THE METABOLISM OF LYSOPHOSPHATIDYLCHOLINE IN THE MAMMALIAN HEART

by

Thomas Mock

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of Graduate Studies

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
BHT	butylated hydroxytoluene
cAMP	cyclic adenosine 5'-monophosphate
cm	centimeter
CMC	critical micellar concentration
CoA	Coenzyme A
DAD	delayed afterdepolarizations
DEM	diethylmaleimide
dpm	disintegrations per minute
EDTA	ethylinediaminetetraacetic acid
FFA	free fatty acid
g	gram
g	gravity
GSH	glutathione
HEPES	(N-2-Hydroxyethylpoperazine-N'-2-ethanesulfonic Acid)
hr	hour
i.p.	intraperitoneal
I_{si}	slow inward current
Ki	inhibitory constant
Km	Michaelis-Menten constant
LCAC	long chain acyl carnitine
LCAT	lecithin-cholesterol acyl transferase
LPC	lysophosphatidylcholine
M	molar

mg	milligram
min	minute
ml	milliliter
mm	millimeter
nmol	nanomole
PC	phosphatidylcholine
pHMB	parahydroxymercuribenzoic acid
PLA	phospholipase A
rpm	rotations per minute
S.E.	standard error
U.V.	ultraviolet
μCi	microCurie
μg	microgram
μl	microliter
μM	micromolar
μmol	micromole
v	volume
Vmax	maximum velocity

ACKNOWLEDGMENTS

An all too common yet possibly naive perception of successful research as being an ordered series of experiments which progresses along a well-defined path broken only by the occasional uncooperative test subject or dropped test tube proved to be mere fantasy early on in my stay in the department. To my wife Marianne and the rest of my family, whose timely reminders that the often all-consuming frustration and attendant alopecia which stemmed from that realization are integral parts of the learning process, I owe a huge debt of gratitude.

Special thanks to my supervisor, Dr. Ricky Man, for providing me with the opportunity to conduct my research in an environment that encourages learning. I valued the freedom to make my own mistakes. I also wish to thank the staff and fellow trainees in the department who perhaps unknowingly have provided the incalculable educational benefit of being at once teacher and student.

To past and present members of Dr. Man's laboratory including Anne Blanchard, Ila McNicholl and Drs. Weimin Hu and Antoine Bril I extend my warmest appreciation for all the help and support.

Lastly I wish to anknowledge a person unknown to me yet whose sage advice has been a guiding principle in the completion of this thesis. To quote, " In der Kürze liegt die Würze" whose sentiment, loosely translated, is "The appeal lies in its brevity".

ABSTRACT

The metabolism of lysophosphatidylcholine (LPC) was investigated in canine heart rendered regionally ischemic. Significant increases in LPC content in ischemic tissue *vs* non-ischemic control tissue were detected at 3 and 5 hr of ischemia. The hypothesis that the increases in LPC levels are the result of an imbalance between production and elimination which is reflected in changes in enzyme activity was subsequently tested. Cytosolic, mitochondrial and microsomal fractions prepared from non-ischemic and ischemic myocardium were assayed *in vitro* for the LPC-producing enzyme phospholipase A and the LPC-eliminating enzymes acyl CoA:LPC acyl transferase, LPC:LPC transacylase and lysophospholipase. No significant alterations in the activity of phospholipase A in any fraction were detected over the course of 5 hr of ischemia. In contrast significant time-dependent decreases in the activity of the microsomal LPC:LPC transacylase, microsomal acyl CoA:LPC acyl transferase and cytosolic and microsomal lysophospholipase were found. In each case a level of statistical significance was achieved at 3 hr of ischemia which was temporally related to the accumulation of LPC. Therefore the present results suggest that impaired catabolism of LPC rather than enhanced production is the biochemical mechanism for LPC accumulation in the ischemic canine heart. Reduced pH and long-chain acyl carnitine are two metabolic determinants which were found to inhibit catabolic enzymes although the extent and type of inhibition varied among the microsomal acyl CoA:LPC acyl transferase and cytosolic and microsomal lysophospholipase. The myocardial content of long chain acyl carnitine increased during ischemia in parallel with the decrease in catabolic enzyme activity and LPC accumulation indicating a possible role for LCAC in control of LPC catabolism.

