BIOCHEMICAL ALTERATIONS OF LYSOPHOSPHOLIPIDS IN THE ISCHEMIC CANINE MYOCARDIUM DURING ACUTE ARRHYTHMIAS

BY

MARC P.J. PELLETIER

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

Department of Biochemistry
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To my aunt, Suzanne Pelletier, my mother and my sister Louise, a big kiss for their determined effort in typing this thesis and other important documents.

Finally, to my wife and daughter, an extra special thank you for the hard work and patience with which you endured me for the past two years. Without your support this end could not have been realized.

For Louise and Pier-Andree

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ABSTRACT

The perpetuation of malignant cardiac arrhythmias after the onset of ischemia is well documented. However, the exact biochemical cause(s) for these electrophysiological disturbances remain(s) unclear. Recently several investigators have reported an accumulation of lysophosphoglycerides in the ischemic myocardium. The production of cardiac arrhythmias by exogenous lysophospholipids in isolated perfused mammalian hearts is also well documented. Due to the various lipid extraction procedures used, different lysophospholipid levels have been reported in the mammalian heart. In this study we present an improved lysophospholipid extraction and estimation procedure, and report the lysophospholipid content of the canine heart. Ischemia in the canine heart was produced by a two-stage occlusion of the left anterior descending coronary artery. Severe arrhythmias were observed at 24 h after occlusion. Intracellular lysophosphatidylcholine and lysophosphatidylethanolamine levels were elevated by more than 2-fold at 24 h. The enzymes responsible for the synthesis and catabolism of lysophospholipids in the ischemic tissues were assayed and compared with those in control tissues. There was no significant change in lysophosphatidylcholine: acyl CoA acyltransferase activity, whereas an increase in the phospholipase A activity was detected. A significant decrease in the activities of lysophospholipase and transacylase was observed in the ischemic tissue. Hence, the accumulation of lysophospholipids during ischemia with acute arrhythmias is probably due to an enhancement of synthesis and a reduction in the rate of catabolism of these lysophospholipids.

ABBREVIATIONS

ACS	aqueous counting scintillant
ATP	adenosine 5' triphosphate
ANSA	1-amino-2 naphthol 4 sulfonic acid
C	degrees Celsius
cAMP	adenosine 3' - 5' monophosphate
coA	coenzyme A
FA	fatty acid
g	gram
g	gravitational force
GPC	glycerophosphocholine
h	hour
kg	kilogram
LPC	lysophosphatidylcholine
M	molar
mM	millimolar
min	minute
mg	milligram
ml	millilitre
N	normal
nm	nanometre
nmol	nanomole
pmol	picomole
S	second
TLC	thin layer chromatography
ul	microlitre
uM	micromolar
umol	micromole
U.V.	ultraviolet
v	volume
W	weight
%	percent

INTRODUCTION

I. THE BIOLOGICAL MEMBRANE

a) Structure and Function

Biological membranes are indispensable entitities for proper cellular function and life. They are organized non-covalent assemblies composed of proteins embedded in a matrix of lipids as proposed by Singer and Nicolson (1972) (Fig. 1). Membranes are sheetlike structures that form highly selective permeability barriers between organelles and cells. Membranes contain electrically charged surface groups, which help support a difference of electrical potential across the membrane structure. Membranes are fluid structures that allow rapid diffusion of lipids and proteins unless these are anchored by specific interactions. Membranes are fluid because the hydrophobic tails of their polar lipids consist of an appropriate mixture of saturated and unsaturated fatty acids that is fluid at the normal temperature of the cell. Cholesterol also is a key regulator of membrane fluidity by fitting between the fatty acyl chains and preventing their crystallization. The biological membrane has many functions. Other than its obvious structural role, it plays a central role in biological communication. The biological membrane contains specific receptors for external stimuli such as hormones. Some membranes generate signals which can be chemical or electrical. The

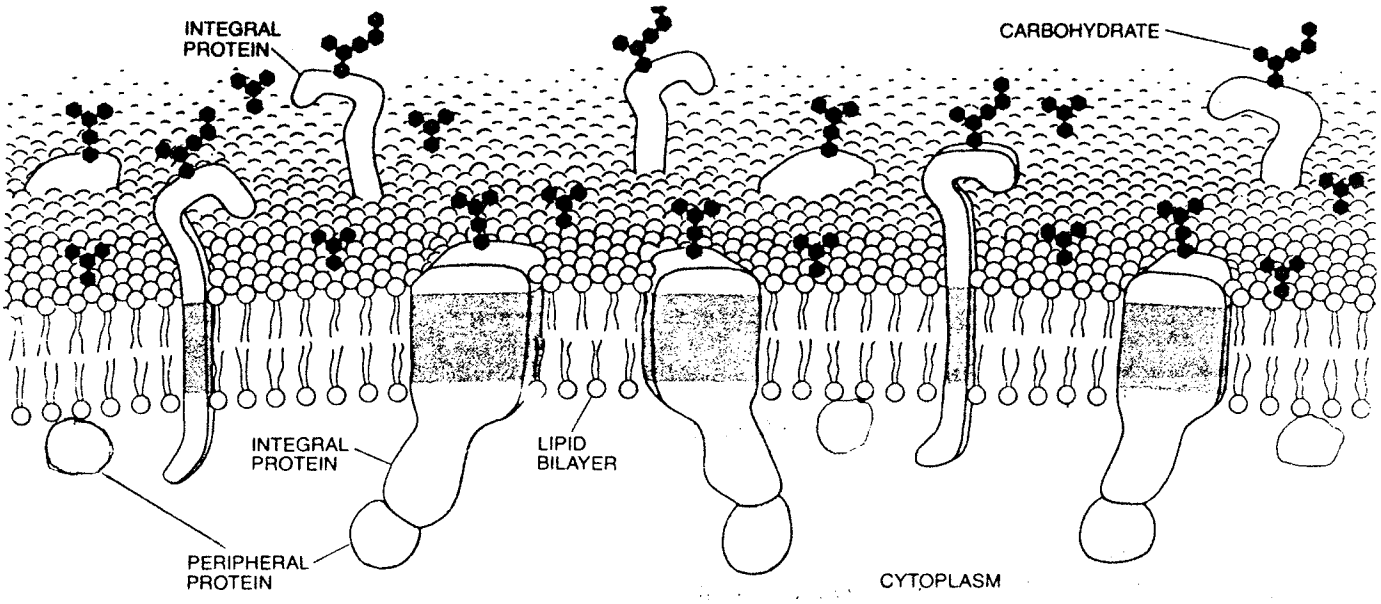


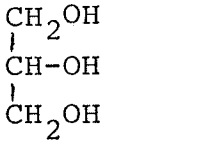
Fig. 1 Model of Plasma Membrane includes proteins and carbohydrates as well as lipids. Integral proteins are embedded in the lipid bilayer. Peripheral proteins are merely associated with the membrane surface. The carbohydrates consist of simple sugars strung together in chains that are attached to proteins (forming glycoproteins) or to lipids (forming glycolipids). The asymmetry of the membrane is manifested in several ways. Carbohydrates are always on the exterior surface and peripheral proteins are almost always on the cytoplasmic surface. The two lipid monolayers include different proportions of the various kinds of lipid molecule. Most important, each species of integral protein has a definite orientation which is the same for every molecule of that species (Lodish and Rothman 1979).

biological membrane also provides sites for isolation of many biochemical processes. Photosynthesis and oxidative phosphorylation, two very important energy conversion processes are carried out by membrane systems. (Stryer 1981).

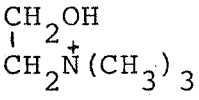
b) Phospholipids Are Important Entities of the Biological Membrane

Phospholipids are the major class of membrane lipids. The phospholipid types as well as the proteins are asymmetrically distributed in the biological membrane (Op den Kamp 1979; Vance et al 1977; Bell et al 1981). The maintenance of this assymetry as well as the maintenance of the phospholipid composition is essential for cellular integrity and proper function.

Phospholipids derived from glycerol are called phosphoglycerides (Fig. 2). A phosphoglyceride consists of a glycerol backbone, two fatty acid chains and a phosphorylated alcohol. The fatty acid chains usually contain an even number of carbon atoms. These chains may be saturated or unsaturated and are ester linked to the glycerol backbone. In mammalian hearts and brains, a good proportion of the fatty acid chains are ether-linked to the first carbon of the glycerol backbone, thus forming a phospholipid plasmalogen. Hydrolysis of one of the fatty acid chains yields a lysophosphoglyceride.



glycerol



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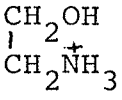
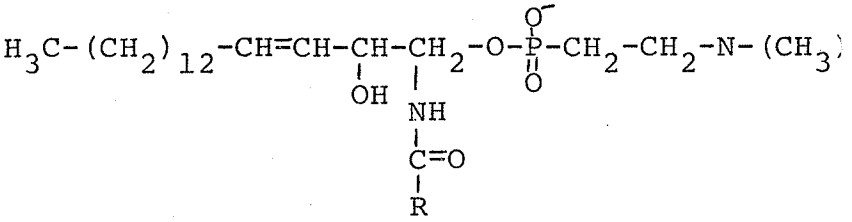
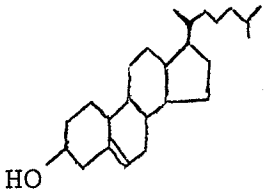


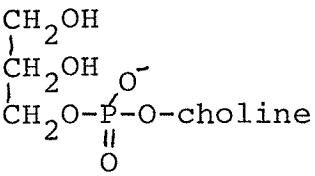
ethanolamine



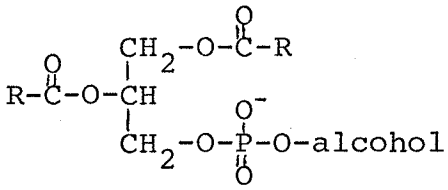
sphingomyelin



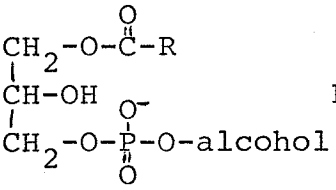
cholesterol



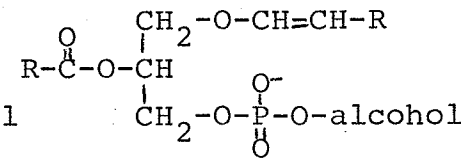
glycerophosphocholine



phosphoglyceride



lysophosphoglyceride



phosphoglyceride plasmalogen

Fig. 2 Structures of the major lipids.

c) Lysophosphoglycerides Are Phospholipids with Unique Functional Characteristics

Due to their structural characteristics, lysophosphoglycerides are amphiphiles with polar heads and non polar fatty acyl chains. These fatty acyl chains may be ether (plasmalogen) or ester linked to the glycerol backbone. Lysophosphoglycerides are therefore phospholipids with unique functional characteristics. Lysophosphatidylcholine is the major lysophosphoglyceride (White 1972) and it is soluble in both aqueous and organic solvents. Lysophosphatidylcholine exists in monomeric form in solution at low concentrations. However at concentrations exceeding the critical micellar concentration of the phospholipid, lysophosphatidylcholine forms micelles. The critical micellar concentration depends on fatty acyl chain length as well as temperature (Katz and Messineo, 1981). Lysophosphatidylcholine is a detergent and therefore aids in the aqueous dissolution of the other phospholipids such as phosphatidylcholine (Helenius and Simons, 1975).

The cytolytic nature of lysophosphatidylcholine has drawn much attention in the past few years (Weltzein, 1979). The presence of lysophospholipids in most biological membranes have been demonstrated. Although lysophospholipids make up a significant component of blood plasma, the levels of lysophospholipid found in most biological membranes are very low making up 1-3% of the total

phospholipid content of these membranes (White 1972). The levels of lysophospholipid in biological membranes were demonstrated to be below the critical cytolytic concentration of lysophospholipids (White 1973). However when lysophospholipids were present in large enough quantities lysis of the biological membrane occurred (Weltzien, 1979).

The roles of lysophospholipids in cellular membranes are not restricted to membrane lysis. Their roles are in fact very widespread. The rapid turnover of lysophospholipid in the biological membrane has been well established (Brockerhoff and Jenson 1974; Robertson and Lands 1964; Van den Bosch and Van Deenen 1966). However, the appearance of lysophospholipids in multiple biological functions suggests that they are more than simply intermediates in phospholipid metabolism. The following effects of lysophospholipids were caused by variations of lysophospholipid concentrations well within their critical cytolytic concentration. Lysophosphatidylcholine has been speculated to be involved in cell fusion and cell shape. Lucy and co-workers (1970) first detected a lysophosphatidylcholine-mediated cell fusion but its involvement in systems in vivo is unclear. The contribution of lysophosphatidylcholine in determining the shape of human erythrocytes of various cholesterol content was examined by Lange and Slayton (1982). This effect was first observed in 1935 (Bergenham and Farhraeus, 1935). Studies conducted in the early 70's revealed that a slight increase in membrane lysophospholipid

resulted in a loss of the biconcave shape of the erythrocyte which adopted a highly crenated echinocytic form (Sato and Fujii 1974; Lichtman et al 1974; Marikovsky et al 1976). This effect was accompanied by an increased in osmotic fragility. The original shape was restored by washing the cells with albumin (Klibanksy and de Vries, 1963). The reasons for these effects of lysophospholipids are still not clear although many explanations have been proposed (Sheetz et al 1976; Painter et al 1975; Lawrence et al 1974; Weltzein 1979; Marikovsky et al 1976).

A wide variety of membrane-associated enzymes have been reported to be affected by lysophosphatidylcholine. Rat liver phenylalaninehydroxylase (Fisher and Kaufman, 1973), alkaline phosphatases (Hung and Melnykovych, 1976 and 1977), CDP synthesis in rat intestinal mucosa (Odoherty et al, 1977) and 3', 5' cyclic nucleotide phosphodiesterase (Pichard and Cheung, 1977) were all shown to be stimulated by lysophosphatidylcholine. More recently, lysophosphatidylcholine had been shown to inhibit rabbit and dog heart sarcolemmal Na^+ K^+-ATPase activity (Karli et al 1979). An augmentation of cAMP content was induced by lysophosphatidylcholine in rabbit hearts (White and Lad, 1975). This could lead to further changes in cellular reactivity. Lysophosphatidylcholine has also been shown to potentiate Ca^{++}-accumulation in rat cardiac myocytes (Sedlis et al 1983). Perfusion of the cat gallbladder in the presence of 1mM lysophosphatidylcholine in the perfusate resulted in

the immediate contraction of the gallbladder associated with a change in net fluid transport (Neiderhiser 1983).

