REGULATION OF PHOSPHOLIPID BIOSYNTHESIS IN THE HEART

BY

EDMUND FRANCIS T.C. LEE

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in partial fulfillment of the requirements for the degree of
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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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DEDICATED TO

MY FATHER- CLEMENT TAT-PONG, LEE

for his uncompromising principles that influence my life

MY MOTHER- CECILIA SAM-FUNG, LEE

for leading her son into intellectual pursuits

Without your love and sacrifice, it would not be possible for me to complete the programme.

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LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
ADP	adenosine-5'-diphosphate
ATP-γ-S	adenosine-5'-O-(3-thiotriphosphate)
cAMP	adenosine-3',5'-monophosphate
CDP	cytidine-5'-diphosphate
CTP	cytidine-5'-triphosphate
DAG	1,2-diacylglycerol
DTT	dithiothreitol
db-cAMP	N^6,2'-O-dibutyrl-adenosine-3':5'-cyclic monophosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-amino-ethyl-ether)N,N'-tetraacetic-acid
HCl	hydrochloric acid
IBMX	3-isobutyl-1-methylxanthine
$MgCl_2$	magnesium chloride
$MnCl_2$	manganese chloride
NaF	sodium fluoride
PMSF	phenylmethylsulfonyl fluoride
PKI	protein kinase A specific inhibitor
PI	phosphatidylinositol
SD	standard deviation
t.l.c.	thin layer chromatography
Tris	trizma base

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ABSTRACT

Phospholipids are the principal structural components of the biological membrane. Changes in the content and composition of the membrane phospholipids have profound effects on activities of membrane bound proteins. In addition, the conduction of electrical signals across the membrane in the heart appears to be dependent on the appropriate composition of the cardiac membrane phospholipids.

Part 1: Effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis in rat cardiac myocytes.

Phosphatidylcholine is a major component of membrane phospholipids in the heart. In the heart, the CDP-choline pathway accounts for over 90% of phosphatidylcholine biosynthesis. Although the biosynthetic pathway of phosphatidylcholine is known, its control mechanisms remain unclear.

Recently, many hormones have been shown to regulate phosphatidylcholine biosynthesis. Steroid hormones such as glucocorticoids and diethylstillbestrol have been shown to enhance the synthesis of phosphatidylcholine in fetal and rooster lungs. Others, such as noradrenaline and vasopressin have been reported to inhibit phosphatidylcholine biosynthesis in lung and liver. The inhibitory effect induced by vasopressin is believed to be a result of the reduction of CTP:phosphocholine cytidylyltransferase activity. Although vasopressin has been shown to inhibit phosphatidylcholine biosynthesis in lung and liver, its effect in the heart remains

unestablished.

The purpose of this study was to investigate the effect of vasopressin on phosphatidylcholine biosynthesis in rat cardiac myocytes. The initial approach was to determine the effect of vasopressin on choline uptake. Vasopressin caused a biphasic effect on choline uptake. Choline uptake was enhanced (25%) by a low vasopressin concentration (0.2 μM), but was attenuated (19%) by a higher vasopressin concentration (1.0 μM). The second approach was to examine the effect of the hormone on phosphatidylcholine biosynthesis by measuring the incorporation of labelled choline into the phosphatidylcholine. Results revealed that vasopressin regulated phosphatidylcholine biosynthesis in a biphasic manner. The mechanism of the observed biphasic response was explored by assaying the phosphatidylcholine biosynthetic enzymes in the CDP-choline pathway. It was found that the regulation of phosphatidylcholine biosynthesis induced by vasopressin was at the step catalyzed by CTP:phosphocholine cytidylyltransferase. At lower concentrations of vasopressin (0.05-0.2 μM), a general increase in CTP:phosphocholine cytidylyltransferase activity was observed which resulted in an enhanced conversion of phosphocholine to phosphatidylcholine via the CDP:choline pathway. At higher vasopressin concentrations (0.3-1.0 μM), a decrease in the activity of cytidylyltransferase was detected, which was caused by the translocation of the enzyme from the microsomal to the cytosolic fraction. In view of the fact that phospholipid biosynthesis in rat hepatocytes is inhibited by

vasopressin at all concentrations, the biphasic modulation of phosphatidylcholine biosynthesis in rat cardiac myocytes illustrates the diverse effects of this hormone in different mammalian tissues.

Part 2: Modulation of phosphatidylinositol biosynthetic enzymes by methyl lidocaine in isolated hamster hearts.

A number of amphiphilic compounds have been shown to exhibit regulatory effects on the biosynthesis of phospholipids in mammalian tissues. Methyl lidocaine, an experimental antiarrhythmic drug synthesized by Astra Pharmaceuticals, is a stable amphiphilic compound with local anaesthetic properties. In an earlier study, the *de novo* biosynthesis of phosphatidylinositol (PI) in hamster hearts was shown to be enhanced by methyl lidocaine. However, the mechanism involved in the increased biosynthesis of PI by methyl lidocaine was not clarified. In the present study, the mechanism for the modulation of PI biosynthesis by methyl lidocaine in the hamster heart was investigated.

The effect of methyl lidocaine on enzymes involved in the biosynthesis of PI in the heart was examined. Perfusion of the hamster heart with labelled methyl lidocaine showed that two-thirds of the radioactivity associated with the heart was bound extracellularly and was exchangable with unlabelled methyl lidocaine. The remainder of the radioactivity was taken up by the cardiac cell and recovered in subcellular fractions as methyl lidocaine. The direct action of methyl lidocaine was

studied by its presence in enzyme assays, whereas the indirect action of the drug was studied on enzyme activities in the heart after methyl lidocaine perfusion. CTP:phosphatidic acid cytidylyltransferase, a rate-limiting enzyme in PI biosynthesis, was stimulated by methyl lidocaine in a direct manner. Kinetic studies revealed that methyl lidocaine caused a change in the affinity between the enzyme and phosphatidic acid and resulted in the enhancement of the reaction. Alternatively, acyl-CoA:lysophosphatidic acid acyltransferase, another key enzyme for PI biosynthesis, was not activated by the presence of methyl lidocaine. However, the enzyme activity was stimulated in hearts perfused with methyl lidocaine. The enhancement of the acyltransferase by methyl lidocaine perfusion was found to be mediated via the adenylate cyclase cascade with the elevation of the cAMP level. The stimulation of protein kinase A activity by cAMP resulted in the phosphorylation and activation of the acyltransferase. Interestingly, the activity of protein kinase C was not stimulated by methyl lidocaine perfusion. We conclude that the enhancement of PI biosynthesis by methyl lidocaine was resulted from the direct activation of the cytidylyltransferase, as well as the phosphorylation and subsequent activation of the acyltransferase.

Introduction

I. The Biological Membrane

1. Membrane Structure and Function

In the eucaryotic cell, the shape of the cell is defined by the plasma membrane and the organelles are defined by the intracellular membrane system. The integrity of the biological membrane is crucial to cellular functions. Beyond its structural role as a protective barrier, the biological membrane also plays an important role in mediating many intracellular events. In this section, the structure and function of the biological membrane will be discussed.

The plasma membrane defines the geographical limits of the cell (Alberts *et al.* 1994). It separates the cell from its environment. The plasma membrane also acts as a highly selective barrier which contains pumps and gates so that the cellular electrochemical gradients can be maintained (Walter and Gutknecht 1986; Sweadner and Goldin 1980). Furthermore, the plasma membrane contains numerous transport systems made up of proteins which allow the entrance of nutrients and permit the removal of waste and metabolites (Stein 1986). In addition, the membrane participates in endocytosis, exocytosis, and pinocytosis (Alberts *et al.* 1994). The membrane also functions with respect to the transmission of cell signals. Hydrolysis of membrane lipids produces second messengers which elicit a wide variety of cellular responses (Liscovitch and Cantley 1994).

Eucaryotic cells contain a set of internal membranes that form boundaries for organelles including the Golgi apparatus, endoplasmic recticulum, mitochondria and perixosomes. Each subcellular organelle has its own local environment which is designed to carry out certain specific function(s). For example, mitochondria is an ATP generator, whereas endoplasmic recticulum serves as a reservoir for intracellular Ca^{2+}.

Despite the diverse functional and structural differences between membranes, they share some common features (Cullis and Hope 1991). First, all biological membranes in eucaryotic cells have a sheet-like structure which is only a few molecules thick. This structure forms closed boundaries between compartments of different composition. Second, lipids and proteins are the major component of all biological membranes. The weight ratio of protein to lipid, however, varies according to the cell type; generally, the ratio is between 1:4 to 4:1. The biological membrane usually contains a small amount of carbohydrate which is associated with the lipid or the protein moiety. Third, most membrane lipids are amphipathic in nature. These lipids form a closed bimolecular layer in aqueous media which is impermeable to any polar molecules. This bimolecular layer is also known as the membrane bilayer. Fourth, membrane lipids and proteins are distributed asymmetrically across the membrane surfaces. Finally, the bimolecular layers of membranes have a fluid-like property which allows lipid and protein molecules to diffuse freely in the plane of the membrane (Cullis and Hope 1991; Singer and

Nicolson 1972).

2. Membrane Lipids

By definition, lipids are insoluble in water but are highly soluble in organic solvents such as chloroform. Apart from its biological role as a major component of the membrane, lipids are also important as biological fuels and precursors for signal molecules such as steroid hormones and eicosanoids. There are three groups of membrane lipids: phospholipids, glycolipids, and cholesterols (Cullis and Hope 1991).

Phospholipids are the most abundant lipids in all biological membranes (White 1973). In eucaryotic cells, phospholipids can be derived from glycerol or sphingosine. Phospholipids derived from glycerol are known as phosphoglycerides, whereas phospholipids derived from sphingosine are known as sphingomyelin.

Fig.1 depicts the structure of a phosphoglyceride which consists of a glycerol backbone. The hydroxyl groups of the glycerol backbone at sn-1 and sn-2 are esterified to carboxyl groups of two fatty acids, whereas the hydroxyl group at sn-3 is esterified to a phosphate. The resulting compound is called phosphatidic acid. The phosphorylated group of phosphatidic acid is esterified to the hydroxyl group of one of the following alcohols: choline, serine, ethanolamine, glycerol, and inositol which forms phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol (Fig. 2).

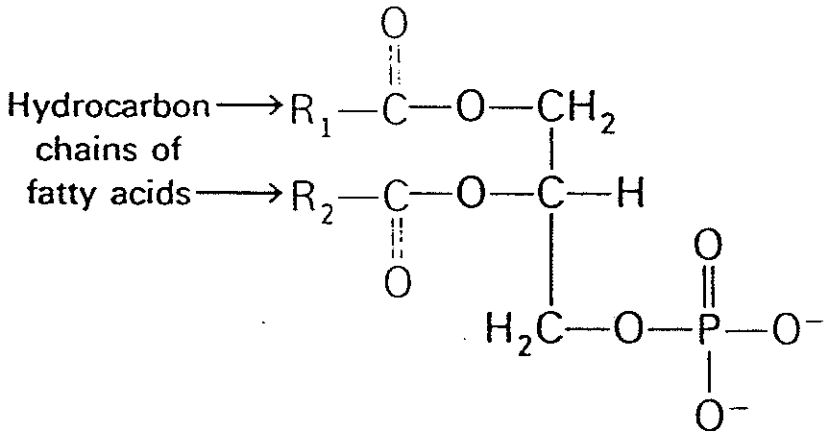


Figure 1: Basic structure of a phosphoglyceride

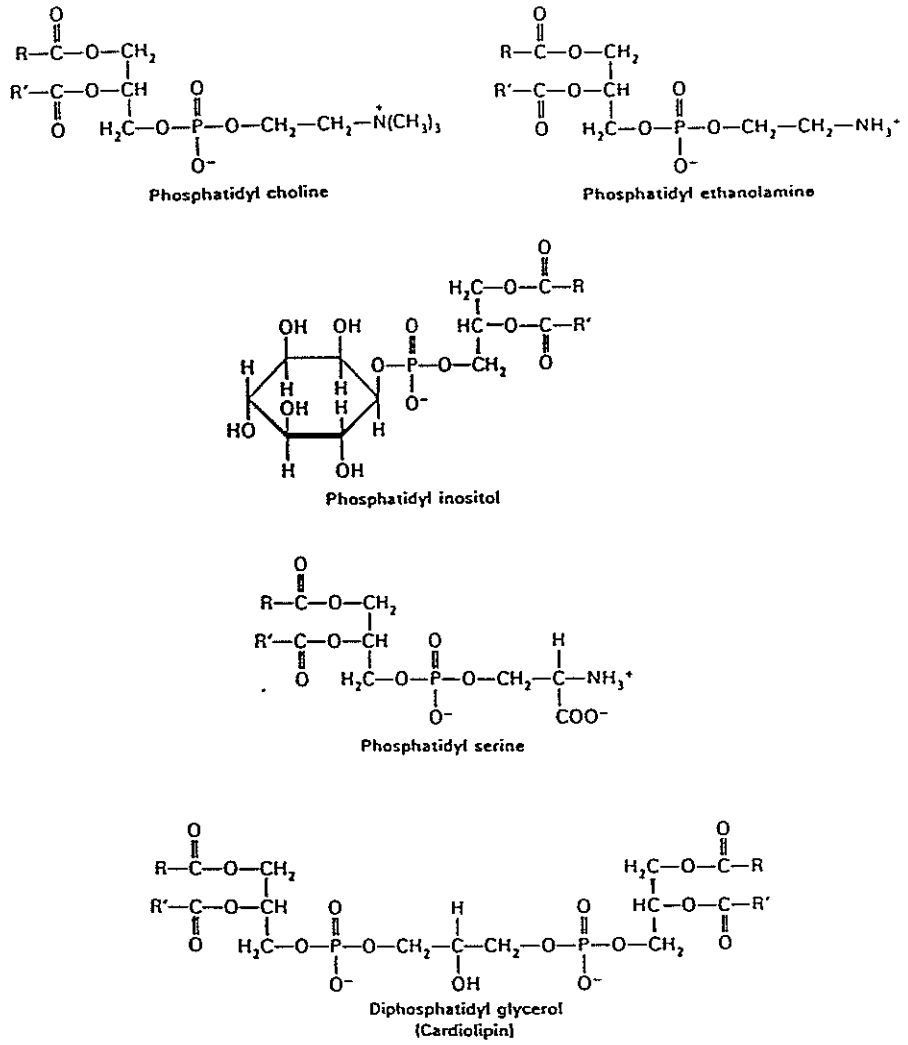
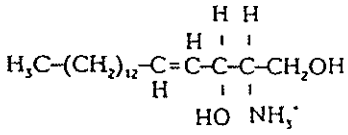


Figure 2: The five major types of phosphoglycerides in the mammalian heart

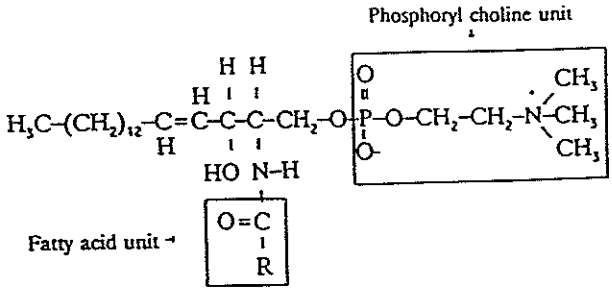
The base portion of phosphoglycerides is regarded as the polar head group, whereas the two fatty acid chains are regarded as the nonpolar hydrophobic tails. The fatty acid chain usually contains an even number of carbon atoms, ranging from 14 to 24. A large proportion of fatty acids have 16 and 18 carbon chains. These acyl chains are unbranched but can be saturated (no double bond) or unsaturated (with one or more double bond(s)). In general, the acyl chain at the sn-1 position is usually saturated, whereas the chain at the sn-2 is usually unsaturated. The degree of unsaturation of the acyl chain plays an important role in the regulation of membrane fluidity (Cullis and Hope 1991).

As mentioned earlier, phospholipids can be derived from glycerol as well as sphingosine. Shingomyelin is the only major phospholipid found in membranes that is derived from sphingosine (Sweeley 1991). In sphingomyelin (Fig. 3), the primary hydroxyl group of sphingosine is esterified to phosphocholine, whereas the amino group of the sphingosine backbone is associated with a fatty acid chain by an amide bond. Both sphingosine and phosphoglyceride contain polar head groups and nonpolar hydrophobic tails.

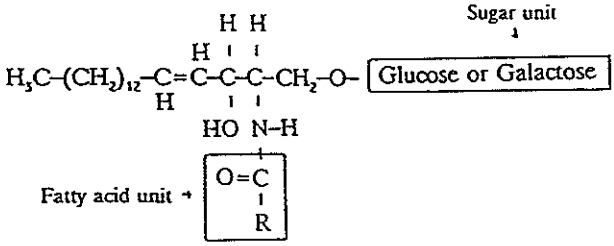
Glycolipid is a sugar-containing lipid which is structurally similar to sphingomyelin. The simpliest form of glycolipid is cerebroside (Fig. 3), which contains one sugar residue, either galactose or glucose. Ganglioside is another form of glycolipid which contains a branched chain of as many as seven sugar residues (Sweeley and Siddiqui 1977; Hakomori 1983). Glycolipids play a major role in



Sphingosine



Sphingomyelin



Cerebroside

Figure 3: Structures of sphingosine, sphingomyelin, and cerebroside

cell-cell adhesion and recognition (Sweeley 1991).

Cholesterol is the third type of lipid present in biological membranes. As shown in Fig. 4, it contains a bulky steroid moiety with a hydroxyl group that may interact with the polar head group of phospholipids, whereas the hydrocarbon tail interacts with the nonpolar core of the membrane bilayer. Cholesterol is a key modulator of membrane fluidity. It prevents crystallization of fatty acid chains by fitting between them (Bloch 1991).

3. Membrane Proteins

Membrane lipids form compartments and permeability barriers, whereas membrane proteins carry out most membrane processes. As mentioned in the preceding section, the protein content in the plasma membrane varies according to the cell type. For example, myelin, a special membrane which serves as an insulator around the nerve fiber, has a protein content of 18%. The inner membranes of mitochondria and chloroplast which serve as energy transducers have a protein content of up to 75%. Membrane proteins are believed to be associated with the lipid bilayer in five different ways (Alberts *et al.* 1994).

