HORMONE AND AMINO ACID REGULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS

By

Adrian S. Man

A thesis submitted to the Faculty of Graduate Studies

in partial fulfilment of the requirements for the degree of

Master of Science.

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Pat, thank you for giving me the opportunity to study and work in biochemical research. Being a part of your lab gave me the skills and knowledge needed to become a scientist. But more importantly, you have shown me how challenging and personally rewarding scientific research can be. That enthusiasm will certainly remain with me. Thanks for your guidance and patience.

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TO MY PARENTS

" Let your imagination go, guiding it by judgement and principle, but holding it in and directing it by experiment." - Michael Faraday

" Concern for man himself and his fate must always form the chief interest of all technical endeavors ... in order that the creations of our minds shall be a blessing and not a curse to mankind. Never forget this in the midst of your diagrams and equations." - Albert Einstein

" I just want to do the best I can and have fun." - Monica Seles

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ABSTRACT

Phosphatidylcholine is the principal phospholipid in mammalian tissues. In mammalian hearts, the vast majority of phosphatidylcholine is synthesized via the CDP-choline pathway. In this pathway, choline taken up by the heart is rapidly phosphorylated into phosphocholine by the action of choline kinase. Phosphocholine is then converted into CDP-choline by the action of CTP:phosphocholine cytidylyltransferase. The CDP-choline is condensed with diacylglycerol by the action of CDP-choline: diacylglycerol cholinephosphotransferase for the formation of phosphatidylcholine. Although the first committed step in this pathway is catalyzed by choline kinase, the rate-limiting step is catalyzed by cytidylyltransferase. This enzyme is located in both the microsomal and the cytosolic fractions, and translocation of the enzyme from one compartment to another has been postulated as an important mechanism for the *in vivo* regulation of the enzyme activity.

The choline transport mechanism has been documented to be a site of regulation by various effectors. A number of compounds including hemicholinium-3, benzylcholine and chlorocholine have been identified as potent inhibitors of choline uptake. The influence of amino acids on choline uptake and phosphatidylcholine biosynthesis in renal cortical slices has also been reported. In addition, the effects of hormones on phosphatidylcholine biosynthesis have been investigated in several systems. Steroids including diethylstilbestrol and glucocorticoids stimulate phosphatidylcholine biosynthesis in the rooster and fetal lung. Alternatively,

norepinephrine and vasopressin have been shown to inhibit phosphatidylcholine biosynthesis. Enzymes involved in the biosynthesis of phosphatidylcholine are subject to regulation by various agents. However, the effect of various hormones on phosphatidylcholine biosynthesis in cardiac cells has not been elucidated.

The effect of vasopressin, oxytocin, and 17ß-estradiol on choline uptake and phosphatidylcholine biosynthesis in isolated rat heart myocytes was investigated. Myocytes were incubated with labelled choline in the presence of the hormones. Uptake of choline was enhanced by low concentrations (0.05-0.2 μM) of vasopressin but was attenuated by high vasopressin concentrations (0.5-1.0 μM). The biosynthesis of phosphatidylcholine was also affected by vasopressin in a biphasic manner. At low concentrations of vasopressin, the conversion of phosphocholine to CDP-choline was enhanced by a general increase in CTP:phosphocholine cytidylyltransferase activity. At high vasopressin concentrations, a decrease in the activity of cytidylyltransferase was detected which was caused by the translocation of the enzyme from the microsomal fraction to the cytosolic fraction. These observed effects of vasopressin were negated by pertussis toxin, suggesting the involvement of a G-protein. Phosphatidylcholine biosynthesis in cardiac myocytes was unaffected by oxytocin. The steroid hormone 17ß-estradiol inhibited phosphatidylcholine biosynthesis in the myocytes.

The uptake of choline in mammalian hearts in the presence of amino acids was also examined. Isolated hamster, guinea pig, rat and rabbit hearts were perfused with labelled choline in the presence and absence of amino acids. Neutral amino

acids enhanced choline uptake in the hamster heart, but not in the guinea pig, rat and rabbit hearts. Phosphatidylcholine biosynthesis in these hearts was not affected by the presence of amino acids. Choline uptake in hamster myocytes was also enhanced by neutral amino acid. The enhancement of choline uptake suggests a direct interaction between the amino acid and the transport of choline into the myocardial cells. The different responses in choline uptake to neutral amino acids indicate that the regulation of choline uptake in the heart may be different between mammalian species.

INTRODUCTION

I. The Biological Membrane and Phospholipids

A) Membrane structure and function

The plasma membrane and the membranes of subcellular organelles determine the structural organization of the eukaryotic cell. The membrane defines the cells dimensions and differentiates the external environment from intracellular contents. The integrity of the plasma membrane is critical to cellular function. Many biological processes including reception of external signals, signal transduction, secretion and transport are mediated via the membrane (Alberts *et al.* 1989).

The functions of the plasma membrane are numerous and detailed. Such roles are hereafter discussed briefly. The generation of the membrane is essential in the biogenesis of the cell. The geographical limits of the cell are defined by the membrane. In addition to the separation of intracellular contents from the external environment, the membrane acts as a selective barrier. This barrier permits the entrance of cell nutrients, maintains the ion concentration gradient and allows excretion of waste products. The membrane is also the site of transport processes such as endocytosis, exocytosis and pinocytosis. Some proteins bound within the lipid membrane are involved in active transport of macromolecules. The plasma membrane also functions with respect to the transmission of cell signals. Hydroylysis of lipids such as phosphatidylinositol-4,5-bisphosphate produce intracellular

messengers which elicit responses within the cell (Alberts *et al.* 1989).

Membranes of intracellular organelles have the same structural integrity as the plasma membrane and thus have similar functions. These organelles include the endoplasmic reticulum, golgi apparatus, mitochondria, peroxisomes, lysosomes, nuclei, chloroplasts and other vesicles.

The present concept of membrane structure is described by the fluid mosaic model derived by Singer and Nicholson (1972)(Fig.1). This model states that the membrane exists as a lipid bilayer of approximately 6 -9 nm in thickness. Lipids constitute approximately 50 % of the mammalian cell membrane mass. These bilayers are fluid, thus allowing lateral diffusion of lipid and protein within the plane of the membrane. The ability of lipids to maintain a bilayer organization, in an aqueous environment, is indicative of the amphipathic nature of membrane lipids (Cullis and Hope 1985). The lipids contain a hydrophilic (polar) head group region and a hydrophobic (non-polar) region. Macromolecular organization is thermodynamically favourable when the polar regions orient toward the aqueous phase and the non-polar regions are sequestered from water. Proteins within the membrane can have carbohydrate moieties which face the extracellular space.

The fluidity of the membrane is dependent on the nature of the hydrophobic region of the lipid. Most lipid species can undergo a temperature dependent transition between a viscous gel (frozen) state to a fluid (liquid-crystalline) state (Silvius 1982). However, most if not all lipid membranes are fluid at physiological temperatures.

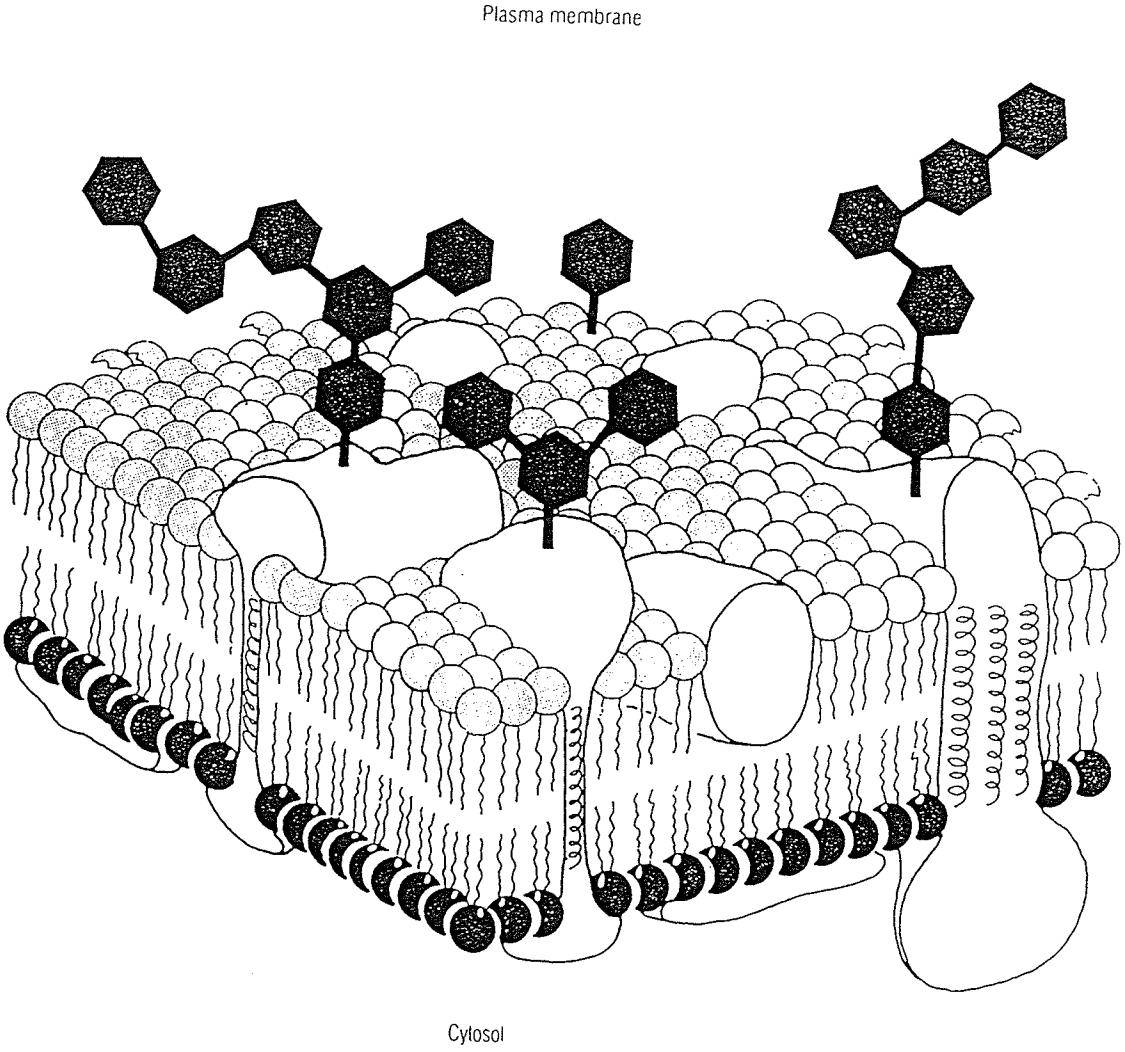


FIGURE 1. Fluid Mosaic Model of the Eukaryote Cell Membrane. (Cullis and Hope 1985)

B) Mammalian membrane lipid structure

The major class of lipids found in biological membranes are phospholipids (Lehninger 1982). Phospholipids consist of a glycerol backbone with attached acyl groups and a polar head group containing phosphate (Fig.2). Sphingolipids also constitute a significant fraction of the membrane. This second large group of lipids contains two nonpolar tails and a polar head group. Sphingosine is the basis of this class of lipids. One long-chain fatty acid and a polar head alcohol are attached to the sphingosine molecule (Fig.3). Both phospholipids and sphingolipids are termed saponifiable i.e. they yield soaps of their fatty acid components upon heating with alkali. In addition to these two lipid classes, the biological membrane also possesses nonsaponifiable lipids. These include those lipids belonging to the steroid class. Steroids are complex fat-soluble molecules with a perhydrocyclophenanthrene ring structure. The most abundant steroids are steroid alcohols (sterols). Cholesterol is the major sterol in animal tissues. Cholesterol and its esters are important components of plasma lipoproteins and the outer cell membrane (Table 1).

The major phospholipids found in membranes are phosphoglycerides (Lehninger 1982). These molecules consist of a glycerol backbone to which two fatty acids are esterified to the first and second hydroxyl groups of glycerol. The third hydroxyl group forms an ester linkage to phosphoric acid. The resulting molecule is phosphatidate which is the parent compound of all phosphoglycerides. A second alcohol is esterified to the phosphoric acid group of phosphatidate to form phosphoglycerides. All phosphoglycerides contain two nonpolar tails attributed to

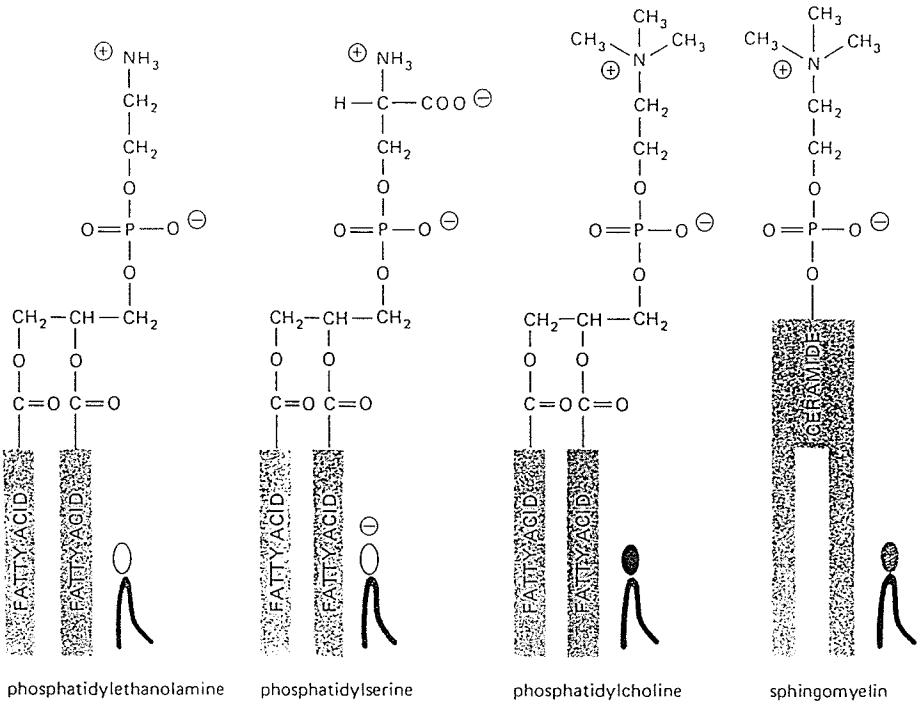


FIGURE 2. Structure of Various Phosphorus-containing Lipids. (Alberts *et al.* 1989)

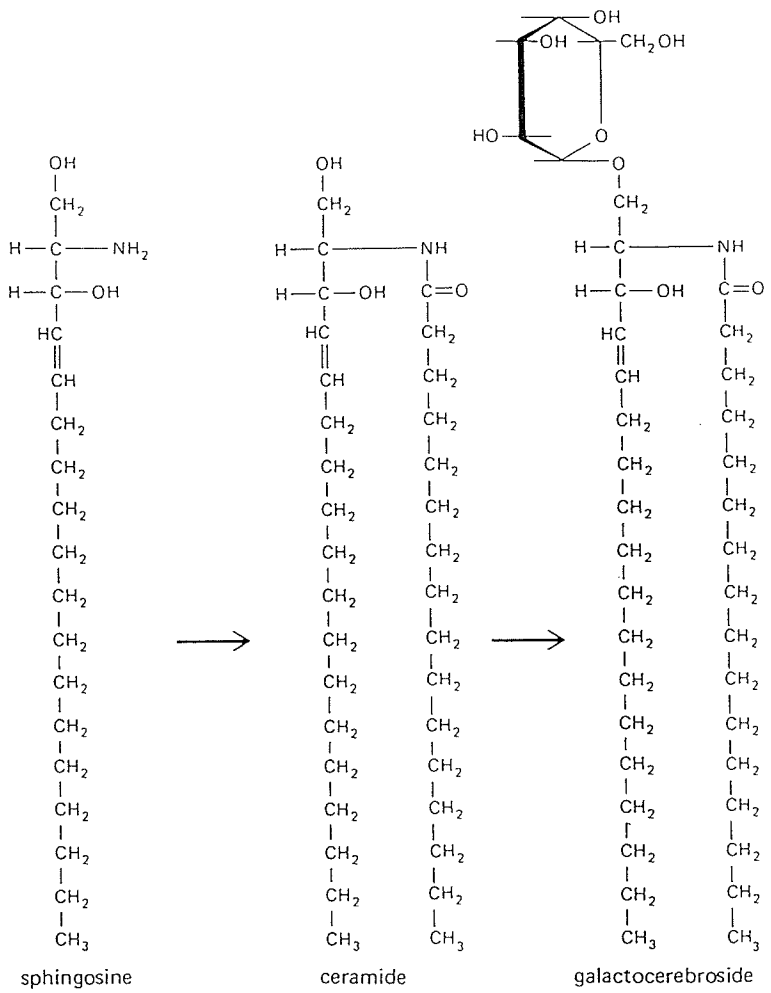


FIGURE 3. Structural Components of Sphingolipids.

(Alberts *et al.* 1989)

TABLE 1

Lipid Composition of Various Biological Membranes.

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

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

(Cullis and Hope 1985)

their long-chain fatty acids. The most abundant fatty acids found in phosphoglycerides contain 16 or 18 carbon atoms. Normally one of the fatty acids is saturated and the other unsaturated; the latter esterified at the 2-hydroxyl group of glycerol.

Phosphoglycerides are named according to the alcohol located on the polar head of the molecule. The most prominent phosphoglycerides in the membrane are phosphatidylcholine and phosphatidylethanolamine although their distribution varies among cell types and organelles. Other phosphoglycerides include phosphatidylinositol and phosphatidylserine with inositol and serine as their respective alcohol head groups. Cardiolipin differs from other phosphoglycerides in that it possesses a "double" phosphoglyceride. Cardiolipin is characteristic of the inner mitochondrial membrane.

