in the activation $cPLA_2$ by agonists (Waterman *et al.* 1996; Kramer *et al.* 1996).

Reported plasma concentrations of lysophosphatidylcholine range from approximately 130 to 150 μM in healthy subjects (Okita *et al.* 1997; Rabini *et al.* 1994) to 1.7 mM in hyperlipidemic patients (Rodriguez *et al.* 1987), while reported

concentrations of human serum albumin ranged from approximately 185 to 850 μM (Viani *et al.* 1992; Veering *et al.* 1990). These figures would correspond to theoretical molar lysophosphatidylcholine:albumin ratios ranging from 0.15 to 9.2 in serum. In our studies, arachidonic acid release was stimulated by lysophosphatidylcholine at concentrations that correspond to lysophosphatidylcholine:albumin ratios of 6.2 to 25 (Fig. 23). We selected a ratio of 12.5 (50 μM lysophosphatidylcholine and 0.025% or approximately 4 μM albumin) to use for the subsequent experiments because this ratio represented the lowest lysophosphatidylcholine concentration that elicited the maximum effect on arachidonic acid release. Although the complexities of other serum components would likely complicate the *in vivo* situation, the lysophosphatidylcholine:albumin ratios used in this study may mimic conditions found in physiological or pathophysiological situations.

Lysophosphatidylcholine is a natural amphiphile, and incorporates into lipid membranes and affects membrane fluidity and permeability (Weltzein 1979; Kitagawa *et al.* 1976; Fink and Gross 1984). Indeed, lysophosphatidylcholine (at concentrations higher than those used in this study) has been used as an agent for permeabilizing cells (Miller *et al.* 1979). However, the detergent properties of lysophosphatidylcholine do not fully account for its myriad of biological effects. For example, lysophosphatidylcholine increases intracellular Ca^{2+} levels (Su *et al.* 1995; Chen *et al.* 1995; Inoue *et al.* 1992) and yet inhibits receptor-mediated Ca^{2+} mobilization (Kugiyama *et al.* 1992; Inoue *et al.* 1992). It exhibits both vasorelaxant (Saito *et al.* 1988) and vasoconstrictive (Murohara *et al.* 1994) properties. It perturbs nitric oxide synthase mRNA and protein levels in endothelial cells, and whether increases or decreases in mRNA or protein were observed

depended on the concentration and incubation conditions used in each study (Hirata *et al.* 1995; Dudek *et al.* 1995; Zembowicz *et al.* 1995). Lysophosphatidylcholine increases the expression of various growth factors and adhesive molecules in endothelial cells (Kume and Gimbrone 1994; Kume *et al.* 1992; Nakano *et al.* 1994; Ochi *et al.* 1995), and it was shown recently that lysophosphatidylcholine can modulate gene expression independently of PKC and MAPK (Ochi *et al.* 1995; Fang *et al.* 1997; Zhu *et al.* 1997). In our study, lysophosphatidylcholine was the only lysolipid tested that stimulates arachidonic acid release (Fig. 24), despite the fact that other lysolipids also possess detergent properties and perturb intracellular Ca^{2+} levels (Christiansen and Carlsen 1983; Mendz *et al.* 1990; Xu *et al.* 1995; Falasca *et al.* 1995; Thomson *et al.* 1994). Furthermore, the stimulation of arachidonic acid release by lysophosphatidylcholine parallels earlier observations that the ability of lysophosphatidylcholine to stimulate PKC is unique among lysolipids (Oishi *et al.* 1988; Sasaki *et al.* 1993). To our knowledge, the findings of the current study demonstrate a novel role of lysophosphatidylcholine in the modulation of endothelial cell function.