From these observations it is easy to see how even small variations of lysophospholipid content in a biological system could result in further signals with respect to membrane structure, lipid dependant enzymes, etc. For this reason the level of lysophospholipids is well regulated by lipid metabolizing enzymes which are themselves subject to regulation by inhibitors and activators. The metabolism of lysophosphatidylcholine is depicted in Fig. 3.

II. LYSOPHOSPHATIDYCHOLINE METABOLISM

a) Lysophosphatidylcholine: acyl CoA acyltransferase

The acylation of lysophosphatidylcholine by lysophosphatidylcholine: acyl CoA acyltransferase was first noted by Lands (1960). This enzyme specifically catalyses the esterification of a fatty acyl CoA with a lysophosphatidylcholine molecule to form phosphatidylcholine. The formation of the fatty acyl CoA requires ATP and a CoA. This enzyme is usually found to be more active in the microsomes than any other subcellular membranes of mammalian tissues (Gross and Sobel 1982; Van Heusden and Van den Bosch 1981).

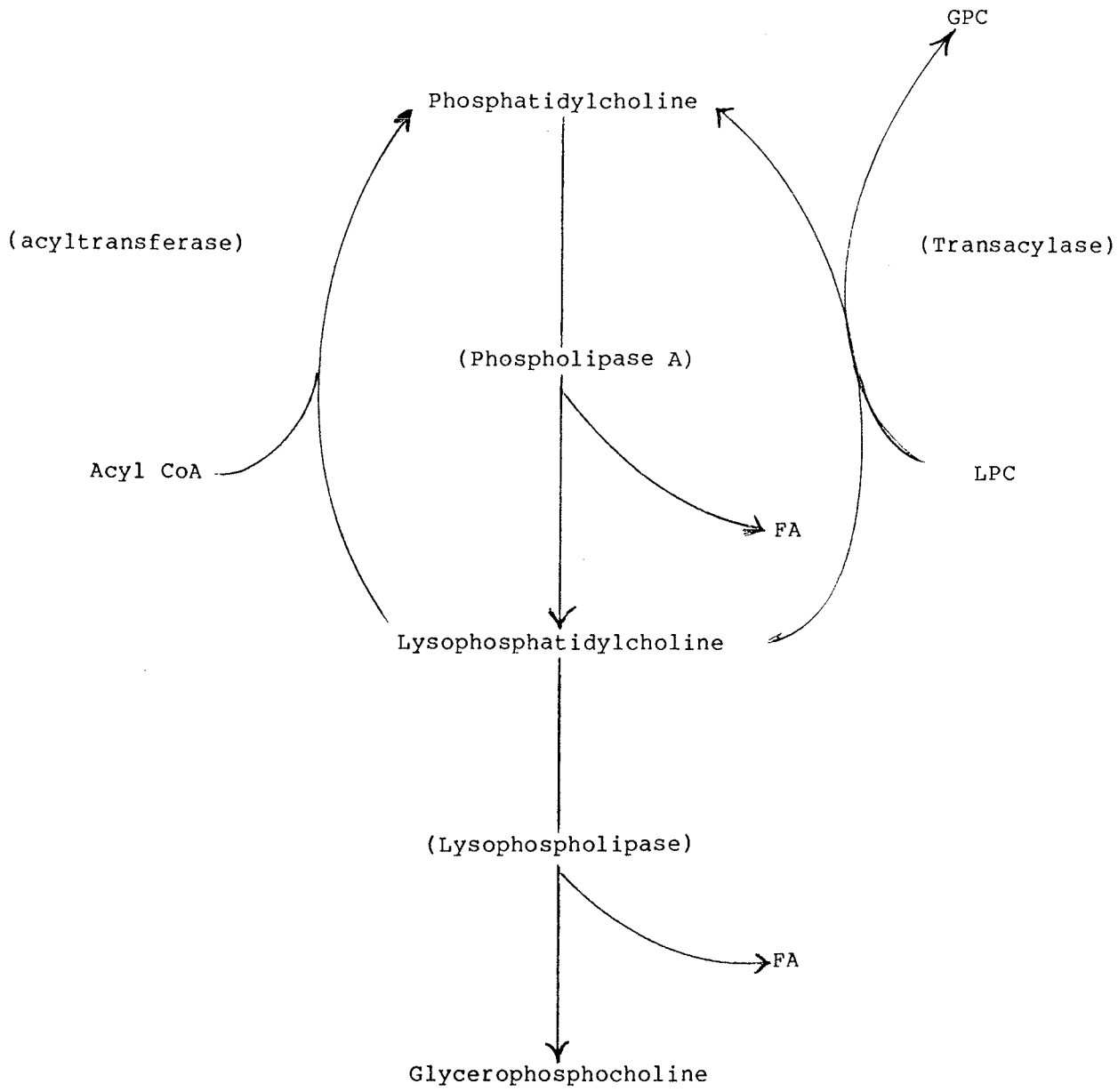


Fig. 3 Lysophosphatidylcholine Metabolism in Canine Heart

b) Lysophosphatidycholine: Lysophosphatidycholine Transacylase

The transacylation of lysophosphatidylcholine catalysed by lysophosphatidylcholine: lysophosphatidylcholine transacylase was discovered by Erbland and Marinetti (1965) and Van den Bosch et al (1965). This reaction requires two molecules of lysophosphatidylcholine and yields phosphatidylcholine and glycerophosphocholine. This enzyme does not require ATP, CoA or acyl CoA. The absence of an energy requirement suggests that this mechanism of lysophosphatidylcholine reacylation is not as important as the acyltransferase activity under conditions when ATP levels are high. However, in energy-starved tissues this enzyme's activity could prove to be significant. This activity has been found to co-purify with a lysophospholipase activity and has been shown to be localized in the cytosol of some mammalian tissues (Gross and Sobel 1982; Van Heusden and Van den Bosch 1981; Casals et al 1982).

c) Lysophospholipase

Lysophospholipase catalyses the hydrolysis of acyl ester bonds in lysophosphoglycerides to yield free fatty acids and glycerophosphocholine. This enzyme activity is ubiquitous in nature and its presence has been found not to be restricted to a single subcellular site in eucaryotic cells (Marples and Thompson 1960; Van den Bosch et al 1968; Erbland and Marinetti 1965; Bjornstad 1966). This enzyme has been shown to have unusual kinetics when reaction rates were plotted as a function of substrate concentration in rat

brains and rabbit heart cytosol. The resulting biphasic curves suggests that the enzyme may use the monomers and probably not the micelles of the substrate (Gatt et al 1972).

d) Phospholipase A

The hydrolysis of one of the fatty acyl chains of the phosphatidylcholine molecule yields a lysophosphatidylcholine molecule. This reaction which is catalysed by either phospholipase A_1 or A_2 represents the major biosynthetic pathway for the production of lysophosphatidylcholine. The phospholipase A_1 specifically catalyses the hydrolysis of the fatty acid ester bond in the first position of the glycerol backbone. The phospholipase A_2 specifically catalyses the hydrolysis of the fatty acid ester bond in the second position of the glycerol backbone.

Phospholipases have been shown to be ubiquitously distributed in nature (Robertson and Lands 1964; Van den Bosch 1974; Pfeiffer et al 1979). They have been grouped into three classes based on their origin: venom phospholipases, digestive phospholipases and intracellular phospholipases occurring in the tissues of animals. The action of phospholipase A (A_1 and A_2) in the mammalian heart has been reported, however the activity was very low (Corr et al 1982). The low observed activity may be real or may have resulted from a poor enzyme substrate interaction. The dissolution of phosphatidylcholine in an aqueous medium is very

difficult due to the hydrophobic fatty acyl chains on the phosphatidylcholine molecule. Also the endogenous phosphatidylcholine pool is very large, therefore observed phospholipase activity may only be a small reflection of the real total activity.

Phospholipase A activity in mammalian tissues has been found in cardiac sarcolemma and in the mitochondrial and microsomal membranes (Franson et al 1978; Weglicki et al 1971; Waite and van Deenen 1967). Phospholipase A_2 of the dog cardiac sarcolemma had an alkaline (8.5) pH optimum and a Ca^{++} requirement. Phospholipase A_1 and A_2 activities were found in myocardial microsomal fractions. They both had pH optimums of 7.5. Many factors have been shown to influence phospholipase activity. A decreased blood supply to many organs has been shown to activate their phospholipase A activity although the exact mechanisms are not known (Edgar et al 1982; Chien et al 1978). In rat blood platelets phospholipase A activity was shown to originate from the association of an "activable" phospholipase and an unknown "activating factor" (Etienne et al 1982). Phospholipases A from other mammalian tissues were found to be modulated by such factors as bradykinin, calmodulin, mepacrine, adrenergic agonists as well as other factors (Wichert and Meyer 1983: Chan et al 1982; Thomas et al 1981; Weglicki et al 1971; Franson et al 1979).

Phospholipase A has been shown to affect many other biochemical phenomena such as an increased spectrin-actin dissociation of human erythrocyte membranes (Gottlieb 1982), decreased depolarization – dependant Ca^{++}-uptake of brain synaptosomes (Noremberg and Lazarewicz 1982), inactivation and solubilization of opiate receptors (Ruegg et al 1982), as well as others. The exact mechanisms are not known, however, it has been hypothesized that a membrane disfunction was involved in all cases. This membrane disfunction may have been caused by the phospholipase itself or by the products resulting from action of phospholipase A.

III. ISCHEMIA AND CARDIAC ARRHYTHMIA

a) Ischemia Affects the Normal Electrophysiology and Biochemistry of the Heart

Severe disturbances of cardiac rate and rhythm account for the majority of deaths secondary to coronary artery disease (Armstrong et al 1972). The perpetuation of malignant cardiac arrhythmias after onset of ischemia is well documented (Bigger et al 1977; Elharrar and Zipes 1977; Williams et al 1974). Many biochemical and physiological changes have been observed during ischemia. However the exact biochemical causes for these disturbances in cardiac rhythm remain obscure. Coronary sinus plasma potassium levels are elevated after ligation of a coronary vessel (Case et al 1969). This loss of intracellular potassium

appears to be a general tissue response to hypoxia. Myocardial loss of tissue magnesium and an influx of sodium parallel this loss of potassium (Downar et al 1977). The primary metabolic disturbance in myocardial ischemia is related to an inadequate supply of oxygen. The heart must shift to an anaerobic metabolism resulting in an increase in lactic acid (Downar et al 1977). There is also evidence of a depletion of glycogen and ATP as well as a hydrogen ion efflux (Vial et al 1982; Hollis et al 1978). Coronary sinus blood pH becomes markedly acidic in severe ischemia (Tait et al 1982). This presumably causes a myocardial acidosis which may inhibit enzymatic activity. From an electrophysiological viewpoint, one has observed such changes as reduction of action potential duration and amplitude, and an increased refractory period (Elharrar and Zipes 1977). Therefore a membrane defect is indicated. In 1976, Eugene Downar's group obtained blood from a local coronary occlusion (Downar et al 1977). He compared the effects of the "ischemic" blood to that of control blood on transmembrane potentials of muscle strips taken from the same heart. Whereas the control blood had no effect on the tissue physiology, the "ischemic" blood produced electrophysiological changes mimicking those caused by coronary occlusion. These effects could not be attributed to changes in potassium concentration even in combination with acidosis, hypoxia and hypoglycemia. Therefore it appeared that an unknown metabolite was being released into the venular effluent of the ischemic area of the heart that had potent depressant effects on the excitability of

the myocardium. This study lead to further evaluation of the potential role of several classes of metabolites that may mediate the electrophysiological derangements observed upon the onset of ischemia (Watanabe et al 1982; Sugiyama et al 1982; Katz and Messineo 1982).

b) Ischemia Results in an Accumulation of Lysophosphoglycerides

Recently, several investigators have reported on accumulation of lysophosphoglycerides in the ischemic myocardium of several mammalian species. In 1978, Sobel et al first reported a 69% increase in lysophosphatidylethanolamine and lysophosphatidylcholine 60 min after occlusion of the left anterior descending coronary artery of the rabbit heart. In 1981, Shaikh and Downar reassessed Sobel et al's findings (1978) and found that Sobel et al's lipid extraction procedure artifactually produced lysophospholipid. In their study of the ischemic porcine myocardium, Shaikh and Downar reported lysophospholipid levels to be approximately 25 times lower than those reported by Sobel, however they also observed a 70% increase in the lysophospholipid levels in ischemic areas. Other investigators also had reported sgnificant increases in lysophospholipid levels in other mammalian ischemic myocardia (Vasdev et al 1979; Chien et al 1981). The question remained as to whether the lysophosphatidylcholine was arrhythmogenic and whether the levels of lysophosphatidylcholine found in the ischemic tissues were significant enough to elicit the electrophysiological alterations observed in the ischemic myocardium.

The cytolytic and membrane perturbing properties of lysophosphatidylcholine (Weltzein 1979) made this metabolite an attractive candidate as a generator of cardiac arrhythmia. In experiments conducted in 1982, Man et al (1983) studied whether lysophospholipid could have a direct effect on the production of cardiac arrhythmia. Hamster hearts were perfused with Krebs-Henseleit buffer (1932) in the presence of various concentrations of lysophospholipid as well as other compounds in order to assess arrhythmogenic nature of these compounds. All lysophospholipid were found to be arrhythmogenic. Phosphatidylcholine and glycerophorycholine (the immediate metabolites of lysophosphatidylcholine) were found to have no effect on cardiac rhythm. Free fatty acids were also shown not to produce the significant alterations of the transmembrane action potential produced by lysophospholipids. Since lysophospholipids are detergents, the effects of some common detergents were also investigated. Both detergents studied proved to be arrhythmogenic in the same concentrations as lysophosphatidylcholine. It was postulated that the lysophospholipid, which are transiently associated with the sarcolemma prior to their transport into the cells, alter the characteristics of the sarcolemma during their brief association with the membrane. Changes in membrane fluidity by detergent-like agents could cause the observed depression of action potentials which is a prerequisite for cardiac arrhythmia.