The sphingolipids which are found in the biological membrane can be subdivided into three types of molecules: sphingomyelins, cerebrosides, and gangliosides (Lehninger 1982). Of these molecules, only sphingomyelin contains phosphorus. Sphingomyelins are the most abundant sphingolipid in the membrane. The polar head groups characteristically associated with these molecules are phosphocholine and phosphoethanolamine. Apart from the absence of the glycerol backbone, sphingomyelin closely resembles other phosphoglycerides in structure and electric charge.

Cerebrosides are characterised by the presence of sugar units on the polar head region and are thus referred to as glycosphingolipids. Glucocerebrosides are normally found in cell membranes of nonneural tissues whereas galactocerebrosides

are present in brain cells. Some cerebrosides contain more than one sugar unit. These complex cerebrosides are usually located in the outer layer of the cell membrane.

The most complex sphingolipids are gangliosides. These lipids possess a large polar head region containing several sugar units. N-acetylneuraminic acid (sialic acid) is characteristically the terminal sugar unit. Gangliosides compose less than 6 percent of mammlian cell membranes. These specialized lipids are known to function as specific receptor sites on the cell membrane. In neurons, gangliosides become bound to neurotransmitter molecules during transmission of a chemical impulse.

II. Phosphatidylcholine Biosynthesis and Catabolism

A) Biosynthesis of Phosphatidylcholine

The first identification of phosphatidylcholine (lecithin) was reported in 1847 (Gobley 1850). He demonstrated the presence of phospholipid in egg yolk; thus the name lecithin after the Greek *lekithos* for egg yolk. In the 1860s, Diakonow and Strecker showed that lecithin contained two fatty acids linked to glycerol and that choline was attached to the third hydroxyl by a phosphodiester linkage (Diakonow 1868; Strecker 1868).

The importance of phosphatidylcholine was first established by Eagle in 1955 when choline was found to be essential for cell survival in culture. Choline-

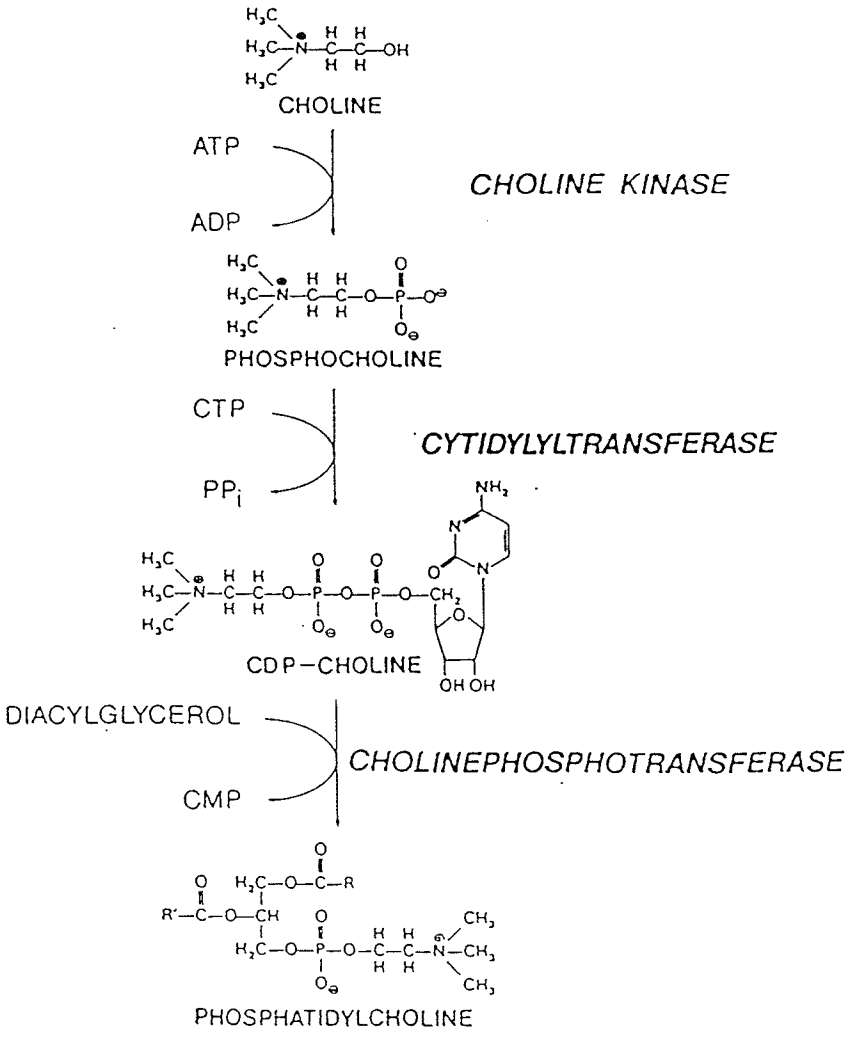


FIGURE 4. Biosynthesis of Phosphatidylcholine via the CDP-Choline Pathway. (Pelech and Vance 1984)

deficient animals developed a number of pathological conditions. No genetic mutations of phosphatidylcholine biosynthesis have been isolated in higher eukaryotes. Phosphatidylcholine is also important as a component in lung surfactant (Klaus *et al.* 1961). This compound is essential in preventing collapse of the alveoli when air is expelled during respiration.

The major route for phosphatidylcholine biosynthesis occurs via the CDP-choline pathway (Fig.4). The pathway was first elucidated by Kennedy who determined that CTP was required for *de novo* phosphatidylcholine biosynthesis (Kennedy 1962). Choline is taken up into the cell through a specific transport mechanism. Choline is subsequently phosphorylated to phosphocholine through the action of choline kinase. Phosphocholine reacts with a molecule of CTP to form CDP-choline via the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase. The final step of the pathway is catalyzed by CDP-choline:1,2-diacylglycerol phosphocholinetransferase which involves the condensation of CDP-choline and diacylglycerol to produce phosphatidylcholine and CMP.

Other known routes of phosphatidylcholine biosynthesis include calcium-mediated base exchange and methylation of phosphatidylethanolamine. Methylation of phosphatidylethanolamine is of quantitative importance in liver tissue. The first evidence of this pathway came when Stetten in 1941 isolated [^{15}N]choline from rats that were fed [^{15}N]ethanolamine in their diet. Bremer and Greenberg later discovered a microsomal enzyme (phosphatidylethanolamine-N-methyltransferase) which converts phosphatidylethanolamine to phosphatidylcholine via the transfer of

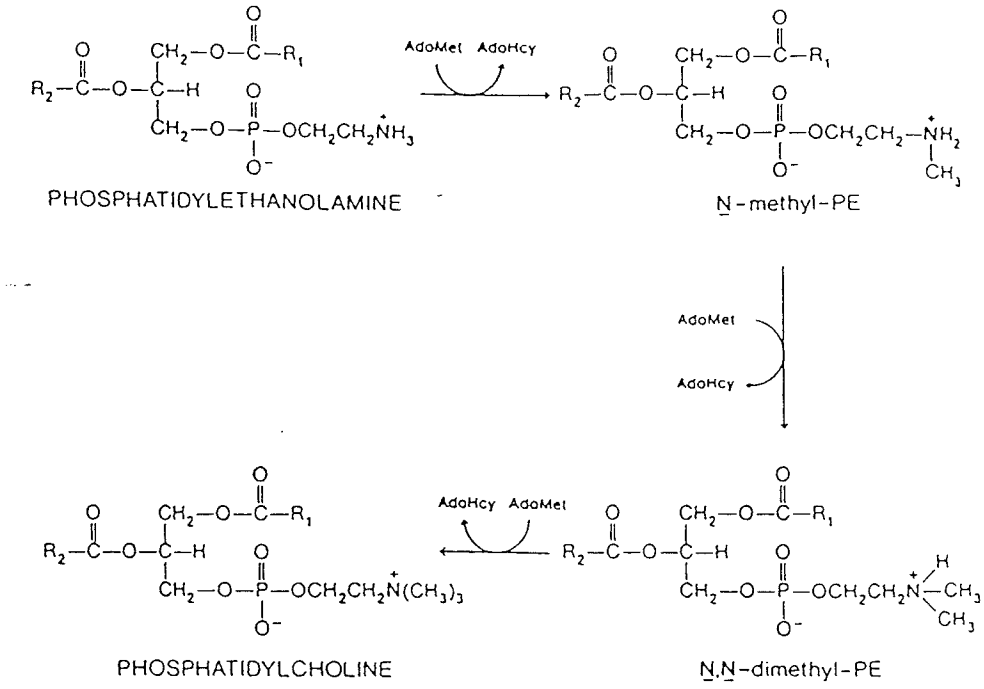


FIGURE 5. Biosynthesis of Phosphatidylcholine via Methylation of Phosphatidylethanolamine

(Pelech and Vance 1984)

methyl groups from S-adenosylmethionine (Bremer and Greenberg 1961) (Fig.5). Calcium-mediated base exchange involving exchange of choline for other phospholipid head groups was demonstrated in rat liver microsomes (Dils and Hubscher 1961). However, this pathway is believed to constitute a minor fraction of total phosphatidylcholine biosynthesis in most tissues.

Choline uptake has been shown to occur via two possible transport mechanisms. One being a high affinity (K_m < 5μM) sodium-dependent system and the other a low affinity (K_m > 30μM) sodium-independent mechanism (Sung and Johnstone 1965). The high affinity system is present in cholinergic cells where it is tightly coupled to acetylcholine synthesis. For noncholinergic mammalian cells, the K_m for choline varies between tissues ranging from 3μM in chick fibroblasts (Barald and Berg 1978) to 200-300μM in rat lens cells (Jernigan *et al.* 1981). Heart cells possess a single, sodium-independent, choline uptake system (Zelinski *et al.* 1980). A non-saturable component for choline transport has also been detected in perfused rat liver (Zeisel *et al.* 1980) and human erythrocytes (Askari 1966). However, this diffusion mechanism only appears to operate at high extracellular choline concentrations. Since plasma concentrations of choline are in the range of 10 to 15μM, uptake probably occurs through a specific transport mechanism (Bligh 1952). Once taken up into the cell, choline is normally phosphorylated by choline kinase. However, in the liver, choline can also be oxidized to betaine where it functions in donating methyl groups for methionine biosynthesis (Pelech and Vance 1984).

Choline kinase is a cytosolic enzyme found in all eukaryotic cells (Ishidate

1989). It catalyzes the conversion of choline to phosphocholine. The enzyme has been purified from lung and liver and has a molecular weight of approximately 120000-160000 daltons. Choline kinase has an optimal alkaline pH between 8.0 - 9.5. Although the reported K_m values for choline and ATP vary depending on cell type, the enzyme displays a high apparent K_m for ATP (2.0mM) and a low apparent K_m for choline (30μM). The divalent cation Mg^{2+} is necessary for choline kinase activity. Kinetic studies have indicated that the enzyme initially binds ATP-Mg^{2+} followed by choline with subsequent activation by free Mg^{2+}. Release of phosphocholine occurs before that of ADP-Mg^{2+}. The mechanism of action is thus an ordered bi-bi reaction (Dixon and Webb 1979). Recently, several isoenzymes of choline kinase have been isolated. Physiological functions for these multiple enzymic forms have yet to be determined.

The rate-limiting step of the pathway is catalyzed by CTP:phosphocholine cytidylyltransferase. In 1963, Schneider recovered enzyme activity in both microsomal and cytosolic fractions (Schneider 1963). The enzyme was later purified to homogeneity in rat liver by Feldman and Weinhold (1987). Cytidylyltransferase consists of two nonidentical proteins, with a molecular weight of 45000 and 38000 daltons respectively. The 45kd subunit contains catalytic activity. The functional role of the 38kd protein as part of the cytidylyltransferase enzyme complex is not yet determined. The microsomal form of the enzyme is immunologically similar to the cytosolic form. Since cytidylyltransferase has a lipid requirement to maintain activity, it is the microsomal form that is considered the active species. The pH optimum is

between 6.0 and 7.0. The apparent K_m for CTP and phosphocholine range between 0.2mM-0.3mM although only the K_m for CTP is sensitive to associated phospholipid.

The final reaction in the *de novo* biosynthesis of phosphatidylcholine is catalyzed by CDP-choline:1,2-diacylglycerol phosphocholinetransferase. Van Golde found that the enzyme had highest activity in the smooth and rough endoplasmic reticulum (Van Golde *et al.* 1971). These results indicate that phosphatidylcholine is exclusively synthesized in the endoplasmic reticulum. However, there is other evidence to suggest that phosphocholinetransferase exists, to a lesser extent, in the Golgi and the outer layer of the mitochondria (Jelsema and Morré 1978). The enzyme is an integral membrane protein. The active site is believed to be located on the cytosolic domain of the enzyme. The extent to which the enzyme penetrates through the bilayer and into the lumen is not yet determined (Coleman and Bell 1983). Phosphocholinetransferase has preference for diacylglycerol species with palmitate on C-1 and linoleate on C-2 of the glycerol moiety (Sarzala and Van Golde 1976). The mechanism of action is still undetermined. Although solubilized from microsomal membranes, phosphocholinetransferase remains to be purified to homogeneity.

The enzyme phosphatidylethanolamine-N-methyltransferase converts phosphatidylethanolamine to phosphatidylcholine via methylation of the amine group of the ethanolamine moeity. Transfer of methyl groups occurs through S-adenosyl-L-methionine. This methylation reaction is important in the liver where it accounts for 20 to 40% of phosphatidylcholine biosynthesis (Sundler and Åkesson 1975). Activity

of methyltransferase in heart, kidney, lung and testis are low but detectable (2 to 6% of liver). The exact subcellular localization of methyltransferase, in rat liver, remains unidentified. It has been reported that methylation activity was confined to the endoplasmic reticulum (Van Golde *et al.* 1974). In contrast, Higgins and Fieldsend (1987) found twice the enzyme activity in the cis-Golgi compared to the endoplasmic reticulum or the trans-Golgi. The topology of methyltransferase has been equally argumentative. Successive transfer of methyl groups was shown to occur on the cytosolic surface of the microsomal membrane (Vance *et al.* 1977). However, it was also reported that the initial methylation of phosphatidylethanolamine occurs in the microsomal lumen and subsequently converted to phosphatidylcholine on the cytosolic side (Higgins 1981). The enzyme has been purified to homogeneity and has a molecular weight of 18.3 kd as determined by SDS-polyacrylamide gel electrophoresis (Ridgeway and Vance 1987).

B) Catabolism of Phosphatidylcholine

Phosphatidylcholine exists in a dynamic state within the cell membrane. The biosynthesis of phospholipids is balanced by the actions of various phospholipases. These enzymes control the degradation of phospholipids. Phospholipases are characterized according to the phospholipid bond they hydrolyze.

The initial step in the catabolism of phosphatidylcholine can be catalyzed by any one of four phospholipases: phospholipase A_1, phospholipase A_2, phospholipase C, and phospholipase D (Roberts and Dennis 1989)(Fig.6). Phospholipase A_1 and

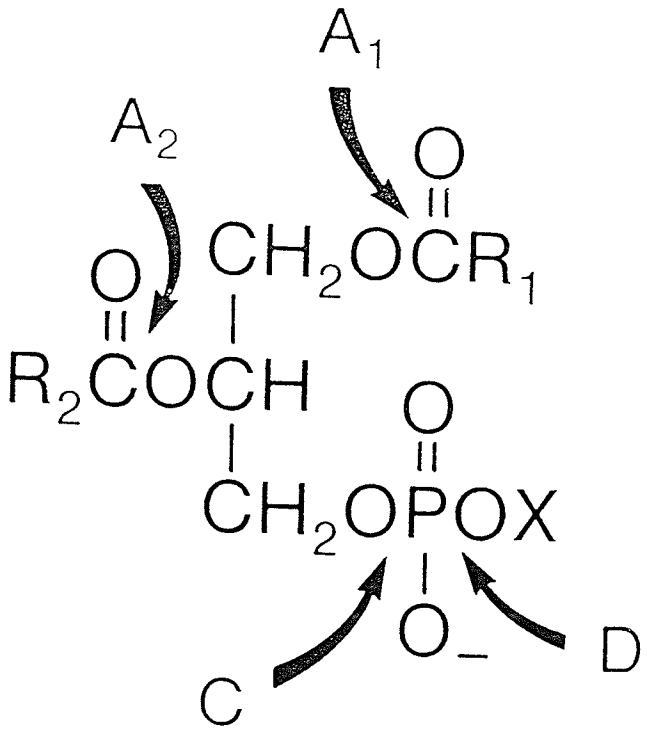


FIGURE 6. Site of Action of Phospholipases.

A1: Phospholipase A1
A2: Phospholipase A2
C: Phospholipase C
D: Phospholipase D
X: Polar head group

(Roberts and Dennis 1989)

phospholipase A_2 hydrolyze the sn-1 and sn-2 fatty acyl bond respectively. Phospholipase C cleaves the glycerophosphate bond. Phospholipase D hydrolyzes the choline phosphate ester bond of phosphatidylcholine. The products of these reactions are free fatty acid, lysophosphatidylcholine, diglyceride, phosphatidic acid, phosphocholine and choline. These compounds can be utilized in other biosynthetic pathways or degraded further. Lysophosphatidylcholine is the substrate for lysophospholipase which hydrolyzes the remaining fatty acyl bond to form free fatty acid and glycerophosphocholine. Phosphodiesterase can subsequently cleave either the glycerol phosphate bond or the cholinephosphate bond. The resulting fatty acids of phosphatidylcholine are metabolized by the ß-oxidation pathway. Glycerol, choline, and inorganic phosphate are the final catabolic products, and are used as precursers in various anabolic pathways.

