Regulation of Polyglycerophospholipid Biosynthesis

A Thesis

Presented to the Faculty of Graduate Studies, University of Manitoba,

in partial fulfillment of the

requirements for the degree of

Master of Science

in the

Department of Biochemistry

by

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c. October 10, 1997. T. K. Ross

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REGULATION OF POLYGLYCEROPHOSPHOLIPID BIOSYNTHESIS

BY

TIMOTHY KIERAN ROSS

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

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ABBREVIATIONS

μCi: Microcurie

μg: Microgram

μL: Microlitre

μM: Micromolar

μmol: Micromole

ADP: Adenosine-5'-diphosphate

ATP: Adenosine-5'-triphosphate

Ba^{2+}: Barium

BMP: Bis(monoacylglycerol)phosphate

BSA: Bovine serum albumin

Ca^{2+}: Calcium

$CaCl_2$: Calcium chloride

CAD: Cationic amphiphilic drug

CDP-DG: Cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol

CDP: Cytidine-5'-diphosphate

$CHCl_3$: Chloroform

Ci: Curie

CL: Cardiolipin

cm: Centimetre

CMP: Cytidine-5'-monophosphate

Co^{2+}: Cobalt

CO_2: Carbon dioxide

CoA: Coenzyme A

CQ: Chloroquine

CSA: Cyclosporin A

CTP: Cytidine-5'-triphosphate

CTP:PA: Cytidine-5'-triphosphate:Phosphatidic acid

DDW: Double Distilled Water

DG: 1,2-diacyl-*sn*-glycerol

DMEM: Dulbecco's modified essential medium

dpm: Disintegrations per minute

EI_2: Enzyme-inhibitor

EKG: Echocardiograph

g/L: grams/litre

GTP: Guanosine-5'-triphosphate

H: Hydrogen

HCl: Hydrochloric acid

KCl: Potassium chloride

Kg: Kilogram

KH_2PO: Potassium phosphate

KHB: Krebs Henseleit Buffer

L: Litre

LPC: Lysophosphatidylcholine

LPE: Lysophosphatidylethanolamine

LPG: Lysophosphatidylglycerol

M: Molar

MeOH: Methanol

mg: Milligram

Mg^{2+}: Magnesium

$MgCl_2$: Magnesium chloride

$MgSO_4$: Magnesium sulfate

min.: Minute

mL: Millilitre

mM: Millimolar

NBCS.: New born calf serum

N_2: Nitrogen

NaCl: Sodium chloride

NAD: Nicotinamide adenine dinucleotide

$NaHCO_3$: Sodium bicarbonate

NF-ATc: Cytosolic Subunit of the Nuclear Factor of Activated Transcription

NH_4Cl: Ammonium chloride

NH_4OH: Ammonium hydroxide

nm: Nanometre

nmol: Nanomole

O_2: Oxygen

PA: Phosphatidic Acid

PAP: Phosphatidate Phosphohydrolase

PC: Phosphatidylcholine

PE: Phosphatidylethanolamine

PG: Phosphatidylglycerol

PGP: Phosphatidylglycerol phosphate

Pi: Inorganic phosphate

PI: Phosphatidylinositol

PLA_2: Phospholipase A_2

pmol: Picomole

PS: Phosphatidylserine

rpm: Revolutions per minute

sec: Second

SM: Sphingomyelin

t.l.c.: thin layer chromatography

TG: 1,2,3-triacyl-*sn*-glycerol

vol.: Volume

ABSTRACT

Polyglycerophospholipids are an important class of phospholipid which include cardiolipin, phosphatidylglycerol, and bis(monoacylglycero)phosphate. Polyglycerophospholipids are characterized as phospholipids comprised of 2 or more glycerol molecules joined via phosphodiester linkage with 2, 3 or 4 fatty acids esterified to glycerol moieties. Cardiolipin, an important polyglycerophospholipid comprises approximately 14% of the heart phospholipid mass. Cardiolipin was first discovered in 1942 by Mary Pangborn in the bovine heart and has been isolated to the mitochondrion where it comprises approximately 25% of the phospholipid mass. Cardiolipin is produced via the CDP-diacylglycerol pathway on the inner mitochondrial membrane. The mitochondrion requires cardiolipin for energy metabolism in that cardiolipin regulates enzymes such as cytochrome c oxidase, creatine kinase, among other enzymes of the electron transport chain.

Cardiac ischaemia is a condition in which there exists an imbalance between the myocardial oxygen demand and coronary arterial supply. Biochemical complications arising from cardiac ischaemia include ATP depletion, a net loss of adenine nucleotides, as well as an increase in $[Ca^{2+}]$, $[P_i]$, and $[H^+]$. Ironically, cells may endure a non-lethal period of ischaemia, only to succumb to reperfusion injury. Reperfusion injury is a consequence of cell reoxygenation whereby the proliferation of oxygen free radicals (and resultant oxidative stress), increased $[Ca^{2+}]$, continued ATP deprivation, and higher pH all contribute to the reversible formation of a non-selective permeability transition pore in the inner mitochondrial membrane. The opening of this pore results in the further decoupling of the cell's oxidative phosphorylation mechanisms, resulting in a vicious cycle

of pore formation and decreased cellular metabolic efficiency. Cyclosporin A (CSA), an immunosuppressant, has been found to reverse pore formation in the post-ischaemic heart. Our goal was to study the effects of the opening and closing of these pores on cardiolipin biosynthesis.

Hearts perfused in the Langendorff mode with Krebs Henseleit buffer with or without 0.2 μM CSA, were subjected to 30 minutes of ischaemia-reperfusion injury, and then reperfused with or without 0.2 μM CSA in the presence of [1,(3)-^{3}H]glycerol for a further 30 minutes. Radioactivity incorporated into cardiolipin did not change significantly in injured hearts or those protected by CSA relative to controls. In addition the pool size of cardiolipin was unaltered.

Chloroquine is an important anti-malarial agent. It is a lysomotropic weak base and a member of a group of drugs known as cationic amphiphillic drugs (CADs). Chloroquine is also known to cause phospholipid storage disease by inhibiting phospholipid degradative enzymes. We examined the effect chloroquine had on the *de novo* synthesis of cardiolipin. We incubated rat liver hepatocytes in the absence or presence of 0.1 mM chloroquine with [1,(3)-^{3}H]glycerol for up to 2 hours. We found a dramatic increase in the production of cardiolipin and its precursors in the cells incubated in the presence of chloroquine relative to controls. Furthermore, we noticed a dramatic decrease in the production of neutral phospholipids phosphatidylcholine and phosphatidylethanolamine. Finally, there was an increase in radioactive linoleic acid incorporated into cardiolipin in hepatocytes incubated with chloroquine though there was little change in linoleic uptake or incorporation into phospholipids.

We also examined the effect that chloroquine had on phospholipid production in H9c2 cardiac myoblasts. We incubated H9c2 cells for 2 and 18 hours in the presence or

absence of 0.1 mM chloroquine, and then incubated the cells for another 2 hours in the continued presence or absence of chloroquine with [1,(3)-^{3}H]glycerol. We found that chloroquine inhibited the transport of glycerol into the cells, exhibiting mixed-type inhibition. This resulted in a decrease in *de novo* cardiolipin formation. Linoleic acid uptake was not inhibited, however incorporation into cardiolipin and phosphatidylcholine was decreased in H9c2 cells incubated with chloroquine.

INTRODUCTION

I. The Biological Membrane

The biological membrane is a fluid, dynamic aggregation of proteins and lipids required of all living organisms. In both prokaryotes and eukaryotes, it serves to define the cell, encapsulating them with a physical boundary. The plasma membrane maintains essential differences between the cytosol and the extracellular environment. In eukaryotes, biological membranes compartmentalize organelles such as the nucleus, mitochondrion and microsomes, maintaining their integrity. The biological membrane sustains ion gradients required for the production of ATP, as well as the transmembrane movement of selected solutes and the production and transmission of electrical signals. The biological membrane provides an impermeable barrier to most water soluble molecules, regulating the flow of metabolites into and out of the cell by use of pumps and gates (Voet and Voet, 1990). The importance of this regulation is evident in the mitochondria where an electro-chemical proton gradient is required for the performance of chemiosmotic processes used to drive synthesis of ATP. Furthermore, the membrane provides a scaffolding for the enzymes of this process (Fig. 1).

II. Introduction to Lipids

1. Lipids

Lipids (Greek: *lipos*, fat) are the major biological molecules comprising the membrane. They are soluble in organic solvents such as ether or chloroform, but insoluble in water. The term lipids refers to a wide variety of compounds including fats, oils, most non-protein membrane components, and some vitamins and hormones, with phospholipids constituting the most abundant form of lipid. A bilayer of these lipids

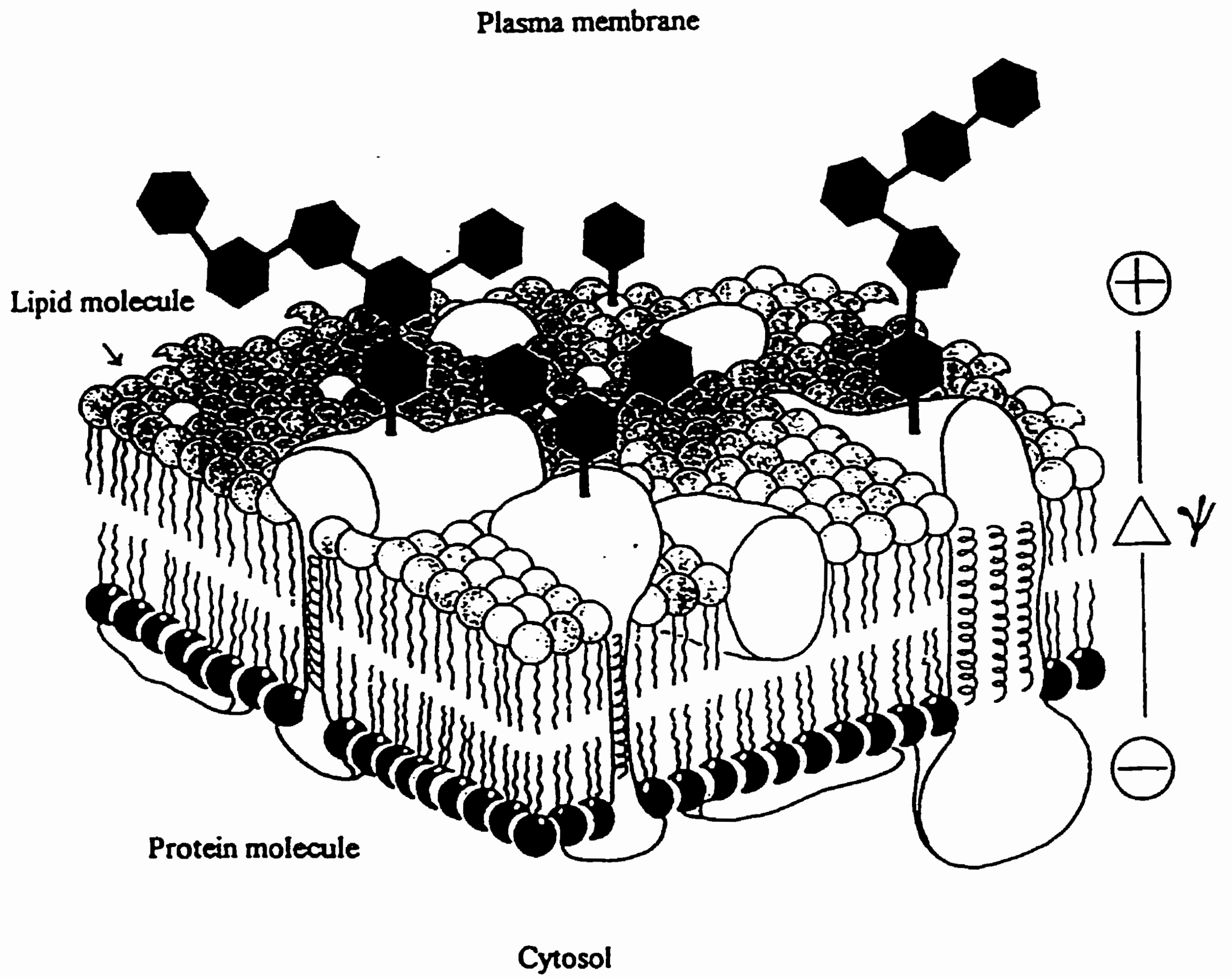


Figure 1: Fluid Mosaic Model of the Eukaryotic Plasma Membrane

provides the structure enveloping the cell, and its organelles. These lipids also help regulate membrane proteins required by the cell including receptors, enzymes and transport proteins.

2. Phospholipids

Phospholipids are lipids containing a phosphate group. They are described as being amphipathic (dual sympathy) molecules, consisting of a hydrophilic, or water-loving, phosphoryl-X "head", and a hydrophobic, or water hating, aliphatic "tail". These properties cause phospholipids to aggregate into micelles and bilayers when immersed in water. These energy favorable structures allow contact between the heads of the phospholipids and water and prevent unfavorable interactions between the hydrophobic tails and the aqueous medium. Although the term phospholipid refers to a wide range of molecules, there are 3 main types: sphingomyelin, plasmalogens, and glycerophospholipids.

3. Sphingomyelin

Sphingomyelin is a major phospholipid in mammalian cells, comprising the myelin sheaths surrounding nerve cells. Sphingomyelin is the only phospholipid containing ceramide as its hydrophobic tail, however, it has a phosphocholine as the head group and has a similar charge distribution and conformation to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Fig. 2) (Voet and Voet, 1990). Sphingomyelin is produced by the joining of PC and ceramide in the following reaction:

PC + CERAMIDE → SPHINGOMYELIN + DIACYLGLYCEROL (Vance, 1991).

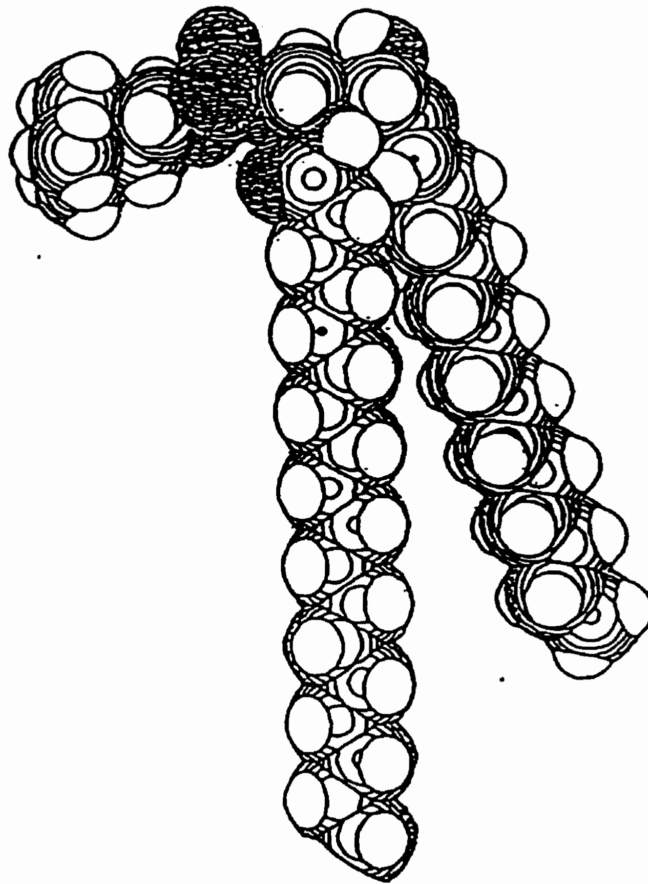


Figure 2: The Structure of Sphingomyelin

4. Plasmalogens

Plasmalogens are phospholipids consisting of a polar head group such as choline, ethanolamine, or serine attached via phosphodiester linkage to a glycerol moiety. The hydrophobic region consists of 2 acyl chains, one joined via a β-unsubstituted ether linkage at the C1 position, the other esterified at the C2 position. Plasmalogens are found predominantly in human nervous tissue (Voet and Voet, 1990).

III. Introduction to Phosphoglycerides

Phosphoglycerides comprise the third and major phospholipid. They are comprised of one or two fatty acid chains esterified at the *sn*1 and *sn*2 positions to a glycerol moiety. Phosphoglycerides are classified by the polar head group attached via phosphodiester linkage to the *sn*3 position of the glycerol. This head group may include choline or ethanolamine, or in the case of cardiolipin, another phospholipid, phosphatidylglycerol (Fig. 3). Gregor Cevc has shown that the head groups of phospholipids contribute to surface polarity and interfacial hydration and are the most important factors determining the characteristics of membranes (1987). This means that the head groups of phospholipids affect the thermodynamic and functional nature of the lipid bilayer membranes. The fatty acid chain composition changes as body mass changes and affects permeability of lipid vesicles and tumor cells. For example, n-3 polyunsaturated fatty acids are most likely to increase bilayer permeability (Porter *et al.* 1996). Degree of unsaturation tends to vary among different phospholipids as well, and the number of double bonds in phospholipids directly influences membrane permeability. The majority of phosphoglycerides have a saturated fatty acid chain at the C1 position, while typically the acyl chains at the C2 position have one or more *cis*-double bonds. The degree of unsaturation of the fatty acid chains can affect the degree of fluidity a

$$R_1-C(=O)-O-{}^1CH_2-{}^2CH(O-C(=O)-R_2)-{}^3CH_2-O-P(=O)(O^{\ominus})-O-X$$

X = rest of polar head

Precursor of X (HO — X)	Formula of X	Name of resulting glycerophospholipid family
Water	$-H$	Phosphatidate
Choline	$-CH_2CH_2\overset{\oplus}{N}(CH_3)_3$	Phosphatidylcholine (Lecithin)
Ethanolamine	$-CH_2CH_2\overset{\oplus}{N}H_3$	Phosphatidylethanolamine (Cephalin)
Serine	$-CH_2-CH(\overset{\oplus}{N}H_3)COO^{\ominus}$	Phosphatidylserine
Glycerol	$-CH_2CH(OH)-CH_2OH$	Phosphatidylglycerol
Phosphatidyl-glycerol	$-CH_2CH(OH)-CH_2-O-P(=O)(O^{\ominus})-O-CH_2-CH(OC(=O)R_4)-CH_2OC(=O)R_3$	Diphosphatidylglycerol (Cardiolipin)
myo-Inositol	cyclohexane ring, C1 = bond site; C1–C6 each bearing H and OH	Phosphatidylinositol

Figure 3: Phospholipid Structures and Head Groups

membrane might have, as well as other properties such as melting point.

Biosynthesis of PC, PE, PS and TG requires dephosphorylation of PA by a phosphatidic acid phosphohydrolase (PAP) which produces DG. The acylation of DG produces TG. In the production of PC and PE, a choline or ethanolamine condenses with CTP producing CDP-choline or CDP-ethanolamine with the elimination of CMP. CDP-choline or CDP-ethanolamine then attacks the DG at the *sn*3 position, producing PC or PE. PS may be created from PE or PC via a base exchange reaction (Fig. 4). The second path PA may take results in the formation of polyglycerophospholipids and PI via the CDP-DG pathway.

IV. Introduction to Polyglycerophospholipids

Polyglycerophospholipids are a class of phospholipid that have 2 or 3 glycerol molecules joined together via phosphodiester linkage. Two, three or four fatty acid chains of varying composition and species are also present, joined via ester linkage to the glycerol moieties. The 3 principal polyglycerophospholipids include diphosphatidylglycerol, hereafter referred to by its common name, cardiolipin (CL), phosphatidylglycerol (PG), and bis(monoacylglycerol)phosphate (BMP) (Fig. 5).