Lysophosphatidylcholine was shown therefore to cause arrhythmias but it was not known as to whether or not physiological levels of lysophospholipid would suffice to produce arrhythmia. This question was investigated by Man and Choy (1982). Hamster hearts perfused in the Langendorff mode (1895), in the presence of as little as 0.02 mM lysophosphatidylcholine developed cardiac arrhythmia. This concentration was lower than the 0.29 mM lysophosphatidylcholine concentration found in hamster serum. However, the hamster serum was not arrhythmogenic. It was found that all of the serum lysophosphatidylcholine was protein bound and that when bound to protein this phospholipid was not arrhythmogenic. Only the interstitial free lysophosphatidylcholine was arrhythmogenic. It was therefore postulated that the level of total lysophospholipid had to exceed the binding capacity of serum proteins and that free lysophospholipid levels had to surpass a threshold concentration before the production of cardiac arrhythmia was observed.

During the past five years other metabolites that could potentially mediate electrophysiological alterations induced by ischemia have been evaluated by several investigators but the lysophospholipids have gained much support as the specific mediators of cardiac arrhythmia. Reduction of pH to 6.7, comparable to that seen early after the onset of ischemia <u>in vivo</u>, resulted in a 3-fold

increase in sensitivity to the electrophysiological effects of exogenous lysophosphatidylcholine in perfused tissues (Corr et al 1981). The content of lysophosphatidylcholine in effluents from ischemic cat heart increased by 88% to levels sufficient to induce marked electrophysiological changes when the pH was reduced to 6.7 (Snyder et al 1981).

Lysophospholipids are amphiphilic and are therefore readily incorporated into membranes. Therefore, it is not surprising that exposure of the sarcolemma to lysophosphatidylcholine impairs membrane function, when one considers its various membrane perturbing properties described previously. Increased levels of lysophosphatidylcholine in effluents from ischemic zones suggests that there is a considerable amount of lysophosphatidylcholine located extracellularly thereby having direct access to the external surface of the sarcolemma. Studies conducted by Gross et al (1982) indicated that incorporation of as little lysophosphatidylcholine as 1% of total cellular phospholipid induced marked electrophysiological changes mimicking those of ischemia. Reversal of these electrophysiological effects was associated with a decrease in sarcolemal lysophosphatidylcholine and an increase in phosphatidylcholine and free fatty acids.

IV. RESEARCH AIMS

From observations reported in 1978, Sobel and Corr (1979) derived their lysolipid hypothesis. After short periods of occlusion of the left anterior descending coronary artery, they observed significant increases in lysophospholipids and declines in phosphatidylcholine and phosphatidylethanolamine. They believed that the accumulated lysophospholipids were derived from the catabolism of the parent membrane phospholipid. They suspected that certain factors, such as a change in pH or a depletion in ATP resulted in the activation of phospholipases or a decrease in phospholipid susceptibility toward phospholipases (Gazitt *et al* 1975). The lysophospholipids released were potentially capable of producing sarcolemmal damage in the ischemic heart due to their detergent properties. Damage to the sarcolemma would have resulted in the electrophysiological changes observed in the ischemic heart. The elevation of lysophospholipid was confirmed by a number of independent studies, however a discrepancy arose about the exact level of myocardial lysophosphatidylcholine concentration. Shaikh and Downar (1981) reported that the mode of lipid extraction used by Sobel *et al* (1978) artifactually produced lysophospholipids therefore Shaikh and Downar (1981) reported lysophosphatidylcholine levels that were many fold lower than those of Sobel *et al* (1978). These reports led to the aims of this study:

a) to examine different modes of extraction and analysis of lysophosphatidylcholine and study the effects of storage on this phospholipid in order to correctly assess the true amount of lysophospholipids present in the heart;

b) to compare the levels of lysophospholipids in the normal and ischemic canine heart;

c) if a change in lysophosphatidylcholine was evident, it was our aim to further assess this change by examining the enzyme activities involving lysophosphatidylcholine;

d) before any of these studies could be attempted, a working model of the ischemic heart had to be developed. We proposed to artificially occlude the left anterior descending coronary artery of the canine heart in an attempt to mimic the electrophysiological and biochemical effects of severe ischemia.

Hopefully this work will lead to a better understanding of a biochemical cause of cardiac arrhythmia. The correct assessment of a biochemical abnormality contributing to a disease is imperative fo the proper diagnosis and treatment of the disease.

MATERIALS

a) Chemicals

Tris (hydroxy methyl) amino methane hydrochloride, 1-palmitoyl lysophosphatidylcholine, 1-palmitoyl lysophosphatidylethanolamine, 2-dipalmitoyl phosphatidylcholine, Molybdenum Blue, bovine serum albumin and the total cholesterol determination kit were obtained from Sigma Chemical Company (St. Louis). The Sil G25 thin layer chromatography plates were the products of Brinkmann (Westbury, N.Y.). The ACS aqueous counting scintillant was obtained from Amersham Corporation (Oakville, Ont.). Palmitaldehyde sodium bisulfite, a generous gift of Dr. E.T. Pritchard, was originally purchased from K & K Laboratories (Plainsville, N.Y.). Sodium pentobarbital and morphine sulfate were obtained from Allen and Hanburys (Toronto). Diazapam was the product of Horner (Montreal). All radioactive compounds were purchased from New England Nuclear (Boston). All other chemicals and solvents used, were of reagent grade and were obtained from Fisher Chemical Company (Don Mills, Ont.).

b) Experimental Animals

Mongrel dogs of either sex, weighing 8-15 kg, were obtained from Central Animal Care Unit, University of Manitoba. The animals were kept in a light- and temperature-controlled room.

METHODS

a) Harris Model of the Ischemic Heart (Harris 1950)

Mongrel dogs weighing 8-15 kg were used throughout this study. The dogs were anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg body weight). The dogs were then intubated and ventilated with air. The dogs' chests were shaved then disinfected with a povidine solution. An incision was made through the skin down to the ribs. The larger blood vessels were cauterized when required. An incision was made through the intercostal muscle between the 4th and 5th ribs. A rib spreader was introduced and the 4th and 5th ribs were separated from one another to expose the heart. An incision of the pericardium exposed the left anterior descending coronary artery (Fig. 4). The left anterior descending coronary artery was isolated and occluded by the Harris two-stage technique (Fig. 5). This involved a partial occlusion of the artery followed by a 10 min waiting period in order to let the heart adjust to the occlusion. The artery was then fully occluded. The occlusion of the left anterior descending coronary artery was performed in two stages because of the reported increase in the survival rate of the animals from this manipulation (Harris 1950). Air was forced out of the chest cavity. The chest cavity was closed and the dog was allowed to recover. Appropriate dosages of morphine sulfate (1 mg/kg body weight) and diazapam (10 mg/15 kg body weight) were administered intra-muscularly as analgesic and sedative. The cardiac rhythm after surgery was monitored by EKG recording (lead 2).

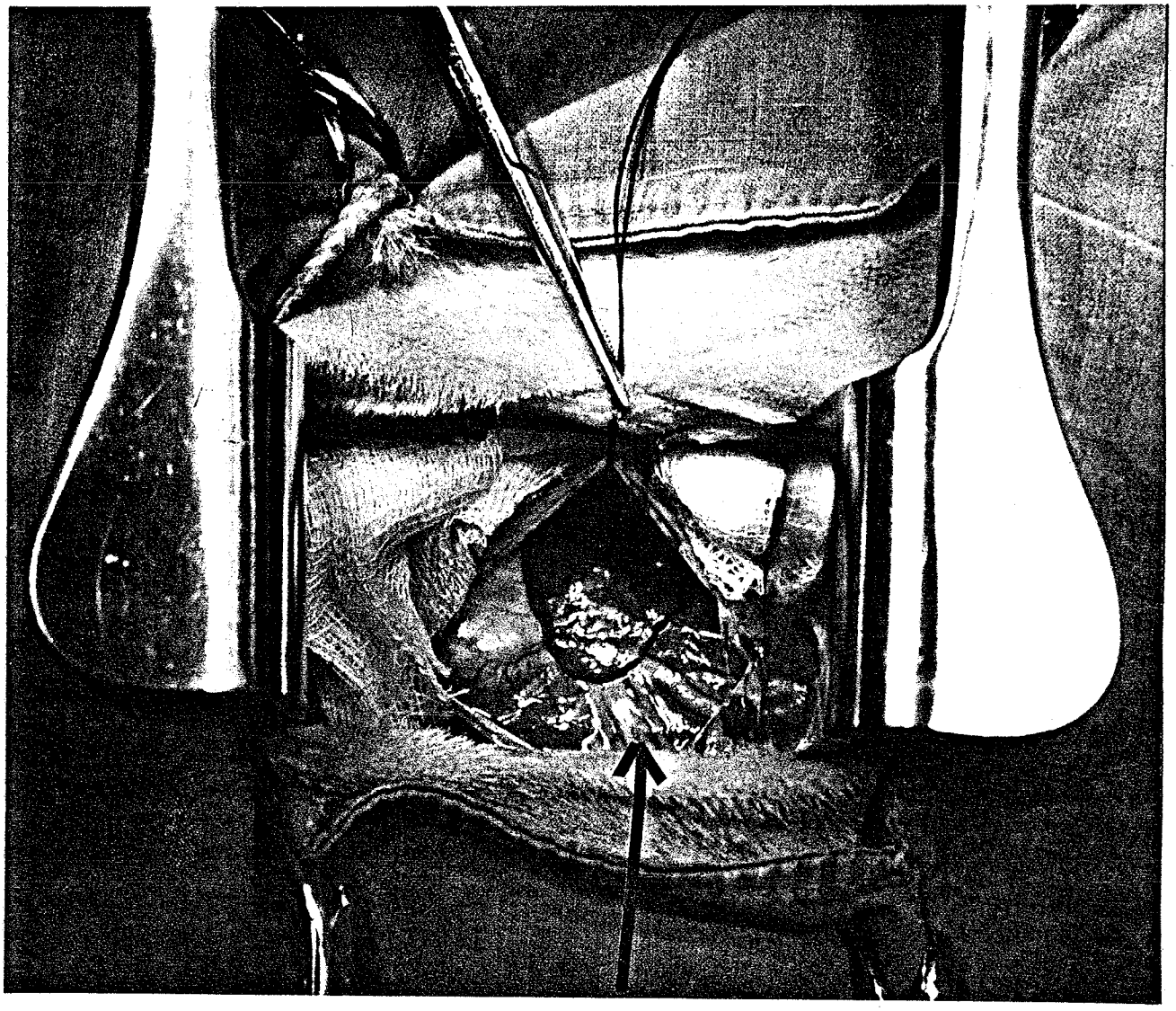


Fig. 4 The exposed left anterior decending coronary artery

Dogs were anesthetized, intubated and ventilated with air. An incision made through the intercostal muscle between the 4th and 5th ribs exposed the heart. An incision through the pericardium exposed the left anterior descending coronary artery which is the red blood vessel indicated by the arrow.

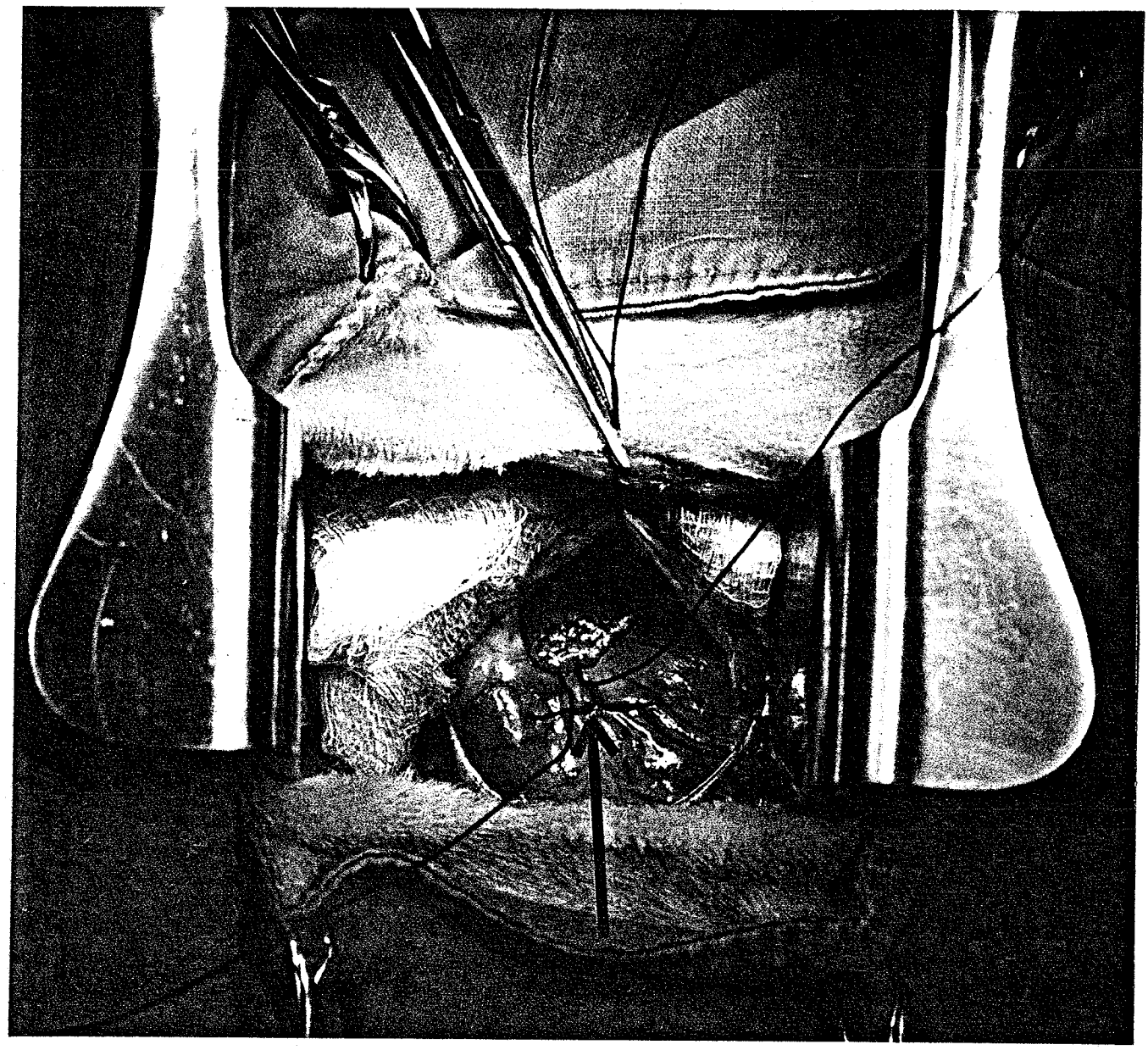


Fig. 5 First occlusion of the left anterior decending coronary artery

The left anterior descending coronary artery was isolated and occluded by a two stage technique. This involved the partial occlusion of the artery, which is indicated by the arrow, followed by a 10 min stabilization period. This was then followed by the total occlusion of the artery.