In addition to the established role as catabolic enzymes, phospholipases are involved in the remodeling of existing phospholipids (Choy and Arthur 1989). Structural studies of phosphatidylcholine have indicated that an asymmetrical distribution of acyl groups exists. These molecular species are different between tissues and animal species. Various pathological and nutritional states in animals can also effect the acyl compositions in phosphatidylcholine. These observations suggest that the molecular species of phospholipids correlate with specific cellular functions. It has been suggested that mechanisms exist which account for the asymmetric distribution of these phospholipid acyl groups.

The pathways concerning the deacylation and reacylation of

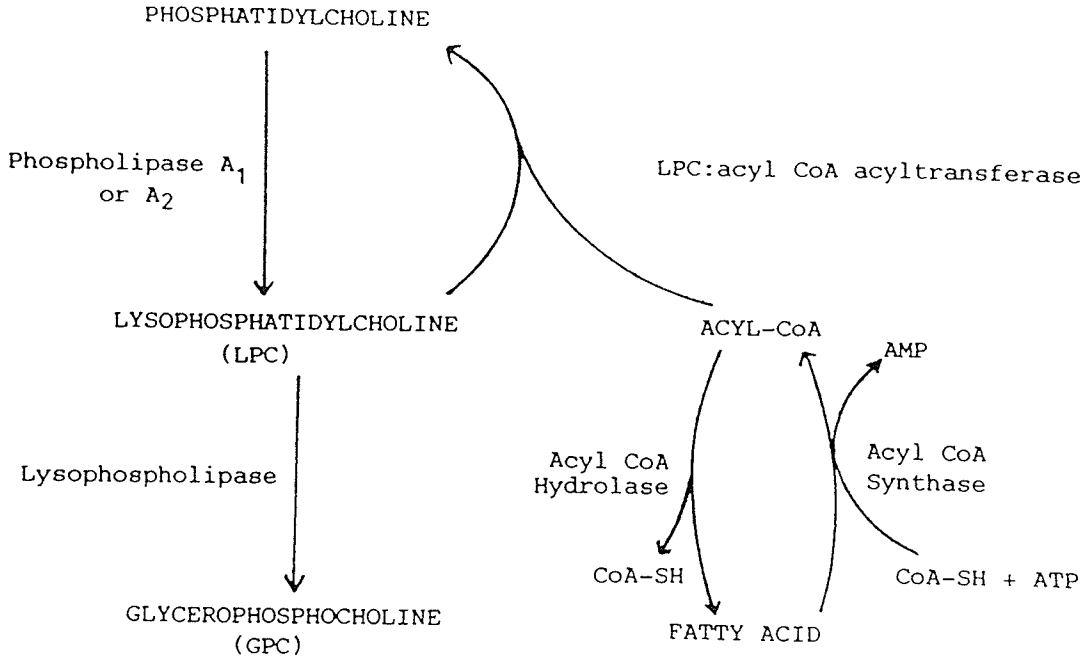


FIGURE 7: Lands Pathway.

(Choy and Arthur 1989)

phosphatidylcholine was first elucidated in 1960 (Lands 1960)(Fig.7). Phospholipases were responsible for the cleavage of phospholipids into lysophospholipid and free fatty acid. The formation of phosphatidylcholine from lysophosphatidylcholine is catalyzed by lysophosphatidylcholine:acyl CoA acyltransferase. This is a membrane-bound enzyme and has been purified to homogeneity in bovine brain (Deka *et al.* 1986) and bovine heart (Sanjanwala *et al.* 1988). Solubilization has also been reported in rat liver (Mukherjee *et al.* 1992). Evidence suggests that acylation of 1-acyl-glycerolphosphocholine and 2-acyl-glycerolphosphocholine are catalyzed by two different types of acyltransferases (Lands and Hart 1965). The general concensus indicates that specificities of the two acyltransferases account for the observed distribution of saturated and unsaturated acyl groups on phosphatidylcholine.

Biosynthesis of phosphatidylcholine can also occur through transacylation. This pathway involves the transfer of an acyl group from a donor phospholipid to lysophosphatidylcholine. There are three known types of transacylation pathways: 1) CoA-dependent, 2) CoA-independent, and 3) lysophosphatidylcholine-lysophosphatidylcholine transacylation. Both the CoA-dependent and CoA-independent pathways have been demonstrated in several tissues and cells (Irvine *et al.* 1979). The acyl groups involved in the transfer process are mostly polyunsaturated in nature. This leads to the postulation that phosphatidylcholine species containing polyunsaturated fatty acids are formed via the actions of transacylases rather than acyltransferases.

C) Role of Phosphatidylcholine in Signal Transduction

Among the enzymes involved with phosphatidylcholine catabolism, phospholipase C has been implicated in signal transduction. It is generally accepted that phospholipase C catalyzes the turnover of phosphoinositides which leads to the production of the second messengers *sn*-1,2-diacylglycerol and inositol 1,4,5-triphosphate (Voet and Voet 1990)(Berridge 1987). Both these second messengers eventually lead to the activation of a protein kinase C. However, recent evidence has suggested the existence of a phosphatidylcholine-specific phospholipase C which produces diacylglycerol and phosphocholine (Fig.8). The resulting diacylglycerol formed is also believed to act as a second messenger. This leads to the possibility that phosphatidylcholine breakdown is involved in the transmission of cellular responses (Exton 1990).

Evidence of phosphatidylcholine hydrolysis was first demonstrated in response to Ca^{2+}-mobilizing agents (Bocckino *et al.* 1985). When exposed to agonists, the accumulated diacylglycerol in hepatocytes was enriched in palmitic, oleic, and linoleic acids. This is in contrast to diacylglycerol derived from the breakdown of phosphoinositides which contain predominately stearic and arachidonic acids. It is therefore suggested that phosphatidylcholine was the source of the accrued diacylglycerol.

The stimulation of phosphatidylcholine hydrolysis has been associated with G-proteins. This is supported by observations that stimulation is induced by submicromolar concentrations of GTP analogues but not other nucleotides, requires

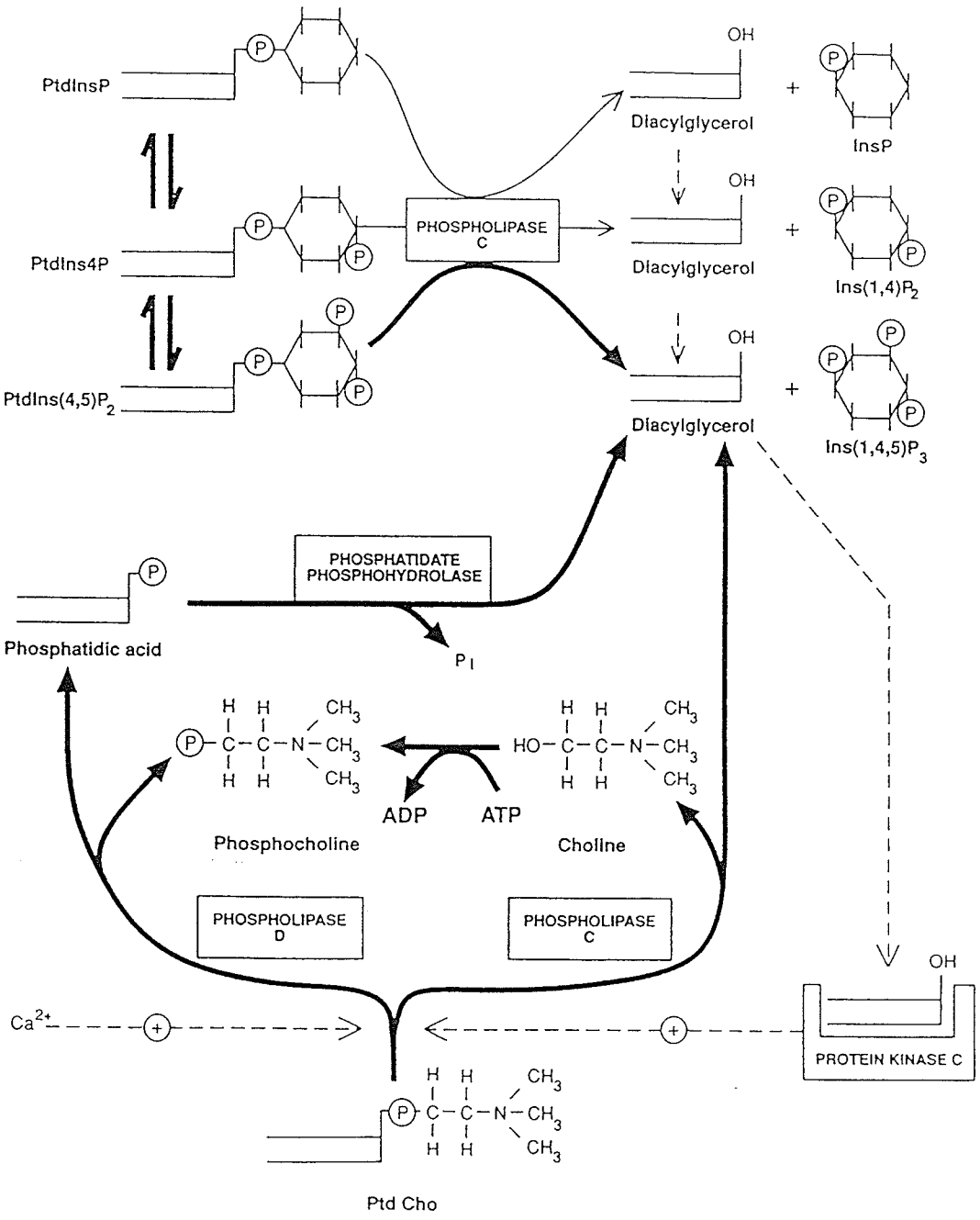


FIGURE 8: Receptor-mediated Breakdown of Phosphatidylcholine and Phosphatidylinositol.

(Shears 1991)

millimolar Mg^{2+}, and is inhibited by an analogue of GDP (Irving and Exton 1987).

The widespread occurence of agonist induced phosphatidylcholine hydrolysis suggests some physiological significance. Species of diacylglycerol originating from phosphatidylcholine are effective activators of protein kinase C *in vitro* (Go *et al.* 1987). Also, the transformation of some cells by certain oncogenes is associated with increased phosphatidylcholine breakdown, without any changes in inositol phosphates (Wolfman and Macara 1987). The reason for having two pathways of diacylglycerol formation remains speculative. Some studies indicate that phosphoinositide hydrolysis produce relatively small amounts of diacylglycerol for a short duration compared with phosphatidylcholine hydrolysis (Pessin and Raben 1989). This may be due the hundred-fold higher cellular content of phosphatidylcholine. Although the precise mechanism of intracellular signalling and other physiological effects are yet to be elucidated, studies indicate that phosphatidylcholine hydrolysis may be involved in this process.

III. Regulation of Phosphatidylcholine Metabolism

A) Choline Uptake

The biosynthesis of phosphatidylcholine, via the CDP-choline pathway, is dependent on the supply of extracellular choline. The dietary intake of choline can therefore have a regulatory role in the biosynthesis of phospholipids (Haines and Rose 1970). Choline from the diet is absorbed by the small intestine and is delivered

to the the liver where it is cleared from the portal circulation. A saturable and non-saturable mechanism exist, in some tissues, for choline uptake depending on the the concentration of choline. The non-saturable is attributed to simple diffusion and occurs with high extracellular choline concentration. The saturable component has an apparent K_m of 10 μM for choline in isolated rat hepatocytes (Pritchard and Vance 1981).

Normally, the rate of phosphatidylcholine biosynthesis in the liver is not influenced by choline transport. However, there is evidence in Novikoff hepatoma cells that choline transport serves as the rate-limiting step in the CDP-choline pathway (Plagemann 1971).

Various compounds have been shown to inhibit choline transport. Hemicholinium-3 (Diamond and Kennedy 1969) and chlorocholine (Haeffner 1975) inhibit choline uptake *in vitro*. However, *in vivo* regulation by these modulators has yet to be determined. Other studies have shown that ethanolamine, a metabolite structurally similar to choline, does regulate choline uptake. Ethanolamine is present in mammalian plasma at concentrations of approximately 50 μM (Zelinski and Choy 1984). In the isolated hamster heart, concentrations of 0.1-0.5 mM ethanolamine inhibited 26-63% the incorporation of 0.5 mM choline into phosphatidylcholine (Zelinski and Choy 1984). The nature of ethanolamine inhibition was determined to be competitive. Ethanolamine did not affect any of the steps involved in the conversion of intracellular choline to phosphatidylcholine. Despite decreased uptake of choline, the intracellular choline pool size did not change. This suggests that

choline is recycled within the cell and that phosphatidylcholine biosynthesis can be maintained for at least short periods of time, ie. 1 hour.

In addition to ethanolamine, amino acids have been implicated in regulating choline uptake. Amino acids were reported to influence choline uptake and phosphatidylcholine biosynthesis in renal cortical slices (Havener and Toback 1980). In the isolated hamster heart, the presence of glycine increased choline uptake by 30%. Glycine, a neutral amino acid, did not affect phosphorylation of choline or the rate of phosphatidylcholine biosynthesis (Hatch and Choy 1986). Subsequent studies revealed that other neutral amino acids, L-alanine, L-serine, and L-phenylalanine, also enhanced choline uptake to the same extent. Basic or acidic amino acids did not affect the transport of choline. Enhancement of choline uptake by neutral amino acids was not additive nor dose-dependent, but required a threshold concentration greater than 0.1 mM (Hatch *et al.* 1988). It is postulated that neutral amino acids facilitate uptake by direct interaction with the choline transport system.

B) Energy Requirements

The synthesis of phosphatidylcholine via the CDP-choline pathway requires adenosine-5'-triphosphate (ATP) and cytidine-5'-triphosphate as cofactors. Therefore, the availability of these energy supplying compounds can affect the rate of phosphatidylcholine biosynthesis (Vance and Choy 1979). ATP is involved in the phosphorylation of choline by choline kinase and CTP serves as a substrate for CTP:phosphocholine cytidylyltransferase to form CDP-choline (Zelinski *et al.* 1980).

In polio-infected HeLa cells, increased levels of CTP caused an enhancement of phosphatidylcholine biosynthesis. However, the corresponding increase in ATP did not affect the phosphorylation of choline (Choy *et al.* 1980). The effects of ATP and CTP pools on phosphatidylcholine biosynthesis in the heart were also examined. A pathological model was employed in which Syrian hamsters developed cardiomyopathy through autosomal recessive inheritance. These animals exhibited degenerative lesions in the myocardium with 100% incidence (Bajusz 1971). A 34% decrease in ATP and CTP concentrations were observed in these animal hearts (Choy 1982). When the myopathic hearts were perfused with labelled choline, a 22% increase in the incorporation of radioactivity into phosphatidylcholine was observed compared with the controls. However, the total level of cardiac phosphatidylcholine remained unchanged. A decrease in CDP-choline levels in the myopathic hearts was also observed.

The low levels of CTP accounted for the decreased CDP-choline levels. This decrease in CDP-choline concurred with the increase in specific radioactivity of CDP-choline and consequently the increased labelling in phosphatidylcholine. The decrease in ATP levels did not affect the conversion of choline to phosphocholine. Apparently, choline kinase is not sensitive to a 34% decrease in ATP levels. It is evident that CTP plays an important role in regulating phosphatidylcholine metabolism (Hatch *et al.* 1989).

C) Regulation of CTP:phosphocholine cytidylyltransferase

The present view of cytidylyltransferase (CT) regulation involves the translocation of the enzyme between subcellular compartments (Pelech and Vance 1984)(Fig.9). It is postulated that CT in cells is enhanced through a translocation of inactive enzyme from the cytosol to the endoplasmic reticulum where it is activated by phospholipids. This translocation can be regulated by phosphorylation/dephosphorylation reactions, supply of fatty acids, or increased amounts of diacylglycerol in the endoplasmic reticulum (Vance 1989). Also, synthetic compounds such as phorbol esters have been suggested as possible regulators of CT activity through translocation (Pelech *et al.* 1984).

There have been several studies which demonstrate that CT associated with the microsomal fraction is the active form. There is a correlation between the rate of phosphatidylcholine biosynthesis in cellular systems and the association of CT with microsomal membranes. The addition of 1 mM oleic acid to primary hepatocytes caused a twofold increase in phosphatidylcholine biosynthesis with a corresponding 1.8-fold increase of CT activity in microsomes (Pelech *et al.* 1983). Other studies using different cell systems and whole animals support this observation. Cytosolic CT appears to be an inactive source which can be mobilized should the need arise.