Fig. 5 depicts different interactions between membrane proteins and the lipid bilayer. Most transmembrane proteins are believed to extend across the bilayer as a single alpha (α) helix **(A)** or as multiple alpha (α) helices **(B)**; some of these single-pass and multipass proteins have a covalently attached fatty acid chain

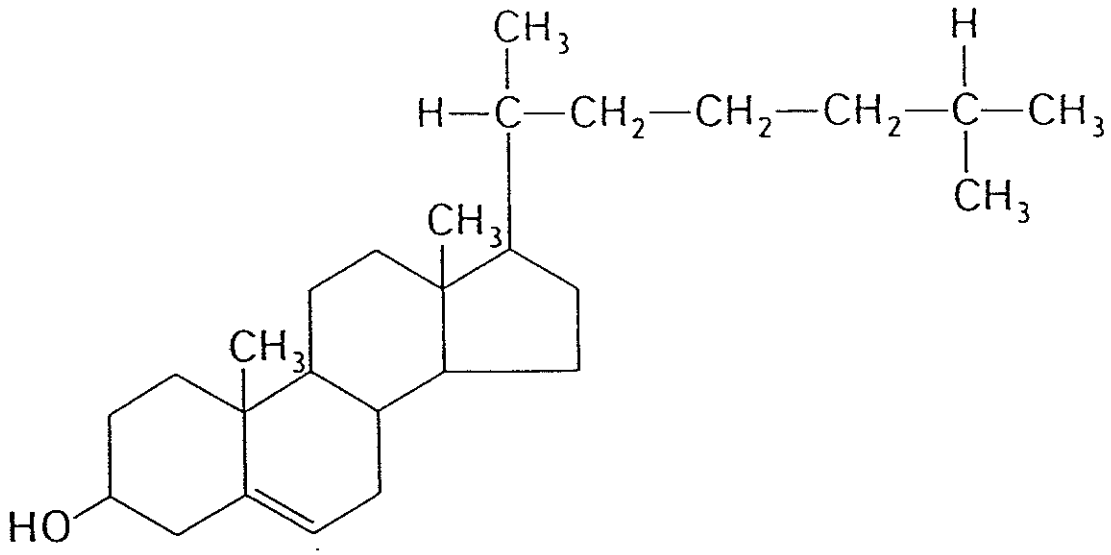
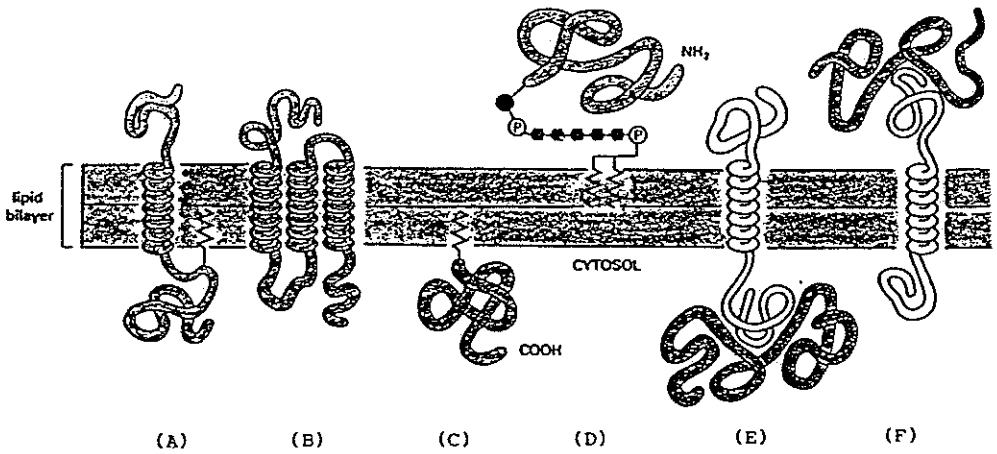


Figure 4: Chemical structure of cholesterol



(Alberts *et al.* 1994)

Figure 5: Interactions between membrane proteins and the lipid bilayer

inserted in the cytoplasmic monolayer. Removal of these transmembrane proteins from the bilayer usually requires the use of detergents or organic solvents. On the other hand, some membrane proteins are attached to the bilayer via a covalent interaction with a fatty acid chain located in the cytoplasmic side of the bilayer **(C)**, whereas some other membrane proteins contain an oligosaccharide which is linked to a phospholipid molecule in the outer leaflet of the membrane bilayer **(D)**. Peripheral proteins either on the inner or outer leaflet of the bilayer **(E,F)** are attached to the membrane by noncovalent interactions with transmembrane proteins. Peripheral proteins can be easily solublized by increasing the ionic strength of the environment by adding salts or changing the pH (Alberts *et al.* 1994)

4. Membrane Carbohydrates

Membranes of eucaryotic cells usually have a small carbohydrate content ranging from 2% to 10% (Alberts *et al.* 1994). In glycolipids, sugar residues are linked to the hydroxyl group of the sphingosine backbone. In glycoproteins, sugars are linked to the oxygen atom in the side chain of threonine or serine (O-linkage) or to the amide nitrogen atom in the side chain of asparagine (N-linked). The distribution of these carbohydrate groups in membranes can be readily detected by specific labelling techniques using lectins, a plant protein which has a high affinity for specific sugar residues. It was found that sugar residues of membrane

glycolipids and glycoproteins are always located on the extracellular side of membranes (Alberts *et al.* 1994).

The carbohydrate groups in glycoproteins serve to orient glycoproteins in membranes and to maintain the asymmetric character of biological membranes. Since sugar residues are highly hydrophilic, both glycoproteins and glycolipids prefer to be located near the aqueous surface rather than in the hydrophobic core. Carbohydrates on cell surfaces are believed to have a fundamental role in membrane phenomena such as cell-cell adhesion, cell-cell recognition, modulation of receptor activity, antigenic specificity, and regulation of growth (Sweeley 1991).

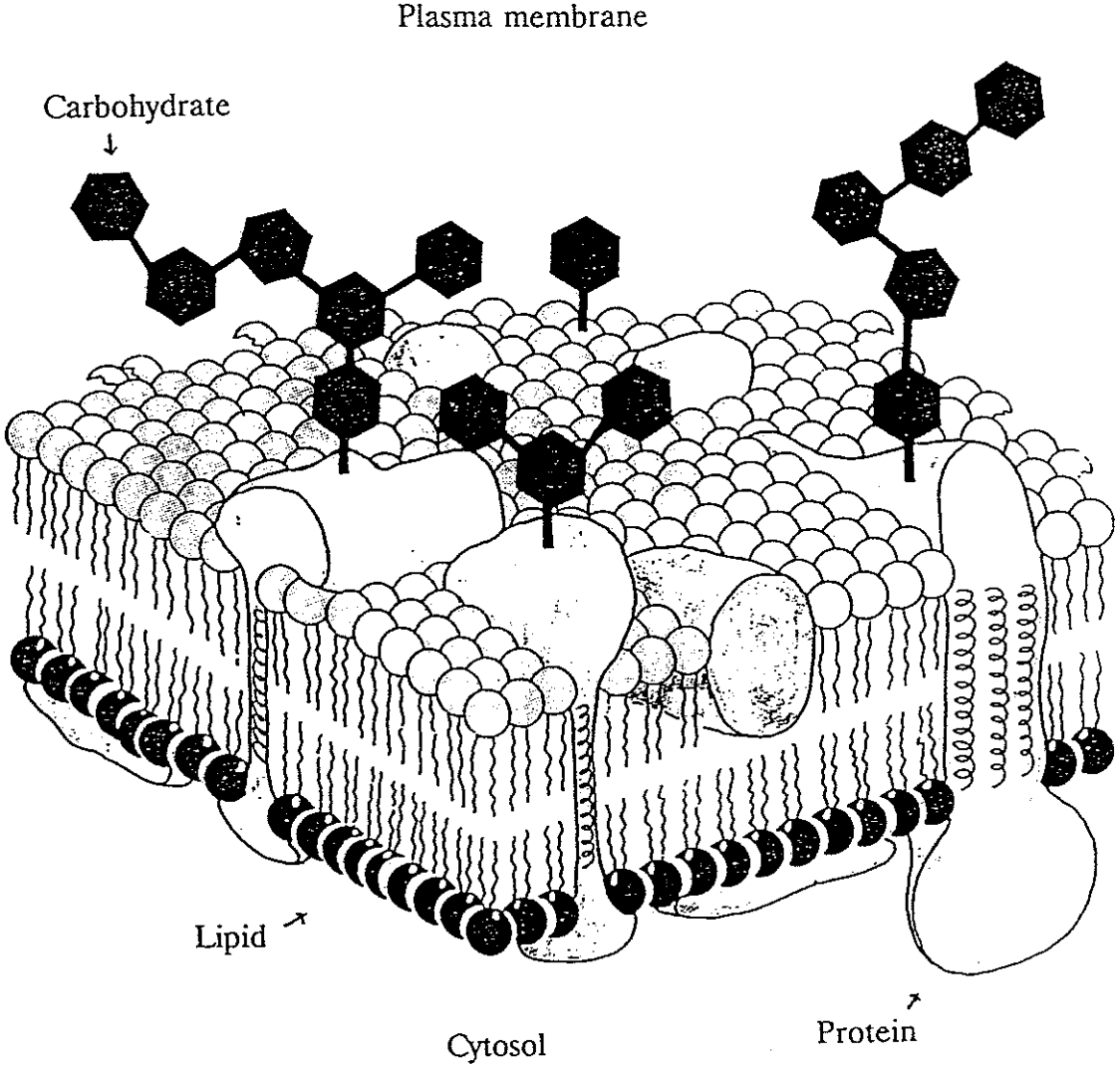
5. Membrane Asymmetry

The components of the biological membrane are distributed asymmetrically (Cullis and Hope 1991). The outer and inner leaflets of the membrane bilayer have different lipid and protein compositions. Membrane proteins have a unique orientation because they can be synthesized and inserted into the membrane in an asymmetric manner. Asymmetric distribution of membrane proteins is essential to maintain the regular cellular function. For example, the Na^+/K^+ pump, which functions to maintain the electrochemical gradient, is located asymmetrically such that K^+ is continuously pumped into the cell, whereas Na^+ is pumped outside the cell (Sweadner and Goldin 1980). Lipids are also distributed asymmetrically. For example, phosphatidylcholine is preferentially located in the outer leaflet of the

bilayer, whereas phosphatidylethanolamine and phosphatidylserine are located in the inner leaflet (Chap *et al.* 1977; Rothman and Lenard 1977).

6. Fluid Mosaic Model of Plasma Membrane

The present concept of membrane structure can be interpreted by the fluid mosaic model proposed by Singer and Nicholson in 1972. Fig. 6 shows the topography of membrane proteins, lipids, and carbohydrates in the fluid mosaic model of a typical eucaryotic plasma membrane. Membrane exists as a phospholipid bilayer of 60 - 100 Angstroms in thickness. Phospholipids constitute about 50% of the mass of mammalian cell membranes. The ability of phospholipids to form the bilayer organization is a result of their amphipathic character, i.e. phospholipids contain a polar region (hydrophilic) and a non-polar region (hydrophobic). It is thermodynamically favourable when the polar regions orient towards the aqueous phase and the non-polar regions are sequestered from water. Phospholipids which are arranged in a bimolecular layer play two important roles as: 1) a permeablility barrier, and 2) a solvent for integral membrane proteins. In addition, the fluid property of lipids allows membrane proteins to diffuse laterally in the bilayer plane. A membrane protein can diffuse through a distance of several microns in approximately one minute. The lateral mobility depends on the size and charge of the protein. Transverse movement which refers to the flip-flop movement of membrane lipids and proteins, however, is not observed,



(Cullis and Hope 1991)

Figure 6: Fluid mosaic model of the eucaryotic plasma membrane

indicating that the asymmetry of membranes can be preserved (Singer and Nicolson 1972).

II The *de novo* biosynthesis of phosphatidylinositol and phosphatidylcholine

1. Introduction

The backbone of phosphoglycerides such as phosphatidylcholine and phosphatidylinositol are derived from glycerol. There are at least 25 enzymes involved in the biosynthesis of phosphoglycerides. Most of these enzymes are membrane bound and contain transmembrane domains which interact with the hydrophobic core of the membrane bilayer. In addition, some of these enzymes simultaneously act with the lipid-soluble substrates as well as water soluble compounds. Owing to the difficulty in solubilizing these membrane bound enzymes, many of their kinetic parameters have not yet been identified. In biological membranes, about 80% of phospholipids are present as zwitterionic phospholipids: phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, choline-containing and ethanolamine-containing plasmalogens. The remaining 20% of phospholipids are present as acidic phospholipids at pH 7: phosphatidylserine, phosphatidylglycerol, cardiolipin, and PI. The composition of phospholipid species in the membrane varies between tissues and animal species. However, it is believed that the compositional differences are greater between tissues than between animal

species (White 1973). Fig. 7 shows the pathways for the *de novo* biosynthesis of phosphatidylcholine and PI. In most tissues, choline-containing lipids such as phosphatidylcholine, sphingomyelin, and choline plasmalogen represent 40-60% of the total membrane phospholipid content. Among these choline-containing lipids, phosphatidylcholine is the most abundant. Although the final step for the biosynthesis of phosphatidylcholine takes place in the endoplasmic recticulum, phosphatidylcholine is found in the plasma membrane and other subcellular locations. The cellular distribution of phosphatidylcholine is believed to be achieved by co-transport of lipids with excreted proteins from the endoplasmic recticulum to other subcellular membranes (Esko and Raetz 1983).

2. *sn*-Glycerol-3-phosphate

sn-Glycerol-3-phosphate is the precursor for the *de novo* biosynthesis of all phosphoglycerides. The plasma membrane is freely permeable to glycerol but not *sn*-glycerol-3-phosphate. When glycerol is taken up by the cell, it is readily converted into *sn*-glycerol-3-phosphate through the action of glycerol kinase. The kinase is a cytosolic enzyme which contains two subunits, and its activity varies greatly between tissues and species. In the rat heart, there is very little glycerol kinase activity (Robinson and Newsholme 1967). In the rat liver, glycerol kinase accounts for the synthesis of up to 20% of the total *sn*-glycerol-3-phosphate pool (Esko and Raetz 1983). In the chinese hamster ovary cell, no phosphorylation of

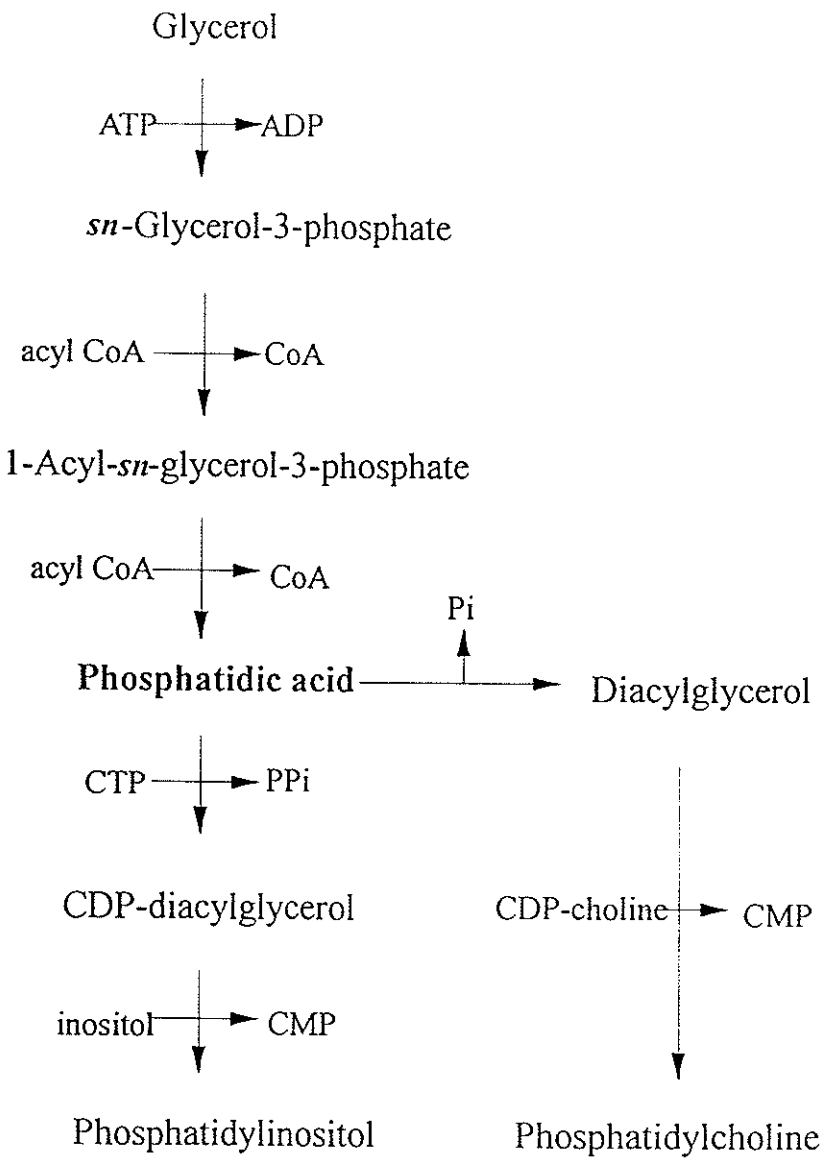


Figure 7: Pathways for the *de novo* biosynthesis of phosphatidylcholine and phosphatidylinositol in the mammalian heart

glycerol takes place due to the absence of the kinase (Esko and Raetz 1983). Alternatively, the formation of *sn*-glycerol-3-phosphate can be achieved via the reduction of dihydroxyacetone phosphate (Brindley 1991).