1. Bis(monoacylglycerol)phosphate

BMP, also known as lysobisphosphatidic acid is found mostly in alveolar macrophages where it comprises 14-18% of the lipid phosphorus (Huterer and Wherrett, 1979). BMP may serve to stabilize lysosomal phospholipid bilayer (Weglicki *et al.*

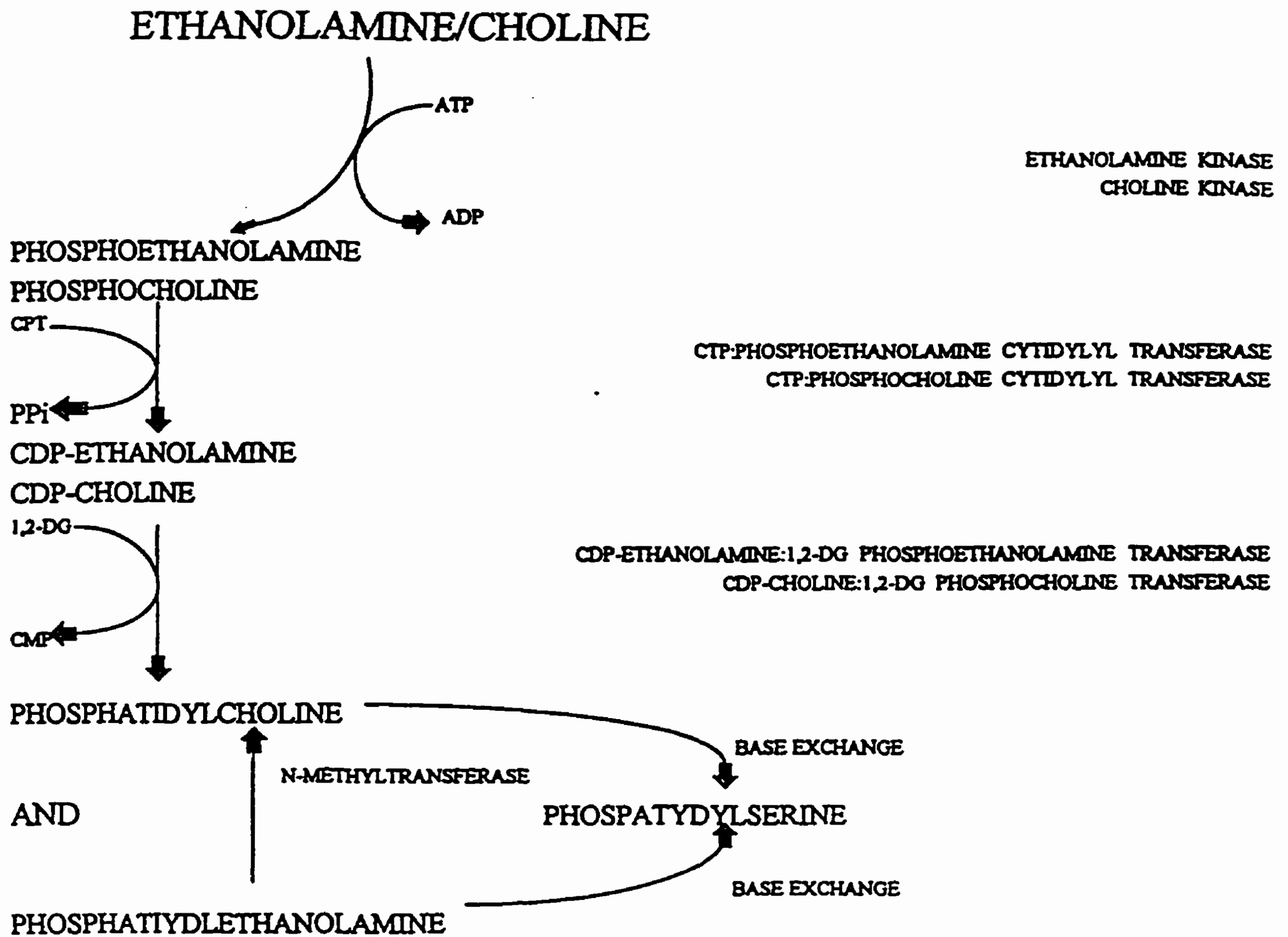
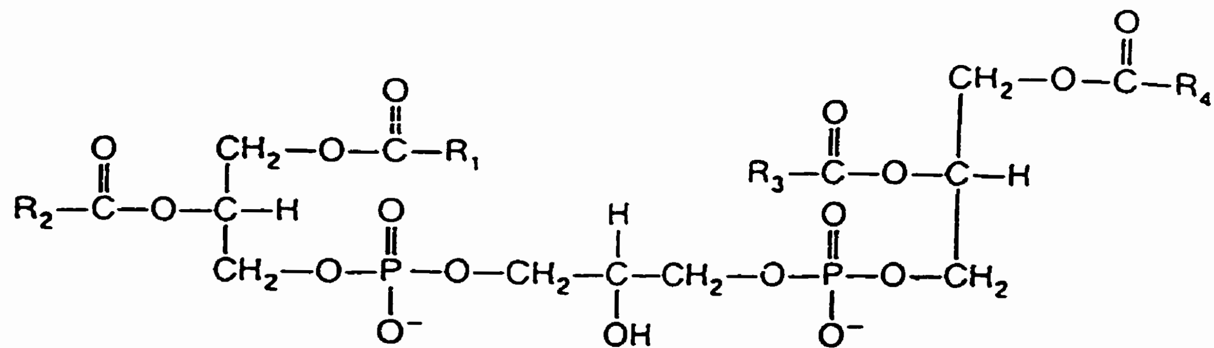
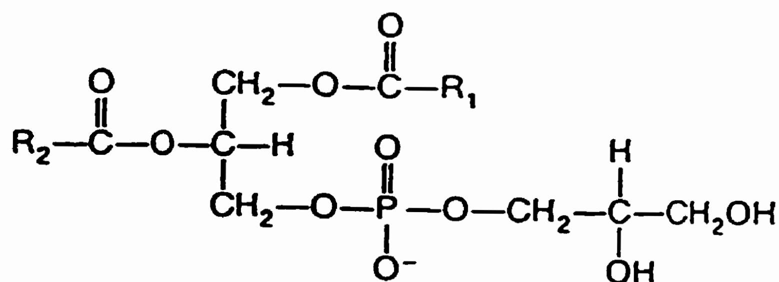


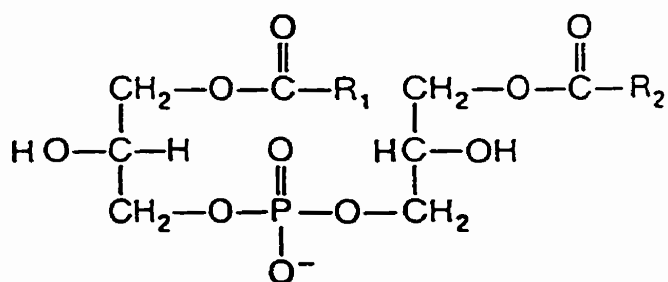
Figure 4: Biosynthetic Pathways of Phosphatidylchloline, Phosphatidylethanolamine, and Phosphatidylserine



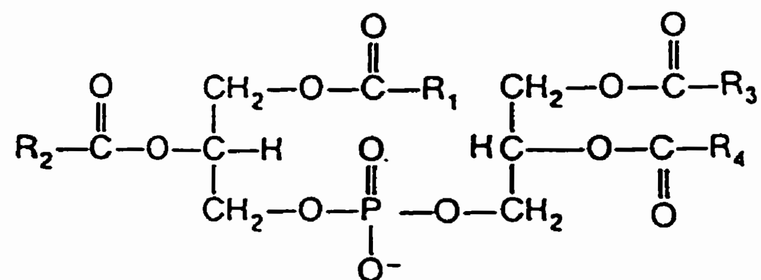
Cardiolipin



Phosphatidylglycerol



Bis(monoacylglycero)phosphate



Bisphosphatidic acid

Figure 5: Structural Formula of Four Polyglycerophospholipids

1973). BMP is located principally in lysosomes (Wherrett and Huterer 1972) where it is formed and makes up 12.8% of the phospholipid mass (Poorthuis and Hostetler, 1976). Production requires the uptake of PG into the lysosomes, where it is degraded to LPG. LPG then undergoes several different reactions to form BMP. BMP is not typically observed in the mammalian heart (Simon and Rouser, 1969, and Poorthuis *et al.* 1976).

2. Phosphatidylglycerol

PG was first discovered in *Scendesmus* sp. by Maruo and Benson (1957), and its structure was elucidated soon after (Benson and Maruo, 1958). PG was identified, isolated and characterized in rat liver mitochondria by Gray in 1964. PG comprised about 1.5% of the phospholipid phosphorus of the rat heart, 0.2% of the bovine heart, and 0.6% of mouse and human heart (Simon and Rouser, 1969 and Poorthuis *et al.*, 1976). PG comprised 0.6% of the liver, 1.5% of the kidney and 1.1% of the spleen phospholipids in the rat. The lung contained the most PG (4.1%) because PG is a component of lung surfactant (Poorthuis *et al.* 1976). PG comprised 11% and 4% of the phospholipid of the mitochondria of bovine and sheep heart, respectively. Rat liver mitochondria, lysosomes and microsomes were shown to consist of 1%, 2%, and 5% PG, respectively. Rat kidney mitochondria contained 3% PG, but PG was not detected in the microsomes (Strickland and Benson, 1960). Gray proposed that PG was likely a precursor to CL due to the similarity in their structures (1964). This was later confirmed by Hostetler *et al.* in 1971.

3. Cardiolipin

was elucidated (MacFarlane and Gray, 1957 and Gray and MacFarlane, 1958). CL was

shown to have 2 pKa's, the second above physiological pH due to the free hydroxyl of the connecting glycerol (Kates *et al.* 1993). CL has been identified in eukaryotic and prokaryotic cells (Hostetler, 1982) and comprised 15% of the total phospholipid phosphorus in the rat heart (Simon and Rouser, 1969, Poorthuis *et al.* 1976 and Hatch, 1994), 9% in the human heart and 12% in bovine heart (Simon and Rouser, 1969). In the pig heart, CL comprised 18.1% of the mitochondrial phospholipid phosphorus, including 25.4% of the total phospholipid of the inner mitochondrial membrane (Comte *et al.* 1976). Studies that involved PLA_2 and immunoreaction techniques using CL antibodies in the beef heart showed CL to comprise 20% of the phospholipid mass including 25% of the inner mitochondrial membrane (Krebs *et al.* 1979). Furthermore, 6% of the mitoplasts, or "right-side-out" oriented inner mitochondrial preparations, and 18% of submitochondrial particles, or "inside-out" oriented membranes, were shown to be comprised of CL (Krebs *et al.* 1979). Beef heart, liver and kidney contain 20.6%, 17% and 19% of their total phospholipid phosphorus as CL, respectively (Fleischer *et al.* 1967), whereas rat liver and kidney contained 5.7% and 7% of their phospholipid phosphorus as CL, respectively (Poorthuis *et al.* 1976).

a) Biosynthetic Pathway

The *de novo* biosynthetic pathway of PG in eukaryotes follows the CDP-DG pathway proposed by Kiyasu *et al.* (1963). The determination of this pathway was accelerated when it was realized that L-glycerol-3-phosphate was the active intermediate rather than free glycerol in the production of PG. Initially, CTP:PA cytidylyltransferase catalyses the formation of CDP-DG from substrates PA and CTP (Kiyasu *et al.* 1963). Glycerol-3-phosphate and CDP-DG then condense to form PGP via the enzyme PGP synthase. This is followed by the rapid removal of phosphate by PGP phosphatase,

producing PG. PG and CDP-DG condense in a reaction catalyzed by CL synthase to form CL (Hostetler *et al,* 1971) (Fig. 6). Studies concur that production of CL as well as PG requires only the mitochondrial fraction, and that CL may not be produced anywhere else in the cell (Jelsema and Moore, 1978, Schlame and Halder, 1993, and Hostetler and van den Bosch, 1972). PG synthesis, although found primarily in the inner mitochondrial membrane (86% of PG was produced here), may also be synthesized in the microsomes (Hostetler and van den Bosch, 1972, Davidson and Stanacev, 1971 and Hauser and Eichberg, 1975).

b) CTP:PA Cytidylyltransferase

In mammalian tissues, CDP-DG was first isolated in the pineal gland of the rat (Hauser and Eichberg, 1975), and then isolated and purified from rat liver (Thompson and MacDonald, 1975). The amount recovered from the liver was 5.8 - 17.4 mol/Kg of liver (as compared with 780 mol PA/Kg of liver where PA comprised only 0.7% of the total lipid phosphorus) (Thompson and MacDonald, 1975). The enzyme that catalyses the formation of CDP-DG, CTP:PA cytidylyltransferase, has been solubilized from bovine brain (Lin *et al.* 1991). The activity of CTP:PA cytidylyltransferase in rat liver mitochondrial and microsomal fractions had different properties (Mok *et al.* 1992). For example, treatment with GTP increased the affinity of the microsomal isoform for PA,

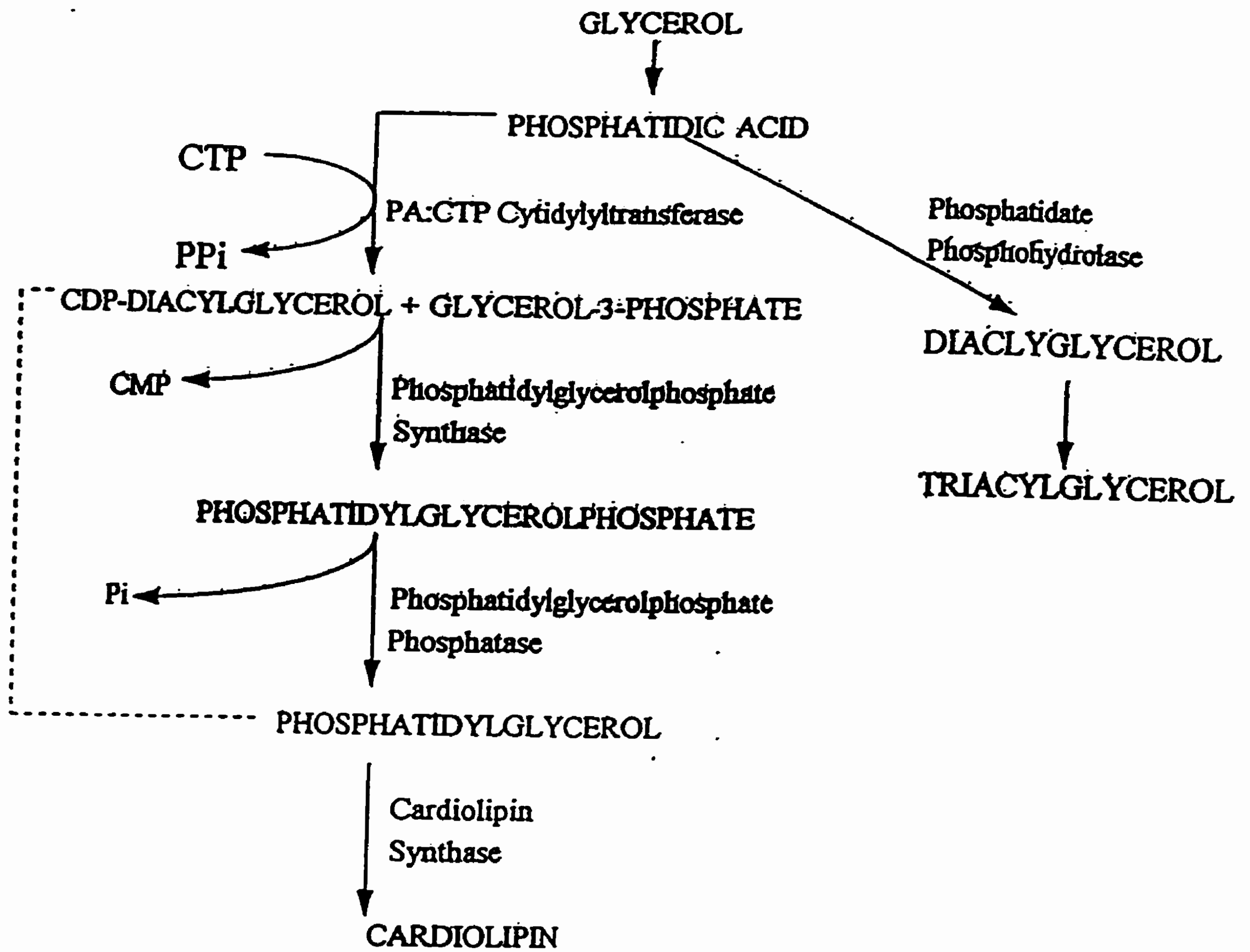


Figure 6: Biosynthetic Pathways of Cardiolipin, Phosphatidylglycerol, Diacylglycerol and Triacylglycerol

whereas GTP treatment inhibited the activity of the mitochondrial CTP:PA cytidylyltransferase. Since the microsomal and mitochondrial enzymes appeared to be distinct isoforms, they may have access to different pools of CTP and PA *in vivo*. Therefore it is possible that the mitochondrial isoform of the enzyme may be regulated differently from the microsomal enzyme. Maximum enzyme activity occurred primarily in the microsomes, and to a lesser extent, the inner mitochondrial membrane. The activity of Triton X-100 solubilized bovine heart mitochondrial CTP:PA cytidylyltransferase has been recently characterized in our laboratory. The enzyme had a pH optimum of 8.5, was activated by PC, SM and LPE, inhibited by LPC and was found to be heat and acid labile (unpublished observations, Taylor, W., Hatch, G. M.). In the rat heart, CDP-DG production occurred in both microsomal and mitochondrial membranes (Hatch, 1994). The production of CDP-DG is the rate-limiting step of CL biosynthesis in the heart.

c) Phosphatidylglycerol Synthesis

PGP synthase and PGP phosphatase have both been partially purified from rat liver (McMurray and Jarvis, 1978, MacDonald and McMurray, 1980). PGP synthase, the enzyme responsible for catalyzing the synthesis of PGP from CDP-DG and glycerol-3-phosphate, is an integral component of the mitochondrial inner membrane. The free enzyme was extracted from rat and pig liver mitochondria with Triton X-100 or Nonidet p-40 and was activated by Mg^{2+}, Ba^{2+}, Ca^{2+} as well as by PE (McMurray and Jarvis, 1978). In the rat heart, PGP synthase was heat labile with K_m values for CDP-DG and glycerol-3-phosphate of 46 μM and 20 μM respectively (Cao and Hatch, 1994). The heat sensitivity of PGP synthase was demonstrated in an experiment where a 5 min preincubation at 55°C reduced activity by 70%. PGP phosphatase isolated from rat liver

mitochondria was also an integral mitochondrial membrane protein with a pH optimum of 6.3 (MacDonald and McMurray, 1980). PGP phosphatase had catalytic activity at temperatures as high as 72°C although its maximum reaction rate was at 50°C (MacDonald and McMurray, 1980). However, in heart mitochondria, a time dependent inactivation of PGP phosphatase activity occurred at 55°C (Cao and Hatch 1994). Inhibition of enzyme activity was attenuated by the addition of 0.5 mM oleate.

d) Cardiolipin Synthesis

CL synthase catalyses the condensation of CDP-DG and PG in eukaryotic cells (Hostetler *et al.* 1971, Schlame *et al.* 1993). It is membrane associated and is found exclusively in the mitochondria, and more specifically in the inner mitochondrial membrane (Hostetler *et al.* 1971, Schlame *et al.* 1993) where the hydrophilic domains are exposed to the matrix side, interacting with effectors in the matrix compartment (Schlame and Halder, 1993). This way, newly synthesized CL and CDP-DG, which are both manufactured in the inner leaflet of the inner mitochondrial membrane, are accessible to newly imported proteins in the matrix (Schlame and Halder, 1993). CL synthase has been purified from rat liver and its properties examined extensively by Schlame and Hostetler (1991). Their studies indicated a strong anionic monomeric 50 kDa protein with K_m values of 45 μM for PG and 1.6 μM for CDP-DG. CL synthase had a requirement for divalent ions, particularly cobalt, an isoelectric point at pH 4-5, and a pH optimum of 8-9. Phospholipids, especially PE, were necessary for reconstitution of CL synthase activity. In yeast, CL synthase was stable (retaining 75% activity) up to 55°C, after which it lost activity. Direct association of Mg^{2+} or Co^{2+} with the enzyme formed an active complex which had a high affinity for CDP-DG and a lower affinity for PG (Schlame *et al.* 1995).

e) Functions of Cardiolipin

Goormaghtigh *et al.* (1980) reported that adriamycin complexed with negatively charged phospholipids including CL. This relationship has been exploited in exploring intra-membrane properties of CL. The use of adriamycin has helped determine that roughly 56% of mitochondrial CL is located on the cytoplasmic face of the inner mitochondrial membrane and 44% has been found on the matrix side of the inner mitochondrial membrane in liver and heart (Cheneval *et al.* 1985). Adriamycin has been shown to affect many sites of the electron transport chain (Gosalvez *et al.* 1974). It was therefore deemed likely that CL plays a role in regulating the enzymes located here (Goormaghtigh *et al.* 1980).

Use of adriamycin as a probe has helped identify other enzymes regulated by CL, such as cytochrome c oxidase (Goormaghtigh *et al.* 1982, Robinson, 1993 and Goormaghtigh and Ruysschaert, 1984). The importance of CL with respect to cytochrome c oxidase has been assessed in experiments where the removal of CL resulted in the loss of activity. The activity was regained upon replacement of CL. It has been demonstrated that cytochrome c oxidase requires two to three CL molecules per molecule of cytochrome c oxidase to function (Robinson *et al.* 1990, Vik *et al.* 1981). Studies using adriamycin on membranes have also demonstrated the importance of the presence of CL. CL was required for creatine kinase binding to the mitochondrial membrane (Muller *et al.* 1985 and Cheneval and Carafoli, 1988), and maintaining the active conformation of L-glycerol-3-phosphate dehydrogenase (Beleznai and Jancsik, 1989). CL has been shown to activate the rat liver multicatalytic proteinase complex (or proteasome) (Ruiz de Mena *et al.* 1993). The phosphate-transport system also required CL (Mende *et al.* 1982 and Kadenback *et al.* 1982). Triton X-100 abolished the activity

of the phosphate transport system, but the activity was restored when CL was added (Mende *et al.* 1983). Other enzymes associated with CL include the tricarboxylate carrier (Stipani *et al.* 1980), whereby thyroid hormone treatment may affect the carrier by elevating CL levels (Paradies and Ruggiero, 1990). This may also occur with pyruvate translocase since CL and pyruvate translocase activity levels dropped in hypothyroid rats (Paradies and Ruggiero, 1989) and reconstituted pyruvate carrier requires CL (Nalecz *et al.* 1986). Carnitine palmitoyl transferase binds CL tightly (Fiol and Bieber, 1984). CL was shown to be essential for oxidative phosphorylation in a Chinese hamster ovary cell line that possessed a thermolabile PGP synthase (Ohtsuka *et al.* 1993). When shifted to the restrictive temperature, the cells were deficient in CL and PG, and cell growth was attenuated. This study demonstrated a reliance of rotenone-sensitive NADH ubiquinone reductase (Complex I) upon CL.

