THE EFFECT OF METHYL LIDOCAINE ON CARDIAC LIPID METABOLISM

By

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TO MY WIFE ANITA

AND MY PARENTS

For all your love and support

throughout my university years,

this thesis is dedicated to you.

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(Keith you will graduate sooner if you stay on this continent).

To Greg Patton. Thanks for fixing my car, but quit eating all my food.

To my wife Anita, you're the best.

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ACS	Aqueous counting scintillant
ADP	Adenosine-5'-diphosphate
ANSA	1-Amino-2-napthol-4-sulphonic acid
ATP	Adenosine-5'-triphosphate
C	Carbon
°C	Degrees Celsius
Ca^{2+}	Calcium
cAMP	Adenosine-3',5'-monophosphate
$CaCl_2$	Calcium chloride
CDP	Cytidine-5'-diphosphate
Ci	Curie
Co.	Company
CoA	Coenzyme A
Corp.	Corporation
CTP	Cytidine-5'-triphosphate
Da	Daltons
dpm	Disintegrations per minute
DTT	Dithiothreitol
EC	Enzyme classification
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(ß-amino-ethyl ether)N,N'-tetraacetic acid
GC	Gas chromatography

GDP	Guanosine-5'-diphospate
GTP	Guanosine-5'-triphosphate
GPC	Glycerophosphocholine
hr	Hour
KDa	Kilodalton
K_m	Michaelis-Menten constant
K_t	Transport constant
l	Litre
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
M	Molar
min	Minute
mCi	Millicurie
mg	Milligram
Mg^{2+}	Magnesium
ml	Millilitre
mm	Millimetre
mM	Millimolar
Mn^{2+}	Manganese
NaF	Sodium fluoride
N_2	Nitrogen
NaCl	Sodium chloride
nm	Nanometre
nM	Nanomolar

O_2	Oxygen
P	Statistical probability
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
Pi	Phosphate
PI	Phosphatidylinositol
pmol	Picomole
PS	Phosphatidylserine
R_f	Relative mobility
s	Second
TAP	Theoretical aqueous phase
tlc	Thin-layer chromatography
TOP	Theoretical organic phase
Tris	Trizma base
μ	Micron
μCi	Microcurie
μl	Microlitre
μM	Micromolar
μmol	Micromole
UV	Ultraviolet
v	Volume
w	Weight

ABSTRACT

Biological membranes are composed of lipids and proteins. The lipid composition of cellular membranes is found to be highly conserved. The mechanisms that are responsible for the control of lipid metabolism are poorly understood. One mechanism for maintaining the lipid composition of cell membranes involves acyl chain remodelling via the Lands pathway. This deacylation-reacylation pathway is believed to be responsible for the generation of phospholipids with specific acyl compositions. Why this reaction occurs and what regulates the reaction is not known. In this study, the role of the reacylation reaction in the hamster heart was addressed. This reaction is essential in the heart since elevated levels of lysophospholipids can result in the formation of arrhythmias. The enzymes involved in lysophospholipid metabolism were assayed in hearts perfused with the antiarrhythmic drug methyl lidocaine. The drug was found to inhibit the lysophosphatidylcholine and lysophosphatidylethanolamine acyltransferase reaction without altering the activities of the phospholipase A or the lysophospholipase. This selective inhibition of the acyltransferase allowed us to study the role of the acyltransferase in lysophospholipid metabolism. Perfusions with lysophospholipids, in the presence of methyl lidocaine resulted in a decrease in the formation of the parent phospholipid. What was surprising was the fact that the labelling of the lysophospholipid fraction was unchanged and an increase in the radioactivity associated with the fatty acid was detected. The results suggest that when the acyltransferase reaction is inhibited, the lysophospholipase reaction will remove lysophospholipids before they become

cytolytic. Since methyl lidocaine proved to be a useful tool to study lysophospholipid metabolism, it was used further to study *de novo* lipid biosynthesis in the hamster heart.

In order to study the complete *de novo* biosynthetic pathway, radiolabelled glycerol was used as a precursor. When hamster hearts were perfused with glycerol and methyl lidocaine, there was an increase in the uptake of radioactivity by the heart. The enhanced radioactivity found in the glycerol perfused, methyl lidocaine treated heart was due to increased labelling in the phosphatidylinositol, diacylglycerol and triacylglycerol fractions. Analysis of the enzymes involved in the synthesis of these lipids via *in vitro* enzyme assay revealed that methyl lidocaine directly stimulated the activity of phosphatidic acid phosphatase and CTP: phosphatidic acid cytidylyltransferase. This stimulation of enzyme activity by the drug resulted in enhanced labelling in the phosphatidylinositol and diacylglycerol fractions. However, the enhanced labelling of the triacylglycerol fraction was not due to direct stimulation of diacylglycerol acyltransferase by methyl lidocaine. To further investigate the mechanism involved in the enhanced labelling of triacylglycerol, the levels of long-chain acyl-CoA were determined in hearts perfused with methyl lidocaine. Since long-chain acyl-CoA is a substrate in the formation of triacylglycerol, an increase in acyl-CoA level in methyl lidocaine treated hearts could account for the increase in the labelling of triacylglycerol. Analysis of long-chain acyl-CoA levels revealed that drug treatment did not alter the pool size of this compound.

The *in vitro* effect of methyl lidocaine on lipid enzyme activities did not account for all the changes observed in the study on *de novo* lipid biosynthesis. It is plausible that the drug also mediates its effect on lipid biosynthesis indirectly through second messengers. To assess this possibility, hamster hearts were perfused for 5 min with methyl lidocaine and then the hearts were assayed for lipid enzyme activities. Surprisingly, lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase activities were stimulated in hearts perfused with methyl lidocaine. This suggested that methyl lidocaine may be altering the activity of these enzymes in an indirect fashion. A possible second messenger activation of these enzymes was explored. Assessment of the phosphoinositide cascade during methyl lidocaine perfusion indicated that protein kinase C activity was unchanged in the presence of the drug. This suggested that the phosphoinositide cascade was probably not stimulated during methyl lidocaine perfusion. The activity of the cAMP cascade was assessed by determining the pool size of cAMP in hearts perfused with methyl lidocaine. In hearts perfused with the drug, there was an increase in the level of cAMP. When the lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase fractions were incubated with active cAMP-dependent protein kinase there was a stimulation of enzyme activity. This suggests that methyl lidocaine enhances cAMP levels and the concomitant increase in cAMP-dependent protein kinase activity may lead to the activation of lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase. Furthermore, indirect activation of diacylglycerol acyltransferase could account for the enhanced labelling of triacylglycerol by methyl lidocaine that was previously observed.

INTRODUCTION

I. The Biological Membrane

1. The Function of the Biological Membrane

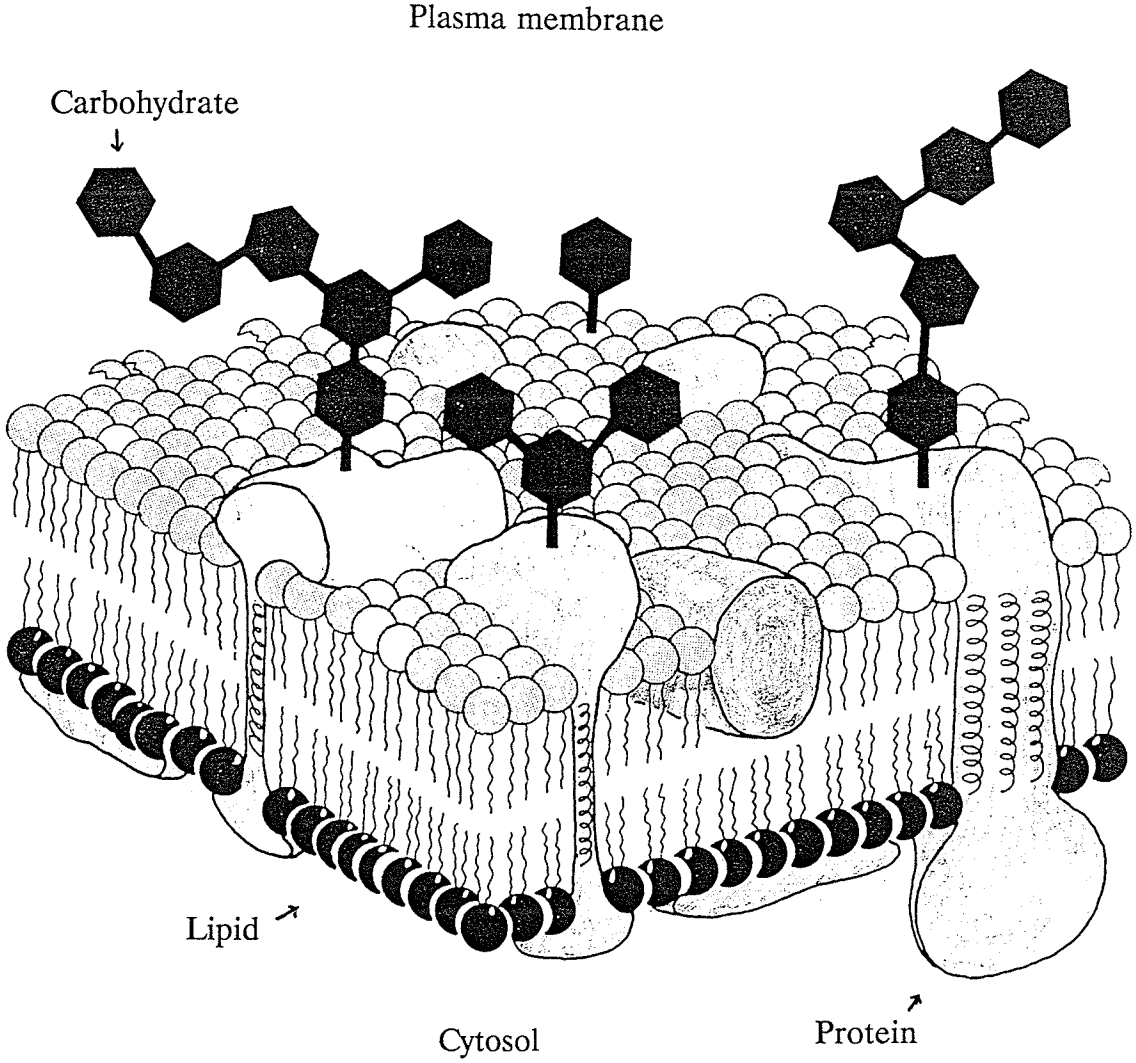
The plasma membrane and the intracellular membranes of eukaryotic cells are collectively known as biological membranes. The development of the plasma membrane is a key step in the generation of the earliest forms of life (Alberts *et al.* 1989). The plasma membrane defines the geographical limit of the cell and provides a barrier which is selective to the outer environment. The eukaryotic cell contains a set of internal membranes which form closed boundaries resulting in different compartments within the cells known as organelles. The major types of organelles found in eukaryotic cells are: endoplasmic reticulum, Golgi apparatus, nucleus, mitochondria, lysosomes and peroxisomes. These organelles carry out various functions that are essential for the maintenance of cell viability.

The function of membrane lipids has been elucidated in broad outline since the early experiments of Gorter and Grendell (1925), who extracted lipids from the erythrocyte membrane and measured the area these lipids were able to cover as a monolayer at an air-water interface. Although a number of assumptions were made with the data, the errors compensated for each other and led to the correct

conclusion that the erythrocyte membrane contained enough lipid to form a lipid bilayer matrix surrounding the red blood cell (Cullis and Hope 1985). This bilayer organization, which provides a permeability barrier between exterior and interior compartments, was further characterized by Danielli and Davson (1935). Subsequently further observations were made; that bilayers are fluid, they allow the rapid lateral diffusion of lipid and protein in the plane of the membrane, and that membrane proteins are often inserted into and through the lipid matrix. These observations have further contributed to our present understanding of membranes, resulting in the Singer and Nicolson (1972) fluid mosaic model (Figure 1).

Biological membranes can perform very diverse functions but contain many common features. Membranes consist of three basic components: lipid, protein and carbohydrate. Lipids are able to form a bilayer organization due to their amphipathic character (Cullis and Hope 1985). This property is due to a hydrophilic (head group) region and a hydrophobic (tail) region. As shown in Figure 2, a typical phospholipid molecule has a polar (head) group attached to the *sn*-3 position of a glycerol backbone through a phosphodiester linkage. At the *sn*-1 and *sn*-2 positions of the glycerol backbone, fatty acyl groups are attached. The acyl groups vary in length from 14-24 carbons. The acyl group located at the *sn*-1 position is usually saturated (no double bonds) while the acyl group at the *sn*-2 position is usually unsaturated (one or more double bonds). The fluidity of the membrane is affected by the length of the acyl chain and degree of unsaturation of the fatty acid (Cullis and Hope 1985). The lipid bilayers form a barrier for polar molecules and small ions such as Na^+, K^+ and

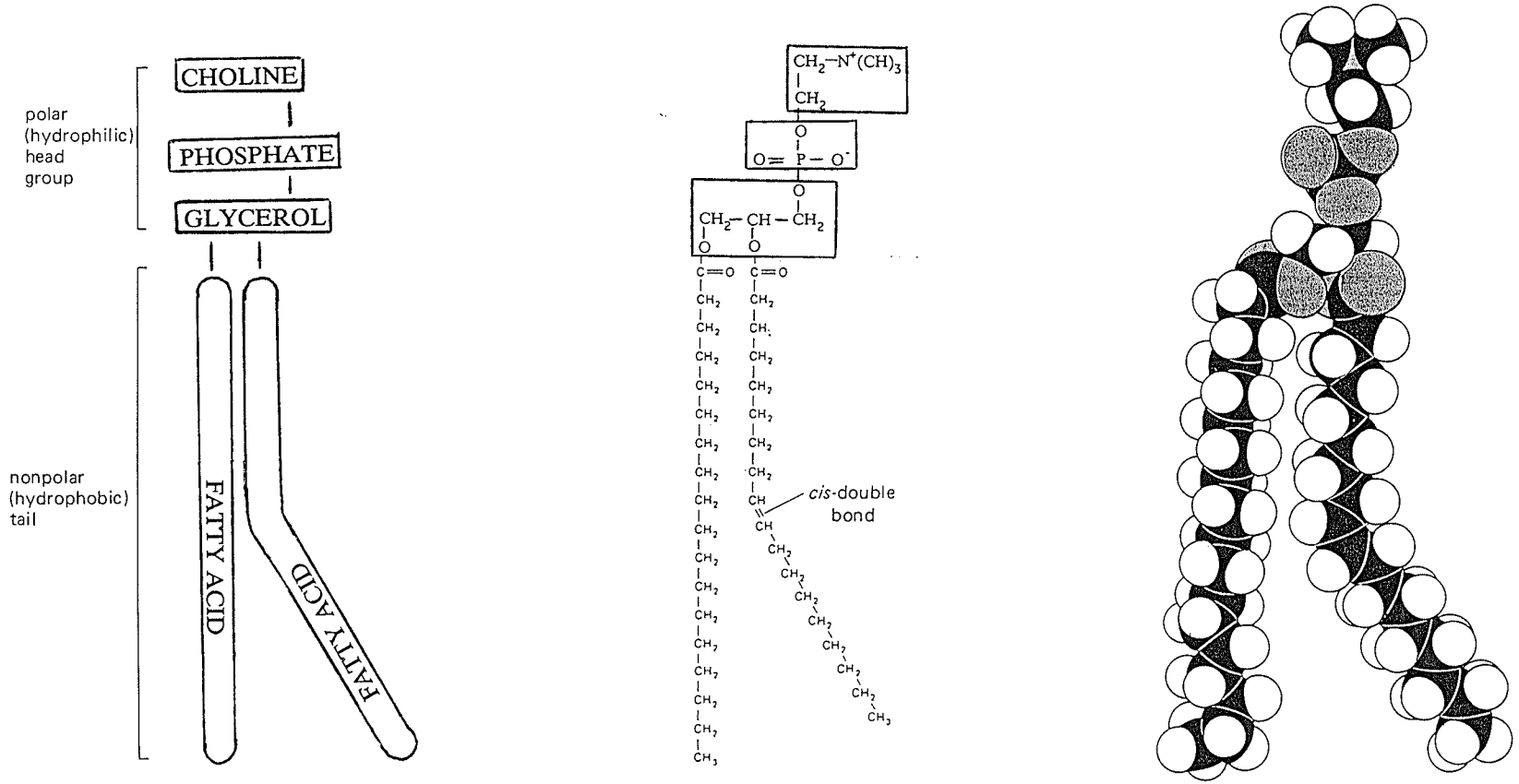
Figure 1



Fluid mosaic model of the eucaryotic plasma membrane

(Cullis and Hope 1985)

Figure 2



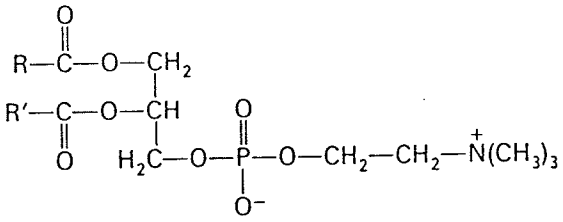
The parts of a phospholipid molecule, phosphatidylcholine, represented schematically in three different models.

H^+. This barrier is essential for the maintenance of cellular electrochemical gradients.

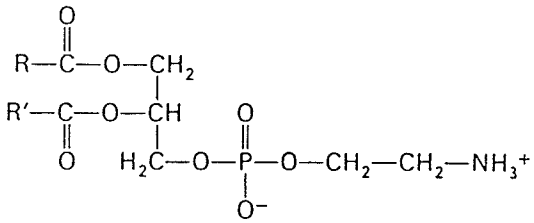
2. Membrane Lipids

There are three major classes of lipids found in biological membranes: phospholipids, glycolipids and cholesterol (Cullis and Hope 1985). In mammalian membranes, glycerol-based phospholipids are the most abundant. These phospholipids include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cardiolipin (Figure 3). Glycolipids are sugar-containing lipids. Glycolipids and sphingomyelin are derived from a sphingosine backbone. Glycolipids differ from sphingomyelin in the type of unit linked to the hydroxyl group of the sphingosine. In sphingomyelin, the hydroxyl group is linked to a phosphorylcholine moiety while glycolipids are linked to one or more sugar residues (Figure 4). Cerebroside, another glycolipid, contains only one sugar residue while gangliosides contain up to seven sugar residues. The glycolipids play major roles in cell-cell recognition and antigenic specificity (Sweeley 1985). Neural tissues contain large amounts of sphingosine-based lipids. Cholesterol is a major component of mammalian membranes and functions to maintain membrane fluidity. Cholesterol content of membranes varies greatly. In erythrocytes and myelin plasma membranes, cholesterol and phospholipids are present in approximately equimolar quantities while in membranes of the endoplasmic reticulum or inner mitochondria there is little or no cholesterol (White 1973).

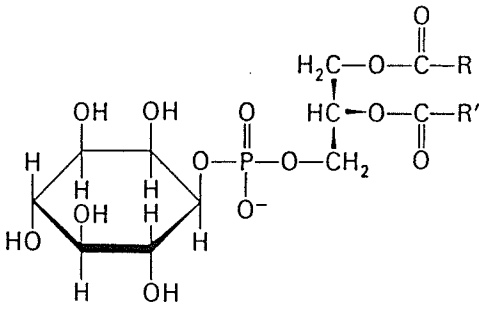
Figure 3



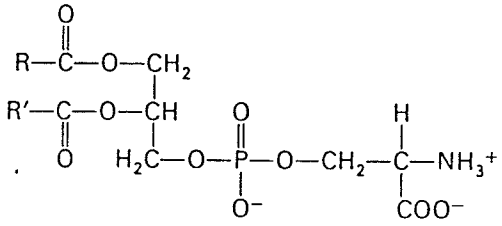
Phosphatidyl choline



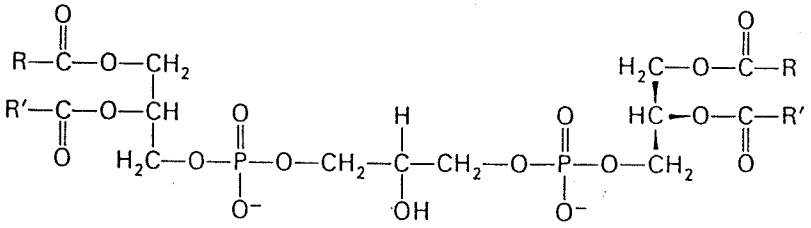
Phosphatidyl ethanolamine



Phosphatidyl inositol



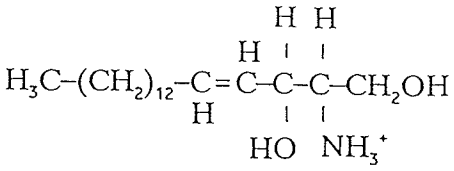
Phosphatidyl serine



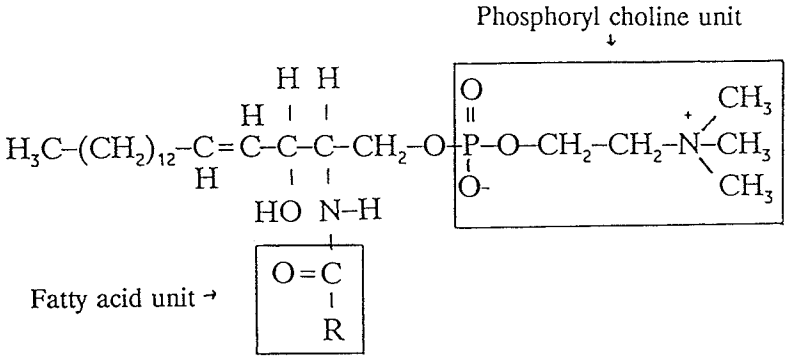
Diphosphatidyl glycerol
(Cardiolipin)

The 5 major types of phospholipids found in the mammalian heart

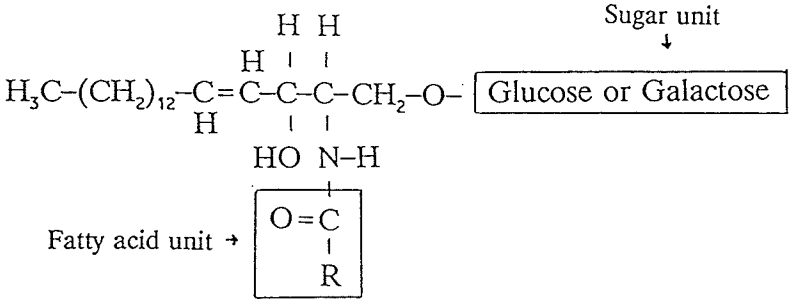
Figure 4



Sphingosine



Sphingomyelin



Cerebroside

Structural features of sphingosine, sphingomyelin and cerebroside

3. Membrane Proteins

Lipids in membrane bilayers form permeability barriers while proteins perform most other membrane functions. Many of the roles of proteins include serving as pumps, gates, receptors, energy transducers and enzymes. The protein content in membranes can vary from a low of about 18% in myelin and up to 75% in mitochondria. Membrane proteins can be classified as either peripheral or integral. Peripheral proteins are bound to membranes through noncovalent interactions (electrostatic and hydrogen bonding) with the membrane as well as other membrane proteins. These interactions can occur on the cytosolic or extracellular side of the membrane. Peripheral proteins can be removed from the membrane with gentle procedures such as the addition of salt or altering the pH. Integral membrane proteins interact extensively with the hydrophobic fatty acid chains of phospholipids. These proteins contain hydrophobic amino acid stretches which can span the membrane bilayer many times. The removal of integral membrane proteins from the bilayer is more difficult and requires the use of detergents or organic solvents to disrupt the membrane bilayer. Numerous membrane proteins are found to be regulated by the phospholipids that they interact with (Yeagle 1989). Due to the fluidity of the membrane bilayer, the proteins in the membrane are free to diffuse laterally throughout the membrane. The chances of a protein undergoing transverse diffusion (moving from one lipid plane to another) is very low and hence the asymmetry of the membrane can be maintained, in a model membrane, for very long periods of time. The asymmetry of the membrane is essential for proper cellular function.

4. Membrane Carbohydrate

Membranes of eukaryotic cells have a carbohydrate content that varies from 2%-10%. The majority of the carbohydrate is contained within glycolipid and glycoproteins. Glycolipids contain sugar residues linked to sphingosine as discussed above. Glycoproteins contain sugars which are attached in either N-linkages or O-linkages. If a sugar molecule is attached to the nitrogen of asparagine then it is N-linked, while the sugar attached to the side chain of serine or threonine is O-linked. Through the use of lectins, which are plant proteins that bind specific sugar residues, sugar residues of membrane glycolipids and glycoproteins are found to be located exclusively on the extracellular side of the membrane. Carbohydrates on cell surfaces play important roles in cell-cell recognition.

II. The *de novo* Biosynthesis of Phospholipids and Neutral Lipids in Mammalian Tissues

1. Introduction

The formation of phospholipids is achieved from the union of fatty acylthioesters and water-soluble precursors through a system of 25 or more enzymes (Esko and Raetz 1983). Lipid enzymes are usually hydrophobic, membrane bound and often act simultaneously on membrane bound substrates as well as water soluble compounds. Due to the difficulty of purifying membrane bound enzymes, many of the enzymes involved in the biosynthesis of lipids are poorly characterized. The choline-linked lipids (phosphatidylcholine, choline plasmalogen and sphingomyelin) represent about 40-60% of the total phospholipid but their contribution varies considerably between tissues (Table 1) (White 1973). In most tissues, the membrane is composed of about 80% zwitterionic phospholipids (phosphatidylcholine, phosphatidylethanolamine, choline and ethanolamine plasmalogens and sphingomyelin). The remaining 20% is made up of phospholipids which are acidic and negatively charged at neutral pH (phosphatidylserine and phosphatidylinositol). The compositional differences are usually greater between tissues than they are between the same tissue in different animals (White 1973).

Almost all of the cell cardiolipin can be found in the mitochondria (Table 1). The lipid composition of the endoplasmic reticulum is similar to that of the whole tissue.

Table 1

The lipid composition of various biological membranes

Lipid	*Erythrocyte**	*Myelin**	*Mitochondria*† *(inner and outer membrane)*	*Endoplasmic reticulum*†
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

Note: The data are expressed as weight % of total lipid.
* Human sources.
† Rat liver.

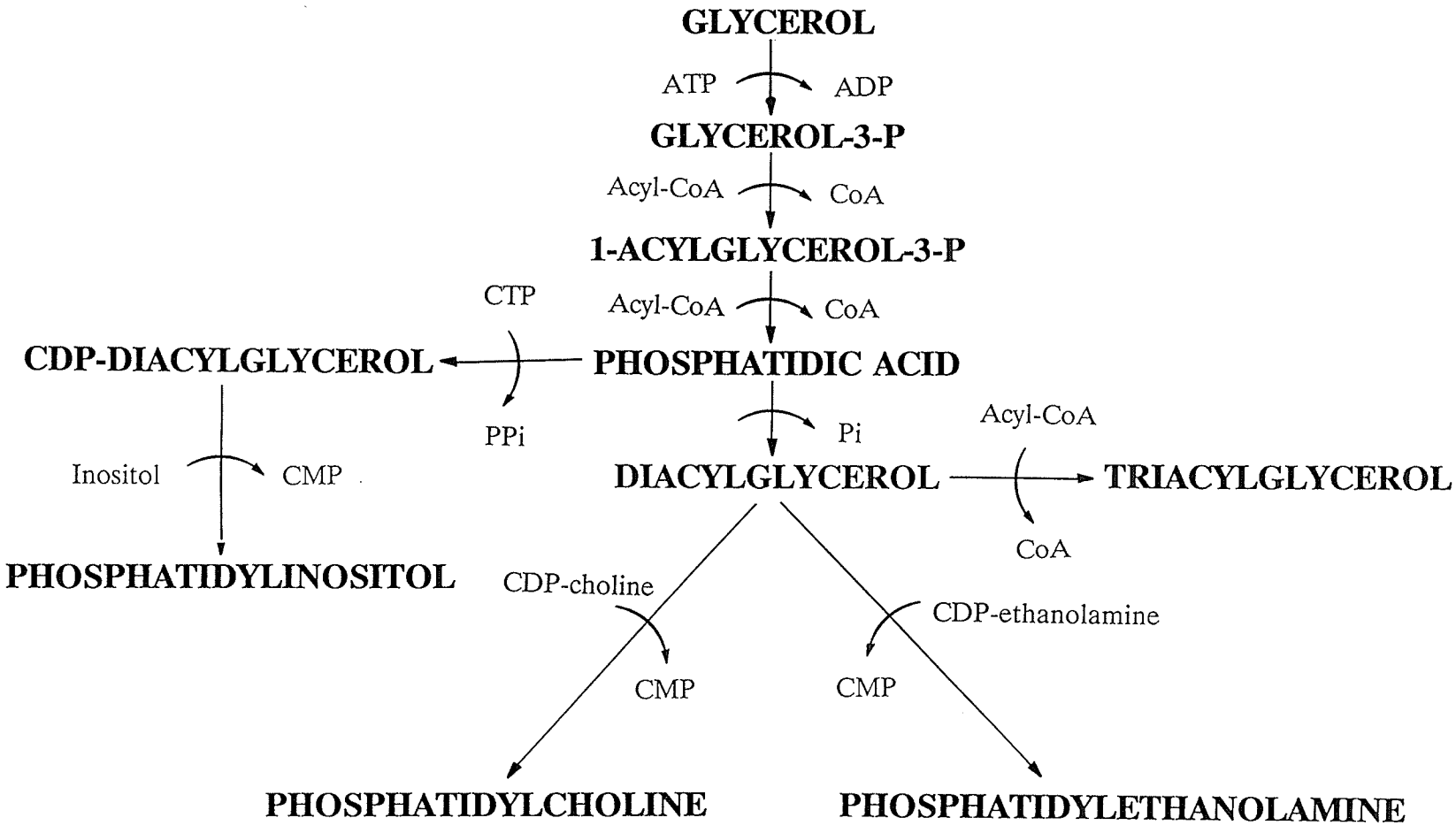
(Cullis and Hope 1985)

The subcellular distribution of the various phospholipids reflects to an extent the location of the enzymes involved in their biosynthesis. Cardiolipin synthesis occurs exclusively in the mitochondria where most of this lipid is located. Although phosphatidylcholine biosynthesis is localized to the endoplasmic reticulum it can be found in other subcellular locations. This cellular distribution of phosphatidylcholine may be achieved via co-transport of lipids with proteins excreted from the endoplasmic reticulum and targeted to other subcellular membranes (Esko and Raetz 1983).