Enzyme phosphorylation was also implicated as a possible regulator of CT activity. Glucagon, a hormone which increases cAMP levels, caused a 40% decrease in phosphatidylcholine biosynthesis in cultured hepatocytes after a 2 hour treatment (Pelech *et al.* 1981). This observation correlates well with inhibition of the CT reaction. However, the activity of CT was not changed in either the microsomal or

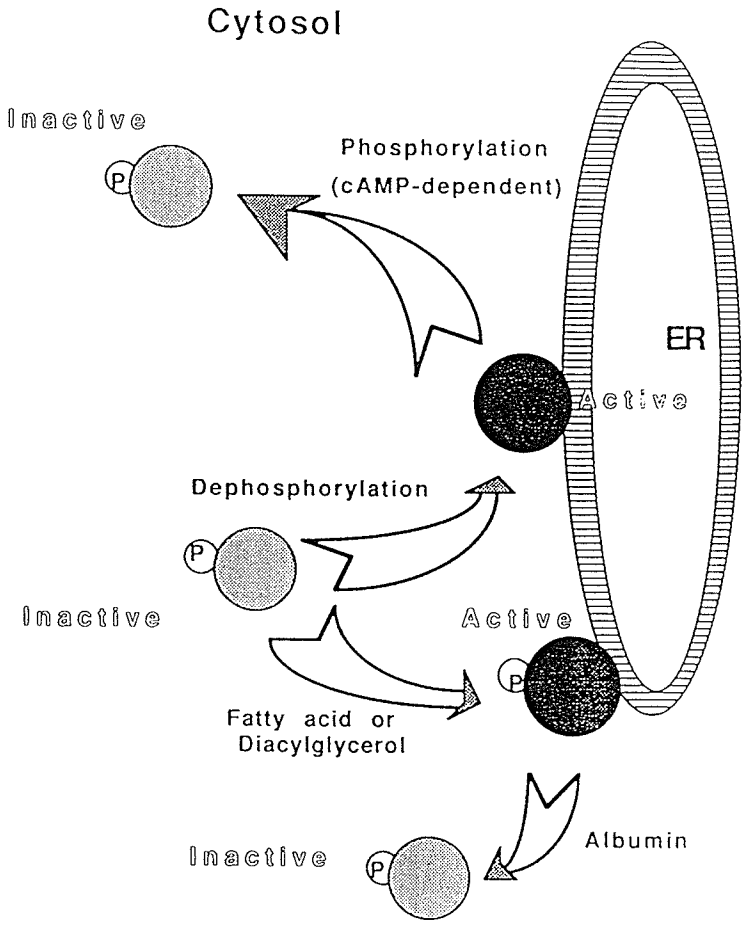


FIGURE 9: Translocation Model of CTP:Phosphocholine Cytidylyltransferase (Vance 1989)

cytosolic. This suggests that the increase in cAMP caused an inhibition of phosphatidylcholine biosynthesis by release of CT from the membrane to the cytosol. It is postulated that a cAMP-dependent protein kinase C phosphorylates the enzyme which alters the binding affinity for the membrane. Studies using protein kinase C inhibitors support this hypothesis. *In vitro* experiments have shown that CT is a substrate for the pure catalytic subunit of cAMP-dependent kinase (Sanghera and Vance 1989).

Fatty acids can also facilitate CT binding to microsomal membranes. The evidence for fatty acid regulation of CT in cultured cell systems is substantial (Vance 1989), but the effect of fatty acids on CT in whole animal systems remains speculative. In isolated rat hepatocytes, addition of albumin-bound fatty acids caused a two- to threefold stimulation of both the microsomal CT reaction and phosphatidylcholine biosynthesis (Pelech *et al.* 1983). A concomitant decrease in cytosolic CT activity was observed. Most long-chain fatty acids (saturated and unsaturated) stimulated phosphatidylcholine synthesis but short-chain fatty acids were ineffective. The only evidence for *in vivo* fatty acid regulation was observed in pre- and postnatal rat lung. A correlation between CT activity and the fatty acid content in fetal lung microsomal membranes was demonstrated (Weinhold *et al.* 1984).

In vitro experiments have also demonstrated that diacylglycerol mediates the translocation of CT from the cytosol to the membrane. Diacylglycerol present in the cytosol promotes the aggregation of the enzyme (Choy *et al.* 1979). Other neutral lipids such as cholesterol and triacylglycerol exhibit no effect on the activity of the

enzyme. In another approach, cultured cells were treated with exogenous phospholipase C which degrades phosphatidylcholine to diacylglycerol and phosphocholine (Sleight and Kent 1983). This treatment resulted in the reversible translocation of CT to cellular membranes which was not dependent on protein synthesis.

Other studies indicate that activation of CT requires the presence of lipids (Vance 1989). Neutral lipids exhibit no stimulatory effect on the enzyme activity. Neutral phospholipids such as phosphatidylcholine and phosphatidylethanolamine are poor activators. However, phospholipids which carry a negative charge (acidic) such as phosphatidylinositol and phosphatidylserine stimulate CT activity. The enzyme is activated by lysophosphatidylethanolamine and inhibited by lysophosphatidylcholine (Choy and Vance 1978). It is clear that CT has a requirement for phospholipids or phospholipid/fatty acid mixtures in order to have full catalytic activity (Vance 1989). Since phospholipids are usually found only in cellular membranes, this requirement gives additional support to the hypothesis that the membrane-associated enzyme is the active form of the enzyme.

D) Regulation of Phosphocholinetransferase

The majority of phosphatidylcholine formed in mammalian tissues has a saturated acyl group at the C-1 position and an unsaturated acyl group at the C-2 position of the glycerol moiety (Ansell and Spanner 1982). The composition of the acyl groups varies between tissues. The required molecular composition within a

specific tissue has been postulated to be mediated via 1,2-diacylglycerol:CDP-choline phosphocholinetransferase selectivity (Lands 1960). In hepatocytes, phosphocholinetransferase has a preference for palmitate at the C-1 and linoleate at the C-2 position. Phosphocholinetransferase in the lung is indiscriminate in its selection for diacylglycerol. In the hamster heart, maximum enzyme activity has been observed with diacylglycerol containing a monoenoic acyl group at the C-2 position, regardless of the acyl content at the C-1 position. Lower enzyme activity with polyunsaturated acyl groups at either C-1 or C-2, and very low activity with disaturated diacylglycerol have been reported (Arthur and Choy 1984). However, selectivity of the enzyme for diacylglycerol is broad when the hamster heart is perfused with labelled diacylglycerol of known acyl content. The difference of specificity compared to the *in vitro* assay may be attributed to the presence of detergent in the assay mixture.

Since diacylglycerol is a substrate for phosphocholinetransferase, the supply of diacylglycerol may regulate phosphatidylcholine biosynthesis. During starvation, diacylglycerol is preferentially channelled to phospholipid biosynthesis at the expense of triacylglycerol synthesis (Groener and Van Golde 1977). However, the level of diacylglycerol does not act as a rate-limiting factor in rat liver. Phosphatidylcholine biosynthesis is not affected by increased levels of diacylglycerol (Groener *et al.* 1979). Diacylglycerol apparently acts as a positive effector of cytidylyltransferase (Pelech and Vance 1984).

IV) Hormonal Effects on Phospholipid Metabolism

A) Mechanisms of Hormone Action

Hormones are biochemical compounds which can elicit various effects in living systems. These effects are initiated through binding of the hormone to a specific cellular receptor. Binding causes the production of second messengers which in turn precipitate a series of intracellular events. Hormones are classified according to structure, receptor type, and the nature of the second messenger produced (Granner 1990a)(Table 2).

One class of hormones is lipophilic and with the exception of the thyroid hormones, is derived from cholesterol. These substances can cross the cell membrane and bind to intracellular receptors in either the cytosol or nucleus of target cells. The ligand-receptor complex is believed to mediate intracellular effects.

A second class of hormones consist of water-soluble compounds which bind to a cell membrane associated receptor. These hormones elicit intracellular effects through second messengers. This classification can thus be subdivided based on the second messenger produced. The best studied of these messengers are cAMP, cGMP, and calcium and/or phosphatidylinositides.

The characterization of hormones and their effects are far from complete and not surprisingly, a hormone can fit into more than one category as more information is obtained. Present information suggests that a hormone can have more than one mechanism of action depending on the cell type.

TABLE 2

Classification of Hormones

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

(Granner 1990a)

There are two general mechanisms of hormone action which have been studied extensively. The first involves the binding of a hormone to intracellular receptors with subsequent effects on gene expression. The second mechanism involves the production of second messengers upon hormone binding to a membrane-bound receptor.

When lipophilic molecules diffuse through the plasma membrane, they bind to specific intracellular receptors. The location of the receptor within the cell depends on the hormone involved and the tissue type. The resulting complex undergoes conformational changes (activation) which enable it to bind to chromatin. The nuclear DNA contains regions called the hormone response elements to which the ligand/receptor complex can bind. This process results in altered gene expression (Granner 1990a). By selectively affecting gene transcription and production of mRNA, amounts of specific proteins are altered. This can consequently elicit a metabolic response.

Some water-soluble hormones initiate responses by binding to cell surface receptors and causing an increase in intracellular cAMP concentration (Granner 1990a). cAMP is produced through the action of adenylate cyclase and activates protein kinases by binding to regulatory subunits of the enzyme. Four molecules of cAMP are needed to activate the protein kinase. The activated protein kinase phosphorylates other proteins involved in metabolic processes.

There is ample evidence which suggests that the production of cAMP is

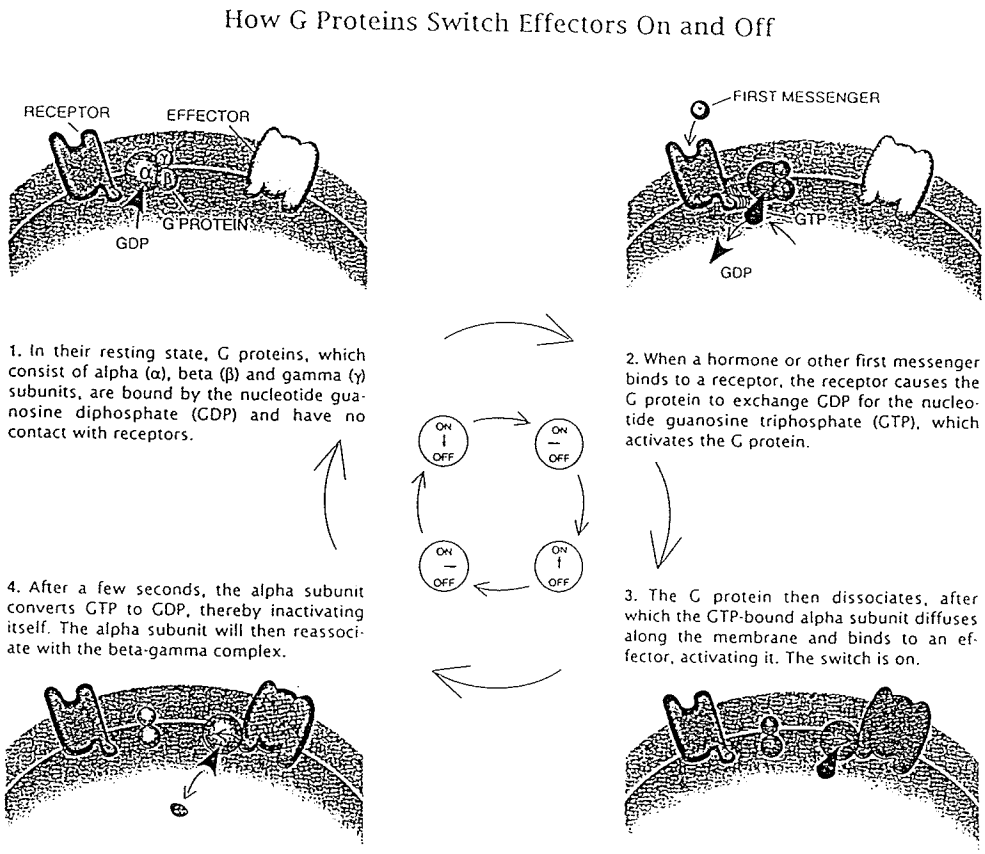


FIGURE 10: G-Protein Involvement in Receptor/Effector Coupling.

(Linder and Gilman 1992)

coupled to G-proteins (Linder and Gilman 1992). These G-proteins are linked to the hormone receptor and activated upon binding of the ligand (Fig.10). G-proteins are known to activate adenylate cyclase although other effectors such as phospholipase C, cyclic GMP phosphodiesterase, and potassium channels are believed to be affected. At present, there have been several G-proteins identified and some of these proteins are sensitive to inhibition by pertussis or cholera toxin. Inhibition by these toxins is caused by the ADP-ribosylation of the G-protein α-subunit.

Other hormones act through calcium and/or phosphoinositides as second messengers (Granner 1990a). Certain hormones enhance membrane permeability to calcium thereby increasing calcium influx. Others increase calcium concentration through the production of inositol 1,4,5-triphosphate and diacylglycerol from phosphatidylinositol-4-5-bisphosphate. Inositol 1,4,5-triphosphate releases calcium from intracellular stores. The calcium ion forms a complex with calmodulin and subsequently activates various protein kinases. Diacylglycerol also acts as a second messenger by stimulating a calcium-phospholipid-dependent protein kinase.

B) Characteristics of Vasopressin, Oxytocin, and Estrogen

The core of this research focuses on the effects of arginine-vasopressin, oxytocin, and 17ß-estradiol on phosphatidylcholine biosynthesis. The following is a general overview of the three hormones.

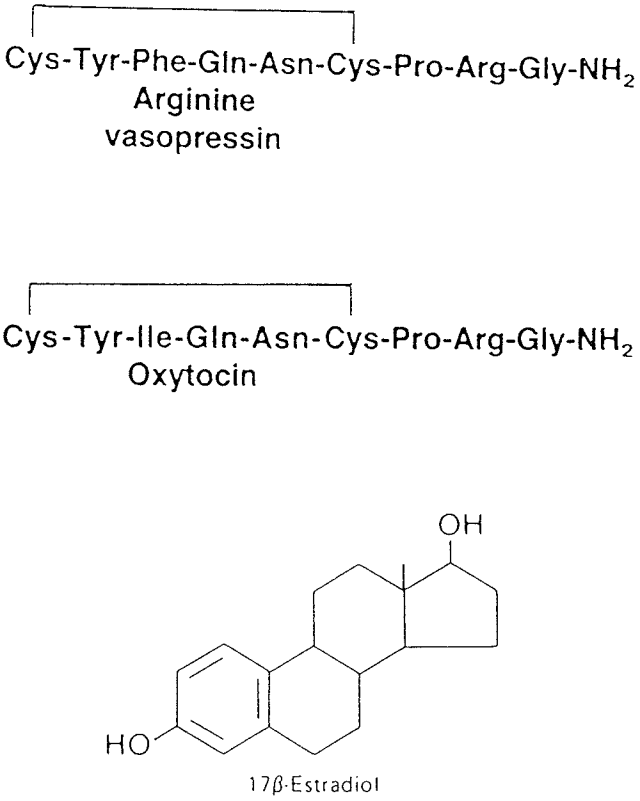


FIGURE 11: Hormone Structures.

(Granner 1990 b,c)

1. Vasopressin and Oxytocin

Vasopressin (antidiuretic hormone) and oxytocin are peptide hormones consisting of nine amino acids with cysteine molecules at position one and six linked by a disulfide bridge (Fig.11). Vasopressin is synthesized in the supraoptic nucleus and oxytocin in the paraventricular nucleus. Both hormones are transported to nerve endings in the posterior pituitary where, upon stimulation, they are released into circulation (Granner 1990b).

Vasopressin release is stimulated by a number of factors. Increased osmolality of plasma is the primary physiological stimulus. Other stimuli include emotional and physical stress and chemical agents including acetylcholine, nicotine, and morphine. Oxytocin release results from stimulation of the nipples. Vaginal and uterine distension are secondary stimuli.

The most understood of the many actions of vasopressin is its effect on water reabsorption in the kidney (Hays 1991). Cells of the renal collecting duct and the ascending limb of Henle's loop contain V_2 receptors which bind vasopressin. These receptors are coupled to adenylate cyclase and the production of cAMP. The collecting duct is responsible for the conservation of water. During conditions of dehydration, vasopressin concentration increases and segments of the collecting duct become permeable. An osmotic gradient causes water to be reabsorbed from the tubule. Binding of vasopressin to V_2 receptors promotes cAMP accumulation which in turn initiates a series of events that ultimately increases luminal membrane

permeability.

Another documented action of vasopressin is its vasoconstrictor effect on the cardiovascular system (Hays 1991). This cardiovascular effect occurs at significantly higher concentrations than required for maximal antidiuresis. Decreased coronary blood flow and increased pulmonary arterial pressure are a few examples of the vasoconstrictor effect. These effects are mediated by V_1 receptors and are coupled to a phospholipase C that is responsible for the hydrolysis of phosphatidylinositol-4,5-bisphosphate. Thus, many of the cellular effects of vasopressin are accompanied by a increase in calcium concentration.

The complementary DNA encoding the hepatic V_1 arginine-vasopressin receptor has recently been cloned (Morel *et al.* 1992). The liver DNA codes for a protein containing seven putative transmembrane domains. This protein binds vasopressin and related compounds with similar affinities to the native rat liver V_1 receptor. The identities between the V_1 receptor and other members of the G-protein coupled receptor superfamily are found in the membrane-spanning domains. A cytoplasmic loop in the C-terminal region of the protein is believed to contain residues subject to phosphorylation. This suggests that the receptor may be regulated by protein kinases. The transmembrane domains of the protein are structurally similar to bacterial rhodopsin and other G-protein coupled receptors.

The mechanism of oxytocin action is much less understood (Granner 1990b). The hormone causes contraction of uterine smooth muscle and thus is used in pharmacologic amounts to induce labor. The physiological function of oxytocin is

believed to involve the contraction of myoepithelial cells surrounding the mammary alveoli. This promotes the movement and ejection of milk. Membrane receptors are found in uterine and mammary tissues. These receptors are increased in number by estrogens.

2. Estrogen

The estrogens are a family of steroid hormones synthesized in ovarian and extraovarian tissues (Granner 1990c). 17ß-Estradiol (Fig.11) is the primary estrogen of ovarian origin. All estrogens are formed via the aromatization androgens. During secretion, estrogens are bound to a plasma transport protein called a sex-hormone binding globulin. The rate of ovarian steroid secretion varies considerably during the menstral cycle. This class of hormones exert major effects through binding to intracellular receptors which then bind to specific regions of chromatin.