3. 1-Acyl-*sn*-glycerol-3-phosphate (Lysophosphatidic acid)

sn-Glycerol-3-phosphate undergoes an acylation reaction to form 1-acyl-*sn*-glycerol-3-phosphate. This reaction is catalyzed by *sn*-glycerol-3-phosphate acyltransferase. The enzyme is believed to be a rate-limiting enzyme in the biosynthesis of phosphatidic acid since the enzyme exhibits by far the lowest specific activity in the pathway (Söling *et al.* 1989). It is regulated by a fatty acid binding protein and Ca^{2+}. Two isozymes have been identified; one of which is in the microsomal and the other in the mitochondrial fractions. The two enzymes respond differently to heat treatment and magnesium activation (Bell and Coleman 1980). The exact physiological function of the mitochondrial isozyme remains unclear (Brindley 1991). In the liver, the microsomal isozyme has ten times higher specific activity than the mitochondrial enzyme (Van Den Bosch 1974). In terms of acyl specificity, the rat mitochondrial isozyme demonstrates a preference for the transfer of saturated acyl-CoA over unsaturated acyl-CoA. Conversely, the microsomal isozyme displays no selectivity for the acyl species. Furthermore, the mitochondrial isozyme has lower Km values for the substrates than the microsomal isozyme. Alternatively, acyl-*sn*-glycerol-3-phosphate can be formed by the

acylation of dihydroxyacetone-phosphate and the subsequent reduction of acyl-dihydroxylacetone-phosphate (Hajra and Agranoff 1968). The acylation reaction catalyzed by dihydroxylacetone-phosphate acyltransferase appears to be a second catalytic function of *sn*-glycerol-3-phosphate acyltransferase. Dihydroxyacetone-phosphate acyltransferase and *sn*-glycerol-3-phosphate acyltransferase have virtually identical pH dependencies, acyl-CoA chain length dependencies, thermolabilities, Km and Ki values, and susceptibilities to N-ethylmaleimide, trypsin, and detergents. Interestingly, *sn*-glycerol-3-phosphate is a competitive inhibitor of dihydroxylacetone-phosphate acyltransferase; whereas *sn*-glycerol-3-phosphate acyltransferase is competitively inhibited by dihydroxylacetone-phosphate (Schlossman and Bell 1976).

4. Phosphatidic acid

The next step in the biosynthesis of phospholipids is the subsequent acylation of 1-acyl-*sn*-glycerol-3-phosphate to phosphatidic acid. Phosphatidic acid is the key intermediate for the biosynthesis of all phospholipids. The acylation reaction of 1-acyl-*sn*-glycerol-3-phosphate is accomplished through the action of acyl CoA:lysophosphatidic acid acyltransferase. This enzyme is distinct from 2-acyl-*sn*-glycerol-3-phosphate acyltransferase and 1-acyl-glycerophosphocholine acyltransferase based on their differences in substrate specificity (Yamashita *et al.* 1973; Miki *et al.* 1977). Acyl CoA:lysophosphatidic acid acyltransferase is present

in rat liver microsomes and has an optimal pH at 7. In the rat liver, the acyltransferase demonstrates high substrate selectivity for monoenoic acyl-CoA and dienoic acyl-CoA. In addition, the enzyme activity is dramatically induced five to eight times during perinatal development (Coleman and Haynes 1983). *In vitro* studies reveal that the acyltransferase obtained in the microsome from guinea pig cerebellum is activated by protein kinase A and calmodulin-dependent protein kinase (Söling *et al.* 1989).

5. CDP-diacylglycerol

The formation of CDP-diacylglycerol is accomplished through a reaction between phosphatidic acid and cytidine triphosphate. This reaction is catalyzed by CTP:phosphatidic acid cytidylyltransferase. Since CDP-diacylglycerol represents a very small amount of the total lipid pool, the cytidylyltransferase is believed to be a rate-limiting enzyme for the biosynthesis of acidic phospholipids. The enzyme has an absolute requirement for Mg^{2+}. In the eucaryotic cell, CTP:phosphatidic acid cytidylyltransferase is found in both the microsomal and the mitochondrial fractions (Esko and Raetz 1983). *In vitro* studies show that direct addition of GTP results in a four-fold increase in the microsomal cytidylyltransferase activity. Fluoride blocks the GTP-stimulation, whereas ATP directly inhibits the enzyme activity. The physiological importance of the activation and inhibition of the enzyme by GTP and ATP, however, remain to be established (Liteplo and Shribney 1980).

Furthermore, the enzyme has limited specificity for the acyl composition of phosphatidic acid which may explain why the acyl composition of CDP-diacylglycerol is very similar to that of phosphatidic acid (Carter and Kennedy 1966).

6. Phosphatidylinositol

PI is formed through the condensation of CDP-diacylglycerol and *myo*-inositol. The latter substrate is derived from glucose. The condensation process is catalyzed by PI synthase. Similar to CDP:phosphatidic acid cytidylyltransferase, the PI synthase displays little acyl specificity, which may explain why the acyl composition of newly synthesized PI contains mainly monoenoic and dienoic species of fatty acids. These newly synthesized PI undergoes a deacylation-reacylation process which functions to remodel the acyl composition of PI (Esko and Raetz 1983). After remodelling, PI contains abundant polyenoic fatty acid species at the sn-2 position.

Successive phosphorylation of PI produces PI-4,5-bisphosphate which is an important molecule involved in the transduction of biological signals across the membrane. The hydrolysis of PI-4,5-bisphosphate generates two second messengers upon agonist stimulation: namely diacylglycerol and inositol-1,4,5-triphosphate (Berridge and Irvine 1989). Inositol 1,4,5-triphosphate causes an increase in the intracellular Ca^{2+} level. Ca^{2+} together with diacylglycerol cause the activation of

protein kinase C which in turn cause the phosphorylation of different intracellular proteins, leading to a wide variety of cellular responses.

7. Phosphatidylcholine

Phosphatidylcholine was first identified in the egg yolk in 1847 by Gobley. Hence, phosphatidylcholine was originally named lecithin after the Greek *lekithos* for egg yolk. Diakonow and Strecker in 1868 reported that lecithin contained two fatty acids linked to the first two hydroxyl groups of the glycerol backone, whereas a choline was attached to the third hydroxyl group (Vance 1991).

The physiological importance of phosphatidylcholine was first implicated by Eagle in 1955 who found that cells growing in a medium depleted of choline would result in subsequent cell death. It is now known that phosphatidylcholine is an important component in lung surfactant which prevents collapse of the alveoli while air is propelled during respiration (Klaus *et al.* 1961). In addition, phosphatidylcholine is a major component of lipoproteins which regulate the movement of lipids into and out of specific target tissues (Davis 1991).

Three separate routes for the *de novo* biosynthesis of phosphatidylcholine have been established: a) CDP-choline pathway, b) Methylation pathway, and c) the Base-exchange pathway. The CDP-choline pathway is the major route for phosphatidylcholine biosynthesis in the heart, whereas the other two pathways are minor (Fig. 8). (Zelinski 1980; Ansell and Spanner 1982).

a) CDP-choline pathway

The pathway was first elucidated by Kennedy who found that CTP is required for the biosynthesis of phosphatidylcholine (Kennedy 1962). As depicted in Fig. 8, choline is first taken up by the cell through a specific transport system. Once choline enters the cell, it is readily phophorylated by choline kinase to form phosphocholine. Phosphocholine then reacts with CTP to generate CDP-choline. This reaction is catalyzed by CTP:phosphocholine cytidylyltransferase which is the rate-limiting enzyme in this pathway. The final product, phosphatidylcholine, is formed by the condensation of CDP-choline and diacylglycerol through the action of CDP-choline:1,2-diacylglycerol cholinephosphotransferase. The uptake of choline has been shown to occur via two transport systems. The first system is a sodium-dependent system with a high affinity for choline (Km < 5μM). The second system is sodium-independent with a low affinity for choline (Km > 30μM) (Sung and Johnstone 1965). The high affinity system, which is associated with acetylcholine biosynthesis, is only observed in the cholinergic cell, whereas the sodium-independent system is observed in most mammalian tissues. Cardiac cells appear to contain only the sodium-independent choline uptake system (Hatch and Choy 1986). In the liver, choline taken up by the cell has two fates. Choline is either phosphorylated to form phosphocholine or oxidized to form betaine which serves as a methyl group donar for methionine biosynthesis (Pelech and Vance 1984). In the heart, choline is utilized for the biosynthesis of phosphatidylcholine

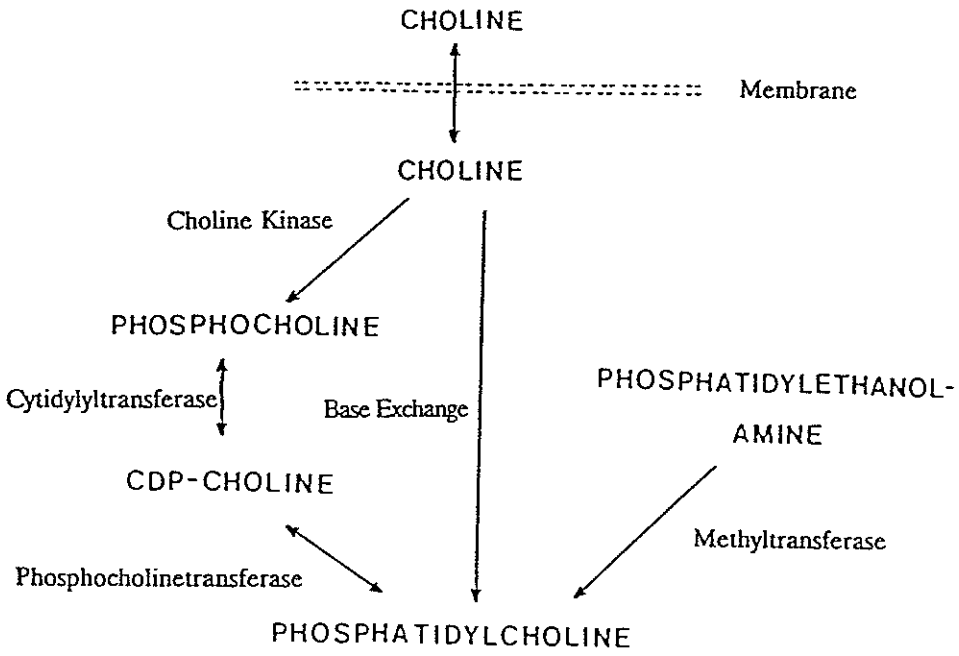


Figure 8: Pathways for the biosynthesis of phosphatidylcholine

(Zelinski *et al.* 1980).

Unlike many other lipid biosynthetic enzymes, choline kinase is present in the cytosol (Ishidate and Nakazawa 1992). The kinase has an absolute requirement for Mg^{2+} and ATP. The Km value of this enzyme for choline varies between tissues. In the brain, the Km for choline is 2.6 mM, whereas in the lung, the Km is 44 μM. This enzyme reaches its optimal activity at pH between 9 and 10, and the activity is greatly inhibited by Ca^{2+}.

CTP:phosphocholine cytidylyltransferase is present in both the cytosolic and microsomal fractions (Schneider 1963). The intracellular level of its catalytic product, CDP-choline, is very low suggesting that phosphocholine is rapidly transferred to phosphatidylcholine and that the cytidylyltransferase is a rate-limiting enzyme in the CDP-choline pathway (Zelinski *et al.* 1980). The enzyme was purified to homogeneity in rat liver by Feldman and Weinhold in 1987. It is a heterodimer with two non-identical subunits having a molecular weight of 45 and 38 KDa respectively. The 45 KDa subunit contains catalytic activity, whereas the actual function of the 38KDa subunit has not been identified. In the liver, the enzyme has an absolute requirement for Mg^{2+} with Km values of 0.17 mM and 0.3 mM for phosphocholine and CTP, respectively. The pH optimum of the enzyme is between 6 and 7. It is postulated that the activity of cytidylyltransferase in mammalian cells is enhanced by translocation of inactive enzyme from the cytosolic pool to the endoplasmic recticulum where it is activated by certain

phospholipids (Fig. 9) (Vance and Pelech 1984). The translocation mechanism is believed to be regulated by a phosphorylation/dephosphorylation reaction. Recently, the enzyme activity has been found to be localized in the nucleus of several cell types (Wang *et al.* 1993).

CDP-choline:1,2-diacylglycerol cholinephosphotransferase catalyzes the final step of the CDP-choline pathway. The highest activity of the enzyme is found in the smooth and rough endoplasmic recticulum, suggesting that phosphatidylcholine is synthesized locally in the endoplasmic recticulum (Cornell 1992). The enzyme is an integral membrane protein, and the active site is located in the cytosolic domain of the enzyme. Furthermore, the transferase displays high preference for diacylglycerol species with palmitic acid at the sn-1 and linoleic acid at the sn-2 positions of the glycerol moiety (Sarzala and Van Golde 1976). The enzyme is distinct from CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase based on differences in ionic charge, pH optima and heat stabilities (O and Choy 1990).

b) Methylation pathway

The first evidence which showed the existence of this pathway was given by Stetten in 1941. In his study, [^{15}N]choline was obtained from rats fed with [^{15}N]ethanolamine, indicating that ethanolamine might be modified to choline by a stepwise methylation reaction (Vance 1991). In bovine adrenal medulla, two

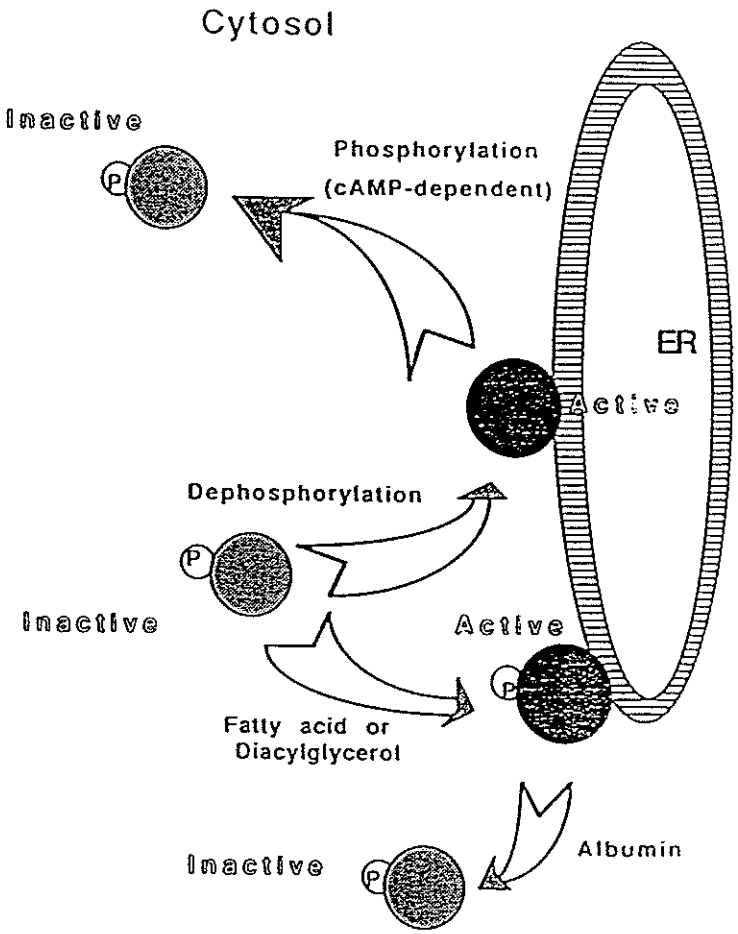


Figure 9: Translocation model of CTP:phosphocholine cytidylyltransferase

methyltransferases were isolated (Hirata *et al.* 1978) which were later found to be widely distributed in other tissues. These two methyltransferases require S-adenosyl-L-methionine as the methyl group donar and are believed to display different substrate specificity. The first methyltransferase is responsible for the transfer of a methyl group from S-adenosyl-L-methionine to phosphatidylethanolamine. The second methyltransferase plays a role in the subsequent methylation reactions which convert phosphatidylmonomethylethanolamine into phosphatidylcholine. The first methyltransferase has an optimum pH at 6.5, has an absolute requirement for Mg^{2+}, and a low Km for S-adenosyl-L-methionine (1.4 μM). Alternatively, the second methyltransferase does not require Mg^{2+} for activation, has an optimum pH at 10, and has a high Km for S-adenosyl-L-methionine (Hirata *et al.* 1978). The methylation pathway only reflects a minor contribution for the biosynthesis of phosphatidylcholine in the heart. The formation of phosphatidylcholine by this pathway is of significance only in the liver which accounts for about 20-40% total phosphatidylcholine synthesis (Vance 1990). The enzyme in rat liver has been purified to homogeneity and has a molecular weight of 18.3 kDa as determined by SDS-polyacrylamide gel electrophoresis (Ridgway and Vance 1992). Interestingly, the purified enzyme displays high pH optimum and has the ability to catalyze the three sucessive transmethylation reactions. Recently, a cDNA for phosphatidylethanolamine N-methyltransferase from a rat liver cDNA library has

been cloned and expressed in several cell lines. The calculated molecular mass of the enzyme is 22.3 kDa, which is equivalent to that of the pure enzyme isolated from rat liver (Cui *et al.* 1993).

c) Base-exchange reaction

The base-exchange reaction only accounts for a very minor contribution for the net synthesis of phosphatidylcholine. The mechanism of this reaction involves an exchange of free choline with the phosphatidylethanolamine head group. This exchange reaction is catalyzed by a base-exchange enzyme. The enzyme has an absolute requirement for Ca^{2+} and has an optimum pH at 7. The major contribution of this reaction is to remodel phospholipids that are newly synthesized in the cell (Kanfer 1989). Two base exchange enzymes have been identified in the mammalian cell (Tijburg *et al.* 1989). The first enzyme is responsible for an exchange reaction between serine and phosphatidylcholine or phosphatidylethanolamine. The second system is exclusive for the exchange of ethanolamine with other phospholipids, and the enzyme is located on the cytoplasmic side of the endoplasmic recticulum (Bell *et al.* 1981).

III. Signal Transduction

1. Introduction: hormonal effects

In the mammalian system, communication between organs is a very important mechanism for maintaining physiological homeostasis. One way of facilitating this communication is through the use of biochemical messengers known as hormones. Hormones are made in specific tissues and are released to the blood circulation. The secreted hormone is transported by the blood stream to the target organ, and the hormone carries out its action by altering the activity of the target organ. The effect of a hormone is initiated by binding to a specific cellular receptor located either on the outer leaflet of the plasma membrane or intracellularly. Such a binding elicits a profound effect on cellular functions.

As shown in Table 1, hormones are classified according to their specific mechanisms of action, the second messenger generated, and the type of membrane receptors (Granner 1990). In general, there are two distinct classes of hormones. One class refers to hormones which are lipid soluble, whereas another class refers hormones which are water soluble.

2. Hormones: mechanisms of action

a) All lipophilic hormones, except thyroid hormones, are derived from cholesterol and are collectively known as steroid hormones. Hormones of this class are permeable to the plasma membrane bilayer, and they bind to their specific intracellular receptors in the cytoplasm. Upon binding, the hormone-receptor complex undergoes a dimerization process followed by a phosphorylation reaction.