2. *sn*-Glycerol-3-Phosphate

Glycerol forms the backbone of all glycerophospholipids. Glycerol has been used previously as a precursor to study lipid biosynthesis in lymphocytes (Allan and Michell 1975) and rat liver (Akesson *et al.* 1970; Brindley and Bowley 1975). The cell membrane is freely permeable to glycerol. Once inside the cell it is converted to *sn*-glycerol-3-phosphate by glycerol kinase (Figure 5). Glycerol kinase activity between tissues and species is quite variable. In rat liver, the action of glycerol kinase accounts for up to 20% of the *sn*-glycerol-3-phosphate pool (Esko and Raetz 1983). Rat heart was initially believed to contain no glycerol kinase activity (Wieland and Suyter 1957). However, subsequent work has shown low enzyme activity in rat myocardial tissue (Robinson and Newsholme 1967). In chinese hamster ovary cells, the phosphorylation of glycerol does not take place due to an absence of glycerol kinase activity (Esko and Raetz 1983).

Figure 5



Pathways for the biosynthesis of neutral lipids and phospholipids in the mammalian heart

The other source of *sn*-glycerol-3-phosphate in the cell is through the action of the enzyme *sn*-glycerol-3-phosphate dehydrogenase (Brindley 1985). This reaction converts dihydroxyacetone phosphate, which is produced in the cell during glycolysis, into *sn*-glycerol-3-phosphate. This reaction accounts for the majority of the *sn*-glycerol-3-phosphate produced in the cell.

3. 1-Acyl-*sn*-Glycerol-3-Phosphate (Lysophosphatidic Acid)

The formation of 1-acyl-*sn*-glycerol-3-phosphate occurs through an acyltransferase reaction using acyl-CoA and *sn*-glycerol-3-phosphate. The enzyme involved in this reaction is glycerol-3-phosphate acyltransferase (EC 2.3.1.15). In the liver, the acyltransferase activity is divided between the mitochondrial and microsomal fractions. In the heart, the microsomal enzyme activity is approximately 10 times the activity of the mitochondrial enzyme. This is consistent with a very low glycerol-3-phosphate acyltransferase activity in the mitochondrial fraction (van den Bosch 1974). The microsomal and mitochondrial enzymes appear to be isoenzymes as determined by their different responses to heat treatment, sulfhydryl reagents and Mg^{2+} (Bell and Coleman 1980). The rat mitochondrial acyltransferase shows a selectivity for the transfer of saturated acyl-CoA species over unsaturated to the glycerol-phosphate backbone. In comparison, the microsomal enzyme does not display as much selectivity. Although these enzymes have not been purified, the K_m values for the two substrates in the reaction; glycerol-3-phosphate and acyl-CoA are different. Studies have shown that glycerol-3-phosphate concentrations alone do not regulate glycerol-

3-phosphate acyltransferase activity *in vivo* (Bell and Coleman 1980). The mitochondrial enzyme displays lower K_m values for its substrates indicating its ability to work at low substrate concentrations. The physiological function, however, of the mitochondrial acyltransferase is not clear at this time (Brindley 1985). The 1-acyl-*sn*-glycerol-3-phosphate acyltransferase activity is enhanced more than 70-fold when comparing 5-day old rats to perinatal. The induction of large changes in enzyme activity suggest the enzyme is inducible. Evidence suggests that the enzyme may be regulated by fatty-acid binding protein and calcium (Bell and Coleman 1983).

4. Phosphatidic Acid

The formation of phosphatidic acid occurs through an acyltransferase reaction using acyl-CoA and 1-acyl-*sn*-glycerol-3-phosphate. This reaction is catalyzed by 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (EC 2.3.1.20). This enzyme activity can be separated from a 2-acyl-*sn*-glycerol-3-phosphate acyltransferase (Yamashita *et al.* 1973) and 1-acyl-glycerophosphorylcholine acyltransferase in rat liver microsomes (Miki *et al.* 1977). The enzyme is found predominantly in the microsomes with a pH optimum at 7. This enzyme prefers the use of monoenoic and dienoic acyl-CoA esters as substrates for the transfer of the acyl group onto the *sn*-2 position of the glycerol. Although 1-acyl-*sn*-glycerol-3-phosphate acyltransferase does not appear to be rate limiting, the activity of the enzyme has been shown to increase from 5-8-fold during perinatal development in rat liver (Coleman and Haynes 1983). The product of 1-acyl-*sn*-glycerol-3-phosphate acylation (phosphatidic acid) is the key branch

metabolite between acidic phospholipid biosynthesis and neutral lipid biosynthesis. A considerable portion of phosphatidic acid is converted into diacylglycerol where it serves as a precursor for the biosynthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol.

5. CDP-Diacylglycerol

Cytidine diphosphodiacylglycerol (CDP-diglyceride) is formed through a reaction which utilizes CTP and phosphatidic acid. This reaction is catalyzed by CDP-diglyceride synthetase (CTP: phosphatidic acid cytidylyltransferase) (EC 2.7.7.41) which requires magnesium for activity (Sturton and Brindley 1977). This enzyme has been purified from *Saccharomyces cerevisiae* (Kelley and Carman 1987). In the mammalian system, activity has been found in both the microsomal and mitochondrial fractions (Esko and Raetz 1983). Liteplo and Sribney (1980) have reported that the addition of GTP to the assay caused a 4-5-fold increase in enzyme activity *in vitro*. It is not known if this has any relation to enzyme activity *in vivo*. The enzyme displays little specificity for the acyl composition of the phosphatidic acid substrate (Carter and Kennedy 1966). This may explain why the acyl composition of CDP-diacylglycerol is very similar to that of the phosphatidic acid.

6. Phosphatidylinositol

Phosphatidylinositol is formed by the condensation of CDP-diacylglycerol and *myo*-inositol. This reaction is catalyzed by phosphatidylinositol synthetase (EC 2.7.8.11) which has been purified to near homogeneity by Takenawa and Egawa (1977). The *myo*-inositol is generated in most tissues from glucose. In the proximal tubule of the kidney, the levels of intracellular *myo*-inositol were higher than the K_m (2 mM) for the enzyme, indicating that the levels of myo-inositol do not play a role in regulating the synthesis of phosphatidylinositol *in vivo* (Galvao and Shayman 1990). Experiments using labelled glycerol indicate that newly synthesized phosphatidylinositol contains mainly monoenoic and dienoic species of fatty acids which are subsequently converted to polyenoic forms such as arachidonic acid through a deacylation-reacylation process (Esko and Raetz 1983).

Phosphatidylinositol has been shown to be intimately associated with signal transduction. In this process phosphatidylinositol becomes phosphorylated on the inositol moiety by inositol kinases (Berridge and Irvine 1989). Once phosphorylated, phospholipase C cleavage results in the production of 1,2-diacylglycerol and 1,4,5-trisphosphate. The presence of phosphorylated phosphatidylinositol is essential for phospholipase C-dependent signal transduction. Signal transduction will be discussed in detail in section IV.

7. Phosphatidylserine

Phosphatidylserine is made *de novo* in procaryotes via the CDP-diacylglycerol pathway. This pathway has not been detected in animals or plants but has been found in yeast (Vance 1985). A known pathway for the biosynthesis of phosphatidylserine in mammalian tissue is through a base exchange reaction. Phosphatidylserine in the liver is synthesized by a microsomal Ca^{2+}-dependent base exchange between L-serine and the ethanolamine of phosphatidylethanolamine. The base exchange enzyme was purified from brain and found to contain no phospholipase D activity. The enzyme was also found to be specific for phosphatidylethanolamine. Studies using Chinese hamster ovary cells have shown that phosphatidylserine may also be made through a serine exchange with phosphatidylcholine and lysophosphatidylcholine (Esko and Raetz 1983). Phosphatidylserine can be decarboxylated to phosphatidylethanolamine and then undergo base exchange with serine to reform phosphatidylserine. Whether the existence of this futile cycle in the cell is for the formation of ethanolamine remains to be determined. Very limited information is available on the regulation of phosphatidylserine biosynthesis in the mammalian system.

8. Phosphatidylcholine

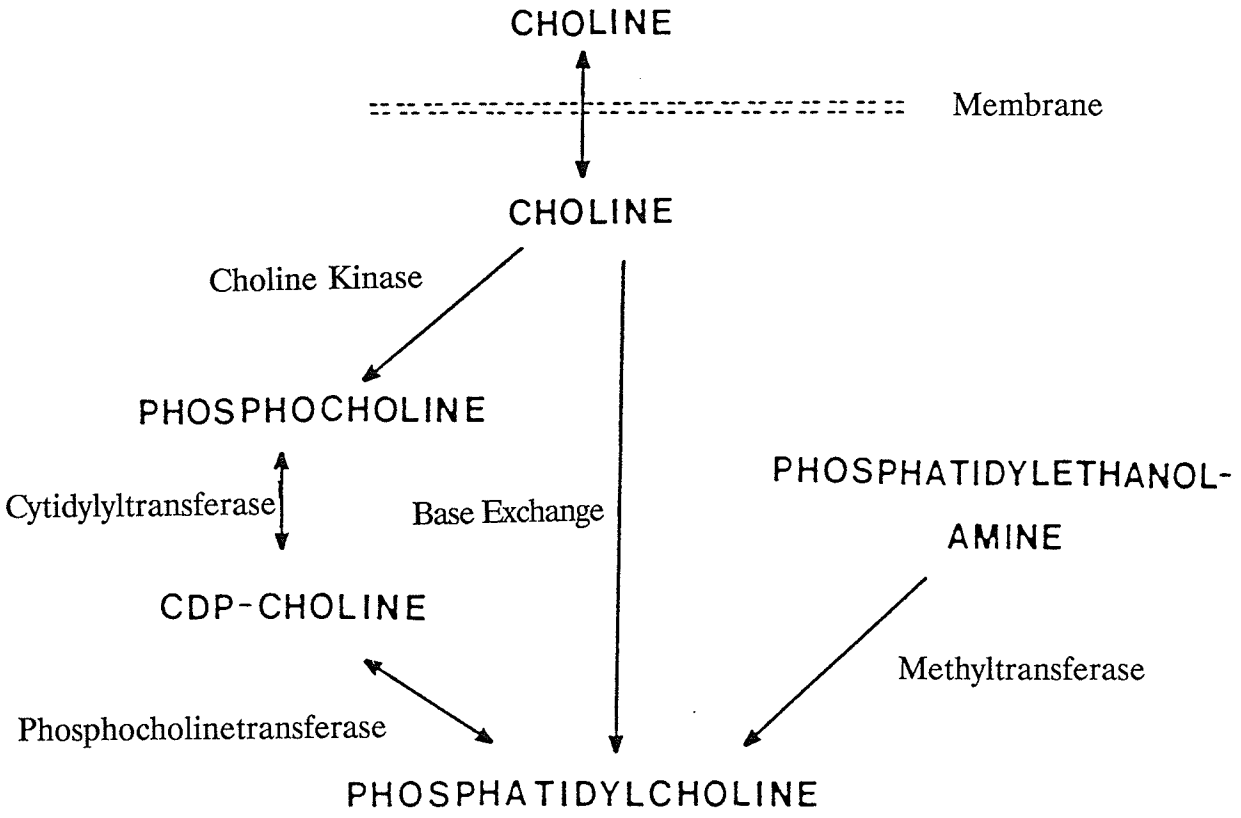
Phosphatidylcholine is the most abundant phospholipid in the mammalian heart and can be formed through 3 known pathways. These pathways include the CDP-choline pathway, the methylation of phosphatidylethanolamine and the Ca^{2+}-mediated base

exchange pathway (Figure 6) (Vance and Choy 1979). The contribution of these pathways to the formation of phosphatidylcholine is dependent on the type of tissue analyzed. In the liver, the methylation of phosphatidylethanolamine accounts for 20-40% of the phosphatidylcholine formed (Vance and Choy 1979) while this pathway only contributes 2.5% in the hamster heart (Zelinski *et al.* 1980). The base exchange pathway is a minor pathway for the production of phosphatidylcholine in both the heart and liver. The major pathway for phosphatidylcholine biosynthesis is the CDP-choline pathway (Vance and Choy 1979; Zelinski *et al.* 1980).

A. CDP-Choline Pathway

Unlike other polar head groups, choline is a dietary requirement of most mammals and is obtained from the diet. Choline is taken up by the cell through a saturable transport system. Once choline enters the cell, it is phosphorylated to phosphocholine. This step is catalyzed by choline kinase (EC 2.7.1.32) and requires ATP and Mg^{2+} for activity. The enzyme exists in the cytosol of many tissues (Pelech and Vance 1984). The purified enzyme consists of two subunits with a molecular weight of 75,000 - 80,000 Da (Ishidate *et al.* 1984). In some cases choline kinase activity exists on a different protein than ethanolamine kinase. However, other studies indicate that both activities share a common protein but have separate active sites (Ishidate 1989).

Figure 6



Pathways for the biosynthesis of phosphatidylcholine

The next step in the CDP-choline pathway involves the conversion of phosphocholine to CDP-choline. This rate-limiting step of the pathway requires CTP and is catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15). The rate-limiting role of this enzyme in the CDP-choline pathway has been shown in the isolated hamster heart (Zelinski *et al.* 1980). The cytidylyltransferase enzyme was found to be located in both the cytosolic as well as the microsomal fraction in various tissues (Vance 1989). When the enzyme translocates from the cytosol to the microsomes, there is a concomitant increase in the activity of the enzyme. This enzyme has been purified from rat liver cytosol and found to consist of two nonidentical subunits with a mass of 38 and 45 KDa. The 45 KDa subunit contains the catalytic activity of the enzyme while the function of the 38 KDa subunit remains undefined (Feldman and Weinhold 1987). This enzyme has been cloned from rat liver and found to lack a hydrophobic domain large enough to span the membrane. It has been proposed that hydrophobic residues in an amphipathic helix may allow the enzyme to interact with membrane phospholipids (Kalmar *et al.* 1990).

The final step of the pathway is the formation of phosphatidylcholine from CDP-choline and 1,2-diacylglycerol. The reaction is catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) (Cornell 1989). The enzyme activity has been found in both the microsomal as well as the mitochondrial fractions (Ghosh *et al.* 1990). In the hamster heart, the enzyme has a pH optimum of 7.5 - 8.5 and displays an absolute divalent cation requirement of Mg^{2+} or Mn^{2+} (O *et al.* 1989).

B. Methylation of Phosphatidylethanolamine

This pathway is responsible for 20-40% of phosphatidylcholine biosynthesis in the liver but contributes very little in other tissues (Vance 1990). The methylation pathway involves three successive methylation reactions where the transfer of the methyl group comes from S-adenosyl-L-methionine. The pathway begins with phosphatidylethanolamine and progresses through phosphatidyl-N-monomethylethanolamine, phosphatidyl-N,N-dimethylethanolamine and finally phosphatidylcholine. The enzyme responsible for the conversion of phosphatidylethanolamine to phosphatidylcholine is phosphatidylethanolamine-N-methyltransferase (PE N-methyltransferase). This enzyme has been purified from rat liver microsomes and has a molecular mass of 18,300 Da (Ridgway and Vance 1987). The rate of the reaction appears to be controlled by the availability of the substrates phosphatidylethanolamine and S-adenosylmethionine. The methylation pathway appears to be inversely regulated with the CDP-choline pathway. When the methylation pathway in hepatocytes was blocked, the CDP-choline pathway compensated for the loss of phosphatidylcholine biosynthesis by increasing the activity of cytidylyltransferase (Vance and Ridgway 1988).

C. Base Exchange Reation

The base exchange reaction plays a very minor role in the net synthesis of phosphatidylcholine. The synthesis of phosphatidylcholine in this manner involves the

exchange of free choline with the head group of phosphatidylethanolamine. This base exchange reaction requires Ca^{2+} and results in the formation of free ethanolamine and phosphatidylcholine (Kanfer 1980). The major function of this reaction is to remodel phospholipids that have already been synthesized in the cell (Kanfer 1989).

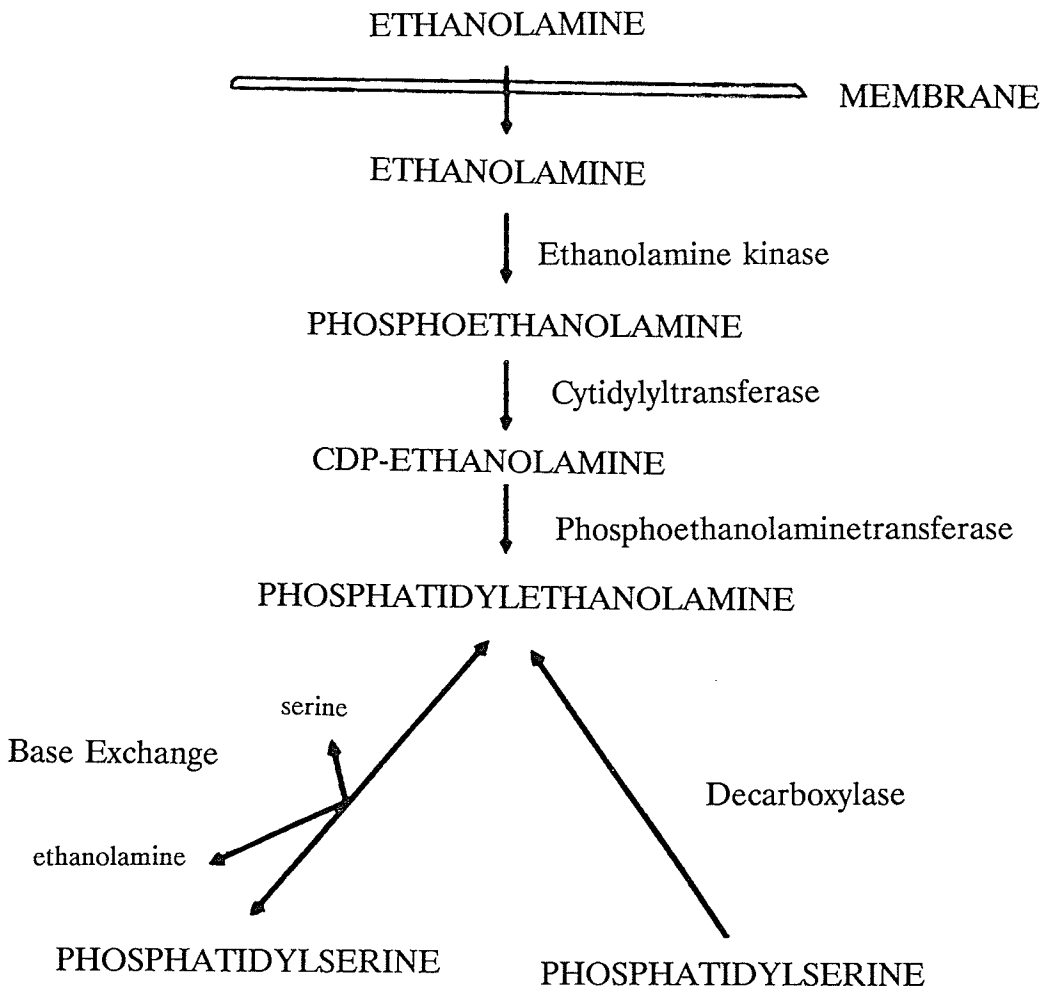
9. Phosphatidylethanolamine

Phosphatidylethanolamine is one of the major phospholipids in mammalian tissues. This phospholipid can be synthesized either through the CDP-ethanolamine pathway, the decarboxylation of phosphatidylserine or a Ca^{2+}-mediated base exchange reaction (Figure 7) (Vance 1985). The contribution of these pathways in the biosynthesis of phosphatidylethanolamine varies between cell types. In Chinese hamster ovary cells, the decarboxylation of phosphatidylserine is the major pathway for the synthesis of phosphatidylethanolamine (Miller and Kent 1986). In tissues such as the hamster heart, the CDP-ethanolamine pathway is the main route for the biosynthesis of phosphatidylethanolamine (Zelinski and Choy 1982b).

A. CDP-Ethanolamine Pathway

This pathway begins with the uptake of ethanolamine into the cell. In the hamster heart, the uptake system for ethanolamine has a K_t of 170 μM (Zelinski and Choy 1982b). Upon uptake, ethanolamine is rapidly phosphorylated to phosphoethanolamine by ethanolamine kinase (EC 2.7.1.82). This cytosolic enzyme

Figure 7



Pathways for the biosynthesis of phosphatidylethanolamine

requires ATP and Mg^{2+} as cofactors for the reaction. The purified enzyme from the mammalian liver has a molecular weight of 47,000 (Porter and Kent 1990). Purification of this enzyme usually results in the co-purification of choline kinase. Hence, it is still unclear if the kinase that phosphorylates ethanolamine is the same kinase that phosphorylates choline.

Phosphoethanolamine is then converted to CDP-ethanolamine by the enzyme CTP:phosphoethanolamine cytidylyltransferase (EC 2.7.7.14). This cytosolic enzyme catalyzes the rate limiting step in the CDP-ethanolamine pathway. The enzyme requires CTP for the conversion of phosphoethanolamine into CDP-ethanolamine. Unlike phosphocholine cytidylyltransferase, the phosphoethanolamine cytidylyltransferase does not translocate from the cytosol to the membrane fraction. The enzyme was purified from rat liver and exists as a dimer with a molecular weight of 100,000. The K_m values for CTP and phosphoethanolamine are 65 μM and 53 μM respectively (Sundler 1975).

The final step of the pathway involves the condensation of CDP-ethanolamine and diacylglycerol to form phosphatidylethanolamine. This reaction is carried out by the enzyme CDP-ethanolamine:1,2-diacylglycerol phosphoethanolaminetransferase (EC 2.7.8.1). The enzyme is an integral membrane protein which faces the cytosolic side of the endoplasmic reticulum (Ballas and Bell 1980). The phosphoethanolaminetransferase activity has been separated from phosphocholinetransferase activity through DEAE-sepharose column chromatography

(O *et al.* 1989). The enzyme requires phosphatidylcholine for maximal activity and is competitively inhibited by the product of the reaction; phosphatidylethanolamine (Hjelmstad and Bell 1991).

B. Base Exchange Reaction

Phosphatidylethanolamine can be synthesized from other phospholipids through a base exchange mechanism which can account for up to 10% of its synthesis (Zelinski and Choy 1982b). There have been two base exchange activities identified in mammalian cells (Tijburg *et al.* 1989). The first exchange system involves an exchange between serine and phosphatidylcholine or phosphatidylethanolamine (Kuge *et al.* 1991), while the other system is exclusive for ethanolamine. The latter system is believed to be located on the cytoplasmic side of the endoplasmic reticulum (Bell *et al.* 1981). This activity has been purified from rat brain microsomes and has a pH optimum of 7 and requires Ca^{2+} for activity (Suzuki and Kanfer 1985).

C. Phosphatidylserine Decarboxylation

Phosphatidylethanolamine synthesis through the decarboxylation of phosphatidylserine is carried out by the enzyme phosphatidylserine decarboxylase (EC 4.1.1.65). This enzyme is located on the inner membrane of the mitochondria. In most mammalian tissues, the decarboxylation of phosphatidylserine is not considered a main route for phosphatidylethanolamine synthesis (Arthur and Page

1991; Xu *et al.* 1991; Zelinski and Choy 1982b). However, in a cultured baby hamster kidney cell line (Voelker 1984) and in Chinese hamster ovary cells (Miller and Kent 1986), phosphatidylserine decarboxylase is touted as the main route for phosphatidylethanolamine biosynthesis. Addition of increasing concentrations of ethanolamine to Chinese hamster ovary cells (Miller and Kent 1986) resulted in an increased contribution of the CDP-ethanolamine pathway for phosphatidylethanolamine biosynthesis.

10. Diacylglycerol

Diacylglycerol is formed from phosphatidic acid through the action of the enzyme phosphatidic acid phosphatase (EC 3.1.3.4). This enzyme is responsible for controlling the branch between acidic phospholipid biosynthesis and neutral lipid biosynthesis. Changes in enzyme activity have been found to be proportional to the amount of biosynthesis of triacylglycerol in the liver. The activity of phosphatidic acid phosphatase changes to a greater extent than does the other enzymes involved in triacylglycerol synthesis under different physiological conditions (Brindley 1985). This enzyme exists in both soluble and membrane bound forms. The microsomal form of the enzyme displays a higher activity than the soluble form of the enzyme. The enzyme can be translocated *in vitro* from the cytosol to the membrane by the addition of fatty acid (Cascales *et al.* 1984). Phosphatidic acid phosphatase activity has also been shown to be dependent on magnesium for activity (Walton and Possmayer 1985). It is not known at this time whether the requirement of phosphatidic acid

phosphatase for magnesium is absolute or whether the presence of the cation improves the interaction between phosphatidate and the enzyme (Tijburg *et al.* 1989). In the liver, this enzyme has also been shown to be inhibited by a cAMP-dependent phosphorylation reaction which is reversible in the presence of fatty acids (Pelech *et al.* 1983). Conversely, cAMP was shown to increase protein synthesis and result in enhanced phosphatidic acid phosphatase activity by increasing the half-life of the enzyme (Pittner *et al.* 1986). The possible phosphorylation of this enzyme is currently under debate as Butterwith *et al.* (1984) was not able to repeat the experiments of Berglund *et al.* (1982) who showed that cAMP-dependant protein kinase altered enzyme activity.

Diacylglycerol plays a central role in the biosynthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol. All of these lipids are regulated to some extent through the availability of diacylglycerol. Previous studies have indicated that more than one pool of diacylglycerol exists within the cell (Binaglia *et al.* 1982; Rustow and Kunze 1985). These authors suggest that *de novo* diacylglycerol pools are distinct from existing pools of diacylglycerol in the membrane. In the case of phosphatidylcholine, the enzymes involved in its biosynthesis from glycerol-3-phosphate are organized in a multienzyme complex, in which the metabolic intermediates are channelled directly from one enzyme active site to the next without mixing with the endogenous membrane lipid (Rustow and Kunze 1987).

11. Triacylglycerol

Triacylglycerol is formed from diacylglycerol through the action of diacylglycerol acyltransferase. Diacylglycerol acyltransferase is located in the microsomal subcellular fraction of various tissues including the heart (Tijburg *et al.* 1989). In rat liver, the active site for diacylglycerol acyltransferase is found on the cytoplasmic surface of microsomal vesicles (Bell and Coleman 1983). A report by Goldberg *et al.* (1981) has suggested that diacylglycerol acyltransferase can act as the rate limiting enzyme in the biosynthesis of triacylglycerol. The stimulation of triacylglycerol synthesis is the result of enhanced diacylglycerol acyltransferase activity without stimulation of phosphatidic acid phosphatase activity. This finding has been duplicated when an inhibitor of diacylglycerol acyltransferase was used to determine the role of this enzyme in triacylglycerol biosynthesis (Tijburg *et al.* 1989). Other reports indicate that both phosphatidic acid phosphatase and diacylglycerol acyltransferase act to regulate the biosynthesis of triacylglycerol. If fatty acid supplies are low, phospholipid biosynthesis occurs at the expense of triacylglycerol (Geelen *et al.* 1978; Butterwith *et al.* 1984)

The regulation of diacylglycerol acyltransferase activity may also occur through covalent modification. It has been suggested that the diacylglycerol acyltransferase can be regulated through a phosphorylation-dephosphorylation mechanism (Haagsman *et al.* 1981). In rat hepatocytes, glucagon caused a 53% decrease in enzyme activity which was mimicked by cAMP. Further studies revealed that cAMP-

dependent protein kinase did not affect enzyme activity (Haagsman *et al.* 1982). This finding is in contrast with the report of Soling *et al.* (1989) who showed that diacylglycerol acyltransferase activity could be stimulated 5-11-fold by cAMP-dependent protein kinase and calmodulin-dependent protein kinase. Several other factors have been reported to alter diacylglycerol acyltransferase activity including fatty acid binding protein, lysophospholipids and some soluble proteins (Bell and Coleman 1983). The physiological significance of these factors is yet to be defined.

Triacylglycerol is the final step in acylglycerol formation. This lipid is stored within triacylglycerol droplets or secreted as very-low-density lipoproteins in the liver. In the heart, triacylglycerol is the major source of ATP for the mechanical function of the heart. Even when exogenous glucose is present, more than 50% of the oxygen consumed by the heart is due to the utilization of triacylglycerol (Jesmok *et al.* 1976; Crass 1977). The hydrolysis of triacylglycerol is regulated by various lipases. It has been suggested that lipases are activated by cAMP-dependent protein kinase. Other investigators have challenged this idea and suggest that lipase activity is actually regulated by fatty acids which result in a feedback inhibition of the lipase activity (de Groot *et al.* 1989).

From the results presented in this section it is clear that the regulation of lipid biosynthesis in mammalian tissue is highly complex. Lipid biosynthesis is regulated through the activity of enzymes involved in their synthesis as well as the pool sizes of the substrates. In some cases, the study of pool sizes of the substrates can be

misleading due to the fact that some intermediates may be channelled from one enzyme to another generating distinct intracellular pools. It is clear that more work must be done in this area in order to obtain a complete understanding of glycerophospholipid biosynthesis in mammalian tissues.