It is clear that lysophosphatidylcholine may modulate a pathway that is responsible for its generation *in vivo*. It is therefore tempting to speculate that the activation of phosphatidylcholine hydrolysis by PLA_2 could be regulated via a positive feedback mechanism. Lysophosphatidylcholine may thus function as an intracellular messenger molecule. A prolonged and over-activation of the system may subsequently create adverse effects to the cells, although accumulation of excess levels of lysophosphatidylcholine can be avoided by reacylation to re-form the parent phospholipid or by hydrolysis of the lysolipid by lysophospholipases (Savard and Choy 1982; Gross and Sobel 1982; Severson

and Fletcher 1985). Hence, the physiological consequences of the stimulation of arachidonic acid release in endothelial cells by lysophosphatidylcholine will be an interesting area for further study. Since arachidonic acid and its metabolites have many biological properties related to vascular homeostasis, the perturbation of arachidonic acid release by lysophosphatidylcholine may be a further mechanism whereby this lysolipid could contribute to vascular dysfunction.

5 SUMMARY

This study investigated the regulation of the biosynthesis and catabolism of lipids in mammalian tissues. In the first part of this study, phospholipid biosynthesis was investigated in the hamster heart. In the second part of this study, the regulation of the hydrolytic release of arachidonic acid was investigated in endothelial cells. Certain aspects of the regulation of lipid metabolism was revealed under the various experimental conditions.

At least two levels of control of phospholipid biosynthesis in the hamster heart were demonstrated: (a) the activities of key biosynthetic enzymes; and (b) the energy status of the heart. Perfusion of the hearts with lidocaine, resulted in increases in the incorporation of radioactive glycerol into most phospholipid fractions, with the sole exception of phosphatidylcholine, whose radioactive labelling was decreased. *In vitro* enzyme assays revealed the ability of lidocaine to stimulate acyl-CoA:lysophosphatidic acid acyltransferase and to inhibit CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase in a direct manner. The enhancement of phosphatidic acid phosphatase and CTP:phosphatidic acid cytidylyltransferase was revealed only when the activities of these enzymes were assayed following lidocaine perfusion, suggesting an indirect mechanism of modulation of these enzyme activities. Thus, lidocaine appears to affect the regulation of phospholipid biosynthesis in the hamster heart via both direct and indirect modulation of key enzymes. Hypoxia lowered the energy status of the heart, which lead to depressed levels of the metabolites ATP and CTP, ultimately resulting in a general decrease in phospholipid synthesis. Thus, phospholipid biosynthesis is

intimately tied to the energy status of the heart, as was previously demonstrated for phosphatidylcholine. The partial restoration of phospholipid synthesis by lidocaine appeared to be mediated at least in part via the modulation of enzyme activities, indicating that perturbation of one level of control of phospholipid synthesis could be partially counteracted by modulation of another.

In the second part of this study, we investigated the regulation of arachidonic acid release under the influence of a potentially deleterious compound, lysophosphatidylcholine. We found that exposure of the cells to lysophosphatidylcholine containing long saturated acyl chains induced a dose-dependent increase in the release of arachidonic acid, and that the effect was mediated through $cPLA_2$. Our findings support a model in which the induction of arachidonic acid release by lysophosphatidylcholine is dependent on Ca^{2+} influx and the activation of PKC. A possible way in which PKC may induce arachidonic acid release is via a MAPK. MAPK is known to phosphorylate and activate $cPLA_2$. These processes result in the stimulation of $cPLA_2$ activity to give rise to an enhanced arachidonic acid release. The results of this study add to the growing number of biological properties of lysophosphatidylcholine, which may come to be regarded as an intracellular signalling molecule. This study has also demonstrated a mechanism whereby a product of phospholipid catabolism can itself modulate further catabolism of phospholipids.

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IMAGE EVALUATION
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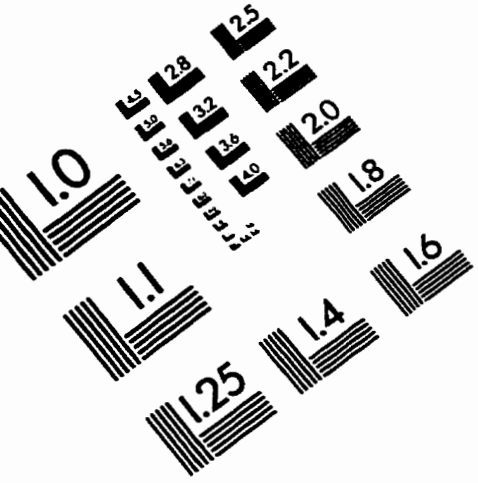