The sequences of the human and chicken estrogen receptor proteins have been deduced from sequence analysis of full-lengh cDNA molecules (Granner 1990c). Each has three highly conserved regions, the second and third being the DNA- and hormone binding domains. The hormone binding domain is highly hydrophobic and the DNA binding region is rich in cysteine and basic amino acid residues. These two domains are conserved in the glucocorticoid, progesterone, and calcitriol receptors.

The major function of estrogens is to prepare the structural determinants of the female reproductive system (Granner 1990c). They are responsible for the

maturing of germ cells, providing hormonal timing for ovulation, the maintenance of pregnancy, and providing hormonal influences for parturition and lactation. Estrogens stimulate the development of tissues involved in reproduction. The rate of protein, rRNA, tRNA, mRNA, and DNA synthesis is increased consequently stimulating the size and number of cells.

C) Hormonal Effects on Phosphatidylcholine Metabolism

The effects of hormones on phosphatidylcholine metabolism have been investigated in several systems. Steroid hormones including diethylstilbestrol and glucocorticoids stimulate synthesis in rooster (Vigo and Vance 1981) and fetal lung (Rooney *et al.* 1986). Injection of the synthetic estrogen into roosters appears to induce choline kinase activity with a corresponding increase in phosphocholine. The glucocorticoids stimulate pulmonary phosphatidylcholine biosynthesis by increasing the amount of cytidylyltransferase associated with microsomes. Subsequent studies indicate that glucocorticoid treatment increase both cytosolic and microsomal cytidylyltransferase activity by an unknown mechanism (Post 1987). Estrogen has also been implicated in the stimulation of phosphatidylcholine biosynthesis in fetal rabbit lung. This is attributed to an increase in the amount of cytosolic phospholipid which activates cytosolic cytidylyltransferase (Chu and Rooney 1985).

Alternatively, other hormones are known to inhibit phosphatidylcholine biosynthesis. Vasopressin inhibits phospholipid synthesis in isolated rat hepatocytes via an effect on the cytidylyltransferase reaction (Tijburg *et al.* 1987). Vasopressin

also inhibits phosphocholinetransferase through an increase in calcium concentration (Alemany *et al.* 1982). Similarly, norepinephrine appears to elicit its effect on cytidylyltransferase activity (Haagsman *et al.* 1984). Phosphatidylcholine biosynthesis in neonatal bone was unaffected by hormones known to stimulate other processes in bone (Stern and Vance 1987). The peptide hormone prolactin induces a threefold elevation of choline kinase but does not increase the rate of phosphatidylcholine biosynthesis or the activities of cytidylyltransferase or phosphocholinetransferase (Ko *et al.* 1986).

In addition to hormones, the effect of mitogenic growth factors have been investigated. In 3T3 fibroblasts, an eightfold increase in phosphatidylcholine biosynthesis was reported which was thought to arise from an induction of choline kinase with a concomitant increase in the phosphocholine pool (Warden and Friedkin 1984). This serves as an example for regulation occurring at the level of gene expression.

Despite the above mentioned observations, mechanisms of hormonal regulation in specific tissues remains to be elucidated. Also, hormonal effects in other cell systems has not yet been investigated.

RESEARCH AIMS

The objective of this research is to expand our limited understanding of hormonal effects on phosphatidylcholine biosynthesis. Some of the control

mechanisms of phospholipid metabolism have been elucidated. However, our current knowledge on the hormonal regulation of phospholipid metabolism is based on studies performed in liver and lung tissue. Information on the hormonal regulation of phospholipid metabolism in other tissues such as heart and brain remain scant by comparison.

The goal of this study is to define the role of hormones on phosphatidylcholine biosynthesis in the mammalian heart. It can be argued that information gained from experimentation in other tissues such as liver and lung may be applicable to the heart. However, the majority of phosphatidylcholine synthesized in the liver and lung are secreted as lipoproteins or surfactant (King and Clements 1972; Post and van Golde 1988). In the heart, no phosphatidylcholine is known to be exported (Zelinski *et al.* 1980). The difference in the utilization of phosphatidylcholine between these tissues implies that metabolism may be regulated differently.

To examine the effects of various agents on the heart, two approaches were used in this study. The perfusion of the isolated heart and the isolation of myocardial cells were both employed. The isolated heart model was used to study the uptake of choline and phosphatidylcholine biosynthesis in the whole organ. The myocyte model was used to analyze the effect of hormones on phosphatidylcholine metabolism where such studies could not be performed in the intact heart.

This treatise contains data regarding the effects of estrogen, a steroid hormone and two peptide hormones, vasopressin and oxytocin on phosphatidylcholine

metabolism in the heart. These two classes of hormones were selected based on previous information indicating an effect on phospholipid metabolism in other tissues. The mechanism of hormone action by these different compounds in the heart was also investigated.

In addition, the effect of amino acids on phosphatidylcholine biosynthesis was examined. This was done to further investigate mechanisms of phosphatidylcholine regulation in the mammalian heart. Both approaches of studying agent effects in cardiac tissue were used with respect to the amino acid study.

MATERIALS AND METHODS

I. Materials

A) Chemicals

Phospho[Me-^{14}C]choline, CDP[Me-^{14}C]choline and [tyrosyl-^{3}H] arginine vasopressin were obtained from Amersham Canada Ltd (Oakville, Ontario). [Me-^{3}H] choline was purchased from New England Nuclear Division of Dupont (Mississauga, Ontario). Oxytocin and [arginine]-vasopressin were the product of 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). Lyophilized pertussis toxin was purchased from Gibco Laboratories (Burlington, Ontario). Minimal essential medium (Joklik modified), Medium 199, Dulbecco's phosphate buffered saline, phosphocholine, choline, cytidine 5'-diphosphocholine,

Tris-base, CTP, ATP, hyaluronidase and EDTA were obtained from Sigma Chemial Co. (St. Louis, Missouri). Phosphatidylcholine (pig liver) and 1,2-diacylglycerol (pig liver) were purchased from Serdary Research Laboratory (London, Ontario). Magnesium acetate and magnesium chloride were products of Fisher Scientific Co. (Fair Lawn, New Jersey). Sephadex G-25 (fine) gel filtration media was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Collagenase (Class 2) was obtained from Worthington Biochemical Corporation (Freehold, New Jersey). All other chemicals were of analytical grade and obtained locally from Canlab, a division of Baxter.

B) Experimental Animals

Male Sprague Dawley rats, obtained from Charles River Canada Inc. (St, Constante, Quebec), were used during the course of experimental study. The rats (250 ± 40 g) were maintained on Agway rodent chow and tap water *ad libitum*, in a light and temperature controlled room. Syrian golden hamsters (110 ± 20 g) and guinea pigs (280 ± 20 g), from Charles River Canada Inc. (St. Constante, Quebec), were used in intact heart perfusion studies. New Zealand White rabbits (1.8 ± 0.5 g) were obtained from the central animal care unit of the University of Manitoba (Winnipeg, Canada).

II. Methods

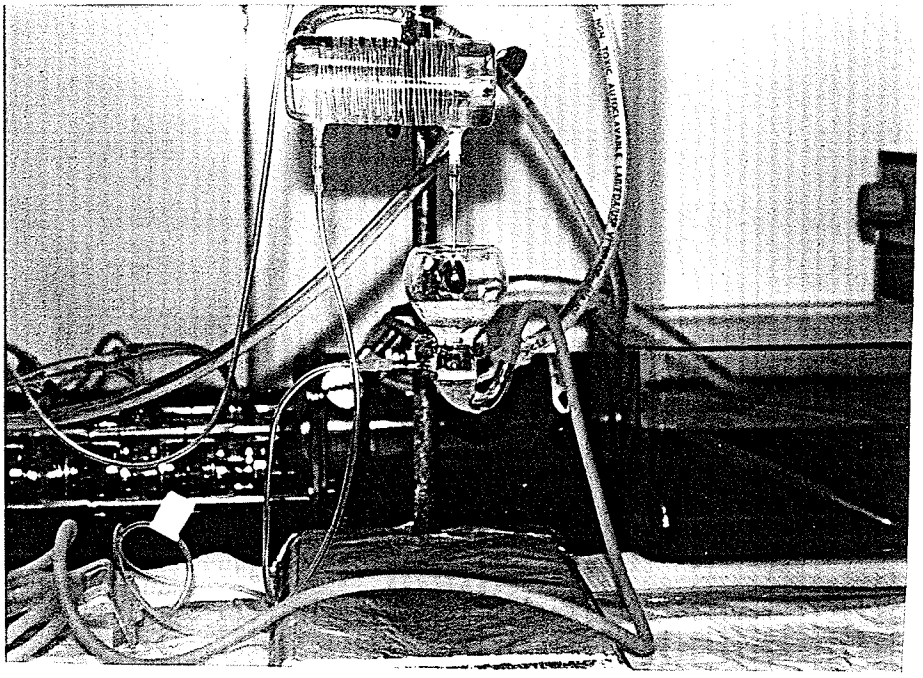
A) Preparation of Buffer Solutions

The perfusion of the rat heart in obtaining isolated myocardial cells required the appropriate buffer solutions. Buffer A contains 11 g/l MEM-Jokliks modified buffer preparation, 60.0 mM taurine, 2.0 mM Dl-carnitine, 1.0 mM adenosine, 3.4 mM magnesium chloride, 50.0 mM sodium bicarbonate, 15.0 mM glucose, 0.1 mM octanoic acid, 8.0 mM L-glutamate, 0.1 g/l penicillin and 0.1 g/l streptomycin. Buffer B contains 0.1 mM calcium chloride dissolved in buffer A. Buffer C contains 9.8 g/l of Medium 199 preparation, 2.0 mM L-glutamine, 2.2 g/l sodium bicarbonate, 0.1 g/l penicillin and 0.1 g/l streptomycin. Solutions were sterile filtered through 0.22 μm Falcon 7150 bottle top filters. Prior to rat heart perfusions, all buffers were maintained at 37° and saturated with 95% oxygen/5% carbon dioxide.

A modified Krebs-Henseleit bicarbonate buffer was used during experiments with the perfused intact heart. This buffer contained 118 mM sodium chloride, 4.7 mM potassium chloride, 2.5 mM calcium chloride, 0.5 mM EDTA, 1.2 mM magnesium sulfate, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$. The buffer was prepared within an hour prior to use, equilibrated with O_2/CO_2 (95:5), and adjusted to pH 7.4.

B) Isolation of Rat Cardiac Myocytes

Myocardial cells were obtained from the heart of Sprague Dawley rats. Myocyte isolation was performed by the procedure of Langer *et al.* (Langer *et al.* 1987). The rat was sacrificed by decapitation and the heart removed. The heart was subsequently perfused in the Langendorff manner with buffer A for 7 minutes, at a flow rate of 5 mL/minute (Fig.12). The ensuing step was perfusion of the heart with



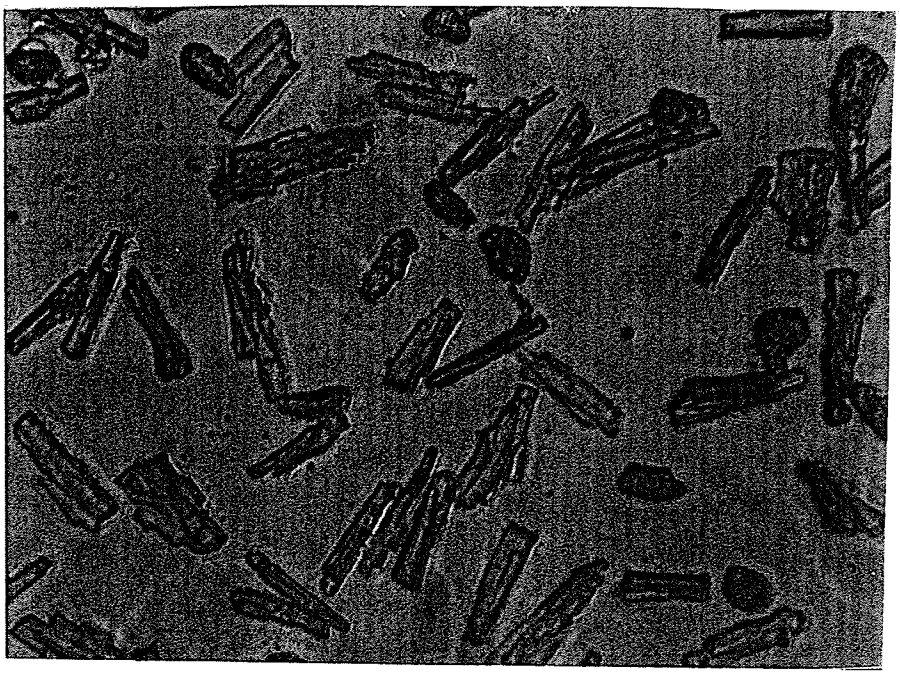


FIGURE 12. Perfusion Apparatus and Isolated Myocytes

buffer B containing 4 mg/mL collagenase (class 2) and 2 mg/mL hyaluronidase, at a flow rate of 2.5 mL/minute. Perfusate was recycled over the course of the perfusion. Perfusion with the enzyme solution continued until digestion of heart tissue was complete. The digested tissue was then placed in a culture dish containing buffer B.

The myocardial cells were subsequently dislodged from the tissue by gentle agitation with sterile forceps. The cell suspension was then transferred to a culture tube. Myocytes were pelleted by gravitation and supernatant containing dead cells and non-myocardial cells were removed. Cells were then suspended in buffer B (15 mL) and transferred to a culture dish (100 x 20 mm). Cells were incubated for 30 minutes at 37° in an incubator continously aerated with 5% CO_2. Myocyte viability was determined by trypan blue exclusion. Cell homogeneity was observed under a light microscope. The myocardial cells were rod-shaped and exhibited contractile ability (Fig.12).

Following the 30 minute incubation, buffer C (5 mL) was added to the cell suspension consequently increasing the calcium concentration to 0.5 mM. The myocytes were further incubated for 15 minutes. The cell suspension was then transferred to a culture tube and suspended in buffer C. The myocytes were pelleted by gravitation and the supernatant removed. An aliquot of myocyte suspension was then pipetted into petri dishes (60 x 15 mm) containing buffer C supplemented with 5% fetal calf serum. Isolated myocytes were further incubated for 20 minutes to allow attachment to the plate. Non-adhering cells were removed and the remaining

attached cells were resuspended in buffer C.

C) The Determination of Choline Uptake and Analysis of Choline-containing Metabolites from Myocytes

Myocytes isolated from the rat heart were incubated in 60 x 15 mm petri dishes for 15 minutes prior to use. Each dish of cells (containing 200-300 μg protein) was incubated with 2 ml of growth medium containing 10 μM labelled choline (0.15 mCi/μmol) for the prescribed time period. The medium containing labelled choline was removed and the cells were washed twice with 2 ml of Dulbecco's phosphate buffered saline followed by the addition of 1 ml methanol. The cell suspension was transferred to a glass test tube and an equal volume of chloroform was added. The suspension was mixed to ensure cell disruption. The cell samples were subsequently centrifuged at 500 g. Supernatant was transferred to another set of glass test tubes and the cell pellet was washed with an additional aliquot of chloroform/methanol (1:1, v/v). The supernatants were combined to a total of 6 mL. A 2 mL aliquot from each sample was taken for the determination of total choline uptake.

Extraction of lipids from the myocytes was performed using the method developed by Folch *et al*, 1957. Phase separation was initiated upon the addition of distilled water and chloroform to the supernatant to form a chloroform/methanol/water (4:2:3, v/v/v) mixture. Centrifugation of the mixture at 500 g facilitated phase separation. Subsequent to phase separation, the lower (organic) phase was removed and the solvent evaporated under nitrogen gas flow.

Phospholipids in the lower phase was resuspended in 500 μL of chloroform and applied to a silica G-25 chromatography plate. Phosphatidylcholine was then isolated by thin-layer chromatography with a solvent containing chloroform/methanol/water/acetic acid (70:30:4:2, v/v/v/v). The location of the phosphatidylcholine band was visualized by iodine staining. Iodine was removed by heating the plate for 20 minutes. The solvent in the upper phase was first removed in a Savant Speedvac SC100 rotary evaporator and subsequently resuspended in 200 μL of distilled water. The radioactive choline-containing metabolites were separated by thin-layer chromatography with a solvent system containing methanol/0.6% sodium chloride/ammonium hydroxide (50:50:5, v/v/v). Non-radioactive carrier choline (1 mM), CDP-choline (1 mM), and phosphocholine (1 mM) were spotted with the radioactive samples to ensure proper separation of the metabolites. The location of the radioactive metabolites on the thin-layer chromatographic plate was detected by a Bioscan System 200 Imaging Scanner. The fractions of silica gel containing radioactive metabolites were transferred into scintillation vials and suspended in 1 mL distilled water, 10 mL Ecolite(+), and 50 μL acetic acid. Radioactivity in fractions was determined by scintillation counting using channels ratio calibrating method.

D) Perfusion of Isolated Heart

Hearts were removed from the animal immediately after decapitation. Perfusion in the Langendorff mode was employed where the aorta was cannulated

to a perfusion apparatus. Krebs-Henseleit buffer containing labelled choline was introduced through the aorta, thus effectively perfusing the coronary vessels. The buffer was recycled during the perfusion period. The hearts were perfused for 60 minutes at 37°. The flow rate was maintained at 6-8 mL/g heart weight, producing an aortic pressure of 60-80 mm Hg. The perfusate contained 10 μM [methyl-^{3}H] choline (0.35 μCi/mL) and was saturated with 95% O_2/5% CO_2.