This suggested link between modulation of catabolism and cardiac LPC level was studied in the isolated intact heart. In a comparative analysis of microsomal LPC catabolism in the rat and guinea pig heart it was clear that specific activity measured *in vitro* is of no value in predicting relative participation of acyl CoA:LPC acyl transferase and lysophospholipase in clearance of exogenous labeled LPC in the intact organ. In rat heart microsomes equal participation of acyl CoA:LPC acyl transferase and lysophospholipase in LPC clearance was established. Impairment of deacylation by pretreatment of rat hearts with pHMB, a sulfhydryl agent, led to increased levels of unmetabolized labeled LPC. A similar result was obtained with the use of diamide, an agent widely used to deplete glutathione. The rapid reversal of the inhibitory effect obtained by removal of diamide revealed additional regulation of the LPC level by acyl CoA:LPC acyl transferase. Perfusion of the rat heart under "ischemic" conditions, where the composition of perfusate and pO_2 were altered to mimic ischemia, led to an increased level of LPC which was coupled exclusively to a decrease in lysophospholipase-mediated free fatty acid (FFA) release. In guinea pig microsomes deacylation by lysophospholipase is clearly the preferred route of LPC clearance. In contrast with rat heart microsomes a reduction in acylation by acyl CoA:LPC acyl transferase induced by pHMB and diamide, did not result in any significant change in the amount of unmetabolized labeled LPC because of compensatory deacylation by lysophospholipase. A reduction in lysophospholipase activity by diamide, however, did impair catabolism of LPC and led to a greater level of unmetabolized LPC. In contrast to rat heart no effect of perfusion under "ischemic" conditions on the microsomal profile of radioactive LPC and the products of its catabolism was found. The data obtained suggest that inhibition of catabolic enzymes is one mechanism which governs the level of LPC in heart microsomal membranes. Furthermore, variability in LPC catabolism between rat and guinea pig microsomes is likely the result of the relative extent to which lysophospholipase participates in LPC clearance.

"A moment's insight is sometimes worth a life's experience"

Oliver Wendell Holmes

INTRODUCTION

Lysophospholipids are phospholipid metabolites which have been detected in virtually all biological membranes (Ansell *et al.*, 1973). Unlike their diacyl counterparts, which possess two fatty acid residues and provide the structural framework of the membrane, lysophospholipids contain only one fatty acid residue and as such display the properties of an amphiphile. This amphiphilicity imparts upon lysophospholipids the property of a natural detergent. Insertion into the biological membrane under appropriate circumstances affects membrane dynamics the consequences of which may alter cellular physiology, a focus of investigation for more than a decade (Weltzien, 1979). The importance of maintaining low concentrations of lysophospholipids is underscored by their ability to lyse cell membranes (Weltzien, 1979), hence the trivial name. Regulation of lysophospholipid levels through precise coordination of production and elimination is therefore crucial to the integrity of the membrane.

Lysophosphatidylcholine (LPC) is formed from phosphatidylcholine (PC) by phospholipases A_1 and A_2 which release the fatty acid residue at the C-1 and C-2 positions, respectively (Fig. 1). Various cardiac phospholipases A have been described and differentiated on the basis of subcellular locus, pH optimum, ion requirements and substrate specificity (Weglicki and Low, 1987). An alternate source of LPC may include hydrolysis of PC by lecithin-cholesterol acyltransferase in plasma (Aron *et al.*, 1978). The removal of LPC is effected by a number of enzymes, LPC:acyl CoA acyltransferase, lysophospholipase and LPC:LPC transacylase. The patterns of subcellular distribution and specific activities and, in some cases, the actual presence of these enzymes in cardiac tissue among various species are remarkably diverse and hence, the extent and preferrred routes of elimination of LPC are in all likelihood species-specific (Gross and Sobel, 1982, Severson and Fletcher, 1985, Savard and Choy, 1982)