b) Modes of Tissue Extraction

The animals were anesthesized by intravenous injection of sodium pentobarbital (30 mg/kg body weight) and the hearts were rapidly excised. The left ventricle muscle was removed from the beating heart. A portion of the tissue was rapidly frozen by Wollenberger Tongs (Wollenberger et al 1960) and stored in liquid N_2. A second portion was placed in a sealed container and stored at -20^{o}. The rest of the tissue was stored in an ice-cooled container for 20 or 60 min prior to extraction.

c) Extraction of Lipids for Tissue Storage Studies

Lipids were extracted from the tissue by homogenization in one of three solvents with a Polytron homogenizer. The tissue was placed in 10 volumes (w/v) of either $CHCl_3/CH_3OH$ (2/1), $CHCl_3/CH_3OH$ (1/1) or $CHCl_3/CH_3OH$ (1/2) and homogenized twice for 30 seconds each at 6/10 full power. The homogenate was centrifuged and the insoluble pellet was re-extracted with one-half the volume of the original solvent. The extracts were pooled and the solvent was removed by evaporation under reduced pressure. The extract was reconstituted in 10 ml $CHCl_3/CH_3OH$ (2/1) and a biphasic mixture was caused by the addition of 0.1M KCl. The upper phase was removed, the lower phase was evaporated, and the lipids were reconstituted in $CHCl_3/CH_3OH$ (2/1) to a final volume concentration of 1 mg wet weight heart/ml solvent. L-α-[palmitoyl-1-^{14}C] 1 — palmitoyl lysophosphatidylcholine was

added to the reconstituted lipid extract to monitor the degradation of the lysophospholipid during storage. In some experiments L-α-dipalmitoyl-[2 palmitoyl-1-^{14}C] phosphatidylcholine was added to monitor the parent phospholipid degradation. The reconstituted lipid samples were stored at -20^{0}. Recovery of the lysophospholipid was determined by adding internal standards of 1-palmitoyl lysophosphatidylcholine.

d) Extraction of Phospholipid for Phospholipid class determinations

One gram of tissue was homogenized in 10 ml $CHCl_3/CH_3OH$ (1/2) by polytron 2 x 15s at 6/10 full power. The homogenate was centrifuged at 1000 x g for 10 min. The pellet was re-extracted with 5 ml of the same solvent, centrifuged, and the supernatants were pooled. A biphasic mixture was caused by the addition of 15 ml $CHCl_3$ and 14 ml H_2O. The upper phase was removed and washed with 5 ml theoretical lower phase ($CHCl_3/CH_3OH/H_2O$; 86/14/1) (Folch et al 1956). The lower phases were pooled and dried under N_2. The extract was reconstituted in 1 ml $CHCl_3/CH_3OH$ (1/2).

e) Histological Examination

The tissue from the left ventricle was fixed in 10% buffered formaldehyde solution, and subsequently embedded in paraffin. The tissue sections were stained with eosine and hematoxylin.

f) Determination of Tissue Water Content

Fresh tissue samples were obtained and the tissue wet weight was determined. The tissue samples were then dried at 100° under reduced pressure for 24 h. The dried samples were then weighed again. This procedure was repeated until consistent values of tissue dry weight were obtained.

g) Isolation of the Phospholipid Classes

Heart lipid extracts were spotted on 2, 4 or 8 cm bands (Camag Linomatt III, Terochem Lab Ltd., Winnipeg) on precoated sil-G25 Brinkmann thin layer chromatography plates. The plates were then developed in one of the three solvents. The solvent containing $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75/60/10/8; v/v) (Shaikh and Downar 1981) was used for the isolation of lysophosphatidylcholine. The solvent containing $CHCl_3/CH_3OH/H_2O/NH_4OH$ (70/30/4/1; v/v) (Choy and Vance 1978) was used when lysophosphatidylethanolamine was to be isolated. The solvent containing $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (70/30/4/1; v/v) (Zelinski et al 1980) was used when all phospholipids were to be analyzed. The phospholipids were stained by exposure to iodine vapour. In some cases ninhydrin or Molybdenum Blue sprays were used to stain the phospholipid.

h) Homogenization and Subcellular Fractionation of Tissues for Enzymatic Analysis

Tissue samples were kept on ice-cooled saline until ready for use. The tissue was homogenized in 10 ml of the buffer to be used in the enzyme assay with a Waring blender for 2 x 15 sec. (hi speed). The homogenate was centrifuged at 5000 x g for 5 min. The pellet was discarded, and the supernatant was designated as the post nuclear fraction. The post nuclear fraction was centrifuged at 10,000 x g for 20 min to obtain the post mitochondrial fraction. The post mitochondrial fraction was centrifuged at 100,000 x g for 1 h. The supernatant was designated as cytosol. The pellet was designated as the microsomal fraction. In order to avoid contamination of the microsomal fraction in the cytosol, the microsomal pellet was resuspended in the assay buffer and resedimented by centrifugation twice before use.

i) Scintillation Counting

1.5 ml H_2O, 200 µl acetic acid and 10 ml ACS were added to the samples for radioactivity determination. Prior to counting the vials were sonicated in a Ultrasonic bath for 10 min, vortexed, and stored in a dark place for several hours. Counting efficiencies were determined by the channel ratio calibration method.

j) Elution of Phospholipid from the Silica Gel Plates

Subsequent to the separation of the phospholipid by thin layer chromatography in some cases the phospholipid had to be eluted from the silica gel. This was achieved by grinding the silica gel into a very fine powder and placing this powder in a small column. The lipids were eluted with 10-20 ml $CHCl_3/CH_3OH$ (1/2). In some cases, $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50/39/1/10; v/v) (Arvidson 1968) was used. The solvent was dried under N_2 and the lipid extract was reconstituted in a known volume of $CHCl_3/CH_3OH$ (2/1). Yields were determined with internal standards.

k) Quantitation of Lipid Phosphorus by Acid Digestion

Phospholipid concentrations were determined by several methods. The most extensively used method was the method developed by Bartlett (1959). After the phospholipid classes were isolated by thin layer chromatography, the corresponding bands were placed in test tubes and 0.5 ml of H_2SO_4 were added. The mixture was incubated at 160° overnight and then allowed to cool to room temperature. Subsequently, 1 ml of H_2O_2 was added to each tube and the test tubes were returned to the 160° block for 2 h. Subsequent to cooling, 9.1 ml of 0.26% ammonium molybdate were added. The test tubes were well vortexed, and 0.4 ml of freshly prepared ANSA (1-amino-1-naphthol-4-sulfonic acid) reagent was added to each tube. The tubes were vortexed and placed in a 100° water bath for 10 min. Subsequent to cooling, the silica gel was

sedimented by centrifugation and the supernatant was read at 820 nm against a silica gel blank. Potassium phosphate, 1-palmitoyl lysophosphatidylcholine or 1,2-dipalmitoyl phosphatidylcholine was used as standard.

When a greater degree of sensitivity was required such as for the determination of lysophosphatidylcholine, a "micro Bartlett" assay was used (Bartlett 1959). In most cases the phospholipid was first eluted from the gel before assaying for lipid-P as per Methods (j) because of the interference of the silica gel. A volume of 1.1 ml of perchloric acid was added to each sample and the sample was digested at 160^{0} for 3 h. The sample was cooled and 8 ml of water were added. Subsequent to the addition of 0.8 ml of 5% ammonium molybdate, the samples were vortexed and 0.2 ml of freshly prepared ANSA were added. The samples were vortexed and placed in a boiling water bath for 10 min. Subsequent to cooling, the samples were centrifuged at 1000 x g for 10 min and the supernatant was read at 820 nm against a blank. Potassium phosphate, 1-palmitoyl lysophosphatidylcholine or 1,2-dipalmitoyl phosphatidylcholine was used as a standard.

l) Determination of Lipid Phosphorus Without Acid Digestion

When a quick assay of moderate sensitivity was required such as in the evaluation of total lipid phosphorus in the myocardial tissue, the method described by Raheja et al was used (1973).

In this method, 40 ml of concentrated HCl were added to 10 ml mercury and 80 ml of 13.3% ammonium molybdate. The mixture was vortexed and filtered to yield solution A. Two hundred ml of concentrated H_2SO_4 were added to 40 ml 13.3% ammonium molybdate and this mixture was added to solution A to yield solution B. Forty-five ml of methanol, 5 ml of chloroform and 20 ml of water were added to 25 ml of solution B to yield the chromogenic solution. A volume of 0.4 chloroform and 0.1 ml chromogenic solution were added to dry and silica gel-free phospholipid samples. The samples were placed in a boiling water bath for 1 - 1.5 min. Subsequent to cooling (5 min), 5 ml chloroform were added and the samples were vortexed. The samples were centrifuged at low speed and the lower chloroform layer was read at 710 nm against a blank. 1,2-dipalmitoyl phosphatidylcholine was used as standard.

m) Protein Determinations

Protein concentrations were determined by the method developed by Lowry et al (1951). Tissue was well homogenized in 20 volumes saline (w/v) and an aliquot was used for protein determination. In some cases aliquots of subcellular fractions were used. Samples were incubated in 0.5 ml of 0.66 N NaOH at 37^0 overnight. Solution A was prepared by adding 3 ml 2% $CuSO_4$ and 3 ml 4% sodium potassium tartrate to 100 ml 13% Na_2CO_3. A volume of 1.5 ml of solution A was added to each sample which was then

vortexed vigorously. The mixture was allowed to sit at room temperature for 10 min after which 0.5 ml of 2 N phenol reagent (Fisher Chemical) were added. The mixture was again vortexed vigorously and allowed to sit at room temperature for 1 h. Absorbance was measured at 625 nm against a blank. Bovine serum albumin was used as standard.

n) Alternate Methods of Lysophosphatidycholine Quantitation

Alternate methods were used for the quantitation of lysophosphatidylcholine in order to further support our data. A first method involved the acetylation of lysophosphatidylcholine by the method developed by Blank et al (1979). Lysophosphatidylcholine was isolated by thin layer chromatography and the phospholipid was eluted off the silica gel with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50/39/10/1; v/v) then dried under N_2. A volume of 100 µl pyridine and 20 µl [1-^{14}C] acetic anhydride was added to the samples. The reaction mixtures were capped, vortexed and incubated at 80°. The samples were then dried under N_2 then lyophilized for at least 2 h. The samples were reconstituted in $CHCl_3/CH_3OH$ (1/2) and 1/2 the entire volume was spotted on a thin layer chromatography plate. The plate was developed in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75/60/10/8). The band corresponding to the acetylated lysophosphatidylcholine was scraped and counted. 1-palmitoyl lysophosphatidylcholine was used as standard.

A second alternative involved isolating the lysophosphatidylcholine by thin layer chromatography and staining the thin layer chromatography plate with Molybdenum Blue. Samples and standards spotted on the same plate, were quantitated by absorbance at 630 nm with a Shimadzu thin layer chromatography scanner. 1-palmitoyl lysophosphatidylcholine was used as standard.

o) Phospholipase A assay

This assay is a modification of the procedure used by Winter et al (1982). A buffer solution of 0.05M Tris HCl, 0.145 M NaCl, 1mM $CaCl_2$, pH 8.5 was prepared. Approximately 1g tissue was homogenized in 10 ml ice cold buffer. A volume of 100 µl of the post nuclear fraction was used per assay. The preparation was kept on ice until ready for use. Ten nmol labelled L-α-dipalmitoyl [choline-Me-^{3}H] phosphatidylcholine (1,143,000 dpm/nmol) and 200 nmol 1-palmitoyl lysophosphatidylcholine were dried in a silanylated test tube under a gentle stream of N_2 and reconstituted in 1 ml ice cold buffer just prior to use. A volume of 100 µl of this substrate preparation was used per assay. The reaction samples therefore each contained 1 nmol phosphatidylcholine, 20 nmol lysophosphatidylcholine and 400 mg protein in 200 µl buffer. The samples were introduced into a 37° water bath and the reaction was initiated by the addition of substrate solution to the tissue preparation. The incubation was for 30 min at 37°. The reaction

was stopped by the addition of 600 µl $CHCl_3/CH_3OH$ (1/2). The samples were dried under N_2 and reconstituted in 4 ml $CHCl_3/CH_3OH$ (2/1) and 2 ml H_2O A biphasic mixture was therefore produced. The upper phase was removed and 1/2 the lower phase was spotted on a thin layer chromatography plate which was subsequently developed in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75/60/10/8). The band corresponding to the lysophosphatidylcholine was counted.

p) Lysophospholipase Transacylase assay

This assay is a modification of the assay used by Van Heusden et al (1980). A buffer solution of 50mM phosphate and 0.145M NaCl was prepared by titrating a 1M K_2PO_4 solution with a 1M KH_2PO_4 solution until the desired pH was achieved. This 1M phosphate buffer was diluted 20 times with 0.145M NaCl to produce the 50mM working solution. This enzyme activity was assayed at pH 7.3 in the control and ischemic tissue comparative studies. The tissue (1-2g) was homogenized in 10 ml ice cold buffer. The post nuclear, post mitochondrial and on some occasions the cytosolic and microsomal fractions were obtained and kept on ice until ready for use. A volume of 100 µl of each fraction was used per assay.