E) Determination of Choline Uptake and Analysis of Choline-containing Metabolites from the Perfused Heart

Subsequent to perfusion, the heart was homogenized in 10 mL of chloroform/methanol (1:1, v/v) using a Polytron homogenizer from Brinkmann Instruments. The homogenate was centrifuged at 2000 g for 10 minutes. The resulting pellet was re-extracted twice with the same solvent. An aliquot of the pooled extract was taken to determine choline uptake. Chloroform and water were added to the lipid extract to achieve a 4:2:3 (v/v/v) chloroform/methanol/water ratio. Subsequent to phase separation, the volumes of the upper and lower phases were reduced by evaporation *in vacuo*. An aliquot of the upper phase was analyzed for radioactivity in the choline-containing metabolites by thin-layer chromatography (TLC) with a solvent containing methanol/0.6% sodium chloride/ammonium hydroxide (10:10:1, v/v/v). An aliquot of the lower phase was analyzed for radioactivity associated with phosphatidylcholine by TLC with a solvent containing choroform /methanol /water /acetic acid (70:30:4:2, v/v/v/v).

F) The Determination of Enzyme Activities

1. Choline Kinase

Choline kinase activity was assayed by determining the conversion of [Me-^{3}H] choline to phosphocholine (Ishidate *et al.* 1984). The reaction mixture contained 0.1M Tris-HCl (pH 8.5), 10mM magnesium chloride, 10mM ATP, 0.25mM [Me-^{3}H] choline iodide and enzyme preparation to a final volume of 0.5 ml. The reaction was initiated by the addition of labelled choline. The mixture was incubated at 37° for 20 min. The reaction was terminated by placing the assay test tubes in a boiling water bath for two minutes. The samples were subsequently centrifuged at 5000 g for 10 minutes. An aliquot (250μL) was applied to a Dowex AG 1-X8 (OH-) column (0.5 x 2 cm). The column was then washed with 2.5 mL of 5 mM choline chloride followed by 6 mL of distilled water. The labelled phosphocholine was eluted from the column with 0.5 mL of 1.0 M NaOH followed by 1.5 mL of 0.1 M NaOH. Fractions were collected in scintillation vials. Acetic acid (0.1 mL) and 10 mL of Ecolite(+) was added to the fractions. Radioactivity associated with phosphocholine was determined by liquid scintillation counting.

2. CTP:phosphocholine cytidylyltransferase

CTP:phosphocholine cytidylyltransferase activity was assayed by the conversion of phospho[Me-^{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[Me-^{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° for 20 minutes. The reaction was terminated by placing assay tubes in a boiling water bath for two minutes. The samples were subsequently centrifuged at 5000 g for 10 minutes. An aliquot of supernatant was then applied to a thin-layer chromatography plate (silica G-25). The labelled CDP-choline was separated from the labelled substrate by thin-layer chromatography with a solvent system containing CH_3OH/0.6% NaCl/NH_3OH (10:10:1, v/v/v) The location of the CDP-choline fraction was identified with the Bioscan System 200 Imaging Scanner. Silica gel containing labelled product was removed and placed in scintillation vials containing water (1 mL), acetic acid (50 μL), and Ecolite(+) (10 mL).

3. CDP-choline:1,2-diacylglycerol Phosphocholinetransferase

CDP-choline:1,2-diacylglycerol Phosphocholinetransferase activity was determined by the conversion of CDP-[Me-^{14}C]choline to phosphatidylcholine (O and Choy 1990). The reaction mixture contained 100mM Tris-HCl (pH 8.5), 10mM magnesium chloride, 1mM EDTA, 0.4mM CDP-[Me-^{14}C] choline, 1.0mM diacylglycerol (prepared in 0.015% Tween 20 by sonication) and enzyme preparation to a final volume of 1.0 ml. The reaction was initiated by the addition of labelled substrate and carried out at 37° for 20 minutes. Termination of the reaction occurred by the addition of 3 ml of chloroform/methanol (2:1, v/v) to the mixture. Water (0.5ml) was added to cause phase separation. The lower phase (containing the

labelled phosphatidylcholine) was washed twice with 40% methanol. An aliquot was taken for radioactivity determination.

G) Subcellular Fractionation

Microsomal and cytosolic fractions were obtained from cardiac myocytes 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 douncing was verified by observation under a light microscope. The cellular suspension was centrifuged at 20,000 g for 10 min and the supernatant obtained was centrifuged at 100,000 g for 60 min. The supernatant (cytosol) was collected and the precipitate (microsomal pellet) was resuspended in the homogenizing buffer.

H) Gel Filtration

Vasopressin and other peptides were identified via a gel filtration column. Sephadex G-25 Fine media was used in the column (1.25 x 50 cm). The gel filtration material was first allowed to swell in excess Tris-HCl buffer (10 mM) for a minimun of 3 hours. The suspension was subsequently de-gassed. The gel suspension was poured into the column to a volume of 40 mL ensuring an even distribution of media. The column was equilibrated with 120 mL Tris-HCl (10 mM) at a flow rate of 5mL/minute. A LKB Bromma 2120 varioperpex peristaltic pump was used to control rate of eluent buffer flow. Homogeneity of the packed bed was examined by

running through a sample of Blue Dextran 2000 (2 mg/mL). Quality of the packing was represented by a narrow band of the coloured substance as it progressed though the bed. Absorbance of the eluent was read at 280 nm using an Isco UA-5 absorbance/fluorescence detector.

I) Other Analytical Procedures

1. Lowry Protein Assay

Analysis of protein content was determined by the method of Lowry *et al.* (1951). Protein samples and bovine serum albumin (BSA) standards were initially digested in 0.66 M NaOH (1.5 mL) for a mimimum of 2 hours at 37°. After digestion, 1.5 mL of reagent A (containing 13% Na_2CO_3, 2% $CuSO_4$, 4% Na^+K^+tartrate, 33:1:1 v/v) was added to the protein samples and the solution mixed thoroughly. Subsequent to a 10 minute incubation period, 0.5 mL of 2 N Folin & Ciocalteu's phenol reagent was added to the samples. Protein samples were then mixed and allowed to incubate for 30 minutes at 25°. Absorbance was then read at 625 nm using a Bausch & Lomb Spectronic 2000 spectrophotometer.

2. Radioactivity Determination

Radioactivty associated with samples was determined by liquid scintillation counting using the channels ratio calibrating method. Radioactive samples were contained in scintillation vials with Ecolite(+) scintillation fluid. A LKB Wallac 1211

Minibeta liquid scintillation counter was employed in determining radioactivity.

3. Statistical Analysis

Student's t-test was used for statistical analysis of data. The significance level was set at $p < 0.05$.

RESULTS

I. The Effect of Vasopressin on Choline Uptake and Phosphatidylcholine Biosynthesis

A time course experiment was performed to determine the effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis. Cardiac myocytes were incubated in growth medium containing 10 μM [Me-^{3}H] choline in the presence of 0.2 and 1.0 μM arginine-vasopressin for 20-120 min. Myocytes incubated in the same medium without vasopressin were used as controls. Subsequent to incubation, total uptake of choline by myocytes was determined. As depicted in Fig. 13, choline uptake by myocytes was linear up to 60 min in the presence or absence of vasopressin. The presence of 0.2 μM vasopressin caused an enhancement of choline uptake at all time points whereas the presence of 1 μM vasopressin caused a significant reduction in choline uptake. An increase in the incorporation of choline into phosphatidylcholine was observed in the presence 0.2 μM vasopressin (Fig.14). An initial lag phase in the appearance of label associated with phosphatidylcholine

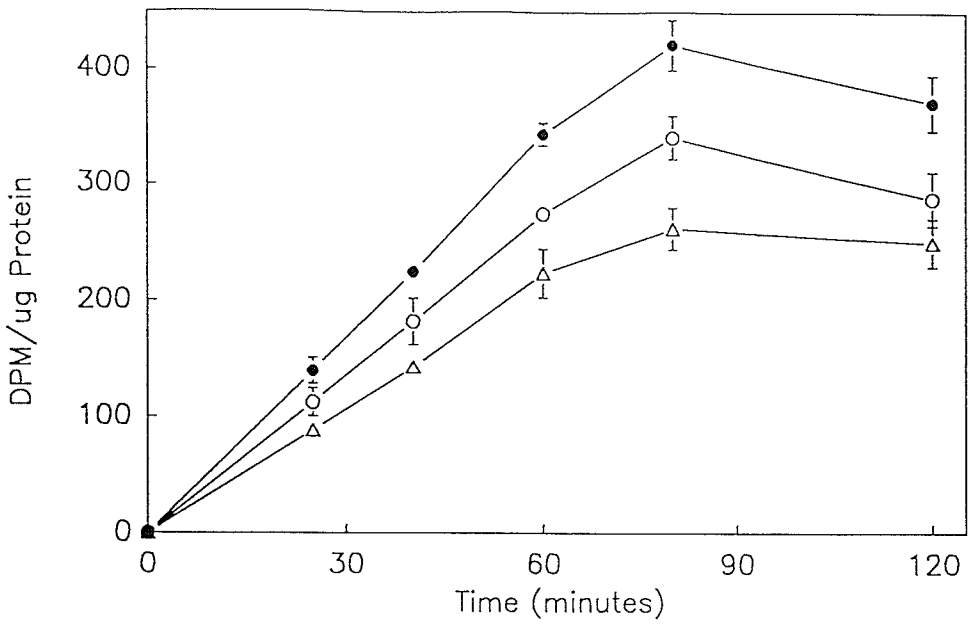


FIGURE 13. Time Course of Choline Uptake in Rat Cardiac Myocytes.

Rat cardiac myocytes were incubated with 10 μM [Me-^{3}H]choline (150 μCi/μmol) in the absence (○) and presence of 0.2 μM (●) or 1.0 μM (△) vasopressin. The total amount of radioactivity incorporated into the myocytes at each time point was determined. Each point represents the mean value of two separate experiments.

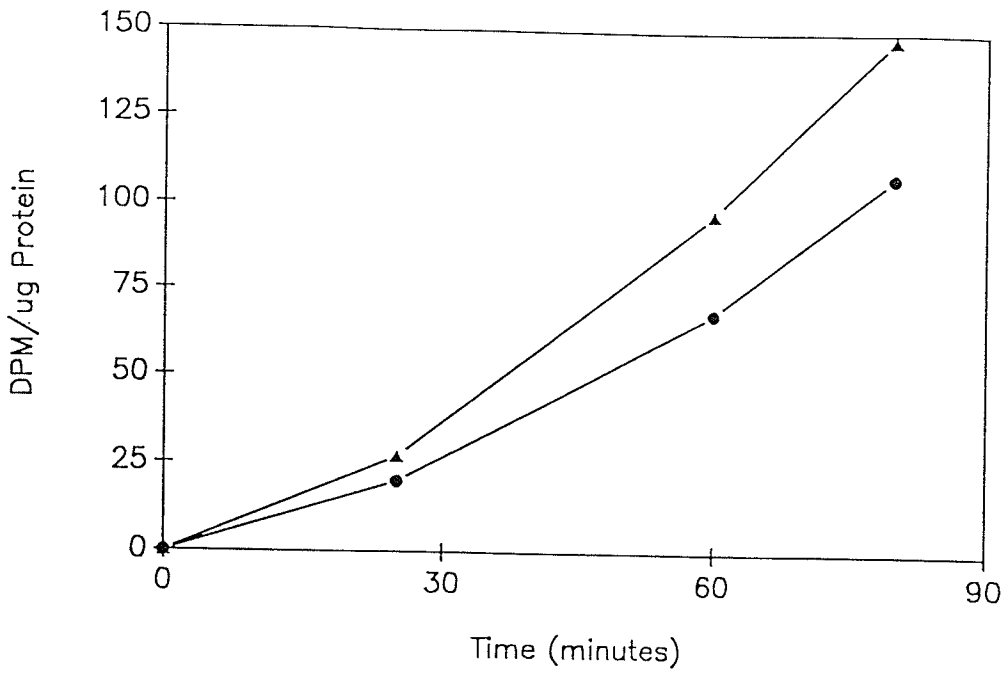


FIGURE 14. Time Course of Incorporation of Radioactivity into Phosphatidylcholine in rat Cardiac Myocytes.

Rat cardiac myocytes were incubated with 10 μM [Me-^{3}H] choline (150 μCi/μmol) in the absence (●) and presence of 0.2 μM (▲) vasopressin. The total amount of radioactivity associated with phosphatidylcholine at each time point was determined. Each point represents the mean value of two separate experiments.

was observed in all cases due to the progressive labelling of intermediates in the CDP-choline pathway. Alternatively, a decrease in the incorporation of radioactivity into phosphatidylcholine was observed in the presence of 1.0 μM hormone (Data not shown).

The effect of other vasopressin concentrations on choline uptake and phosphatidylcholine biosynthesis was investigated. Myocytes were incubated with growth medium containing 10 μM labelled choline for 60 min at various vasopressin concentrations. Subsequently, the uptake of choline and the amount of radioactivity in the phosphatidylcholine fraction in the myocytes were determined. As shown in Table 3, an enhancement of choline uptake was observed with a corresponding increase in the labelling of phosphatidylcholine at 0.05-0.2 μM vasopressin. At higher vasopressin concentrations (0.5-1.0 μM), choline uptake was attenuated, together with a reduction in the labelling of phosphatidylcholine. It appears that choline uptake in myocytes was modulated by vasopressin in a biphasic manner, with a transition occurring between 0.3-0.4 μM. However, the change in the labelling of phosphatidylcholine was not proportional to the change in choline uptake which indicates that vasopressin might also affect phosphatidylcholine biosynthesis in myocytes.

The nature of the vasopressin action was investigated by using a specific V_1 receptor antagonist [d$(CH_2)_5$, D-Tyr$(OEt)^2$, Val^4, Cit^8]-vasopressin. Myocytes were incubated with 10 μM [Me-^{3}H] choline and 0.2 μM and 1.0 μM arginine-vasopressin for 60 minutes in the presence and absence of 0.1 μM vasopressin antagonist.

TABLE 3

Effect of Vasopressin on Choline Uptake and Phosphatidylcholine Biosynthesis.

Hormone	Total Choline Uptake	Phosphatidylcholine
	(dpm/μg protein)	
Control	274 ± 8 (5)	68 ± 3 (8)
0.05 μM vasopressin	301 ± 10 (5)[a]	79 ± 3 (8)[a]
0.1 μM vasopressin	304 ± 14 (3)[a]	82 ± 3 (3)[a]
0.2 μM vasopressin	343 ± 10 (5)[a]	97 ± 11 (6)[a]
0.3 μM vasopressin	324 ± 15 (3)	73 ± 9 (3)
0.4 μM vasopressin	229 ± 7 (3)	58 ± 8 (3)
0.5 μM vasopressin	232 ± 19 (3)[a]	60 ± 2 (3)[a]
1.0 μM vasopressin	223 ± 21 (4)[a]	41 ± 4 (9)[a]
0.1 μM antagonist	287 ± 3 (3)	78 ± 6 (4)

Cardiac myocytes in 60 mm petri dishes were incubated with growth medium containing 10 μM [Me-^{3}H] choline (150 μCi/μmol) for 60 min in the presence and absence of vasopressin, and with both 0.2 μM vasopressin and 0.1 μM [d$(CH_2)_5$, D-tyr$(OEt)^2$, val^4, cit^8] vasopressin. Subsequently, the total choline uptake and the incorporation of label into phosphatidylcholine were determined. Each set of values represents the mean ± standard deviation (number of experiments).

[a] $p < 0.05$

TABLE 4

Effect of Vasopressin on the Incorporation of Radioactivity into Choline-containing Metabolites.

Vasopressin	Choline	Phosphocholine	CDP-choline
		(dpm/μg protein)	
Control	53 ± 3 (6)	163 ± 9 (4)	8.8 ± 2.9 (6)
0.2 μM	66 ± 4 (6)[a]	187 ± 14 (5)[a]	9.4 ± 1.8 (3)
1.0 μM	41 ± 11 (13)[a]	152 ± 7 (3)	3.4 ± 1.3 (9)[a]

Cardiac myocytes were incubated with growth medium containing 10μM [Me-^{3}H] choline for 60 min in the presence of 0.2 or 1.0 μM vasopressin. Subsequently, the radioactivity associated with the choline-containing metabolites of the CDP-choline pathway in the myocytes was determined. Each set of values represents the mean ± standard deviation (number of experiments)

[a] $p < 0.05$

Myocytes incubated in the presence of only 10 μM [Me-3H] choline were used as controls. As shown in Table 3, the presence of 0.1 μM vasopressin antagonist attenuated the stimulation by 0.2 μM vasopressin with respect to choline uptake and subsequent incorporation into phosphatidylcholine. The presence of 0.1 μM antagonist also attenuated the effect of 1.0 μM vasopressin (Data not shown).

II. Analysis Choline-containing Metabolites

The effect of vasopressin on the radioactivities associated with the choline-containing metabolites in myocytes was determined. The choline-containing metabolites in the upper phase of the cellular extract were separated by thin-layer chromatography with a solvent system containing methanol/0.6% sodium chloride/ammonium hydroxide (50/50/5; v/v/v). In the presence of 0.2 μM vasopressin, a 24% increase in the labelling of choline was observed (Table 4) which is commensurate with the increase in choline uptake. However, only 15% in the labelling of phosphocholine was detected. In the presence of 1 μM vasopressin, the decrease in the labelling of choline parallels the decrease in choline uptake, but the labelling of phosphocholine was not changed when compared with the control. The labelling of CDP-choline was greatly reduced.