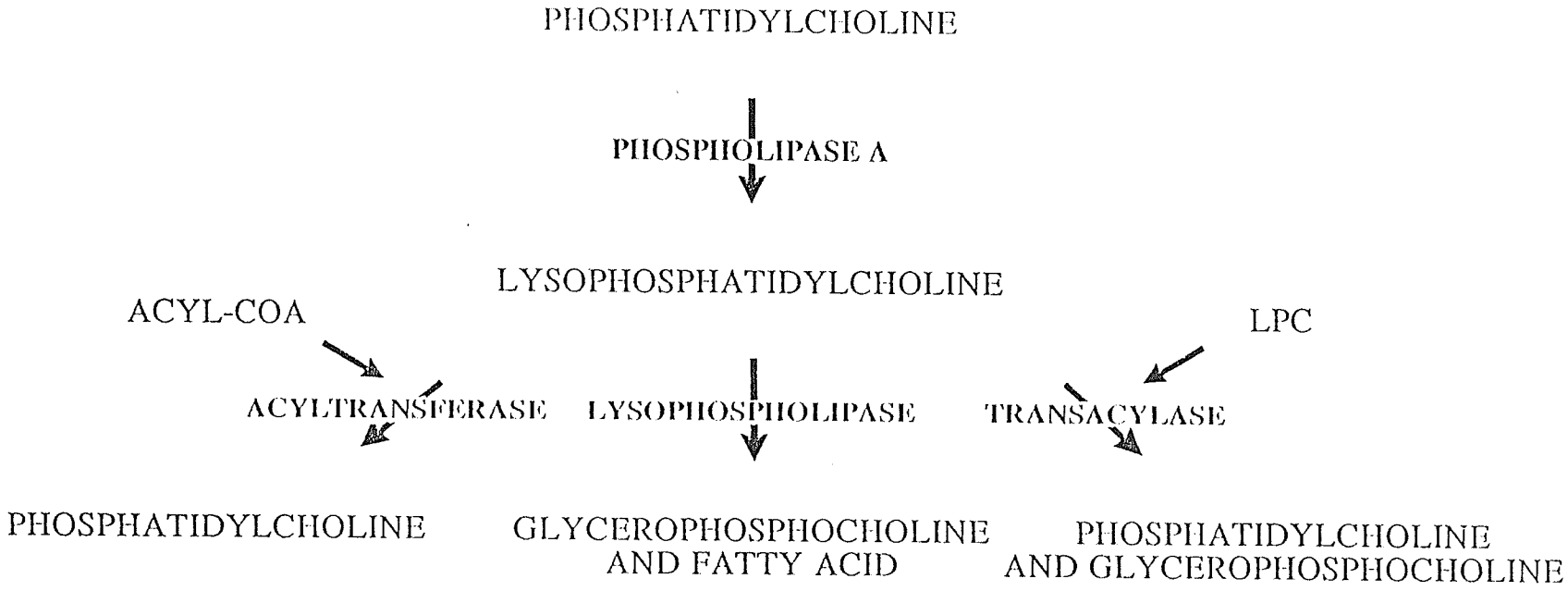
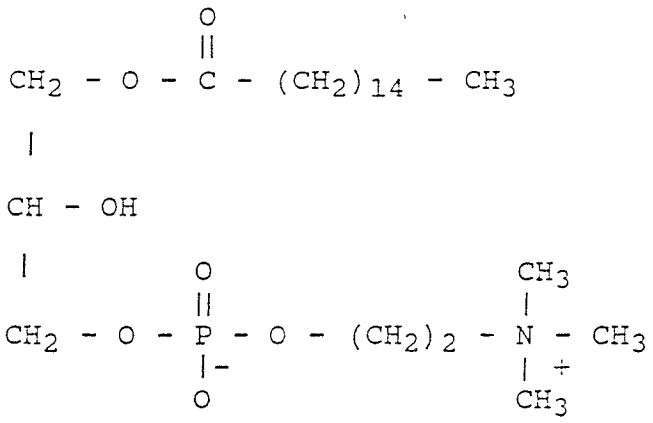


Figure 1. Myocardial LPC metabolism

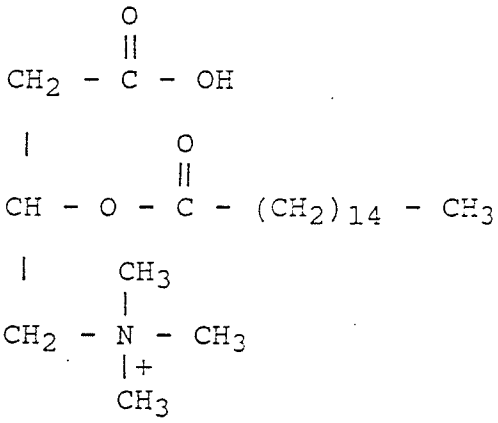
As early as 1957 it was suggested that lysophospholipids contribute to ischemic heart damage (Hajdu *et al.*, 1957) . Intense interest in LPC as a biochemical mediator of membrane dysfunction was sparked by the seminal observation by Sobel *et al.* (1978) of increased levels of LPC in the ischemic rabbit myocardium. Since then confirmation of increased LPC during myocardial ischemia has been provided in various models (Snyder *et al.*, 1981, Shaikh and Downar, 1981, Akita *et al.*, 1986, Kinnaird *et al.*, 1988) including man (Sedlis *et al.*, 1989). Several lines of evidence have shown LPC to markedly affect properties of the excitable membrane (Corr *et al.*, 1979, Arnsdorf and Sawicki, 1981, Clarkson and Ten Eick, 1983, Kiyosue and Arita, 1986, Burnashev *et al.*, 1989). Those studies argue strongly for a role for LPC in the production of malignant arrhythmias during myocardial ischemia. The last several years have witnessed a resurgence in interest in LPC as a mediator of various physiological and biochemical processes at various levels in a large number of tissues and organs (Ahumada *et al.*, 1979, Owens *et al.*, 1982, Sedlis *et al.*, 1983, Ambudkar *et al.*, 1988, Oishi *et al.*, 1988, Saito *et al.*, 1988, Ginsburg *et al.*, 1989). Indeed the evidence thus far obtained has prompted a number of investigators to describe LPC as a putative second messenger in some instances based on its likely participation in signal transduction (Oishi *et al.*, 1988).