Table 1: Classification of hormones (Granner 1990)

Classification of hormones by mechanism of action.

Group I. Hormones that bind to intracellular receptors	
Estrogens	Calcitriol (1,25[OH]$_2$-D$_3$)
Glucocorticoids	Androgens
Mineralocorticoids	Thyroid hormones (T_3 and T_4)
Progestins	
Group II. Hormones that bind to cell surface receptors	
A. The second messenger is cAMP.	
Adrenocorticotropic hormone (ACTH)	Parathyroid hormone (PTH)
Angiotensin II	Opioids
Antidiuretic hormone (ADH)	Acetylcholine
Follicle-stimulating hormone (FSH)	Glucagon
Human chorionic gonadotropin (hCG)	α_2-Adrenergic catecholamines
Lipotropin (LPH)	Corticotropin-releasing hormone (CRH)
Luteinizing hormone (LH)	Calcitonin
Melanocyte-stimulating hormone (MSH)	Somatostatin
Thyroid-stimulating hormone (TSH)	β-Adrenergic catecholamines
B. The second messenger is cGMP.	
Atrial natriuretic factor (ANF)	
C. The second messenger is calcium or phosphatidylinositides (or both):	
α_1-Adrenergic catecholamines	Acetylcholine (muscarinic)
Cholecystokinin	Oxytocin
Gastrin	Gonadotropin-releasing hormone (GnRH)
Substance P	Angiotensin II
Thyrotropin-releasing hormone (TRH)	
Vasopressin	
D. The intracellular messenger is unknown:	
Chorionic somatomammotropin (CS)	
Growth hormone (GH)	Nerve growth factor (NGF)
Insulin	Epidermal growth factor (EGF)
Insulinlike growth factors (IGF-I, IGF-II)	Fibroblast growth factor (FGF)
Prolactin (PRL)	Platelet-derived growth factor

Subsequently, this dimeric complex migrates to the nucleus where it binds to the hormone response elements of the chromatin. This interaction results in a change in gene expression and transcription (Granner 1990).

b) Another class of hormones are those which are water-soluble. These hormones do not cross the membrane bilayer but are able to alter cellular activities through a process known as signal transduction. Signal transduction is initiated by the binding of the hormone to its specific membrane receptor. Such a binding results in the formation of second messengers which initiates a series of intracellular events. Second messengers such as cAMP, Ca^{2+}, cGMP, diacylglycerol, and inositol-1,4,5-trisphosphate have been studied extensively. There is growing evidence which shows that membane lipids serve as a major source of second messengers upon hormonal stimulation (Liscovitch and Cantley 1994).

Receptor molecules that span the membrane bilayer seven times allow signals to be transmitted to G-proteins. G-protein is named because of its association with the guanine nucleotide. G-protein consists of three protein subunits: alpha (α), beta (β), and gamma (γ). The alpha subunit possesses GTPase activity and contains a guanine nucleotide binding site. Beta and gamma subunits do not display any guanine nucleotide binding activity and are believed to associate with each other as a complex (Gilman 1987). Adrenergic receptors refer to a family of receptors which have a strong interaction with G-proteins. Four adrenoceptor

family members (α1, α2, β1, β2) have been identified according to their specific actions in response to agonists/hormones (Hardie 1991a).

As shown in Fig. 10, the binding of an agonist to an adrenergic receptor results in the activation of the receptor. The activated receptor induces an exchange of GTP for GDP on the alpha subunit and dissociation of alpha from the beta-gamma dimer. The GTP-bound alpha subunit diffuses laterally in the plane of the membrane bilayer where it interacts with various effector molecules such as adenylate cyclase or phospholipase C. The interaction induces a transient activation of the effector. After a short period of time, the GTP molecule is hydrolyzed into GDP by the GTPase in the alpha subunit, resulting in the inactivation of the subunit. The inactivated alpha subunit looses its association with the effector and finally reassociates with the beta-gamma complex (Gilman 1987).

In general, the activation of adrenergic receptor triggers one of the following two G-protein mediated signal transduction cascades: namely adenylate cyclase cascade, and the phosphoinositide cascade (Linder and Gilman 1992; Granner 1990).

3. Adenylate Cyclase Cascade

As shown in Fig. 10, adenylate cyclase cascade is initiated by the activation of adenylate cyclase in response to beta(β)1-adrenergic activation (Schramm and Selinger 1984). Adenylate cyclase which converts ATP into cAMP is a membrane

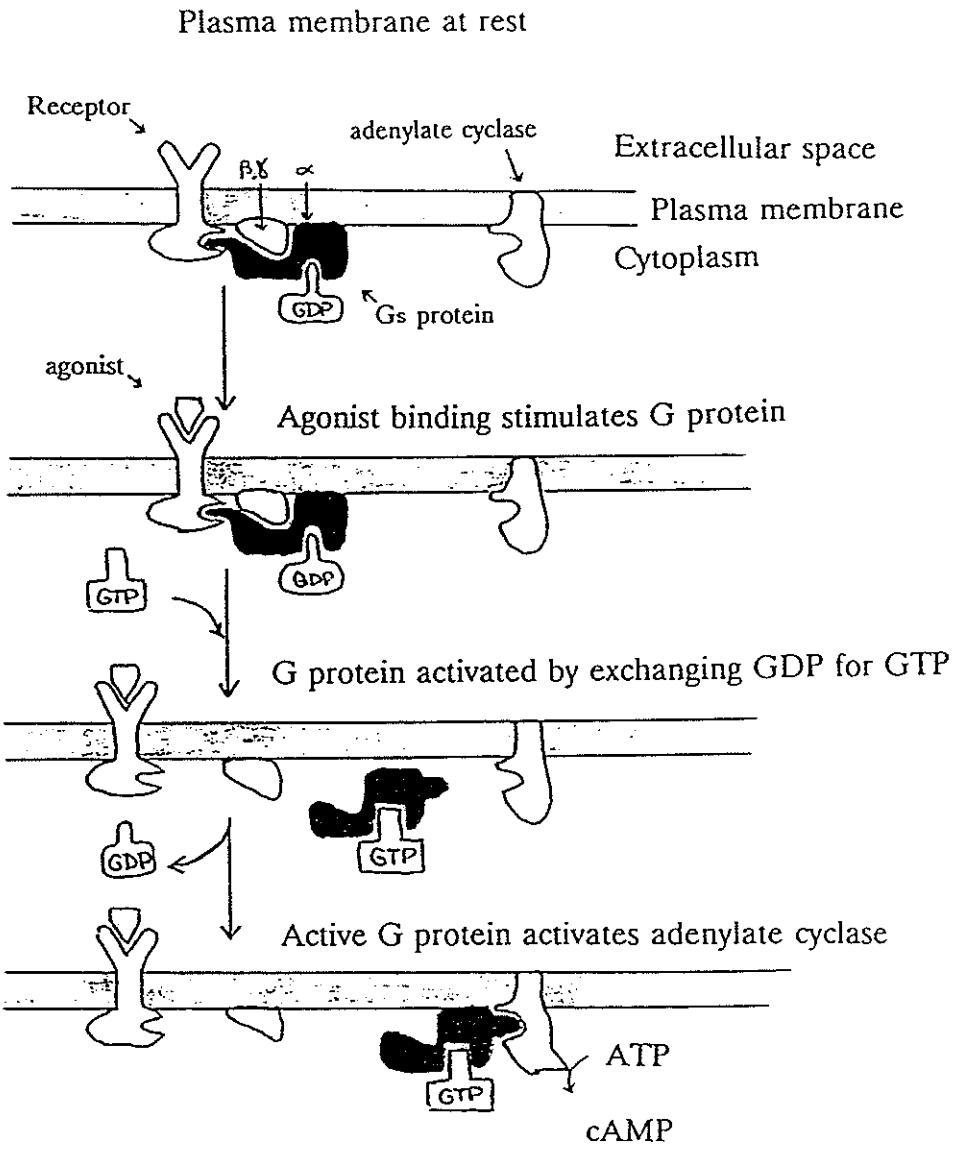


Figure 10: G-protein and adenylate cyclase cascade

bound enzyme located in the inner leaflet of the plasma membrane bilayer. cAMP is hydrolyzed by cAMP-specific phosphodiesterase. In addition, the elevated level of cAMP results in the activation of protein kinase A. Protein kinase A, a serine/theorine kinase, is a tetramer containing two catalytic (C) and two regulatory (R) subunits. The regulatory subunits keep the enzyme inactive by blocking the access of substrates to the catalytic site. cAMP binds to the regulatory subunit of protein kinase A, resulting in a conformation change of the subunit. As a result, the regulatory subunit is dissociated from the catalytic subunit. The active catalytic subunit phosphorylates various cellular proteins and enzymes, leading to different intracellular responses. Recently, the role of cAMP and protein kinase A in regulating gene transcription and cell proliferation have been implicated (Roesler *et al.* 1988; Dumont *et al.* 1989). The classical example of the adenylate cyclase cascade is the breakdown of glycogen by glucagon when the glucose level in the body is low. In liver, glucagon activates protein kinase A through the adenylate cyclase cascade. The activation of protein kinase A in turn phosphorylates and activates glycogen phosphorylase which is an enzyme responsible for the breakdown of glycogen into glucose (Palasi *et al.* 1971).

4. Phosphoinositide Cascade

The binding of an agonist to an alpha(α)1-adrenergic receptor triggers another signal transduction cascade, namely phosphoinositide cascade (Berridge

1987). As depicted in Fig. 11, the activation of a receptor coupled G-protein activates a phosphatidylinositol-4,5-bisphosphate specific phospholipase C. The activated phospholipase in turn hydrolyzes the membrane phosphatidylinositol-4,5-bisphosphate, resulting in the formation of diacylglycerol and inositol-1,4,5-trisphosphate (Berridge and Irvine 1989). Inositol-1,4,5-trisphosphate is a potent Ca^{2+} mobilizer which stimulates the release of Ca^{2+} from the endoplasmic recticulum into the cytosol. Diacylglycerol is an activator of protein kinase C. Protein kinase C, a serine/theorine kinase, is localized in both the microsome and the cytosol. The cytosolic form is believed to be the inactive form, whereas the microsomal form is the active form. Inositol 1,4,5-trisphosphate and diacylglycerol act synergistically to modulate protein kinase C. The kinase requires diacylglycerol for activation and Ca^{2+} for translocation from the cytosolic to the microsomal compartment (Nishizuka 1992). Phosphatidylserine has been shown to cause direct activation of the kinase (Bell and Burns 1991).

IV. Vasopressin

1. Characteristics of Vasopressin

Figure 12 depicts the amino acid sequence of vasopressin. Vasopressin is a peptide hormone consisting of nine amino acids with cysteine residue at position one and six forming a disulfide bridge. It is synthesized in the supraoptic nucleus

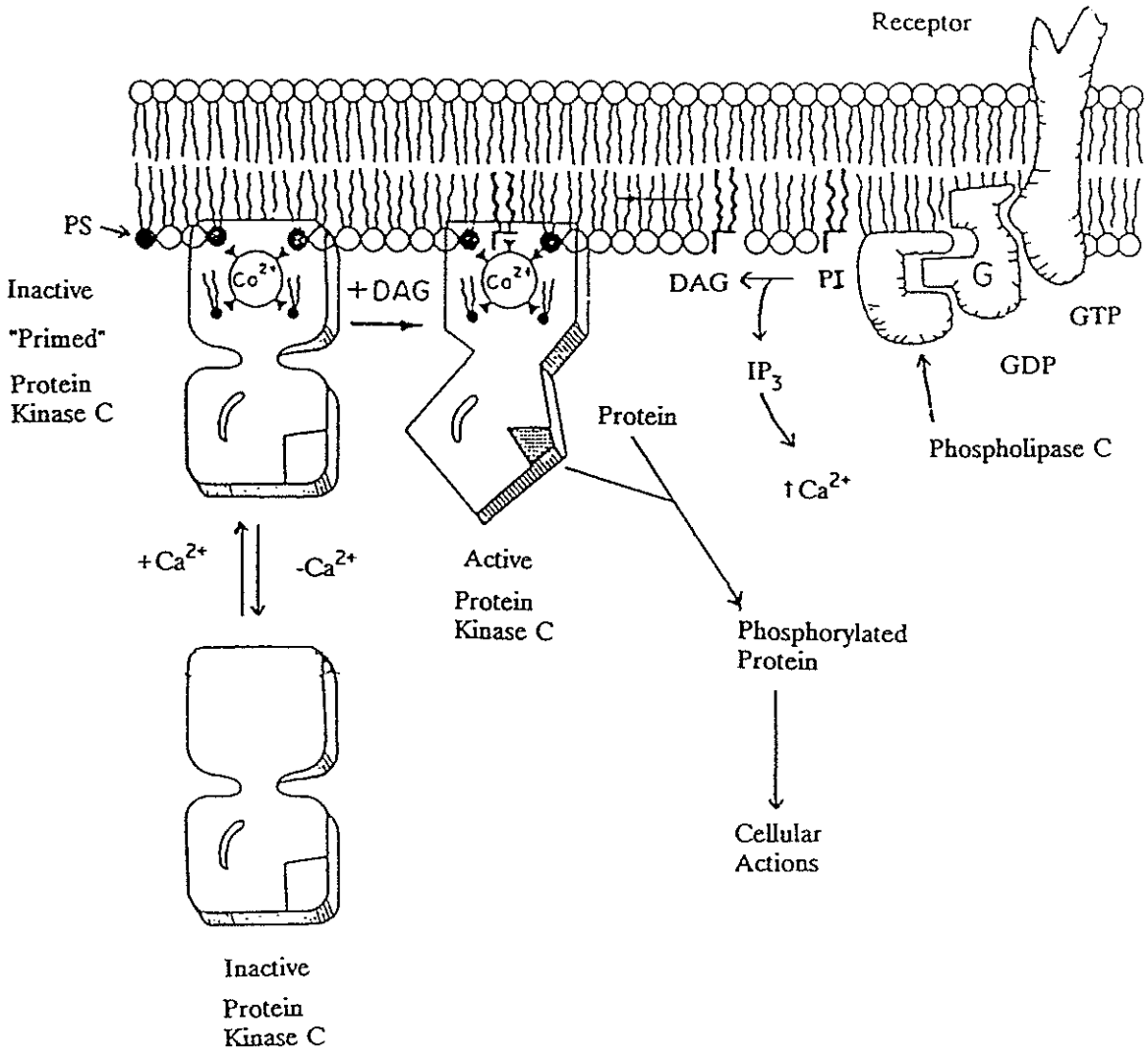


Figure 11: Phosphoinositide cascade

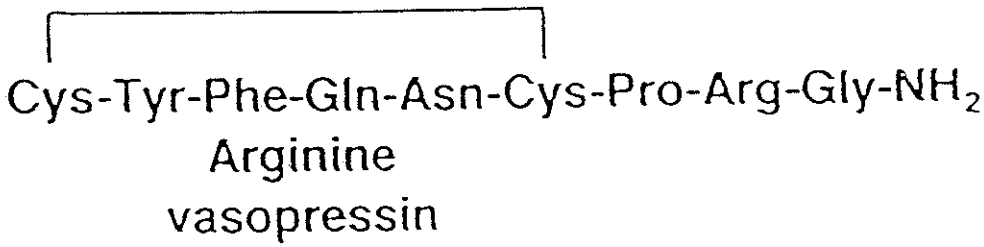


Figure 12: Amino acid sequence of vasopressin

and is transported to nerve endings in the posterior pituitary gland, where it is released into the circulation upon stimulation. The hormone is released by numerous factors, for example, its release may be caused by an increase in plasma osmalarity, emotional stress, or chemicals such as nicotine and morphine (Landau 1986; Hardie 1991b).

As mentioned in the signal transduction section, stimulation of the beta(β)1-adrenergic receptor results in the activation of adenylate cyclase, whereas activation of an alpha(α)1-adrenoceptor leads to the stimulation of phosphatidylinositol-4,5-bisphosphate specific phospholipase C. Interestingly, vasopressin is an agonist which exhibits dual binding activities to the α1- and β1- adrenergic receptors (Hays 1990; Morel *et al.* 1992).

In the kidney, during condition of dehydration, vasopressin concentration increases in the circulation. Vasopressin carries out its anti-diuretic action by binding to the V2 receptors located in the collecting duct and the ascending limb of Henle's loop (Hays 1990). V2 receptor which is a member of the β1 -adrenergic receptor family is coupled to adenylate cyclase. Activation of adenylate cyclase results in the production of cAMP. The elevated level of cAMP activates protein kinase A which in turns initiates a series of cellular events, leading to an increase in the luminal membrane permeability. The increase in permeability results in the reabsorption of water in the collecting duct.

In the cardiovascular tissue, vasopressin exerts its effect on vasoconstriction

by decreasing coronary blood flow and increasing pulmonary arterial pressure (Hays 1990). This effect is mediated through the V1 receptor. V1 receptor which is a member of the α1-adrenergic receptor family is tightly coupled to phosphatidylinositol-4,5-bisphosphate specific phospholipase C. Activation of the phospholipase results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate and the subsequent release of inositol-1,4,5-trisphosphate and diacylglycerol. The increase in diacylglycerol level causes the activation of protein kinase C, and the kinase is believed to play an essential role in causing vasoconstriction. Recently, the cDNA of the V1 receptor in the liver has been cloned (Morel *et al.* 1992). The cDNA reveals that the receptor protein contains seven putative transmembrane domains and a cytoplasmic loop in the C-terminal. The transmembrane domain has structure similar to bacterial rhodopsin and other G-protein coupled receptors. Furthermore, the receptor may be regulated by phosphorylation since the cytoplasmic loop contains several potential phosphorylation sites.

2. Effects of Hormones on Phosphatidylcholine Metabolism

Steroid hormones such as estrogen and glucorcorticoids have been shown to enhance phosphatidylcholine biosynthesis in lung. In rooster lung, estrogen enhanced phosphatidylcholine biosynthesis is a result of the activation of choline kinase (Vigo and Vance 1981). Alternatively, in fetal rabbit lung, the enhancement of phosphatidylcholine biosynthesis by estrogen is due to the stimulation of

CTP:phosphocholine cytidylyltransferase (Chu and Rooney 1985). In rooster lung, glucocorticoids induce phosphatidylcholine biosynthesis by increasing the biosynthesis of CTP:phosphocholine cytidylyltransferase (Rooney *et al.* 1986; Post 1987).