A substrate solution of 10 nmol L-α-[palmitoyl-1-^{14}C] 1-palmitoyl lysophosphatidylcholine (8.0×10^4 dpm/nmol)/ml buffer was prepared by reconstituting 1-palmitoyl lysophosphatidylcholine

in the ice cold buffer solution just prior to use. A volume of 100 µl was required per assay. Therefore, the reaction samples each contained 1 nmol lysophosphatidylcholine and 300-400 mg protein in a volume of 200 µl.

The reaction was initiated by the addition of the substrate solution to the tissue preparation. The samples were incubated at 37° for 15 min. The reaction was stopped with 600 µl $CHCl_3/CH_3OH$ (1/2). Subsequent to drying under N_2, the samples were reconstituted in 4 ml $CHCl_3/CH_3OH$ 2/1 and 2 ml water which resulted in a biphasic mixture. The upper phase was removed and 1/2 the lower phase was spotted on a thin layer chromatographic plate which was subsequently developed in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75/60/10/8). The bands corresponding to free fatty acids and phosphatidylcholine were counted.

q) Lysophosphatidylcholine: Acyl CoA acyltransferase

This assay is a modification of the assay used by Van Heusden et al (1980). A 50mM Tris HCl, 10mM $MgCl_2$ buffer (pH 7.4) was prepared. The tissue (1-2g) was homogenized in 10 ml ice cold buffer and 100 µl of the post nuclear fraction was used per assay. This preparation was kept on ice until ready for use. Eight nmol [oleoyl-1-^{14}C] oleoyl Coenzyme A (1.26×10^5 dpm/nmol) and 80 nmol 1-palmitoyl lysophosphatidylcholine were reconstituted in 1 ml ice cold buffer just prior to use. A volume of 100 µl was used per assay.

The reaction was initiated by adding the substrate to the tissue preparation. A typical reaction sample therefore contained 0.8 nmol oleoyl coA, 8 nmol lysophosphatidylcholine and 400 mg protein in 200 µl buffer. The reaction was incubated at 37° for 10 min and stopped by adding 600 µl chloroform/methanol (1/2). Subsequent to drying under N_2, the samples were reconstituted in 4 ml chloroform/methanol (2/1) and 2 ml water which resulted in a biphasic mixture. The upper phase was removed and 1/2 the lower phase was spotted on a thin layer chromatographic plate. Subsequent to development in chloroform/methanol/acetic acid/water (75/60/10/8), the phosphatidylcholine band was identified, removed and counted.

r) Total Cholesterol

Total tissue cholesterol was estimated using the Sigma kit No. 350 which is based on the procedure developed by Allain et al (1974) depicted in Fig. 6. Lipids were extracted from the hearts as per Methods (d) and reconstituted in $CHCL_3/CH_3OH$ (1/2) for a final concentration of 0.5 g heart/ml solvent. Nine test tubes were prepared as follows:

a. 10 µl of the cholesterol standard (2 mg cholesterol/ml), 15 ul H_2O and 1.0 ml enzyme solution (containing cholesterol ester hydrolase, cholesterol oxidase, hydrogen peroxidase, 4-aminoantipyrene and phenol) were added to 3 of the test tubes which were labelled "standard".

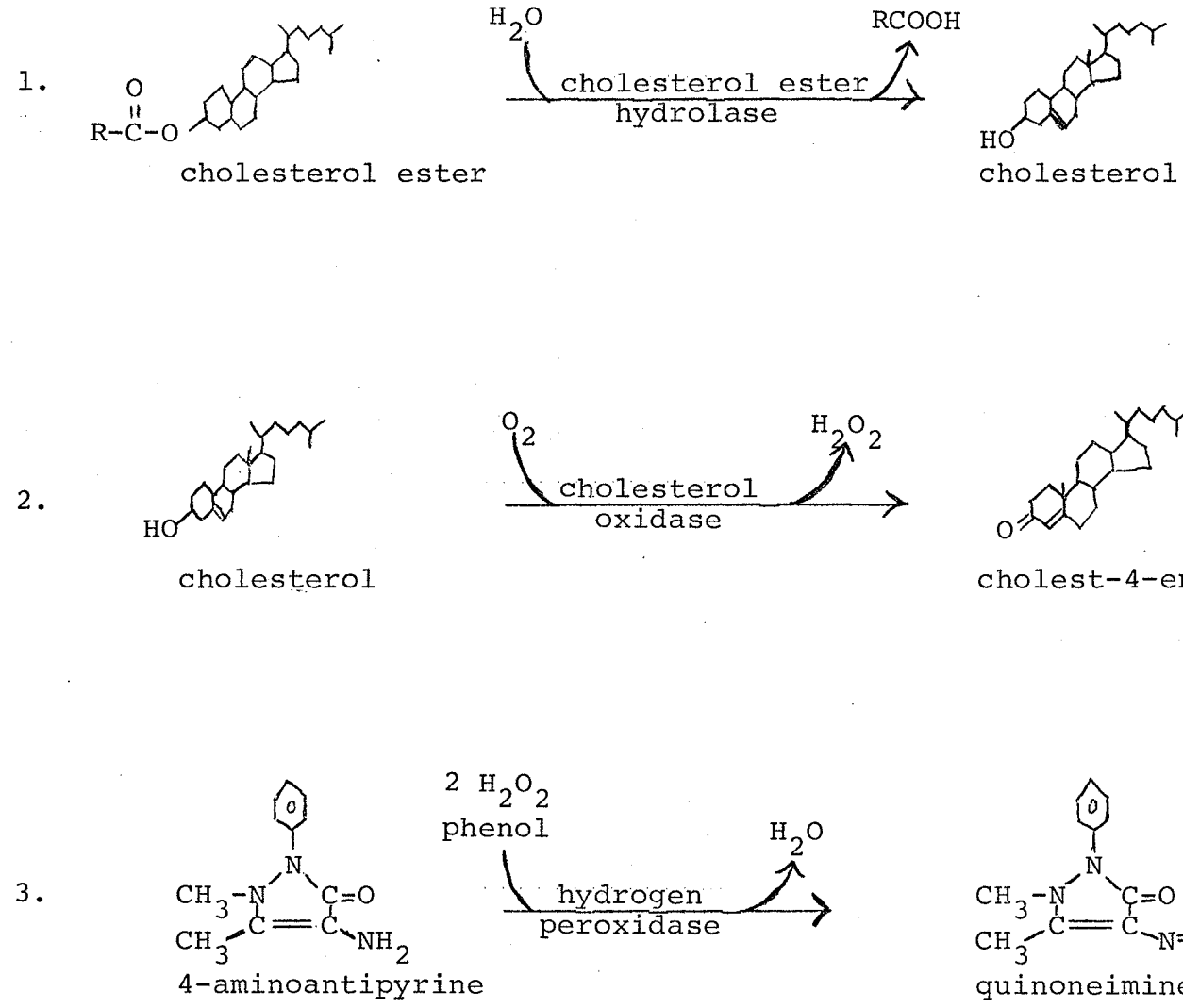


Fig 6 Reaction scheme for the enzymatic measurement of total cholesterol.

b. 20 µl of the lipid extract were dried in four test tubes and reconstituted in 25 µl H_2O. 1.0 ml enzyme solution was added to each tube which were subsequently labelled "sample".

c. Two blanks were prepared by adding 1 ml enzyme solution to 25 µl H_2O.

The test tubes were incubated at 37° for 13 min then read at 500 nm.

s) Determination of Plasmalogen Content

The plasmalogen content of the lipid classes was determined by the procedure of Wittenberg et al (1956). The phospholipid classes were isolated as per Methods (g), and eluted off the silica gel as per Methods (j) with $CHCl_3/CH_3OH$ (1/2).

Aliquots of the eluted phospholipid were analyzed for plasmalogen as well as phosphorus (Bartlett). Samples were dried along with palmitaldehyde sodium bisulfite standards. Volumes of 1.6 ml 96% ethanol, 0.2 ml 0.3% p-nitrophenyl hydrazine/96% ethanol and 0.2 ml 2N H_2SO_4 were added to samples and standards. The test tubes were capped and incubated at 60° for 30 min. The samples were cooled for 5 min on ice and 1 ml H_2O and 2 ml hexanes were added. The samples were vortexed hard and centrifuged at 1000 x g

for 60 seconds. The lower phase was removed and the upper phase was washed twice with 2 ml 35% ethanol. One ml of the upper phase was dried and reconstituted in 3 ml 96% ethanol and read at 396 nm.

RESULTS

a) Effect of occlusion on the left anterior decending coronary artery on theelectrophysiology of the left ventricle

Periodic ventricular arrhythmias developed spontaneously after the occlusion of the left anterior descending coronary artery and lasted up to 5 h. A decrease in the resting potential and in the upstroke velocity of action potential as well as loss of cellular excitability and alterations in refractory periods were observed. The ventricular arrhythmias consisted of single and multiple extrasystoles and periods of ventricular tachycardia. An example of the EKG readings is depicted in Fig. 7. A quiescence period was observed between 4-8 h post surgery in which no arrhythmias were evident. This was followed by another onset of ventricular arrhythmias. Periods of ventricular arrhythmias were recorded at 24 post-surgery which persisted for several days. In experiments where the left anterior descending coronary artery was not occluded, no arrhythmias were observed which clearly established that the arrhythmias were a result of ischemia and not due to surgical manipulation.

b) Morphological and Histological Changes in the Ischemic Tissue

Morphological examination of the ischemic area in the left ventricle revealed a discoloration as well as a softer texture when

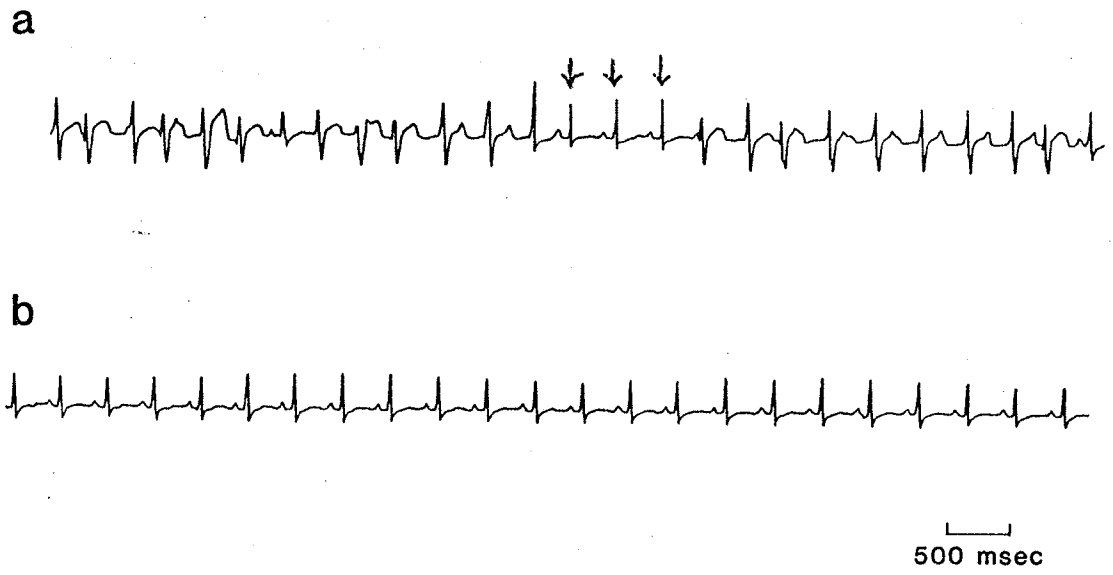


Fig. 7. The lead II EKG of a dog 24 h after complete occlusion of the left anterior descending coronary artery

The arrows indicate the beats which followed the normal sequence of activation, where the p wave precedes the QRS complex. In the remainder of the tracing the ventricular depolarizations are independent of the atria.

a) this tracing illustrates the ventricular arrhythmia present at 24 hours after left anterior descending coronary artery ligation.
b) this tracing illustrates the lead II EKG of a dog, 24 h after the complete occlusion of the left anterior descending coronary artery following the administration of lidocaine.

compared to normal heart tissue (Fig. 8). The ischemic area covered approximately 1/4 the size of the left ventricle and extended from the upper 1/3 of the left papillary muscle down to the apical region. The tissue from the posterior side of the left ventricle appeared to be similar to normal cardiac tissues under morphological and histological examination and was therefore used as control tissue in the subsequent biochemical analysis. Histological comparison between control and ischemic tissues showed that most fibers in the ischemic area underwent coagulative necrosis (fig. 8). The nuclei of the cells were not visible in some areas and interstitial inflammation with edema was apparent. Neutrophil infiltration of the ischemic tissue was observed in some areas.

c) Alterations of Total Lipid, Protein and Water Content in the Ischemic Heart

Since alterations in lipid composition have been implicated as one of the biochemical causes of cardiac arrhythmias, the lipid composition of control and ischemic tissues was compared. A decrease in total cholesterol and phospholipids was observed in the ischemic tissue based on tissue wet weight (Table 1). However histological examination of the ischemic tissue revealed local edema. Therefore the observed decrease in total lipid may only have been a reflection of an increased water content of the ischemic tissue. Estimation of total water and total protein content of of the ischemic tissue is depicted in Table 2. When the dry weight to