Physiochemical properties of lysophospholipids

The structure of LPC, as a representative lysophospholipid, is shown in Figure 2. In simple terms LPC is composed of a hydrophilic portion, which comprises the glycerol phosphate backbone and the bound choline head group and a hydrophobic moiety, the aliphatic chain. In Nature we find differences in the length and degree of saturation in the aliphatic chain which are manifest in the physical property known as the critical micelle concentration (CMC). This parameter defines the concentration in aqueous solution at



palmitoyl lysophosphatidylcholine



palmitoyl carnitine

Figure 2. Structures of palmitoyl lyosphosphatidylcholine and palmitoyl carnitine.

which monomers spontaneously aggregate to form micelles (Weltzien, 1979). The CMC has proved to be a valuable index in predicting the lytic activity of various species of LPC and is proportionally related to the detergent potential of the lipid. The micellar form of the lysophospholipid is hypothesized to act as a "sink" which provides lipid to the surrounding medium when the equilibrium between the monomeric and micellar form is upset. It is the monomeric form of LPC which is believed to bind to the membrane and become incorporated into the lipid matrix (Fink and Gross, 1984). The subsequent disruption of the molecular packing as evidenced by the increase in transbilayer fluidity gradient and possible dissolution of membrane components are believed to underlie many of the physiological and biochemical actions ascribed to LPC (Poole *et al.*, 1970, Lee and Chan, 1977). It is widely held that the composition and physical state of the lipid annulus exert a profound influence on the conformation and accessibility of integral membrane proteins and as a result affect the catalytic performance of enzymes (Smith and Stubbs, 1987). Not surprisingly LPC has repeatedly been shown to influence greatly the activity of membrane bound proteins such as adenylate and guanylate cyclase (Shier *et al.*, 1976, Zwiller *et al.*, 1976), glycosyltransferase (Shier and Trotter, 1976), Na^+/K^+-ATPase (Kelly *et al.*, 1986) and Ca^{2+}-uniporter (Rustenbeck and Lenzen, 1989). It would be misleading, however, to suggest that the actions of LPC are entirely the result of its detergent properties. In an exhaustive study on the LPC-induced depolarization of cat ventricular muscle, Clarkson and Ten Eick (1983) have shown that LPC acts as a depressant of membrane channels independent of its ability to compromise membrane integrity. They have offered compelling evidence to show that instead of the production of a "porous" or "leaky" membrane which would be anticipated from its detergent potential, LPC actually increases membrane resistance in the concentration range studied (50-200 μM). Two recent studies (Engelberger *et al.*, 1987, Lenzen *et al.*, 1989) have demonstrated that the amphiphilic nature of various lysophospholipids alone does not render them equally effective in production of the effects studied. They have clearly established that the class of headgroup is a critical determinant of

activity. In one study (Lenzen *et al.*, 1989) differences in the potencies of lysophospholipids in influencing mitochondrial Ca^{2+}-uptake (LPC being most potent) were attributed to the ability to form intermolecular hydrogen bonds with nearby membrane lipids. Clearly then, the effects of lysophospholipids must be considered in the context of both physical and chemical interactions.

Cardiovascular effects of LPC

The cardiac and vascular effects of LPC are varied and extensive. After it was demonstrated that LPC levels rise in ischemic myocardium (Sobel *et al.*, 1978), a great deal of effort was directed towards determining the functional consequences of this abberation. Various models and approaches were employed to establish the biochemical, physiological and electrophysiological correlates of raised cardiac LPC. Subsequent studies demonstrated that the elevation of LPC is also apparent in ischemic venous drainage (Snyder *et al.*, 1981) and in cardiac lymph (Akita *et al.*, 1986) which means that it is likely that the effects of LPC are wide in scope and may involve interaction at a number of different levels.

LPC is a normal constituent of blood (Kelly *et al.*, 1986) and has been found to be effectively extracted by the heart (Stein and Stein, 1966); the extent of incorporation is a function of the amount of LPC and the concentration of albumin, which binds LPC. Bergmann *et al.* (1981) have shown that perfusion of isolated rabbit heart with both sub-CMC and supra-CMC concentrations of LPC produces profound effects on several functional characteristics. Sub-CMC concentrations of LPC dose-dependently elicited arrhythmias and contracture. An increase in perfusion pressure was caused by constriction of coronary arteries and consequently increased resistance to flow. Supra-CMC concentrations led to virtually immediate contracture and constriction of isolated coronary arteries. Parallel studies with bile salts, which are also found in blood, produced divergent

results at sub-CMC concentrations, an indication that the nonspecific detergent property of LPC did not contribute to the observed effects. In a recent study Saito *et al.* (1988) have provided evidence that at concentrations lower than those used by Bergmann *et al.* (1981), LPC can also effect relaxation of aortic strips, an effect unlike that mediated by endothelium-derived relaxation factor. The extent of relaxation appeared to be closely related to activation of guanylate cyclase. Cardiac cAMP levels are also regulated by LPC. Ahumada *et al.* (1979) have shown that in broken cell preparations and in isolated perfused rabbit heart stimulation of adenyl cyclase by LPC increases cAMP levels, an effect unrelated to ß-adrenergic stimulation. These authors have proposed that elevation of LPC may contribute to the augmented cAMP levels seen in ischemic myocardium despite complete ß-adrenergic blockade.