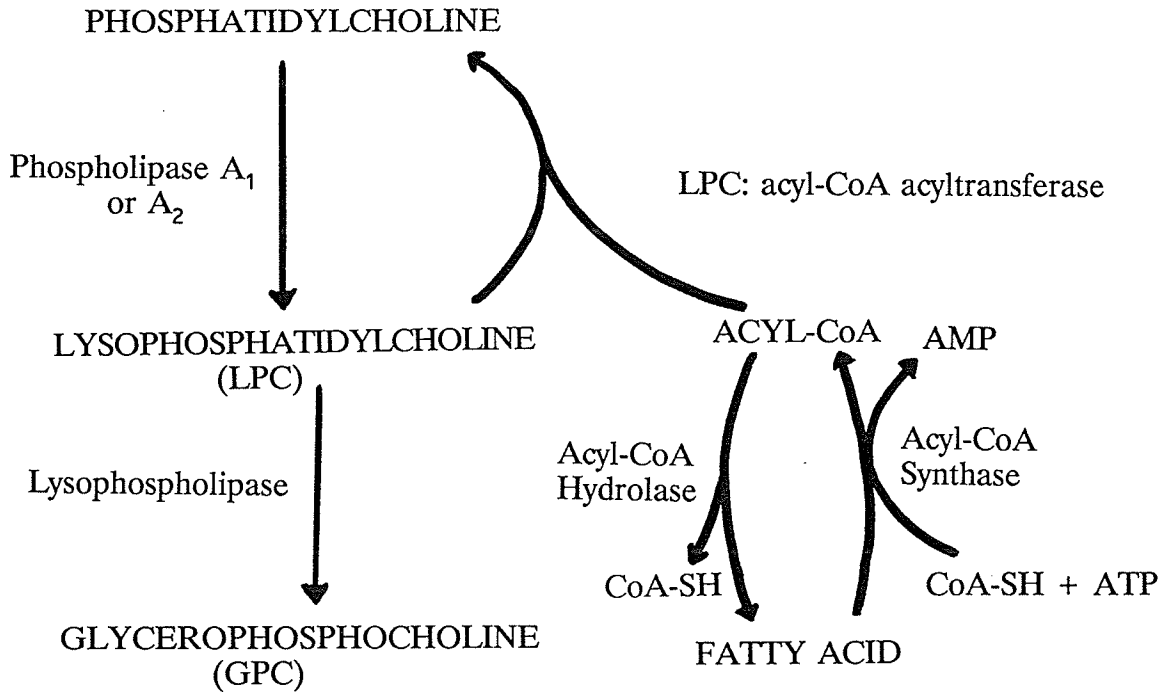
III. The Deacylation-Reacylation Cycle (Lands Pathway)

1. Introduction

Structural analysis of phosphatidylcholine (PC) indicates that saturated fatty acids are usually esterified at the C-1 position and unsaturated fatty acids at the C-2 position (Choy and Arthur 1989). The observed fatty acid distribution does not seem to result from the limited selectivity of CDP-choline: diacylglycerol cholinephosphotransferase for diacylglycerol species during the *de novo* biosynthesis of PC (Arthur and Choy 1984). The remodelling of newly synthesized PC via deacylation-reacylation has been regarded as an important mechanism for the selection of PC acyl groups in mammalian tissues.

The pathway for the remodelling of PC was first identified by Lands (1960). In this pathway, PC is first deacylated by phospholipase A_1 or A_2, and the resultant lysophosphatidylcholine is acylated back to PC by the action of lysoPC: acyl-CoA acyltransferase. The lysoPC can also be degraded by the action of lysophospholipase to form glycerophosphocholine (Figure 8). LysoPC: acyl-CoA acyltransferase activity has been detected in various mammalian tissues (Choy and Arthur 1989), bacteria, protozoa (Okuyama *et al.* 1977) and plants (Devor and Mudd 1971; Stymne and Stobart 1984). The transfer of acyl groups from acyl-CoA to 1-acyl-glycerophosphocholine (1-acyl-GPC) is catalyzed by 1-acyl-GPC: acyl-CoA acyltransferase, while transfer of fatty acids to 2-acyl-GPC is catalyzed by 2-acyl-

Figure 8



The deacylation-reacylation pathway of phosphatidylcholine

GPC: acyl-CoA acyltransferase (Morash *et al.* 1989). These two reactions appear to be carried out by separate enzymes (Lands and Hart 1965; Arthur 1989). Most of the studies on acyltransferase have been focused on the acylation of 1-acyl-GPC, and only limited information is available on the acylation of 2-acyl-GPC.

2. Subcellular Localization and Purification

1-Acyl-GPC: acyl-CoA acyltransferase has been found in all mammalian organs. Most of the enzyme activity is located in the microsomal fraction (Eibl *et al.* 1969) The enzyme appears to have a transmembrane orientation in the microsomal membrane vesicle (Renooij and Snyder 1981). Acyltransferase activity has also been reported in the mitochondrial and plasma membrane fractions (Colard *et al.* 1980; Arthur *et al.* 1987). Low enzyme activity has been detected in the cytosolic fraction of the rabbit heart (Needleman *et al.* 1985).

Attempts to purify the 1-acyl-GPC: acyl-CoA acyltransferase were hampered by difficulties in its solubilization from the membrane domain. The use of detergent for this purpose has not been very effective since enzyme activity is inevitably inhibited by the presence of detergents. For example, when microsomes were treated with 1% cholate, 0.25% deoxycholate or 0.05% Triton X-100, over 90% of enzyme activity was inhibited. The solubilization of the enzyme with 1-acyl-GPC and its analogues appears to be a more viable approach (Weltzien *et al.* 1979; Gavino and Deamer 1982; Deamer and Gavino 1983). The purification of the enzyme from bovine brain

heart has been reported (Deka *et al.* 1986; Sanjanwala *et al.* 1988).

3. Acyl Specificity

Since dipalmitoyl-PC is the major form of PC in the lung, the selectivity of the acyltransferase towards palmitoyl-CoA was expected to be higher than oleoyl-CoA. However, no difference in specificity of the acyltransferase towards palmitoyl-CoA and oleoyl-CoA has been detected (Choy and Arthur 1989). In rat liver, the enzyme has a definite specificity towards acyl-CoA with unsaturated acyl groups (van den Bosch *et al.* 1967; Colard *et al.* 1980) and 1-palmitoyl-GPC is more effective than 1-stearoyl-GPC as an acyl acceptor (Holub *et al.* 1979).

In the guinea pig heart, the microsomal enzyme is more active towards unsaturated acyl-CoAs but displayed little selectivity with respect to the degree of unsaturation (Arthur and Choy 1986). Interestingly, the mitochondrial enzyme appears to have a very high specificity towards linoleoyl-CoA (Arthur *et al.* 1987). In addition, the acyl specificity of the enzyme appears to be affected by the presence of detergent (Okuyama *et al.* 1975) and during enzyme purification. For example, the purified acyltransferase from bovine brain displays a higher degree of selectivity towards arachidonate than the microsomal enzyme (Deka *et al.* 1986). Stimulation of smooth muscle cells with phorbol myristate results in the enhancement of arachidonate incorporation into 1-acyl-GPC (Kanzaki *et al.* 1989).

4. Characteristics and Kinetics

The acyltransferase reaction is reversible and is dependent on the concentrations of the substrates. The pH optimum for the mammalian enzyme is between 7 and 8 and may be dependent on the transfer of a specific acyl group (Jezyk and Lands 1968). Using the partially purified enzyme from the rat liver, the transfer of oleoyl-CoA was found to be more active at pH 7 while arachidonoyl-CoA transfer was more rapid at a higher pH (Hasegawa-Sasaki and Ohno 1980). Recently, the molecular weight of the purified enzyme was determined to be 43,000 for bovine brain (Deka *et al.* 1986) and 64,000 for bovine heart (Sanjanwala *et al.* 1988). The partially purified enzyme from rabbit lung displayed a nonclassical iso-ping-pong mechanism and the K_m for palmitoyl-CoA and 1-acyl-GPC were found to be 8.5 and 61 μM, respectively (Arche *et al.* 1987). In human platelets, the K_m values for saturated and unsaturated acyl-CoA vary from 1.05 to 5.70 μM in the presence of 100 μM of 1-acyl-GPC (McKean *et al.* 1982). In most mammalian tissues, the K_m values for acyl-CoA and 1-acyl-GPC vary between 1-15 μM and 50-100 μM, respectively.

5. Regulation of Enzyme Activity

1-Acyl-GPC acyltransferase activity was inhibited by high concentrations of 1-acyl-GPC and palmitoyl-CoA, but high concentrations of other acyl-CoAs were not inhibitory (Hasegawa-Sasaki and Ohno 1975). Enzyme activity in the liver microsomes was relatively unaffected by sulfhydryl-binding reagents such as

iodoacetate, N-ethylmaleimide and p-chloromercuriphenylsulfonic acid, but the activity of the partially purified enzyme was inhibited by these reagents (Hasegawa-Sasaki and Ohno 1980). The compounds WY-14643 and clofibric acid were found to inhibit mitochondrial 1-acyl-GPC: acyl-CoA acyltransferase (Riley and Pfeiffer 1986). In addition, divalent metallic cations such as Mg^{2+} or Ca^{2+} were inhibitory to enzyme activity (Arthur and Choy 1986; Yashiro *et al.* 1989). Acyltransferase activity *in vivo* was affected by long term administration of clofibric acid and chronic administration of isoproterenol (Kawashima *et al.* 1989; Yashiro *et al.* 1989). Enzyme activity in rat liver, rat heart and rabbit gastric mucosa microsomes were inhibited by detergents and inhibitors of cyclic nucleotide phosphodiesterase (Shier 1977). The regulation of enzyme activity by a phosphorylation / dephosphorylation cycle has been postulated (Reinhold *et al.* 1989).

IV. Signal Transduction and Second Messengers

1. Introduction

In order for cells in a tissue to work together they must communicate. The way in which they can communicate is through the use of chemical messengers (known as hormones) found in the blood known as hormones. Hormones are molecules synthesized by a specific tissue and secreted into the blood. They are then carried by the blood to a target organ where they cause a change in the activity of the tissue. Steroid hormones are synthesized from cholesterol and can pass through the target cell's plasma membrane and cause a direct effect on cellular function. Non steroid hormones, however, are found to exert their effects without entering the target cell. This effect is achieved through a process known as signal transduction. The process begins with the binding of the hormone to a receptor on the surface of the cell. The receptor molecule spans the cell membrane and allows the message to be transmitted by an intermediate, usually a G protein. The G proteins are named as such because of their binding to the guanine nucleotide.

The two general mechanisms in which non steroid hormones effect cellular functions are described. The first pathway is referred to as the adenylate cyclase cascade. In this scheme the binding of a hormone to its receptor results in increased levels of cAMP (adenosine 3',5'-monophosphate). The elevation of cAMP results in the stimulation of cAMP-dependent protein kinase. The second pathway is known as the

phosphoinositide cascade. In this pathway the binding of the hormone to the receptor results in the hydrolysis of membrane phospholipids. This in turn produces two intracellular messengers: diacylglycerol and inositol trisphosphate. These products result in the activation of protein kinase C as well as the release of intracellular Ca^{2+}, respectively.

2. Adrenergic Receptors

Adrenergic receptors (α_1, α_2, $ß_1$, $ß_2$) belong to a family of receptors which mediate their actions by interacting with G proteins. The signal transduction pathway is initiated by an agonist binding to the receptor located on the plasma membrane of the cell. This stimulates the interaction of the receptors with specific G proteins. The receptor-G protein interaction results in the loss of GDP from a guanine nucleotide binding site found on the G protein, resulting in GTP binding. The GTP-G protein is the active form of the G protein which dissociates from the receptor and activates the effector protein. Effector proteins identified thus far include adenylate cyclase (EC 4.6.1.1), guanylyl cyclase (EC 4.6.1.2), phospholipase C (EC 3.1.4.3), phospholipase A_2 (EC 3.1.1.4), phosphodiesterases, Ca^{2+} and K^+ channels.

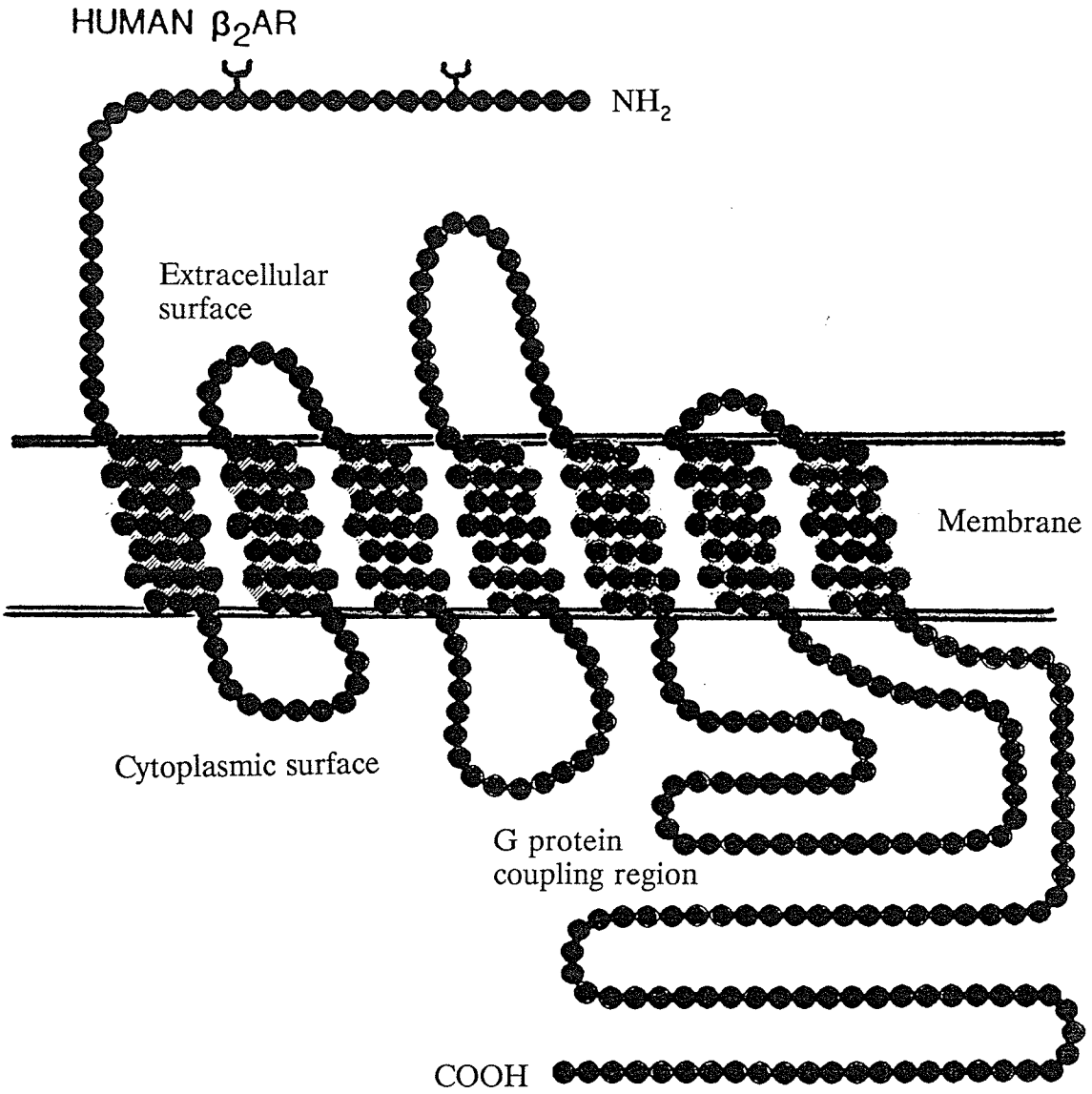
Despite diversity in receptor functions, they share structural homology. The receptors on the plasma membrane that are activated by epinephrine (adrenaline) or related catecholamines are called adrenergic receptors. These receptors have been subclassed according to their pharmacological properties (Dohlman *et al.* 1987). The stimulation

of α_1-adrenergic receptors results in the activation of the phosphoinositide cascade resulting in increased Ca^{2+} levels. The activation of the α_2-adrenergic receptor causes an inhibition of adenylate cyclase activity, while $ß_1$- and $ß_2$-adrenergic receptor activation results in the stimulation of adenylate cyclase.

The ß-adrenergic receptor has been purified and found to be an integral membrane protein with a molecular mass of approximately 64 KDa (Figure 9). Sequence analysis of the receptor molecule reveals seven hydrophobic stretches of about 20-25 amino acids in length. The actual membrane structure was then predicted as a transmembrane protein with seven transmembrane hydrophobic α-helices. This feature is now found to be common among receptors coupled to G-proteins. The amino-terminus of the receptor faces the extracellular side of the cell and was found to contain N-linked oligosaccharides. The ligand binding domain of the ß-adrenergic receptor is believed to reside as a pocket buried within the membrane bilayer as determined by deletion mutagenesis (Strader *et al.* 1989). The ligands of the adrenergic receptors are protonated amines. These compounds undergo an ionic interaction with the conserved aspartic acid residue located within the binding site (Strader *et al.* 1988). The ligand binding is also stabilized through hydrogen bonds and hydrophobic interactions between the aromatic ring and the side chains present in the putative binding area (Strader *et al.* 1989).

The interaction between the receptor and the G protein occurs on the cytosolic side of the plasma membrane. Deletion analysis has indicated that the internal loops of

Figure 9



Structure of the human ß-adrenergic receptor

(Dohlman *et al.* 1987)

the receptor as well as the carboxyl terminus, play important roles in the interaction between receptor and G protein. The cytosolic portion of the receptor is also involved in the desensitization of the receptor. Under prolonged exposure to an agonist, the ß-adrenergic receptor becomes desensitized and becomes less responsive to the stimulation. This is due to phosphorylation of the receptor by protein kinase A and ß-adrenergic receptor kinase (Hausdorff *et al.* 1990). This phosphorylation event prevents the activation of the G protein and thus stops the pathway. Activation of the pathway will be restored when the residues are dephosphorylated by a phosphatase.

3. G Proteins

The middle step in the signal transduction process is carried out by G proteins. They are bound to the inner surface of the plasma membrane and consist of three protein subunits called alpha (α), beta (ß) and gamma (γ) with the molecular weight of approximately 42,000, 35,000 and 10,000, respectively. The alpha subunit is different in all the G proteins currently isolated while the beta-gamma combination may not be unique. At this time there have been numerous G proteins identified which act on different effectors. The more common G proteins include: G_s (stimulates adenylate cyclase), Gi (inhibits adenylate cyclase), G_p or G_q (activates phospholipase C), G_t (activates cGMP phosphodiesterase) and G_o (regulates calcium and potassium channels) (Casey and Gilman 1988; Linder and Gilman 1992). After a hormone binds to the receptor, the conformational change in the receptor causes the alpha subunit

of the G protein to release bound GDP and replace it with GTP. The binding of GTP activates the alpha subunit and results in its dissociation from the beta-gamma complex. The active GTP-alpha subunit then diffuses along the inner surface of the plasma membrane and activates an effector such as adenylate cyclase. After a short period of time the intrinsic GTPase activity found in the alpha subunit hydrolyzes the GTP into GDP resulting in the inactivation of the alpha subunit. Upon inactivation, the α-subunit reassociates with the beta-gamma complex once again.

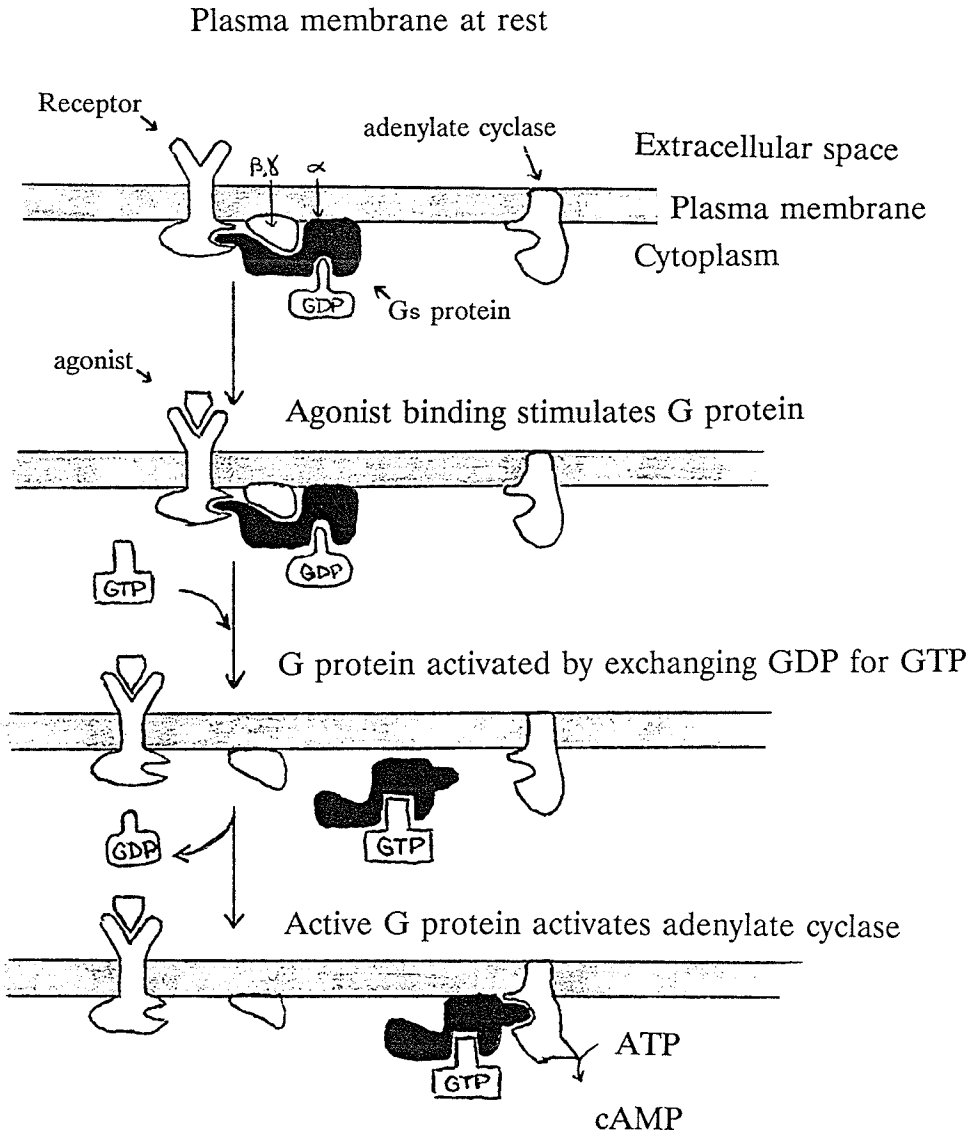
Bacterial toxins isolated from *Vibrio cholera* and *Bordetella pertussis* irreversibly modify G proteins. The cholera toxin enters the cytosol of the cell where it catalyzes the covalent addition of an ADP-ribosyl group from intracellular NAD+ to the alpha subunit of G_s. This altered G_s allows the activation of adenylate cyclase by blocking the intrinsic GTPase activity of the α-subunit. Therefore, the active GTP-alpha form of the G protein remains in the cell to continuously activate the adenylate cyclase, resulting in high levels of cAMP. The pertussis toxin also works in a similar fashion. It also ADP-ribosylates the alpha subunit of a G protein. In this case it blocks the GTPase activity of the Gi protein which prevents this G protein from inhibiting the adenylate cyclase. The end result is the same since both toxins result in the increase in intracellular cAMP levels (Gilman 1987).

4. Adenylate Cyclase Cascade

The first step in this cascade involves the binding of an agonist to the ß-adrenergic receptor located on the extracellular side of the plasma membrane. The binding of the agonist causes the activation of G_s which in turn activates adenylate cyclase. Cyclic AMP is produced by the enzyme adenylate cyclase which converts ATP into cAMP (Figure 10). The enzyme cyclic nucleotide phosphodiesterase converts cAMP to ATP, limiting the lifetime and activity of cAMP (Edelman *et al.* 1987).

The second stage in the cascade is the activation of a protein kinase by cAMP. cAMP-dependent protein kinases exist as tetramers composed of two catalytic (C) and two regulatory (R) subunits. The catalytic subunits are inactive when bound to the regulatory subunits. When cAMP binds the regulatory subunits, a conformational change in R occurs that lowers the affinity of the R subunit for the C subunit. This causes the tetramer to dissociate into a regulatory protein-cAMP complex and two active catalytic subunits. The active C subunit catalyzes the phosphorylation of numerous cellular enzymes and proteins. The protein kinase will phosphorylate proteins on either serine or threonine residues. The newly phosphorylated proteins generally generate a physiological or metabolic response. A phosphatase, that is specific for the protein phosphorylated, reverses the effects of the cAMP-dependent protein kinase (Taylor 1989).

Figure 10

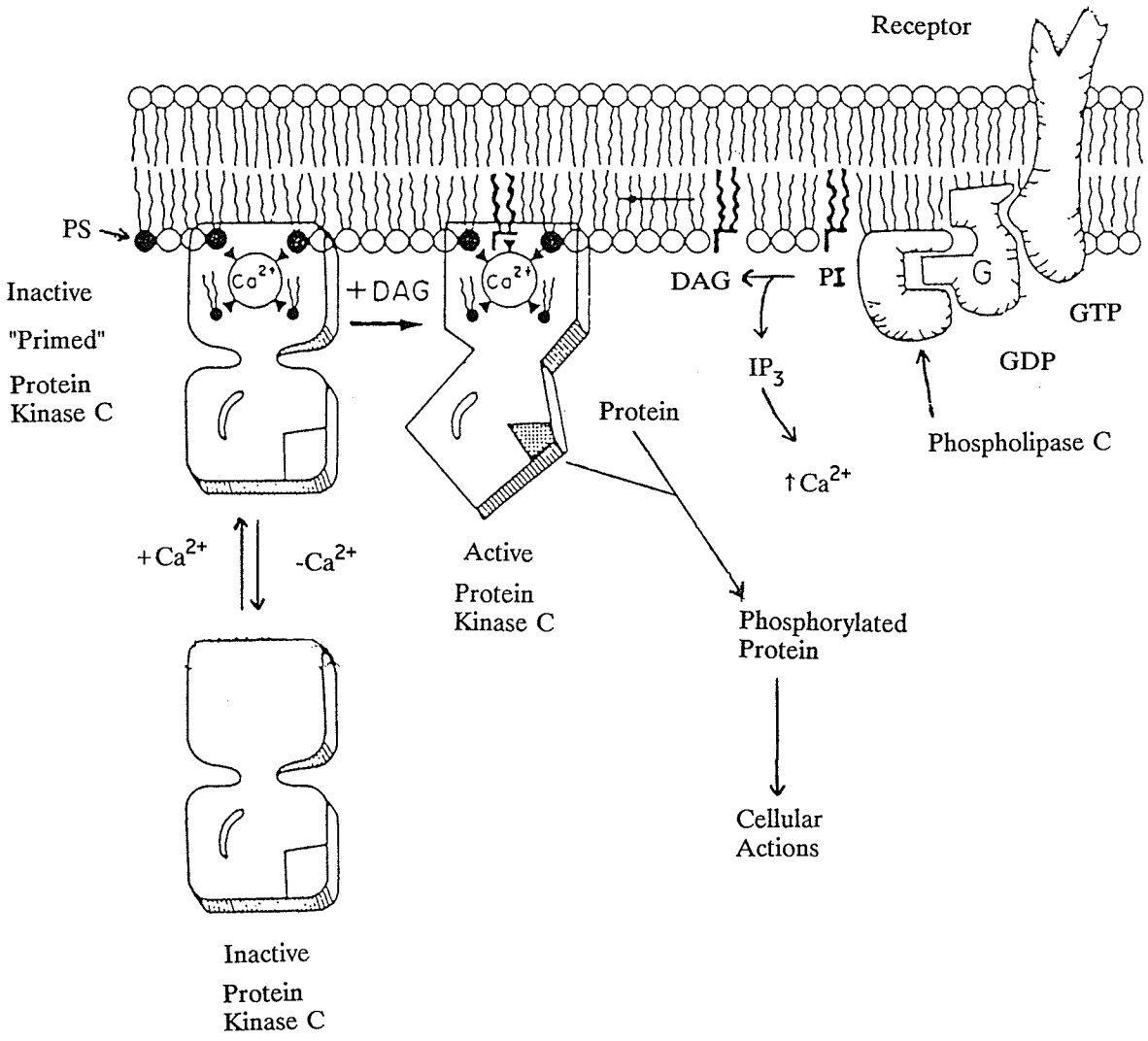


Activation of the adenylate cyclase cascade

5. Phosphoinositide Cascade

As with the adenylate cyclase cascade, the pathway of signal transduction via inositol lipids is also initiated by the binding of an agonist to a receptor. Again, the stimulation of a receptor by an agonist will result in the activation of a specific G protein. The G protein will in turn activate phosphatidylinositol specific phospholipase C. The phospholipase C will catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate into the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (Figure 11) (Berridge and Irvine 1989). Recent information indicates that phosphatidylinositol may not be the only phospholipid which is involved in signal transduction. Phosphatidylcholine has also been shown to be hydrolysed by phospholipase C or D resulting in the production of diacylglycerol (Figure 12) (Exton 1990; Shears 1991). The diacylglycerol produced acts as an activator for protein kinase C. Protein kinase C is a peripheral membrane protein which can be removed from membranes in the presence of EDTA/EGTA (inactive). The kinase becomes activated and tightly bound to the membrane when in the presence of 1,2-diacylglycerol and Ca^{2+}. This increased affinity with the membrane fraction is associated with the active form of the kinase (Bell 1986). Phosphatidylserine has also been shown to be a very strong activator of the kinase (Bell and Burns 1991). Active protein kinase C phosphorylates a multitude of cellular proteins and thereby controls numerous cellular processes.