III. Activities of Enzymes in the CDP-choline Pathway

The disproportionate changes between choline uptake and labelling of some choline-containing metabolites suggest that vasopressin might also affect the

conversion of metabolites in the CDP-choline pathway. Hence, the activities of the enzymes in the CDP-choline pathway were also determined. Myocytes were incubated with 0.2 μM or 1.0 μM vasopressin for 60 min, and the cells were homogenized in a glass douncer. Myocytes incubated in the absence of vasopressin were used as controls. The activities of choline kinase, CTP:phosphocholine cytidylyltransferase and CDP-choline:diacylglycerol cholinephosphotransferase were determined in the homogenates and the results are shown in Table 5. Incubation with 0.2 μM vasopressin caused a two-fold enhancement of the cytidylyltransferase activity, but the other enzymes in the CDP-choline pathway were not affected. Incubation with 1.0 μM vasopressin caused a severe (55%) reduction in the cytidylytransferase activity. The activities of choline kinase and cholinephosphotransferase were not significantly changed by this treatment.

The mechanism for the change of cytidylyltransferase activity at different vasopressin concentrations was investigated. Myocytes incubated with 0.2 or 1.0 μM vasopressin were homogenized, and subcellular fractions were obtained by differential centrifugation. Enzyme activities in the microsomal and cytosolic fractions were determined and the results are depicted in Table 6. In the presence of 0.2 μM vasopressin, enhancement of enzyme activities in both the microsomal and cytosolic fractions were observed. In the presence of 1.0 μM vasopressin, there was a severe reduction in enzyme activity in the microsomal fraction, with a corresponding increase in enzyme activity in the cytosolic fraction.

TABLE 5

The Effect of Vasopressin on the Activities of the Phosphatidylcholine Biosynthetic Enzymes.

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

Cardiac myocytes were incubated with growth medium in the presence and absence of vasopressin. Subsequently, the myocytes were homogenized and enzyme activities in the homogenate were determined. Each set of values represents the mean ± standard deviation (number of experiments).

[a] $p < 0.05$

TABLE 6

Effect of Vasopressin on the Specific Activity of CTP:phosphocholine cytidylyltransferase in the Microsomal and Cytosolic fractions.

Vasopressin	Cytidylyltransferase Activity	
	Microsomal	Cytosolic
	(pmol/min/mg protein)	
Control	0.415 ± 0.073 (4)	0.319 ± 0.086 (3)
0.2 μM	0.612 ± 0.071 (3)[a]	0.543 ± 0.048 (3)[a]
1.0 μM	0.169 ± 0.029 (3)[a]	0.605 ± 0.041 (3)[a]

Cardiac myocytes were incubated with growth medium in the presence and absence of vasopressin. Subsequently, the myocytes were homogenized and subcellular fractions were obtained by differential centrifugation. The cytosolic enzyme activity was assayed in the presence of phospholipids. Each set of values represents the mean ± standard deviation (number of experiments).

[a] $p < 0.05$

IV. The Uptake of Vasopressin in Cardiac Myocytes

The ability of the cardiac myocytes to take up vasopressin was investigated. Myocytes were incubated with 0.2 or 1.0 μM [^{3}H-tyr] vasopressin (1.5 μCi/nmol) for 60 min in growth medium followed by incubation with growth buffer containing 10 μM non-labelled vasopressin for another 10 min. After incubation, the cells were homogenized in 0.25 M sucrose buffer and an aliquot of the homogenate was taken for radioactivity determination (Table 7). Uptake of vasopressin by myocytes was found to be dose-dependent but not linear. Five-fold increase of vasopressin in the medium does not result in a five-fold increase in hormone uptake. The fate of the vasopressin taken up by the cells was also examined. The myocyte homogenate was centrifuged at 100,000 x g for 60 minutes to obtain the cytosolic fraction. An aliquot of the cytosol was applied to a Sephedex G-25 (fine) column equilibrated with 10 mM Tris-HCl (pH 7.5)-0.1 M KCl. The elution profile of the labelled materials in the cytosol is depicted in Fig.15. For comparative purposes, the elution profile of labelled vasopressin and tyrosine were also obtained. The labelled vasopressin taken up by the cells appears to be metabolized into labelled tyrosine and its metabolites.

V. The Effect of Oxytocin on Choline Uptake and Phosphatidylcholine Biosynthesis

The effect of vasopressin on choline uptake may be a result of a direct interaction of the hormone with the choline transport site. Hence the effects of a structurally similar hormone, oxytocin, on choline uptake and phosphatidylcholine

TABLE 7

The Uptake of Vasopressin by Rat Cardiac Myocytes.

Vasopressin in medium	Vasopressin uptake
	(fmol/μg protein)
0.2 μM	5.5 ± 1.1 (6)
1.0 μM	22.2 ± 8.0 (6)

Cardiac myocytes were incubated in the presence of [^{3}H-tyr] vasopressin (1.5 μCi/nmol) for 60 min. After incubation, the medium was removed and cells were washed with unlabelled vasopressin (10 μM). The myocytes were homogenized and the amount of radioactivity in the homogenate was determined. Each set of values represents the mean ± standard deviation (number of experiments).

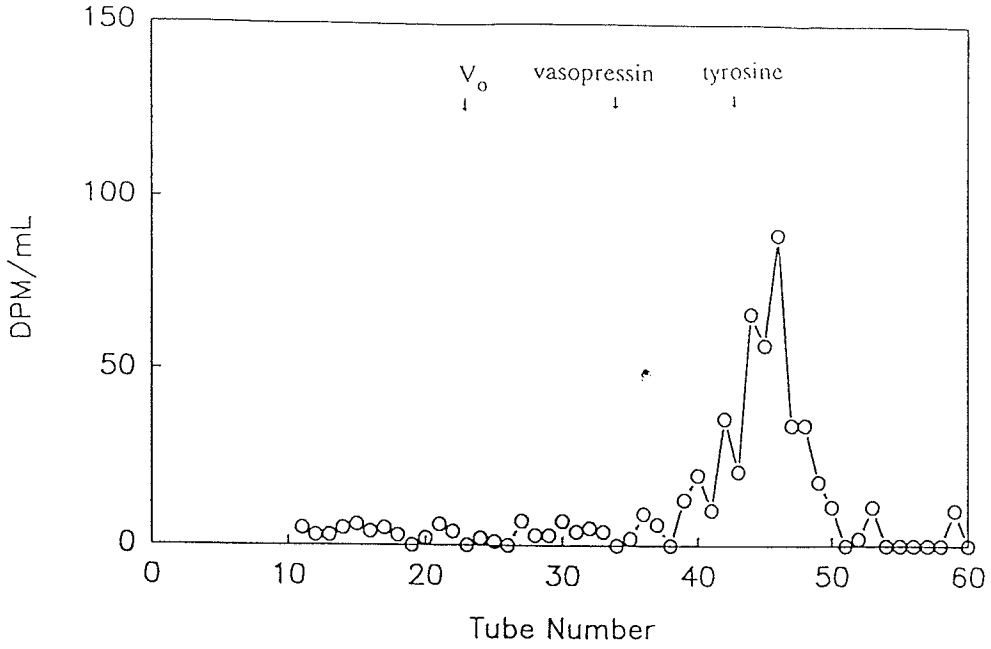


FIGURE 15. Sephadex G-25 Chromatography of the Cytosol from Rat Cardiac Myocytes incubated with labelled Vasopressin.

Rat cardiac myocytes were incubated with [^{3}H-tyr] vasopressin for 60 min. The cytosol obtained from the myocytes was applied to a Sephadex G-25 (fine) column of 1.25 x 45 cm. Subsequent to sample application, the column was washed with a buffer containing 10 mM Tris-HCl/0.1 M KCl, pH 7.5. The flow rate was 6 ml/h and 1 ml fractions were collected. The amount of radioactivity in each fraction was determined.

biosynthesis was determined. Cardiac myocytes were incubated with 2 mL growth medium containing 10 μM [Me-^{3}H] choline (150 μCi/μmol) in the presence and absence of 0.05, 0.2, and 1.0 μM oxytocin for 60 minutes. After incubation, the total uptake of choline and the incorporation of radioactivity into phosphatidylcholine were determined. Oxytocin, at these concentrations, did not have any effect on the uptake of choline or on the biosynthesis of phosphatidylcholine (Table 8).

VI. The Effect of 17ß-Estradiol on Choline Uptake and Phosphatidylcholine Biosynthesis The effect of a steroid hormone, 17ß-estradiol, on phospholipid metabolism was investigated. Cardiac myocytes were prepared and incubated in growth medium containing 10 μM [Me-^{3}H] choline (150 μCi/μmol). The cells were treated with and without 1.0 μM estradiol for 60 minutes. Subsequently, the total choline uptake and the radioactivity associated with phosphatidylcholine were determined and the results are depicted in Table 9. In the presence of 1.0 μM estradiol, choline uptake was decreased by 31%. The incorporation of choline into phosphatidylcholine was attenuated by 50%.

The effect of a higher concentration of 17ß-estradiol (50μM) was also determined. Concentrations at this level were observed to be cytotoxic. The rat myocardial cells lost viability after an extended period of estrogen exposure (1 hour).

VII. The Effect of Pertussis Toxin on Vasopressin Regulation of Phosphatidylcholine Biosynthesis

TABLE 8

Effect of Oxytocin on Choline Uptake and Phosphatidylcholine Biosynthesis

Concentration	Total Choline Uptake	Phosphatidylcholine
		(dpm/μg protein)
control	274 ± 8 (5)	68 ± 3 (8)
0.05 μM oxytocin	229 ± 20 (4)	57 ± 7 (4)
0.2 μM oxytocin	275 ± 17 (5)	68 ± 10 (10)
1.0 μM oxytocin	282 ± 30 (4)	65 ± 9 (4)

Cardiac myocytes in 60 mm petri dishes were incubated with 2 mL growth medium containing 10 μM [Me-^{3}H] choline (150 μCi/μmol) in the presence and absence of oxytocin for 60 minutes. Subsequent to incubation, the total uptake of choline and the incorporation of label into phosphatidylcholine were determined. Each set of values represents the mean ± standard deviation (number of experiments).

TABLE 9

Effect of 17ß-Estradiol on Choline Uptake and Phosphatidylcholine Biosynthesis

Concentration	Total Choline Uptake	Phosphatidylcholine
	(dpm/μg protein)	
control	470 ± 42 (4)	101 ± 9 (5)
1 μM estradiol	323 ± 51 (4)[a]	50 ± 12 (5)[a]

Cardiac myocytes in 60 mm petri dishes were incubated with growth medium containing 10 μM [Me-^{3}H] choline (150 μCi/μmol) for 60 min in the presence and absence of estradiol. Subsequently, the total choline uptake and the incorporation of label into phosphatidylcholine were determined. Each set of values represents the mean ± standard deviation (number of experiments).

[a] $p < 0.05$

Experiments were conducted to investigate the modulation of phosphatidylcholine biosynthesis by vasopressin. Since vasopressin binds to membrane associated receptors, it can be concluded that transmission of an intracellular signal must occur in order to elicit its effect. G-proteins have been linked to several types of membrane-bound receptors and are involved in receptor-effector coupling (Casey and Gilman 1988). Pertussis toxin is known to inhibit various G-proteins, including G_i, via ADP-ribosylation of an α-subunit. Cardiac myocytes were pre-treated with and without 200ng/mL pertussis toxin for 1 hour. Subsequently, cardiac myocytes were incubated with 10 μM [Me-^{3}H] choline (150 μCi/μmol) in the presence and absence of 0.2 and 1.0 μM vasopressin. The uptake of choline and radioactivity associated with phosphatidylcholine were determined after the 60 minute incubation period.

In myocytes pre-treated with 200 ng/mL pertussis toxin, 0.2 μM vasopressin did not cause any increase in choline uptake or stimulation of phosphatidylcholine biosynthesis (Table 10). Similarly, in myocytes pre-treated with 200 ng/mL pertussis toxin, 1.0 μM vasopressin did not have any effect on choline uptake or consequent incorporation into phosphatidylcholine.

VIII. The Effect of Amino Acids on Choline Uptake and Phosphatidylcholine Biosynthesis in Mammalian Hearts

Previous studies have indicated that choline uptake in the heart is affected by amino acids (Hatch *et al.* 1988). The uptake of choline by hamster, guinea pig, rat

TABLE 10

Effect of Pertussis Toxin on Vasopressin Regulation of Phosphatidylcholine Biosynthesis

Hormone	Total Choline Uptake	Phosphatidylcholine
	(dpm/μg protein)	
Control	280 ± 13 (4)	70 ± 10 (4)
0.2 μM vasopressin	362 ± 9 (4)[a]	92 ± 8 (4)[a]
0.2 μM vasopressin + 200ng/mL pertussis toxin	265 ± 24 (4)	65 ± 10 (4)
1.0 μM vasopressin	223 ± 21 (4)[a]	41 ± 4 (4)[a]
1.0 μM vasopressin + 200ng/mL pertussis toxin	270 ± 19 (4)	76 ± 5 (4)

Cardiac myocytes in 60 mm petri dishes were incubated with growth medium containing 10 μM [Me-^{3}H] choline (150 μCi/μmol) for 60 min. The medium also contained 0.2 μM or 1.0 μM vasopressin in the presence and absence of 200 ng/mL pertussis toxin. Myocytes exposed to only labelled choline were used as controls. Subsequently, the total choline uptake and the incorporation of label into phosphatidylcholine were determined. Each set of values represents the mean ± standard deviation (number of experiments).

[a] $p < 0.05$

TABLE 11

The Effect of Amino Acids on Choline Uptake in Mammalian Hearts.

Amino acid (1 mM)	Hamster heart	Guinea pig heart	Rat heart	Rabbit heart
		(dpm x 10^{-6}/g heart wet weight)		
Control	3.60±0.28 (6)	2.12±0.19 (7)	2.57±0.30 (8)	2.48±0.34 (4)
L-Glycine	4.72±0.17 (4)[a]	2.19±0.18 (4)	2.80±0.25 (3)	--
L-Alanine	4.66±0.21 (4)[a]	1.90±0.13 (4)	2.88±0.39 (4)	2.31±0.27 (4)
L-Lysine	3.54±0.33 (4)	2.06±0.06 (3)	2.41±0.29 (3)	2.05±0.33 (3)
L-Aspartate	3.47±0.25 (4)	2.09±0.18 (4)	2.79±0.23 (4)	2.38±0.27 (3)

Hamster, guinea pig, rat and rabbit hearts were perfused with 10 μM labelled choline (0.35 μCi/mL) for 60 min, and the amount of radioactivity in the tissue extracts after perfusion were determined. The values represent the mean ± standard deviation (number of experiments).

[a] $p < 0.05$ when compared with control.

and rabbit hearts in the presence of amino acids was examined. Hearts were perfused with 10 μM labelled choline (0.35 μCi/mL) in Krebs-Henseleit buffer for 60 min at 37°C. Subsequent to perfusion, the amount of radioactivity in the tissue extracts was determined (Table 11). In the presence of 1.0 mM L-lysine and 1.0 mM L-aspartate, choline uptake was unaffected in the hearts of all species when compared with the control. In the presence of 1.0 mM glycine or 1.0 mM L-alanine, the uptake of choline was elevated in the hamster heart but not in the guinea pig, rat, or rabbit hearts. Increasing the amino acid concentration to 5.0 mM had no effect on choline uptake in the guinea pig, rat, or rabbit hearts (Data not shown).

The effect of amino acids on the labelling of phosphatidylcholine was also studied. Subsequent to perfusion, the phosphatidylcholine fraction was isolated from the tissue extract by thin-layer chromatography, and the amount of radioactivity in this fraction was determined (Table 12). As expected, no significant change was observed in the labelling of phosphatidylcholine in the guinea pig, rat or rabbit heart when amino acid was present in the perfusate. Although choline uptake was enhanced in the hamster heart perfused with neutral amino acids, there was no corresponding increase in the labelling of phosphatidylcholine.

The neutral amino acid L-alanine was investigated further in these experiments. A time course of L-alanine uptake in the perfused hamster and rat heart was performed (Fig.16). It was observed that the neutral amino acid enhanced choline uptake at all time points between 15 and 60 minutes in the hamster heart. L-alanine did not affect the uptake of choline in the rat heart.

TABLE 12

The Effect of Amino Acids on the Incorporation of Labelled Choline into Phosphatidylcholine in Mammalian Hearts.

Amino acid (1 mM)	Hamster heart	Guinea pig heart	Rat heart	Rabbit heart
		(dpm x 10^{-5}/g heart wet weight)		
Control	7.75±0.63 (6)	5.47±1.25 (7)	4.00±0.80 (8)	4.12±0.91 (4)
L-Glycine	7.47±0.54 (4)	6.10±0.58 (4)	3.85±0.77 (3)	--
L-Alanine	7.89±0.32 (4)	4.79±0.71 (4)	4.26±1.76 (4)	4.86±0.47 (4)
L-Lysine	8.04±0.66 (4)	5.51±0.46 (3)	5.11±0.78 (3)	4.41±0.65 (3)
L-Aspartate	7.33±0.71 (4)	4.05±1.18 (4)	3.95±0.24 (4)	4.04±0.54 (3)

Hamster, guinea pig, rat and rabbit hearts were perfused with 10 μM labelled choline (0.35 μCi/mL) for 60 min, and the amount of radioactivity incorporated into the phosphatidylcholine fractions after perfusion were determined. The values represent the mean ± standard deviation (number of experiments).