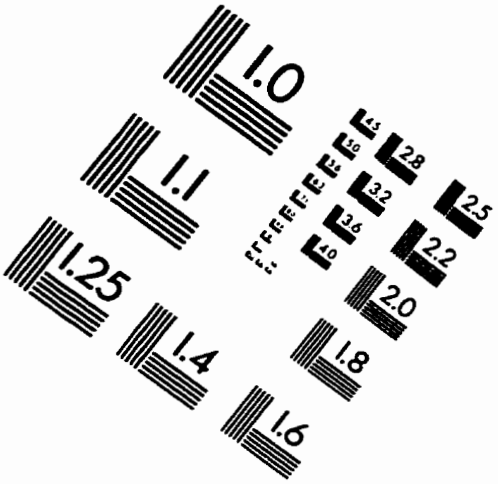

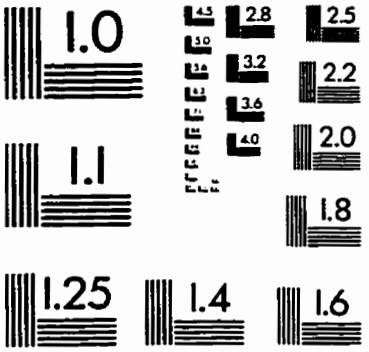

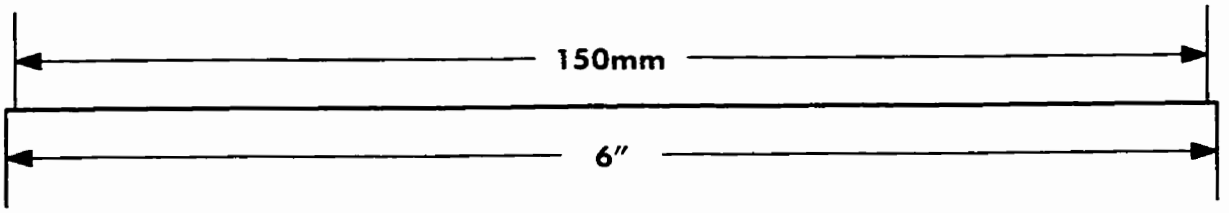

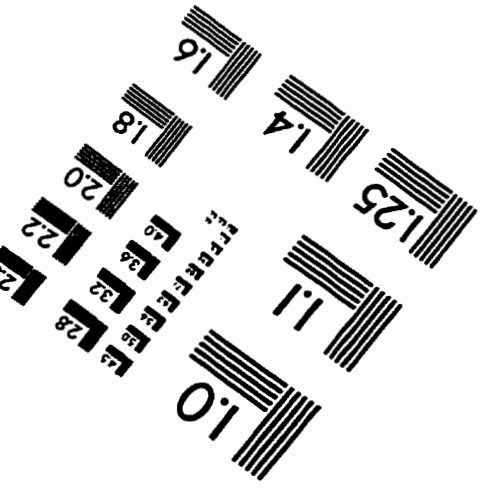

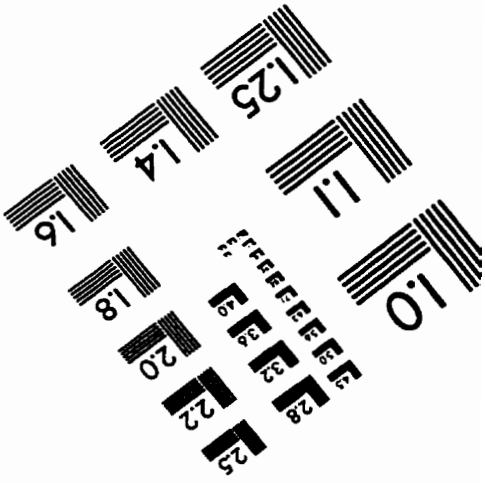

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