In contrast to estrogen and glucocorticoids, noradrenaline and vasopressin have been shown to inhibit phosphatidylcholine biosynthesis. Noradrenaline suppresses phosphatidylcholine biosynthesis by inhibiting CTP:phosphocholine cytidylyltransferase (Haagsman *et al.* 1984). Similiar to noradrenaline, vasopressin also inhibits phosphatidylcholine biosynthesis by reducing the activity of CTP:phosphocholine cytidylyltransferase in rat hepatocytes (Tijburg *et al.* 1987). In addition, vasopressin lowers the CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity by increasing the intracellular Ca^{2+} concentration (Alemany *et al.* 1982). Although vasopressin appears to inhibit phosphatidylcholine biosynthesis in tissues such as liver and lung, its role in the regulation of phosphatidylcholine biosynthesis in the heart remains unclear.

V. Methyl lidocaine

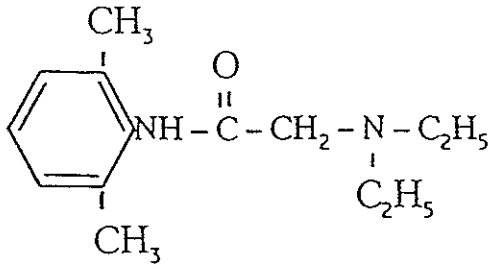
1. Introduction: Methyl Lidocaine

Methyl lidocaine is an experimental antiarrhythmic drug with local anesthetic property. Most local anesthetics contain a tertiary nitrogen atom which

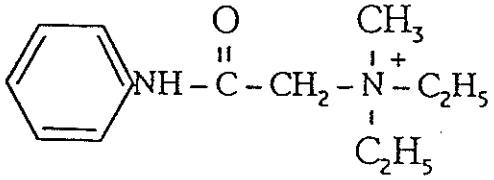
exists either as a charged ammonium ion or an uncharged amine, depending on the pH state of the solution. The charged form is believed to carry out active pharmacological action, whereas the uncharged form is the inert form. As depicted in Fig. 13, methyl lidocaine is an amphiphilic compound and has an external methyl group which covalently binds to the tertiary nitrogen atom, thus giving rise to a permanent positive charge. This experimental drug has a structure which is very similar to lidocaine. Methyl lidocaine was developed by Astra Pharmaceuticals to examine whether the charged group has a role in the antiarrhythmic action of lidocaine.

2. Antiarrhythmic Actions

The clinical effectiveness between lidocaine and methyl lidocaine has been compared. Despite the successful clinical application of lidocaine, the drug carries two major drawbacks. Lidocaine is rapidly metabolized; therefore, continuous intravenous administration is required to maintain its concentration in the circulation. In addition, the metabolites of lidocaine may elicit certain toxic effects which lead to the impairment of the central nervous system (Smith and Duce 1971). In contrast, methyl lidocaine does not display any side effect and is found to be an effective antiarrhythmic agent (Gillis *et al.* 1973; Kniffen *et al.* 1974). A study conducted by Patterson *et al.* in 1988 showed that methyl lidocaine is a more effective depressant than lidocaine in terms of the ability to depress conduction in



Lidocaine



Methyl lidocaine

Figure 13: Chemical structures of lidocaine and methyl lidocaine

ischemically injured myocardium.

3. Methyl lidocaine and Lipid Biosynthesis

Many amphiphilic compounds have been shown to regulate lipid metabolism in mammalian tissues (Kanfer and McCartney 1993; Yada and Nakazawa 1986). Methyl lidocaine also exerts a profound effect on lipid metabolism. An earlier study showed that perfusion of hamster hearts with labelled glycerol in the presence of methyl lidocaine resulted in a pronounced increase in the total uptake of labelling in the organic fraction. Such an increase was attributed to an increase in the labelling of diacylglycerol, triacylglycerol, and PI. These earlier findings indicate that methyl lidocaine may have the ability to enhance the biosynthesis of neutral lipids and acidic phospholipids (Tardi *et al.* 1992).

MATERIALS AND METHODS

I. Materials

1. Experimental Animals

Male Sprague Dawley rats (250 ± 40g) of either sex were used during the course of experimental study. Rats were obtained from Charles River Canada Inc. (St. Constante, Quebec) and were maintained on the Agway rodent chow (Agway Inc., Syracuse, N.Y.), and tap water *ad libitum*, in a light- and temperature controlled room.

Syrian golden hamsters (120 ± 20g) of either sex were used during the studies. The animals were obtained from Charles River Canada Inc. (St. Constante, Quebec). Hamsters were maintained on the Agway rodent diet RMH 3000 (Agway Inc., Syracuse, N.Y.), and tap water *ad libitum*, in a light- and temperature controlled room.

2. Chemicals

Phospho[*methyl*-^{14}C]choline, CDP[*methyl*-^{14}C]choline, [*tyrosyl*-^{3}H] arginine vasopressin, and the reagents for the determination of cAMP level and protein kinase C activity were obtained from Amersham Canada Ltd (Oakville, Ontario).

[*methyl*-^{3}H] Choline, [γ-^{32}P]-ATP (30 Ci/mmol), oleoyl-1-[^{14}C]-Coenzyme A(56.0 mCi/mmol) and [5-^{3}H]-CTP (24 Ci/mmol), and [U-^{3}H]-methyl lidocaine were obtained from New England Nuclear Division of Dupont (Mississauga, Ontario). Arginine-vasopressin and ATP-γ-S were purchased from Boehringer Mannheim Canada Ltd (Laval, Quebec). [d$(Ch_2)_5$, D-Tyr$(OEt)^2$, Val^4, Cit^8]-Vasopressin was obtained from Peninsula Laboratories Inc. (Belmont, California). Minimal essential medium (Joklik modified), Medium 199, Dulbecco's phosphate buffered saline, phosphocholine, choline, cytidine 5'-diphosphocholine, ATP, CTP, hyaluronidase, EDTA, EGTA, oleoyl-CoA, ADP, PMSF, benzamidine, β-glycerol-3-phosphate, NaF, IBMX, Tris[hydroxyymethyl]aminomethane, DTT, Triton X-100, db-cAMP, bovine serum albumin, and the catalytic subunit of protein kinase A (from bovine heart) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Collagenase (Class 2) was obtained from Worthington Biochemical Co. (Freehold, New Jersey). Phosphatidylcholine (pig liver), 1,2-diacylglycerol (pig liver), lysophosphatidic acid, and phosphatidic acid were products of Doosan Serdary Research Laboratories (London, Ontario). Sephadex G-25 (fine) gel filtration media was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Methyl lidocaine was a gift from Astra Pharmaceutical Products (Mississauga, Ontario, Canada). P81 papers were obtained from Whatman (Clifton, NJ, USA). SQ 22536 (adenylate cyclase inhibitor) was the product of Calbiochem Co. (Windsor, Ontario, Canada). Glacial acetic acid, $MgCl_2$, and t.l.c. plates (Redi plate silica gel G25) were

purchased from Fisher Scientific (Winnipeg, Manitoba, Canada). Kemptide and PKI (specific protein kinase A inhibitor) were obtained from Bachem Inc. (Torrance, CA, USA) All other chemicals and solvents were of reagent grade and obtained from the Canlab Division of Baxter Co. (Mississauga, Ontario, Canada).

II. Methods

1. Preparation of Buffer Solutions for Rat Myocyte Isolation

Buffer A contained MEM-Jokliks modified buffer preparation (11g/l), 60 mM taurine, 2 mM DL-carnitine, 1.0 mM adenosine, 3.4 mM magnesium chloride, 50 mM sodium bicarbonate, 0.1 mM octanoic acid, 8 mM glutamate, 15 mM glucose, penicillin (0.1 g/l) and streptomycin (0.1 g/l). Buffer B contains 0.1 mM calcium chloride dissolved in Buffer A. Buffer C contains Medium 199 preparation (9.8g/l), 2 mM L-glutamine, 2.2 g/l sodium bicarbonate, penicillin (0.1 g/l) and streptomycin (0.1 g/l). All solutions were sterilized by 0.22 μm Falcon 7150 bottle top filters. All buffers were maintained at 37°C and saturated with 95% oxygen/5% CO_2 prior to rat heart perfusions. Buffer B containing collagenase and hyaluronidase were saturated with 95% oxygen/5% CO_2 (aerated for 30 min).

2. Isolation of Rat Cardiac Myocytes

Isolation of myocytes was performed according to the method of Langer *et*

al. (Langer *et al.* 1987). In brief, the rat was sacrificed by decapitation and the heart was removed. The isolated heart was perfused in the Langendorff mode with Buffer A for 5 min at a flow rate of 9.5 ml/min. Susequently, the heart was further perfused in Buffer B containing 4 mg/ml collagenase and 2 mg/ml hyaluronidase at a flow rate of 9.5 ml/min. The perfusate was recycled over the course of the perfusion. Perfusion was terminated when the heart was completely digested. The digested heart was placed in a culture dish containing Buffer B. The myocytes were dislodged from the heart tissue by gentle shaking. The cell suspension was transferred to a culture tube. Buffer B was added to bring the volume to 50 ml. Myocytes were sedimented by gravitation, and the supernatant containing dead cells and non-myocardial cells were removed. Cells were suspended in Buffer B to a total volume of 15 ml and then transferred to a 100 mm petri-dish. The dish containing myocytes was incubated for 30 min in an incubator at 37°C. Susequent to incubation, 5 ml of Buffer C was added to the cells followed by 15 min incubation. After incubation, the myocytes were transferred to a 50 ml polystyrene tube where they were washed twice with Buffer C. After the second wash, 10 ml of Buffer C containing 5% fetal bovine serum was added and the cell suspension was distributed in even aliquots to ten culture dishes containing 1 ml Buffer C supplemented with 5% fetal bovine serum. Incubation for 30 min was required to allow attachment of myocytes to the dishes. Myocytes are rod-shaped cells which exhibit contractibility. Myocyte viablilty was monitored by trypan blue exclusion.

3. Perfusion of the isolated hamster hearts

a) Preparation of Krebs-Henseleit Buffer

Krebs-Henseleit Buffer (1 L) 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 water. Kreb-Henseleit Buffer was prepared fresh and was aerated with 95% O_2/5% CO_2 for 30 min prior to perfusion.

b) Perfusion of isolated hamster hearts

The hamsters were sacrificed by decapitation and the hearts were removed. The isolated hamster hearts were first perfused at 37°C in the Langendorff mode with Krebs-Henseleit Buffer for 10 min in order to remove the remaining blood in the hearts. The isolated hamster heart was then perfused with Kreb-Henseleit buffer in the presence or absence of 0.5 mg/ml methyl lidocaine. After perfusion, 5 ml of Krebs-Henseleit buffer was forced through the canula, followed by 5 ml of air. The heart was cut open, blotted dry, and homogenized in the appropriate buffer as indicated.

4. Determination of Choline Uptake and Analysis of Choline-containing Metabolites from Myocytes

The isolated rat myocytes in growth medium were incubated in 60 mm petri-dishes for 15 min. The medium was removed and 2 ml of Buffer C- 10 μM [^{3}H]-choline (0.15 mCi/μmol) was added and the cell was incubated from 0 - 120 min. Subsequently, the medium was removed and the myocytes were washed twice with 2 ml Dulbecco's phosphate buffered saline. Methanol (1 ml) was added into each dish and the cells were scraped using a rubber policeman. The cell suspension was transferred to a silianized test tube. The dish was rinsed with an additional 1 ml methanol. Chloroform (2 ml) was added to the methanol wash which contains the cell suspension. The cell samples were subsequently centrifuged at 500 x *g* and the supernatant was transferred to another tube. The cell pellet was rewashed with 2 ml of chloroform/methanol (1:1, by vol). The supernatants were pooled to a total volume of 6 ml. A 2 ml aliquot from each tube was taken for the determination of total choline uptake.

Phase separation of the chloroform/methanol extract was initiated by the addition of water and chloroform to form a chloroform/methanol/water mixture in a ratio of 4:2:3. The mixture was centrifuged at 500 x *g* for 5 min. After centrifugation, the organic phase was removed and the solvent was evaporated under nitrogen. Phospholipids in the nitrogen dried sample were resuspended in 200 μl chloroform and were applied on a silica G-25 t.l.c. plate. The t.l.c. plate

was developed in a solvent system containing chloroform/methanol/water/acetic acid (70:30:4:2, by vol). Pig liver phosphatidylcholine was used as a standard. The phosphatidylcholine fraction after t.l.c. was visualized by exposing the plate to iodine vapour.

The solvent in the aqueous phase obtained after phase separation was removed and the residue was dissolved in 200 µl distilled water. The [^{3}H]-choline-containing metabolites were separated by t.l.c. using a solvent system containing methanol/0.6% sodium chloride/ ammonium hydroxide (50:50:5, by vol). Different choline-containing compounds such as CDP-choline and phosphocholine were used as standards. The location of the radioactive metabolites on t.l.c. was detected using a Bioscan System 200 Imaging Scanner. The fractions of silica gel containing specific radiolabelled choline-containing metabolites were transferred to scintillation vials. One millilitre of distilled water, 50 µl of acetic acid, and 10 ml of scintillation fluid were added to each vial. Radioactivity was determined by scintillation counting using channels ratio calibration method.

5. Enzyme Assays

a) Choline kinase

Choline kinase was assayed by determining the conversion of [*methyl*-^{3}H]choline to phosphocholine (Ishidate and Nakazawa 1992). The reaction mixture

contained 0.1 M Tris-HCl (pH 8.5), 10 mM magnesium chloride, 10 mM ATP, 0.25 mM [*methyl*-^{3}H]choline iodide and enzyme preparation to a final volume of 0.5 ml. The reaction was initiated by the additon of [*methyl*-^{3}H]choline. The reaction mixture was incubated at 37 °C for 20 min. The reaction was terminated by placing the assay tubes in a boiling water bath for 2 min. The assay mixtures were subsequently centrifuged at 5000 x *g* for 10 min. An aliquot of the supernatant (250 µl) was applied to a Dowex AG 1-X8 (OH-) column (0.5 x 2 cm). The column was washed with 2.5 ml of 5 mM choline chloride followed by 6 ml of water. 0.5 ml of 1.0 M NaOH and 1.5 ml of 0.1 M NaOH were collected in scintillation vials. Acetic acid (0.1 ml) and 10 ml of Ecolite(+) were added to the fractions. Radioactivity associated with phosphocholine was determine by liquid scintillation counting.

b) CTP:phosphocholine cytidylyltransferase

CTP:phosphocholine cytidylyltransferase activity was determined by the conversion of phospho[*methyl*-^{14}C]choline to CDP-choline (Vance *et al.* 1981). The reaction mixture contained 100 mM Tris-succinate (pH 7.5), 12 mM magnesium acetate, 2.5 mM CTP, 1.0 mM phospho[*methyl*-^{14}C]choline and enzyme preparation to a final volume of 100 µl. The reaction was initiated by the addition of labelled phosphocholine and carried out at 37°C for 20 min. The reaction was terminated by placing assay tubes in a boiling water bath for 2 min. The samples were

centrifuged at 5000 x *g* for 10 min. An aliquot of supernatant was then applied to a t.l.c. plate (silica G-25). The labelled CDP-choline was separated from the labelled substrate by t.l.c. with a solvent system containing CH_3OH/0.6% NaCl/NH_3OH (10:10:1, by vol). The radioactivity of the CDP-choline fraction was identified using a Bioscan System 200 Imaging Scanner. Silica gel containing the labelled product was removed and placed in scintillation vials containing water (1 ml), acetic acid (50 ul), and Ecolite(+) scintillation cocktail (10 ml).

c) CDP-choline:1,2-diacylglyerol cholinephosphotransferase

CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity was determined by the conversion of CDP-[*methyl*-^{14}C]choline to phosphatidylcholine (O and Choy 1990). The reaction mixture contained 100 mM Tris-HCl (pH 8.5), 10 mM magnesium chloride, 1 mM EDTA, 0.4 mM CDP-[*methyl*-^{14}C]choline, 1.0 mM diacylglycerol and enzyme preparation to a final volume of 1.0 ml. The reaction was initiated by the addition of the labelled substrate and carried out at 37 °C for 20 min. The reaction was terminated by the addition of 3 mL chloroform/methanol (2:1, by vol) to the mixture. Water (0.5 ml) was added to cause phase separation. The lower phase containing labelled phosphatidylcholine was washed twice with 40% methanol. An aliquot was taken for the determination of radioactivity.

d) CTP:phosphatidic acid cytidylyltransferase

The assay mixture contained 50mM Tris/maleate (pH 6.5), 20 mM $MgCl_2$, 15 mM Triton X-100, 0.5 mM phosphatidic acid, 0.2 mg of protein and 1.0 mM [5-^{3}H]-CTP (10,000 dpm/nmol) (Kelley and Carman 1987). The reaction was initiated by the addition of the enzyme protein into the assay mixture. The assay mixture was incubated at 37^{0}C for 15 min and the reaction was terminated by the addition of 2.5 ml of 0.1 M HCl in methanol. After cooling, 5 ml chloroform was added to the mixture. The chloroform/methanol mixture was washed three times with 10 mL 2 M $MgCl_2$ (Belendiuk *et al.* 1978). CDP-diacylglycerol in the organic phase was separated by t.l.c. with a solvent containing chloroform/methanol/ acetic acid/ water (25:14:2:4, by vol). The radioactivity in the CDP-diacylglycerol fraction was determined by scintillation counting.

e) Acyl-CoA:lysophosphatidic acid acyltransferase assay

The assay mixture contained 100 mM Tris/HCl (pH 7.5), 0.2 mM 1-palmitoyl-*sn*-glycerol-3-phosphate, 90 mM sucrose, 1 mg/ml bovine serum albumin, 1 mM DTT, 40 μM [1-^{14}C]oleoyl-CoA (4,000 dpm/nmol) and 0.2 mg of protein in a final volume of 0.5 ml (Batenburg *et al.* 1986). The reaction was initiated by placing the enzyme protein into the assay mixture. The mixture was incubated at 30^{0}C for 30 min. The reaction was terminated by the addition of 3 ml chloroform/methanol (2:1, by vol). Water was then added to the mixture to cause

phase separation, and the labelled phosphatidic acid in the organic phase was isolated by t.l.c. with a solvent system containing chloroform/methanol/ammonium hydroxide/water (70:30:4:2, by vol). The phosphatidic acid fraction was visualized by exposure to iodine vapour and the radioactivity in the fraction was determined by scintillation counting.