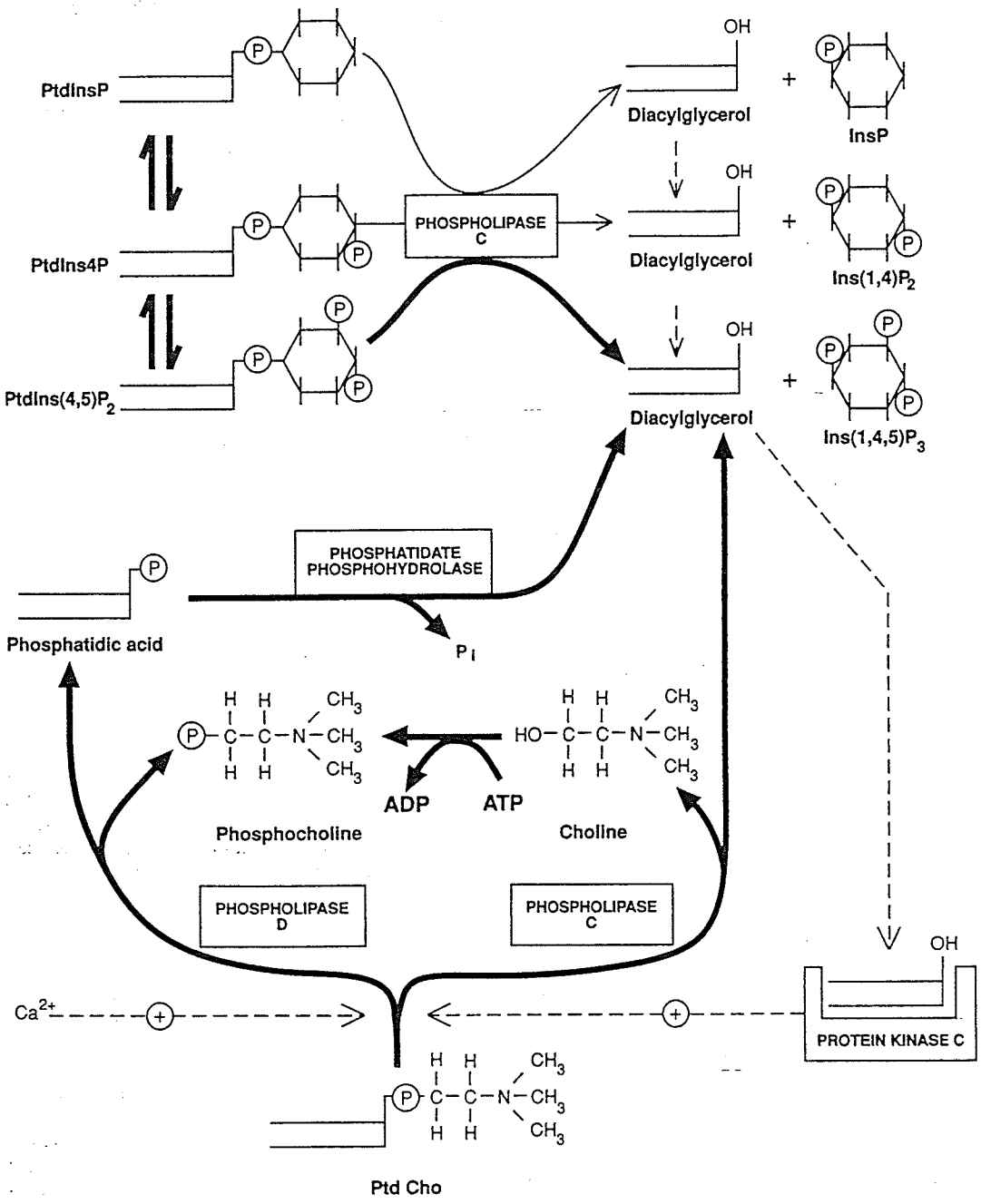
Figure 11



Activation of protein kinase C during the activation of the phosphoinositide cascade

(Bell 1986)

Figure 12



Shears 1991

Phosphatidylcholine involvement in the activation of protein kinase C

Inositol 1,4,5-trisphosphate (IP_3) is the other second messenger formed through this signal transduction process. The production of IP_3 mobilizes Ca^{2+} from intracellular stores and helps to activate protein kinase C. The prolonged increase in intracellular Ca^{2+} is believed to arise from the stimulation of both phospholipase C and D activities specific for phosphatidylcholine. This process is thought to extend the signalling response by allowing for the continued release of diacylglycerol from the much larger phospholipid pool (Billah and Anthes 1990; Exton 1990). The release of Ca^{2+} from intracellular stores may occur in a wave-like fashion, but the physiological significance of this event is not fully understood (Berridge 1990).

V. Methyl Lidocaine

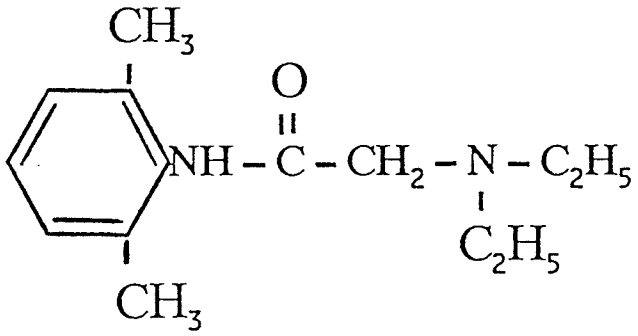
1. Introduction

The development of methyl lidocaine arose from a study dealing with local anaesthetics. In 1970, Oppenheim synthesized methyl lidocaine in order to investigate the charge effect of an anaesthetic. Most local anaesthetics contain a tertiary nitrogen atom and exist as uncharged amines or charged ammonium ions depending on the pH of the solution. The purpose of the Oppenheim study was to determine if the charged or uncharged form of an anaesthetic was the active form. In order to resolve the question, an antiarrhythmic drug was synthesized which contained an extra methyl group at the tertiary nitrogen. The extra methyl group resulted in the formation of a quaternary nitrogen with a permanent positive charge. This new antiarrhythmic drug was structurally similar to lidocaine and was called methyl lidocaine. The result of the study between lidocaine and methyl lidocaine showed that the charged form of a local anaesthetic appears to be the active form.

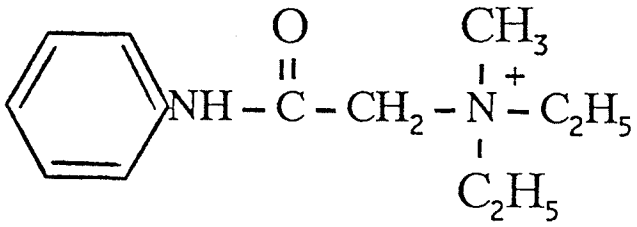
2. Antiarrhythmic Action

In the following years, the antiarrhythmic actions of methyl lidocaine were compared to lidocaine (Figure 13). Although the clinical application of lidocaine has been most successful, its limitations include the fact that it is rapidly metabolized and must be administered as a continuous intravenous infusion in order to maintain proper blood

Figure 13



Lidocaine



Methyl lidocaine

levels. Lidocaine is also known to break down into a toxic metabolite (Smith and Duce 1971) which is associated with central nervous system toxicity. Studies comparing methyl lidocaine to lidocaine found that methyl lidocaine was an effective antiarrhythmic agent that did not possess the negative central nervous system effects found with lidocaine (Gillis *et al.* 1973; Kniffen *et al.* 1974). Research over the next decade indicated that the two drugs had quite different effects on cardiac conduction. Analysis of atrial and ventricular conduction showed that methyl lidocaine was a much stronger depressant then lidocaine (Man and Dresel 1977). Recent work has indicated that methyl lidocaine could actually be arrhythmogenic due to its preferential depression of conduction in ischemically injured myocardium (Patterson *et al.* 1988).

3. Methyl Lidocaine and Lipid Metabolism

The focus of this thesis is not to determine if methyl lidocaine is a viable antiarrhythmic agent. Instead this drug was used as a probe to study the complex regulation of lipid metabolism in the heart. Our initial interest in this drug stemmed from work discussed in section I of the results. This study involved the analysis of lysophospholipid levels in the heart. Lysophospholipids are known to cause arrhythmias in the heart when present at high concentrations (Katz and Messineo 1981b; Man and Choy 1982) therefore, the effect of various antiarrhythmic drugs on lysophospholipid levels was investigated. Preliminary results showed that methyl lidocaine proved to be a very useful drug to study the phospholipid metabolism in

the heart. This encouraged us to continue the use of methyl lidocaine to study the lipid biosynthesis in the mammalian heart.

MATERIALS AND METHODS

I. Materials

1. Experimental Animals

Male Syrian golden hamsters (120 ± 20 g) and male Sprague-Dawley rats (250 ± 50 g) were used during the studies of lysophospholipid metabolism and lipid biosynthesis. The animals were obtained from Charles River Canada Inc. (St. Constante, Que.). Both hamsters and rats were maintained on the Agway rodent diet RMH 3000 (Table 2) (Agway Inc., Syracuse, N.Y.), and tap water *ad libitum*, in a light- and temperature-controlled room.

2. Chemicals

Hexanoyl-CoA (C6:0), myristoyl-CoA (C14:0), palmitoyl-CoA (C16:0), stearoyl-CoA (C18:0), oleoyl-CoA (C18:1), arachidonoyl-CoA (C20:4), Triton X-100, sodium deoxycholate, iodine, butylated hydroxytoluene, 2',7'-dichlorofluorescein, DL-α-glycerophosphate, Tris-HCl, Tris-maleate, EDTA, EGTA and heptadecanoic acid methyl ester were obtained from Sigma Chemical Co.(St. Louis, Mo.).

Phosphatidylcholine (pig liver), phosphatidylethanolamine (pig liver), lysophosphatidylcholine (pig liver), lysophosphatidylethanolamine (pig liver),

Table 2

REPRESENTATIVE PROXIMATE ANALYSIS	R-M-H 3000 3200
Protein %	22.5
Fat %	5.5
Linoleic Acid %	1.5
Fiber %	4.5
Ash %	5.5
NFE %	52.0
Gross Energy Kcal/gm	4.4
Digestible Energy Kcal/gm	3.7
Metabolizable Energy Kcal/gm	3.5

REPRESENTATIVE VITAMIN CONTENT	R-M-H 3000 3200
Vitamin A IU/kg	20,229.00
Vitamin D_3 IU/kg	1,045.00
Vitamin E IU/kg	55.62
Thiamine mg/kg	11.50
Riboflavin mg/kg	10.38
Pantothenic Acid mg/kg	21.92
Niacin mg/kg	72.45
Pyridoxine mg/kg	8.84
Folic Acid mg/kg	1.67
Biotin mg/kg	0.40
Vitamin B_{12} mg/kg	0.06
Choline mg/kg	1,542.00
Menadione mg/kg	0.97

Agway rodent diet RMH 3000 composition

phosphatidylserine (beef brain), phosphatidylinositol (pig liver), 1,2-diacylglycerol (pig liver), 1,2,3-triacylglycerol (pig liver), phosphatidic acid (egg lecithin), 1-palmitoyl glycerol-3-phosphate and dipalmitoyl CDP-diacylglycerol were purchased from Serdary Research laboratories (London, Ontario). The purity of the above lipid standards were >98% as determined by the supplier based on chromatographic methods.

L-α-Dipalmitoyl-[2-palmitoyl-9,10-^{3}H]-phosphatidylcholine (50 Ci/mmol) and [1-^{14}C]arachidonyl-CoA (46.3 mCi/mmol) were obtained from NEN division, Dupont Co. (Dorval, Que.). [1-^{14}C]Palmitoyl-CoA (58 mCi/mmol), [1-^{14}C]stearoyl-CoA (55 mCi/mmol), [1-^{14}C]oleoyl-CoA (52.2 mCi/mmol), [1-^{14}C]linoleoyl-CoA (53.9 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine (57 mCi/mmol), 1-[1-^{14}C]-palmitoyl-lysophosphatidylcholine (59 mCi/mmol), 1,2-di-[1-^{14}C]-palmitoyl-phosphatidylethanolamine (52 mCi/mmol), myo-[2-^{3}H]inositol (18.2 Ci/mmol), [9,10(n)^{3}H]palmitic acid (500 mCi/mmol), 1-[^{14}C]-palmitoyl carnitine (57 mCi/mmol), tri-[9,10-^{3}H]olein (26.8 Ci/mmol), cholesteryl [1-^{14}C]oleate (57 mCi/mmol), [1<3>-^{3}H]glycerol (2.8 Ci/mmol), [5-^{3}H]cytidine 5'-triphosphate (24 Ci/mmol), [U-^{3}H] acetic anhydride (500 mCi/mmol), [U-^{3}H] methyl lidocaine (13.25 mCi/mg) (prepared through tritium gas exchange labelling), [γ-^{32}P] adenosine 5'-triphosphate and aqueous counting scintillant (ACS) were obtained from Amersham Canada Ltd. (Oakville, Ont.).

BF_3 methanol (12% w/w) and gas-liquid chromatographic columns consisting of 15% DEGS on 80/100 Chromosorb W AW were purchased from Supelco Canada Ltd. (Oakville, Ont.). Thin-layer chromatographic plates (Sil-G25) were products of Macherey-Nagel and purchased through Brinkmann Instruments (Rexdale, Ont.).

The protein kinase C and the cAMP assay systems were purchased from Amersham Canada Ltd. (Oakville, Ont.). All of the reagents used during their assay were provided in these kits.

Methyl lidocaine was a gift from Astra Pharmaceutical Products (Worcester, MA). All other chemicals were of analytical grade and were obtained through the Canlab Division of Baxter Diagnostics Corporation (Mississauga, Ont.) All solutions were prepared with glass distilled water and were adjusted to the desired pH.

3. Enzymes

Phospholipase A_2 (*Crotalus adamanteus* venom) and the catalytic subunit of cAMP-dependent protein kinase (bovine heart) was obtained from the Sigma Chemical Co. (St. Louis, Mo.). Phospholipase D (cabbage) was purchased from Boehringer Mannheim Canada Ltd.

II. Methods

1. Studies on Lysophospholipid Metabolism

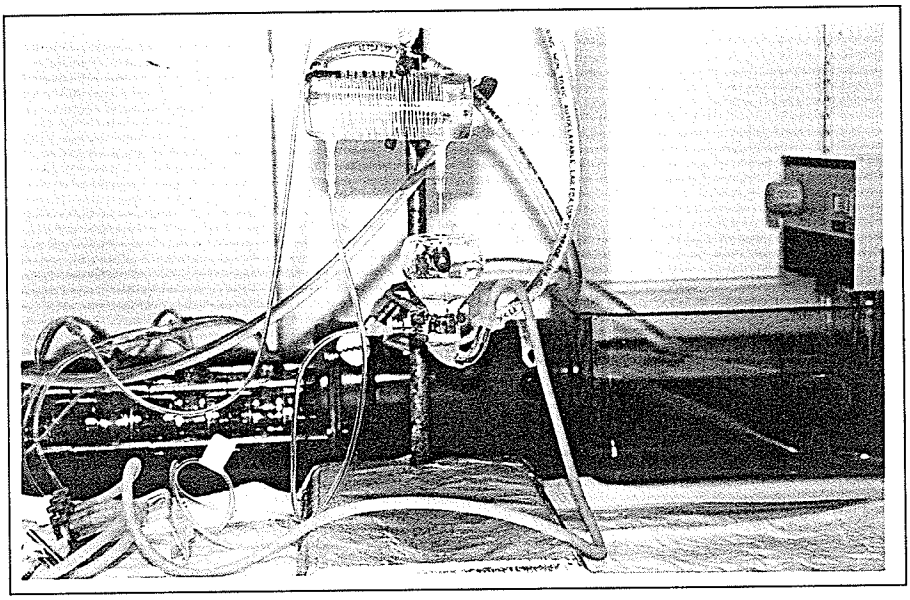
A. Preparation of Lysophosphatidylethanolamine

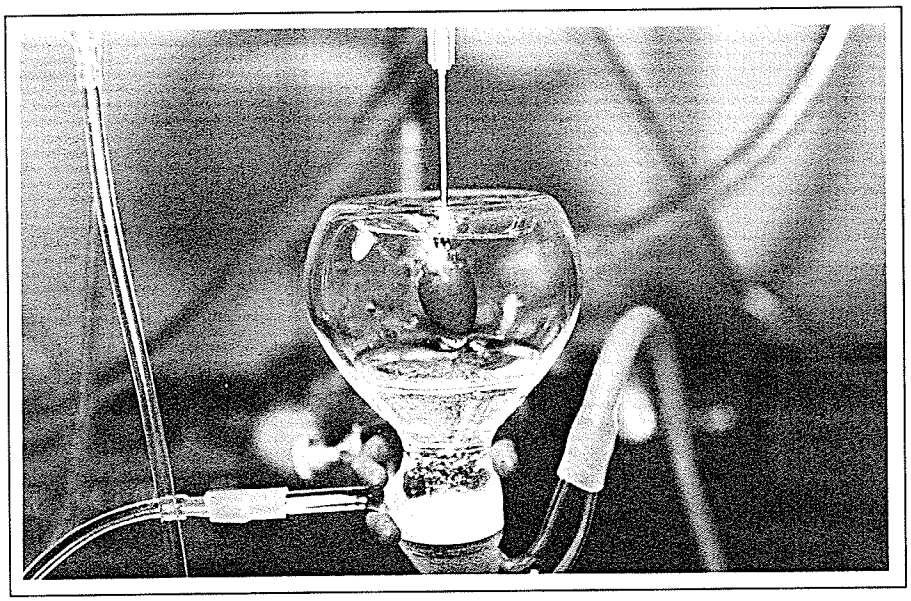
Labelled 1-[1-^{14}C]-palmitoyl-glycerophosphoethanolamine was prepared by the hydrolysis of 1,2-[1-^{14}C]-dipalmitoyl-phosphatidylethanolamine with *Crotalus adamanteus* venom phospholipase A_2 (Hanahan *et al.* 1954). Labelled phosphatidylethanolamine (7.5 μmoles) was dried down under a stream of nitrogen and resuspended in 6 ml of diethyl ether. The reaction was initiated by adding 150 μl of 0.1 M Tris-HCl (pH 7.4), 10 mM $CaCl_2$ and 100 units of phospholipase A_2 (295 units/mg protein). The reaction mixture was incubated at 25°C for 3 hours with continuous mixing. Subsequent to the reaction, labelled lysophosphatidylethanolamine was separated from other lipids by thin-layer chromatography (Sil-G25) in a solvent system of chloroform : methanol : acetic acid : water (70:30:2:4, v/v) (Zelinski *et al.* 1980). The hydrolysis was over 80% as determined from the radioactivity associated with the lysophosphatidylethanolamine fraction.

B. Perfusion of the Isolated Hamster Heart

The isolated hamster heart was perfused in the Langendorff mode (Langendorff 1895) with Krebs-Henseleit buffer (Krebs and Henseleit 1932) (Figure 14). The

Figure 14





Perfusion of the isolated hamster heart in the Langendorff mode

Krebs-Henseleit buffer (1000 ml) was prepared fresh prior to perfusion and contained 100 ml of solution A (containing 70.1 g/l sodium chloride, 21.0 g/l sodium bicarbonate and 9.91 g/l dextrose), 10 ml of solution B (containing 3.55 g/100 ml potassium chloride, 2.94 g/100 ml magnesium sulfate and 1.63 g/100 ml sodium phosphate, monobasic), 5 ml of solution C (containing 3.73 g/100 ml calcium chloride, dihydrate) and 885 ml of distilled water. The buffer was then saturated with 95% O_2 and 5% CO_2 (Zelinski and Choy 1982a). Perfusion took place at 37°C with a perfusion pressure of 80 mm Hg (at the cannulated aorta) and a coronary flow rate of 4.0-4.5 ml/min. The isolated heart was stabilized with Krebs-Henseleit buffer for 10 min and then perfused with 0.5 mg/ml methyl lidocaine for 30 min. In another set of experiments, 10 μM 1-[1-^{14}C]-palmitoyl-glycerophosphocholine (20 μCi/μmol) or 10 μM 1-[1-^{14}C]-palmitoyl-glycerophosphoethanolamine (5 μCi/μmol) was added to the perfusate along with bovine serum albumin (0.1 mg/ml) and 0.5 mg/ml methyl lidocaine for 30 min. Perfusions with the labelled lysophospholipid in the absence of methyl lidocaine were used as a control. Subsequent to perfusion, the heart was washed with 10 ml Krebs-Henseleit buffer, cut into small pieces, blotted dry and weighed. At this point the hearts were either used for the preparation of subcellular fractions or analyzed for their phospholipid content.

C. Preparation of Subcellular Fractions

Hamsters were sacrificed by decapitation and the hearts were removed and placed on ice or used immediately for perfusion as mentioned above. The hearts were

washed, weighed, cut into pieces, and homogenized in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA at 4°C to give a 10% w/v crude homogenate. The crude homogenate was centrifuged at 100 g for 10 min in order to remove the connective tissues and other cell debris. The remaining supernatant was collected and used as homogenate for enzyme assays. The microsomal fraction was prepared from the cellular homogenate by differential centrifugation (Zelinski *et al.* 1980). The cell homogenate was centrifuged at 10,000 g at for 10 min at 4°C and the resulting supernatant was recentrifuged at 100,000 g for 60 min. The resulting supernatant formed the cytosolic fraction. The microsomal pellet was resuspended in 0.25 M sucrose in 10 mM Tris-HCl (pH 7.4) using a Dounce homogenizer. The homogenate was used to assay lysophosphatidylcholine (LPC): acyl-CoA acyltransferase, lysophosphatidylethanolamine (LPE): acyl-CoA acyltransferase, phospholipase A and lysophospholipase activities. The microsomal fraction was used to assay the activity of both the LPC: acyl-CoA acyltransferase as well as the LPE: acyl-CoA acyltransferase. The protein concentrations in the subcellular fractions were determined by the method of Lowry *et al.* (1951).

D. Determination of Phospholipid Composition

Following perfusion, the hearts were weighed; and then chloroform : methanol (1:1, v/v) was added to give a 10% homogenate (w/v). Homogenization involved two 10 sec bursts with a Polytron homogenizer (Brinkmann Instruments; Rexdale, Ont.) equipped with a PT-30 probe. The homogenate was centrifuged at 2000 g for 10 min,

and the pellet was washed twice with chloroform : methanol (1:1, v/v). The supernatants were pooled and phase separation was achieved by the addition of water and chloroform until a chloroform : methanol : water ratio of 4:2:3 (v/v) was obtained. The aqueous phase was removed and the solvent in the organic phase was removed by evaporation *in vacuo*. The organic phase was resuspended in 3 ml of chloroform : methanol (2:1) and filtered through Whatman 1PS filter paper. The filtrate was dried under a stream of N_2 and resuspended in 1 ml of chloroform : methanol (2:1) and stored at -20°C. An aliquot of the total lipid extract was spotted onto a thin-layer chromatographic plate (Sil-G25) using known standards as carriers and markers. In the experiments where hearts were perfused with labelled lysophospholipids, fatty acid was separated from the other lipids by thin-layer chromatography in a solvent system containing petroleum ether : diethyl ether : acetic acid (70:30:2, v/v) (Skipski and Barclay 1969). The phospholipid fractions were visualized by the exposure of the plate to iodine vapour in a closed chamber. For the determination of radioactivity, the selected silica gel fractions were scraped into a scintillation counting vial along with 1 ml of water. Acetic acid (0.1 ml) was added to prevent chemiluminescence. The radioactivity was determined by liquid scintillation counting.

In experiments where phospholipid pool sizes were determined, the thin-layer chromatography plates were developed with a solvent system containing chloroform : methanol : acetic acid : water (70:30:2:4, v/v) (Zelinski *et al.* 1980). The various lipid fractions were visualized by spraying the plates with 0.25% 2'-7'-

dichlorofluorescein in ethanol (w/v) under UV light according to the method of Arvidson (1968). Lipid samples were scraped from the plates and the lipids were eluted by washing the silica gel with 4 ml of chloroform : methanol : water : acetic acid (50:39:10:1, v/v). The samples were centrifuged at 1000 g for 10 min and the supernatant was collected. The extraction was repeated twice more and 4 ml of 4M ammonium hydroxide 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 of methanol : water (1:1, v/v). The solvent in the lower phase was removed under a stream of N_2 and resuspended in 1 ml of chloroform : methanol (2:1, v/v). An aliquot of the sample was used for the determination of lipid phosphorus according to the method of Bartlett (1959). Recovery of phospholipids from the thin-layer chromatographic plate was over 95% based on the recovery of radioactivity and lipid phosphorus from known standards.

E. Acyl-CoA:1-acyl-glycerophosphocholine Acyltransferase (Lysophosphatidylcholine acyltransferase) (EC 2.3.1.23)

The enzyme activity in the hamster heart homogenate and microsomes was determined by using labelled oleoyl-CoA as an acyl donor (Arthur and Choy 1986; Arthur *et al.* 1987). The reaction mixture contained 20 mM Tris-HCl (pH 8.5), 100 nmol of 1-acyl-glycero-phosphocholine (pig liver), 86 nmol of 1-[1-^{14}C]oleoyl-CoA and the appropriate amount of enzyme protein. Methyl lidocaine (0 - 2.0 mg/ml) was added into the reaction mixture and the reaction was initiated by the addition of

labelled oleoyl-CoA to a final volume of 0.7 ml. The mixture was incubated at 25°C for 30 min and the reaction was terminated by the addition of 3 ml of chloroform : methanol (2:1, v/v). Phase separation was caused by the addition of 0.8 ml of 0.9% KCl to the mixture. After brief centrifugation, the upper phase was removed, and aliquots of the lower phase were separated by thin-layer chromatography (Sil Gel G) using unlabelled phosphatidylcholine (pig liver) as a carrier. The plate was developed in a solvent containing chloroform : methanol : water : acetic acid (70:30:4:2, v/v) (Zelinski *et al.* 1980). After development, the phosphatidylcholine fraction on the plate was visualized by exposure of the plate to iodine vapour. The silica gel containing the phosphatidylcholine fraction was removed from the plate and placed in a scintillation vial for radioactivity determination. The enzyme activity was calculated from the radioactivity associated with the phosphatidylcholine fraction.

F. Acyl-CoA:1-acyl-glycerophosphoethanolamine Acyltransferase (Lysophosphatidylethanolamine acyltransferase)

The enzyme activity in the hamster heart homogenate and microsomes was determined by using labelled oleoyl-CoA as an acyl donor (Arthur and Choy 1986, Arthur *et al.* 1987). The reaction mixture contained 20 mM Tris-HCl (pH 8.5), 100 nmol of 1-acyl-glycerophosphoethanolamine (pig liver), 86 nmol of 1-[1-^{14}C]oleoyl-CoA and the appropriate amount of enzyme protein. Methyl lidocaine (0 - 2.0 mg/ml) was added into the reaction mixture and the reaction was initiated by the addition of labelled oleoyl-CoA to a final volume of 0.7 ml. The mixture was

incubated at 25°C for 30 min and the reaction was terminated by the addition of 3 ml of chloroform : methanol (2:1, v/v). Phase separation was caused by the addition of 0.8 ml of 0.9% KCl to the mixture. After brief centrifugation, the upper phase was removed, and aliquots of the lower phase were separated by thin-layer chromatography (Sil Gel G) using unlabelled phosphatidylethanolamine (pig liver) as a carrier. The plate was developed in the same solvent as the 1-acyl-glycerophosphocholine assay and the radioactivity associated with the phosphatidylethanolamine band was used to calculate enzyme activity.

G. Preparation of Labelled Phosphatidylcholine

Phosphatidyl-[Me-^{3}H]choline was prepared by perfusion of isolated hamster heart in the Langendorff mode with labelled choline as previously described (Tam *et al.* 1984). The hamster heart was stabilized for 10 min in Krebs-Henseleit buffer followed by a 90 min perfusion in 5 μM [Me-^{3}H]choline (6 μCi/ml). Subsequent to perfusion, the heart was cut in half and homogenized in chloroform : methanol (1:1, v/v). The homogenate was centrifuged at 1000 g for 10 min and the resulting pellet was washed twice more. Chloroform and water were added to the pooled supernatant to form a final ratio of chloroform : methanol : water (4:2:3, v/v). After phase separation, the solvent in the lower phase was removed *in vacuo* and the lipids resuspended in chloroform : methanol (2:1, v/v). Labelled phosphatidylcholine was separated from the other lipids by thin-layer chromatography (Sil-G25) with a solvent system of chloroform : methanol : acetic acid : water (70:30:2:4, v/v). The

phosphatidylcholine fraction was identified by spraying the plate with 0.25% 2'-7'-dichlorofluorescein in ethanol (w/v). The labelled phosphatidyl[Me-^{3}H]choline eluted from the silica gel was found to have a specific radioactivity between 3000 - 4000 dpm/nmol.

H. Phospholipase A

Phospholipase A activity was determined in the hamster heart homogenate using phosphatidyl[Me-^{3}H]choline as a substrate (Cao *et al.* 1987b). The reaction mixture (0.5 ml) contained 20 mM Tris-HCl (pH 8.5), 5 mM calcium chloride and 1 μmol phosphatidyl[Me-^{3}H]choline dispersed in 0.2 ml water by sonication. Since the assay was performed using homogenate as an enzyme source, lysophospholipase activity in this fraction may affect the results. Hence, 200 nmol of unlabelled lysophosphatidylcholine was included in the reaction mixture. The presence of lysophosphatidylcholine inhibited any further hydrolysis of the newly formed lysophosphatidyl[Me-^{3}H]choline but did not inhibit the hydrolysis of phosphatidylcholine (Tam *et al.* 1984). The assay was initiated by the addition of 0.5 - 0.7 mg homogenate protein and incubated at 37°C for 30 min. The reaction was terminated by the addition of 1.5 ml chloroform : methanol (2:1, v/v). Water was added to the mixture to cause a phase separation. The solvent in the lower phase was removed by evaporation under nitrogen. The labelled lysophosphatidylcholine product was separated from the other lipids by thin-layer chromatography (Sil-G25) in a solvent containing chloroform : methanol : acetic acid : water (70:30:2:4, v/v).

I. Lysophospholipase

Lysophospholipase activity in the hamster heart homogenate was assayed using 1-[1-^{14}C]palmitoyl-glycerophosphocholine as a substrate (Cao *et al.* 1987a). The reaction mixture contained 20 mM Tris-HCl (pH 7.0), 0.2 mM labelled lysophosphatidylcholine (2000 dpm/nmol) and 0.5 - 0.7 mg homogenate protein. The reaction was initiated by the addition of labelled substrate. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 1.5 ml chloroform : methanol (2:1, v/v). Water was added to the mixture to cause phase separation. The solvent in the lower phase was removed under a stream of nitrogen. The labelled fatty acid released by the reaction was separated from other lipids by thin-layer chromatography (Sil-G25) in a solvent system of chloroform : methanol : acetic acid : water (70:30:2:4, v/v).

2. Studies on *de novo* Lipid Biosynthesis

A. Perfusion of the Isolated Hamster Heart

The isolated hamster heart was perfused in the Langendorff mode with Krebs-Henseleit buffer (pH 7.4) saturated with 95% O_2 - 5% CO_2 at a flow rate of 4.0 - 4.5 ml/min as previously described in section 1-B. After an initial 10 min stabilization period, the hearts were perfused with the same buffer containing 1 mM ^{3}H glycerol (3 μCi/μmol) and 0.5 mg/ml methyl lidocaine for various times. Perfusions with ^{3}H glycerol without methyl lidocaine were used as controls. Subsequent to perfusion the hamster hearts were washed with 10 ml Krebs-Henseleit buffer.