A finding which has aroused considerable interest, particularly because of its implication in the initiation and propagation of the electrical signal and also contraction of the cardiac muscle, has been the ability of LPC to alter cellular Ca^{2+} fluxes. Using cultured rat cardiac myocytes, Sedlis *et al.* (1983) have demonstrated that exposure to LPC at concentrations less than 100 μM increased the exchangeable pool of cellular Ca^{2+}. This resulted in augmented Ca^{2+} accumulation as a result of altered fluxes across the cell membrane. Concentrations above 100 μM led to an absolute increase in cellular calcium as stimulation of influx exceeded that of efflux. At the membrane level LPC has been found to inhibit microsomal Ca^{2+} transport (Ambudkar *et al.*, 1988) apparently by uncoupling Ca^{2+} transport and ATP hydrolysis. Similarly, LPC has been shown to inhibit mitochondrial Ca^{2+} uptake likely by dissipation of the mitochondrial membrane potential (Lenzen *et al.*, 1989). Moreover, a net Ca^{2+} efflux was noted which in conjunction with impaired uptake led to increased extramitochondrial Ca^{2+}.

Na^+/K^+-ATPase inhibition by LPC and other amphiphiles has been well characterized in cardiac sarcolemma (Owens *et al.*, 1982). The effects are strongly dependent on temperature, concentration and the ratio of incorporated amphiphile to sarcolemmal protein. In the aforementioned study palmitoyl CoA and LPC were shown to markedly inhibit Na^+/K^+-ATPase, whereas palmitoyl carnitine was without effect. Thus the complex inhibitory profile obtained from these lipid intermediates preclude a single mechanism of inhibition. The physiological relevance of Na^+/K^+-ATPase inhibition by LPC awaits further study.

The involvement of free-radicals in ischemic/reperfusion injury is currently the topic of intense investigation (Kako, 1987, Tsushima and Moffat, 1989). It is believed that the presence of oxygen coupled with compromised cellular defenses (eg. reduced glutathione peroxidase and superoxide dismutase activities) leads to the production of several highly reactive oxygen species (O_2^-, $OH\cdot$) which can cause severe damage to membranes via peroxidation of unsaturated fatty acids and proteins. Several studies have provided a link between oxygen free radical production and functional and electrophysiological derangements (Burton *et al.*, 1984, Basu and Karmazyn, 1987, Okabe *et al.*, 1989, Tsushima and Moffat, 1989). Administration of free radical scavengers such as α-tocopherol and superoxide dismutase (Jolly *et al.*, 1984, Kim and Akera, 1987, Janero and Burghardt, 1989) can to a large extent protect against free radical-induced damage. A number of recent studies (Mak *et al.*, 1986, Kihlström *et al.*, 1987) have demonstrated that free radical formation *in vitro* is potentiated by LPC. Malondialdehyde formation, which is routinely used as an index of free radical production, was increased 210% in sarcolemmal vesicles treated with 50 μM LPC using dihydroxyfumarate/ Fe^{3+}-ADP as the free radical generating system (Mak *et al.*, 1986). Thus the accumulation of LPC in ischemic cardiac tissue may predispose the tissue to enhanced damage via free radicals. Moreover, the depressant effects of LPC on contractile parameters on cultured myocytes is enhanced by

superoxide radical (Sedlis *et al.*, 1990). The damaging effects of LPC and free radicals may be therefore synergistic.

Electrophysiological effects of LPC

In an important study Downar *et al.*, (1977) have convincingly argued for the presence of an "arrhythmogenic" factor in ischemic venous drainage. Manipulation of other indices of ischemia such as pH, K^+, lactate, glucose etc. was insufficient to induce the electrophysiological changes typical of myocardial ischemia in isolated porcine myocardial strips. Application of blood collected from the ischemic tissue, however, consistently and reproducibly elicited a shortening of action potential duration, a decrease in resting membrane potential, post repolarization refractoriness and, ultimately, unresponsiveness. By inference then, these authors concluded an as yet unidentified factor(s) present in ischemic blood was responsible for the observed effects.