f) Acyl-CoA:glycerol-3-phosphate acyltranserase

The activity of acyl-CoA:glycerol-3-phosphate acyltransferase was determined by the formation of the labelled lysophosphatidic acid with [1-^{14}C]-oleoyl-CoA as substrate (Batenburg *et al.* 1986). The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 1.5 mM glycerol-3-phosphate, 90 mM sucrose, 1 mg/ml bovine serum albumin, 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 by the addition of 3 ml of chloroform:methanol (2:1, by vol). Water was added to cause phase separation, and the labelled lysophosphatidic acid in the organic phase was isolated by t.l.c. with a solvent system containing chloroform:methanol:ammonia hydroxide: water (70:30:4:2, by vol). The lysophosphatidic acid fraction was visualized by iodine vapour and the radioactivity was determined. The amount of radioactivity associated with the lysophosphatidic acid fraction was used to determine the activity of acyl-CoA:glycerol-3-phosphate acyltransferase.

g) Phosphatidylinositol synthase

The activity of phosphatidylinositol synthase was determined by the formation of labelled phosphatidylinositol with *myo*[^{3}H]-inositol as substrate (Tardi *et al.* 1992). 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. The reaction was carried out at 37°C for 30 min and was terminated by the addition of 1 ml chloroform: methanol: concentrated HCl (100:100:1, by vol) and 0.4 mL of 0.9% KCl. The mixtures were centrifuged at 2000 x *g* for 5 min and the labelled phosphatidylinositol in the organic phase was analyzed by t.l.c. with a solvent system containing chloroform: methanol: acetic acid: water (50:30:8:4, by vol). The phosphatidylinositol fraction was visualized by iodine vapour staining and the radioactivity in the fraction was determined by scintillation counting. The radioactivity associated with the phosphatidylinositol fraction was used to determine the activity of the phosphatidylinositol synthase.

h) Protein kinase A and protein kinase C

The protein kinase A assay is adapted from the procedure of Corbin and Reimann (Corbin and Reimann 1974). The hamster heart after perfusion was homogenized in a buffer containing 50 mM Tris/HCl, 5 mM EDTA, 10 mM EGTA, 0.3% (w/v) β-mercaptoethanol, 10 mM benzamidine, 50 μg/ml PMSF, 100

mM β-glycerol-3-phosphate, 0.1 μg/ml IBMX, 2 mM DTT and 20 mM NaF. β-glycerol-3-phosphate was added to prevent cAMP from being hydrolysed by the phosphodiesterase, whereas NaF was used as a phosphatase inhibitor and IBMX was employed as a cAMP-phosphodiesterase inhibitor. Equal volumes of 50 mM potassium phosphate buffer (pH 6.8), 4.24 mM Kemptide (specific substrate for protein kinase A), and Mg[γ-^{32}P]ATP (25 μCi/μmol) containing 1 mM ATP and 18 mM magnesium acetate were mixed to a total volume of 50 μl. The reaction was initiated by adding 20 μl of enzyme (5 μg) to the mixture. A reaction without enzyme was employed as the blank. The reaction mixture was incubated at 30^{0}C for 20 min, and the reaction was terminated by spotting the sample onto the p81 paper. The paper was immersed in a 5% acetic acid solution. The paper was then washed three times and the protein kinase A activity was determined by the amount of phosphorylated kemptide bound to the paper. In the control experiment, 2 μM of PKI (a protein kinase A inhibitor) was added to determine the amount of phosphorylation due to other endogenous kinases.

The protein kinase C assay was a modification of the method of Hannun *et al.* (Hannun *et al.* 1985) and was carried out with the aid of an enzyme assay kit (Amersham). 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, 0.9 mM of a specific peptide for protein kinase C, 30 mM DTT and 0.05% (w/v) sodium azide in a total volume of 25 μl. Microsomal or cytosolic fraction

(50 μg protein) in a volume of 25 μl was added to the mixture, followed by the addition of 25 μl of magnesium/ATP buffer containing 50 mM Tris/HCl (pH 7.5), 150 μM [γ-^{32}P]ATP (66.6μCi/μmol), 45 mM magnesium acetate and 0.05% (w/v) sodium azide. The blank for the assay was represented by a reaction without enzyme. The reaction was carried out at 25°C for 15 min, and terminated with 100 μl of a stopping solution. The reaction mixture was spotted on the binding paper. The paper was washed three times with 15 ml of a 5% acetic acid solution. Subsequently, the paper was washed in 95% ethanol for 5 min, dried and the radioactivity determined. Protein kinase C activity was calculated from the phosphorylated peptide bound to the paper.

6. Binding and uptake of methyl lidocaine in hamster hearts

Isolated hamster hearts were perfused with Krebs-Henseleit buffer (pH 7.4) containing 0.01-0.1 mg/ml [^{3}H]methyl lidocaine (1μCi/nmol) for a 30 min period. In some experiments, hearts were perfused (chase) for another 30 min with 1 mg/ml unlabelled methyl lidocaine. After perfusion, hearts were homogenized and subcellular fractions were prepared. The radioactivity obtained in each subcellular fraction was determined by liquid-scintillation spectroscopy.

7. The pool size of cAMP

The pool size of cAMP in hamster heart was determined with the aid of a

kit from Amersham. Subsequent to perfusion, hamster hearts were immediately placed in liquid N_2. The frozen hearts were weighed and homogenized in 95% ethanol. The homogenate was centrifuged at 1,000 x *g* for 10 min. The supernatant, containing cAMP, was dried under a stream of N_2 and assayed according to the procedure described in the cAMP assay kit. Briefly, the dried sample was resuspended in 50 mM Tris/HCl (pH 7.5) and 4 mM EDTA. The assay mixture contained 4.5 nM [8-^{3}H]cAMP (27.8 μCi/nmol), cAMP binding protein and protein sample in a volume ratio of 1:2:1. A standard curve ranging from 0-6 pmoles of cAMP was prepared. The reaction mixture was incubated at 4^{0}C for 2 h. 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 12,000 x *g* for 2 min at 4^{0}C. The resulting supernatant was taken and the radioactivity determined. The amount of cAMP present in the sample was calculated from the cAMP standard curve.

8. Subcellular Fractionation

a) Microsomal and cytosolic fractions were obtained by differential ultracentrifugation. Myocytes were suspended in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA and homogenized in a glass douncer. Myocyte disruption after homogenization was verified by observation under a light microscope. The cellular suspension was centrifuged at 20,000 x *g* for 10 min and the supernatant

obtained was centrifuged at 100,000 x *g* for 60 min. The supernatant (cytosol) was collected and the precipitate (microsomal pellet) was resuspended in the homogenizing buffer.

b) The hamster heart was homogenized in a buffer containing 0.25 M sucrose and 10 mM Tris/HCl (pH 7.5) at 4°C to give a crude homogenate. The homogenate was centrifuged at 100 x *g* for 10 min. The pellet is the connective tissue, unbroken cells, and other cell debris, whereas the supernatant refers to the post-nuclear fraction. Subcellular fractions were prepared from the post-nuclear fractions by differential centrifugation as previously described (Zelinski *et al.* 1980). In brief, the post-nuclear fraction was centrifuged at 100,000 x *g* for 60 min. The resulting supernatant formed the cytosolic fraction, whereas the pellet was the microsomal fraction. The microsomal pellet was resuspended in 0.25 M sucrose/10 mM Tris-HCl (pH 7.4) buffer using a Dounce homogenizer.

9. Protein Determination

The protein content in each fraction was determined by the modified method of Lowry *et al.* in 1951. Standards containing 0, 10, 20, 40, 80, and 160 μg of bovine serum albumin were prepared as standards. The sample/standard was digested with 1.5 ml of 0.66 M NaOH for three hours at 37°C. Subsequently, 1.5 ml of reagent A (containing 1 ml of 2% $CuSO_4$, 1 ml of 4% Na-K tartrate, and 33 ml of 13% Na_2CO_3) was added to the sample/standard. The reaction was allowed

to sit for 10 min and 0.5 ml of 1 M Folin's reagent was then added. The mixture was incubated for 30 min and the absorbance was determined at 625 nm.

10. Statistical determination

Student's t test was used for all statistical analysis. The level of significance was defined as $P < 0.05$.

Results

Part 1: Effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis in rat cardiac myocytes

1. Effect of Vasopressin on Choline Uptake

The effect of vasopressin on the choline uptake in rat cardiac myocytes was studied. Isolated myocytes were incubated in medium containing 10 μM [*methyl*-^{3}H]choline in the absence or presence of 0.2 or 1.0 μM arginine-vasopressin from 0 to 120 min. Subsequent to incubation, myocytes were homogenized and the uptake of labelled choline was determined in the homogenate. Fig. 14 shows the time course for labelled choline uptake. The uptake of labelled choline was linear for up to 60 min in both the arginine-vasopressin-treated and the control cells. Choline uptake appeared to reach the maximum at about 80 min. Interestingly, 0.2 and 1.0 μM arginine-vasopressin induced different effects on labelled choline uptake. Myocytes treated with 0.2 μM arginine-vasopressin exhibited a higher total choline uptake (25%) than the control cells throughout the time course. In contrast, cells treated with 1.0 μM arginine-vasopressin displayed a reduction of choline uptake (19%) as compared to the control cells.

In order to further investigate the effect of arginine-vasopressin on the total choline uptake, myocytes were incubated in medium containing 10 μM [*methyl*-

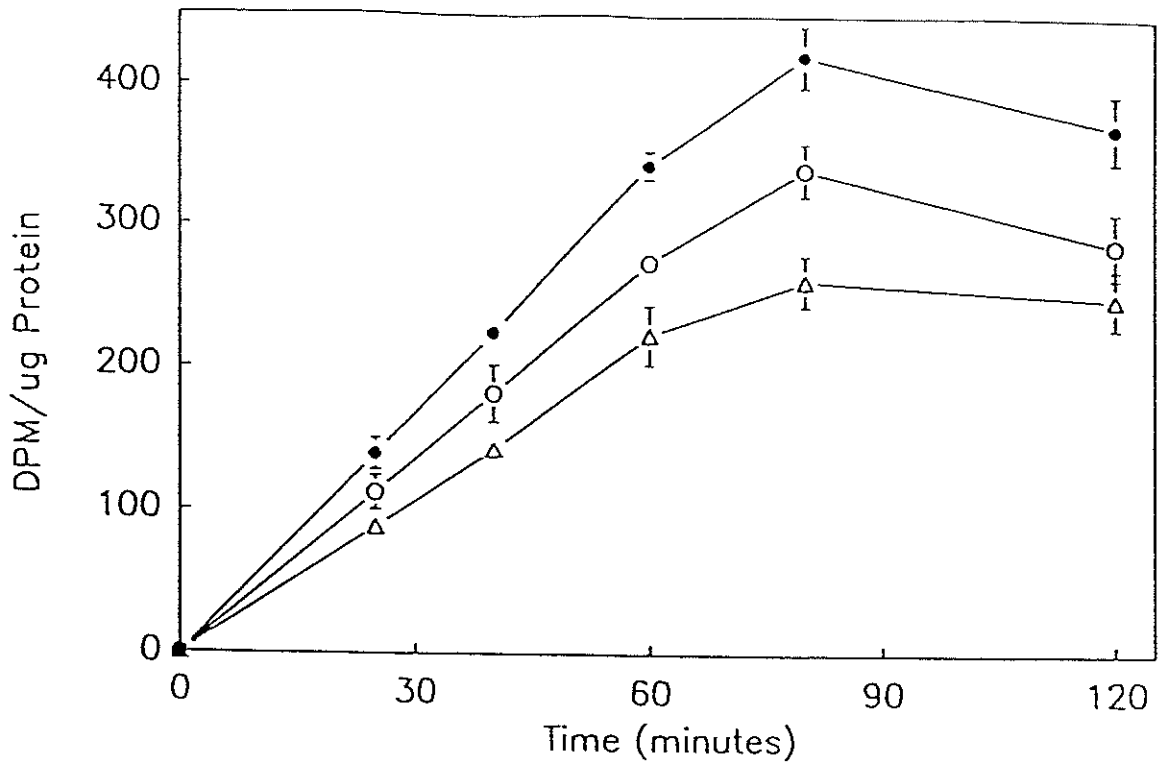


Figure 14: Time course study on labelled choline uptake in rat cardiac myocytes. Myocytes were incubated in medium containing 10 μM [*methyl*-^{3}H]choline in the presence of 0.2 (●) or 1.0 μM (△) arginine-vasopressin for up to 120 min. The control (○) referred to incubation in the absence of arginine-vasopressin. After the indicated time points, myocytes were homogenized and the uptake of labelled choline was determined in the homogenate. Each point represents the mean value of four determinations from two separate experiments, and the vertical bar represents the SD.

^{3}H]choline in the presence of various concentrations of arginine-vasopressin, ranging from 0.05 to 1.0 μM. As depicted in Table 2, an enhancement of choline uptake was observed in myocytes treated with lower concentrations of arginine-vasopressin (0.05 - 0.3 μM). In contrast, higher concentrations of vasopressin (0.4 - 1.0 μM) resulted in an attenuation of choline uptake.

The mechanism of action of arginine-vasopressin was explored by using a V1-vasopressin antagonist, [d(Ch_2)$_5$, D-Tyr(OEt)^2, Val^4, Cit^8]-vasopressin. The rationale of this study is based on the notion that the modulation of choline uptake by vaopressin is mediated through the V1- vasopressin receptor in the myocyte. In this study, myocytes were incubated in growth medium containing 10 μM [*methyl*-^{3}H]choline and 0.1 μM V1-receptor antagonist in the presence of 0.2 or 1.0 μM arginine-vasopressin. As shown in Table 2, the enhancement of choline uptake induced by 0.2 μM arginine-vasopressin was totally abolished in myocytes treated with the arginine-vasopressin antagonist. The attenuation of choline uptake caused by 1.0 μM arginine-vasopressin was also abrogated in the presence of the V1-antagonist (data not shown). These results indicate that arginine-vasopressin regulates choline uptake in cardiac myocytes through the V1-receptor.

Table 2: Effect of arginine-vasopressin on choline uptake in rat cardiac. myocytes Myocytes were incubated with 10 μM [*methyl*-^{3}H]choline for 60 min in the absence or presence of different concentrations of arginine-vasopressin, ranging from 0.05-1.00 μM. After incubation, the total choline uptake was determined. Each set of values represents the mean ± SD (number of experiments).

Arginine-vasopressin (μM)	Total choline uptake
	dpm/μg protein
Control	274 ± 8 (5)
0.05	301 ± 10 (5)*
0.10	304 ± 14 (3)*
0.20	343 ± 10 (5)*
0.30	324 ± 15 (3)*
0.40	229 ± 3 (3)*
0.50	323 ± 19 (3)*
1.0	223 ± 21 (4)*
0.1 μM V1- antagonist	267 ± 8 (8)
0.1 μM V1- antagonist + 0.2 μM arginine-vasopressin	287 ± 3 (3)

*P< 0.05

2. Effect of Vasopressin on the Incorporation of Choline into Phosphatidylcholine.

As shown in the preceding section, choline uptake was modulated by vasopressin in a biphasic manner. Lower doses of vasopressin (0.05 - 0.3 μM) enhanced choline uptake, whereas higher doses of vasopressin (0.4 -1.0 μM) reduced choline uptake. It is plausible that the modulation of choline uptake by vasopressin may affect phosphatidylcholine biosynthesis. Therefore, the effect of vasopressin on the *de novo* biosynthesis of phosphatidylcholine was investigated. Myocytes were incubated in growth medium containing 10 μM [*methyl*-^{3}H]choline in the presence or absence of various concentrations of arginine-vasopressin (0.05 - 0.1 μM) for 60 min. Phosphatidylcholine biosynthesis was determined by the incorporation of labelled choline into phosphatidylcholine.

Table 3 shows that the effect of vasopressin on the incorporation of labelled choline into phosphatidylcholine was biphasic in cardiac myocytes. Lower concentrations of vasopressin (0.05 - 0.3 μM) caused an increase in the labelling of phosphatidylcholine. Alternatively, higher vasopressin concentrations (0.04 - 0.1 μM) resulted in a reduction of the labelling of phosphatidylcholine. Furthermore, the vasopressin response was totally abolished when the cells were treated with 0.1 μM vasopressin antagonist, suggesting that V1-vasopressin receptor was involved in the regulation of phosphatidylcholine biosynthesis. Taken together, it appears that vasopressin induces a biphasic effect on both the choline uptake and the incorporation of labelling into phosphatidylcholine, with a transition occuring

between 0.3 - 0.4 μM vasopressin. It can be argued that the change in choline uptake by vasopressin might lead to the change in the incorporation of the labelled choline into phosphatidylcholine. However, it does not seem to be the case since the change in the labelling of phosphatidylcholine was not proportional to the change in labelled choline uptake. Based on this finding, we postulate that vasopressin may not only play a role in modulating choline uptake, but it may also have the ability to regulate phosphatidylcholine biosynthesis via the CDP-choline pathway.

Table 3: Effect of arginine-vasopressin on the incorporation of labelled choline into phosphatidylcholine in rat cardiac myocytes. Myocytes were incubated in medium containing 10 μM [*methyl*-3H]choline for 60 min in the absence or presence of different concentrations of vasopressin, ranging from 0.05-1.00 μM. After incubation, the incorporation of labelled choline into phosphatidylcholine was determined. Each set of values represents the mean ± SD (number of experiments).

Arginine-vasopressin (μM)	Total choline uptake
	dpm/μg protein
Control	68 ± 3 (8)
0.05	79 ± 3 (8)*
0.10	82 ± 3 (3)*
0.20	97 ± 11 (6)*
0.30	73 ± 9 (3)
0.40	58 ± 8 (3)*
0.50	60 ± 2 (3)*
1.0	41 ± 4 (9)*
0.1 μM V1- antagonist	68 ± 4 (3)
0.1 μM V1- antagonist + 0.2 μM arginine-vasopressin	74 ± 6 (4)

*P< 0.05

3. Effect of Vasopressin on Phosphatidylcholine Biosynthesis

The effects of vasopressin on (1) the incorporation of labelled choline into choline-containing metabolites, and (2) the activities of the biosynthetic enzymes in the CDP-choline pathway were investigated. Since 0.2 μM and 1.0 μM of vasopressin were shown to induce the most pronouced effect on the incorporation of labelled choline into phosphatidylcholine, these concentrations were used throughout the study.