B. Preparation of Subcellular Fractions

Hamsters were sacrificed by decapitation and the hearts were removed and placed on ice. The hearts were washed, weighed, cut into pieces, and homogenized in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA at 4°C to give a 10% w/v crude homogenate. The crude homogenate was centrifuged at 100 g for 10 min in order to remove the connective tissues and other cell debris. The supernatant was collected and used as homogenate for enzyme assays. Subcellular fractions of the heart were prepared by differential centrifugation as described in section 1-C. The protein concentrations in the subcellular fractions were determined by the method of Lowry *et al.* (1951).

C. Determination of Phospholipid Composition

Following 60 min of perfusion, hamster hearts were weighed, cut into pieces, and homogenized in chloroform : methanol (1:1, v/v). The homogenate was centrifuged at 1000 g for 10 min, and the pellet was washed twice with chloroform : methanol (1:1, v/v) as described in section 1-D. The supernatants were pooled, and water and chloroform were added to cause phase separation. Aliquots of the aqueous and organic phases were taken for scintillation counting. The lower phase was collected and the lipid components were separated by thin-layer chromatography (Sil-G25). Separate aliquots of the lower phase were used to identify and quantitate the components making up the phospholipid and neutral lipid fractions. The phospholipid fraction was separated in a solvent containing chloroform : methanol : ammonium hydroxide : water (70:30:4:2, v/v) while the neutral lipids were separated in petroleum ether : diethyl ether : acetic acid (60:40:1, v/v). For the determination of radioactivity, the lipid fractions in the chromatogram were visualized by exposure to iodine vapour and the radioactivity associated with each band was determined by scintillation counting. For the determination of pool sizes, the thin-layer chromatographic plate was sprayed with 0.25% 2'-7'-dichlorofluorescein in ethanol (w/v) and the lipid fractions were visualized under UV light. The fractions were scraped from the chromatogram and the lipids were eluted from the silica gel as previously described in section 1-D. The lipid phosphorus content of each phospholipid class was determined by the method of Bartlett (1959).

D. Preparation of ^{14}C Phosphatidic Acid

Phosphatidic acid was synthesized enzymatically through the hydrolysis of 1-palmitoyl-2-[^{14}C]oleoyl-phosphatidylcholine by phospholipase D. The hydrolysis is a modification of the procedure of Comfurius and Zwaal (1977). The substrate, ^{14}C phosphatidylcholine (50 μmoles @ 2000 dpm/nmol) was dried down under N_2 and resuspended in 2 ml of diethyl ether. Phospholipase D (1.33 mg) was then dissolved in 2 ml of 100 mM sodium acetate (pH 5.6) in the presence of 100 mM $CaCl_2$. The phosphatidylcholine was then mixed with the enzyme suspension and placed in a screw capped tube. After 30 min of incubation at 42°C, the reaction mixture was cooled in ice and 1.33 mg of fresh enzyme was added to the mixture. The reaction mixture was reincubated at 42°C for another 30 min. The reaction was terminated by removing the diethyl ether under N_2. Phase separation was achieved by the addition of 6 ml of chloroform : methanol (2:1, v/v). The lower phase was analyzed by thin-layer chromatography with a solvent system of chloroform : methanol : water : acetic acid (50:37:2:3, v/v). The thin-layer chromatographic plate was sprayed with 0.25% 2'-7'-dichlorofluorescein in ethanol (w/v) and the phosphatidic acid fraction was visualized under UV light. The phospholipid was eluted from the silica gel as described in section 1-D.

E. Glycerol-3-phosphate Acyltransferase (EC 2.3.1.15)

Hamster heart homogenate was assayed for glycerol-3-phosphate acyltransferase activity (Batenburg *et al.* 1986) in the presence of various concentrations of methyl lidocaine (0.0 - 2.0 mg/ml). The assay buffer consisted of 100 mM Tris-HCl (pH 7.5), 1.5 mM glycerol-3-phosphate, 90 mM sucrose, 1 mg/ml BSA, 1 mM DTT, 40 μM [1-^{14}C]oleoyl-CoA (4000 dpm/nmol) and 0.1 - 0.2 mg of homogenate protein in a volume of 0.5 ml. The reaction was initiated with the addition of the enzyme source and the mixture was incubated at 30°C for 30 min. The reaction was terminated by the addition of 3 ml of chloroform : methanol (2:1, v/v). Water was added to cause phase separation, and the labelled lysophosphatidic acid in the lower phase was isolated by thin-layer chromatography with a solvent containing chloroform : methanol : ammonia hydroxide : water (70:30:4:2, v/v). The lysophosphatidic acid fraction was visualized by iodine vapour and the radioactivity determined. The amount of radioactivity associated with the lysophosphatidic acid fraction was used to calculate the glycerol-3-phosphate acyltransferase activity.

F. Lysophosphatidic Acid Acyltransferase (EC 2.3.1.20)

Hamster heart homogenate was assayed for lysophosphatidic acid acyltransferase activity (Batenburg *et al.* 1986) in the presence of various concentrations of methyl lidocaine (0.0 - 2.0 mg/ml). The assay buffer consisted of 100 mM Tris-HCl (pH 7.5), 0.2 mM 1-palmitoyl-glycerol-3-phosphate, 90 mM sucrose, 1 mg/ml BSA, 1 mM

DTT, 40 μM [1-^{14}C]oleoyl-CoA (4000 dpm/nmol) and 0.1 - 0.2 mg of homogenate protein in a volume of 0.5 ml. The reaction was initiated with the addition of the enzyme source and the mixture was incubated at 30°C for 30 min. The reaction was terminated by the addition of 3 ml of chloroform : methanol (2:1, v/v). Water was added to cause phase separation, and the labelled phosphatidic acid in the lower phase was isolated by thin-layer chromatography with a solvent containing chloroform : methanol : ammonia hydroxide : water (70:30:4:2, v/v). The phosphatidic acid fraction was visualized by iodine vapour and the radioactivity determined. The amount of radioactivity associated with the phosphatidic acid fraction was used to calculate the lysophosphatidic acid acyltransferase activity.

G. Phosphatidic Acid Phosphatase (EC 3.1.3.4)

Enzyme activity was assayed by determining the production of [^{14}C]diacylglycerol from mixed vesicles containing 1-palmitoyl-2-[^{14}C]oleoyl phosphatidic acid (prepared in section 2-D) and phosphatidylcholine (Martin *et al.* 1987) in the presence of various concentrations of methyl lidocaine (0.0 - 2.0 mg/ml). The assay buffer contained 100 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.2 mg of BSA, 0.6 mM 1-palmitoyl-2-[^{14}C]oleoyl phosphatidate (2000 dpm/nmol), 0.4 mM phosphatidylcholine, 1-20 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA and 0.75 mM oleic acid in an assay volume of 0.1 ml. Hamster heart post mitochondrial fraction (0.2-0.3 mg protein) was used as the enzyme source and was preincubated for 20 min in the presence of oleic acid at 37°C. Phosphatidylcholine, 1-palmitoyl-2-[^{14}C]oleoyl phosphatidate, EDTA

and EGTA were all sonicated together at 30 KHz until a uniform suspension was achieved. The reaction was initiated by the addition of $MgCl_2$. The reaction was carried out at 37°C for 60 min and stopped with 2 ml of chloroform : methanol (2:1, v/v). Water was added to cause phase separation and the labelled diacylglycerol product in the organic phase was analyzed by thin-layer chromatography in petroleum ether : diethyl ether : acetic acid (60:40:1, v/v). The diacylglycerol fraction was visualized by iodine vapour staining and the radioactivity of the fraction determined by scintillation counting. The amount of radioactivity associated with the diacylglycerol fraction was used to determine the activity of the phosphatidic acid phosphatase.

H. 1,2-Diacylglycerol Acyltransferase

Hamster heart homogenate was assayed for 1,2-diacylglycerol acyltransferase activity (Brindley and Bowley 1975) in the presence of various concentrations of methyl lidocaine (0.0-2.0 mg/ml). The assay system consisted of 25 mM Tris-HCl (pH 7.4), 50 μM [1-^{14}C]palmitoyl-CoA (2000 dpm/nmol), 2 mM 1,2-diacylglycerol, 18 mM $MgCl_2$, 1 mg/ml BSA and 1 mM DTT in a total volume of 0.5 ml. A 1,2-diacylglycerol emulsion was prepared by sonication in the above buffer at 30 KHz until a uniform suspension was obtained. The reaction was initiated by the addition of 0.2-0.3 mg homogenate protein. The reaction was carried out at 37°C for 10 min and stopped with 3 ml of chloroform : methanol (2:1, v/v). Water was added to cause phase separation, and the labelled triacylglycerol product in the organic phase

was separated and identified by thin-layer chromatography with a solvent containing petroleum ether : diethyl ether : acetic acid (60:40:1, v/v). The triacylglycerol fraction was visualized by iodine vapour staining and the radioactivity of the fraction determined by scintillation counting. The amount of radioactivity associated with the triacylglycerol fraction was used to calculate the 1,2-diacylglycerol acyltransferase activity.

I. CTP: Phosphatidic Acid Cytidylyltransferase (EC 2.7.7.41)

Hamster heart homogenate was measured for CTP: phosphatidic acid cytidylyltransferase activity in the presence of various concentrations of methyl lidocaine (0.0-2.0 mg/ml). The reaction mixture contained 50 mM Tris-maleate (pH 6.5), 20 mM $MgCl_2$, 1% Triton X-100, 1.0 mM [5-^{3}H]CTP (10,000 dpm/nmol), 0.5 mM phosphatidic acid and 0.3-0.4 mg of homogenate protein (Kelley and Carman 1987). The phosphatidic acid, in the buffer, was sonicated at 30 KHz until an even suspension was achieved. The reaction was initiated by the addition of the enzyme source and the mixture was incubated at 30°C for 5 min. The reaction was stopped with 2.5 ml of 0.1 M HCl in methanol and placed on ice for 15 min. Controls were stopped immediately prior to the incubation. After cooling, 5 ml of chloroform was added to each mixture and allowed to sit at room temperature for 30 min. The resultant chloroform : methanol mixture was washed 3 times with 10 ml of 2 M $MgCl_2$ (Belendiuk *et al.* 1978). The labelled CDP-diacylglycerol in the organic phase was analyzed by thin-layer chromatography with a solvent containing chloroform :

methanol : acetic acid : water (25:14:2:4, v/v). The CDP-diacylglycerol fraction was visualized by iodine vapour staining and the radioactivity in the fraction determined by scintillation counting. The radioactivity associated with the CDP-diacylglycerol fraction was used to determine the activity of the CTP: phosphatidic acid cytidylyltransferase.

J. Phosphatidylinositol Synthase (EC 2.7.8.11)

Hamster heart post mitochondrial fraction was assayed for phosphatidylinositol synthase in the presence of various concentrations of methyl lidocaine (0.0-2.0 mg/ml). The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 1 mM EGTA, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 0.2% Triton X-100, 5 mM CDP-dipalmitoyl glycerol and 5 mM *myo*-[^{3}H]inositol (Imai and Gershengorn 1987). The reaction was carried out at 37°C for 30 min. The reaction was terminated by the addition of 1 ml of chloroform : methanol : concentrated HCl (100:100:1, v/v) and 0.4 ml of 0.9% KCl. The mixtures were centrifuged at 2000 g for 5 min and the labelled phosphatidylinositol in the organic phase was analyzed by thin-layer chromatography with a solvent containing chloroform : methanol : acetic acid : water (50:30:8:4, v/v). The phosphatidylinositol fraction was visualized by iodine vapour staining and the radioactivity in the fraction determined by scintillation counting. The radioactivity associated with the phosphatidylinositol fraction was used to determine the activity of the phosphatidylinositol synthase.

K. Serine Base Exchange

Hamster heart microsomal fraction was assayed for the serine base exchange reaction in the presence of various concentrations of methyl lidocaine (0.0-2.0 mg/ml). The reaction mixture contained 10 μM Hepes (pH 7.3), 0.8 μmol phosphatidylcholine, 20 nmol ^{3}H serine (78000 dpm/nmol), 6 μmol $CaCl_2$, 50 μg of bovine serum albumin and the microsomal fraction in a total volume of 250 μl (Miura and Kanfer 1976). The reaction was carried out at 37°C for 15 min in a shaking water bath. The reaction was stopped with 2 ml of chloroform : methanol (2:1, v/v) and water was added to cause phase separation. The tubes were centrifuged at 2000 g for 5 min and the labelled phosphatidylserine in the organic phase was analyzed by thin-layer chromatography with a solvent containing chloroform : methanol : ammonia hydroxide : water (70:30:4:2, v/v). The phosphatidyserine fraction was visualized by iodine vapour staining and the radioactivity in the fraction determined by scintillation counting.

L. Quantitation of 1,2-Diacylglycerol Levels

Hamster hearts were perfused for 60 min in the presence or absence of 0.5 mg/ml methyl lidocaine. Following perfusion, a total lipid extraction was performed on the heart as detailed in section 1-D. The diacylglycerol fraction was isolated by thin-layer chromatography in a solvent system containing petroleum ether : diethyl ether : acetic acid (60:40:1, v/v). The diacylglycerol was identified by iodine vapour and

eluted from the silica gel with 5 ml of chloroform : methanol (1:1, v/v) and 100 μl of water. The suspension was centrifuged at 2000 g for 5 min and the supernatant decanted into a fresh tube. The pellet was resuspended in 5 ml of chloroform : methanol (1:1, v/v) and recentrifuged. The supernatants were combined and dried under N_2. The purified diacylglycerol was suspended in 1 ml of chloroform. The diacylglycerol fraction was quantitated by acetylation using ^3H acetic anhydride (Ishidate and Weinhold 1981). Various aliquots of diacylglycerol were dried under N_2, resuspended in 125 μl of pyridine and subsequently 260 μmol ^3H acetic anhydride (200-500 dpm/nmol) and 2 μl of 70% perchloric acid. The reaction was carried out at 70°C for 30 min, mixed for 10 sec and reincubated for another 30 min. After cooling the reaction on ice, 1 ml water was added and the tubes were mixed vigorously. The reaction mixture was extracted three times with 1.5 ml hexane. The hexane extract containing the acetylated diacylglycerol was washed three times with 1.5 ml of 50% methanol. The radioactivity associated with the hexane phase was determined by scintillation counting.

M. Quantitation of Triacylglycerol Levels

Hamster hearts were perfused for 60 min in the presence or absence of 0.5 mg/ml methyl lidocaine. Following perfusion, a total lipid extraction was performed on the heart as detailed in section 1-D. The triacylglycerol fraction was isolated by thin-layer chromatography in a solvent system containing petroleum ether : diethyl ether : acetic acid (60:40:1, v/v). Triacylglycerol was identified by iodine vapour and eluted

from the silica gel with 5 ml of chloroform : methanol (1:1, v/v) and 100 μl of water. The suspension was centrifuged at 2000 g for 5 min and the supernatant decanted into a fresh tube. The pellet was resuspended in 5 ml of chloroform : methanol (1:1, v/v) and recentrifuged. The supernatants were combined and dried under N_2. The purified triacylglycerol was suspended in 1 ml of chloroform and quantitated by determining the fatty acid content (Metcalfe and Schmitz 1961: Groener *et al.* 1979). An aliquot of the purified triacylglycerol fraction was dried under N_2 in a screw capped tube. BF_3-methanol (0.5 ml) was added to the triacylglycerol and sonicated for 1 min to disperse the lipid. The samples were covered with teflon, capped and placed in boiling water for 5 min. The reaction was terminated by cooling the tubes on ice, followed by the addition of 0.5 ml of water and 1.5 ml of petroleum ether. The tubes were mixed and centrifuged at 2000 g for 5 min to achieve phase separation. The lower phase was washed with another 1.5 ml of petroleum ether. The upper phase from the wash was pooled with the original upper phase, and the solvent in the pooled fraction was removed by N_2. The efficiency of methylation was found to be over 95%. The resulting fatty acid methyl esters were suspended in 100 μl of heptane and injected into 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 quantitation. The data obtained was analyzed by a Shimadzu Chromatopac CR601 integrator.

N. Quantitation of CDP-Diacylglycerol Levels

Hamster hearts were perfused for 60 min in the presence or absence of 0.5 mg/ml methyl lidocaine. Following perfusion, a total lipid extraction was performed on the heart as detailed in section 1-D. The CDP-diacylglycerol fraction was isolated by thin-layer chromatography in a solvent system containing chloroform : methanol : ammonia hydroxide : water (70:30:4:2, v/v). The CDP-diacylglycerol was visualized with iodine vapour and eluted from the silica gel. CDP-diacylglycerol content was determined by fatty acid methyl ester quantitation as discussed in section 2-M.

O. Quantitation of Long-Chain Acyl-CoA Levels

Hamster hearts were perfused in the presence or absence of 0.5 mg/ml methyl lidocaine for 60 min. Long-chain acyl-CoA levels were determined by the method of Tardi *et al.* (1992). Following perfusion, the hearts were placed into liquid N_2. The long-chain acyl-CoA levels were then determined according to the method described in section 3.

3. Determination of Long-Chain Acyl-CoA Levels

A. Preparation of Sample

Mature male Sprague Dawley rats and Syrian Golden hamsters were sacrificed by decapitation, and the organ was rapidly removed and placed in liquid nitrogen. The frozen tissue (1 g) was placed in a test tube containing 10 ml of chloroform : methanol : water (1:1.5:0.2, v/v). Labelled long-chain acyl-CoA (0.1 μCi) or 100 nmol of long-chain acyl-CoA was added as an internal standard. The tissue was homogenized by a PT-30 polytron generator. In some experiments, 1 μCi of labelled phosphatidylcholine, palmitic acid, palmitoyl carnitine, cholesteryl oleate or triolein was added prior to homogenization.

B. Extraction of Long-Chain Acyl-CoA

The homogenate was centrifuged at 2000 g for 5 min. The supernatant was removed and the pellet was extracted twice with 5 ml of chloroform : methanol : water (1:1.5:0.2, v/v). The supernatants were pooled and 1 mg of butylated hydroxytoluene was added to minimize the oxidation of the unsaturated long-chain acyl-CoA. Chloroform and water were added until the ratio of chloroform : methanol : water was 4:2:3 (v/v). Phase separation of the mixture was achieved by centrifugation at 2000 g. The aqueous phase, containing the long-chain acyl-CoAs, were removed and the organic phase was extracted twice with 10 ml of theoretical aqueous phase (TAP)

containing chloroform : methanol : water (3:48:47, v/v) (Sheltawy and Dawson 1969). The pooled aqueous phases were reduced to 3 ml by evaporation under reduced pressure. The long-chain acyl-CoA was then partitioned from the aqueous phase into the organic phase with ammonium sulphate. The pooled aqueous phase was shaken with 6 ml chloroform and 0.75 ml saturated ammonium sulphate, and centrifuged at 2000 g for 10 min. The organic phase (containing long-chain acyl-CoAs and some white precipitate) was removed and the aqueous phase was re-extracted twice with 10 ml of theoretical organic phase (TOP) containing chloroform : methanol : water (86:14:1, v/v) (Sheltawy and Dawson 1969). The extracts were pooled, with the original lower phase, and centrifuged at 2000 g for 5 min (to pellet the white precipitate). The volume of the supernatant was reduced under N_2 and the long-chain acyl-CoAs were extracted back into the aqueous phase by the addition of 5 ml of TAP. After centrifugation, the aqueous phase was removed and the organic phase was re-extracted two times with 5 ml TAP. Vigorous mixing was essential for high acyl-CoA recovery during the extraction.

C. Analysis of Long-Chain Acyl-CoA

Samples obtained after phase partition were applied to a thin-layer chromatographic plate (G-25). The plate was developed in *n*-butanol : acetic acid : water (4:1:2, v/v). The fraction containing long-chain acyl-CoA (R_f 0.43) was identified by comparison with acyl-CoA standards. The R_f for long-chain acyl-CoA ($>C_{12}$) is different from hexanoyl-CoA and octanoyl-CoA (0.20), fatty acids (0.85), acyl carnitine (0.52),

phosphatidylcholine (0.52) and phosphatidylethanolamine (0.65). The acyl-CoA in the fraction was eluted from the silica gel with 10 ml of the developing solvent. Acyl-CoA content in the sample was determined by gas-liquid chromatography. The acyl groups in the sample were converted into methyl esters in the presence of boron trifluoride (Metcalfe and Schmitz 1961; Barron and Mooney 1968). The methylation procedure was previously described in section 2-M.

4. Mechanism of Action

A. Perfusion of the Isolated Hamster Heart

The isolated hamster heart was perfused in the Langendorff mode with Krebs-Henseleit buffer (pH 7.4) saturated with 95% O_2 - 5% CO_2 at a flow rate of 4.0 - 4.5 ml/min as previously described in section 1-B. After an initial 10 min stabilization period, the hearts were perfused with the same buffer containing 0.01 mg/ml or 0.02 mg/ml ^3H methyl lidocaine (6000 dpm/nmole) for 30 min. Subsequently, the hearts were perfused for 1 min in Krebs-Henseleit buffer and then for 30 min with fifty times the concentration of unlabelled methyl lidocaine. The hearts were then transferred back to Krebs-Henseleit buffer for a final 15 min wash. The hearts were homogenized and subcellular fractions were prepared. In another set of experiments, hamster hearts were used to study the effect of methyl lidocaine on lipid enzyme activity *in vivo*. In this study, hearts were stabilized and then perfused for 5 min in the presence of 0.5 mg/ml methyl lidocaine, with or without 1 mM glycerol. Following perfusion, subcellular fractions were prepared in the presence of 25 mM NaF. These perfusion conditions were also used for the determination of protein kinase C acitivity and cAMP levels within the hamster heart.

B. Preparation of Subcellular Fractions

For the subcellular distribution of ^{3}H methyl lidocaine following perfusion, the hearts were cut into pieces, and homogenized in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA at 4°C to give a 10% w/v crude homogenate. Subcellular fractions were prepared and the radioactivity associated with these fractions was determined through liquid scintillation counting. For studies on lipid enzyme activity *in vivo*, the homogenizing buffer was supplemented with 25 mM NaF to inhibit the action of phosphatases (Holmes *et al.* 1990). This buffer was also used for the cAMP-dependent protein kinase experiments. The crude homogenate was centrifuged at 100 g for 10 min in order to remove the connective tissue and other cell debris. The remaining supernatant was collected and used as homogenate for enzyme assays. The remaining subcellular fractions were prepared as previously described in section 1-C. For experiments involving protein kinase C analysis, the homogenizing buffer consisted of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA and 0.3% DTT (w/v). The microsomal fraction was resuspended in 1 ml of the homogenizing buffer using a dounce homogenizer. The protein concentrations in the subcellular fractions were determined by the method of Lowry *et al.* (1951).

C. Protein Kinase C

Hamster heart microsomal and cytosolic fractions were assayed for protein kinase C activity subsequent to methyl lidocaine perfusion. In this assay, the γ-phosphate of

adenosine-5'-triphosphate (ATP) was transferred to the threonine group on a peptide that is specific for protein kinase C. The assay was a modification of the method of Hannun (1985) and was carried out according to the procedure described in the Amersham protein kinase C assay system. The reaction mixture consisted of 50 mM Tris-HCl (pH 7.5), 12 mM calcium acetate, L-α-phosphatidyl-L-serine, 24 μg/ml phorbol 12-myristate 13-acetate, 900 μM peptide, 30 mM dithiothreitol and 0.05% w/v sodium azide in a total volume of 25 μl. Microsomal or cytosolic fractions were then added (25 μl) followed by the addition of 25 μl of magnesium/ATP buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 μM [γ-^{32}P] ATP (66.6 μCi/μmol), 45 mM magnesium acetate and 0.05% w/v sodium azide. The blank for the assay was represented by reactions lacking any subcellular fraction. The reaction mixture was mixed and incubated at 25°C for 15 min. The reaction was terminated with 100 μl of the stop solution and then spotted onto binding paper. The sample in the binding paper was washed in 15 ml of 5% acetic acid and 10 mM tetra sodium pyrophosphate for 15 min. The binding paper was then transferred to a fresh wash solution for another 15 min. Subsequently, the paper was washed in 95% ethanol for 5 min, dried and the radioactivity associated with the paper determined (Lin *et al.* 1982). Protein kinase C activity was measured as the phosphorylated peptide bound to the paper.

D. Cyclic AMP

Cyclic AMP (cAMP) levels were determined in hamster hearts perfused with or without 0.5 mg/ml methyl lidocaine. Following perfusion, hamster hearts were

immediately immersed in liquid N_2. Once frozen, the hearts were weighed and homogenized in 5 ml of 95% ethanol. The samples were mixed occasionally at room temperature for 5 min and then centrifuged at 1000 g for 10 min. The supernatant was collected and the pellet was resuspended in 3 ml of 95% ethanol : water (2:1, v/v). The sample was then re-centrifuged and the supernatants pooled. The samples were dried under a stream of N_2 and assayed for cAMP according to the methods described in the Amersham cAMP assay system. Briefly, the sample was resuspended in 1 ml of 50 mM Tris-HCl (pH 7.5) and 4 mM EDTA. The assay mixture contained 50 μl of [8-^{3}H]adenosine 3',5'-cyclic phosphate (27.8 μCi/nmol), 100 μl of cAMP binding protein and 50 μl of sample. A standard curve of cAMP also was prepared, ranging from 0 - 16 pmoles. The reaction mixture was mixed and incubated at 4°C for 2 hours. Separation of the protein bound cAMP from the unbound nucleotide was achieved by adsorption of the free nucleotide onto 100 μl of coated charcoal, followed by centrifugation at 12000 g for 2 min at 4°C. From the resulting supernatant a 200 μl aliquot was removed and the radioactivity determined. The amount of cAMP present in the sample was determined by using the cAMP standard curve.

E. Cyclic AMP-Dependent Protein Kinase

Hamster heart homogenate was prepared from unperfused hearts as discussed in section 4-B. The effect of cAMP-dependent protein kinase (catalytic subunit) on lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase was

investigated. The reaction mixture contained 60 μl of homogenate, 2 mM ATP, and the presence or absence of 100 units of the catalytic subunit of cAMP-dependent protein kinase (1 unit of kinase activity will transfer 1 pmole of phosphate onto casein in 1 min). The homogenate was incubated with the kinase for 10 min at 37°C and then assayed for enzyme activity.

F. Enzyme Assays

All lipid enzyme assays were performed as described in section 2 with the exception of phosphatidic acid phosphatase and CTP: phosphatidic acid cytidylyltransferase. In the case of the phosphatase, the reaction was carried out in the presence of 1 mM magnesium chloride. The cytidylyltransferase reaction was carried out in the presence of 7.5 mM magnesium chloride.

5. Other Procedures

A. Determination of Lipid Phosphorus

The lipid phosphorus content in the samples were determined by the method of Bartlett (1959). An aliquot of the sample was taken and the solvent was evaporated under nitrogen. Inorganic phosphorus was used as a standard (0-10 μg/tube). The inorganic phosphorus standard was prepared by dissolving 0.011 g of potassium phosphate in 250 ml water (10 μg phosphorus/ml). Perchloric acid (1.1 ml) was added to each sample and the mixture was incubated at 160°C for 2 hours. The mixture was cooled to room temperature and the appropriate amount of inorganic phosphorus was added to the standard tubes in a total volume of 1 ml. Subsequently, 8 ml of water and 800 μl of 5% ammonium molybdate (w/v) was added and the contents of the tubes were mixed. A 200 μl solution of ANSA (1-amino-2-napthol-4-sulfonic acid) was added and the tubes contents were again mixed. These tubes were placed in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 830 nm. The ANSA reagent was prepared by dissolving 11.7 g sodium sulfite (anhydrous), 80 mg sodium metabisulfite and 0.2 g of ANSA in 100 ml of water. This solution is stable in the dark for up to two months.

B. Protein Determination

Protein concentrations of subcellular fractions were determined by the modified method of Lowry *et al.* (1951). Bovine serum albumin (1 mg/ml) was used as standard (0-100 μg). The volume of each tube was made up to 1 ml by the addition of 0.1 ml 5% sodium deoxycholate (w/v) and water. Solution A was made by dissolving equal volumes of 1% copper sulphate (w/v) and 2% potassium sodium tartrate (w/v). To each tube, 4 ml of solution B (containing 1 ml of solution A in 50 ml of 2% sodium carbonate (w/v) in 0.1 M sodium hydroxide) was added and mixed. The reaction mixture was incubated at room temperature for 10 min followed by the addition of 0.5 ml of 1 N phenol reagent. The tubes were immediately mixed and incubated at 60°C for 10 min. The absorbance was measured at 730 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer.

C. Radioactivity Determination

Radioactivity was determined using an LKB liquid scintillation counter (1211 MiniBeta) and the counting efficiency was calculated by the channels' ratio calibration method.

D. Statistical Analysis

Student's t-test was used for all statistical analysis. The level of significance was set at $P < 0.05$. All the results are expressed as the mean ± standard deviation of at least three separate experiments done in duplicate unless otherwise stated.

EXPERIMENTAL RESULTS

I. Studies on Lysophospholipid Metabolism

1. Introduction

The acylation of lysophospholipids serves a number of important cellular functions. It is part of the mechanism for the remodelling of the fatty acyl chains of cellular phospholipids (Lands 1960), as well as an alternate pathway for the removal of the cellular lysophospholipids. Lysophospholipids have been shown to be potent cytolytic agents at high concentrations (Weltzein 1979). Indeed, the accumulation of lysophospholipids in the ischemic heart has been suggested as a biochemical cause for cardiac dysfunctions, including the development of cardiac arrhythmias (Katz and Messineo 1981b; Man and Choy 1982; Corr *et al.* 1984).

Although the acyltransferases responsible for the acylation of lysophosphatidylcholine and lysophosphatidylethanolamine have been studied by a number of investigators, most of these studies were directed towards the acyl specificity of the enzymes (Choy and Arthur 1989). In some other studies, local anesthetics (Shier 1977; Sanjanwala *et al.* 1988), clofibric acid (Kawashima *et al.* 1986) and neuroanabolic drugs (Parthasarathy *et al.* 1981) were used to modulate acyl-CoA acyltransferase activities. However, the effects of these compounds on the other lysophospholipid metabolic enzymes or on the acylation of lysophospholipids *in vivo* are not known. In this study,

methyl lidocaine was used as a probe to study the physiological role of the enzymes involved in the maintenance of lysophospholipid levels in the mammalian heart.