4. Remodeling of Cardiolipin

As with most phospholipids, CL is in a constant state of degradation and resynthesis. This is particularly evident with regard to the acyl moieties of CL which exhibit a faster turnover rate than the other molecular components of CL (Landriscina *et al.* 1976). In the remodeling of CL fatty acyl chains, two steps are required. First a deacylation occurs by the actions of a phospholipase A_1 (PLA_1) or PLA_2. Van den Bosch *et al.* (1965) determined that PLA_1 is a heat labile enzyme that cleaves the *sn*1 position of the phospholipid glycerol moiety, and PLA_2 cleaves the *sn*2 position. Cleavage by either of these enzymes results in a lysophospholipid whereas cleavage by other phospholipases results in DG or PA (Fig. 7). William Lands discovered that the fatty acid portion of PC is subject to turnover without the synthesis and breakdown of the rest of PC (1960).

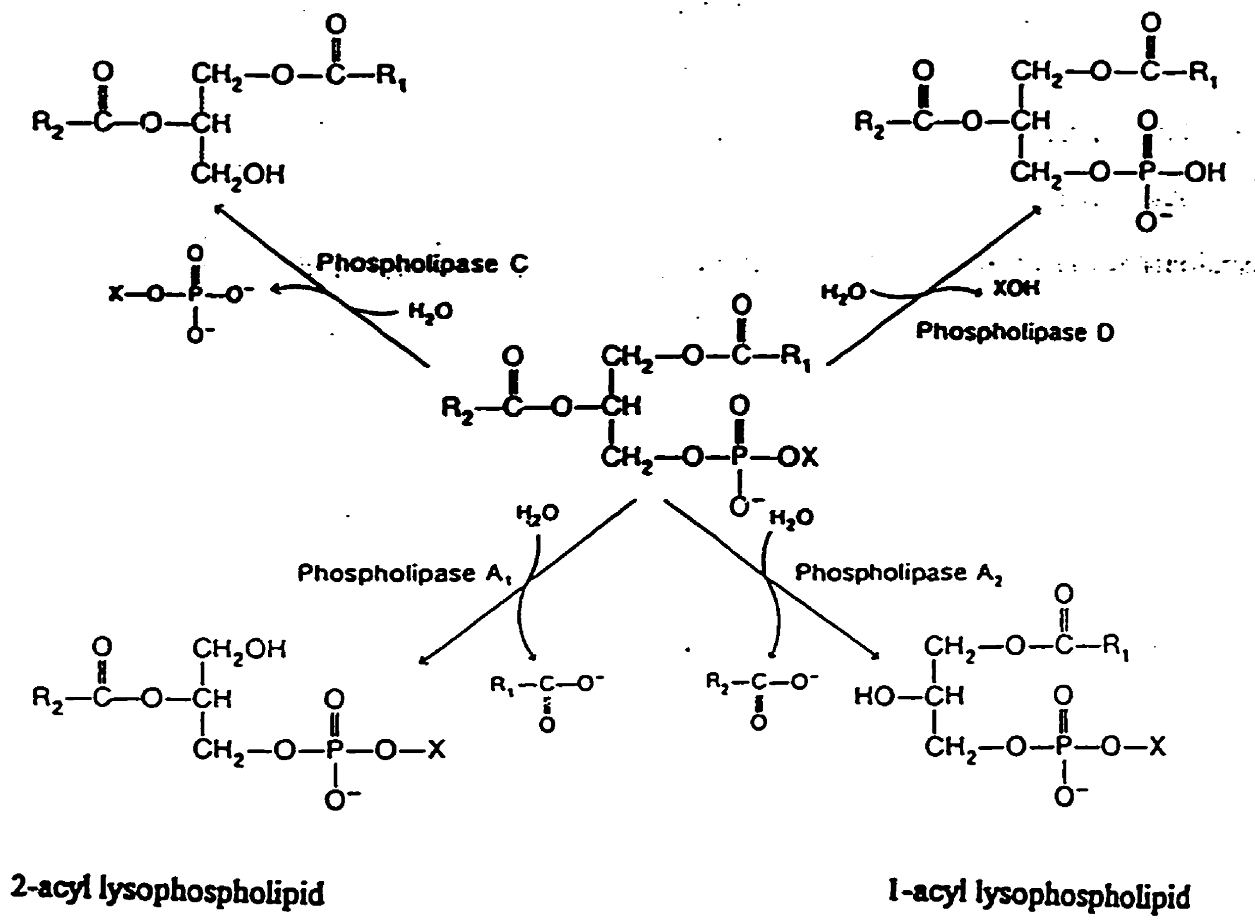


Figure 7: Reactions Catalyzed by Phospholipases

The second step required in remodeling of phospholipids requires the activation of the fatty acid to yield acyl-Coenzyme A. The lysophospholipid attacks the acyl-CoA molecule in a bimolecular nucleophilic substitution reaction (Fig. 8). This kind of remodeling is essential for CL. Even though CL is 75-80% linoleic acid, its precursors have a considerably different molecular species.

a. Molecular Species of Polyglycerophospholipids

Many studies have been performed to determine the fatty acid molecular species of CL and its precursors. Schlame *et al.* (1993) determined that the 4 acyl positions were occupied by monounsaturated and diunsaturated chains of 16-18 carbons in length. In bovine heart mitochondria, the predominant tetra-acyl species were (18:2-18:2)-(18:2-18:2) at 47.7%, (18:3-18:2)-(18:2-18:2) at 21.0%, and (18:1-18:2)-(18:2-18:2) at 14.6%. Other tetra-acyl species included (18:3-18:2)-(18:3-18:2), and (18:1-18:2)-(18:1-18:2). Rat liver CL primarily consisted of 18:2 (83.6%), 18:1 (10.0%), 16:1 (3.3%), and 20:3 (1.3%) (Gray, 1964). The fatty acid composition of PG was found to be considerably different from CL whereas PG consisted of 16:0 (12%), 18:0 (14%), 18:1 (21%), 18:2 (20%), and C20 polyenoic acids (20%). The predominant diacyl species of PG in the rat liver included (16:0-18:1) at 16.7%, (16:0-18:2) at 13.3%, (16:0-16:0) at 12.7%, (16:0-20:4) at 8%, (18:1-18:2) at 7.2%, and (16:0-20:4) at 6.5%, with others comprising the remaining 35% (Rustow *et al.*, 1989). Although it is not known if CTP:PA cytidylyltransferase exhibits acyl selectivity, the mitochondrial glycerophosphate acyltransferase showed a strong preference for palmitoyl-CoA at the *sn*-1 position. At the *sn*-2 position, linoleoyl-CoA is preferred (Monroy *et al.* 1972, Bjerve *et al.* 1976). It was also thought that enzymes using CDP-DG show selectivity as initial rates of mitochondrial PG and CL synthesis vary with CDP-DG species containing fatty acids

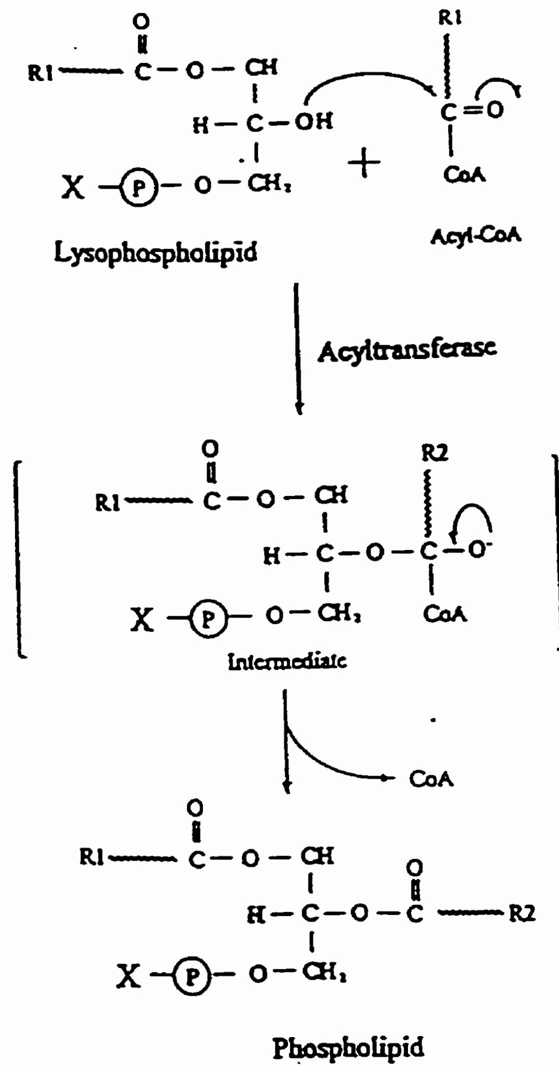


Figure 8: Basic Mechanism of Lysophospholipid Reacylation.

differing in chain length and degree of unsaturation (Hostetler *et al.* 1975). The species patterns of PA, CDP-DG and PG were similar enough to imply that the enzymes of the CL biosynthetic pathway were not species selective (Rustow *et al.* 1989). Catalytic hydrogenation, a tool used to alter the molecular species of the acyl chains of CL, for example, from 18:2 to 18:1 has shown that although (18:2-18:2) may be altered to (18:1-18:2), then (18:1-18:1), then (18:0-18:1), and finally (18:0-18:0), alteration of the acyl species in this manner did not affect the mitochondrial lipid/protein ratio, cytochrome c oxidase, or PLA_2 activity (Schlame *et al.* 1990).

V. Reperfusion Injury

Cardiac ischaemia is a condition in which the supply of myocardial oxygen inadequately meets the demand. Ischaemia consists of hypoxia, acidosis, and substrate deprivation and the accumulation of metabolic wastes (Hagve, *et al.* 1990). During ischaemia, changes occur that would render tissue adversely sensitive to oxygenated blood flow. Net degradation of ATP occurs as the oxygen required to act as the final electron acceptor of the electron transport chain is unavailable. This results in an accumulation of ATP degradation products xanthine, and Pi. Upon reoxygenation of the heart, there are several occurrences which result in reperfusion injury. During ischaemia, little Ca^{2+} is pumped. When reoxygenation occurs, the heart preferentially accumulates Ca^{2+} instead of manufacturing ATP as Ca^{2+} is pumped in down its electrochemical gradient. Energy is required to pump Ca^{2+} against its concentration gradient, however very little energy is available for the recently deprived heart (for review see Gunter and Pfieiffer, 1990). This influx of Ca^{2+} induces a reversible increase in permeability of the inner mitochondrial membrane causing further uncoupling of the mitochondrion (Hunter and Haworth, 1979). The elevated Ca^{2+} also stimulates mitochondrial PLA_2. As

reaction products accumulate, membrane permeability increases (Broekemeier *et al.*, 1989). This permeability transition has been determined to be caused by the opening of a non-selective permeable pore in the inner mitochondrial membrane of 20Å, allowing passage of molecules with a molecular mass of 1500 (Haworth and Hunter, 1979) (Fig. 9). Pore opening is determined by a direct synergism between either Ca^{2+} and Pi, or Ca^{2+} and hydroperoxide caused by oxidative stress (Crompton and Costi, 1988). This permeability is not caused by a defect in the lipid phase, however, but a structurally distinct entity consisting of two molecules. One of them is the ADP/ATP translocase. This is demonstrated by the protective role ADP plays in conditions of pore opening (Hunter and Haworth, 1979). ADP acts at an internal site of the ADP/ATP translocase, preventing pore formation. Furthermore, pore opening is suppressed under physiological levels of ATP despite supraphysiological levels of Ca^{2+}, however low ATP and high Ca^{2+} levels lead to pore formation (Duchen *et al.*, 1993). The other molecule involved in pore formation is cyclophilin, a protein with peptidyl prolyl *cis-trans* isomerase activity. Cyclophilin responds to oxidative stress in the heart, accessing the membrane where it interacts with the adenine nucleotide transporter, stimulating pore opening (Connern and Halestrap., 1994, and Halestrap and Davidson, 1990). This results in lowered cytosolic phosphorylation potential causing Ca^{2+} entry into the cytosol, resulting in further pore formation and ATP dissipation (Crompton and Costi, 1988). Hearts pre-treated with 0.2 μM CSA are typically protected against reperfusion injury.

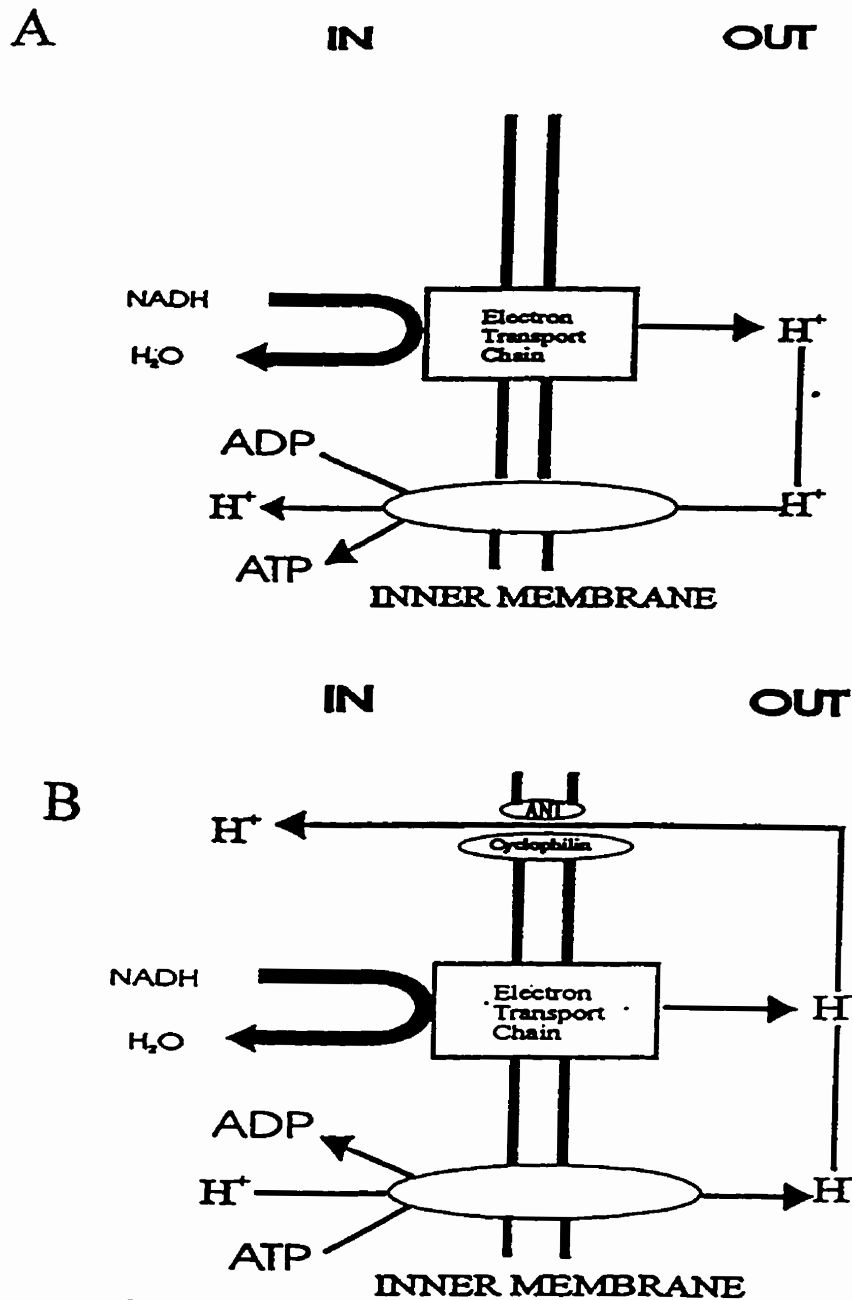
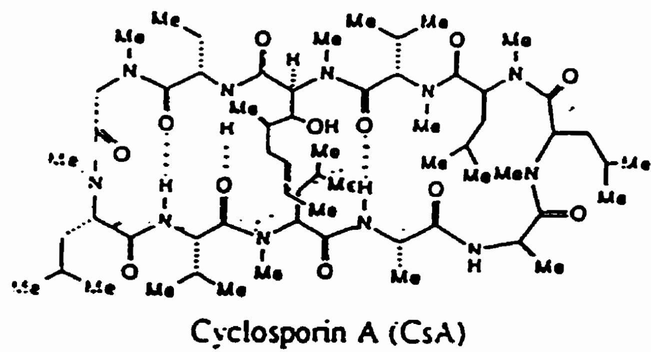


Figure 9: Healthy Inner Mitochondrial Membrane (A) and Unhealthy Inner Mitochondrial Membrane (B). In the healthy scheme (A), protons are pumped out via the electron transport chain into the intermembrane space creating an electrochemical H^+ gradient. A proton pumping ATPase pumps the protons into the matrix along the H^+ gradient, synthesizing ATP. In the unhealthy inner membrane (B), a permeable transition exists allowing protons easy access to the matrix of the membrane, resulting in the net conversion of ADP from ATP.

1. Cyclosporin A

CSA is a lipid soluble, cyclic undecapeptide with a molecular mass of 1203. It is an immunosuppressant produced by *Tolypocladium inflatum* that blocks T-cell activation and proliferation. CSA binds cyclophilin, inhibiting its peptidyl-prolyl *cis-trans* isomerase activity. This "toxic complex" binds calcineurin, inhibiting its serine threonine phosphatase activity which is otherwise required for the dephosphorylation of the cytoplasmic Nuclear Factor of Activated T-cells (NF-ATc) (Fig. 10). This dephosphorylation is required for the NF-ATc to access the nucleus where the nuclear NF-AT is located. Both subunits of NF-AT are required for T-cell activation and proliferation, an occurrence which may otherwise be blocked by the inhibition of calcineurin. CSA enjoys widespread use in preventing graft rejection due to its potent immunosuppressant qualities (Kunz and Hall, 1993).

The benefits of CSA may extend beyond immunosuppresion. Hearts pre-treated with 0.2 μM CSA, subjected to 30 min of ischaemia, and reperfused with 0.2 μM CSA have been shown to recover functional properties including beat, end-diastolic pressure and left-ventricular developed pressure, as well as [ATP]/[ADP][AMP] ratios much better than controls (Griffiths and Halestrap, 1993). This is likely due to CSA's ability to bind cyclophilin which in turn prevents the opening of the pore. When rat hearts were subjected to 60 minutes of hypoxia, radioactivity incorporated into CL and its precursors is decreased significantly (Cheng and Hatch, 1995). Considering the effects of reperfusion injury on the mitochondria and CSA's protective role, it would be interesting to determine how these would affect CL biosynthesis.



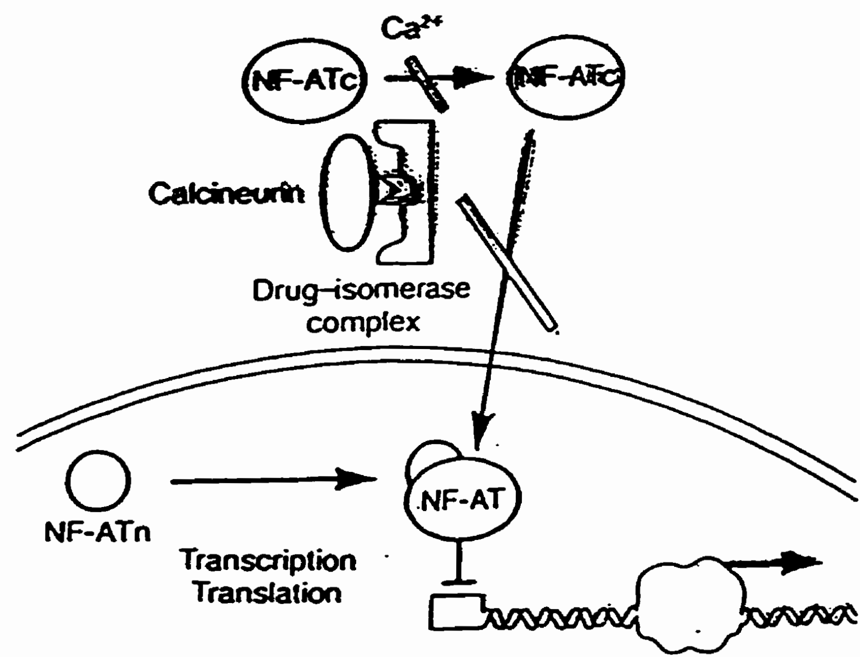
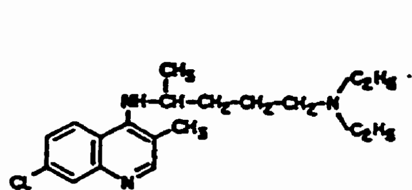


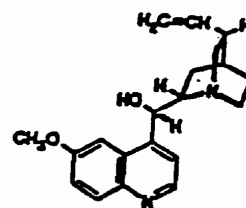
Figure 10: The Role of Cyclosporin A as an Immunosuppressant. Cyclosporin A binds with Calcineurin, inhibiting its phosphatase activity. This prevents the cytoplasmic subunit of the Nuclear Factor of Activated T-cells (NF-ATc) from accessing the nucleus and the nuclear subunit of the NF-ATc, inhibiting transcription and translation.