The effect of vasopressin on the incorporation of labelled choline into choline-containing metabolites in the CDP-choline pathway was first examined. Myocytes were incubated with medium containing 10 μM [*methyl*-^{3}H]choline for 60 min in the absence or presence of 0.2 or 1.0 μM arginine-vasopressin. After incubation, myocytes were placed in a chloroform/methanol mixture to extract phospholipids, followed by phase separation in a system containing chloroform: methanol: water (4:2:3 by vol). The radiolabelled choline associated with the choline-containing metabolites in the aqueous phase were separated by t.l.c. with a solvent system containing methanol: 0.6% sodium chloride: 57% ammonium hydroxide (50:50:5 by vol). As shown in Table 4, 0.2 μM vasopressin induced an increase in labelled choline (24%) which coincided with the increase in the choline uptake as shown previously in Table 2. However, the increase in the labelling of phosphocholine (15%) was less than that shown in choline uptake. Vasopressin at 0.2 μM displayed no effect on the labelling of CDP-choline. The presence of 1.0 μM vasopressin caused a

reduction in the labelled choline (22%) but did not provoke any change in the labelling of phosphocholine and CDP-choline.

The disproportionate increase in the labellings between choline and phosphocholine by vasopressin (0.2 μM) provides additional evidence which suggests that vasopressin may play a role in the regulation of the CDP-choline pathway.

In order to further elucidate the role of vasopressin in the modulation of phosphatidycholine biosynthesis, the activities of phosphatidylcholine biosynthetic enzymes were determined. The biosynthetic enzymes in the CDP-choline pathway are choline kinase, CTP-phosphocholine cytidylyltransferase, and cholinephosphotransferase. In one set of experiments, 0.2 or 1.0 μM vasopressin was added directly to the microsomal fraction obtained from myocytes followed by assaying the enzyme activities. Results indicated that vasopressin did not exhibit any direct effect on the activities of the three enzymes (data not shown). In another set of experiments, myocytes were first incubated with growth medium in the presence or absence of 0.2 or 1.0 μM vasopressin for 60 min. After incubation, the homogenate obtained from the myocytes was used for the determination of enzyme activities. As shown in Table 5, myocytes incubated with 0.2 or 1.0 μM vasopressin did not alter the activities of choline kinase and cholinephosphotransferase. However, incubation with 0.2 μM vasopressin caused a pronounced increase (95%) in the CTP:phosphocholine cytidylyltransferase

activity as compared to the control. In contrast, the activity of the cytidylyltransferase was dramatically reduced (54%) in the vasopressin (1.0 μM) treated cells.

The regulation of CTP:phosphocholine cytidylyltransferase by vasopressin was further investigated. Myocytes were incubated in the presence of 0.2 or 1.0 μM vasopressin for 60 min followed by subcellular fractionation. Myocytes incubated in the absence of vasopressin were employed as the control. The cytidylyltransferase activity was determined in both the cytosolic and microsomal fractions. Table 6 shows that myocytes incubated with 0.2 μM vasopressin caused an increase in the activity of the cytidylyltransferase in both the microsomal (47%) and cytosolic fractions (70%) as compared to the control. Incubation of myocytes with 1.0 μM vasopressin resulted in a reduction of the cytidylytransferase activity in the microsomal fraction (17%) with a corresponding enhancement of the enzyme activity in the cytosolic fraction (90%).

Table 4: Effect of arginine-vasopressin on the incorporation of labelled choline into choline-containing metabolites in rat cardiac myocytes. Myocytes were incubated in medium containing 10 μM [*methyl*-^{3}H]choline for 60 min in the presence of 0.2 or 1.0 μM arginine-vasopressin. The control referred to incubation in the absence of vasopressin. After incubation, the incorporation of labelled choline into choline-containing metabolites was determined. Each set of values represents the mean ± SD (number of experiments).

Arginine-vasopressin (μM)	Choline	Phosphocholine	CDP-choline
	dpm/μg protein		
Control	53 ± 3 (6)	163 ± 9 (4)	8.8 ± 2.9 (6)
0.2	66 ± 4 (6)*	187 ± 14 (5)*	9.4 ± 1.8 (3)
1.0	41 ± 11 (13)*	152 ± 7 (3)	6.8 ± 2.6 (9)

*P< 0.05

Table 5: Effect of arginine-vasopressin on the activities of phosphatidylcholine biosynthetic enzymes in rat cardiac myocytes. Myocytes were incubated in medium containing 0.2 or 1.0 μM arginine-vasopressin. The control referred to incubation in the absence of arginine-vasopressin. After incubation, cell lysates were obtained and the activities of phosphatidylcholine biosynthetic enzymes were determined. Each set of values represents the mean ± SD (number of experiments).

Arginine-Vasopressin (μM)	Choline Kinase	CTP:phosphocholine cytidylyltransferase	Cholinephosphotransferase
		nmol/min/mg protein	
Control	0.018 ± 0.003 (4)	0.254 ± 0.035 (8)	0.116 ± 0.012 (8)
0.2	0.018 ± 0.005 (3)	0.495 ± 0.065 (5)*	0.119 ± 0.008 (5)
1.0	0.015 ± 0.002 (3)	0.118 ± 0.017 (4)*	0.124 ± 0.007 (5)

*$P < 0.05$

Table 6: Effect of arginine-vasopressin on the activity of CTP:phosphocholine cytidylyltransferase in microsomal and cytosolic fractions in rat cardiac myocytes. Myocytes were incubated in the presence of 0.2 or 1.0 μM arginine-vasopressin. The control referred to incubation in the absence of arginine-vasopressin. After incubation, cell lysates were obtained followed by subcellular fractionation. Activities of CTP:phosphocholine cytidylyltransferase were assayed in the microsomal and the cytosolic fractions. Each set of values represents the mean ± SD (number of experiments).

Arginine-vasopressin (μM)	CTP:phosphocholine cytidylyltransferase	
	Microsomal	Cytosol
	nmol/min/mg protein	
Control	0.415 ± 0.073 (4)	0.319 ± 0.086 (3)
0.2	0.612 ± 0.071 (3)*	0.543 ± 0.048 (3)*
1.0	0.169 ± 0.029 (3)*	0.605 ± 0.041 (3)*

*$P < 0.05$

Results

Part 2: Modulation of phosphatidylinositol biosynthetic enzymes by methyl lidocaine in isolated hamster hearts

1. The Binding and Uptake of Methyl Lidocaine in Hamster Hearts

The binding and uptake of methyl lidocaine in the isolated heart were investigated. In the first set of experiments, hamster hearts were perfused with 0.01-0.10 mg/ml [^{3}H]methyl lidocaine for 30 min. After perfusion, hearts were washed and homogenized in a buffer containing 0.25 M sucrose - 10 mM Tris/HCl (pH 7.5). The radioactivity in the homogenate was determined to obtain the total binding and uptake of labelled methyl lidocaine during perfusion (Table 7). In another set of experiments, heart were perfused with 0.01-0.10 mg/ml [^{3}H]methyl lidocaine for 30 min and then perfused with 1.0 mg/ml unlabelled methyl lidocaine (chase) for another 30 min. The chase was designed to remove the labelled methyl lidocaine that was bound but not taken up by the heart. After perfusion, hearts were homogenized in 0.25 M sucrose - 10 mM Tris/HCl (pH 7.5) and subcellular fractions were obtained by differential centrifugation. The radioactivities in the homogenate as well as in each subcellular fraction were determined. As shown in Table 7, the retention (binding and uptake) of the labelled material in the perfused heart appeared to reach a maximum at 0.02 mg/ml methyl lidocaine. However, the

majority of the radioactivity retained in the heart was removed during the chase period, indicating that a large proportion of the labelled methyl lidocaine was bound but not taken up by the heart. The radioactivity taken up by the heart was quantitatively recovered in the nuclear, mitochondrial, microsomal and cytosolic fractions. Analysis of the radioactivity in these subcellular fractions by t.l.c. indicates that the vast majority (92±2%) remained as methyl lidocaine. The maximum amount of uptake was achieved at 0.02 mg/ml methyl lidocaine. Higher concentrations of methyl lidocaine (up to 0.10 mg/ml) in the perfusate did not cause any significant increase the amount of uptake.

Table 7. The binding and uptake of [^{3}H]methyl lidocaine in hamster hearts. In experiment A, isolated hamster hearts were perfused with 0.01-0.10 mg/ml [^{3}H]methyl lidocaine (1μCi/nmol) for 30 min. In experiment B, hearts were perfused (chase) for another 30 min with 1 mg/ml methyl lidocaine. The radioactivity in the homogenate and in each subcellular fraction were determined. Each set of values represents the mean ± SD from four separate experiments in A and three separate experiments in B.

	Methyl lidocaine (mg/ml)			
	0.01	0.02	0.05	0.10
Experiment A		(nmol/g wet weight)		
Total binding and uptake	20.3 ± 1.6	31.0 ± 2.7	30.3 ± 3.1	30.0 ± 3.9
Experiment B		(nmol/g wet weight)		
Total uptake	6.7 ± 1.0	9.6 ± 0.9	10.1 ± 1.2	9.5 ± 1.4
		(% distribution)		
Nuclei and mitochondrial	49 ± 5	47 ± 8	-	-
Cytosolic	31 ± 7	33 ± 5	-	-
Microsomal	10 ± 7	10 ± 5	-	-

2. The Effect of Methyl Lidocaine Perfusion on Enzymes for the Biosynthesis of Phosphatidylinositol

Since the majority of [^{3}H]methyl lidocaine retained by the heart was bound extracellularly in a reversible manner, it was not clear if such binding might affect the activities of the enzymes for the *de novo* biosynthesis of phosphatidylinositol. Hence, the hamster heart was perfused for 30 min in Krebs-Henseleit buffer containing 0.5 mg/ml methyl lidocaine (Tardi *et al.* 1992). After perfusion, the heart was homogenized in 0.25 M sucrose - 10 mM Tris/HCl (pH 7.5), and the postnuclear fraction was obtained from the homogenate by centrifugation. The activities of the enzymes for PI biosynthesis were determined in the postnuclear fraction and the results are depicted in Table 8. The binding of methyl lidocaine did not change the activity of the acyl-CoA:glycerol-3-phosphate acyltransferase, CTP:phosphatidic acid cytidylyltransferase or phosphatidylinositol synthase. However, the activity of acyl-CoA:lysophosphatidic acid acyltransferase was increased by 45% as compared to the control. The activation of acyl-CoA:lysophosphatidic acid acyltransferase by methyl lidocaine perfusion could not be reproduced by the direct addition of methyl lidocaine into the cell-free system (Tardi *et al.* 1992). These findings suggest that the acyltransferase was activated by methyl lidocaine through an indirect mechanism.

Table 8. Effect of methyl lidocaine perfusion on PI biosynthetic enzymes. Hamster hearts were perfused for 30 min with or without 0.5 mg/ml methyl lidocaine. Hearts were homogenized after perfusion and postmitochondrial fractions from homogenates were prepared by centrifugation. Enzyme activities were assayed as described in Materials and Methods. Each set of values represent the mean ± SD of three separate experiments.

Enzymes	Control	Perfusion with methyl lidocaine
	(nmol/h/mg protein)	
Acyl-CoA:glycerol-3-P acyltransferase	6.14 ± 1.20	5.84 ± 1.52
Acyl-CoA:lysophosphatidic acid acyltransferase	0.65 ± 0.14	0.95 ± 0.11*
CTP:phosphatidic acid cytidylyltransferase	0.65 ± 0.09	0.61 ± 0.10
Phosphatidylinositol synthase	0.10 ± 0.03	0.11 ± 0.01

* $P < 0.05$ when compared with the control

3. Mechanism for the Activation of Acyl-CoA:lysophosphatidic Acid Acyltransferase

The activation of acyl-CoA:lysophosphatidic acid acyltransferase by reversible phosphorylation was explored. In an initial set of experiments, the postnuclear fraction obtained from the hamster heart was incubated with 100 μM ATP for 30 min in the presence of NaF and PMSF. After incubation, the acyltransferase activity in the postnuclear fraction was determined and compared with the control (without ATP). The effect of ATP-γ-S (nonhydrolyzable ATP analogue), ADP, or CTP on the enzyme activity was also examined.

As shown in Table 9, the activity of the acyltransferase was significantly elevated (14% as compared to control) in the presence of ATP. However, ATP-γ-S, ADP or CTP did not produce any detectable effect on the enzyme activity. In order to demonstrate that the effect of ATP was not mediated via adenylate cyclase, SQ22536 was added to the assay (Tamaoki *et al.* 1993). Co-incubation with ATP and SQ22536 did not diminish the effect of activation. Our data indicate that ATP, but not its subsequent metabolite, was an important factor for the activation of the acyltransferase, and suggest that the enzyme may be regulated via a phosphorylation-dephosphorylation mechanism.

The ability of methyl lidocaine to cause the phosphorylation of the acyl-CoA:lysophosphatidic acid acyltransferase was explored. Since the phosphorylation of the acyltransferase could arise from the activation of protein

kinase A and/or protein kinase C, the effect of methyl lidocaine perfusion on both protein kinases was examined. Hamster hearts were perfused with 0.5 mg/ml methyl lidocaine for 30 min and were homogenized after perfusion. The microsomal and cytosolic fractions were obtained for the determination of protein kinase A and protein kinase C activities. Our result shows that protein kinase A activity was enhanced by methyl lidocaine perfusion. The activities of protein kinase C in the cytosolic and microsomal fractions were not affected by the same treatment, indicating no translocation of the kinase took place (Table 10). In order to confirm that the activation of acyl-CoA:lysophosphatidic acid acyltransferase could be facilitated by the enhancement of protein kinase A activity, the postnuclear fraction obtained from the homogenate was incubated with 100 units of protein kinase A catalytic subunit. After incubation, the acyltransferase activity was determined. Our result shows that the incubation with protein kinase A catalytic subunit produced a pronouced increase (91%) in the acyltransferase activity. Enzyme activity obtained after protein kinase A incubation was 1.23 ± 0.26 nmol/h/mg protein, whereas the control was 0.65 ± 0.15 nmol/h/mg protein.

The mechanism for the enhancement of protein kinase A activity by methyl lidocaine perfusion was examined. Since protein kinase A activity is modulated by cAMP, the pool size of cAMP in the perfused hamster heart was determined. Table 11 shows that the level of cAMP was greatly elevated in the hamster heart after a 5 min perfusion with 0.5 mg/ml methyl lidocaine, and its level remained

elevated even after 30 min of perfusion.

The involvement of adenylate cylcase in the activation of acyl CoA:lysophosphatidic acid acyltransferase was studied. Hamster hearts were perfused with 0.5 mg/ml methyl lidocaine and 0.25 mM SQ22536 (an adenylate cyclase inhibitor) (Gilman 1987). The presence of SQ22536 completely abolished the elevation of the cAMP level and the enhancement of acyl CoA:lysophosphatidic acid acyltransferase activity (data not shown). Our results suggest that adenylate cyclase is involved in the activation of the acyltransferase by methyl lidocaine perfusion.

The ability of elevated cAMP levels to modulate protein kinase A activity in the heart was examined. The protein kinase A activity in the hamster heart cytosol was found to be significantly elevated by the exogenous addition of 20-100 pmol/ml cAMP, and maximum activation of the enzyme activity was achieved at 80 pmol/ml cAMP (Table 12). Interestingly, the addition of 20-100 pmol/ml cAMP to the cytosol of the hamster heart perfused with methyl lidocaine failed to elicit any significant increase in protein kinase A activity. It is plausible that the elevated level of cAMP in the heart generated by methyl lidocaine perfusion had already produced maximum activation of the protein kinase A activity. Hence, the activity of the enzyme could not be further stimulated by the addition of exogenous cAMP.

Table 9. Effect of ATP on acyl-CoA: lyosphosphatidic acid acyltransferase activity.

The hamster heart was homogenized in a buffer containing 10 mM Tris/HCl (pH 7.5)- 0.25 M sucrose- 20 mM NaF- 2 mM DTT- 50 μg/mL PMSF- 4 mM $MgCl_2$. The postnuclear fraction was incubated with the designated nucleotides (0.1 mM) for 30 min at 37^0 C prior to the assay of acyltransferase activity. Each set of values represents the mean ± SD of four separate experiments.

Preincubation with	Acyl-CoA:lysophosphatidic acid acyltransferase
	(nmol/h/mg protein)
Controls	0.65 ± 0.03
ATP	0.74 ± 0.02*
ATP + SQ22536 (1.25 mM)	0.81 ± 0.05*
ATP-γ-S	0.66 ± 0.04
ADP	0.60 ± 0.04
CTP	0.58 ± 0.04

* $P < 0.05$ when compared with controls

Table 10. Effect of methyl lidocaine on protein kinase A and protein kinase C activities.

Hamster hearts were perfused for 30 min with or without 0.5 mg/ml methyl lidocaine. Hearts were homogenized after perfusion and the microsomal and cytosolic fractions were obtained from homogenates. Protein kinase A and C activities were assayed as described in Materials and Methods. Each set of values represents the mean ± SD of three separate experiments.

	Control	Methyl lidocaine perfusion
	(pmol/min/mg protein)	
Protein kinase C		
Cytosolic	5.50 ± 0.65	5.16 ± 0.24
Microsomal	4.25 ± 0.30	4.00 ± 0.60
Protein kinase A		
Cytosolic	125 ± 14	215 ± 30*

* $P < 0.05$ when compared with the control

Table 11. Effect of methyl lidocaine on the pool size of cAMP

Hamster hearts were perfused with or without 0.5 mg/ml methyl lidocaine for 5 or 30 min. Subsequent to perfusion, the cAMP levels were determined. Each set of values represents the mean ± SD of three separate experiments.

Perfusion Time	cAMP levels (pmol/g wet weight)	
	Control	Methyl lidocaine
5 min	440 ± 15	767 ± 58*
30 min	406 ± 30	602 ± 14*

* $P < 0.05$ when compared with the control

Table 12. Effect of exogenous addition of cAMP on protein kinase A activity in hamster heart cytosol.