2. The Effect of Methyl Lidocaine on the Lysophospholipid Metabolic Enzymes in the Hamster Heart

A. Lysophospholipid Acyltransferases

Since acyltransferases have been shown to be modulated by local anesthetics (Shier 1977), the effects of methyl lidocaine on both lysophosphatidylcholine and lysophosphatidylethanolamine: acyl-CoA acyltransferase activities were examined. Methyl lidocaine was found to inhibit lysophosphatidylcholine (LPC): acyl-CoA acyltransferase in the tissue homogenate as well as the microsomal fraction (Table 3). The effects of methyl lidocaine on lysophosphatidylethanolamine (LPE): acyl-CoA acyltransferase was also investigated. In the presence of increasing concentrations of methyl lidocaine, there was a significant inhibition of acyltransferase activity (Table 4). Inhibition of enzyme activity was observed in the homogenate as well as the microsomal fraction. However, the inhibition of LPC: acyl-CoA acyltransferase was much more severe than the inhibition of the LPE: acyl-CoA acyltransferase. Methyl lidocaine, at 0.5 mg/ml, caused a 71% inhibition of the microsomal LPC: acyl-CoA acyltransferase activity, whereas at the same concentration, a 41% inhibition of the microsomal LPE: acyl-CoA acyltransferase activity was obtained.

TABLE 3

Effect of methyl lidocaine on lysophosphatidylcholine (LPC): acyl-CoA acyltransferase activity

Acyltransferase activity was determined in hamster heart homogenate and microsomal preparations in the presence and absence of methyl lidocaine. Each value represents the mean ± standard deviation of four separate experiments.

Methyl lidocaine (mg/ml)	LPC: acyl-CoA acyltransferase activity	
	Homogenate	Microsomes
	(nmol/hour/mg protein)	
Control (no addition)	72 ± 14	343 ± 20
0.2	50 ± 10 *	214 ± 22 *
0.5	20 ± 6 *	101 ± 21 *
1.0	13 ± 3 *	57 ± 13 *
2.0	5 ± 1 *	25 ± 6 *

* $p < 0.05$

TABLE 4

Effect of methyl lidocaine on lysophosphatidylethanolamine (LPE): acyl-CoA acyltransferase activity

Acyltransferase activity was determined in hamster heart homogenate and microsomal preparations in the presence and absence of methyl lidocaine. Each value represents the mean ± standard deviation of four separate experiments.

Methyl lidocaine (mg/ml)	LPE: acyl-CoA acyltransferase activity	
	Homogenate	Microsomes
	(nmol/hour/mg protein)	
Control (no addition)	7.0 ± 2.4	32 ± 7
0.2	5.4 ± 1.2	25 ± 6
0.5	4.2 ± 1.1	19 ± 5 *
1.0	2.5 ± 0.5 *	12 ± 4 *
2.0	2.0 ± 0.5 *	9 ± 4 *

* $p < 0.05$

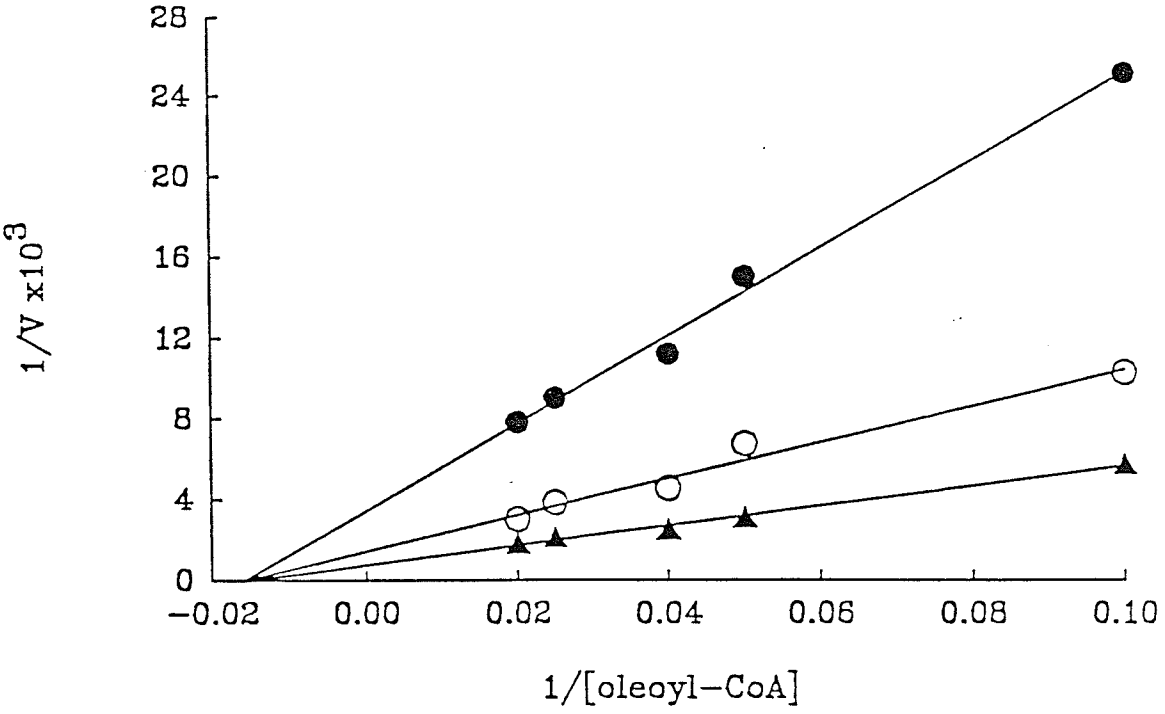
B. Kinetic Studies

The mode of inhibition of the acyltransferases by methyl lidocaine was investigated using enzyme kinetics. Enzyme activities were determined at various substrate concentrations in the presence and absence of methyl lidocaine. The data obtained were analyzed by double reciprocal plots of acyltransferase activity vs different concentrations of oleoyl-CoA (Figure 15) or lysophospholipid (Figure 16). The results indicate that the inhibition of lysophosphatidylethanolamine: acyl-CoA acyltransferase, by methyl lidocaine, was essentially non-competitive. In a separate experiment (data not shown), the inhibition of lysophosphatidylcholine: acyl-CoA acyltransferase by methyl lidocaine was also found to be essentially non-competitive.

C. Effect on Phospholipase A and Lysophospholipase

The effect of methyl lidocaine on the other lysophospholipid metabolic enzymes was also studied. As shown in Table 5, methyl lidocaine had no effect on phospholipase A activity in the hamster heart homogenate at low concentrations (0.2-0.5 mg/ml). However, a small inhibitory effect was displayed by the drug at higher concentrations (1-2 mg/ml). The activity of lysophospholipase in the presence of methyl lidocaine was also investigated (Table 5). At all concentrations tested, methyl lidocaine had no effect on lysophospholipase activity in hamster heart homogenate.

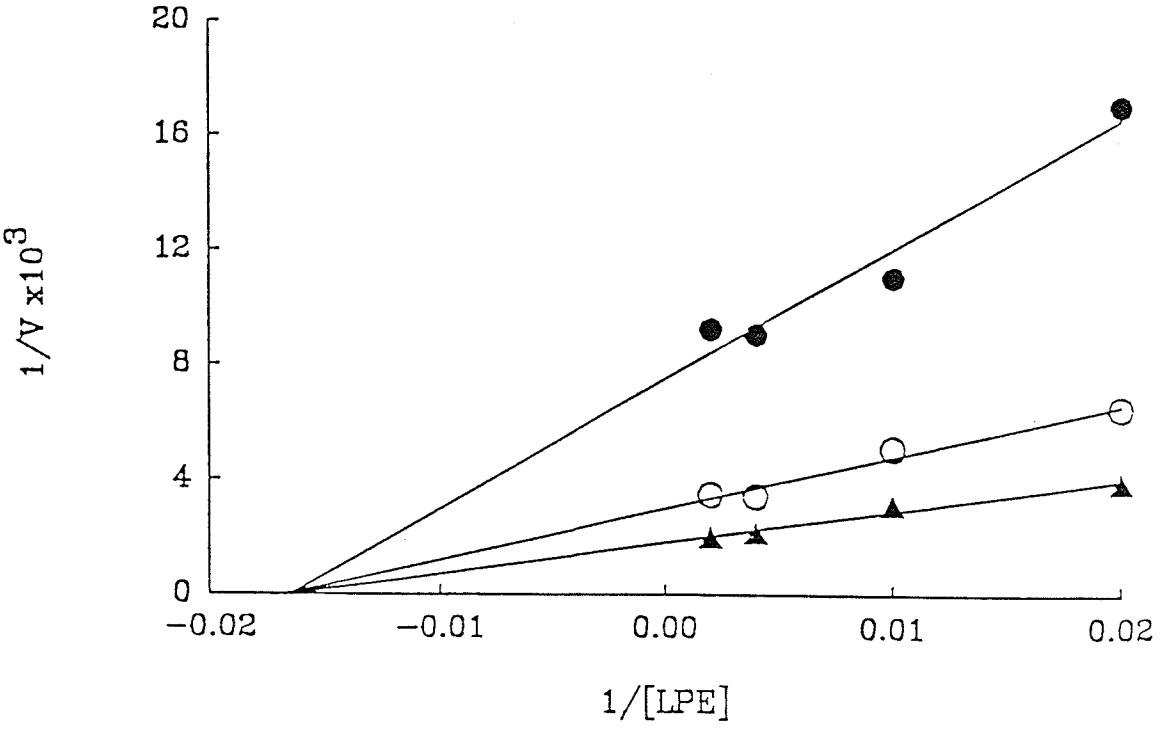
Figure 15



Double reciprocal plot of microsomal lysophosphatidylethanolamine: acyl-CoA acyltransferase activity versus oleoyl-CoA concentrations.

Lysophosphatidylethanolamine: acyl-CoA acyltransferase activity was determined at 25°C with 0.18 mg microsomal protein and was expressed as nmol/hour/mg protein. Enzyme activity was assayed with 140 μM lysophosphatidylethanolamine (LPE) and 10-50 μM oleoyl-CoA. The assays were performed in the absence (▲) and the presence of 0.5 mg/ml (○) or 1.0 mg/ml methyl lidocaine (●).

Figure 16



Double reciprocal plot of microsomal lysophosphatidylethanolamine: acyl-CoA acyltransferase activity versus lysophosphatidylethanolamine concentrations.

Lysophosphatidylethanolamine: acyl-CoA acyltransferase activity was determined at 25°C with 0.18 mg microsomal protein and was expressed as nmol/hour/mg protein. Enzyme activity was assayed with 120 μM oleoyl-CoA and 50-500 μM of lysophosphatidylethanolamine (LPE). The assays were performed in the absence (▲) and the presence of 0.5 mg/ml (○) or 1.0 mg/ml methyl lidocaine (●).

TABLE 5

Effect of methyl lidocaine on phospholipase A and lysophospholipase activities in hamster heart homogenate

Phospholipase A and lysophospholipase activities in hamster heart homogenate were assayed in the presence and absence of methyl lidocaine. Each value represents the mean ± standard deviation of four separate experiments.

Methyl lidocaine (mg/ml)	Phospholipase A activity	Lysophospholipase activity
	(nmol/hour/mg protein)	
Control (no addition)	4.5 ± 0.3	16.3 ± 1.1
0.2	4.2 ± 0.4	16.0 ± 0.8
0.5	4.6 ± 0.3	15.8 ± 0.7
1.0	3.8 ± 0.3 *	16.1 ± 0.8
2.0	3.6 ± 0.4 *	16.4 ± 1.0

* $p < 0.05$

3. The Effect of Methyl Lidocaine on Phospholipid Metabolism in the Isolated Hamster Heart

A. Effect of Methyl Lidocaine on Lysophospholipid Metabolic Enzymes

In order to study the contribution of the acylation process to the metabolism of lysophospholipids, hamster hearts were perfused in Krebs-Henseleit buffer containing 0.5 mg/ml methyl lidocaine. Subsequent to perfusion, the heart was homogenized and activities of the enzymes for the metabolism of lysophospholipids in the homogenate were assayed. As shown in Table 6, no significant change in the activities of phospholipase A and lysophospholipase was detected. However, both lysophosphatidylcholine: acyl-CoA acyltransferase and lysophosphatidylethanolamine: acyl-CoA acyltransferase activities were significantly inhibited by methyl lidocaine perfusion. At 0.5 mg/ml methyl lidocaine in the perfusate, a 43% and 30% decrease in lysophosphatidylcholine: acyl-CoA acyltransferase and lysophosphatidylethanolamine: acyl-CoA acyltransferase activities, respectively were observed. Since the majority of the acyltransferase activities were found to locate in the microsomal fraction, the enzyme activities in the microsomes were also determined. The degree of inhibition observed in the microsomal acyltransferases was similar to that observed in the homogenate.

TABLE 6

Activities of lysophospholipid metabolic enzymes in hamster heart perfused with methyl lidocaine

Hamster hearts were perfused in Krebs-Henseleit buffer in the presence and absence of 0.5 mg/ml methyl lidocaine. Subsequent to perfusion, the hearts were homogenized and microsomal fractions were prepared from the homogenates. Enzyme activities were determined in the homogenates or microsomal preparations. Each value represents the mean ± standard deviation (number of experiments).

Enzyme	Enzyme activities	
	Control	Methyl lidocaine
	(nmol/hour/mg protein)	
Phospholipase A		
(homogenate)	5.1 ± 0.4 (3)	4.8 ± 0.4 (3)
Lysophospholipase		
(homogenate)	18.7 ± 1.5 (3)	18.0 ± 1.2 (3)
LPC: acyl-CoA acyltransferase		
(homogenate)	78.8 ± 11.7 (3)	44.9 ± 12.5 (3) *
(microsomes)	343 ± 20 (8)	205 ± 11 (3) *
LPE: acyl-CoA acyltransferase		
(homogenate)	6.8 ± 0.6 (3)	4.8 ± 0.4 (3) *
(microsomes)	32.0 ± 6.5 (9)	22.4 ± 2.6 (4) *

* $p < 0.05$

B. Effects on Phospholipid Composition

The enzymes involved in the reacylation of lysophospholipids in the hamster heart were inhibited during methyl lidocaine perfusion. This inhibition might result in elevated lysophospholipid levels with a concurrent decrease in phospholipid levels. Therefore, the phospholipid content and composition of the hamster heart perfused with methyl lidocaine were determined and compared with the control. Subsequent to perfusion with 0.5 mg/ml methyl lidocaine, the lipids in the heart were extracted and the phospholipid classes in the extract were separated by thin-layer chromatography. As shown in Table 7, there was no significant difference in the pool sizes of the phospholipids between the experimental and control groups. Subsequent analysis of the acyl compositions of phosphatidylcholine (Figure 17) and phosphatidylethanolamine (Figure 18) fractions by gas-liquid chromatography indicated that perfusion with methyl lidocaine did not produce any detectable change in the acyl profiles of these phospholipids.

4. Acylation of Lysophosphatidylcholine and Lysophosphatidylethanolamine in the Isolated Heart Perfused with Methyl Lidocaine

Since acyltransferase activities were inhibited in the hearts perfused with methyl lidocaine, the ability of these hearts to acylate exogenous lysophospholipids was examined. Hamster hearts were perfused with 1-[1-^{14}C]palmitoyl-glycerophosphocholine (Table 8) or 1-[1-^{14}C]palmitoyl-glycerophosphoethanolamine

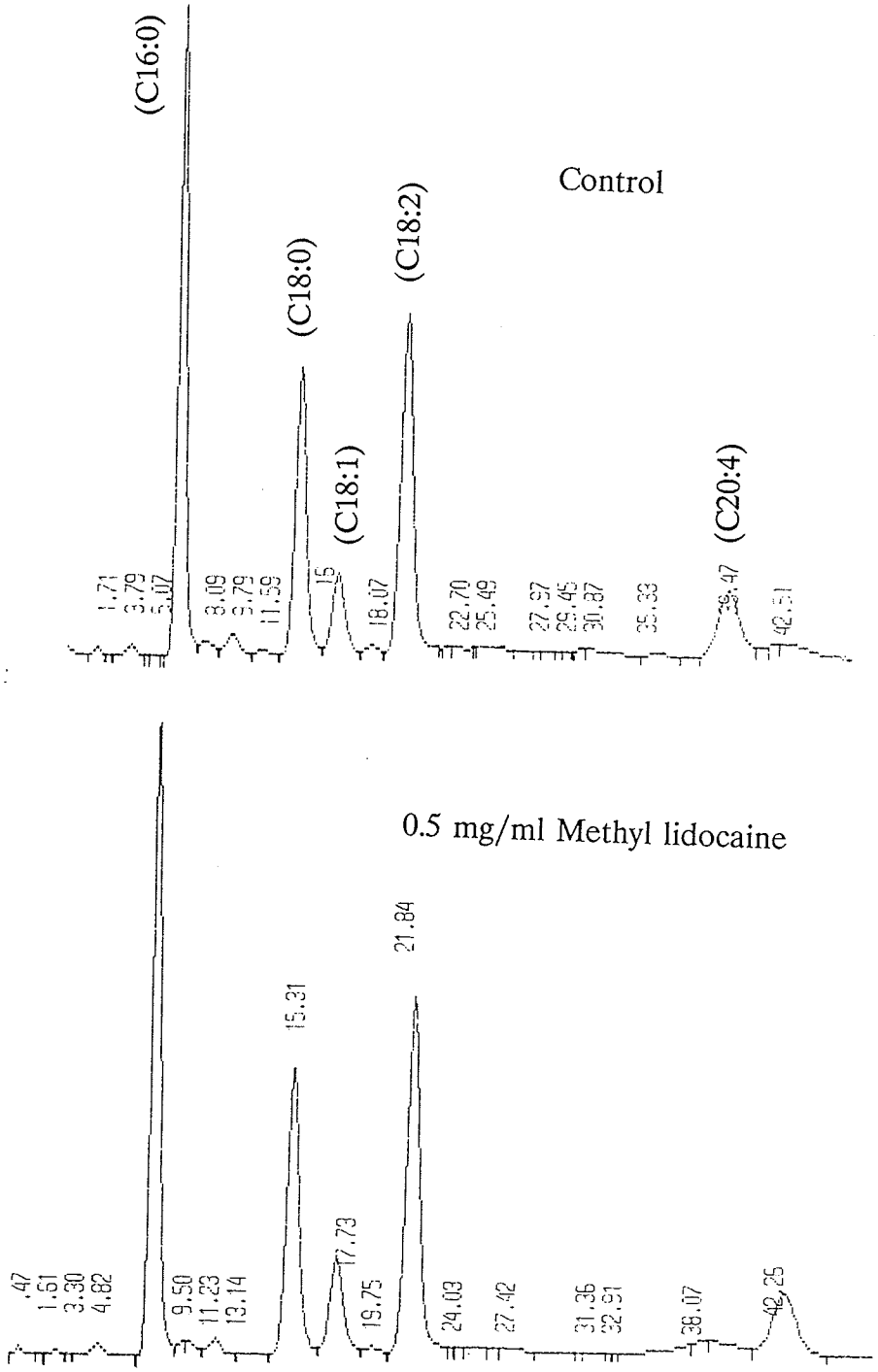
TABLE 7

Phospholipid composition in hamster hearts perfused with methyl lidocaine

Hamster hearts were perfused in Krebs-Henseleit buffer in the presence or absence of 0.5 mg/ml methyl lidocaine. Subsequent to perfusion, the hearts were homogenized in chloroform : methanol (2:1, v/v) and the phospholipid compositions of the hearts were analyzed by thin-layer chromatography. Each value represents the mean ± standard deviation of four separate experiments.

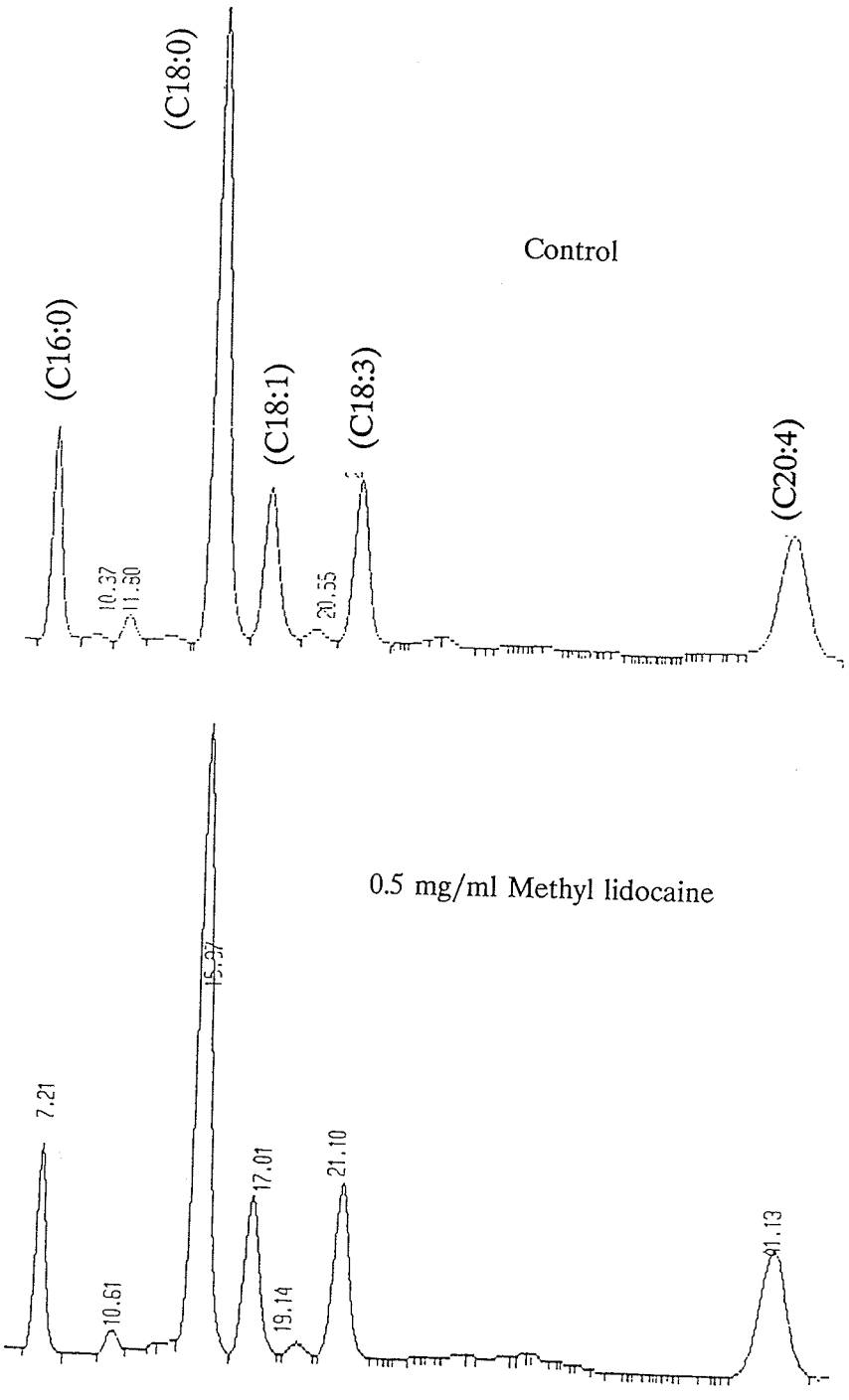
Phospholipid class	Control	Methyl lidocaine
	(μmol lipid-P/g wet weight)	
Lysophosphatidylcholine	0.10 ± 0.02	0.12 ± 0.03
Lysophosphatidylethanolamine	0.04 ± 0.01	0.05 ± 0.02
Phosphatidylcholine	11.7 ± 0.80	10.9 ± 1.15
Phosphatidylethanolamine	9.81 ± 0.88	10.2 ± 1.00
Phosphatidic Acid & Cardiolipin	3.16 ± 0.43	3.55 ± 0.57

Figure 17



Fatty acid methyl ester profile of phosphatidylcholine following perfusion in the presence and absence of 0.5 mg/ml methyl lidocaine

Figure 18



Fatty acid methyl ester profile of phosphatidylethanolamine following perfusion in the presence and absence of 0.5 mg/ml methyl lidocaine

(Table 9) in the presence of 0.5 mg/ml methyl lidocaine. Hearts perfused without methyl lidocaine were used as controls. Subsequent to perfusion, lipids were extracted and the phospholipid classes in the extract were separated by thin-layer chromatography. The presence of methyl lidocaine in the perfusate did not change the total uptake of radioactivity by the hearts. In both studies, over 95% of the radioactivity taken up by the heart was recovered in the lysophospholipid, phospholipid and neutral lipid fractions. The radioactivity associated with the neutral lipid fraction was determined to consist of fatty acids. In the presence of methyl lidocaine, the labelling of phosphatidylcholine was reduced by 38% (Table 8) while the labelling of the phosphatidylethanolamine fraction was reduced 30% (Table 9). This decrease in the labelling of both phospholipids corresponded to an increase in the labelling of the fatty acid. Surprisingly, the labelling of the lysophospholipid was not significantly altered in the methyl lidocaine perfused hearts.

We were concerned that the perfusion with lysophospholipid might result in increased lysophospholipid levels in the heart. Hence, the pool sizes of lysophosphatidylcholine in the perfused heart were determined and compared with the control. The lysophospholipid content in the heart perfused with 10 μM lysophospholipids was very similar to that obtained from the heart perfused with Krebs-Henseleit buffer (Table 7).

TABLE 8

Effect of methyl lidocaine on the metabolism of lysophosphatidylcholine in the isolated hamster heart

Isolated hamster hearts were perfused with 10 μM 1-[1-^{14}C]-palmitoyl-glycero-3-phosphocholine (lysophosphatidylcholine) in the presence or absence of 0.5 mg/ml methyl lidocaine for 30 min. Subsequent to perfusion, the radioactivity associated with each lipid fraction was determined. Each value represents the mean ± standard deviation of four separate experiments.

Lipid fraction	Control	Methyl lidocaine
	(dpm/g wet weight x 10^3)	
Phosphatidylcholine	840 ± 110	521 ± 90 *
Lysophosphatidylcholine	419 ± 75	450 ± 94
Fatty acids	910 ± 135	1180 ± 105 *

* $p < 0.05$

Table 9

Effect of methyl lidocaine on the metabolism of lysophosphatidylethanolamine in the isolated hamster heart

Isolated hamster hearts were perfused with 10 μM 1-[1-^{14}C]-palmitoyl-glycero-3-phosphoethanolamine (lysophosphatidylethanolamine) in the presence or absence of 0.5 mg/ml methyl lidocaine for 30 min. Subsequent to perfusion, the radioactivity associated with each lipid fraction was determined. Each value represents the mean ± standard deviation of four separate experiments.

Lipid fraction	Control	Methyl lidocaine
	(dpm/g wet weight x 10^3)	
Phosphatidylethanolamine	75 ± 13	52 ± 8 *
Lysophosphatidylethanolamine	84 ± 14	79 ± 11
Fatty acids	110 ± 10	138 ± 9 *

* $p<0.05$

II. Studies on *de novo* Lipid Biosynthesis

1. Introduction

The important role of phospholipids in the maintenance of proper membrane function necessitates the rigid control of their biosynthesis and catabolism in mammalian tissues. Phospholipids are actively synthesized in most mammalian tissues, and the pathways for their biosynthesis have been largely elucidated (Vance 1985; Tijburg 1989). However, only limited information is available on the control of their biosynthesis.

Amphiphilic compounds have been used as probes to alter lipid enzymatic activity (Yada *et al.* 1986; Koul and Hauser 1987) and acidic phospholipid biosynthesis (Eichberg and Hauser 1974; Freinkel *et al.* 1975) in mammalian tissues. Since glycerol forms the backbone of all glycerophospholipids, the use of labelled glycerol would enable us to monitor the biosynthesis of all glycerophospholipids in the perfused heart under the influence of methyl lidocaine. Glycerol has been used previously as a precursor to study lipid biosynthesis in lymphocytes (Allan and Michell 1975) and rat liver (Akesson *et al.* 1970; Brindley and Bowley 1975). In the present study, isolated hamster hearts were perfused with ^{3}H glycerol and methyl lidocaine. Following perfusion, the phospholipid groups and their precursors were separated and analyzed.

2. The Effect of Methyl Lidocaine on *de novo* Lipid Biosynthesis

A. ^{3}H Glycerol Uptake

The effect of methyl lidocaine on lipid biosynthesis in the hamster heart was investigated. Isolated hamster hearts were perfused with ^{3}H glycerol for 20-60 min in the presence or absence of 0.5 mg/ml methyl lidocaine. Subsequent to perfusion, the heart was homogenized in chloroform : methanol (1:1, v/v). A clear supernatant was obtained from the homogenate by centrifugation. The pellet was re-extracted with the same solvent, and the radioactivity in the pooled supernatant (tissue extract) was determined. The uptake of labelled glycerol was elevated in hearts perfused with methyl lidocaine (Table 10). The increased uptake was most prominent at 60 min of perfusion. In order to determine the factors responsible for the increased radioactivity found in the methyl lidocaine perfused hearts, the tissue extract was separated into the aqueous and organic phases by the addition of chloroform and water. Aliquots of these two phases were taken for radioactivity determination. Perfusion with methyl lidocaine did not cause any significant change in radioactivity associated with the aqueous phase at any of the time points tested (Table 10). This indicated that the label associated with glycerol and glycerol-3-phosphate (aqueous soluble) were similar between drug and control hearts. Analysis of the organic phase showed a significant increase in radioactivity at every time point of perfusion (Table 10). The results indicate that the enhanced uptake in methyl lidocaine perfused hearts is due to components associated with the organic phase of

TABLE 10

Effect of methyl lidocaine on the uptake of labelled glycerol in the isolated perfused hamster heart

Isolated hamster hearts were perfused in the Langendorff mode for 20-60 min in Krebs-Henseleit buffer containing 1 mM labelled glycerol (3μCi/μmol) in the presence or absence of 0.5 mg/ml methyl lidocaine. Subsequent to perfusion, the hearts were homogenized in chloroform : methanol (1:1, v/v) to obtain the tissue extracts. The tissue extracts were separated into the aqueous and organic phases by the addition of chloroform and water. Aliquots of the tissue extract, aqueous and organic phases were used for radioactivity determination. The results are expressed as means ± standard deviations. The letter n represents the number of individual experiments to each set.