VI. Chloroquine

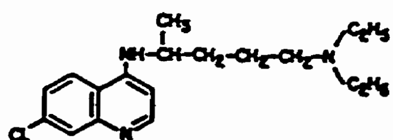
Chloroquine (CQ), also known as 7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline, enjoys widespread use as a chemotherapeutic agent in the treatment of malaria and as an anti-inflammatory agent in the treatment of rheumatoid arthritis. CQ is a drug consisting of a planar, heterocyclic aminoquinoline nucleus, and an aliphatic lipid soluble aminated side chain (Duchen *et al.*, 1993) (Fig. 11). CQ was synthesized in 1934 by chemists working for Bayer AG in Germany, and has been the most widely used antimalarial drug since 1946. It's action is limited to stages of parasitic lifecycle involved in hemoglobin degradation (Fig. 12). CQ, a weak base, follows a pH gradient to enter the food vacuole of the parasite where it accumulates. Here it forms a noncovalent toxic complex with heme (a breakdown product of hemoglobin) which accumulates to toxic levels causing parasites to swell and lyse (Slater 1993 and Homewood *et al.* 1972). CQ has also been demonstrated to be cardiotoxic in that it reduces the excitability and conductivity of cardiac muscles, and results in arrhythmia (Don and Aiwazzadeh, 1970). Chronic administration of CQ may produce negative chronotropy, negative inotropy, a decrease in coronary flow rate, and a decrease in mitochondrial Ca^{2+} binding and accumulation. Its inhibition of Ca^{2+} metabolism results in Ca^{2+} deficiency, causing a disruption in cellular membrane integrity (Essien and Ette, 1986). CQ is also known to cause phospholipidosis, a lipid storage disease. CQ is a weak base lysomotropic agent which enters tissues by one of 2 mechanisms: a) it may be bound non-specifically to membrane phospholipids or b) it may be trapped in acidic compartments. Here CQ is presumably protonated and unable to diffuse through the membrane (Daniel *et al.*, 1995). CQ is also thought to enter the lysosomes by a simple symmetrical carrier (Yayon and Ginsberg, 1982) where it rapidly accumulates



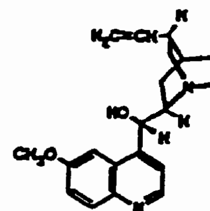
Sontoquine



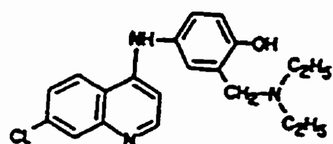
Quinine



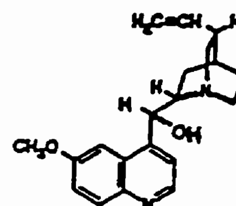
Chloroquine



Quinidine



Amodiaquine



Epiquinine

Figure 11: The Names and Structures of Various Antimalarial Quinolines Including Chloroquine.

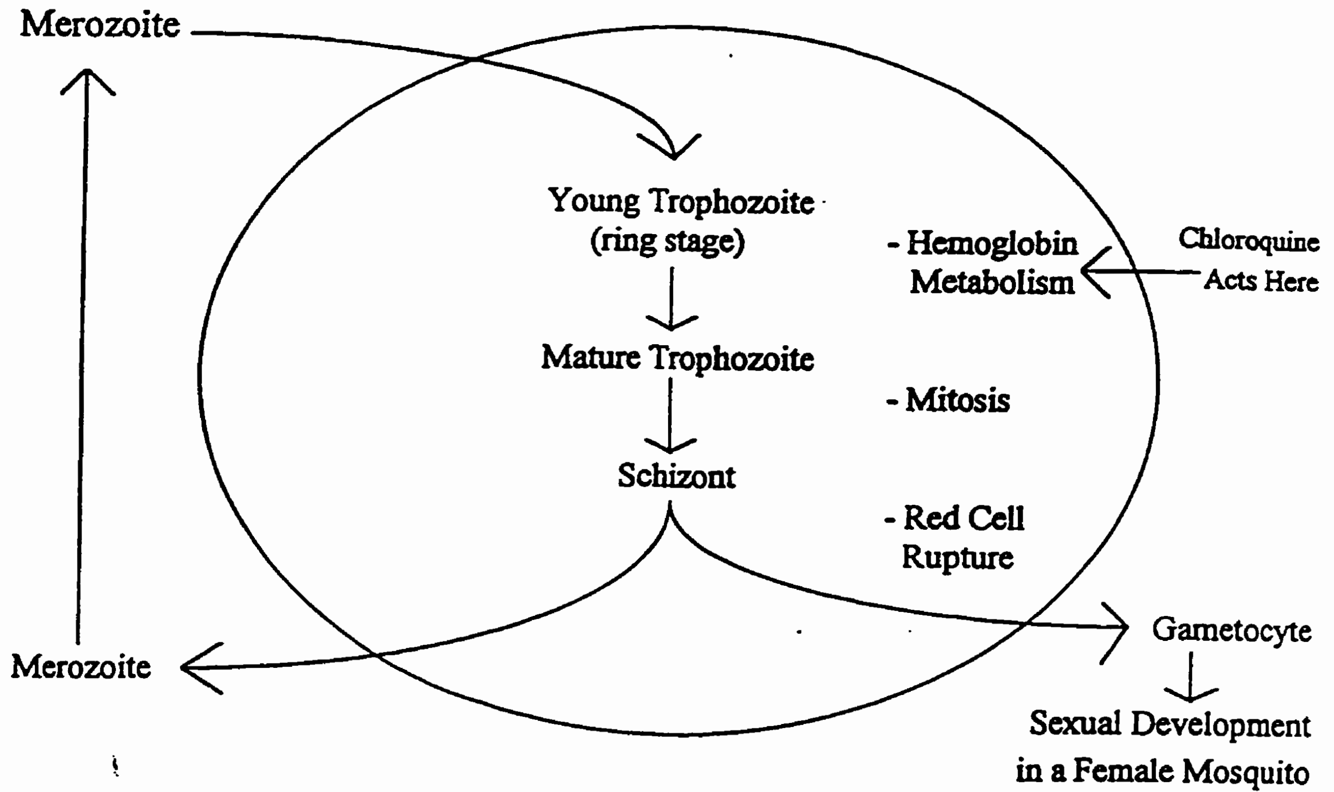


Figure 12: The Life-Cycle of Plasmodium Falciparum

(Hostetler *et al.* 1985). Finally, it has been shown that CQ may bind to lysosomal membranes until the membranes are saturated, another one of its lysomotropic properties (Colombo and Bertini, 1988). CQ seems to work in several ways to elevate phospholipid levels within cells. Matsuzawa and Hostetler (1980a) determined that administration of CQ caused a 1.5-fold increase in phospholipid content which was accounted for by multilamellar bodies induced by CQ. It was later demonstrated that CQ inhibited phospholipase A and C activities (Matsuzawa and Hostetler 1980b) and that CQ competitively inhibited PLA_2 by forming an EI_2 complex with the enzyme (Kubo and Hostetler 1985). CQ has also been shown to stabilize 1-stearoyl, 2-*sn*-arachidonylglycerol induced fluctuations associated with PLA_2 activation. This "bilayer-stabilizing effect" is also thought to inhibit PLA_2 (Zidovetzki *et al.* 1993). CQ has been shown to inhibit all aspects of lysosomal-related processes in the anoxically injured heart (Ridout *et al.* 1986). It inhibits the ability of hearts subjected to anoxia to mount an effective lysosomal response during recovery, a response representing a primary response to ischaemic injury through which myocytes may sequester and degrade damaged organelles. It is by this interference with lysosomal processes, including phospholipase activity that CQ causes the accumulation of phospholipids in the lysosomes. However, it has also been shown that CQ-stimulated increase in cellular lipids depends on the active synthesis of proteins through experiments performed with cyclohexamide, an inhibitor of protein synthesis (Chen *et al.* 1986). It has also been shown that drugs similar to CQ (i.e. other cationic amphiphillic drugs - CADs) induce phospholipid storage, as well as the redirection of phospholipid biosynthesis from neutral to acidic phospholipids (Koul and Hauser, 1987 and Reasor, 1989).

OBJECTIVES

Despite the advances made in phospholipid research, there is still little known about the regulation of CL metabolism. The assessment of the effects that reperfusion injury as well as the protective effect of CSA on the production of CL is an important undertaking. Information from this study will help us to learn how and if the opening and closing of mitochondrial pore regulates CL synthesis. To examine this, isolated rat hearts were perfused in the Langendorf manner and subjected to ischaemia-reperfusion injury in the presence or absence of 0.2 μM CSA and the formation of CL determined.

Chloroquine is a potent lysomotropic agent used in the treatment of malaria. CQ has also been shown to have a significant effect on the biosynthesis of phospholipids. Furthermore, CQ poisoning typically involves cardiomyopathy. We wanted to determine if CQ regulated CL biosynthesis in hepatocytes as well as cardiac cells. To examine this, isolated hepatocytes as well as H9c2 cardiac myoblasts were incubated with [1,3-^{3}H] glycerol and [U-^{14}C] linoleic acid either in the presence or absence of 0.1 mM CQ and the formation of CL determined.

MATERIALS AND METHODS

I. Materials

1. Experimental Animals

Male Sprague Dawley rats (175-225 g, body weight) were used throughout the study. Rats were maintained on Purina Rat Chow and tap water *ad libitum* in a light and temperature controlled environment. Treatment of animals conformed to the Guidelines of the Canadian Council on Animal Care.

2. Chemicals

[1,(3)-^{3}H] Glycerol was obtained from the Amersham, Oakville, Ontario. [1-^{14}C] linoleic acid was obtained from Dupont, Mississauga, Ontario. H9c2 cells were obtained from the American Tissue Type Collection. Cyclosporin A was obtained from Sandoz. Ecolite™ scintillation cocktail was obtained from ICN Biomedicals, Montreal, Quebec, Canada. T.l.c. plates (silica gel 60 Å, 20 cm x 20 cm, 250 µM) were obtained from VWR, London, Ontario, Canada. Chloroquine, sucrose, PC, PE, PI, PS, PA, CDP-DG, PG, CL, and BSA were purchased from Sigma Chemical Co., St. Louis, MO. Trypan Blue was generously donated by Dr. G. Arthur. Hanks' Balanced Salt Solutions and New Born Calf Serum (NBCS) were obtained from Gibco, Grand Island, N.Y., U.S.A. Dulbecco's Modified Essential Medium (DMEM) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All gases were obtained from Welder's Supply, Winnipeg, Manitoba, Canada. All other chemicals and solvents were of analytical grade and obtained from either Fisher Scientific, Edmonton, Alberta, Canada; Sigma Chemical Co., St. Louis, MO, U.S.A.; or CanLab Division of Baxter Co., Mississauga, Ontario, Canada.

II. Methods

A. Effect of Myocardial Reperfusion Injury and the Application of 0.2 μM CSA

1) Langendorf Perfusion of Isolated Rat Hearts

Male Sprague Dawley Rats were sacrificed by decapitation, and their hearts were quickly removed and cannulated via the aorta to a modified syringe needle (18 gauge). In order to remove the remaining blood, the heart was injected with 10 mL of Krebs Henseleit buffer, or KHB (Krebs and Henseleit, 1932). The buffer was prepared by adding to 100 mL of solution A (70 g/L NaCl, 21 g/L $NaHCO_3$, 9.91 g/L Dextrose), 10 mL of solution B (3.55 g/100 mL KCl, 2.94 g/100 mL $MgSO_4$, 1.63 g/100 mL NaH_2PO_4-H_2O, monobasic) and 5 mL of solution C (3.37 g/100 mL $CaCl_2$, dihydrate) in a volumetric flask and adding double distilled water to 1L. After the blood was removed, the heart was attached via the syringe needle to the heat exchanger of the perfusion apparatus in the manner of Langendorf (1985) (Fig. 13). The heart was perfused with KHB for 10 minutes in an open flow manner to stabilize it. Following stabilization, the heart was perfused with 12.5 mL KHB with various compounds or subjected to ischaemia for various time intervals. Following the perfusion experiments, 10 mL of air was injected into the heart to remove the residual perfusate. The hearts were placed in vials, frozen in liquid N_2 and freeze-dried overnight. Buffer was perfused at a rate of 2.5 mL/min with a temperature of 37^oC and was 95%O_2/5% CO_2.

2) Radioactive Labeling Studies in the Isolated Perfused Heart

Hearts were perfused under 3 separate conditions. Control hearts were stabilized for 10 min with KHB. Following stabilization, hearts were perfused for 60 min with

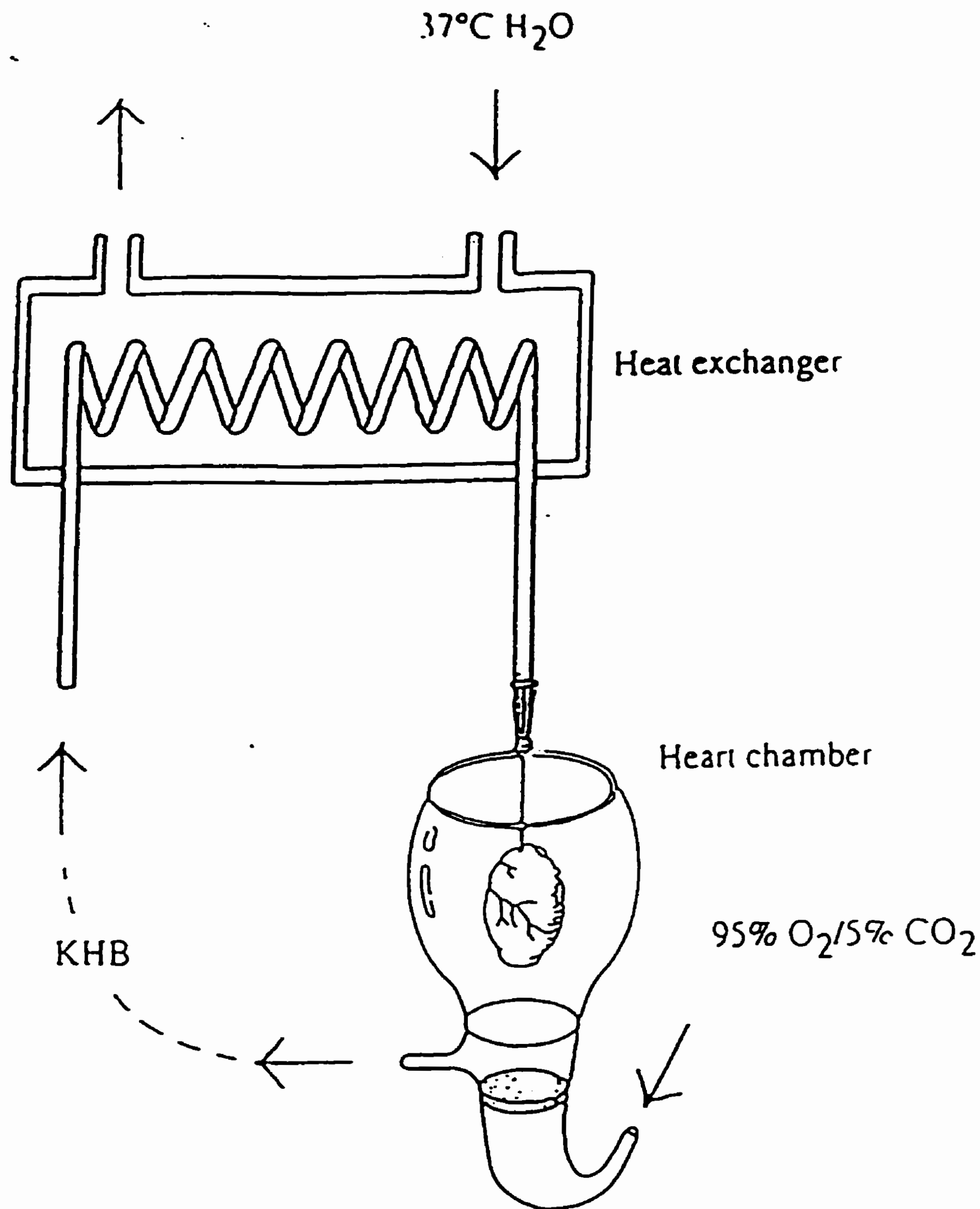


Figure 13: Retrograde Perfusion Apparatus. Krebs Henseleit Buffer is pumped through a heat exchanger where it is warmed to 37°C. The buffer flows through the heart via the aorta in a retrograde manner. The buffer is collected in the heart chamber and is saturated with 95% O_2/5% CO_2. During no-flow ischaemia, the heart is removed from the heat exchanger and allowed to remain in water maintained at 37°C.

KHB using 2.5 μCi/mL [1,(3)-^{3}H] glycerol and 0.6 mM oleate and albumin (Lopaschuk *et al.* 1994). Hearts subjected to reperfusion injury were first stabilized for 10 min with KHB, and then subjected to 25 min of no-flow ischaemia (Griffiths and Halestrap, 1993). This was followed by 60 min of reperfusion with [1,(3)-^{3}H] glycerol (2.5 μCi/mL) in the presence of 1.2 mM oleate and albumin (Cao and Hatch 1995). The final condition began with 10 min stabilization of the heart with 0.2 μM CSA in KHB. Hearts were then subjected to 25 min no-flow ischaemia, followed by 60 min of reperfusion with [1,(3)-^{3}H] glycerol (2.5 μCi/mL) in KHB in the presence of 1.2 mM oleate and 0.2 μM CSA (Griffiths and Halestrap, 1993). Hearts were kept at a constant temperature of 37°, even during ischaemia.

3) Isolation and Separation of Radioactivity Containing Metabolites from Isolated Rat Hearts

After the hearts were freeze dried and weighed, they were placed in a test tube containing 5 mL chloroform:methanol (2:1 by volume). The hearts were then homogenized by a 20 s burst at full speed by a Polytron® Homogenizer (Kinematika, Lucerne, Switzerland). Tissue remaining on the homogenizer probe was removed by another wash with 5 mL of chloroform:methanol (2:1 by volume). The two 5 mL homogenates were combined and centrifuged at 2000 rpm for 10 min in a bench top centrifuge (Model TJ-6 Beckman Instruments, Mississauga Ontario). The homogenate was decanted into a screw-cap tube and 5 mL 0.73% NaCl was added, separating the organic and aqueous phases. Tubes were vortexed until solutions were homogenous, and centrifuged at full speed for 10 min on the bench top centrifuge. The aqueous layer was removed by Pasteur pipette, and 5 mL of theoretical upper phase (chloroform:methanol:0.9% NaCl 3:48:47 by vol.) was added. The tubes were vortexed

and centrifuged as above. The upper layer was removed, and the aqueous phases were combined, dried overnight by air and resuspended in 1 mL DDW. An aliquot of the suspension (100 μL) was removed and placed into a 6 mL scintillation vial. Ecolite™ scintillation fluid was added (5 mL) and radioactivity incorporated into the aqueous layer was determined by scintillation counting in a Beckman model LS 3801 liquid scintillation counter immediately and 48 h later. The organic phase was dried down under N_2 gas and resuspended in 100 μL chloroform:methanol (2:1 by vol.). Thin layer chromatography (t.l.c.) plates permeated with 0.4 M boric acid were heat activated for 1 h at 145°C (Poorthuis *et al.*, 1976). Ten μL of the suspension was removed for scintillation counting and 25 μL was added to the borate treated plates and subjected to two dimensional separation (Poorthuis *et al.*, 1976). The solvent system of the first dimension contained chloroform:methanol:NH_4OH:DDW (70:30:2:3 by vol.), and the second dimension contained chloroform:methanol:DDW (65:35:5). Plates were allowed to dry between dimensions, and overnight after separation. Separated phospholipids were visualized by staining with iodine vapor (Fig. 14). Spots corresponding to phospholipids of interest were removed and placed in scintillation vials, and incorporation of radioactive metabolites was assessed immediately and another 48 hours after addition of scintillation fluid.

4) Determination of Phospholipid Pool Sizes

In order to determine the pool sizes of CL and PG in the isolated perfused rat heart, the phosphorus masses of these phospholipids were measured (Rouser *et al.* 1966). From the organic fraction, 25 μL was removed and added to a borate treated t.l.c. plate. The plate was subjected to two-dimensional chromatography, and placed in an iodine tank in order to identify the location of the phospholipids on the silica gel.