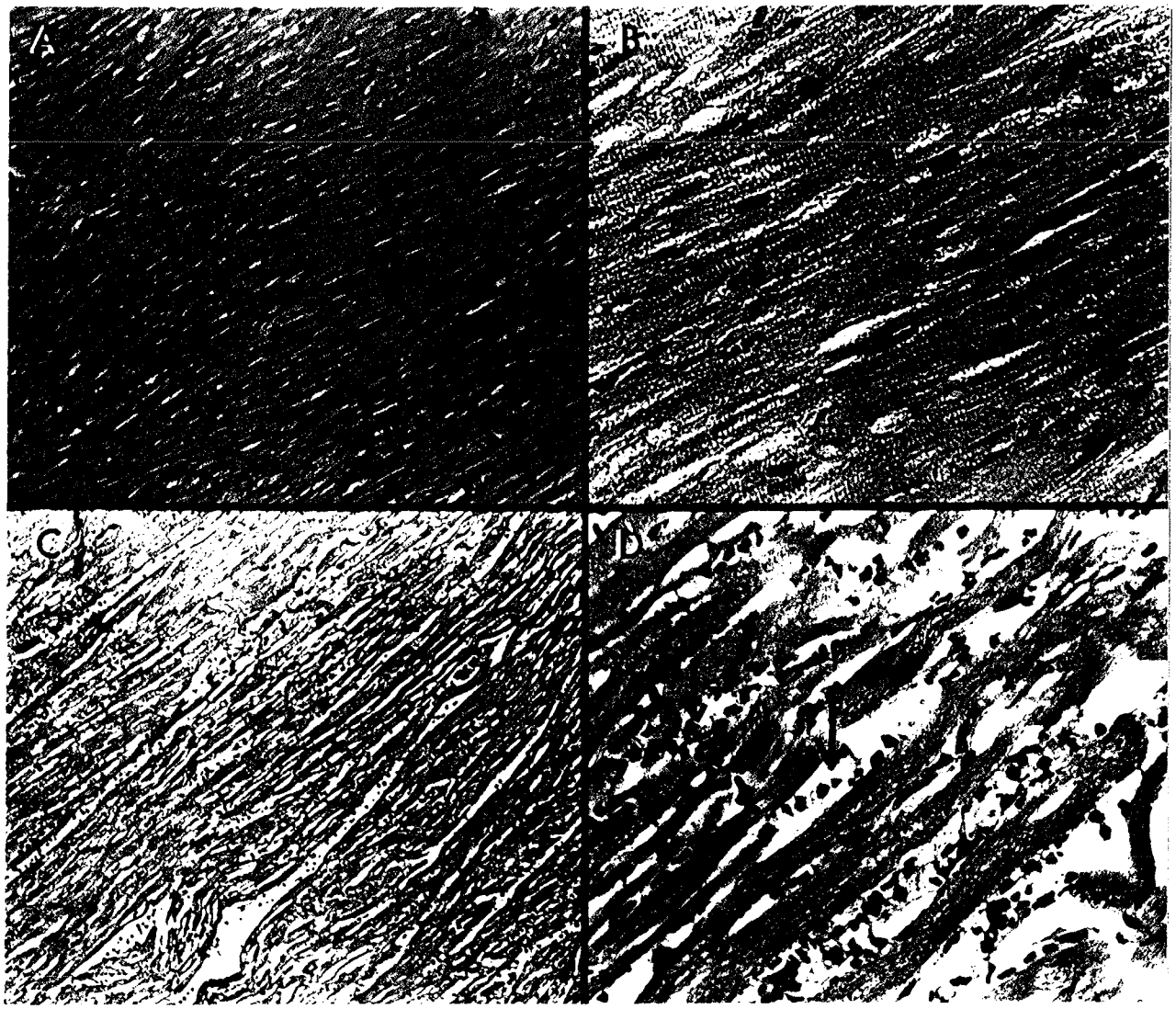


Fig. 8 Histological study of normal and ischemic tissues

Panel A (Low magnification) and panel B (High magnification) depict sections obtained from the normal areas of the heart. Muscle fibers are well organized and the nuclei are clearly visible. Panels C and D show sections obtained from the ischemic areas (24 h). Most fibers have undergone coagulative necrosis and nuclei have disappeared in some cells. Interstitial inflammation with edema and neutrophil infiltration are indicated by arrows (panel D). Calibration bar is 100 μm for panels A and C; 25 μm for panels B and D.

TABLE 1

Total cholesterol and phospholipid content in control and ischemic canine hearts

	Control	Ischemic
Cholesterol		
(mg/g/ wet wt.)	2.21[a] ± 0.24[b] (15)[c]	1.91 ± 0.27 (8)*
(mg/g/ dry wt.)	10.38 ± 1.13 (15)	11.13 ± 1.53 (8)
(mg/g protein)	15.67 ± 1.70 (15)	15.40 ± 2.17 (8)
Phospholipids		
(umol Pi/g wet wt.)	24.85 ± 2.00 (7)	21.42 ± 1.54 (7)*
(umol Pi/g dry wt.)	116.67 ± 9.39 (7)	121.01 ± 8.70 (7)
(umol Pi/g protein)	176.24 ± 14.18 (7)	172.74 ± 12.42 (7)

[a]Mean

[b]Standard deviation

[c]Number of experiments

*$p < 0.05$

TABLE 2

Tissue water and protein contents in control and ischemic canine hearts

	Control	Ischemic
g H_2O/g wet weight	0.787[a] ± 0.021[b](6)[c]	0.823 ± 0.11 (6)
g H_2O/g dry weight	3.880 ± 0.411 (6)	4.671 ± 0.360 (6)
dry weight/wet weight	0.207 ± 0.019 (15)	0.177 ± 0.011 (6)*
g protein/g wet weight	0.141 ± 0.019 (15)	0.124 ± 0.010 (15)*

[a]Mean

[b]Standard deviation

[c]Number of experiments

*$p < 0.05$

wet weight ratio was determined, a 14% increase in water content was observed. A 12% decrease in total protein was also observed when expressed as per g wet weight. Therefore, when total cholesterol and phospholipids were expressed in terms of dry weight of the tissues or by protein content (Table 1), no significant difference was detected between the control and ischemic tissues.

d) Modes of Phospholipid Extraction and Storage

Due to the controversy involving absolute levels of lysolipids in mammalian tissues, a satisfactory mode of tissue lipid extraction and lipid storage had to be established. Total extraction as well as lack of artifactual production of lysophospholipids are essential for their accurate assessment. Since lysolipid levels in mammalian tissues are very low, any artifactual production due to hydrolysis of the large parent phospholipid pools could greatly influence the determined levels. Also, due once again to their low levels, estimation of the absolute levels of lysophospholipids is very difficult, therefore total extraction of this phospholipid is very important. In preliminary experiments, a substantial amount of lysophosphatidylcholine was formed when dog heart phosphatidylcholine was placed in acidified n-butanol for 6 hours. However, no production of lysophosphatidylcholine was detected when phosphatidylcholine was dissolved in chloroform/methanol (2/1 or 1/2) for the same period of time. Therefore all extractions were conducted with neutral

solvents containing only chloroform and methanol. The tissue was homogenized in the solvents as indicated in Table 3 and the homogenates were filtered or centrifuged in order to remove the insoluble materials. These insoluble materials were re-extracted 10X with the same solvent. No significant differences in lysophosphatidylcholine recovery were observed among the solvents used after 5 extractions. Optimal recovery of lysophosphatidylcholine was achieved after three extractions, using $CHCl_3/CH_3OH$ (1/2) as solvent. When an internal standard of labelled lysophosphatidylcholine was added to the tissue homogenate prior to phase separation, 95-100% of the labelled material was recovered in the lower phase. The constituents of the lower phase were isolated by thin layer chromatography. Approximately 96% of the recovered labelled material was recovered as lysophosphatidylcholine.

Analysis of lysophosphatidylcholine levels in the heart subsequent to its removal from the animal is depicted in Table 4. As shown, lysophosphatidylcholine levels in heart tissues obtained by rapid freezing in liquid N_2 were not significantly different than those obtained after storage at 0° for 20 min. However tissue storage at 0° for 60 min or at -20° for 7 days resulted in a significant increase in lysophosphatidylcholine levels.

TABLE 3

Extraction of lysophosphatidylcholine from normal canine heart by various solvents

Number of Extractions	Solvent		
	$CHCl_3/CH_3OH$ (2/1)	$CHCl_3/CH_3OH$ (1/1)	$CHCl_3CH_3OH$ (1/2)
		(nmol lipid-P/g wet wt)	
1	76.8 ± 9.3 (3)	79.8 ± 6.1 (3)	94.5 ± 9.8 (3)
2	101.8 ± 4.3 (3)	104.9 ± 7.4 (3)	117.0 ± 8.0 (3)
3	109.1 ± 7.4 (3)	113.3 ± 6.1 (3)	123.6 ± 4.3 (3)
5	117.1 ± 6.0 (3)	120.0 ± 9.4 (3)	122.0 ± 6.6 (3)
10	111.3 ± 5.8 (3)	122.3 ± 7.2 (3)	118.8 ± 4.6 (3)

a Mean

b Standard Deviation

c Number of expriments

TABLE 4

Lysophosphatidylcholine content in normal canine cardiac tissue under different modes of storage

Model of storage	Period of storage	Lysophosphatidylcholine content
		(nmol lipid-P/g wet wt)
Liquid N_2 frozen (Wollenberger)	60 min	123 ± 26 (6)
0° (in ice)	20 min	133 ± 14 (11)
0° (in ice)	60 min	160 ± 23 (4)*
-20°	7 days	164 ± 6 (6)*

a Mean

b Standard Deviation

c Number of experiments

*P $<$ 0.05 when compared with liquid N_2 frozen sample

Heart tissues were also stored in various solvents or under N_2 without solvent for 14 days. As shown in Fig. 9 no significant change was observed in $CHCl_3/CH_3OH$ (2/1) or without solvent under N_2. However significant increases were observed when the lipid was stored in $CHCl_3/CH_3OH/H_2O$ (86/14/1) which represents the theoretical lower phase (Folch et al 1956). When labelled lysophosphatidylcholine was stored under the conditions, described in fig. 9, 96-100% of the label was recovered as lysophosphatidylcholine. When labelled phosphatidylcholine was stored under the same conditions, no hydrolysis of phosphatidylcholine was detected. The plasmalogen content of phosphatidylcholine and lysophosphatidylcholine in dog heart was determined during prolonged storage in theoretical lower phase. No significant difference was observed among the time points considered.

e) Analysis of Phospholipid Classes in Control and Ischemic Tissues

The complete analysis of phospholipid classes in control and ischemic tissues with respect to tissue protein content is depicted in Table 5. The phospholipid classes were isolated by thin layer chromatography and their levels were estimated by the method developed by Bartlett. The values were compared to those obtained using the alternate estimation methods. All values were comparable. Since tissue water content was observed to increase in

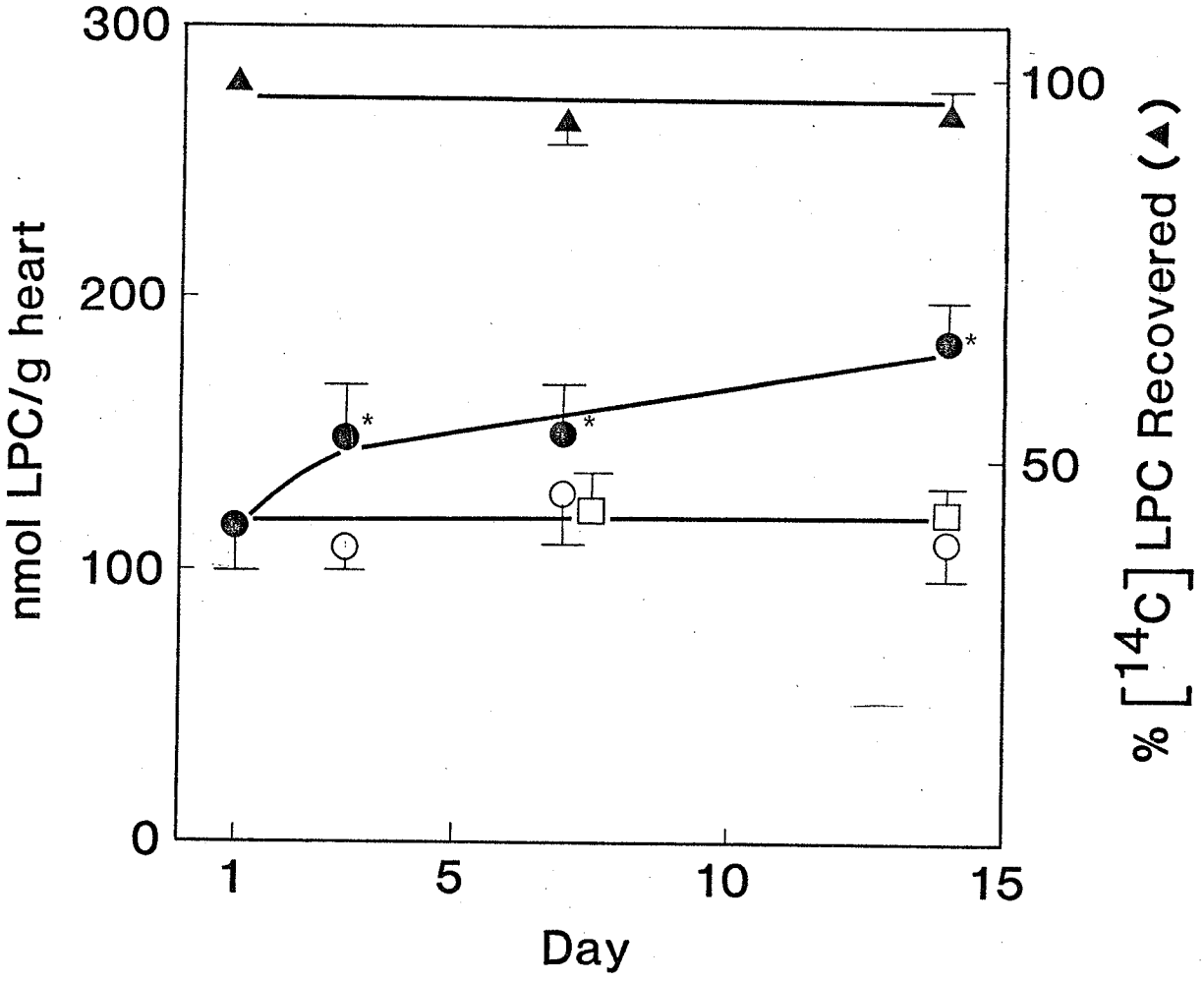


Fig. 9. Stability of lysophosphatidylcholine in lipid extract stored in various media

Lipids extracted from 20 g of canine heart left ventricle were divided into three aliquots and these aliquots were stored at -20 in either chloroform/methanol (2/1) (O), chloroform/methanol/water (86/14/1) (●) or without solvent under nitrogen (□) for the periods indicated. Two microcurries of [1-^{14}C palmitoyl] lysophosphatidylcholine (51 mCi/nmol) were added to each of the aliquot preparations prior to storage. The lysophosphatidylcholine in the lipid extracts was isolated by thin-layer chromatography and assayed as described at the prescribed intervals. Each value represents the mean of six separate experiments. The vertical bars are standard deviation. *$P < 0.05$ when compared with sample from day 1.