Fraction		Time (min)		
		20 (n=3)	40 (n=3)	60 (n=5)
		(dpm/g heart wet weight x 10^3)		
Total uptake	(control)	2282 ± 144	2400 ± 514	3233 ± 960
	(drug)	2828 ± 82 *	3315 ± 41 *	5423 ± 1019 *
Aqueous phase	(control)	1552 ± 194	1203 ± 131	1425 ± 500
	(drug)	1634 ± 82	1387 ± 299	1625 ± 367
Organic phase	(control)	546 ± 58	881 ± 29	1752 ± 534
	(drug)	974 ± 89 *	1568 ± 244 *	3363 ± 430 *

* $p < 0.05$

the tissue extract.

B. Isolation of Phospholipid and Neutral Lipid Fractions

Since perfusion with [^{3}H] glycerol and methyl lidocaine resulted in an increased incorporation of radioactivity in the organic phase of the lipid extract, we subsequently wanted to identify whether any particular component of the organic phase was specifically labelled. The organic phase of the tissue extract contains all the precursors and products of the *de novo* lipid biosynthetic pathway (excluding glycerol and glycerol-3-phosphate) (Figure 5). In order to identify the lipids responsible for the increased radioactivity found in the methyl lidocaine perfused hearts, the lipid fractions in the organic phase were separated by thin-layer chromatography. An aliquot of the sample was used for the analysis of the phospholipid fractions whereas an identical amount was used for the analysis of the neutral lipids. In the analysis of the phospholipid fractions, no change in labelling was observed for the first two components in the biosynthetic pathway. Analysis of the two major phospholipids in the heart also revealed no change in labelling (Table 11). Analysis of the two minor phospholipids revealed elevated labelling of phosphatidylserine at 60 min of methyl lidocaine perfusion, whereas the labelling of phosphatidylinositol was elevated at all time points of perfusion (Table 12). In the analysis of the neutral lipid fraction, the labelling of diacylglycerol and triacylglycerol were found to be substantially increased by methyl lidocaine treatment at all time points (Table 12).

TABLE 11

Effect of methyl lidocaine on the labelling of intermediates and major phospholipids in the hamster heart

Hamster hearts were perfused with labelled glycerol for various times, in the presence or absence of methyl lidocaine as described in Table 10. Subsequent to perfusion, the lipid fractions were separated by thin-layer chromatography and the radioactivity associated with the intermediates and major phospholipids determined. The results are expressed as means ± standard deviations. The letter n represents the number of individual experiments to each set.

Fraction		Time (min)		
		20 (n=3)	40 (n=3)	60 (n=5)
		(dpm/g heart wet weight x 10^3)		
Lysophosphatidic acid	(control)	6.3 ± 1.7	10.1 ± 2.8	22.8 ± 3.9
	(drug)	7.5 ± 1.7	10.2 ± 1.8	29.5 ± 5.6
Phosphatidic acid	(control)	6.8 ± 1.8	3.9 ± 0.2	6.9 ± 1.0
	(drug)	6.6 ± 2.7	3.4 ± 1.0	8.1 ± 2.1
Phosphatidylcholine	(control)	37.2 ± 2.2	78.5 ± 12.7	219.0 ± 46.4
	(drug)	40.7 ± 4.7	79.2 ± 24.8	219.9 ± 81.2
Phosphatidyl-ethanolamine	(control)	27.8 ± 2.6	104.1 ± 9.0	217.7 ± 63.9
	(drug)	37.7 ± 8.7	94.6 ± 22.8	258.6 ± 70.7

TABLE 12

Effect of methyl lidocaine on the labelling of neutral lipids and minor phospholipids in the hamster heart

Hamster hearts were perfused with labelled glycerol for various times, in the presence or absence of methyl lidocaine as described in Table 10. Subsequent to perfusion, the lipid fractions were separated by thin-layer chromatography and the radioactivity associated with the minor phospholipids and neutral lipids determined. The results are expressed as means ± standard deviations. The letter n represents the number of individual experiments to each set.

Fraction		Time (min)		
		20 (n=3)	40 (n=3)	60 (n=5)
		(dpm/g heart wet weight x 10^3)		
Phosphatidylserine	(control)	10.0 ± 1.8	11.5 ± 2.5	20.8 ± 3.9
	(drug)	13.0 ± 2.4	15.7 ± 2.6	27.2 ± 2.3 *
Phosphatidylinositol	(control)	14.8 ± 0.8	38.0 ± 1.2	58.1 ± 11.0
	(drug)	23.5 ± 5.4 *	60.9 ± 1.8 *	90.7 ± 14.5 *
Diacylglycerol	(control)	86.6 ± 8.1	114.9 ± 22.4	139.9 ± 19.4
	(drug)	159.0 ± 7.5 *	162.3 ± 11.9 *	246.7 ± 57.9 *
Triacylglycerol	(control)	213.6 ± 40.6	795.7 ± 39.4	1549.1 ± 354
	(drug)	455.8 ± 103.8 *	1518.9 ± 228 *	3427.4 ± 506 *

* $p < 0.05$

C. Phospholipid and Neutral Lipid Content Following Perfusion

The increased incorporation of radioactivity in components of the biosynthetic pathway, in the presence of methyl lidocaine, might result in changes to the pool size of these lipids. Therefore, the components of the lipid biosynthetic pathway were separated and quantitated. The results indicated that there was no significant change in the contents of the phospholipid fractions (Table 13). Analysis of the neutral lipid fraction, however, did show a significant increase in the levels of diacylglycerol (48%) and triacylglycerol (125%) in the hearts perfused with methyl lidocaine.

3. The *in vitro* Effect of Methyl Lidocaine on the Lipid Biosynthetic Enzymes

The mechanism for the enhancement in the labelling of the cardiac lipids by methyl lidocaine was investigated. The changes in the labelling of the cardiac lipids might be caused by a direct activation of key enzymes involved in the production of these lipids. In order to test this hypothesis, the activities of the enzymes responsible for the synthesis of the acidic phospholipids as well as diacylglycerol and triacylglycerol were determined in the presence of methyl lidocaine. Since the subcellular localization of some lipid biosynthetic enzymes was not well defined (Heathers *et al.* 1985), our initial approach was to use the tissue homogenate as the enzyme source in order to encompass enzyme activity associated with mitochondrial, microsomal and/or cytosolic fractions.

TABLE 13

Effect of methyl lidocaine on the phospholipid and neutral lipid contents in the hamster heart

Hamster hearts were perfused with labelled glycerol in the presence or absence of 0.5 mg/ml methyl lidocaine for 60 min as described in Table 10. Subsequent to perfusion, the lipid fractions were separated by thin-layer chromatography. The amount of lipid in each fraction was determined. The results are expressed as means ± standard deviation from three separate experiments.

Fraction	Control	Methyl lidocaine treated
	(μmol lipid-P/g heart wet weight)	
Lysophosphatidic acid	0.27 ± 0.10	0.25 ± 0.07
Phosphatidic acid	0.33 ± 0.09	0.42 ± 0.13
Phosphatidylcholine	11.71 ± 0.80	10.94 ± 1.00
Phosphatidylethanolamine	9.81 ± 0.80	9.09 ± 1.15
Phosphatidylserine	1.84 ± 0.38	1.41 ± 0.27
Phosphatidylinositol	1.65 ± 0.25	1.48 ± 0.36
	(μmol glycerol/g heart wet weight)	
Diacylglycerol	6.54 ± 0.37	9.71 ± 1.25 *
Triacylglycerol	3.88 ± 0.93	8.74 ± 0.97 *

* $p < 0.05$

The effect of methyl lidocaine on the enzymes involved in the biosynthesis of lipids were studied. The activities of acyl-CoA: glycerol-3-phosphate acyltransferase and acyl-CoA: lysophosphatidate acyltransferase in hamster homogenate were assayed in the presence of 0-2 mg/ml methyl lidocaine. Methyl lidocaine did not cause any changes in the activities of these enzymes (Table 14). The activity of acyl-CoA: diacylglycerol acyltransferase in the hamster heart homogenate was also determined. No enzyme activity was detected when the assay was performed without exogenous diacylglycerol. In the presence of exogenous diacylglycerol, enzyme activity was not affected by methyl lidocaine (Table 14).

Phosphatidate phosphatase is usually regarded as the rate limiting step in the formation of triacylglycerol (Tijburg *et al.* 1989). Due to the low phosphatidate phosphatase activity in the tissue homogenate, enzyme activity was determined in the post-mitochondrial fraction. Enzymatic activity was found to be stimulated by methyl lidocaine (0.2-2.0 mg/ml) at low or near physiological concentrations (1 & 7.5 mM) of Mg^{2+} (Table 15). However, this enhancement was abolished at a higher Mg^{2+} concentration (20 mM). As a positive control, the effect of chlorpromazine on the phosphatase activity was also examined (Table 15). Chlorpromazine is a known inhibitor of the phosphatidate phosphatase enzyme. The biphasic effect of chlorpromazine on the enzyme activity is in agreement with previous studies (Bowley *et al.* 1977).

TABLE 14

Effect of methyl lidocaine on hamster heart acyltransferase activities

Enzyme activities in the hamster heart homogenate were determined in the presence of 0-2 mg/ml methyl lidocaine. The results were expressed as means ± standard deviation of three separate experiments, each of which was done in duplicate.

	Methyl lidocaine (mg/ml)			
Enzyme assayed	Control	0.5	1.0	2.0
	(nmol/hour/mg protein)			
Acyl-CoA: glycerol-3-P acyltransferase	3.83 ± 1.4	3.56 ± 1.7	3.40 ± 1.4	4.11 ± 1.3
Acyl-CoA: lyso-phosphatidic acid acyltransferase	0.68 ± 0.2	0.74 ± 0.1	0.99 ± 0.2	0.55 ± 0.1
Acyl-CoA: diacylglycerol acyltransferase	8.52 ± 2.1	8.28 ± 2.3	8.84 ± 2.3	9.23 ± 1.6

TABLE 15

Effect of methyl lidocaine and chlorpromazine on hamster heart phosphatidate phosphatase activity

Phosphatidate phosphatase activity was determined in the hamster heart post-mitochondrial fraction in the presence of 0-2 mg/ml methyl lidocaine or 1-5 mM chlorpromazine. The results are expressed as means ± standard deviations of four separate experiments, each of which was determined in duplicate.

Drug concentration	Phosphatidate phosphatase		
	1 mM $MgCl_2$	7.5 mM $MgCl_2$	20 mM $MgCl_2$
Methyl lidocaine	(nmol/hour/mg protein)		
0 (Control)	12.41 ± 1.9	13.23 ± 4.6	12.35 ± 1.89
0.2 mg/ml	20.36 ± 1.9 *	26.88 ± 6.1 *	15.11 ± 1.54
0.5 mg/ml	23.25 ± 3.48 *	30.87 ± 8.5 *	18.39 ± 6.78
1.0 mg/ml	23.56 ± 6.59 *	24.57 ± 4.2 *	13.69 ± 3.63
2.0 mg/ml	18.16 ± 1.23 *	32.41 ± 5.0 *	9.82 ± 1.80
Chlorpromazine			
1 mM	18.17 ± 1.07 *		
2 mM	3.70 ± 1.81 *		
3 mM	2.89 ± 1.16 *		
4 mM	3.03 ± 0.63 *		
5 mM	2.09 ± 0.90 *		

* $p < 0.05$

The effects of methyl lidocaine on the enzymes involved in the biosynthesis of the acidic phospholipids were also investigated. These enzymes included the CTP:phosphatidate cytidylyltransferase, phosphatidylinositol synthase and the serine base exchange enzyme. Methyl lidocaine was found to cause a stimulation of CTP: phosphatidate cytidylyltransferase in a dose-dependent manner (Table 16). The presence of methyl lidocaine had no effect on the phosphatidylinositol synthase activity (Table 16). The activity of the serine base exchange enzyme was also unaffected by the presence methyl lidocaine.

4. Quantitation of Long-Chain Acyl-CoA

The enhancement found in the labelling of the lipid groups may be accounted for by changes in the pool size of the acyl donor. Hence, the levels of long-chain acyl-CoA in the isolated perfused heart were determined. Perfusion with methyl lidocaine did not alter the acyl-CoA content in the heart (Table 17). Since the biosynthesis of the acidic phospholipids might be affected by the level of CDP-diacylglycerol, the pool size of this intermediate was also determined. The results indicated that there was a 31% increase in the pool size of CDP-diacylglycerol when perfused with methyl lidocaine for 60 min (Table 17).

TABLE 16

Effect of methyl lidocaine on hamster heart CTP: phosphatidic acid cytidylyltransferase and phosphatidylinositol synthase activities

CTP: phosphatidic acid cytidylyltransferase and phosphatidylinositol synthase activities were assayed with hamster heart homogenate and the post mitochondrial fraction, respectively, in the presence of 0-2 mg/ml methyl lidocaine. The results are expressed as means ± standard deviations of three separate sets of experiments, each of which was determined in duplicate.

Methyl lidocaine (mg/ml)	CTP: phosphatidic acid cytidylyltransferase	Phosphatidylinositol synthase
	(nmol/hour/mg protein)	
0 (Control)	0.648 ± 0.09	0.090 ± 0.006
0.2	0.972 ± 0.16 *	0.089 ± 0.008
0.5	1.032 ± 0.24 *	0.093 ± 0.013
1.0	1.422 ± 0.16 *	0.085 ± 0.007
2.0	1.434 ± 0.19 *	0.084 ± 0.004

* $p < 0.05$

TABLE 17

Effect of methyl lidocaine on the long-chain acyl-CoA and CDP-diacylglycerol pools in hamster hearts

Hamster hearts were perfused with glycerol in the presence or absence of 0.5 mg/ml methyl lidocaine for 60 min. The pool sizes of diacylglycerol and long-chain acyl-CoA were determined. The results are expressed as means ± standard deviations of three separate sets of experiments, each of which was determined in duplicate.

	Control	Methyl lidocaine
Long-chain acyl-CoA (nmol/g wet weight)	61.2 ± 9.2	59.6 ± 7.9
CDP-diacylglycerol (nmol/g wet weight)	12.1 ± 0.1	15.9 ± 0.2 *

* $p < 0.05$

III. Determination of Long-Chain Acyl-CoA Levels

1. Introduction

The intracellular acyl-CoA content and composition have been shown to be important factors for the determination of the acyl groups on the phospholipids during reacylation (Lands and Hart 1965; Choy and Arthur 1989; Yashiro *et al.* 1989). These esters also act as modulators for the regulation of several enzyme systems (Shug *et al.* 1971; Tippet and Neet 1982) including the cardiac sodium pump (Kakar *et al.* 1987) and protein kinase C (Bronfman *et al.* 1988). The identification of acyl-CoA as a metabolic regulator led to the development of several assays for its quantitation (Corkey 1988). In general, the tissue is homogenized and proteins are precipitated from the homogenate. Subsequently, acyl-CoA is purified from the protein-free extract by column chromatography. Enzymatic or chemical analysis can then be employed for the quantitation of total acyl-CoA (Tubbs and Garland 1969). The molecular species of the acyl-CoA can be determined by reverse-phase high-performance liquid chromatography (Woldegiorgis *et al.* 1985; Watmough *et al.* 1989). Since column chromatography is employed to purify acyl-CoA prior to its analysis, the procedure is not only time consuming, but does not allow the simultaneous analysis of more than a few samples.

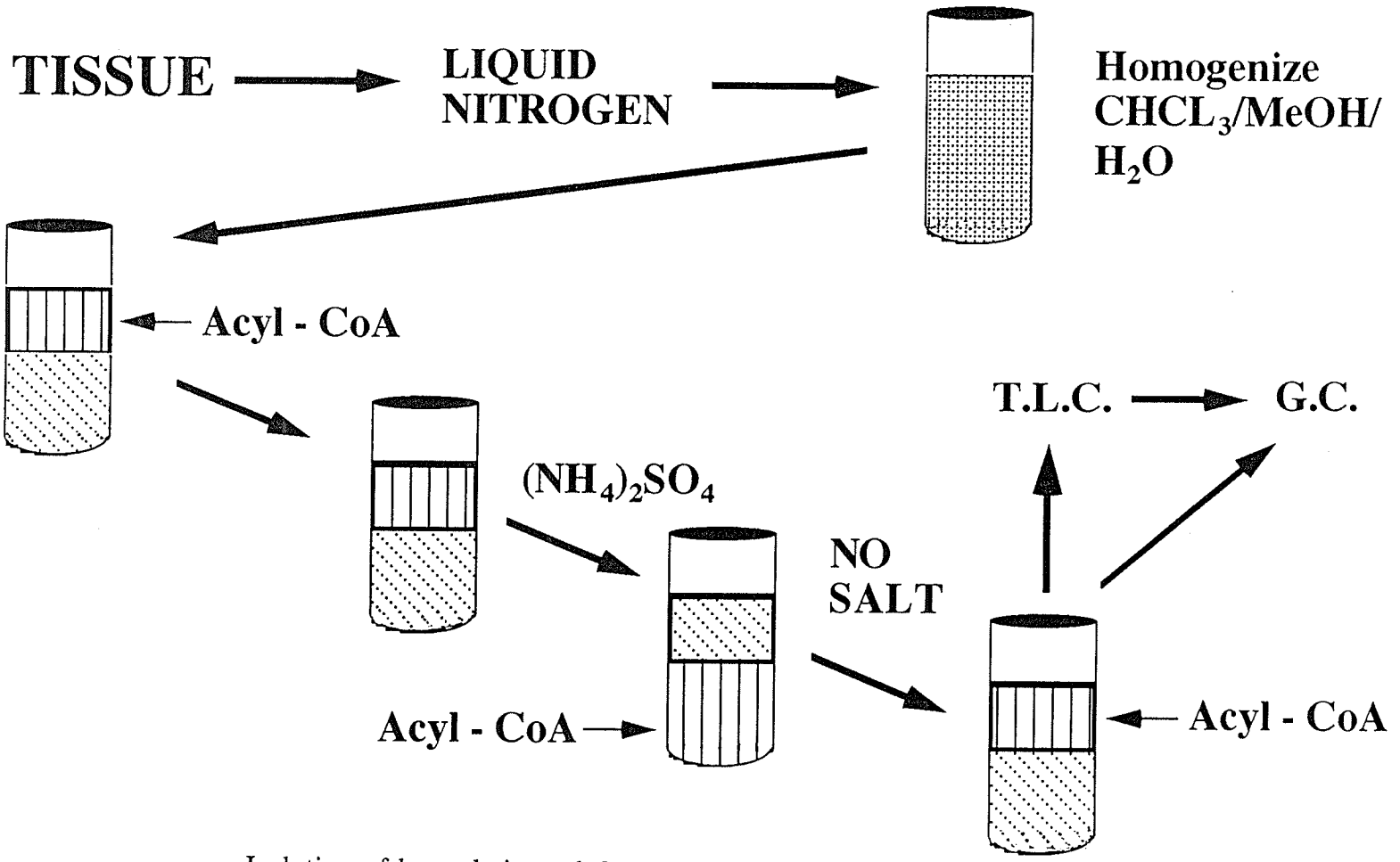
In this study, a novel procedure was developed for the isolation of long-chain acyl-CoA in mammalian tissues. Acyl-CoA was extracted from tissue with chloroform :

methanol and separated from other lipid-containing metabolites by phase partition. Subsequently, the total acyl-CoA content and the molecular species of acyl-CoA were determined by gas-liquid chromatography.

2. Isolation of Long-Chain Acyl-CoA by Phase Partition

The long-chain acyl-CoA in rat liver extract was found to partition in a two phase solvent containing chloroform : methanol : water (4:2:3, v/v). The distribution of acyl-CoA in the aqueous and organic phase was monitored with radiolabelled palmitoyl-CoA (16:0), stearoyl-CoA (18:0), oleoyl-CoA (18:1) and arachidonoyl-CoA (20:4). In all cases, over 95% of the radioactivity was found in the aqueous phase and less than 2% was recovered in the organic phase. This step caused the separation of acyl-CoA from other acyl containing compounds due to its unique solubility in water. When ammonium sulphate was added to the aqueous phase, a substantial portion of the labelled acyl-CoA could be driven into the organic phase. A 30% ammonium sulphate saturation in the aqueous phase was optimal for the partition of labelled acyl-CoA (68 ± 8%) into the organic phase. The majority (72 ± 6%) of the labelled acyl-CoA in the organic phase was then extracted back into the aqueous phase in the absence of ammonium sulphate (Figure 19). The overall recovery of long-chain acyl-CoA was 39-43% (Table 18). The yield obtained from this study is similar to the value reported by Prasad *et al.* (1987) but substantially higher than the value described by Olbrich *et al.* (1981). Based on the results of Table 18, a 40% recovery was used to calculate the total acyl-CoA content.

Figure 19



Isolation of long-chain acyl-CoA by phase partition

TABLE 18

Recovery of radioactivity in samples after phase partition

Rat liver samples were homogenized and extracted as outlined in Materials and Methods. Radiolabelled long-chain acyl-CoAs and lipid (1 μCi) were added to the sample prior to homogenization. The % radioactivity recovered in the sample after solvent partition is shown.

Labelled compound added	Recovery (%)
Palmitoyl-CoA (16:0)	40 ± 4
Stearoyl-CoA (18:0)	43 ± 9
Oleoyl-CoA (18:1)	40 ± 6
Arachidonoyl-CoA (20:4)	39 ± 8
Palmitic acid	<0.1
Phosphatidylcholine	<0.1
Palmitoyl carnitine	<0.1
Cholesteryl oleate	<0.1
Triolein	<0.1

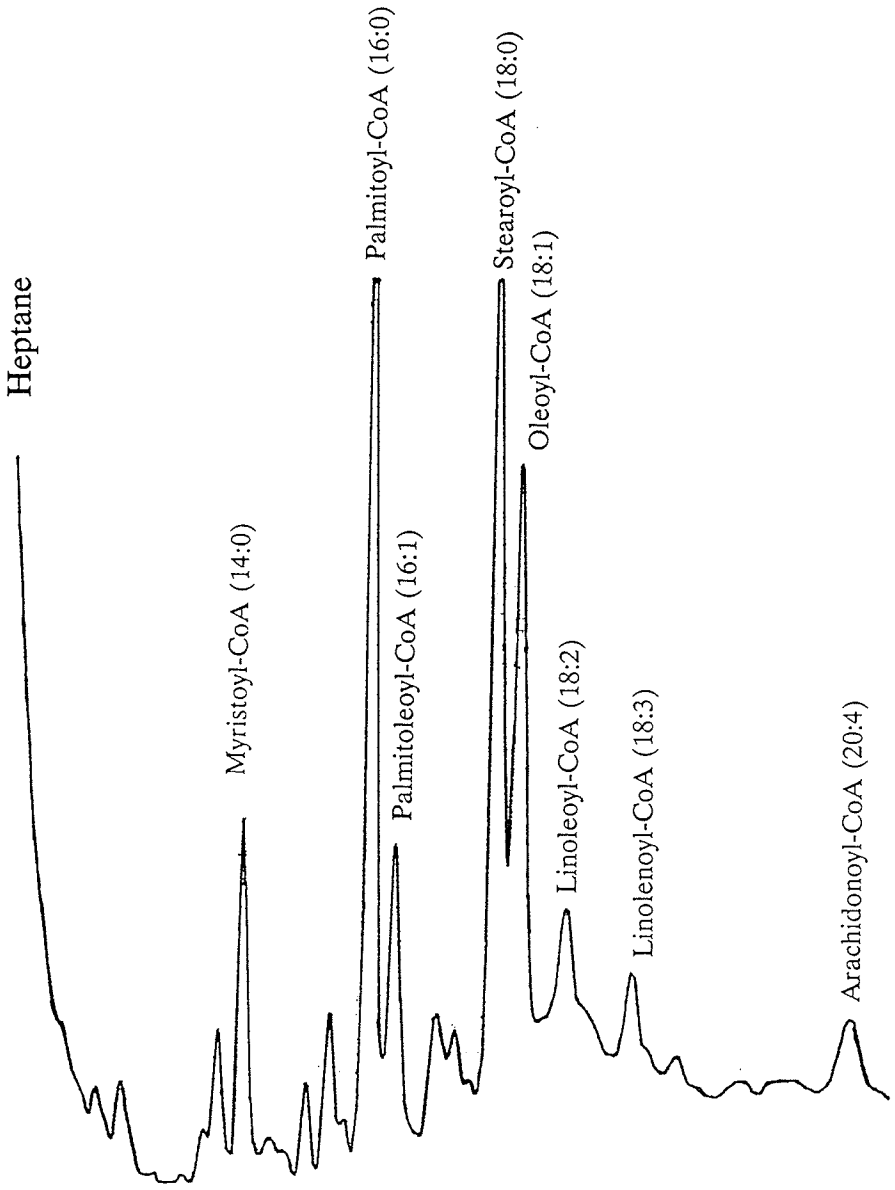
3. Removal of Possible Contaminants

The ability to separate long-chain acyl-CoA from other acyl-containing species by phase partition was examined. Labelled phosphatidylcholine, palmitic acid, triolein, cholesteryl oleate or palmitoyl carnitine was added to the tissue homogenate. Following the final phase partition, the samples were analyzed for radioactivity. Less than 0.1% of the original radioactivity added to the homogenate was detected in the purified acyl-CoA fraction (Table 18). The result suggests that the long-chain acyl-CoA sample obtained after phase partition was not significantly contaminated with other fatty acyl containing compounds.

4. Quantitation of Long-Chain Acyl-CoA

The quantitation of long-chain acyl-CoA in the sample after phase partition was carried out by gas-liquid chromatography. The tissue homogenate was divided into two aliquots. An internal standard (100 nmol of acyl-CoA) was added to one aliquot prior to the isolation of acyl-CoA by phase partition. Subsequently, the acyl-CoA esters in the sample were converted into methyl esters and quantitated by gas-liquid chromatography (Figure 20). The quantity of each methyl ester species was determined by comparing with the methylheptadecanoate standard. The yield of acyl-CoA calculated from the internal standard (100 nmol of acyl-CoA) was in complete agreement with the values obtained from the labelled acyl-CoA (Table 18). The total long-chain acyl-CoA content as well as the distribution of the acyl species

Figure 20



Fatty acid methyl esters derived from long-chain acyl-CoA's

in rat liver are shown in Table 19. Our result (83 ± 11 nmol/ g wet weight) is comparable to those obtained in previous studies (Tubbs and Garland 1964; Berge *et al.* 1984; Prasad *et al.* 1987).

The requirement for further purification of the long-chain acyl-CoA after phase partition was investigated. An aliquot of the acyl-CoA preparation obtained after phase partition was subjected to thin-layer chromatography with a solvent containing n-butanol : acetic acid : water (4:1:2, v/v). Analysis of the chromatogram revealed that the R_f of long-chain acyl-CoA was significantly different from that of free fatty acids, diacylglycerol, triacylglycerol and acyl carnitine (see Materials and Methods section 3-C). The content and composition of the long-chain acyl-CoA fraction obtained from thin-layer chromatography was analyzed by gas-liquid chromatography. As depicted in Table 20, the distribution of acyl species and total acyl-CoA content was similar to that found in the samples after phase partition (Table 19). Hence, the phase partition procedure alone appears to be adequate for the quantitation of long-chain acyl-CoA in mammalian tissues.

TABLE 19

Long-chain acyl-CoA content and distribution of species in samples after phase partition

Acyl-CoA was isolated from rat liver homogenate by solvent partition. The long-chain acyl-CoAs were then converted to fatty acid methyl esters (Materials and Methods, section II-3). The resultant methyl esters were separated and quantitated by gas-liquid chromatography.

Acyl-CoA species	Distribution (%)
Myristoyl-CoA (14:0)	3 ± 2
Palmitoyl-CoA (16:0)	20 ± 5
Palmitoleoyl-CoA (16:1)	3 ± 2
Stearoyl-CoA (18:0)	33 ± 3
Oleoyl-CoA (18:1)	14 ± 6
Linoleoyl-CoA (18:2)	12 ± 4
Linolenoyl-CoA (18:3)	4 ± 2
Arachidonoyl-CoA (20:4)	8 ± 4
Others	3 ± 1
Total long-chain acyl-CoA	83 ± 11 nmol/g wet weight

TABLE 20

Long-chain acyl-CoA content and distribution of species in samples after thin-layer chromatography

Acyl-CoA was isolated from rat liver homogenate by solvent partition, followed by thin-layer chromatography as described in Materials and Methods, section II-3. The resultant methyl esters were separated and quantitated by gas-liquid chromatography.

Acyl-CoA species	Distribution (%)
Myristoyl-CoA (14:0)	3 ± 2
Palmitoyl-CoA (16:0)	16 ± 6
Palmitoleoyl-CoA (16:1)	5 ± 2
Stearoyl-CoA (18:0)	35 ± 5
Oleoyl-CoA (18:1)	14 ± 4
Linoleoyl-CoA (18:2)	12 ± 4
Linolenoyl-CoA (18:3)	3 ± 2
Arachidonoyl-CoA (20:4)	6 ± 4
Others	6 ± 3
Total long-chain acyl-CoA content	81 ± 9 nmol/g wet weight

IV. Mechanism of Action

1. Introduction

The results in section II indicate that the drug, methyl lidocaine, caused changes in the *de novo* biosynthesis of lipids in the hamster heart through direct action on the enzymes involved. However, this action might not account for all the changes. It is plausible that methyl lidocaine also mediated its effect on the lipid biosynthetic enzymes indirectly through activation of second messengers. In this section, the possible involvement of second messengers mediated by methyl lidocaine was explored. In the first part of the study, hearts were perfused with labelled methyl lidocaine to examine uptake of the drug. In the second part of the study, the possible activation of a second messenger system was investigated.

2. Uptake of ^{3}H Methyl Lidocaine in the Perfused Heart

Unlike lidocaine, methyl lidocaine is a positively charged antiarrhythmic drug. Since charged molecules can have difficulty crossing the biological membrane, this study was designed to study the uptake of methyl lidocaine into the cell. Hearts were perfused with 0.01 mg/ml or 0.02 mg/ml [^{3}H] methyl lidocaine for 30 min and then perfused with 0.5 mg/ml or 1.0 mg/ml unlabelled methyl lidocaine for another 30 min. Since concentrations of methyl lidocaine higher than 1.0 mg/ml might result in the heart becoming quiescent or non electrically active, the highest concentration of

methyl lidocaine used in this study was 1.0 mg/ml. Subsequent to perfusion, subcellular fractions were prepared and the radioactivity associated with each fraction was determined. Irrespective of the concentration of methyl lidocaine used, the majority of the radioactivity was found in the fraction containing unbroken cells and nuclei, indicating that a substantial amount of methyl lidocaine was bound to the cell membrane. The remaining radioactivity was found to be associated with the mitochondrial, microsomal and cytosolic fractions (Table 21). An increase in the concentration of methyl lidocaine from 0.01 mg/ml to 0.02 mg/ml did not appear to affect the total binding or uptake of the drug.