Hamster hearts were perfused for 30 min with or without 0.5 mg/ml methyl lidocaine. The heart was homogenized after perfusion and the cytosolic fraction was obtained from the homogenate. Protein kinase A activity was assayed in the presence of cAMP. Each set of values represents the mean of two separate experiments.

cAMP added (nM)	Protein kinase A activity (pmol/min/mg protein)	
	Control	Methyl lidocaine Perfusion
0	125	178
20	155	175
40	173	186
80	187	187
100	189	183

4. The Activation of Acyl-CoA:lysophosphatidic Acid Acyltransferase Activity by db-cAMP

The ability of db-cAMP to activate acyl-CoA:lysophosphatidic acid acyltransferase was studied. db-cAMP mimics the action of cAMP and is transported across the membrane during perfusion (Posternak and Weiman 1974). Hence, the hamster heart was perfused with 1 mM db-cAMP for 30 min and the activities of protein kinase A and acyl-CoA:lysophosphatidic acid acyltransferase in the heart were determined after perfusion. As shown in Table 13, both enzyme activities in the heart were enhanced by db-cAMP. Taken together with the data obtained in preceding sections, it appears that methyl lidocaine caused the generation of cAMP which stimulated the activity of protein kinase A and resulted in the phosphorylation and activation of acyl-CoA:lysophosphatidic acid acyltransferase.

Table 13. Effect of db-cAMP on the activities of acyl-CoA:lysophosphatidic acid acyltransferase and protein kinase A in hamster hearts.

Hamster hearts were perfused with 1 mM db-cAMP for 30 min. After perfusion, the heart was homogenized and the postnuclear fraction was obtained for the determination of enzyme activities. Each set of values represents the mean ± SD of three separate experiments.

Enzyme	Control	db-cAMP
Protein kinase A (pmol/min/mg protein)	101± 5	175 ± 30*
Acyl-CoA:lyosphosphatidic acid acyltransferase (nmol/h/mg protein)	0.61 ± 0.06	0.84 ± 0.09*

* $P < 0.05$ when compared with the control

5. The Direct Effect of Methyl Lidocaine on Enzymes for the Biosynthesis of Phosphatidylinositol

The direct effect of methyl lidocaine on enzymes in the *de novo* biosynthesis of PI was investigated. As shown in our previous study (Tardi *et al.* 1992), the presence of methyl lidocaine in the assay mixture did not have any effect on the activities of acyl-CoA:glycero-3-phosphate acyltransferase, acyl-CoA:lysophosphatidic acid acyltransferase and phosphatidylinositol synthase. However, methyl lidocaine in the assay mixture caused the activation of CTP:phosphatidic acid cytidylyltransferase. These results were confirmed in the present study, and kinetic studies were carried out to examine the activation of the CTP:phosphatidic acid cytidylyltransferase. The enzyme in the microsomal fraction was assayed at different phosphatidic acid (0.01-0.10 mM) and CTP (0.05-0.50 mM) concentrations in the presence of 0.5 or 1.0 mg/ml methyl lidocaine. Subsequently, enzyme activities *vs* concentrations of one substrate were plotted in a double reciprocal manner. As shown in Fig. 15, the Km for phosphatidic acid was changed in the presence of methyl lidocaine, but the Km for CTP was not affected by the same treatment. We conclude that methyl lidocaine caused the activation of CTP:phosphatidic acid cytidylyltransferase by increasing the affinity between the enzyme and phosphatidic acid, but not the affinity between the enzyme and CTP.

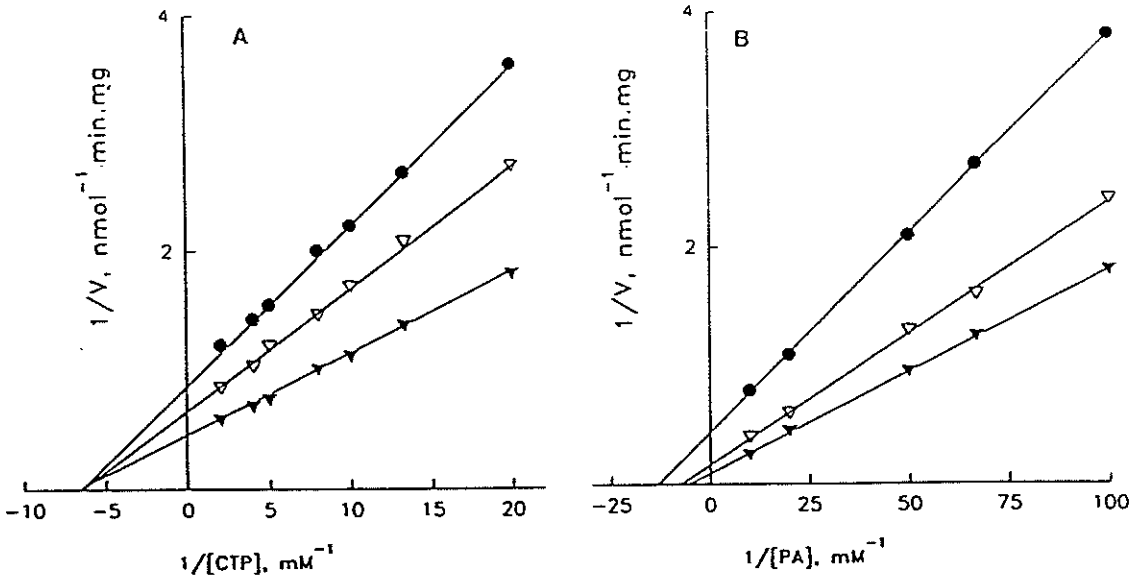


Fig. 15 - Double reciprocal plot of hamster heart CTP:phosphatidic acid cytidylyltransferase activities vs substrate concentrations.

CTP: phosphatidic acid cytidylyltransferase in the microsomal fraction of the hamster heart was assayed in the absence or presence of 0.5 or 1.0 mg/ml methyl lidocaine at different substrate concentrations. In experiment A, the assay mixture contained 0.05-0.5 mM [^{3}H]CTP (10,000 dpm/nmol), 0.5 mM phosphatidic acid, and 0.3 mg enzyme protein. In experiment B, the assay mixture contained 0.01-0.10 mM phosphatidic acid, 1.0 mM [^{3}H]CTP, and 0.3 mg enzyme protein. A double reciprocal plot on the enzyme activity vs substrate concentration was obtained from each set of studies. The symbols represent: (▼) incubation with 1.0 mg/mL methyl lidocaine, (▽) incubation with 0.5 mg/mL methyl lidocaine, and (●) incubation without methyl lidocaine (control).

Discussion

Part 1: Effect of arginine-vasopressin on choline uptake and phosphatidylcholine biosynthesis in rat cardiac myocytes

It is clear from this study that arginine-vasopressin modulates the *de novo* biosynthesis of phosphatidylcholine in a biphasic manner in isolated cardiac myocytes. Phosphatidylcholine biosynthesis is regulated by vasopressin at the levels of (1) choline uptake, and (2) the CTP:phosphocholine cytidylyltransferase activity.

The regulation of choline uptake is a plausible mechanism for the control of phosphatidylcholine biosynthesis. In the heart, choline is taken up through a low affinity Na^+-dependent uptake system and its transport appears to follow the Michaelis-Menton type of kinetics (Zelinski 1980). Choline uptake has been shown to be inhibited by ethanolamine and other choline analogs (Diamond and Kennedy 1969; Zelinski and Choy 1984). In addition, the regulation of choline uptake by amino acids in hamster hearts has been implicated (Hatch and Choy 1986; Chan *et al.* 1992). This study is the first to show that a hormone, vasopressin, has the ability to modulate choline uptake.

The regulation of choline uptake by vasopressin seems to be specific. Our findings illustrate that the effect of choline uptake is attenuated by a vasopressin

antagonist. Additional evidence shows that oxytocin, a peptide hormone which has a similar amino acid structure as vasopressin, fails to elicit such a response (Man 1992). Our results indicate that the regulation of choline uptake by vasopressin is biphasic; hence, it is unlikely that choline uptake is regulated through a direct interaction between vasopressin and the choline transport site. However, the mechanism(s) for the regulation of choline uptake by vasopressin remain undefined.

It is intriguing to note that vasopressin at lower concentration (0.2 μM) results in a disproportionate increase in choline uptake and the incorporation of labelled choline into phosphatidylcholine. It appears that vasopressin (0.2 μM) may have the ability to alter the CDP-choline pathway. Therefore, the effects of vasopressin on (1) the incorporation of labelled choline into choline-containing metabolites and (2) the activities of phosphatidylcholine biosynthetic enzymes in the CDP-choline pathway were investigated. Our results reveal that the regulation of CDP-choline pathway by vasopressin (0.2 μM) is at the step catalyzed by CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme in the pathway.

CTP:phosphocholine cytidylyltransferase is present in both the microsomal and cytosolic fractions. The cytosolic form is believed to be the inactive form, whereas the microsomal form is believed to be the active form. Translocation of the cytidylyltransferase between subcellular compartments by a reversible phosphorylation process is regarded as an important mechanism for the modulation

of the enzyme activity (Pelech and Vance 1984). Our result shows that vasopressin at lower concentration (0.2 μM) causes an enhancement of the cytidylyltransferase activity in both fractions, suggesting that vasopressin may play a role in either the activation of the enzyme or the biosynthesis of the cytidylyltransferase.

Alternatively, higher concentration of vasopressin (1.0 μM) results in an increase in the cytidylyltransferase activity in the cytosolic fraction with a corresponding reduction of the enzyme activity in the microsomal fraction. Although the mechanism for the regulation of the cytidylyltransferase remains unclear, it appears that the enzyme is translocated from the microsome to the cytosol. Since the reduction of phosphatidylcholine biosynthesis by vasopressin (1.0 μM) is abrogated in the presence of the V1- vasopressin antagonist, it is plausible that the translocation of CTP:phosphocholine cytidylyltransferase is mediated through a protein kinase cascade as a result of V1- receptor activation. We hypothesize that V1- receptor triggers the phosphoinositide cascade which results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate and the subsequent release of inositol-1,4,5-trisphosphate and diacylglycerol. The formation of diaycylglycerol activates protein kinase C which in turn phosphorylates CTP:phosphocholine cytidylyltransferase, resulting in its translocation from the microsome to the cytosol. The reduction of the microsomal enzyme activity may contribute to the reduction of phosphatidylcholine biosynthesis. Further work will be needed to support this hypothesis.

One intriguing aspect of this study is the bisphasic effect of vasopressin on phosphatidylcholine biosynthesis. The mechanism(s) involved in the biphasic response remains unidentified. It is, however, possible that the V1- vasopressin receptor can elicit dual actions in response to different concentrations of vasopressin. Recently, the ability of a G-protein coupled receptor to produce dual second messengers has been identified (Thompson 1992; Chabre *et al.* 1992). For example, it has been reported that a calcitonin receptor has the ability to trigger two second messenger pathways, with different ligand concentrations eliciting the two responses. In addition, the alpha(α)2-adrenergic receptor has recently been reported to provoke the activation of phospholipase C together with the inhibition of adenylate cyclase (Cotecchia *et al.* 1990). Hence, we postulate the V1-receptor may be coupled to two different G-proteins associated with two different signal transduction cascades. This postulation may explain the biphasic action of vasopressin.

The biphasic effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis emphasizes the diverse effect of the hormone on different tissues. In isolated rat hepatocytes, vasopressin does not display the biphasic effect as observed in cardiac myocytes (Tijburg *et al.* 1987). In hepatocytes, vasopressin exhibits no effect on choline uptake, but inhibits the incorporation of labelled choline into phosphatidylcholine. The mechanism for the reduction of phosphatidylcholine biosynthesis appears to be at the

cytidylyltransferase level in both hepatocytes and cardiac myocytes.

This study provides additional evidence that a hormone can regulate membrane phospholipid biosynthesis. Studies of phospholipid biosynthesis using other hormones having known myocardial-membrane receptors would enable us to further understand the mechanisms for the regulation of choline uptake and CTP:phosphocholine cytidylyltransferase.

It was once thought that hydrolysis of inositol phospholipids by phospholipase C was the only mechanism to link external stimuli to intracellular events through the activation of protein kinase C. There is growing evidence which shows that hydrolysis of phosphatidylcholine by phospholipase A_2, phospholipase C, and phospholipase D, can generate second messengers which participate in cell signalling. The phosphatidylcholine-derived second messengers such as arachidonic acid, diacylglycerol, phosphatidic acid, and lysophosphatidylcholine have been shown to enhance and prolong the activation of protein kinase C. Such prolonged activation of protein kinase C is important for long-term cellular responses such as cell proliferation and differentiation. In recognition of the recent report which shows that vasopressin has an ability to induce phosphatidylcholine turnover in smooth muscle cell line (Grillone *et al.* 1988), a future study would be to investigate the effect of vasopressin on prolonged activation of protein kinase C. Specifically, our laboratory is interested in addressing the regulation of phosphatidylcholine specific phospholipase A_2, C, or D by vasopressin.

The current understanding on the regulation of the metabolism of other phospholipids is limited. In rat hepatocytes, glucagon and noradrenalin have been reported to mediate the enhancement of phosphatidylethanolamine biosynthesis (Geelen *et al.* 1978; Haagsman *et al.* 1984). However, the regulation of phosphatidylethanolamine by hormones in the heart have not been documented. Hence, the effect of vasopressin on ethanolamine uptake and phosphatidylethanolamine biosynthesis is also an interesting subject for future study.

DISCUSSION

Part 2: Modulation of phosphatidylinositol biosynthetic enzymes by methyl lidocaine in isolated hamster hearts

Methyl lidocaine is an amphiphilic local anaesthetic which has been employed as an experimental anti-arrhythmic drug. It has a methyl group covalently attached to the amino nitrogen of the lidocaine moiety, which causes the molecule to display a permanent cationic charge (Tardi *et al.* 1992; Patterson *et al.* 1988). Presumably, the permanent charge does not affect its binding to the membrane, but may reduce its uptake and subsequent metabolism when compared with lidocaine. In this study we demonstrated that the majority of the labelled methyl lidocaine retained by the heart during perfusion was bound extracellularly and was exchangable with the non-labelled methyl lidocaine. The binding and the uptake of methyl lidocaine appear to be saturable. Although the mode of methyl lidocaine binding to the heart remains unclear, the uptake of methyl lidocaine appears to be carrier mediated (Stein 1986). The portion of methyl lidocaine taken up by the cardiac cell was not actively metabolised, and over 90% was recovered in the subcellular fractions in its original form.

It is clear that a positive effect on the adenylate cyclase cascade similar to that of β-adrenergic agonists (Gilman 1987; Tang and Gilman 1992) was produced

by methyl lidocaine. The activation of adenylate cyclase by methyl lidocaine was first indicated by an increase in cAMP level, and the notion was supported by the ability of SQ22536 (an adenylate cyclase inhibitor) to suppress the increase in cAMP production. The stimulation of protein kinase A activity by the increase in cAMP was detected in a cell free system as well as in the perfused heart with db-cAMP. Finally, the activation of the acyl-CoA:lysophosphatidic acid acyltransferase by phosphorylation was demonstrated by the incubation of the postnuclear fraction with ATP, and more directly, by the addition of the protein kinase A catalytic subunit to the postnuclear fraction. The activation of phospholipid biosynthetic enzymes via this mechanism by β-adrenergic agonists has been well documented. For example, the activation of acyl-CoA:glycerol-3-phosphate acyltransferase by epinephrine has been reported (Sooranna and Saggerson 1976). The activation of acyl-CoA:lysophosphatidic acid acyltransferase by isoproterenol has been shown to be mediated via the adenylate cyclase pathway with the elevation of cAMP and the activation of protein kinase A in guinea pig parotid gland lobules (Söling *et al.* 1989). Alternatively, we are the first to show that the activation of acyl-CoA:lysophosphatidic acid acyltransferase by methyl lidocaine is mediated via the adenylate cyclase cascade. It is tempting for us to speculate that the binding of methyl lidocaine to a receptor is essential in order to elicit its effect on the adenylate cyclase pathway for the enhanced production of cAMP and the subsequent activation of the acyl-CoA:lysophosphatidic acid acyltransferase.

However, the possibility of methyl lidocaine to exert a direct effect on the activation of adenylate cyclase has not been ruled out.

In view that acyl-CoA:lysophosphatidic acid acyltransferase is responsible for the *de novo* synthesis of PI, it is logical to surmise that the activation of acyl-CoA:lysophosphatidic acid acyltransferase should be mediated via the hydrolysis of phosphatidylinositol bisphosphate and the subsequent enhancement of protein kinase C activity (Exton 1990). This does not seem to be the case since protein kinase C activity in the heart was not activated by methyl lidocaine perfusion. The inability to produce a secondary response via the hydrolysis of the phosphatidylinositol biphosphate suggest that the action of methyl lidocaine is specific and may be limited to the modulation of the adenylate cyclase cascade in the hamster heart.

Since a significant amount of methyl lidocaine is taken up by the cardiac cells, the direct activation CTP:phosphatidic acid cytidylyltransferase represents an additional mechanism for the modulation of PI biosynthesis. However, the exact contribution of this mechanism for the enhancement of PI biosynthesis remains undefined. This is because CTP:phosphatidic acid cytidylyltransferase is a microsomal enzyme, and the availability of the endogenous methyl lidocaine to the microsomal system is not known. Interestingly, the presence of methyl lidocaine caused a change in the affinity between the enzyme and phosphatidic acid which was not observed in the presence of lidocaine (Wong *et al.* 1994). It is clear that

the presence of a methyl group in lidocaine would greatly alter its biological property and enable it to play an important role in the direct modulation of PI biosynthesis.

One might have expected that the activity of glycerol-3-phosphate acyltransferase would be affected by methyl lidocaine since the enzyme is believed to be another key enzyme in the PI biosynthetic pathway. In addition, the acyltransferase activity has been reported to be inhibited by protein kinase A (Heathers *et al.* 1985). However, the modulation of the enzyme by protein kinase A could not be reproduced by Rider and Saggerson (Rider and Saggerson 1983) which is in line with our findings.

Beyond the modulation of PI biosynthesis, methyl lidocaine has also been shown to stimulate the biosynthesis of neutral lipids such as diacylglycerol and triacylglycerol. We have shown in a preliminary study that the acyl-CoA:diacylglycerol acyltransferase is also activated by methyl lidocaine perfusion. In a separate study, the enzyme was found to be regulated by phosphorylation (Rodriguez *et al.* 1992). It is possible that the mediation of the adenylate cyclase cascade is a common mechanism by which methyl lidocaine exerts its modulation on lipid biosynthesis in the hamster heart.

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