Since then there have been several studies which indicate a temporal and phenomenological link between LPC and the electrophysiological derangements seen in myocardial ischemia (Corr *et al.*, 1987, Kinnaird *et al.*, 1988). The arrhythmogenic properties of LPC have long been recognized (Sobel *et al.*, 1978, Snyder *et al.*, 1981, Corr *et al.*, 1982). Man *et al.*, (1982) reported that free LPC can cause arrhythmias in the isolated perfused hamster heart. The spectrum of changes typically observed ranges from isolated premature ventricular contractions to ventricular tachycardia and ultimately, ventricular fibrillation. The incidence and severity of arrhythmias are functions of the amount of unmetabolized LPC in the membrane fraction prepared from the heart (Giffin *et al.*, 1988), which clearly indicates that the effects of LPC are concentration-dependent. A more detailed understanding of the electrophysiological effects of LPC has been achieved by investigation at a number of different levels including the study of endocardium (Snyder *et*

al., 1981), papillary muscle (Clarkson and Ten Eick, 1983), Purkinje fibres (Sobel *et al.*, 1978, Corr *et al.*, 1982, Pogwizd *et al.*, 1986) and isolated ventricular cells (Kiyosue and Arita, 1986, Burnashev *et al.*, 1989). Despite the diversity in model the effects of LPC are remarkably consistent. LPC has been shown to affect both the initiation and conduction of the electrical impulse which are critical determinants of arrhythmogenesis (Janse and Wit, 1989). At concentrations calculated to exist during myocardial ischemia *in vivo* LPC induces depolarization in cat ventricular muscle (Clarkson and Ten Eick, 1983) and decreases sodium conductance in isolated canine Purkinje fibres (Corr *et al.*, 1979) which are manifest in altered phase 0 depolarization. LPC changes the contour of the action potential by virtue of its effects on several ion currents thereby modulating action potential duration and effective refractory period (Corr *et al.*, 1979) often producing a marked dissociation of these two parameters. The heterogeneous alterations in action potential repolarization in affected tissue produced by LPC provides the anatomical substrate for reentry circuits which are believed to underlie the early phase of arrhythmias after experimental coronary artery occlusion (Janse and Wit, 1989). Moreover, the effects of LPC on action potential characteristics are potentiated by reduced pH (Snyder *et al.*, 1981), a concomitant of myocardial ischemia (Jennings *et al.*, 1986). The ability of LPC to produce and maintain sustained rhythmic activity at a stable steady-state potential at plateau levels (Arnsdorf and Sawicki, 1981) may be due, in part, to the induction of the slow response (Corr *et al.*, 1982). The slow inward current, I_{si}, has been implicated in the production of reentry arrhythmias during myocardial ischemia (Janse and Wit, 1989) by virtue of conduction delays and altered refractoriness. The link between cAMP and the slow calcium influx channels in the heart has been firmly established (Watanabe and Besch, 1974). Since LPC augments cardiac adenyl cyclase activity and consequently cAMP levels (Ahumada *et al.*, 1979) I_{si} may be directly stimulated by LPC. Delayed afterdepolarizations (DAD) constitute a mechanism whereby triggered rhythms may occur. Pogwizd *et al.* (1986) have demonstrated that LPC can provoke DADs in canine Purkinje fibers, an effect

potentiated by epinephrine and still evident in the presence of acidosis and hyperkalemia. In an elegant study involving microinjection of LPC into canine Purkinje fibers, Akita *et al.* (1986) showed that intracellularly applied LPC does not produce the electrophysiological derangements typically seen. This demonstrated that the arrhythmogenic effects of LPC on this tissue are probably mediated through initial association with the outer side of the plasma membrane.

Metabolism of cardiac LPC

Our basic conceptual understanding of cardiac LPC metabolism has been principally the result of *in vitro* enzyme analysis. This approach has led to the identification of the enzyme activities involved in the production and elimination of LPC. Early on it became apparent that the presence and subcellular localization of enzymes was species-specific. However, typical of similar *in vitro* approaches caution must be exercised in the interpretation of information obtained because of the frequent application of optimal reaction conditions including use of synthetic substrates. Moreover, subcellular fractions from mechanically disrupted cells may not be entirely representative of cellular structures from the standpoint of strict physical and chemical composition which may be of relevance when the dynamics of lipid-lipid, lipid-protein and lipid-water interactions are critical determinants of activity. Nevertheless the *in vitro* approach is indispensable as a tool for studying these enzyme activities under controlled conditions and as such may provide information that will allow the formulation of an hypotheses to explain the rise in cardiac LPC during ischemia.