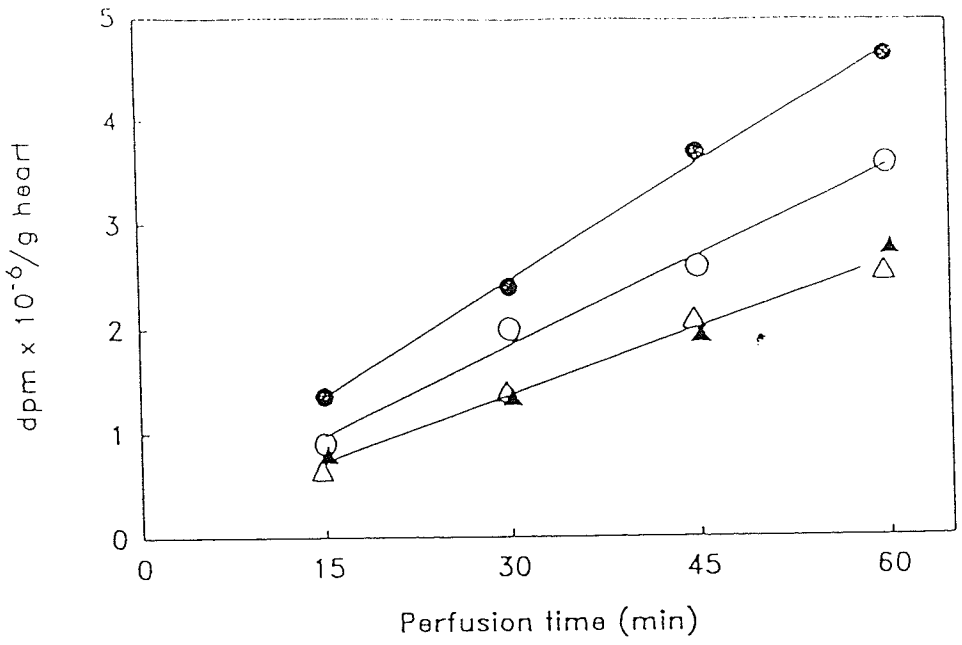


FIGURE 16. Time Course of Choline Uptake in Hamster and Rat Hearts in the Presence of L-Alanine.

Hamster (●, ○) and rat (▲, △) hearts were perfused in Krebs-Henseleit buffer containing 10 μM [Me-^{3}H] choline (0.35 μCi/mL) in the presence (filled symbols) and absence (open symbols) of 1 mM L-alanine for 15-60 minutes. Total choline uptake was determined in the tissue extract after perfusion. Each time point represents the mean of three separate experiments.

IX. Effect of L-Alanine on the Labelling of Choline-containing Metabolites in Mammalian Hearts

Analysis of the choline-containing metabolites revealed that the metabolites in the CDP-choline pathway were not affected by L-alanine in guinea pig, rat or rabbit hearts (Table 13). An accumulation in the labelling of phosphocholine was found in the hamster heart perfused with L-alanine. The increase in the labelling of phosphocholine quantitatively accounted for the increase in labelled choline uptake in the presence of the neutral amino acid.

X. The Effect of L-Alanine on Choline Uptake and Choline Incorporation into Phosphatidylcholine in Myocardial Cells

Studies performed in the isolated perfused heart were extended to myocardial cells. The ability of neutral amino acids to enhance choline uptake in myocytes was investigated. Myocytes were isolated from hamster and rat hearts and incubated with 0.1 μM labelled choline (2 μCi/mL) for 60 min in the presence of L-alanine. Since the viability of the hamster myocytes might not be identical from one batch of cells to another, results were obtained from two separate batches and are shown in Table 14. Choline uptake in hamster myocytes was enhanced by L-alanine in both batches. As a control study, the effect of L-alanine on choline uptake in rat myocytes was investigated. The presence of L-alanine did not enhance the uptake of choline in rat myocytes.

TABLE 13

The Effect of L-Alanine on the Labelling of Choline-Containing Metabolites in Mammalian Hearts.

	Choline	Phosphocholine	CDP-choline
		(dpm x 10^{-5}/g heart wet weight)	
Hamster			
Control	2.47 ± 0.31	25.4 ± 2.9	0.91 ± 0.13
+ L-alanine	2.33 ± 0.30	35.3 ± 2.7[a]	1.00 ± 0.05
Guinea Pig			
Control	2.18 ± 0.68	7.53 ± 1.1	1.08 ± 0.88
+ L-alanine	2.33 ± 0.73	8.40 ± 1.6	0.81 ± 0.35
Rat			
Control	2.73 ± 0.28	14.6 ± 2.7	1.37 ± 0.70
+ L-alanine	2.43 ± 0.48	13.0 ± 3.4	1.26 ± 0.57
Rabbit			
Control	1.77 ± 0.43	11.4 ± 3.0	0.69 ± 0.30
+ L-alanine	1.64 ± 0.22	12.4 ± 1.6	0.84 ± 0.16

Hearts were perfused with labelled choline in the absence and presence of 1 mM L-alanine as described in Table 11. The radioactivities associated with the choline-containing metabolites were determined. The values represent the mean ± standard deviation of four separate experiments.

[a] $p < 0.05$ when compared with control.

TABLE 14

The Effect of L-Alanine on Choline Uptake and Choline Incorporation into Phosphatidylcholine in Isolated Myocytes from Hamster and Rat Hearts.

Myocytes	Choline uptake	Phosphatidylcholine
	(dpm x $10^{-3}/\mu$g protein)	
Hamster (batch A)		
Control	1.13 ± 0.11 (4)	0.29 ± 0.04 (4)
+ L-alanine	1.58 ± 0.15 (4)[a]	0.32 ± 0.06 (4)
Hamster (batch B)		
Control	0.82 ± 0.19 (4)	0.28 ± 0.03 (4)
+ L-alanine	1.60 ± 0.13 (4)[a]	0.29 ± 0.05 (4)
Rat		
Control	0.40 ± 0.01 (4)	0.10 ± 0.01 (4)
+ L-alanine	0.38 ± 0.03 (4)	0.10 ± 0.02 (4)

Myocytes from hamster and rat hearts were incubated with 0.1 μM of labelled choline (2 μCi/ml) for 60 min. The radioactivities in the cell extracts and in phosphatidylcholine after the incubation were determined. The values represent the mean ± standard deviation (number of determinations).

[a] $p < 0.05$ when compared with control.

DISCUSSION

This treatise contains data which show the effect of various hormones and amino acids on phosphatidylcholine biosynthesis and/or choline uptake. Further experiments were done to investigate the mechanism by which these compounds elicited their effect. It appears that neutral amino acids have the ability to regulate choline uptake. The hormones examined in this study have the ability to regulate phosphatidylcholine biosynthesis at both the level of choline uptake and through modulation of enzymes in the CDP-choline pathway.

It is generally accepted that choline uptake is via the low-affinity Na+-independent transport system in non-neural cells (Jenden 1979). In the hamster heart, the uptake of choline follows Michaelis-Menten kinetics and appears to consist of a single uptake system (Zelinski *et al.* 1980). It has been shown earlier that neutral amino acids have the ability to enhance choline uptake in the hamster heart (Hatch *et al.* 1988). This study confirms the previous finding and suggests that the enhancement of choline uptake by amino acids may be limited to the hamster heart. The inability of neutral amino acids to modulate choline uptake in the guinea pig, rat and rabbit hearts implies that the regulation of the uptake mechanism is not the same between mammalian species. Interestingly, the isolated perfused hamster heart and myocardial cells obtained from the hamster also display a higher rate of choline uptake than hearts from the other animals. The reason for this higher rate of choline

uptake is not known. This observation concurs with the hypothesis that regulation of phospholipid metabolism between mammalian species is different.

Since choline uptake in guinea pig, rat and rabbit hearts was not affected by the presence of amino acids, the rate of phosphatidylcholine biosynthesis in these hearts should not be affected. This postulation was confirmed by the analysis of label in phosphatidylcholine and choline-containing metabolites. The ability of the hamster heart to maintain phosphatidylcholine biosynthesis during enhanced choline uptake was documented in previous studies (Hatch *et al.* 1988). The accumulation of labelled phosphocholine in the hamster heart clearly reflects the ability of the rate-limiting step of the CDP-choline pathway to regulate phosphatidylcholine biosynthesis (Hatch *et al.* 1989).

At present, the exact mechanism for the modulation of choline uptake by neutral amino acids in the hamster heart is not known. One hypothesis is that neutral amino acids facilitate the transport of choline across the endothelial wall of the blood vessels and causes an increase in choline concentration in the intercellular fluid. The higher concentration of choline in the intercellular fluid may then cause an increase in choline uptake by myocardial cells. However, the ability of neutral amino acids to enhance choline uptake in hamster myocytes eliminates this possibility and suggests that such an enhancement is a result of a direct modulation of the choline transport site by amino acids.

It is also clear from this study that arginine-vasopressin plays an important role in the regulation of phosphatidylcholine biosynthesis in isolated rat cardiac

myocytes. Two modes of regulation have been identified: (1) the modulation of choline uptake and (2) the regulation of the CTP: phosphocholine cytidylyltransferase activity.

The uptake of choline by the cell is regarded as a plausible mechanism for the control of phosphatidylcholine biosynthesis. The uptake of choline is inhibited by ethanolamine and other choline analogs (Diamond and Kennedy 1969) (Zelinski and Choy 1984). This is the first report that a hormone may also participate in the regulation of choline uptake.

The modulation of choline uptake by vasopressin appears to be quite specific since the action of vasopressin cannot be duplicated by similar amounts of oxytocin. Although no oxytocin receptors have been reported in cardiac cells, the results exclude the possibility of non-specific hormone effects. The biphasic effect of vasopressin on choline uptake implies that the hormone does not directly interact with choline at the transport site. The disproportionate changes between choline uptake and radioactivity incorporated into the phosphatidylcholine fraction suggest that the biosynthesis of the phospholipid is also modulated by vasopressin. It is interesting to note that vasopressin at lower concentrations (0.05-0.2 μM) regulates choline uptake and the synthesis of phosphatidylcholine in unequal proportions. Analyses of the choline-containing metabolites reveal that vasopressin regulates the CDP-choline pathway at the step catalyzed by cytidylyltransferase. Interestingly, the cytidylyltransferase activities in both the microsomal and cytosolic fractions were activated. This leads to the supposition that lower vasopressin concentrations (0.05-

0.2 μM) cause the activation of enzyme activity or an increase in enzyme synthesis in both cellular compartments.

In order to determine whether the hormone may have a direct interaction on the enzyme, the ability of the myocytes to take up vasopressin was determined. Since the nature of the internalized peptide and not the subcellular distribution was of concern, only the cytosol was investigated. Very small amounts (fmol/μg cellular protein) of vasopressin were internalized by the myocytes, and the vast majority of the internalized vasopressin was catabolized into amino acids and their corresponding metabolites. *In vitro* addition of cellular concentrations of vasopressin or its metabolites to the assay mixture did not cause any changes in the cytidylyltransferase activity. We conclude that the activation of the cytidylyltransferase was not caused by a direct interaction between the enzyme and the hormone or its metabolites.

Alternatively, vasopressin at higher concentrations (0.5-1.0 μM) caused the inhibition of total cytidylyltransferase activity. Since the microsomal form of the cytidylyltransferase has been shown to be the more active form of the enzyme, the translocation of the enzyme from one compartment to another is regarded as an important mechanism for the regulation of the cytidylyltransferase activity. The translocation of the enzyme between the cellular compartments is facilitated by phosphorylation-dephosphorylation reactions (Vance and Pelech 1984). The physiological responses to vasopressin are mediated via the V_1 receptor which is a cAMP-independent system coupled to phosphoinositide turnover (Walker *et al.* 1988).

Experiments conducted with a specific V_1 receptor antagonist to vasopressin,

[$d(CH_2)_5$, D-tyr$(OEt)^2$, val^4, cit^8] vasopressin, indicate that V_1 receptors initiate vasopressin response in cardiac myocytes. The vasopressin antagonist competes with vasopressin for binding to the V_1 receptor (Kruszynski *et al.* 1980) and the concentration used was consistent with other cardiac studies (Walker *et al.* 1988). Incubation of vasopressin with the hormone antagonist negated vasopressin effects.

The binding of vasopressin to V_1 receptors can initiate activation of protein kinases through the hydrolysis of phosphatidylinositol-4-5-bisphosphate. Activation of protein kinase C may enhance the phosphorylation and eventual translocation of the cytidylyltransferase from the more active (microsomal) to the less active (cytosolic) form. These biochemical events may offer an explanation to the observed decrease in the conversion of phosphocholine to CDP-choline at high vasopressin concentrations using whole homogenate as the enzyme source. This would also concur with the observed shift in cytidylyltransferase activity from the microsome to the cytosol when exposed to high vasopressin concentrations. In the presence of 1.0 μM vasopressin, a higher specific activity for cytidylyltransferase is observed in the cytosol compared with the microsomal fraction.

Vasopressin mediates its effect through binding to a membrane-associated receptor. G-proteins have been demonstrated to be coupled to these receptors (Casey and Gilman 1988). Inhibition of the G-protein responsible signal transduction would negate the observed vasopressin effects. The observed attenuation of hormonal effects by pertussis toxin establishs a link between vasopressin and a G-protein mediated response. We postulate that the one hour pre-incubation of myocardial

cells with pertussis toxin causes ADP-ribosylation of a G-protein α-subunit responsible for the transmission of intracellular messengers. The most likely second messengers produced in the cardiac cells are diacylglycerol and inositol-triphosphate since V_1 receptors are found in these cells. These messengers could then proceed to elicit the observed responses. Elucidation of the type of G-protein responsible for signal transduction will clarify the mechanism of vasopressin action.

One intriguing aspect of this study is the biphasic action of vasopressin on both choline uptake and phosphatidylcholine biosynthesis. Beyond its ability to activate protein kinase C at high concentrations, vasopressin at lower concentrations may produce a yet unidentified cellular response which would activate the cytidylyltransferase. The ability of a hormone to produce dual second messengers via a single receptor has recently been discussed (Thompson 1992). For example, α-2 adrenergic receptors have the ability to mediate both the stimulation of phospholipase C and inhibition of adenylate cyclase (Cotecchia *et al.* 1990). A calcitonin receptor has been reported to activate both second messenger pathways with different ligand concentrations eliciting the two responses (Chabre *et al.* 1992). The classes of receptors and G-proteins involved in a single receptor/dual pathway have not been determined, although the seven transmembrane-spanning receptor class has been suggested (Chabre *et al.* 1992). Thus, the cardiac V_1 vasopressin receptor may couple to two different G-proteins which may be activated at specific ligand concentrations.

In isolated rat hepatocytes, vasopressin displays no effect on choline uptake

but inhibits the incorporation of labelled choline into phosphatidylcholine (Tijburg *et al.* 1987). Similar to the present study, the mechanism of inhibition appears to be at the level of cytidylyltransferase. Hence, the biphasic effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis observed in this study illustrates the diverse effect of the hormone on different tissues.

The effect of 17ß-estradiol on phosphatidylcholine biosynthesis is also intriguing. The observed decrease of radioactivity incorporated into phosphatidylcholine was greater than the apparent inhibition of choline uptake. This suggests that the estrogen exerts an inhibitory effect on the CDP-choline pathway. The mechanism of inhibition would probably be different from the proposed mechanism of vasopressin. Estrogen is a steroid hormone which has been documented to bind to intracellular but not membrane-associated receptors. The level at which estrogen regulates phosphatidylcholine biosynthesis requires further investigation. The mechanism by which estrogen affects the choline transport site has not been determined.

The study on the effect of amino acids on choline uptake and phosphatidylcholine biosynthesis indicate that the control of cardiac phospholipid metabolism is different between mammalian species. Therefore, the effect of hormones found in this study may be limited to rat cardiac tissue and should not be extrapolated to other tissues or species. We were not able to extend this study to other animal species since protocols for the isolation of myocardial cells in hearts other than rat and hamster are not available.

The physiological relevance of this study is not defined. Others have shown that vasopressin causes coronary effects in the mammalian heart, at circulating concentrations (Walker *et al.* 1988). This indicates that the mammalian heart can be subject to the effects of vasopressin at physiological levels. However, experiments in this research used hormone concentrations above that found in mammalian systems, and therefore are of pharmacological importance.

It can now be established that various hormones have regulatory significance on cardiac phospholipid metabolism. Further studies with other hormones which have myocardial membrane-receptors should be performed. This will clarify the specificity of the observed vasopressin effects on choline uptake and phosphatidylcholine biosynthesis. A difference in phospholipid metabolism between mammalian species is also demonstrated. From the perspective of methodology, this study has confirmed the viability of two procedures in investigating the heart. Both the isolation of myocytes and heart perfusion are reliable methods for studying phospholipid metabolism in the heart.

In this study, a connection between the effect of vasopressin and a pertussis toxin-sensitive G-protein has been established. This G-protein is coupled to a membrane-associated V_1-receptor, but second-messengers involved in the mechanism are not defined. Although modulation of the cytidylyltransferase enzyme occurs upon hormone interaction with the myocardial cells, the mechanism of this enzyme regulation remains unclear. Identification of the G-protein and the second-messengers involved in this process should clarify the action of vasopressin on cardiac

myocytes.

Aspects of hormonal regulation on phosphatidylcholine catabolism should also be considered. Catabolic enzymes involved in phosphatidylcholine degradation are found in both cytosolic and membrane compartments. Other investigations have indicated that hormones are involved in the regulation of these enzymes. Vasopressin has been documented to induce phosphatidylcholine turnover in A-10 cells, a smooth muscle cell line, via the activation of phospholipase C (Grillone *et al.* 1988). It has also been suggested that phorbol esters stimulate the degradation of choline-containing phosphoglycerides (Daniel *et al.* 1986). Hormonal effects on phospholipid catabolism in cardiac systems are currently under investigation.

Data concerning hormonal regulation of the metabolism of other phospholipids is also limited. In rat hepatocytes, glucagon has been shown to stimulate the incorporation of ethanolamine into phosphatidylethanolamine (Geelen *et al.* 1979). This stimulation of phosphatidylethanolamine synthesis was also observed in response to norepinephrine (Haagsman *et al.* 1984). Studies on the regulation of cardiac phosphatidylethanolamine metabolism by hormones have not been documented. Obviously, this study has evoked more questions than answers, an inevitable fate of any scientific research.

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