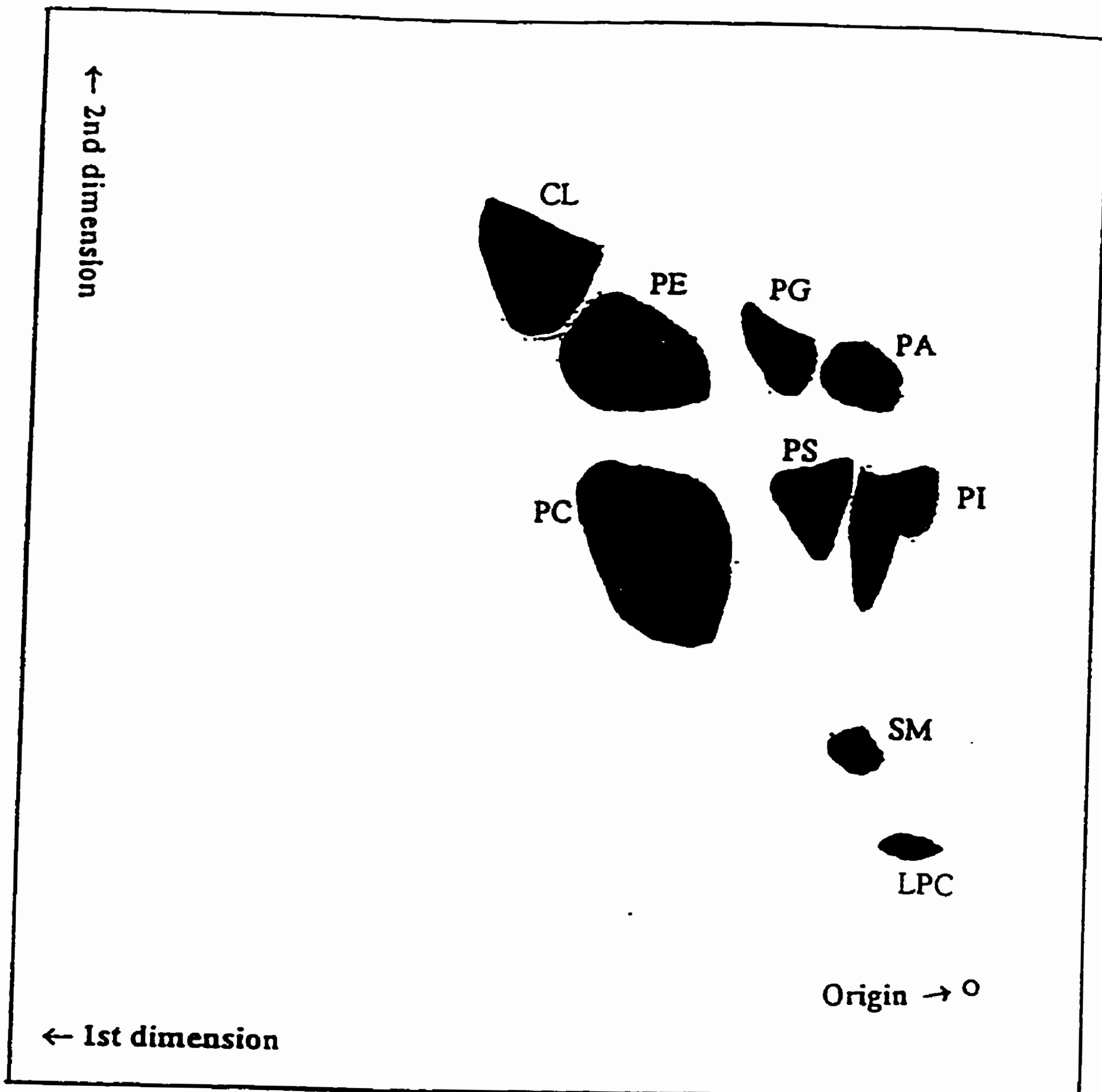


Figure 14: Thin Layer Chromatogram of Two-Dimensional Separation of Phospholipids. Phospholipids include: CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; LPC, lysophosphatidylcholine.

Phospholipids of interest the silica gel were removed from the plate and placed into 13 x 100 mm test tubes. Standards of KH_2PO_4 of 0 to 200 nmol were added to 13 x 100 mm test tubes as well and dried down. Perchloric acid (70%) was added to each tube (450 μl) and the tubes were covered and heated at 180°C for 60 min. The tubes were allowed to cool and 2.5 mL of DDW was added, followed by 0.5 mL of 2.5% ammonium molybdate. The tubes were incubated in a water bath at 95°C for 15 minutes, allowed to cool, and then centrifuged in order to sediment the silica gel. Samples were transferred to disposable cuvettes and the absorbencies were measured on a spectrophotometer (Spectronic 1001 Plus) at 820 nm. Absorbency values were converted to phosphorus mass by comparing readings to those obtained from the standard curve.

B. Chloroquine Studies in Rat Liver Hepatocytes

Hepatocytes were prepared from male Sprague-Dawley rats (175-200 gm) by the collagenase perfusion technique (Davis *et al.* 1979). They were incubated overnight under controlled conditions in DMEM with 10% new-born calf serum (NBCS). Cells were then incubated in DMEM in the absence of 10% NBCS in the presence or absence of 0.1 mM CQ for 2 h. Cells were then incubated for various time periods in the presence or absence of 0.1 mM CQ with either [1,(3)-^{3}H] glycerol (10 μCi/dish) or [U-^{14}C] linoleic acid (1 μCi/dish). Concentration dependent studies were performed by varying the concentrations of CQ while maintaining a constant level of [1,(3)-^{3}H] glycerol.

C. Chloroquine Studies in H9c2 Cardiac Myoblasts

H9c2 cardiac myoblasts were obtain from the American Tissue Type Collection and maintained on DMEM with 10% NBCS. Experiments were conducted following an

overnight fast whereby cells were incubated in the absence of NBCS. Cells were then incubated in the presence or absence of 0.1 mM CQ for various time periods with either [1,(3)-^{3}H] glycerol (10 μCi/dish) or [U-^{14}C] linoleic acid (1 μCi/dish). Inhibition studies were conducted by varying either the concentration of cold glycerol, or the time of incubation while maintaining constant levels of [1,(3)-^{3}H] glycerol in the absence or presence of 0.1 mM CQ.

1) Isolation of Radioactive Metabolites from Cells

After the timed incubations, the medium was removed and cells were washed twice with 1 mL of phosphate buffered saline (prepared by adding 8 gm NaCl, 0.2 gm KCl, 1.15 gm Na_2HPO_4, and 0.2 gm KH_2PO then DDW to 1 L, pH to 7.4 with 1N NaOH). One mL MeOH:DDW (1:1 by vol.) was added and cells were removed from the dish with a rubber policeman. The cells were then transferred with a Pasteur pipette to a screw-cap tube. This procedure was repeated to ensure most of the cells were removed, the screw cap tubes were then vortexed and 50 μL of the whole homogenate was removed for determination of radioactivity and 50 μL was removed for Lowry's protein assay (Lowry *et al.* 1951). To the tubes 0.5 mL of 0.9% NaCl and 2 mL of $CHCl_3$ were added. The tubes were vortexed and centrifuged, the upper layer was removed and Two mL of theoretical upper phase was added (as above). The tubes were vortexed and centrifuged and again the upper layer was removed and the aqueous layers were combined, dried down overnight under air and the radioactivity determined. The organic layer was under by N_2 gas, and resuspended in 100 μL chloroform:methanol (2:1 by vol.). An aliquot of the suspension was placed on a t.l.c. plate (5 cm x 5 cm), as well as CL, PG, PA, PI, PS, and LPC standards and two dimensional t.l.c. was performed as above.

RESULTS

1) Ischaemia-Reperfusion Injury and CSA

To examine if CL biosynthesis was affected by ischaemia-reperfusion injury, isolated rat hearts were subjected to 30 min ischaemia followed by a 30 min reperfusion with [1,(3)-^{3}H] glycerol in KHB in the absence or absence of 0.2 μM CSA. An EKG of the hearts was taken in order to assess functional integrity of the hearts.

Incorporation of radioactivity into phospholipids was assessed relative to heart mass. Subsequent to reperfusion, hearts were freeze dried overnight, and the following day the hearts were homogenized in chloroform:methanol (2:1 by vol.). The organic fraction was extracted, dried by a stream of N_2 gas, and resuspended in 100 μL chloroform:methanol (2:1 by vol.). Of the resuspension, 10 μL was removed and counted. A further 25 μL of the suspension was applied to silica coated t.l.c. plates and phospholipids were isolated. The hearts subjected to ischaemia-reperfusion injury did not demonstrate a significant change in radioactivity incorporated into CL (Fig. 15). The application of 0.2 μM CSA also seemed to have little effect on radioactivity accumulation into reperfused hearts. Furthermore, [1,(3)-^{3}H] glycerol incorporation into the organic fraction was also unaffected by reperfusion injury or CSA.

We examined the effects of ischaemia-reperfusion injury on phospholipid pool sizes of the ischaemic-reperfused and CSA treated hearts. Hearts were subjected to 30 min of ischaemia followed by 30 min of reperfusion. Phospholipids were extracted, subjected to t.l.c. and the phosphorus mass was determined. There was no significant change in the pool sizes of any of the major phospholipids including CL (Fig. 16).

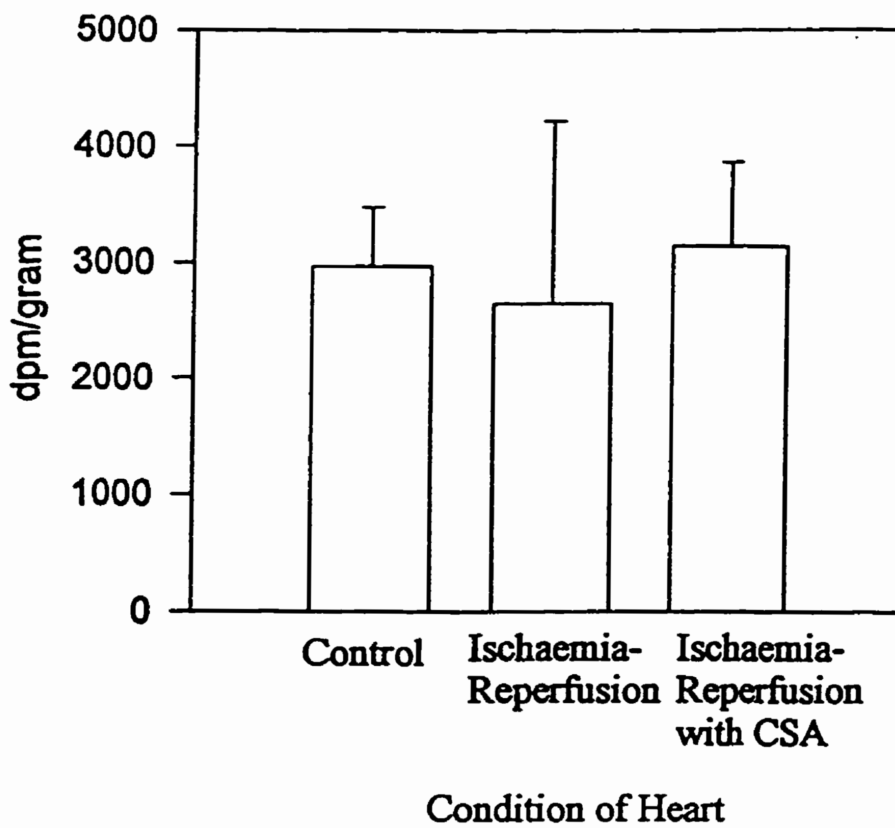


Figure 15: Incorporation of Radioactivity into Cardiolipin in Rat Hearts Perfused with [1, (3)-^{3}H] Glycerol. Isolated rat hearts perfused with Krebs Henseleit Buffer containing [1,(3)-^{3}H] glycerol were subjected to 30 min of ischaemia followed by 30 min of reperfusion in the absence or presence of 0.2 µM CSA. The organic fraction was extracted, and the radioactivity associated with cardiolipin was determined. Vertical bars represent the mean ± SD of at least 3 hearts.

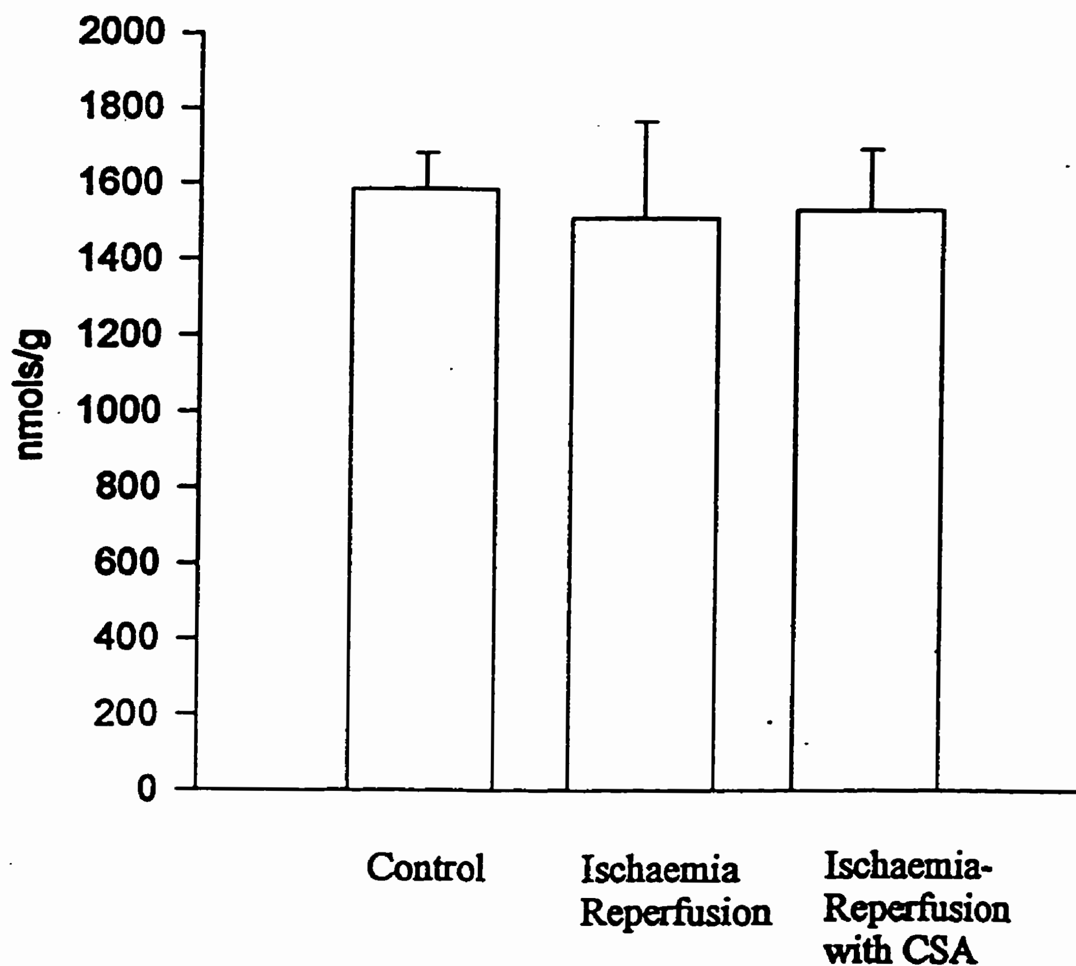


Figure 16: Pool Size of Cardiolipin from Rat Hearts Subjected to Ischaemia-Reperfusion Injury as Determined from Phospholipid Phosphorus Assay. Rat hearts were perfused with Krebs Henseleit buffer and subjected to 30 min of ischaemia followed by 30 min of reperfusion in the presence or absence of 0.2 μM CSA. Cardiolipin was isolated and pool sizes were determined by the sensitive phosphorus assay. Vertical bars represent the mean ± SD of at least three hearts.

2) Chloroquine Studies in Rat Liver Hepatocytes

Rat liver hepatocytes were prepared from Sprague-Dawley rats of 200-250 g and plated at approximately 3×10^6 cells/dish and incubated with DMEM with NBCS (10%). Cells were incubated with DMEM (without NBCS) 2 h prior to the experiment. Cells were then incubated in the absence or presence of 0.1 mM CQ simultaneously with [1,(3)-^{3}H] glycerol or [1-^{14}C] linoleic acid. Cells were removed from the plates with Two mL of MeOH:DDW (1:1 by vol.) and 50 µL was taken for the determination of radioactivity. The organic fraction was extracted, dried down under a stream of N_2, and resuspended in 100 µL chloroform:methanol (2:1 by vol.). Ten µL of the suspension was taken determine radioactivity into the organic fraction. Phospholipids were separated by 2 dimensional t.l.c. on plates treated with 0.4 mM borate (10 cm x 10 cm). Phospholipid standards (CL, PG, PE, PA, PS, PI, and LPC) were spotted on the plates in order to facilitate visualization of the spots. Radioactivity incorporated into DG, TG and CDP-DG was also determined. Cells were also preincubated for 2 h with 0.1 mM CQ, and then [1,(3)-^{3}H] glycerol was added and cells were further incubated for up to 2 h and [1,(3)-^{3}H] glycerol uptake, and incorporation into phospholipids was determined.

Treatment of hepatocytes with CQ resulted in an increase in the amount of radioactivity incorporated into CL (Fig. 17), as well as precursors PG, PA, and CDP-DG (Fig. 18). At the same time, production of other major glycerolipids including TG, PE and PC was much lower in cells incubated in the presence of CQ relative to controls (Fig. 19). After 2 h, [1,(3)-^{3}H] glycerol uptake was decreased in hepatocytes incubated with CQ relative to controls (Fig. 20). Despite increases in [1,(3)-^{3}H] glycerol uptake into CL in the presence of 0.1 mM CQ, 10 mM NH_4Cl (also a CAD) had no effect on [1,(3)-^{3}H] glycerol uptake into CL (Fig. 21).

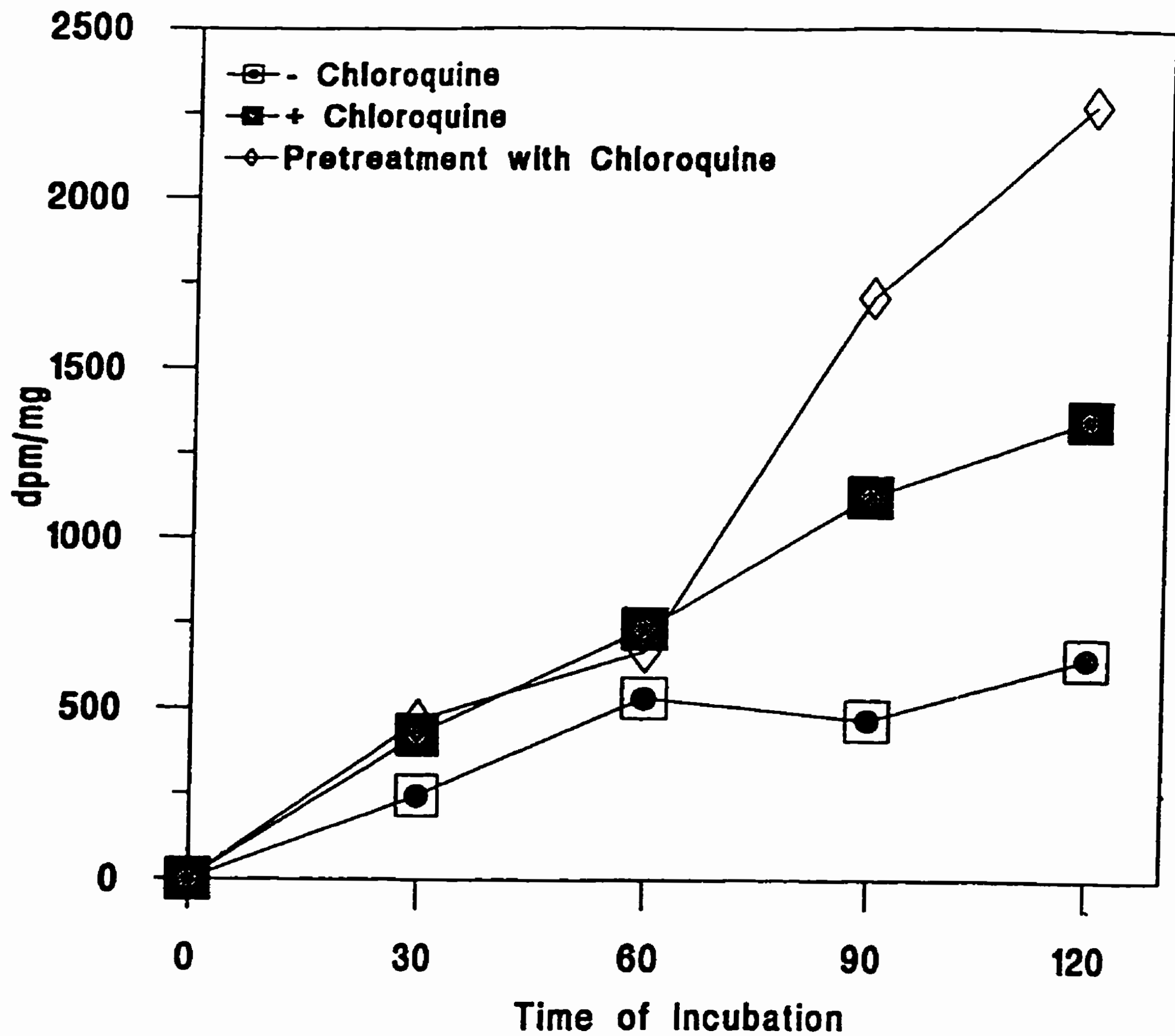


Figure 17: Incorporation of Radioactivity into Cardiolipin in Rat Liver Hepatocytes Treated with Chloroquine. Rat hepatocytes were prepared from rat livers via the collagenase perfusion technique. Cells were then either incubated simultaneously with 0.1 mM chloroquine and [1,(3)-^{3}H] glycerol, or with a 2 hour preincubation of 0.1 mM chloroquine followed by simultaneous incubation with 0.1 mM chloroquine and [1,(3)-^{3}H] glycerol and radioactivity incorporated into cardiolipin was determined relative to cell mass.