Production of LPC

It is widely held that the hydrolysis of PC by phospholipase A (PLA) represents the single most important mechanism for the production of LPC in the heart (Fig.1). To what

extent the production of LPC by lecithin-cholesterol acyltransferase (LCAT) in the plasma and subsequent uptake by the heart may contribute to cardiac LPC is unknown (Aron *et al.*, 1978). The recent finding of a correlation between LPC, LCAT and the severity of coronary atherosclerosis (Wells *et al.*, 1986) suggests the possibility may exist. Hydrolysis of PC by PLA produces equimolar amounts of LPC and free fatty acids; the positional specificity of the PLA (either PLA_1 or PLA_2) determines the species of LPC produced (2-acyl or 1-acyl, respectively). Both PLA_1 and PLA_2 have been detected in the heart (Weglicki *et al.*, 1971); however, the high lysophospholipase activity in some microsomal preparations may preclude the characterization of these A_1 and A_2 activities independently (Nalbone and Hostetler, 1985). PLA activity has been detected in the lysosomes, cytosol, microsomes, mitochondria and sarcolemma (Weglicki *et al.*, 1971, Franson *et al.*, 1978, Nalbone and Hostetler, 1985). Only the lysosomal enzyme displays appreciable activity at acid pH; the others display a preference for neutral to slightly basic pH. Divergent results with respect to ion requirements, particularly Ca^{2+}, and inhibition by EDTA, among subcellular fractions and among species indicates substantial specificity in this regard (Weglicki *et al.*, 1971, Franson *et al.*, 1978). The membrane associated enzymes, however, display an almost universal requirement for Ca^{2+} (Weglicki *et al.*, 1971, Franson *et al.*, 1978). The apparent Km of the rat cytosolic enzyme for PC has been determined to be 0.07 mM while a value of 0.33 mM has been suggested for the microsomal enzyme (Nalbone and Hostetler, 1985). Purified hamster heart cytosolic PLA displays a Km for PC of 0.5 mM (Cao *et al.*, 1987). Partially purified canine cytosolic enzymes with a marked selectivity for PC and plasmenylcholine (choline plasmalogen) had Km's of 3 μM and 7 μM, respectively (Wolf and Gross, 1985). The discrepancy in this important kinetic parameter among various species may be ascribed to species specificity and the extent of enzyme purification, however, different experimental conditions, particularly the use of different substrate species, must also be considered. PLA has been shown to be sensitive to molecular dynamics, conformation and surface charge (Chang *et al.*, 1987). Two striking examples

are the almost exponential increase in enzyme activity with increasing substrate concentration, which is interpreted as indicating PLA_2 preferentially hydrolyzes aggregated over non-aggregated substrate (Chang *et al.*, 1987), and the higher activities obtained with the use of phosphatidylethanolamine as substrate (Tam *et al.*, 1984). Activity of membrane-bound PLA, which by its very nature carries with it a complement of phospholipid, may also conceivably be a function of the method used for subcellular fractionation. Cardiac PLA_2 is known to be inhibited by several drugs including chlorpromazine and mepacrine which has formed the basis for pharmacological manipulation of phospholipid catabolism in several studies in the ischemic heart (Okumura *et al.*, 1983, Das *et al.*, 1986).

Elimination of LPC

Several enzyme activities have been identified in the heart which serve to clear LPC (Gross and Sobel, 1982) Since under normal conditions LPC has not been shown to vary appreciably it appears that clearance of LPC by one or more of these routes is an effective and sufficiently rapid process. Substantial species-specificity exists with respect to the presence of these enzyme activities in the cytosolic, mitochondrial and microsomal fractions of the heart which are most often studied. Moreover, given the marked differences in specific activity among these subcellular fractions it is tempting to speculate that the rates and preferred routes of catabolism are variable.

Acyl CoA:LPC acyl transferase catalyzes the condensation of LPC and acyl CoA to form PC. This represents the second step of the Land's cycle (1960) which in conjunction with deacylation by phospholipase A is thought to be the major regulatory mechanism for the fatty acyl composition of cardiac PC (Arthur *et al.*, 1987). Acyl CoA:LPC acyl transferase activity has been detected in the microsomal fraction of heart from a number of sources including rabbit (Gross and Sobel, 1982), rat (Severson and Fletcher, 1985) and

guinea pig (Arthur *et al.*, 1987). Cytosolic activity has also been reported albeit at a much lower specific activity (Gross and Sobel, 1982). Mitochondrial activity although initially reported to be very low (Gross and Sobel, 1982) has recently been shown to be substantially higher using polyunsaturated fatty acyl CoA substrates (Arthur *et al.*, 1987) which are the preferred fatty acyl donors for acylation at the C-2 position of LPC (1-acyl LPC is predominant in the heart). An apparent Km for LPC of 14 μM and an apparent Km for palmitoyl CoA of 7 μM have been reported for the rabbit microsomal enzyme (Gross and Sobel, 1982). No such kinetic analysis has been performed for the mitochondrial and cytosolic enzymes. Myocardial acyl CoA:LPC acyl transferase activity displays a broad pH profile with maximum activity at about pH 7 (Gross and Sobel, 1982). Several structural analogs have been shown to inhibit activity including glycerophosphocholine (Gross and Sobel, 1981), long chain acylcarntine and free fatty acid (Severson and Fletcher, 1985). Deacylation of PC by PLA and reacylation by acyl CoA:LPC acyl transferase results in no net change in phospholipid content.