TABLE 5

Analysis of phospholipid classes in normal and ischemic canine hearts

Phospholipids	Control	Ischemic
	(μmol lipid-P/g protein)	
Lysophosphatidylcholine	1.00[a] ± 0.21[b](11)[c]	2.07 ± 0.61 (8)*
Sphingomyelin	16.74 ± 4.68 (11)	16.46 ± 3.61 (11)
Phosphatidylcholine	88.93 ± 8.80 (6)	76.07 ± 9.29 (6)*
Phosphatidylserine & Phosphatidylinositol	13.05 ± 2.20 (8)	15.84 ± 3.61 (8)
Lysophosphtidyl-ethanolamine	0.51 ± 0.07 (5)	1.23 ± 0.46 (5)*
Phosphatidylethanolamine	46.03 ± 3.26 (8)	39.23 ± 2.84 (8)*
Phosphatidylglycerol & Cardiolipin	28.37 ± 7.94 (11)	37.30 ± 10.76 (11)*
Others	4.82 ± 1.42 (6)	4.23 ± 1.23 (6)

[a]Mean

[b]Standard deviation

[c]Number of experiments

*$p < 0.05$

the ischemic myocardium the phospholipid classes were expressed in terms of g protein. The high standard deviations are explained in part by differences in phospholipid content of the individual as well as the different degrees of damage to the heart caused by the ischemia. A significant increase in both lysophosphatidylcholine and lysophosphatidylethanolamine was observed in the ischemic areas of the heart with a corresponding decrease in phosphatidylethanolamine and phosphatidylcholine. An increase in phosphatidylglycerol and cardiolipin was also observed but the other phospholipid did not change significantly.

f) Analysis of Phospholipid Plasmalogens in Control and Ischemic Tissues

Since a significant amount of phospholipid in the heart is present in plamalogen form, the plasmalogen content in each phospholipid class was determined (Table 6). Once again high standard deviations were observed. This was not surprising when one considered the tissue differences from animal to animal as well as the artifactual differences arising from the many manipulations involved in the Wittenberg assay. However, it was found that there was no significant change in the plasmalogen content of the lysophospholipids in the ischemic myocardium.

TABLE 6

Plasmalogen content of phospholipids in control and ischemic canine hearts

Phospholipids	Control	Ischemic
	(% plasmalogen)	
Lysophosphatidylcholine	28.5[a] ± 10.6[b] (4)[c]	27.3 ± 8.6 (4)
Lysophosphatidyl-ethanolamine	17.9 ± 11.5 (4)	18.5 ± 8.5 (4)
Phosphatidylcholine	43.6 ± 10.3 (4)	37.8 ± 8.1 (3)
Phosphatidylethanolamine	31.6 ± 5.5 (4)	36.4 ± 5.7 (4)

[a]Mean

[b]Standard Deviation

[c]Number of experiments

g) Lysophosphatidylcholine Metabolism Studies

Having observed a 2-fold increase in lysolipid levels, a study of lysophosphatidylcholine was in order. Both Phospholipase A_1 and A_2 produce lysophospholipids, therefore a dipalmitoyl phosphalidylcholine labelled in the choline position was used. Therefore either of the two phospholipases would produce a radioactive lysophosphatidylcholine. In preliminary studies tissue homogenates were incubated in the presence of radioactive phosphatidylcholine at pH 7, however no phospholipase A activity was observed. The reaction was therefore assayed at pH 8.5. A low but significant phospholipase A activity was observed. When control and ischemic tissues were compared for phospholipase A activity, a significant increase in activity was observed in the ischemic tissue when expressed as per mg protein (Table 7).

Lysophosphatidylcholine has been shown to be quickly reacylated by an active acyl CoA: lysophosphatidylcholine acyltransferase. A decrease in its activity could result in an increase in lysophosphatidylcholine, therefore the acyltransferase activity in the ischemic tissue was compared to the control tissue (Table 7). No significant change was observed however lysophosphatidylcholine and oleoyl CoA were used as substrates. The production of fatty acyl CoA require CoA and ATP. Therefore any differences in the ATP pool could greatly influence the metabolism of lysophosphatidylcholine.

TABLE 7

Enzymes responsible for the metabolism of lysophosphatidylcholine in the normal and ischemic canine myocardium

	Control	Ischemic
	(pmol product/min/mg protein)	
Phospholipase A	0.28[a] ± 0.03[b] (5)[c]	0.53 ± 0.20 (5)*
Lysophospholipase	19.48 ± 5.84 (8)	3.01 ± 3.19 (6)*
LPC:LPC Transacylase	11.13 ± 5.33 (10)	3.65 ± 2.87 (9)*
LPC:ACYL CoA Acyltransferase	26.20 ± 6.56 (4)	20.90 ± 1.81 (4)

All enzymes assays were performed at optimal conditions.

*$p < 0.05$

a mean

b standard deviation

c number of experiments

The other major metabolic pathways involving lysophosphatidylcholine are the lysophospholipase and lysophosphatidylcholine: lysophosphatidylcholine transacylase catalyzed reactions. In preliminary studies, when a radioactive lysophosphatidylcholine was added to a tissue homogenate, one observed an accumulation of labelled fatty acids and labelled phosphatidylcholine representing lysophospholipase and transacylase activities respectively. The lysophospholipase activity was found in the cytosolic and microsomal fractions. It had a very narrow pH profile with an optima pH of approximately 7.0. This enzyme exhibited very unusual type III kinetics in which the plot of velocity vs. substrate concentration was hyperbolic to a certain lysophosphatidylcholine concentration then "broke" and the rate decreased. In the Lineweaver Burke plot, the straight line "broke" and was succeeded by a second straight line with an upward slope. This type of kinetics indicates an enzyme which utilizes monomers but probably not micelles, and where the micelles inhibit the enzyme. An optimal substrate concentration was determined (approximately 5 μM) and used in the comparison of activity between normal and ischemic tissues.

The lysophosphatidylcholine: lysophosphatidylcholine transacylase activity exhibited normal Michaelis Menten kinetics and had a broad pH profile with an optimal pH between 6 and 7. When the microsomes were isolated from the cytosol almost all the activity

was lost. For ths reason the post-nuclear fractions were used to assay this enzyme activity in order to prevent loss of any factors that may influence this activity. When radioactive lysophosphatidylcholine was added to the post-nuclear fractions of tissue homogenates, one observed a decrease in both phosphatidylcholine formation and fatty acid release in the ischemic tissue samples, when the enzyme activities were expressed as per mg protein, reflecting the transacylase and lysophopholipase activities respectively.

DISCUSSION

The appearance of electrophysiological disturbances after the onset of short term ischemia had been reported by many investigators (Ten Eick et al 1976; Sakamoto et al 1982; Watanabe et al 1982; Ikeda et al 1982; Elharrar and Zipes 1977). Also many biochemical alterations were found to accompany these electrophysiological disturbances. Within a few minutes of coronary ligation one observed a loss of intracellular K^+ which was paralleled by a loss of Mg^+ and an influx of Na^+. There was evidence of a drop in pO_2 (Case et al 1969). This inadequate oxygen supply resulted in an increase in lactic acid (Downar et al 1977). Glycogen levels as well as ATP levels were lowered (Vial et al 1982; Podzuweit et al 1978; Hollis et al 1978). Coronary sinus blood becomes markedly acidic. There was an increased tissue level of long chain acyl CoA observed and long chain acylcarnitine acetyl CoA, acetyl carnitine, free CoA and free carnitine levels decreased (Whitmer et al 1978).

The relevance of these changes in biochemistry and electrophysiology in respect to cardiac arrhythmias was unclear. Nevertheless, altered conductivity of electrical signals across the membrane strongly suggested that the cell membrane was affected (structurally or otherwise) upon the onset of ischemia. Since phospholipids are important entities of the biological membrane,

their content and composition in the membrane were further investigated. Several investigators had reported an increase in lysophosphoglycerides in various mamalian ischemic myocardial tissues. However the absolute levels of these lysophospholipids in the tissue was subjected to much debate. Our initial goal was therefore to reinvestigate the various methodologies used for the extraction of these lysophospholipids from the tissue. In our investigations we found that the extraction procedure used by Sobel et al (1978) involving an acidified n-butanol solvent artifactually produced lysophospholipids. This finding substantiated Shaikh and Downar's (1981) report. Therefore this mode of extraction was not used in the present study. We examined the efficiency of three neutral solvents systems for the extraction of lysophosphatidylcholine. A solvent mixture of chloroform/methanol 1/2; (v/v) proved to be the solvent of choice. Lysophosphatidylcholine was optimally extracted with this solvent after only three extractions. Also the lower specific gravity of this solvent permitted the separation of the insoluble material from the solvent by low speed centrifugation.

The possibility of phospholipid alterations caused by removal of the tissue from the heart was also investigated. Storage of the heart tissue at 0^{o} for 20 min did not result in any significant change in intracellular lysophosphatidylcholine levels. However, storage of the tissue at 0^{o} for 1 h or at -20^{o} for 2 weeks

resulted in a significant increase in the intracellular lysophosphatidylcholine levels. Also storage of lipid extracts in $CHCl_3/CH_3OH/H_2O$ 86/14/1; (v/v) which represented the theoretical lower phase of the biphasic mixture described in paragraph Methods (c) (Folch et al 1956), resulted in a net increase in lysophosphatidylcholine. The rise in lysophosphatidylcholine under the conditions described, was probably due to the hydrolysis of a small amount of phosphatidylcholine during storage. Due to the large phosphatidylcholine pool, the hydrolysis of phosphatidylcholine could not be detected. Storage of lipid extracts in $CHCl_3/CH_3OH$ (2/1); (v/v) or dry under N_2 did not result in any significant change in lysophosphatidylcholine levels. All further studies therefore involved removal of the cardiac tissue, storage on ice, weighing of the sample followed by lipid extraction with $CHCl_3/CH_3OH$ (1/2) within 20 min of organ excision. Following phase separation the extracts were dried and reconstituted in $CHCl_3/CH_3OH$ (2/1) until ready for use. The lysophospholipid levels that we report in the canine hearts using this procedure are in close agreement with the levels found in the porcine myocardium (Shaikh and Downar, 1981).

Subsequent to the identification of the problems involved in the lipid extraction and storage, we proceeded to develop an ischemic model of the canine heart. This was achieved by a ligation of the left anterior descending coronary artery. Occlusion of this

artery resulted in periods of acute ventricular arrhythmias as described in paragraph Results (a). Biochemical studies conducted upon removal of the tissue at 24 h post-surgery revealed a two-fold increase in the major lysophospholipid levels in the ischemic area of the heart. This finding agrees with the 50-70% increases reported in other mammalian hearts after short periods (10 min) of ischemia. Also the levels we report are consistent regardless of the estimation procedure used. Since the presence of edema was evident the phospholipid levels were depicted as per mg protein. Beyond the changes observed in lysophosphatidylcholine and lysophosphatidylethanolamine levels, none of the other phospholipids that exhibited level changes were arrhythmogenic in the studies reported by Man et al (1983). In this study it was shown that lysophosphatidylserine and lysophosphatidylglycerol were also able to cause cardiac arrhythmias. However, the levels of these minor lysophospholipids were very low in both control and ischemic tissues and hence may not play a role significant role in the production of cardiac arrhythmias under physiological conditions.

Although a two-fold increase in the levels of lysophosphatidylcholine and lysophosphatidylethanolamine was observed in the ischemic tissue, the percentage of lysophospholipid plasmalogens were similar to those found in control tissues. Since the percentage of plasmalogens in the parent phospholipids was also similar in both control and ischemic tissues, it can be concluded

that the increase in lysophospholipid levels during ischemia was not caused by the preferential hydrolysis of the diacyl phospholipid or the phospholipid plasmalogens.

Having established a link between cardiac arrhythmias and lysophospholipids we wished to further examine the accumulation of lysophosphatidylcholine. The accumulation of a metabolite in a tissue may be attributed to one of four reasons: a) an increased production of the metabolite, b) a decreased catabolism of the metabolite, c) a decreased washout of the metabolite or d) a combination of the above.