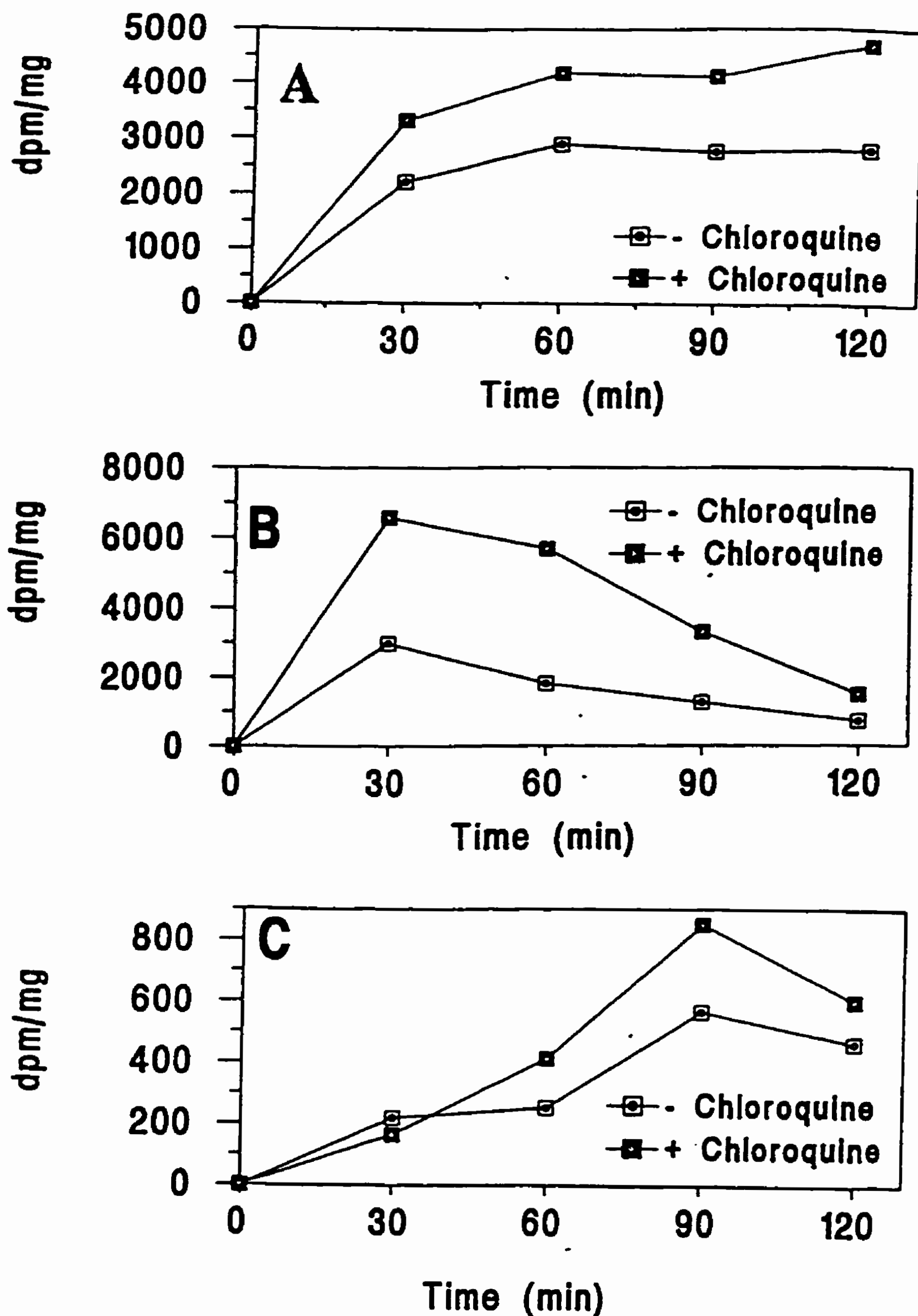


Figure 18: Incorporation of Radioactivity into Cardiolipin Precursors Phosphatidylglycerol (A), Phosphatidic Acid (B) and CDP-DG (C) in Rat Liver Hepatocytes Treated with 0.1 mM Chloroquine. Hepatocytes were prepared from rat livers via the collagenase perfusion technique. Cells were then incubated in the absence or presence of 0.1 mM chloroquine and [1,(3)-^{3}H] glycerol. Phospholipids were removed and isolated and radioactivity incorporated into phosphatidylglycerol, phosphatidic acid and CDP-DG was determined relative to cell mass. Data report the mean of two experiments.

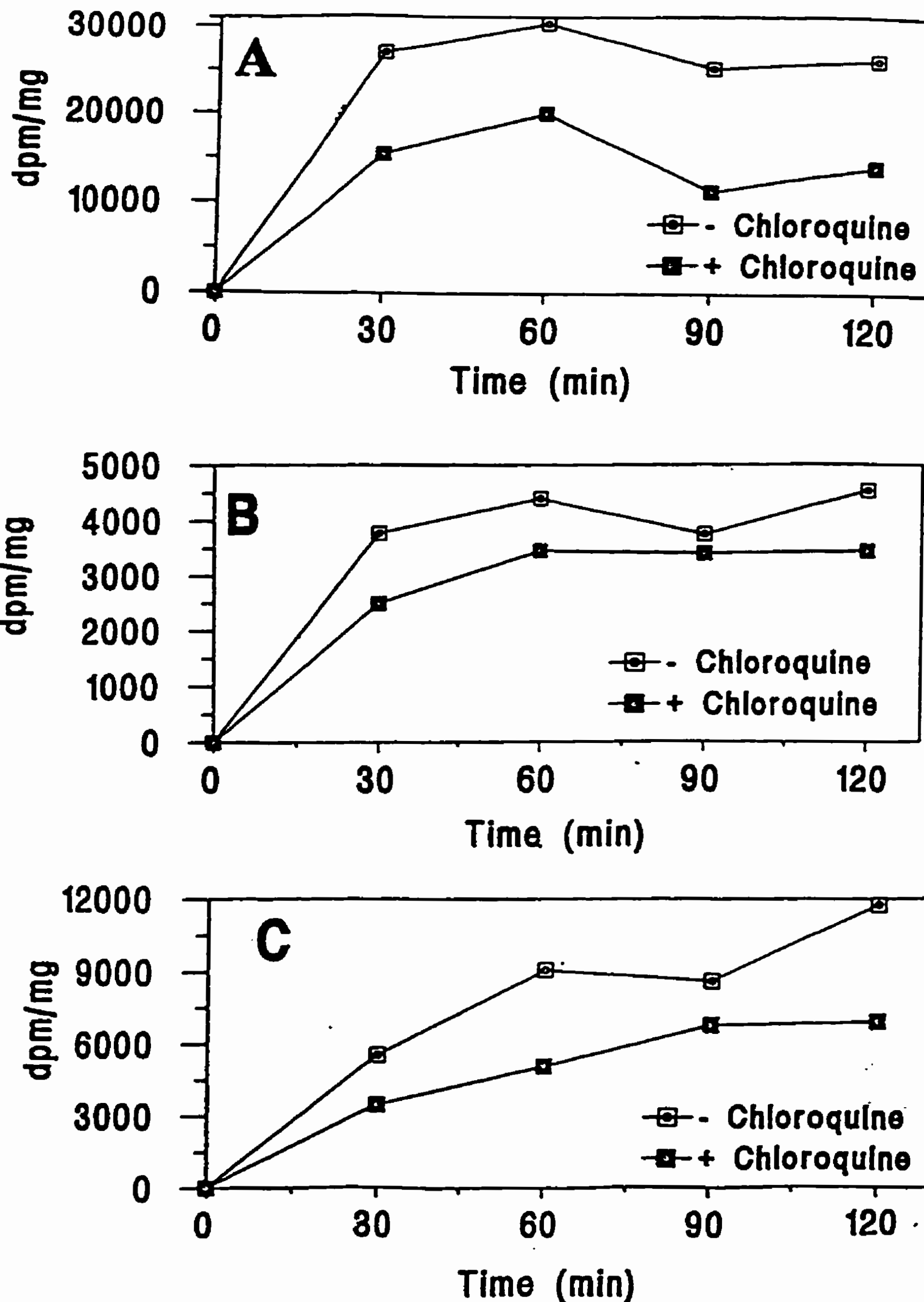


Figure 19: Incorporation of Radioactivity into Diacylglycerol Products Triacylglycerol (A), Phosphatidylcholine (B) and Phosphatidylethanolamine (C). Triacylglycerol, phosphatidylcholine and phosphatidylethanolamine were isolated from cells treated with 0.1 mM chloroquine as in Fig. 18 and incorporation of [1,(3)-^{3}H] glycerol into these glycerolipids was determined. Data represents the mean of two experiments.

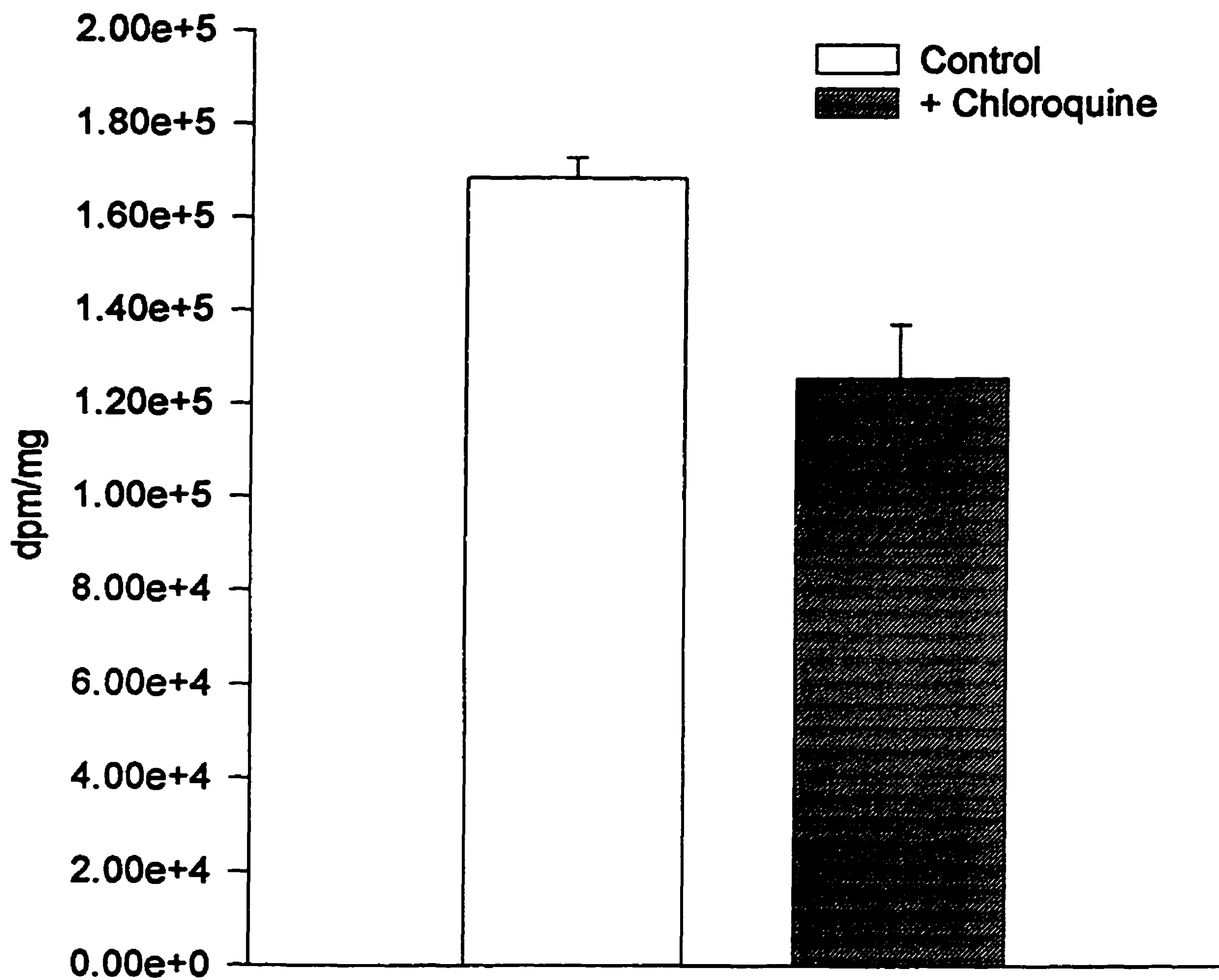


Figure 20: Incorporation of Radioactivity in Hepatocytes Incubated with Chloroquine. Hepatocytes were incubated for 2 h in the absence or presence of 0.1 mM Chloroquine. Radioactivity incorporated into the whole homogenate was determined. Data represent the mean ± of two experiments.

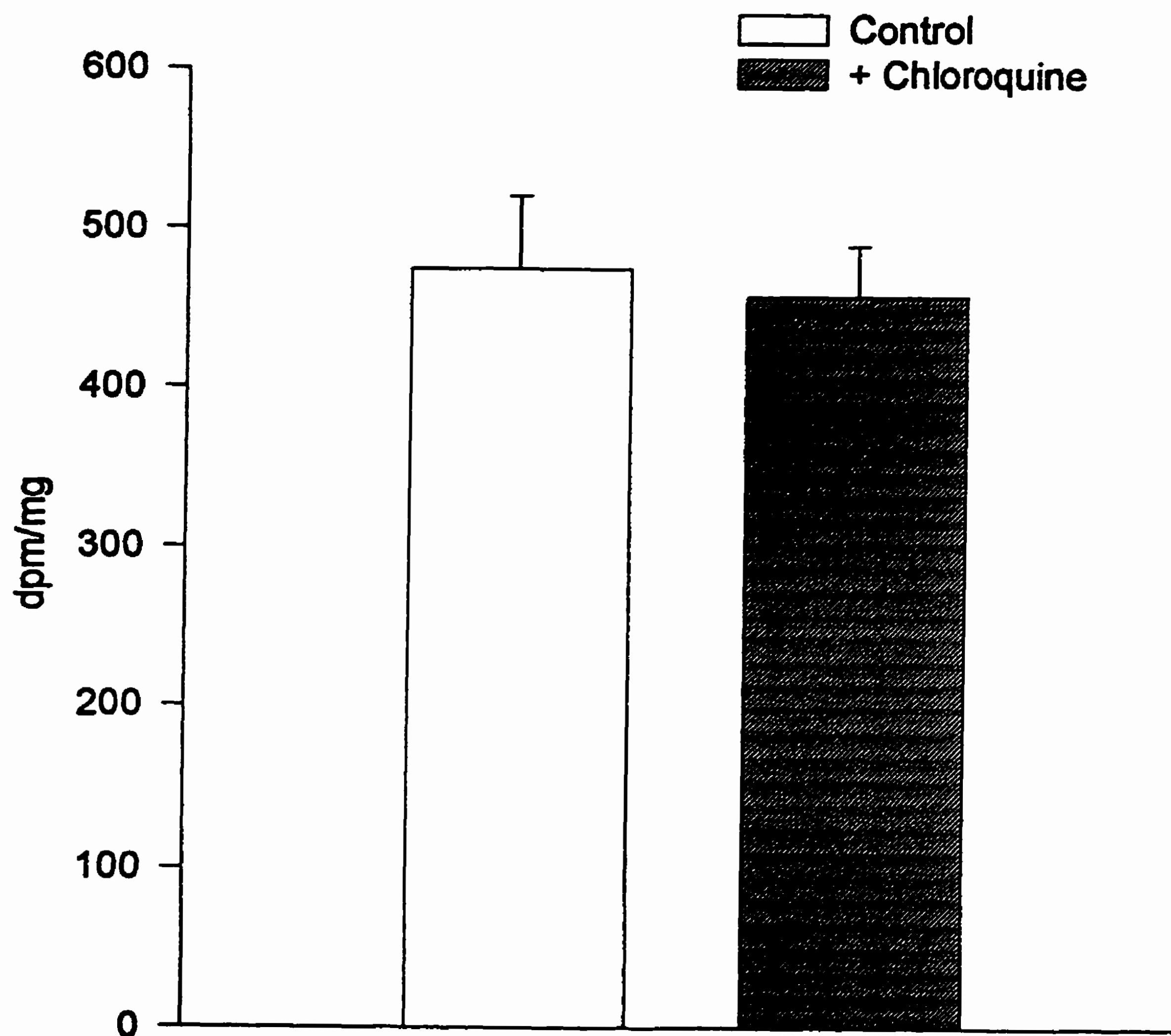


Figure 21: Incorporation of Radioactivity into Cardiolipin in Hepatocytes Incubated with NH_4Cl. Hepatocytes were incubated for 2 h in the absence or presence of 10 mM NH_4Cl. Radioactivity incorporated into cardiolipin was determined. Data represent the mean ± of two experiments.

Incorporation of radioactive linoleic acid was examined in rat liver hepatocytes incubated with CQ. Radioactivity incorporated into CL was increased 32% relative to controls (Fig. 22A). Although uptake of linoleic acid into the cells did not change significantly, nor did overall incorporation of radioactivity into phospholipids. In contrast, the total amount of radioactivity incorporated into the whole homogenate was unaltered (Fig. 22B).

3) Chloroquine Studies in H9c2 Cardiac Myoblasts

H9c2 cardiac myoblasts were rendered quiescent 18 h prior to experimentation when NBCS was removed from the DMEM. The following day, cells were incubated simultaneously with 0.1 mM CQ and [1,(3)-^{3}H] glycerol (10 μCi/dish) for 2 h. Cells were removed with 2 mL methanol:DDW (1:1 by vol.) and 50 μL of the suspension was removed for determination of total uptake of [1,(3)-^{3}H] glycerol. Phospholipids were extracted and suspended in 100 μL chloroform:methanol (2:1 by vol.), and 10 μL of the organic fraction was removed and radioactivity in the organic phase was determined. When H9c2 cells were preincubated for 2 h with 0.1 mM CQ, there was a decrease in [1,(3)-^{3}H] glycerol incorporated into the whole homogenate and the organic fraction. When cells were preincubated for 18 hours with 0.1 mM CQ, [1,(3)-^{3}H] glycerol incorporation into the whole homogenate and the organic phase decreased (Fig. 23). [1,(3)-^{3}H] Glycerol incorporation into CL was also significantly lower in H9c2 cells (Fig. 24).