3. The *in vivo* Effect of Methyl Lidocaine on Lipid Enzyme Activity

It is clear from the preceding studies that a portion of methyl lidocaine might be transported into the cell resulting in the direct stimulation of the phosphatidic acid phosphatase as well as the CTP: phosphatidic acid cytidylyltransferase enzymes. Alternatively, a considerable portion of methyl lidocaine was found to be associated with the cell membrane and might cause the stimulation of a membrane receptor and activate a signal transduction system.

In order to investigate the indirect effect of methyl lidocaine on lipid biosynthesis, hamster hearts were perfused with the drug for 5 min, homogenized in a buffer containing sodium fluoride, and then assayed for lipid enzyme activity. The data in

TABLE 21

Uptake of ^{3}H methyl lidocaine by perfused hamster hearts

Isolated hamster hearts were perfused with 0.01 or 0.02 mg/ml ^{3}H methyl lidocaine for 30 min. Following this period, they were perfused with a fifty-fold increase in methyl lidocaine concentration (unlabelled), for 30 min. The hearts were homogenized and subcellular fractions were prepared. The radioactivity associated with each fraction was determined. Each value represents the mean ± standard deviation of 3 separate experiments.

Subcellular distribution	Methyl lidocaine	
	0.01 mg/ml	0.02 mg/ml
	(% distribution)	
Unbroken cells and Nuclei	73.0 ± 2.5	79.7 ± 2.4
Mitochondrial	11.3 ± 0.9	6.8 ± 0.5
Cytosolic	10.7 ± 1.8	9.1 ± 2.5
Microsomal	5.0 ± 0.7	4.4 ± 0.5
	(nmol/g wet weight)	
Total uptake	26.5 ± 10.4	30.1 ± 3.9

TABLE 22

The *in vivo* effects of methyl lidocaine on the *de novo* lipid biosynthetic enzymes

Hamster hearts were perfused for 5 min in the presence of 0.5 mg/ml methyl lidocaine and 1 mM glycerol. Following perfusion, subcellular fractions were prepared and enzyme activities were determined as described in Materials and Methods, section II-2. The results are expressed as the mean ± standard deviation from 3 separate experiments.

Enzyme assayed	Control	Methyl lidocaine
	(nmol/hour/mg protein)	
Glycerol-3-phosphate acyltransferase	6.14 ± 1.2	5.84 ± 1.5
Lysophosphatidic acid acyltransferase	0.89 ± 0.08	1.59 ± 0.28 *
Phosphatidic acid phosphatase	10.78 ± 3.61	10.91 ± 1.59
Diacylglycerol acyltransferase	7.98 ± 0.83	11.48 ± 1.73 *
CTP: phosphatidic acid cytidylyltransferase	2.97 ± 0.06	2.84 ± 0.60
Phosphatidylinositol synthase	0.093 ± 0.033	0.114 ± 0.012

* $p < 0.05$

Table 22 shows a significant stimulation of lysophosphatidic acid acyltransferase as well as diacylglycerol acyltransferase activities when the heart was perfused with the drug. It should be emphasized that these enzymes were not stimulated when assayed in the presence of methyl lidocaine (Table 14). Taken together, the data suggests that the lysophosphatidic acid acyltransferase and the diacylglycerol acyltransferase were activated in an indirect fashion. Interestingly, phosphatidic acid phosphatase and CTP: phosphatidic acid cytidylyltransferase activities were not significantly changed in the heart perfused with methyl lidocaine. Both of these enzymes were previously shown to be stimulated by methyl lidocaine directly (Tables 15 & 16). No direct or indirect effect by methyl lidocaine was obtained from the assay of glycerol-3-phosphate acyltransferase or phosphatidylinositol synthase activity. While the study was performed with 1 mM glycerol in the perfusate, subsequent experiments showed that the results were not affected by the presence or absence of glycerol.

4. Methyl Lidocaine Effects on Protein Kinase C Activity

In view of the findings in the preceding section, the ability of methyl lidocaine to stimulate the activity of protein kinase C was investigated. The rationale was that methyl lidocaine might have activated a receptor for the stimulation of phospholipase C which would cause the hydrolysis of phosphatidylinositol 4,5-bisphosphate and produce 1,2-diacylglycerol and inositol trisphosphate. The diacylglycerol production would cause the translocation of protein kinase C from the cytosol to the membrane in a Ca^{2+} dependant fashion and result in its activation (Wolf *et al.* 1985). The

activation of protein kinase C could possibly stimulate the activation of lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase by a phosphorylation reaction.

The distribution of protein kinase C was determined in hearts perfused for 5 min in the presence of 0.5 mg/ml methyl lidocaine and the presence or absence of 1 mM glycerol. Following perfusion, subcellular fractions were prepared. The data obtained (Table 23) indicates a fairly equal distribution of protein kinase C activity between the cytosol and the microsomal fraction in the control heart. Perfusion in the presence of methyl lidocaine did not cause any translocation of protein kinase C from one compartment to another. The results were not changed when 1 mM glycerol was included in the perfusate. The results indicate that protein kinase C was not activated during methyl lidocaine perfusion.

5. Methyl Lidocaine Effects on Intracellular cAMP Pool Size

The activation of lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase via the cAMP cascade was examined. In this cascade, the possible binding of methyl lidocaine to a receptor would result in the activation of adenylate cyclase which would increase the level of cAMP. The rise in cAMP levels would result in the activation of a cAMP-dependent protein kinase which might be responsible for the activation of the enzymes.

TABLE 23

Effect of methyl lidocaine perfusion on hamster heart protein kinase C activity

Hamster hearts were perfused for 5 min in the presence of 0.5 mg/ml methyl lidocaine. Following perfusion, the hearts were homogenized and protein kinase C activity associated with the microsomal and cytosolic fractions determined. The results are expressed as the mean ± standard deviation from 3 separate experiments.

Subcellular fraction	Control	Methyl lidocaine	Methyl lidocaine & 1 mM glycerol
		(pmol/min/mg protein)	
Cytosolic	8.76 ± 1.4	9.66 ± 0.9	10.33 ± 2.4
Microsomal	8.22 ± 3.4	6.79 ± 1.0	8.58 ± 1.7

In this study, hamster hearts were perfused with 0.5 mg/ml methyl lidocaine for 5 min and the level of cAMP in the heart was determined (Table 24). A significant increase in the level of cAMP was detected in the heart perfused with methyl lidocaine when compared to the control. The cAMP values reported here are in complete agreement with previous reports using isolated perfused rat hearts (Krause and Wollenberger 1980).

6. cAMP-Dependent Protein Kinase

The elevated levels of cAMP in hearts perfused with methyl lidocaine might result in the activation of cAMP-dependent protein kinase which might be responsible for increased enzymatic activity of lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase through a phosphorylation reaction. In order to determine if the activities of these enzymes were subjected to modulation by cAMP-dependent protein kinase, the heart homogenate was incubated with 100 units of the catalytic subunit of cAMP-dependent protein kinase. Following an incubation period, the enzymes were assayed and the results are shown in Table 25. The addition of cAMP-dependent protein kinase produced a very significant increase in both the lysophosphatidic acid acyltransferase (91%) and the diacylglycerol acyltransferase (103%) activities. It is clear that the increased enzymatic activity seen in Table 22 could be the result of increased cAMP-dependent protein kinase activity.

TABLE 24

Effect of methyl lidocaine perfusion on hamster heart cAMP levels

Hamster hearts were perfused for 5 min in the presence or absence of 0.5 mg/ml methyl lidocaine. Following perfusion, the hearts were immediately frozen in liquid nitrogen. The cAMP levels in the hearts were determined and expressed as the mean ± standard deviation from 3 separate experiments.

Sample	cAMP level
	(pmol/g wet weight)
Control	440 ± 16
Methyl lidocaine	767 ± 58 *

* $p < 0.05$

TABLE 25

Effect of the catalytic subunit of cAMP-dependent protein kinase on enzyme acitivity

Hamster heart homogenate was incubated with 100 units of the catalytic subunit of cAMP-dependent protein kinase. Following a 10 min incubation period, enzyme activities were determined. The results are expressed as the mean ± standard deviation from 3 separate experiments.

Enzyme	Control	cAMP-stimulated
	(nmol/hour/mg protein)	
Lysophosphatidic acid acyltransferase	0.95 ± 0.22	1.80 ± 0.21 *
Diacylglycerol acyltransferase	11.92 ± 1.21	24.25 ± 2.65 *

* $p < 0.05$

DISCUSSION

I. Studies on Lysophospholipid Metabolism

The deacylation reaction catalyzed by phospholipase A is the major route for phospholipid catabolism in mammalian tissues. In the hamster heart, the lysophospholipids formed are further deacylated by lysophospholipase or acylated back to the parent phospholipids by acyl-CoA dependent acyltransferases (van Golde and van den Bergh 1977). Since lysophospholipids are cytolytic at high cellular concentrations, their levels in the cell are normally under rigid control (Weltzein 1979). The regulatory mechanism for the control of lysophospholipid levels in the heart was investigated in rats that were fed diets containing different amounts of vitamin E (Cao *et al.* 1987). The cardiac lysophosphatidylcholine level was altered by dietary treatment and the changes were attributed to an increase in cardiac phospholipase A activities by vitamin E. In another study (Choy *et al.* 1989), lysophosphatidylcholine levels in the heart were altered by ethanol perfusion, and the changes were due to a decrease in phospholipase A activity. These studies clearly indicate the important role phospholipase A plays in the regulation of lysophospholipid levels in the heart. However, the role of acyl-CoA dependent acyltransferase in the maintenance of the cardiac lysophospholipid levels has not been defined. The ability to inhibit the acyltransferases but not phospholipase A or lysophospholipase activities by 0.5 mg/ml methyl lidocaine provided us with an

excellent approach to examine the contribution of acyltransferases to the regulation of lysophospholipid metabolism.

Kinetic studies revealed that the inhibition of acyltransferase activity by methyl lidocaine was not at the substrate level, and both lysophosphatidylcholine: and lysophosphatidylethanolamine: acyl-CoA acyltransferases were inhibited by the same mechanism. One interesting finding is that while the microsomal acyltransferase activities were severely inhibited in the isolated heart perfused with 0.5 mg/ml methyl lidocaine, the phospholipase A and lysophospholipase activities were not affected. At the same time, no accumulation of lysophosphatidylcholine or lysophosphatidylethanolamine was detected. Taken together, the activities of the acyltransferases do not seem to have a direct contribution to the maintenance of the lysophospholipid levels in the heart.

The regulation of lysophospholipid levels in the heart was further examined by perfusion with labelled lysophospholipids. As shown in earlier studies (Savard and Choy 1982) and substantiated in the present study, perfusion of the heart with 10 μM of lysophospholipid did not significantly perturb the cardiac lysophospholipid content. It was not surprising that perfusion with methyl lidocaine caused a reduction in the labelling of the phospholipid. It was surprising, however, that methyl lidocaine perfusion resulted in an increase in the release of labelled fatty acid with no change in the labelling of lysophospholipid. A possible explanation is that the inhibition of the acylation process by methyl lidocaine was compensated by an increase in the

deacylation of the lysophospholipids into glycerophosphocholine and fatty acid. Alternatively, the increase in labelled fatty acid could also be derived from the enhanced deacylation of the labelled phospholipids subsequent to the reacylation process. If the deacylation of the lysophospholipid was indeed enhanced while the acylation process was inhibited by methyl lidocaine, such enhancement may act as an important compensatory mechanism for maintaining the appropriate lysophospholipid levels in the hamster heart.

II. Studies on *De novo* Lipid Biosynthesis

In mammalian tissues, phospholipids are formed *de novo* via the progressive acylation of glycerol-3-phosphate. The formation of glycerol-3-phosphate can occur either through glycolysis or by the phosphorylation of glycerol. In glycolysis, the enzyme glycerol-3-phosphate dehydrogenase converts dihydroxyacetone phosphate into glycerol-3-phosphate. Alternatively, the enzyme glycerol kinase phosphorylates free glycerol to produce glycerol-3-phosphate. Since glycerol-3-phosphate is not readily transported across the membrane, labelled glycerol has been routinely used as a general precursor for the study of phospholipid biosynthesis in mammalian tissues (Akesson *et al.* 1970; Allan and Michell 1975; Brindley and Bowley 1975). In this study, perfusion with labelled glycerol in the presence of methyl lidocaine resulted in an increased incorporation of radioactivity into the neutral lipids and acidic phospholipids. The increase in the labelling of these lipids did not result from a general increase in the specific radioactivities of their precursors since the specific radioactivities of lysophosphatidic acid and phosphatidic acid were not changed in the presence of methyl lidocaine.

Phosphatidate phosphatase and CTP: phosphatidate cytidylyltransferase are regarded as key enzymes in the production of diacylglycerol and acidic phospholipids, respectively (Sturton and Brindley 1977; Sturton *et al.* 1978; Cascales *et al.* 1984). The direct activation of these two key enzymes by methyl lidocaine provided us with a viable explanation for the observed increase in the labelling of diacylglycerol and

phosphatidylinositol. Interestingly, the labelling of triacylglycerol was enhanced but the activity of acyl-CoA: diacylglycerol acyltransferase was not affected by methyl lidocaine. In view of the fact that the level of long-chain acyl-CoA was not elevated, the increase in labelling of triacylglycerol in the methyl lidocaine perfused heart probably resulted from the enhanced labelling and increased pool size of diacylglycerol (Groener *et al.* 1979). Similarly, the increase in labelling of phosphatidylinositol in the methyl lidocaine perfused hearts was probably the result of an increase in the pool size of CDP-diacylglycerol and not due to activation of the phosphatidylinositol synthase.

The labelling of phosphatidylserine was increased only at 60 min of perfusion. The only known pathway for the biosynthesis of phosphatidylserine, in mammalian tissue, is through the base exchange pathway (Tijburg *et al.* 1989). For increased labelling in phosphatidylserine to occur, there should also be an increase in the labelling of phosphatidylcholine or phosphatidylethanolamine. At the 60 min time point, no change in the labellings of these two phospholipids was detected. The exact mechanism for the increased labelling of phosphatidylserine is still unclear.

It has been shown in previous studies that the majority of phosphatidylcholine and phosphatidylethanolamine in the heart is synthesized via the condensation of the CDP-bases with diacylglycerol (Zelinski *et al.* 1980; Hatch *et al.* 1989). The content and specific radioactivity of diacylglycerol was elevated by methyl lidocaine perfusion, yet the labellings and content of these two phospholipids were not significantly

altered. A similar phenomenon was observed in hepatocyte cultures with labelled palmitate in the presence of glucagon (Geelen *et al.* 1978). It appears that the formation of phosphatidylcholine and phosphatidylethanolamine was not dependent on the total intracellular diacylglycerol pool (Groener *et al.* 1979). Indeed, it was suggested that the synthesis of phosphatidylcholine and phosphatidylethanolamine was regulated independently from that of diacylglycerol (Groener *et al.* 1979). This hypothesis is supported by the fact that the total concentration of diacylglycerol in the heart is much higher than the K_m of diacylglycerol for both enzymes (O and Choy 1990). Another factor that may contribute to the observed labelling of the two phospholipids is the possible existence of several intracellular diacylglycerol pools (Soling *et al.* 1987). In addition, the phosphotransferases may display some selective utilization of specific diacylglycerol pools for the synthesis of phosphatidylcholine and phosphatidylethanolamine (Arthur and Choy 1984; Rustow and Kunze 1987).

It is intriguing to note that methyl lidocaine also caused the accumulation (increase in pool sizes) of diacylglycerol and triacylglycerol, but not phosphatidylinositol. One explanation is that the turnover rates of these lipids are different in the hamster heart. Based on the labelling of phosphatidylinositol, and the specific radioactivity of the precursor, only a very small extent of new synthesis occurred. Hence, the increase in synthesis of these phospholipids during the perfusion might represent a very small portion of the total cellular pool size. Alternatively, the antiarrhythmic nature of methyl lidocaine could have reduced the heart rate and consequently, the requirement for neutral lipids as a source of energy. Previous work has shown that

isolated perfused hearts utilize fatty acids in the neutral lipid fraction without affecting phospholipid metabolism (Severson 1979). This might result in decreased turnover rates in diacylglycerol and triacylglycerol, whereas the turnover rates of the phospholipids were not affected by methyl lidocaine treatment.

The effects of several amphiphilic compounds on the *de novo* synthesis of phospholipids in mammalian tissues have been reported (Eichberg and Hauser 1974; Allan and Michell 1975; Freinkel *et al.* 1975; Yada *et al.* 1986; Koul and Hauser 1987). In general, these compounds were shown to inhibit phosphatidate phosphatase activity (Bowley *et al.* 1977; Koul and Hauser 1987). Consequently, the biosynthesis of diacylglycerol was reduced with a concomitant increase in the synthesis of the acidic phospholipids (Eichberg and Hauser 1974; Allan and Michell 1975).

Methyl lidocaine is an amphiphilic compound which stimulates the activities of phosphatidate phosphatase and CTP: phosphatidate cytidylyltransferase, resulting in the stimulation of diacylglycerol and phosphatidylinositol biosynthesis. In view of the fact that the modulation of these enzyme activities is dependent on the Mg^{2+} content; the effect of methyl lidocaine was studied at different Mg^{2+} concentrations. It is clear that methyl lidocaine has the ability to stimulate enzyme activities at the physiological concentration (7.5 mM) of Mg^{2+} (Sturton and Brindley 1977). The ability to stimulate the synthesis of diacylglycerol and phosphatidylinositol by methyl lidocaine may provide us and other investigators with an excellent model to study the

metabolic consequences of enhanced levels of these second messengers (Berridge and Irvine 1989). Similar results have also been found with the ß-adrenergic agonist isoproterenol. This agonist has been found to cause enhanced formation of diacylglycerol as well as increased labelling in phosphatidylinositol and triacylglycerol (Soling *et al.* 1987). Therefore, the changes in lipid metabolism produced by methyl lidocaine are similar to the effects seen with amphiphilic compounds as well as agonists of ß-adrenergic receptors.

III. Determination of Long-Chain Acyl-CoA Levels

The level of intracellular long-chain acyl-CoA plays a very important role in the biosynthesis of lipids in mammalian tissues. Acyl-CoA acts as the acyl donor for the formation of all phospholipids and neutral lipids. It is therefore very important to determine the pool size of this intermediate. Methods for analysis of long-chain acyl-CoA levels within tissue were very laborious or required specialized equipment that was not available (Corkey 1988). The purpose of this study was to develop a simple method that would allow rapid analysis of long-chain acyl-CoA levels in mammalian tissues.

Following the isolation of long-chain acyl-CoA through phase partition, the samples were separated and analyzed by gas-liquid chromatography. The total long-chain acyl-CoA content as well as the distribution of the acyl species in rat liver are shown in Table 19. Our total content of 83 nmol/g wet weight is similar to the values obtained through other procedures (Tubbs and Garland 1964; Berge *et al.* 1984; Prasad *et al.* 1987). However, the value of arachidonoyl-CoA obtained in this study is lower than that reported by Prasad *et al.* (1987). One possible explanation is the difference in the diet of the animals between the two studies. The rodent diet used in the present study contained a 7% lipid supplement (Table 2) whereas the diet used in the other study was a sucrose-casein based diet. It was reported by Masuzawa *et al.* (1987) that the arachidonoyl-CoA level in the rat liver was significantly reduced (from 32% to 11%) by a diet supplemented with 10% soybean oil.

In conclusion, phase partition is a rapid procedure for the determination of long-chain acyl-CoA content and composition in mammalian tissue. The phase partition stage is adequate for the quantitation of acyl-CoA levels as subsequent thin-layer chromatography did not result in any significant changes in long-chain acyl-CoA levels. Using the same procedure, the analysis of long-chain acyl-CoA levels in the hamster heart was found to be 61 ± 9 nmol/g wet weight. The value obtained is similar to the reported value in the rat heart (Masuzawa *et al.* 1987). The relative ease of the procedure permits the determination of acyl-CoA content in a large number of samples. Analysis of long-chain acyl-CoA in smaller samples (biopsy samples) may also be feasible by increasing the sensitivity of the gas-liquid chromatography detector.

IV. Mechanism of Action

Methyl lidocaine is an amphiphilic agent containing a quaternary amine. Due to the positive charge of the drug, it displays a slow lipid permeability which results in a longer circulation half life when compared to its parent compound lidocaine (Gillis 1973). In order to determine the extent of methyl lidocaine uptake into the heart, radioactive methyl lidocaine was used. Hearts were perfused with labelled methyl lidocaine and then chased with unlabelled methyl lidocaine in fifty fold excess. The chase period should facilitate the removal of any remaining non-specifically bound methyl lidocaine. Following the isolation of subcellular fractions, the majority of the drug was found to be associated with unbroken cells, connective tissue and nuclei during low speed centrifugation. The affinity of methyl lidocaine for the membrane fraction may be due to insertion of the drug into the membrane bilayer or it may be the result of direct interaction with membrane proteins. The same type of interaction may be involved in the drug's association with the mitochondrial and microsomal fractions. The drug also displays some degree of solubility since 10% of the drug was associated with the cytosolic fraction.

Total uptake of methyl lidocaine was not significantly different between 0.01 and 0.02 mg/ml perfusions. Higher concentrations could not be tested since concentrations over 1 mg/ml methyl lidocaine have been shown to cause the heart to become physically and electrically inactive.

As mentioned previously, methyl lidocaine is a protonated amine associated with an aromatic ring. These structural features are also common to adrenergic receptor agonists (Strader *et al.* 1989). The changes in lipid metabolism by methyl lidocaine are very similar to those changes elicited by the action of isoproterenol (Soling *et al.* 1987). To investigate the possiblity that methyl lidocaine might be affecting lipid enzyme activity indirectly via activation of a signal transduction system, hearts were perfused for 5 min and the enzymes involved in the *de novo* lipid biosynthetic pathway were assayed. A significant increase in the activity of lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase was detected in hearts perfused with the drug. Interestingly, these enzymes could not be stimulated directly by methyl lidocaine. This suggests that these enzyme activities were enhanced by methyl lidocaine in an indirect manner, possibly via the action of second messengers. In support of a second messenger hypothesis, the ß-receptor agonist isoproterenol, also stimulates the activity of lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase (Yashiro *et al.* 1988; Soling *et al.* 1989).

The possible stimulation of a second messenger system was investigated by determining the status of the protein kinase C and cAMP cascades in methyl lidocaine perfused hearts. The activation of the protein kinase C cascade involves the translocation of protein kinase C from the cytosol to the membrane bound form (Wolf *et al.* 1985). Perfusion of hamster hearts for 5 min in the presence of methyl lidocaine did not result in any change in the activity of protein kinase C. The inability to activate protein kinase C suggests that this enzyme is not responsible for the

enhancement of *in vivo* enzyme activity. When the cAMP cascade was analyzed, there was a significant increase in the level of cAMP in hearts perfused with methyl lidocaine. Therefore, the activation of the cAMP cascade through the receptor mechanism or by direct activation of adenylate cyclase might be responsible for the *in vivo* stimulation of the acyltransferase activity.

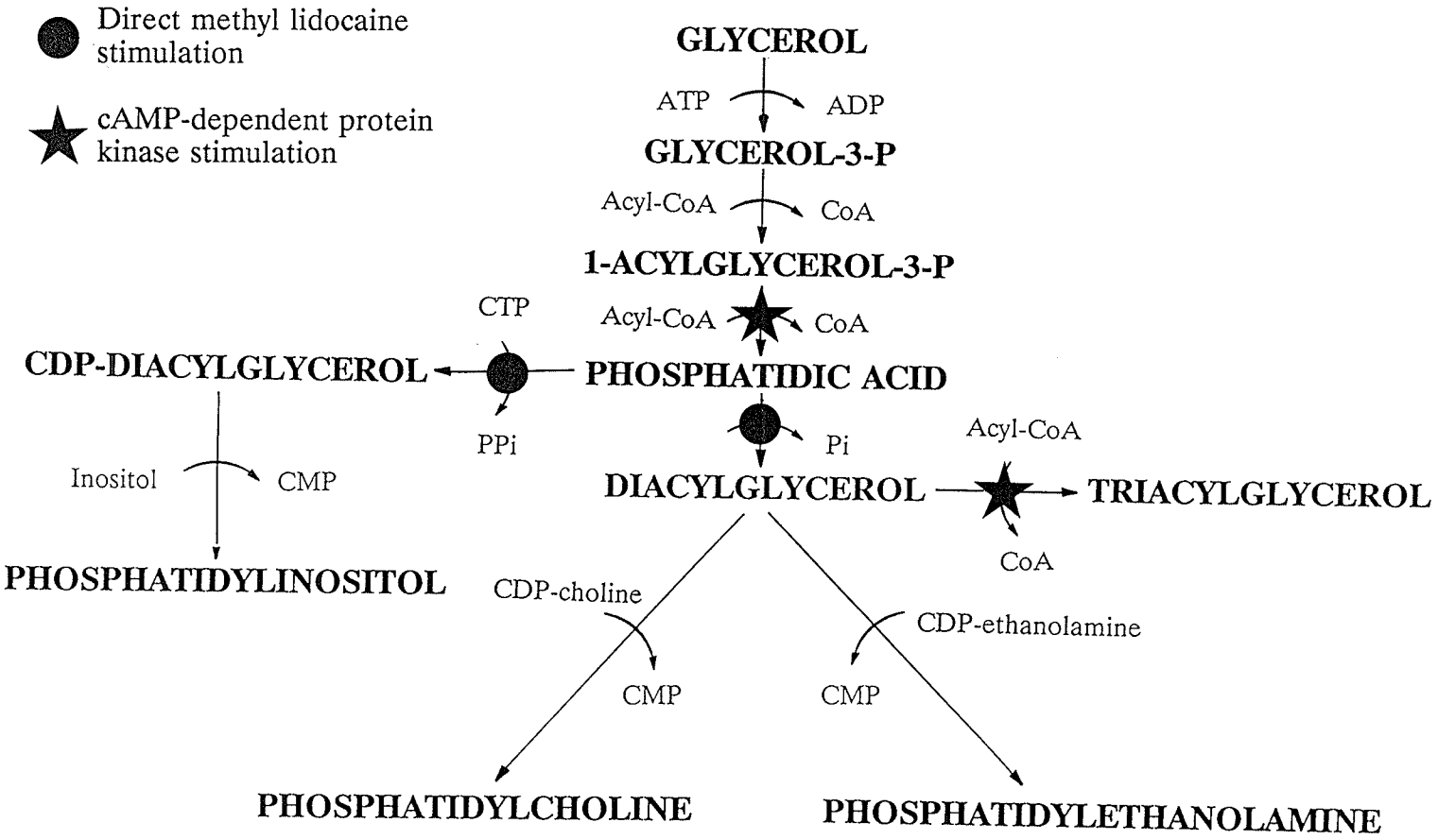
The elevation of cAMP levels in methyl lidocaine perfused hearts could result in the conversion of the inactive cAMP-dependent protein kinase into its active form, which could cause the phosphorylation of the acyltransferases. In order to test this hypothesis, lysophosphatidic acid acyltransferase and diacylglycerol acyltransferase in the post nuclei fraction were incubated in the presence of active cAMP-dependent protein kinase. Our results showed that both enzyme activities were stimulated. Thus, the *in vivo* stimulation of these enzyme activities by methyl lidocaine might be caused by activated cAMP-dependent protein kinase. Similar results were found in studies using the ß-agonist isoproterenol. This agonist was found to activate lysophosphatidic acid acyltransferase as well as diacylglycerol acyltransferase through a cAMP-dependent protein kinase phosphorylation reaction. The same study also found protein kinase C to be ineffective in activating these enzyme activities (Soling *et al.* 1989).

SUMMARY

Methyl lidocaine affects the *de novo* lipid biosynthetic pathway through two distinct mechanisms. Firstly, it was transported across the plasma membrane and entered the cell where it directly simulated phosphatidic acid phosphatase as well as the CTP: phosphatidic acid cytidylytransferase. Secondly, the drug caused an increase in intracellular cAMP levels. The drug may stimulate the production of cAMP either through activation of a receptor, inhibition of phosphodiesterase, or stimulation of adenylate cyclase. The enhanced levels of cAMP may have activated cAMP-dependent protein kinase which in turn stimulated the activity of diacylglycerol acyltransferase and lysophosphatidic acid acyltransferase.

Taken together, the results can explain the distribution of radioactivity following [^{3}H] glycerol perfusion. Methyl lidocaine caused the direct stimulation of phosphatidic acid cytidylyltransferase which resulted in enhanced labelling in the phosphatidylinositol fraction. The activation of phosphatidic acid phosphatase caused increased labelling in the diacylglycerol fraction. The large increase in the labelling of triacylglycerol was not due to a direct stimulation of diacylglycerol acyltransferase activity or to changes in the pool size of long-chain acyl-CoA. The stimulation of diacylglycerol acyltransferase by cAMP-dependent protein kinase is likely responsible for increased triacylglycerol labelling. The activation of lysophosphatidic acid acyltransferase by cAMP-dependent protein kinase may play a small role in increasing the labelling of the phospholipid fractions in methyl lidocaine perfused

Figure 21



Effect of methyl lidocaine on the biosynthesis of glycerophospholipids in the hamster heart

hearts.

Methyl lidocaine was used in this study to perturb lipid biosynthesis in the mammalian heart. In lysophospholipid metabolism, the role of the acyltransferase in the regulation of lysophospholipid levels was determined. In the study of *de novo* lipid biosynthesis, methyl lidocaine may regulate diacylglycerol acyltransferase and lysophosphatidic acid acyltransferase activities via activation of a cAMP-dependent protein kinase. We have also shown that the labelling of phosphatidylcholine and phosphatidylethanolamine was not affected by increased levels of diacylglycerol. The use of [^{3}H] glycerol is an excellent approach for the study of lipid biosynthesis in the mammalian heart. The methods developed in this study are currently in use to determine the effects of selenium and lidocaine on the *de novo* lipid biosynthetic pathway in the mammalian heart.

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