Since phospholipase A_1 and phospholipase A_2 are responsible for the production of lysophospholipid the action of these enzymes was investigated. The use of L-α-dipalmitoyl-[choline-Me-^{3}H]- phosphatidylcholine in our assay would enable us to identify the activity of these two enzymes simultaneously. In a set of preliminary experiments we were not able to detect any phospholipase A activity at the physiological pH. However, enzyme activity was detected at pH 8.5. Since phospholipase A_1 was shown to have a pH optimum of 7.0 and phospholipase A_2 a pH optimum of 8.5 (Franson et al 1978). We conclude that the majority of enzyme activity which caused the hydrolysis of [^{3}H-Me] phosphatidylcholine was phospholipase A_2. A significant increase in the hydrolysis of phosphatidylcholine was

observed in the ischemic tissue when the enzyme activity was expressed as per mg protein. It can be argued that the assay for phospholipase A at pH 8.5 may have little bearing in the in vivo hydrolysis of phosphaditylcholine. An acidic shift of the pH was observed in the ischemic myocardium which should have resulted in a decreased phospholipase A_2 acitivity, and yet phospholipase A activity was increased two-fold in the ischemic myocardium. In order to correctly assess this change in enzyme activity, one must take several factors into consideration. Phospholipase A_2 in cardiac cells are highly compartmentalized, with a considerable amount in the mitochondria and microsomes. It is not known if the pH shift, presumably caused by a lactic acidosis, during ischemia, has any effect on these localized compartments. Another factor to consider is the form of substrate available to the phospholipase A in vivo, which has not been determined. Presumably, the intracellular form of substrate is in mixed form, which may be the preferred substrate, thus facilitating a more rapid rate of catalysis. Since the exact substrate form in vivo is not known, no attempts were made to reproduce it in our assays. In ischemia the initial elevation of the lysophosphatidylcholine level could result in the increased formation of mixed micelles and disruption of the biological membranes. This is because of the detergent-like properties of this phospholipid. The formation of mixed micelles may result in an increased available substrate for the phospholipase A. Also the disrupted membranes could result in an increased Ca^{++}

influx thus potentially influencing phospholipase A activity. The observed increased in phospholipase A activity was further supported by the coresponding decreases in phosphatidylcholine and phosphatidylethanolamine found in the ischemic tissue. The decreases in the parent phospholipid were so substantial in fact, that the increases in the respective lysophospholipid could not account for them. This observation led to several postulations. The possibility of a reduced biosynthesis of the parent phospholipid during ischemia seems to be unlikely since the levels of the other phospholipids either remained unchanged or were elevated in the ischemic tissues. A second possibility is that the lysophosphatidylcholine formed was very rapidly metabolized and the observed increase in lysophosphatidylcholine during ischemia only represents a small portion of the increase in lysophosphatidylcholine production. A third possibility is that lysophosphatidylcholine was rapidly removed from the ischemic area due to its cytolytic and amphiphilic properties, which would allow easy extracellular dispersion. The latter two postulations imply that the observed in vitro phospholipase A activity reflects only a small portion of the in vivo activity. This is not surprising due to the artificial conditions imposed and the hydrophobic nature of the phosphatidylcholine substrate which could result in a poor substrate-enzyme interaction. A good substrate-enzyme interaction is a prerequisite for the success of an enzyme catalyzed reaction.

As a result the two-fold increase in lysophosphatidylcholine may reflect only a fraction of the total increase in lysophospholipid production during prolonged ischemia.

Since the catabolism of lysophospholipid may also play an important role in the elevation of lysophospholipid in the ischemic tissue, the enzymes responsible for lysophosphatidylcholine catabolism were investigated. Three enzyme activities involving lysophosphatidylcholine catabolism were identified in the canine myocardium: a) lysophospholipase, b) lysophosphatidylcholine: lysophosphatidylcholine transacylase, and c) lysophosphatidylcholine: acyl CoA acyltransferase.

A large lysophospholipase activity was observed in the normal myocardium. This enzyme activity could be measured in both the cytosol and microsomes. The post nuclear fraction of myocardial homogenates was used in the normal vs ischemic comparative studies to avoid losses of any activators or inhibitors that may have been present in the tissue homogenates. The lysophospholipase activity in the post nuclear fraction exhibited unusual kinetics in that lysophosphatidylcholine inhibited the enzyme when this substrate's concentration exceeded its critical micellar concentration. This enzyme also had a very narrow pH profile with only 20% activity at pH 6.5, a value comparable to that seen in the ischemic myocardium. These results were in keeping with Gross and Sobel's (1982) recent

characterization of rabbit heart microsomal lysophospholipase. They also have recently purified and characterized a rabbit myocardial cytosolic lysophospholipase (Gross and Sobel 1983) which exhibited normal Michaelian kinetics and had a broader pH profile however this enzyme also rapidly lost activity below pH 6. The cytosolic lysophospholipase activity was competitively inhibited by long chain acylcarnitines, themselves arrhythmogenic amphiphiles, which had been shown previously to accumulate in the ischemic myorcardium (Whitmer et al 1978). These properties made lysophospholipase an attractive candidate as contributor to the accumulation of lysophosphatidylcholine in the ischemic myocardium. When a radioactive lysophosphatidylcholine was introduced to the post nuclear supernatants of tissue honogenates from control and ischemic areas of the heart one observed a significant decrease in the lysophospholipase activity in the ischemic tissue.

The second lysophosphatidylcholine catabolic enzyme activity investigated was the lysophosphatidylcholine: lysophosphatidylcholine transacylase activity. In preliminary studies this enzyme had a very broad pH profile and was found in both the cytosol and the microsomes. This transacylase was found to be ATP and CoA independent. Once again post nuclear supernatants were used in the control vs ischemic comparative studies. In previous studies (Gross and Sobel 1982), this transacylase was found to always co-purify with lysophospholipase and exhibited non-

Michaelian kinetics. The parabolic double reciprocal plot of phosphatidylcholine synthetic activity versus substrate concentration suggested that transacylation of lysophosphatidylcholine occurs only when micellar substrate is present (Gatt et al 1972). When lysophosphatidylcholine: lysophosphatidylcholine transacylase activity in ischemic tissue was compared to control tissue one observed a significant decrease in activity.

The third enzyme activity examined was the ATP and CoA dependent acylation of lysophosphatidylcholine catalyzed by lysophosphatidylcholine: acyl CoA acyltransferase. This enzyme was found to have a broad pH profile and normal Michaelis Menten kinetics in rabbit myocardium (Gross and Sobel 1982) and thus its activity would not be expected to be altered by myocardial ischemia on the basis of pH effects. When this acyltransferase activity in control tissue was compared to ischemic tissue no significant change in activity was observed. However, in our assay, radioactive oleoyl CoA and lysophosphatidylcholine were used as substrates. The formation of fatty acyl CoAs in vivo require ATP, coenzyme A and acyl CoA synthetase (present in the outer mitochondrial membrane). Therefore any drop in ATP levels, which has been reported in the ischemic tissue (Hollis et al 1978; Vial et al 1982; Podzuweit et al 1978) could result in a decrease in available substrate and thus a decrease in enzyme activity. Therefore a reduction in activity of

all three lysophosphatidylcholine catabolic enzymes as well as an increased production of lysophosphatidylcholine could potentially result in the observed augmentation of lysophosphatidylcholine in the ischemic myocardium. In this study, we have observed an increase in lysophosphatidylcholine production and a decrease in its catabolism sufficient enough to account for the increase in lysophospholipid levels in the ischemic tissue. Although an increase in lysophosphatidylcholine production and a decrease in lysophosphatidylcholine catabolism was observed, the mechanisms underlying these changes in lysophosphatidylcholine metabolism are unknown.

This study has provided more support for the implication of lysophospholipid as arrhythmogenic moeities of potential importance in vivo. However in evaluating the potential importance of a metabolite in arrhythmogenisis one must consider a set of "Koch's postulates" (Miller and Keane 1978): a) The metabolite concentration must change upon a change in the normal physiology of the heart. This criteria had been fulfilled by the clear demonstration of a rise in lysophospholipid levels in the ischemic heart. The perpetuation of cardiac arrhythmias upon the onset of ischemia has been clearly established (Bigger et al 1977; Elharrar and Zipes 1977; Williams et al 1974). b) The addition of the exogenously administered metabolite should produce electrophysiological derangements mimicking those produced by

ischemia. This was reported by Man and Choy (1982) who demonstrated that the addition of exogenous lysophospholipid to the perfusate (within the concentrations found in the ischemic heart) of isolated perfused hamster hearts resulted in cardiac arrhythmias. c) Exacerbation or amelioration of the effects of the exogenous metabolite by concomittants of ischemia known to exacerbate or ameliorate arrhythmogenesis. This criteria was fulfilled in part by reducing the pH of the perfusate to 6.7, comparable to that seen after the onset of ischemia (Hollis et al 1978). This resulted in a 3 fold increase in sensitivity of the perfused heart to the electrophysiological effects of exogenous lysophosphatidylcholine (Corr et al 1981). Also Man and Choy (1982) demonstrated that only the free form of lysophosphatidylcholine was arrhythmogenic. They found that when lysophosphatidylcholine was bound to albumin it lost its arrhythmogenicity. They brought forward the hypothesis that the lysophospholipid level had to surpass the binding capacity of serum proteins before the lysophospholipid could express their arrhythmogenic nature. d) There must be evidence of a delineation of enzymes in the ischemic tissue with activity sufficient to account for the biologically active concentration of the metabolite. In this study a decrease in lysophosphatidylcholine catabolism and an increase in lysophosphatidylcholine production was evident although the mechanisms underlying these changes in lysophosphatidylcholine metabolism are unknown. e) the exogenously administered metabolite must be localized to a site comparable to

that of the endogenous metabolite. An increase in lysophospholipid levels was found in effluents from ischemic zones (Snyder et al 1981). This would suggest that the endogenous lysophospholipid had direct access to the external surface of the sarcolemma. This is not surprising due to the amphiphilic nature of lysophospholipid. Results obtained from exposure of ventricular muscle to exogenous lysophosphatidylcholine indicated that when as little as 5% of total sarcolemmal phospholipid was composed of lysophosphatidylcholine, electrophysiological derangements mimicking those of ischemia resulted (Saffitz et al 1982). In our studies, lysophopholipids were shown to make up close to 2% of the total cellular phospholipid in the ischemic myocardium. Lysophospholipid levels were not measured in the sarcolemma, however our values exceed the 1% total cellular phospholipid shown to be required, to elicit the electrophysiological disturbances observed upon ischemia (Gross et al 1982). Therefore exposure of the myocardium to exogenous lysophospholipid results in their incorporation in the sarcolemma eventually resulting in electrophysiological derangements when their content reaches a critical level. f) Before one can claim that lysophospholipids are potential in vivo mediators of cardiac arrhythmias, the final criteria of "Koch's postulates" must be fulfilled. We have not yet been able to modify the concentration of the lysophospholipids either by altering their structure or by delineating their metabolic enzyme activities during severe

ischemia. This modification of the metabolite's concentration should be paralleled by the exacerbation or amelioration of arrhymogenisis despite the presence of severe ischemia. A modification in lysophosphatidylcholine levels in the ischemic heart can only be achieved when a better understanding of the mechanisms involved in the generation of cardiac arrhythmias upon ischemia is realized. A time course study correlating the rise in lysophospholipid, the change in lysophospholipid metabolism and changes in electrophysiology, with time could provide many answers. As of now only a hypothesis can be brought forward. The occlusion of a coronary artery results in a lack of blood, therefore nutrients, feeding the area of the heart that was normally provided for by that artery. The area becomes hypoxic and must therefore resort to an anaerobic metabolism. Glycogen and ATP levels in the area are depleted and lactate accumulates. This accumulation of lactate results in an area that becomes markedly acidic. The reduced pH and ATP as well as other observed factors such as the accumulation of long chain acylcarnitines alters the normal lysophospholipid metabolism. The increase in lysophospholipid production and decrease in lysophospholipid catabolism results in a net increase in lysophospholipids. This high lysophospholipid level may, in fact, further inhibit the lysophospholipase activity thereby further contributing to the increase in this metabolite's accumulation. Due to its amphiphilic property, the lysophospholipid readily diffuses to the external surface of the sarcolemma. Some of

this lysophospholipid is released into the extracellular effluents where it can be transported elsewhere, whereas the rest remains incorporated in the sarcolemma. At low concentrations, amphiphiles such as lysophospholipids exist in solution as monomers that can be inserted into the hydrophobic environment of the lipid membrane. The incorporation of lysophospholipids into the biological membrane can change the physical properties of the lipid bilayer. At high concentrations lysophospholipid monomers aggregate into micelles. These micelles have the ability to incorporate membrane lipids into their structure thereby forming mixed micelles. The incorporation of high concentrations of amphiphiles into membranes can physically disrupt the lipid bilayer, liberating endogenous membrane lipids (Katz and Messineo 1981). This latter effect represents the detergent-like properties of amphiphiles. In this way high and low lysophospholipid concentrations are able to destroy the integrity or at least the physiological function of the biological membrane. The incorporation of the lysophospholipid thereby destroys the membrane potential either by destroying the membrane itself or by causing conformational changes in ion channel proteins. This has been clearly demonstrated by Lawrence et al (1974) who showed that prior to the loss of hemoglobin, lysophosphatidylcholine-treated erythrocytes become leaky for potassium. The altered conductivity of electrical signals across the membrane resulting from the damage to the sarcolemma is a prerequisite for the generation of an irregular heart beat. When the damage is extensive enough the heart

beats irregularly. Eventually the uncontrolled entry of Ca^{++} into the cell leads to cell death.

From this hypothesis one can propose several courses of action in the evaluation of lysophospholipids as possible modulators of cardiac arrhythmias as well in the evaluation of therapies used to manage patients with coronary artery disease. The modification of concentations of lysophospholipids during ischemia is probably the most important factor in the evaluation of lysophospholipids as possible mediators of cardiac arrhythmias. The obvious method used for modifying metabolite levels is by altering their metabolism. Before this can be done, one must characterize the enzyme(s) in question and assure oneself that the method used for modification affects only the enzyme(s) in question. The other procedure used in modifying metabolite levels is to modify the metabolite itself structurally or otherwise in such a way that it affects its arrhythmogenic nature.

A second course of action is to study the effects of antiarrhythmic drugs on lysophospholipid either direct or indirect. These drugs may stabilize the membrane, alter lysophospholipid levels or structures by affecting the lysophospholipid metabolic enzymes or by binding to lysophospholipid much in the same way the serum proteins do. Many physiological studies have been conducted on the effectiveness of Ca^{++} antagonists and other antiarrhythmic

entities on cardiac arrhythmias, however their biochemical mechanisms are unknown (Manning et al 1982; Kupersmith et al 1976; Hashimoto et al 1982; Hashimoto et al 1982b; Chew et al 1982; Fagbemi et al 1983; Singh et al 1983, Patterson et al 1982; Ichihara 1982). Roberts and Walker (1982) have recently reported that feeding of a creatine analogue would delay ATP depletion in the ischemic rat heart. This could play a role in the activity of acyl CoA: lysophosphatidylcholine acyltransferase. Several other directions are being considered in the evaluation of the causes and cures of cardiac arrhythmias. Hopefully this study will lead to the eventual positive evaluation of the biochemical factor(s) involved in the generation of cardiac arrhythmias. Knowledge of the cardiac electrophysiological and biochemical alterations that occur after occlusion of a coronary artery is a prerequisite for understanding the pathophysiology of arrhythmias. Observations made in various animal models and at various times following occlusion of a coronary artery are prerequisites in developing the proper therapeutic management of patients with myocardial infraction.

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