Glycerol uptake was further assessed by incubating H9c2 cardiac myoblasts overnight in the absence or presence of 0.1 mM CQ. Cells were then incubated in the absence or presence of 0.1 mM CQ with 10 μCi of [1,(3)-^{3}H] glycerol and various concentrations of cold glycerol (1 μM, 10 μM, 100 μM, and 1 mM). Cells were

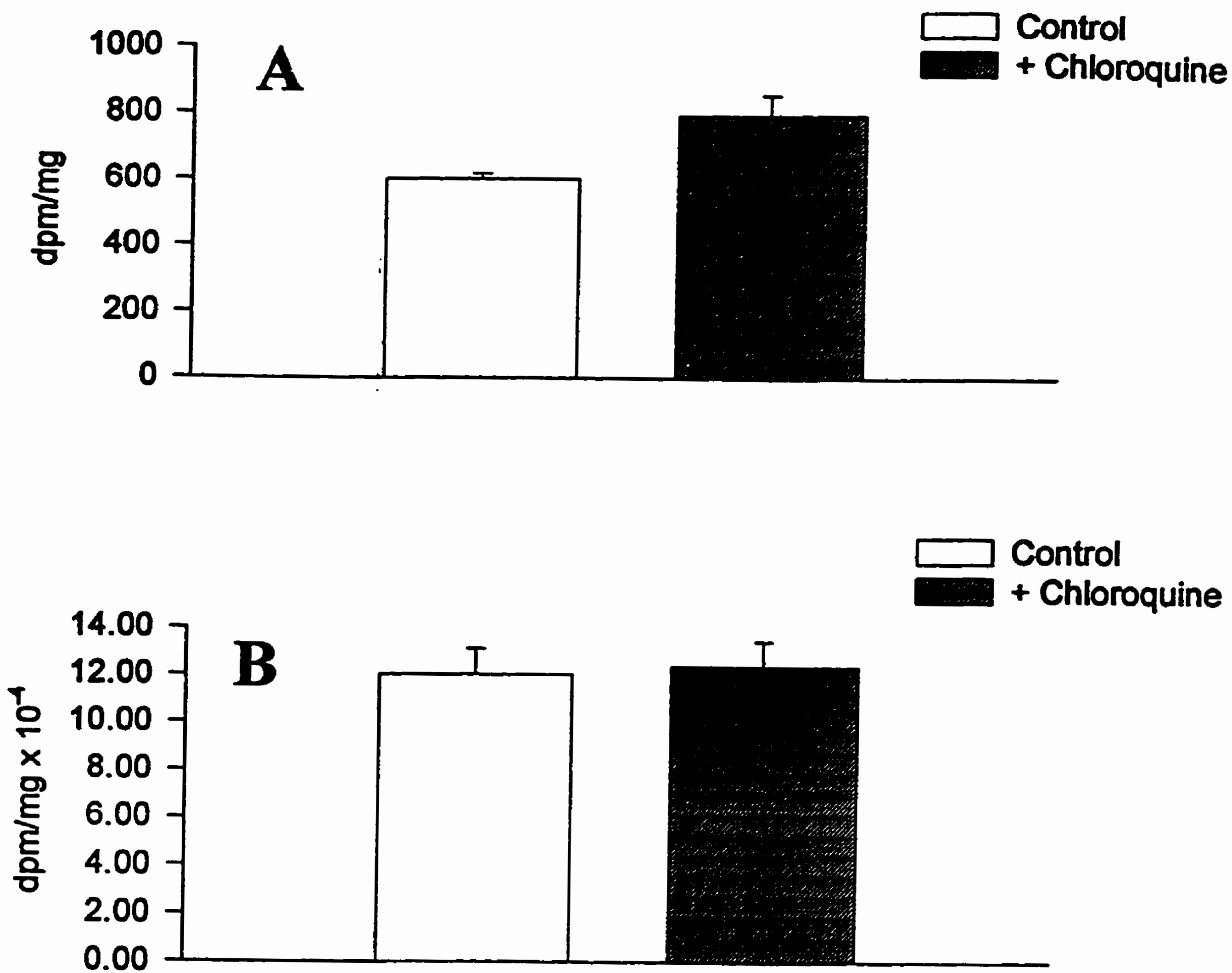


Figure 22: Incorporation of [1-^{14}C]linoleic acid into Cardiolipin (A) and the Whole Homogenate (B). Hepatocytes were incubated with [1-^{14}C]linoleic acid in the presence or absence of 0.1 mM chloroquine for 2 h. Radioactivity incorporated into CL and the whole homogenate was determined. Data represent the mean of two experiments

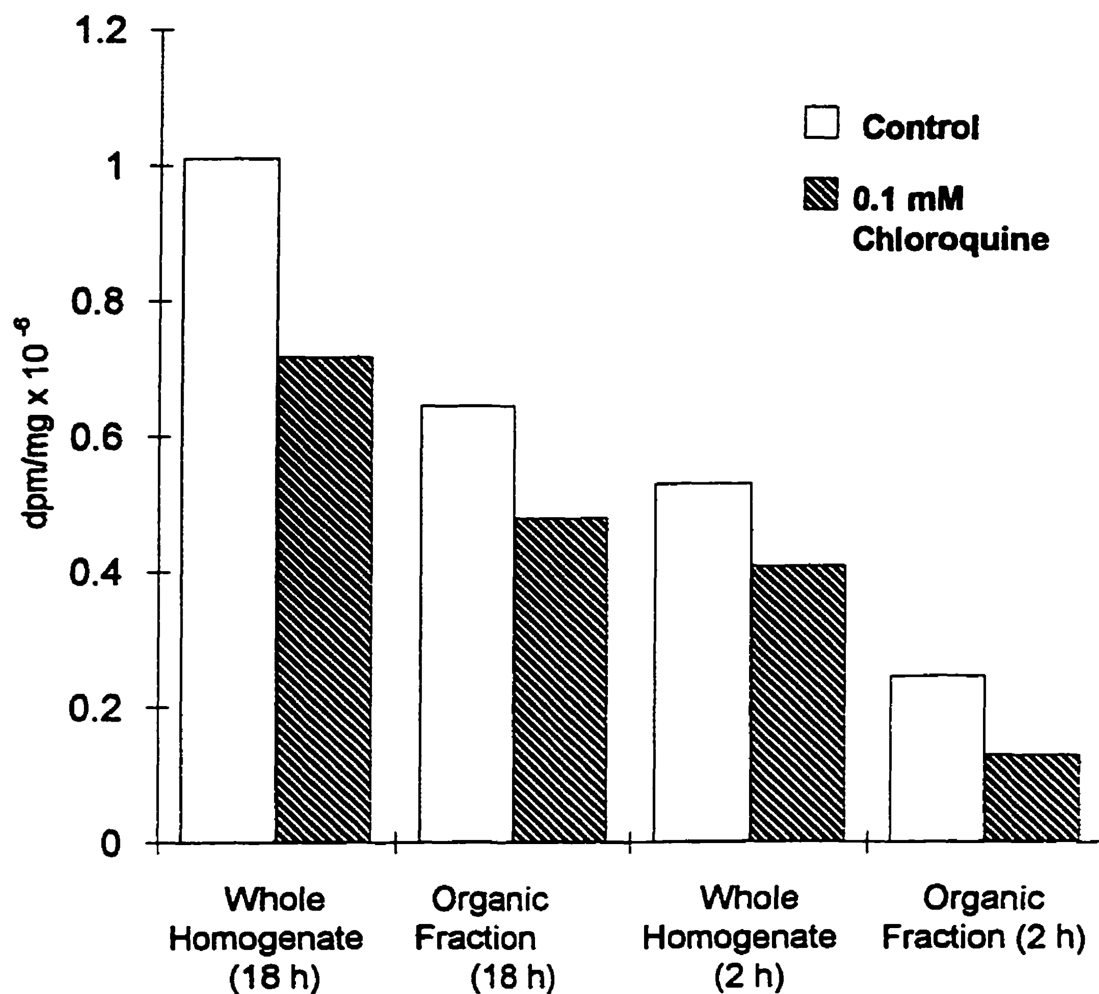


Figure 23: Incorporation of Radioactivity into H9c2 Cardiac Myoblasts Preincubated with Chloroquine. H9c2 cardiac myoblasts were pre-incubated in the absence or presence of 0.1 mM chloroquine for 2 h and 18 h. They were then incubated with [1,(3)-^{3}H] glycerol simultaneously in the absence or presence of 0.1 mM chloroquine. Total uptake of radioactivity (whole homogenate) and radioactivity incorporated into the organic fraction was determined. Data represent the mean of two experiments.

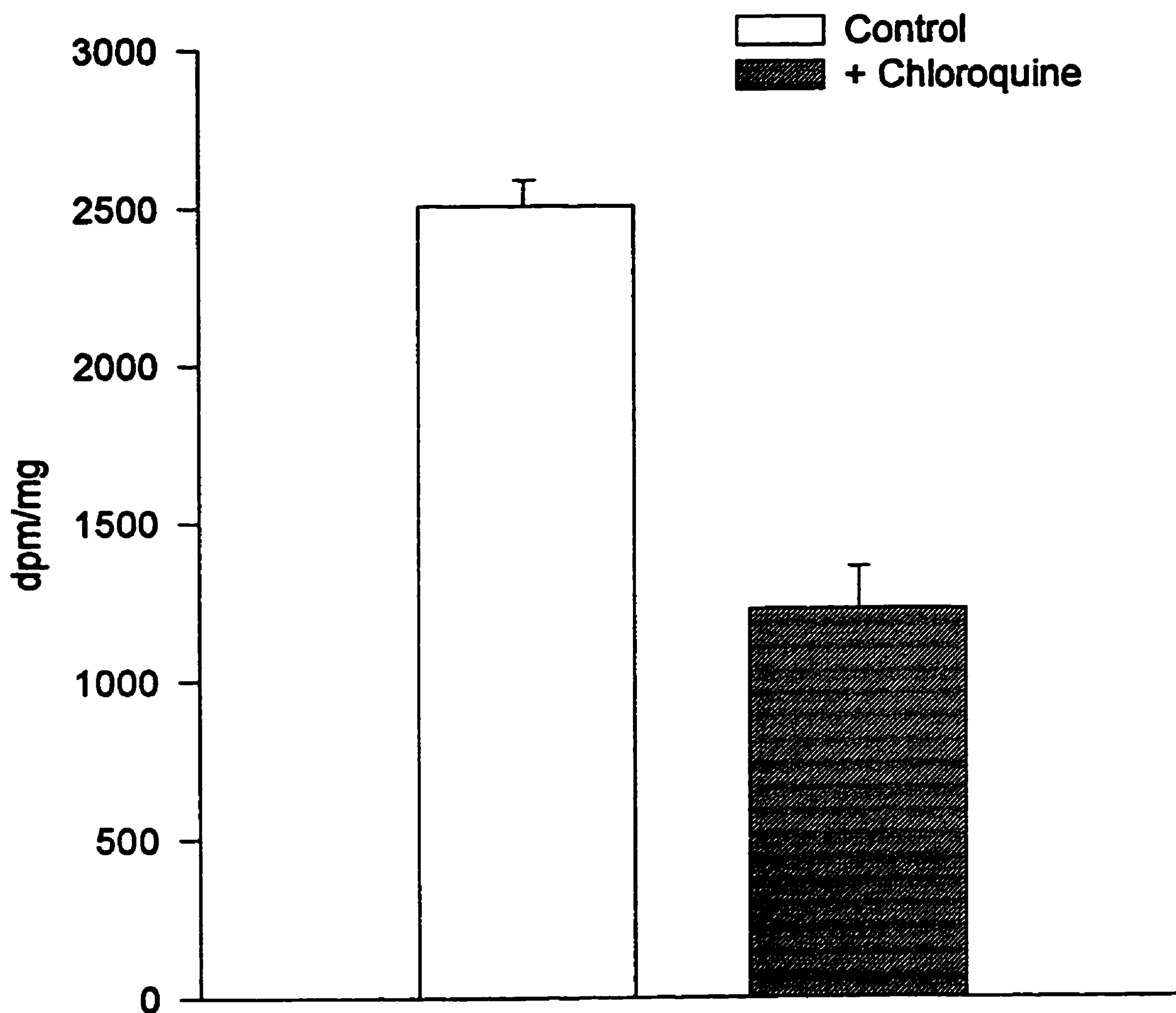


Figure 24: Incorporation of Radioactivity into Cardiolipin in H9c2 Cardiac Myoblasts Preincubated with Chloroquine. H9c2 cardiac myoblasts were pre-incubated in the absence or presence of 0.1 mM chloroquine for 18 h. They were then incubated with [1,(3)-^{3}H] glycerol simultaneously in the absence or presence of 0.1 mM chloroquine for 2 h. Uptake of radioactivity into CL was determined. Data represent the mean of two experiments.

removed from the dishes with methanol:DDW (1:1 by vol.) and a 50 μL aliquot was removed for determination of radioactivity. Uptake of radioactivity was linear in both control and CQ treated cells, however, it was typically lower in cells incubated in the presence of CQ in glycerol concentrations of up to 1 mM (Fig. 25). A Lineweaver-Burke plot of the data (plotting the inverse of the substrate concentrations versus the inverse of the glycerol uptake concentrations) demonstrated chloroquine inhibited glycerol uptake by a mixed inhibition mechanism (Fig. 26).

Cells were also incubated overnight with CQ and the next day they were incubated for one h with [1-^{14}C] linoleic acid. CQ did not affect the amount of linoleic acid taken up into the cell nor into phospholipids, and this may be reflected by label incorporated into CL and PC (Fig. 27).

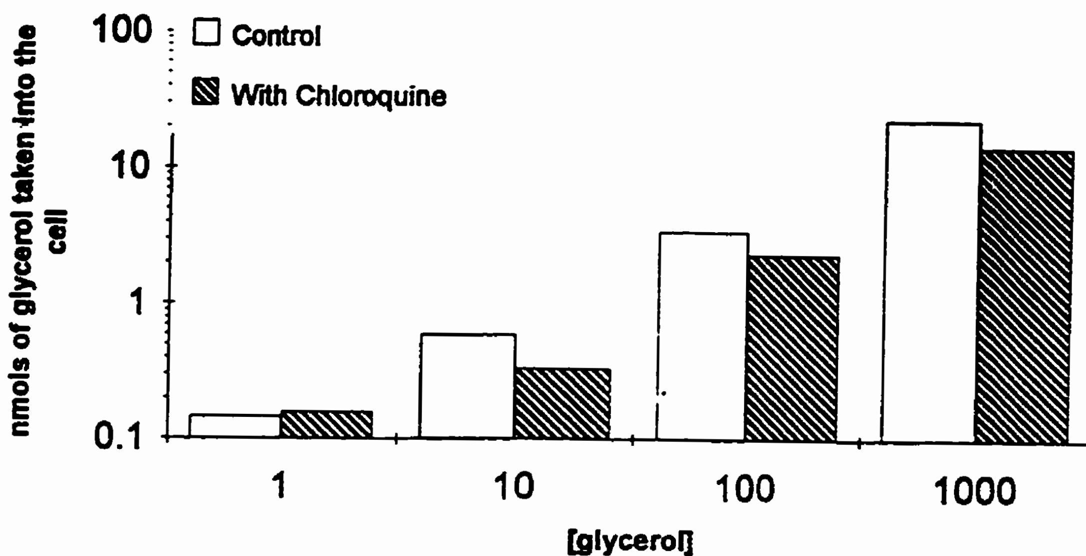


Figure 25: Incubation of H9c2 Cardiac Myoblasts Preincubated with Chloroquine and Various Concentrations of Glycerol. H9c2 cardiac myoblasts were pre-incubated in the absence or presence of 0.1 mM chloroquine for 18 h. They were then incubated with various amounts of [1,(3)-^{3}H] glycerol (1, 10, 100 μM and 1 mM glycerol) simultaneously in the absence or presence of 0.1 mM chloroquine. Cells were harvested and an aliquot was removed for determination of radioactivity. Data report the mean of two experiments.

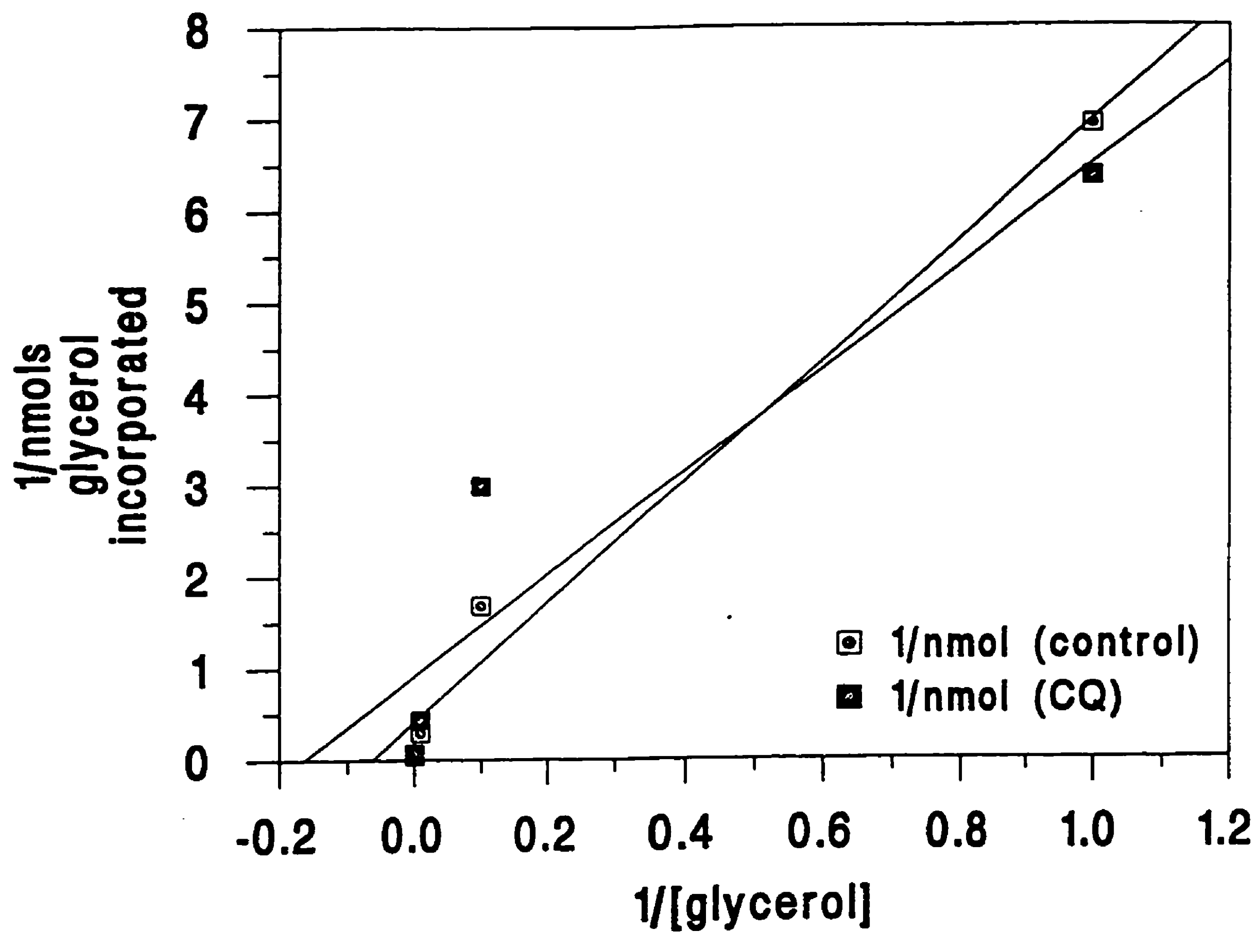


Figure 26: Lineweaver-Burke Plot of Data from Figure 25.

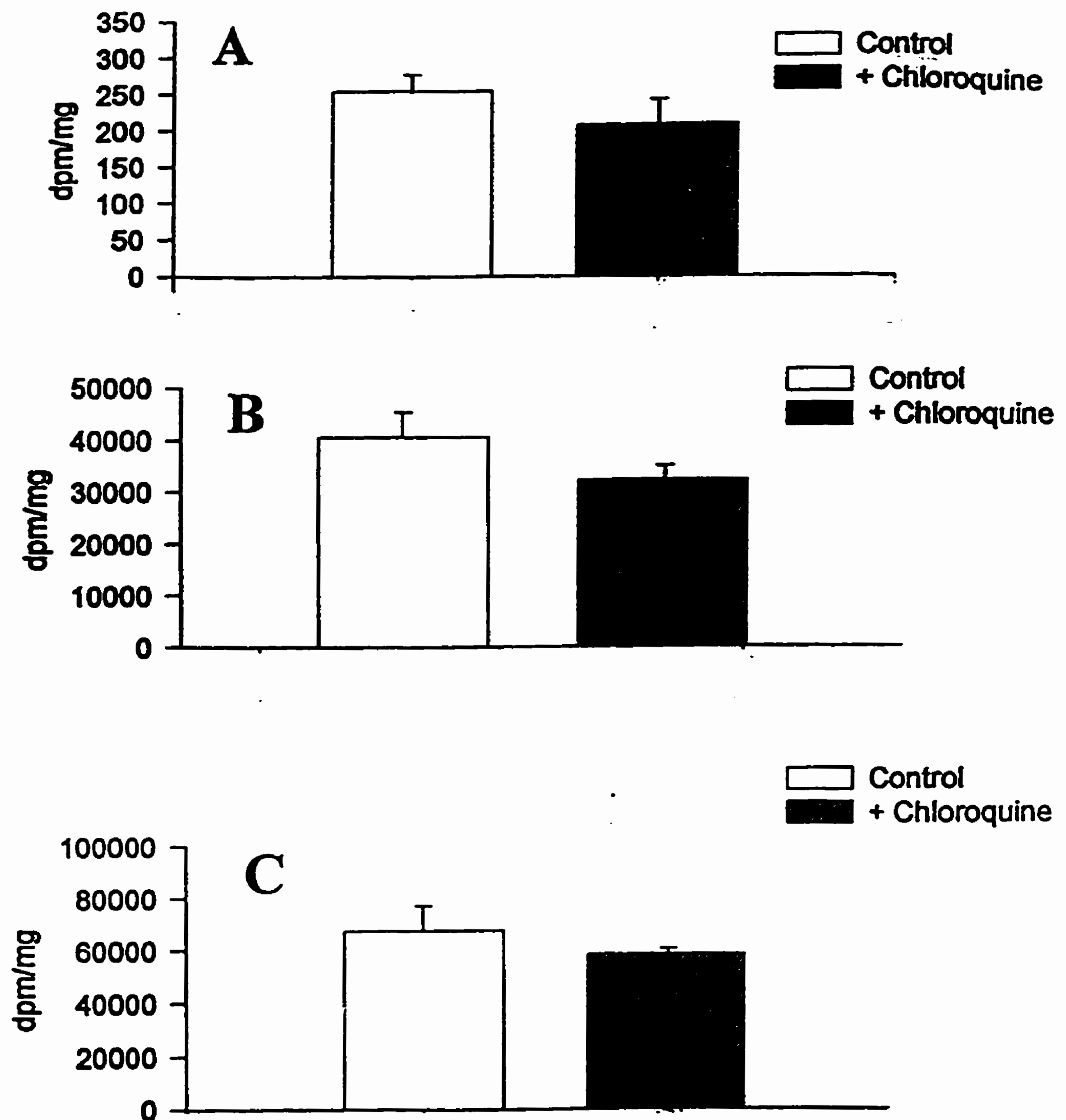


Figure 27: Incorporation of [1-^{14}C]linoleic acid into Cardiolipin (A), Phosphatidylcholine (B), and the Whole Homogenate (C). H9c2 cardiac myoblasts were pre-incubated in the absence or presence of 0.1 mM chloroquine for 18 h. They were then incubated simultaneously with [1-^{14}C]linoleic acid in the absence or presence of chloroquine for 1 h. Data represent the mean ± of three experiments.

DISCUSSION

1) Effects of Ischaemia-Reperfusion Injury and the Application of CSA on Cardiolipin Biosynthesis

The objective of this study was to determine if the mitochondrial permeable transition triggered by ischaemia-reperfusion injury and reversed by 0.2 μM CSA affected CL biosynthesis. To examine this, isolated rat hearts perfused in the Langendorf mode were subjected to 30 min of no-flow ischaemia, followed by 30 min of reperfusion with radioactive glycerol in the absence or presence of CSA. During stabilization, ischaemia and reperfusion, hearts were maintained at a constant temperature of 37°C and a constant flow rate of 2.5 mL/min in order to maintain consistency.

The rate limiting step of CL production is that in which CDP-DG is created from PA and CTP (Hatch, 1994). Previously it was demonstrated that the production of CL requires CTP, a signal for phospholipid biosynthesis (Hatch and McClarty, 1996) and CDP-DG production (Carter and Kennedy, 1968). The production of CTP requires ATP. The permeability transition evoked by ischaemia-reperfusion injury results in a depletion of energy metabolites, specifically ATP. However ATP levels (relative to ADP and AMP levels) are restored in the presence of CSA which reverses pore formation (Griffiths and Halestrap, 1993). From this information one would hypothesize that CL production would mimic the levels of ATP (and CTP). We observed (Fig. 12) that CL production does not drop significantly after reperfusion either in the absence or presence of CSA. These results confirm those of Otani *et al.* (1989) who found that although a drop in radioactive glycerol incorporation into phospholipids occurs when hearts are subjected to reperfusion injury, CL production is unaffected.

Otani's group, however, did not perfuse the hearts in the presence of fatty acid. Lopaschuck *et al.* (1994) showed that hearts subjected to ischaemia-reperfusion injury had a tendency to accumulate fatty acid. This fatty acid accumulation inhibits glucose metabolism and is detrimental to the function of the heart. Lopaschuck's group subjected infant rabbit hearts to ischaemia, and then reperfused the hearts in 0.4 mM palmitic acid, or 1.2 mM palmitic acid in the presence of radioactive glucose and 3% BSA. It was demonstrated that hearts reperfused with the higher fatty acid level showed a decrease in glucose oxidation and function relative to hearts with the lower fatty acid concentration. Cao and Hatch (1995) showed that oleic acid modulates CL biosynthesis in the heart. Levels of 0.6 mM oleic acid bound 1:1 with BSA elevated radioactive glycerol incorporation into CL in the isolated perfused rat heart. We therefore perfused the hearts with oleic acid and BSA concentrations used in Cao and Hatch's experiments. An oleic acid concentration of 0.6 mM was used in control conditions and a concentration of 1.2 mM was used in the pathological condition of reperfusion injury.

Despite the pathological levels of oleic acid used (according to Lopaschuck *et al.* 1994), as well as the 30 min ischaemic insult followed by reperfusion injury and subsequent cellular damage, CL production was unaffected to any significant degree. This may be due to several factors. It is possible that the requirement of the cardiac mitochondria for CL, particularly throughout pathophysiological conditions, is so essential that the production of CL is maintained by a compensatory mechanism. The results of the present study differ from the findings in the study performed by Cheng and Hatch (1995) in which CL production was not compensated for when the heart is subjected to hypoxic perfusion. It is possible that hypoxia, a condition of inadequate oxygen supply resulting in diminished cardiac high-energy phosphate stores, differs from

reperfusion injury in which oxygen is returned to the cell and production of ATP (and therefore CTP) is rapidly restored. This was evident when it was shown that in the rat heart, 30 min of reperfusion following a 30 min period of ischaemia was sufficient to return the energy charge levels to 97% of pre-ischaemic values, even though ATP levels fell to 68% of pre-ischaemic values (Van Bilsen *et al.* 1989). This is in contrast to a hypoxic insult where ATP and CTP levels fell 94% and 92% respectively (Cheng and Hatch, 1995).

Another interesting result is the unaffected CL pool size. This confirms findings that hypoxia does not affect CL pool size (Cheng and Hatch, 1995). This is interesting in view of the fact that reperfusion causes the production of oxygen free radicals, as well as the activation of Ca^{2+} dependent PLA_2. Free radicals peroxidise phospholipids, and PLA_2 removes fatty acid chains from these phospholipids. It is possible that the period of time of ischaemia of the heart was insufficient to induce the reperfusion injury necessary to inflict serious damage to the integrity of the inner mitochondrial membrane. This theory is contrary to studies which suggest that the period of ischaemia, while not damaging in and of itself, was sufficient to result in serious reperfusion damage to the mitochondrion. It is also possible that the time of reperfusion wasn't sufficient to cause noticeable damage to the phospholipid content of the heart by oxygen free radicals or phospholipases. Although the period of reperfusion was likely sufficient to cause some damage to the heart, in our study, significant phospholipid loss did not occur.

2) Effects of Chloroquine on Cardiolipin Biosynthesis in Rat Liver Hepatocytes.

Chloroquine, an important anti-malarial agent, is a cationic amphiphillic drug (CAD), and belongs to a group of drugs that induce phospholipid storage, and are known

to redirect phospholipid biosynthesis from neutral to acidic phospholipids (Koul and Hauser, 1987). Our objective was to determine the effect of CQ on the *de novo* biosynthesis of CL in rat liver hepatocytes. We found that 0.1 mM CQ increases the production of CL as well as CL precursors PA, PG, and CDP-DG relative to controls. Furthermore, production of phospholipids PC, PE, and PS, as well as TG was lowered in the presence of 0.1 mM CQ relative to controls. A concentration of 0.1 mM CQ was used as it was within the range of therapeutic plasma concentrations found in servicemen killed in aircraft crashes in Vietnam (DiMaio and Henry, 1974).

Of course it may be considered that increased autophagy followed by a decrease in lysosomal catabolism may occur, resulting in an accumulation of CL. The effect of the potent autophagy inhibitor 3-methyladenine and CQ on incorporation of [1,3-^{3}H]glycerol into CL was examined in short term incubations of rat liver hepatocytes. The presence of 3-methyladenine had little effect on the production of CL in the absence or presence of CQ. It was determined that 3-methyladenine, and therefore autophagy, had little effect on the stimulation of *de novo* CL biosynthesis in the presence of CQ (Ross and Hatch, 1997).

CQ and other CADs can greatly influence phospholipid synthesis and degradation. There are many reasons for this. CQ is considered a lysomotropic agent and a weak base. Upon entry of the cell, CQ rapidly accumulates into the lysosomes (Hostetler *et al.* 1985). Here it raises the intralysosomal pH (Reasor and Hostetler, 1984). This inhibits pH dependent degradation enzymes. Furthermore, it is known that CQ inhibits the activity of cytosolic phospholipases, mitochondrial PLA_2, and plasma membrane PLA (Loffler *et al.* 1985). CQ also inhibits sarcoplamic reticular PLA_2, and is not an effective

inhibitor of cytoplasmic PLA_2 (Hostetler and Jellison, 1989). By inhibiting these phospholipid degradative enzymes, CQ may cause phospholipids to accumulate. It was also thought that CQ induces phospholipidosis by binding phospholipids, however fluorescence studies have indicated that this does not occur with CQ as it does with other CADs (Joshi *et al.* 1989). It has also been considered that CQ stimulation of phospholipid accumulation requires increased synthesis of cellular proteins (Chen *et al.* 1986). However this was in the presence of 3 μM CQ and lipogenesis at this concentration may involve a different mechanism than at 0.1 mM CQ. Finally, it is thought that CQ may modify certain enzymes and therefore production of phospholipids directly. An example of this would be the inhibition of the phosphatidic acid phosphohydrolase (PAP).

The PAP is an enzyme necessary for controlling the rate of phospholipid biosynthesis. It is an ambiquitous enzyme in that it exists in two forms, cytosolic and membrane bound. Fatty acids activate PAP to match TG synthesis to fatty acid supply by causing PAP to access membranes where it binds, assuming the active form of the enzyme (Martin *et al.* 1986). CADs such as chlorpromazine displaces PAP from ER membranes, decreasing the conversion of PA to DG and TG (Martin *et al.* 1986, Martin *et al.* 1987). PAP exists in 2 isoforms: PAP-1 and PAP-2 (Kanoh *et al.* 1993). These are distinguishable based on sensitivity to N-ethylmaleimide (NEM) and Mg^{2+} dependency. PAP-2 is NEM insensitive, Mg^{2+} independent, regulated by G-proteins, plasma membrane bound, and coupled to phospholipase D. PAP-2 is responsible for signal transduction, however it is not very instrumental in phospholipid synthesis. PAP-1 is soluble, NEM sensitive, Mg^{2+} dependent, and regulates phospholipid synthesis; this is the isoform we are concerned with.

With regard to the effects the presence of 0.1 mM CQ has on phospholipid production, i.e. the increase in production of phospholipids produced via the CDP-DG pathway and decrease in production of DG requiring phospholipids PC and PE, as well as TG, it is possible that CQ acts as other CADs, liberating PAP from the membranes. The mechanism behind this is possibly due to the addition of extra positive charges to the membrane given by the CAD, causing PAP to dissociate from the membrane. This causes PAP to be inactivated and maintains the supply of PA to the CDP-DG pathway, while forbidding PA to be hydrolyzed to DG. Furthermore, we know that PAP translocation to membranes (and subsequent activation) is regulated by fatty acids linoleate, α-linolenate, arachidonate, eicosapentanoate and oleate. Chlorpromazine, a CAD, increases the positive charge on membranes, displacing PAP, resulting in inhibition of the enzyme (Hopewell *et al.*, 1985). Finally, we know that anionic phospholipids, most notably CL, but also CDP-DG and PI have been shown to activate PAP in *Saccharomyces cerevisiae* within range of their cellular concentrations. Conversely, the positively charged sphingoid base sphinganine inhibited PAP activity and diminished the degree of activation by anionic phospholipids (Wu and Carman, 1996). This supports the theory that the positive charge of CQ is responsible for the delocalization of PAP from membranes and the subsequent inactivation of the PAP. Thus it is possible that the increased supply of PA due to the inactivation of PAP is partly responsible for the increased biosynthesis of CL.

It has been shown that an overall increase in acidic phospholipids, including PI and BMP occurs in the presence of CQ and the ratio of acidic to neutral phospholipids was increased 27% (Hostetler *et al.* 1985). This may also be due to decreased hydrolysis of CDP-DG. CQ inhibits the activity of the CDP-DG hydrolase (Taylor and Hatch,

unpublished results). This enzyme works in opposition to the PA:CTP cytidylyltransferase in that it converts CDP-DG to PA. When CDP-DG hydrolase is inhibited by CQ, CDP-DG may be rerouted to PG, CL, and eventually BMP biosynthesis. This may help to explain why radioactive PA is so rapidly depleted in cells incubated in CQ. The excess PA is used up quickly as little is recycled back into the pool as a result of the activity of the CDP-DG hydrolase. Finally, we know that PA donates a negative charge to the membranes, stabilizing PAP on the membrane, and that negative charges and fatty acids promote the binding of the choline-phosphate cytidylyltransferase to membranes (Walton and Possmayer, 1986). It may be possible that the positive charge of CQ would displace the choline-phosphate cytidylyltransferase from the membranes, resulting in lowered production of PC and LPC.

It is not surprising that there was not an overall increase of [1-^{14}C] linoleic acid into phospholipids considering that CQ inhibits phospholipases, decreasing production of lysophospholipids which may be used as substrates for acyltransferases and transacylases. CQ also inhibited the synthesis of long-chain fatty acyl coenzyme A from palmitic acid and other fatty acids in rat brain microsomes (Reddy and Bazan, 1985). At any rate, there was no net change in [1-^{14}C] linoleic acid incorporation into phospholipids.

3) Effects of Chloroquine on Glycerol Uptake in H9c2 Cardiac Myoblasts

As CQ was so effective at regulating phospholipid biosynthesis in rat liver hepatocytes, we wanted to determine its effect on an undifferentiated mammalian heart cell line. We incubated H9c2 cells over a period of 2 hours with radioactive glycerol in the absence or presence of 0.1 mM CQ. CQ had little noticeable effect, so we pre-incubated the cells for 2 h with CQ and then began our 2 h incubations with radioactive

glycerol. Here we began to see an effect as glycerol did not access the cell as readily. The effect was even more dramatic when the cells were incubated overnight (18 h) in the absence or presence of 0.1 mM CQ, followed by a 2 h incubation with radioactive glycerol in the absence or presence of CQ. Here we observed an inhibition of glycerol uptake. We examined the concentration of glycerol versus radioactive glycerol uptake and observed that above physiological glycerol levels, there was a mixed-type inhibition of glycerol uptake by CQ.

The inhibition of glycerol uptake by CQ in H9c2 cardiac myoblasts is interesting when it is considered that where CQ poisoning has been implicated in the death of a person, the cause of death is typically heart failure (DiMaio and Henry, 1974). CQ has been known to inhibit the uptake of ethanolamine and choline in Jurkat T cells (Pelassy and Aussel, 1993). Glycerol uptake in various tissues has been examined, although a concentrative study has not been performed on any mammalian tissues yet (Li and Lin, 1983). Studies by Vom-Dahl and Haussinger (1997) have shown that the permeability of the perfused rat liver to glycerol is high, even comparable to water. There is a rapid equilibration of glycerol between the intra and extracellular space as shown by the increase in liver mass caused by the addition of glycerol. In liver cells, the transport of glycerol is quite specific as the glycerol-related compound 1,3-propanediol had little effect on liver mass. Li and Lin (1983) have shown that the entry of glycerol into isolated rat hepatocytes is catalyzed by a specific carrier and is both saturable and sensitive to inhibition by monoacetin and cytochalasin B. In human red cells, glycerol transport occurs by 2 mechanisms: 1) facilitated diffusion with permeability depending on glycerol concentration. This is a saturable and substrate specific pathway, and 2) an unspecified pathway with permeability independent of glycerol concentration (Carlsen

and Wieth, 1976). Cohen (1968) has also shown through copper inhibition studies that glycerol diffuses across the membranes of erythrocytes by active transport through primary pores and by passive diffusion through secondary pores. These two mechanisms for glycerol transport have been shown to exist at the brush border where glycerol accesses the cell by diffusion and a saturable carrier mediated mechanism (Rubin and Deren, 1974). Surewicz *et al.* (1981) looked at the stabilization properties of propanolol, a CAD, on red-cell membranes and liposomes. Incubation of red-cells with propanolol was shown to promote passive diffusion of glycerol into bovine red-cells, however propanolol inhibited the facilitated uptake of glycerol into human red-cells. If it is assumed that glycerol uptake into H9c2 cells occurs in the same way as human red-cells, that CQ inhibits facilitated diffusion of glycerol in the same way as propanolol, this may explain the inhibition of glycerol uptake that was observed in H9c2 cells. Furthermore, propanolol was shown to stabilize and protect membranes from osmotic lysis by direct drug-lipid interactions. CQ, like other CADs affiliates itself with the membrane, donating a positive charge to the membrane. Perhaps this alignment causes a decrease in passive glycerol uptake as well.

[1-^{14}C] Linoleic acid incorporation into H9c2 cardiac myoblasts was unaffected by CQ. This finding agrees with the study performed by Reddy and Bazan (1985). In that study, similar [U-^{14}C] linoleic acid uptake rates between control cells and those incubated with CQ was observed.

SUMMARY

In the first part of the study, isolated perfused rat hearts were used to study the effects of the mitochondrial permeability transition pore created by ischaemia-reperfusion injury on cardiolipin biosynthesis. We also wanted to evaluate the effects of the application of the pore inhibitor CSA on cardiolipin biosynthesis in the perfused rat heart. The results indicate that neither reperfusion injury nor CSA had any effect on cardiolipin biosynthesis, or pool size. In the second part of the study, the effects of chloroquine on cardiolipin biosynthesis was assessed in rat hepatocytes and H9c2 cardiac myoblasts. Chloroquine caused an increase in *de novo* biosynthesis of phospholipids of the CDP-DG pathway at the expense of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol in rat liver hepatocytes. This is likely due to the ability of chloroquine to inhibit the phosphatidic acid phosphohydrolase, as well as the CDP-DG hydrolase. In H9c2 cardiac myoblasts, chloroquine inhibited the uptake of glycerol in a mixed inhibition fashion. This indicates that there is likely a glycerol transporter in the H9c2 membrane which may be similar to the glycerol transporter in human red blood cells.

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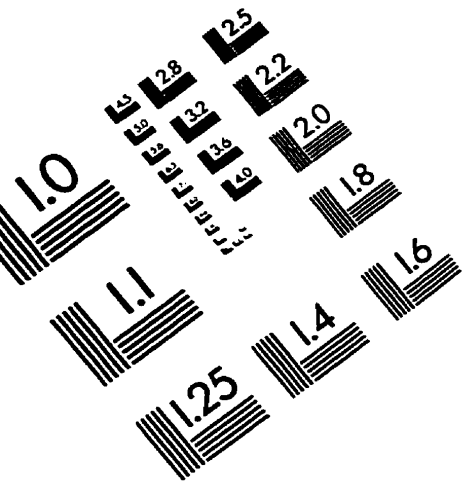
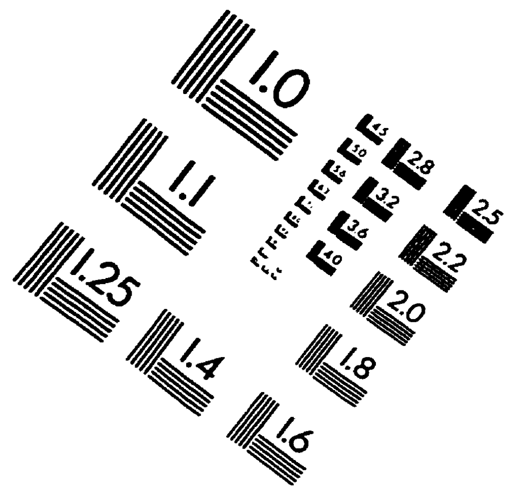
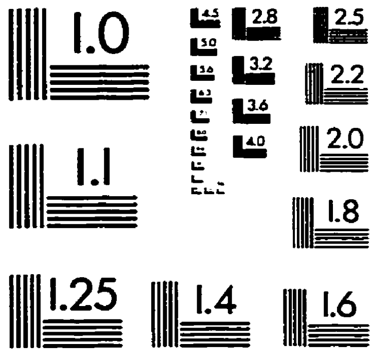
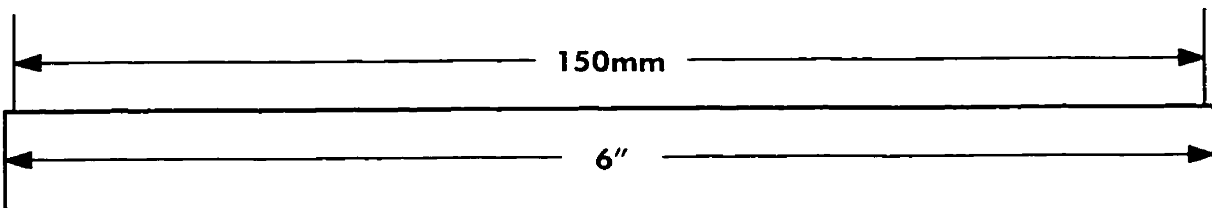
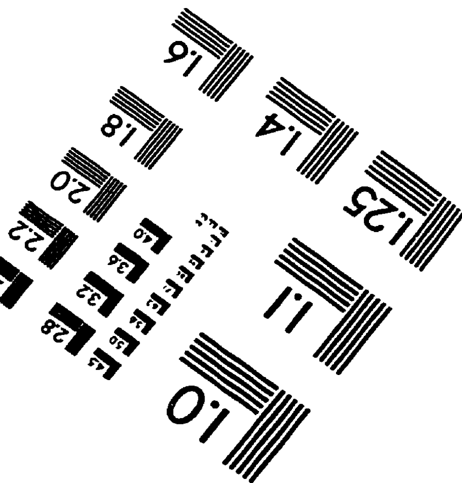
IMAGE EVALUATION TEST TARGET (QA-3)



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