LPC:LPC transacylase catalyzes the disproportionation of two molecules of LPC to form PC and glycerophosphocholine (GPC) and as such is characterized by acyl CoA-independent PC synthesis. In rabbit heart a cytosolic lysophospholipase-transacylase has been described (Gross and Sobel, 1982). A bell-shaped pH profile for both fatty acid release and PC synthesis with maximum activity at pH 7 was reported. No transacylase activity has been detected in the cytosolic fraction of rat heart myocytes (Severson and Fletcher, 1985) or in hamster heart (Savard and Choy, 1982). Rabbit heart microsomal LPC:LPC transacylase activity has been determined to be relatively low compared to other microsomal catabolic enzymes (Gross and Sobel, 1982). Evidently the presence of this enzyme in the heart is strongly species-specific. Conflicting reports have emerged with respect to regulation of transacylase activity by lipids. In rabbit heart cytosolic activity is inhibited by long chain acyl carnitine, palmitoyl CoA and palmitic acid (Gross *et al.*, 1983).

The purified bovine microsomal enzyme, however, is stimulated by various CoA species (Sanjanwala *et al.*, 1989). Further stimulation is observed by palmitic acid in the presence of oleoyl CoA. It must be noted that the product of transacylation is predominantly a disaturated species of PC. Unlike the lung which contains large amounts of dipalmitoyl PC as a constituent of surfactant, the heart contains relatively little disaturated PC. Thus the biological significance of transacylation in LPC clearance is uncertain .

Deacylation of LPC by lysophospholipase has been demonstrated in the cytosol and microsomes from a number of species (Gross and Sobel, 1983, Severson and Fletcher, 1985). Mitochondrial activity is present only in very low levels in rabbit heart (Gross and Sobel, 1982). The specific activity of the microsomal lysophospholipase is greater than the cytosolic enzyme, however, it is still significantly lower than microsomal acyl CoA:LPC acyl transferase specific activity in rabbit heart (Gross and Sobel, 1982) and rat heart myocytes (Severson and Fletcher, 1985). One kinetic feature of the microsomal lysophospholipase that differentiates it from other catabolic enzymes is the potential for inhibition at high substrate/protein ratios. The rabbit enzyme for example has been shown to be very sensitive to modulation at high substrate/protein ratios (Gross and Sobel, 1982). Kinetic analysis of the membrane-associated enzyme has thus proved to be very difficult given the non- Michaelis-Menten nature of its activity. The microsomal enzyme also displays a marked preference for neutral pH; significant inhibition of activity is apparent at minimally reduced pH (Gross and Sobel, 1982). Inhibition of cytosolic and microsomal activity by palmitic acid (Severson and Fletcher, 1985), palmitoyl CoA (Gross, 1983) and long chain acyl carnitine (Gross and Sobel, 1983, Severson and Fletcher, 1985) has been demonstrated. In a recent finding cholesterol was also found to be a competitive inhibitor of the cytosolic lysophospholipase from rabbit aorta (Miyake *et al.*, 1990).

Research objectives

The restriction of blood flow to the myocardium is associated with profound disturbances in phospholipid homeostasis, an index of which is the increase in cardiac LPC levels. Elevated LPC levels have been detected in ischemic tissue *in vivo* (Corr *et al.*, 1982, Corr *et al.*, 1987), in the effluent of the isolated ischemic heart (Sobel *et al.*, 1978), in venular effluents draining ischemic myocardium (Snyder *et al.*, 1981) and in cardiac lymph (Akita *et al.*, 1986) . Elevated levels of LPC have also been reported in the coronary sinus of human subjects suffering from coronary artery disease during atrial pacing-induced ischemia (Sedlis *et al.*, 1989). The accumulation of LPC in the isolated heart indicates a cardiac origin and blood-borne elements, therefore, are not prerequisites (Sobel *et al.*, 1978). Potential sources of LPC include myocytes, endothelial cells, coronary vascular smooth muscle cells and fibroblasts. At present no information is available as to the subcellular site (s) of LPC accumulation as detailed analysis is hampered by the capacity for interorganelle transfer during sample preparation. Moreover, different rates and extents of catalysis among various subcellular compartments may complicate interpretation. One distinguishing feature among species has been the diferent time courses of LPC accumulation (Sobel *et al.*, 1978, Shaikh and Downar, 1981, Corr *et al.*, 1987, Kinnaird *et al.*, 1988) which suggest variability in regulation or coordination of metabolism. The rise in LPC levels may be attributed to increased production, decreased elimination and/or reduced washout. Several lines of evidence from various models have demonstrated that enhanced production of LPC and impaired clearance of LPC may independently, or in concert , give rise to an increase in LPC (Das *et al.*, 1986, Shier, 1977) . Moreover, several concomitants of myocardial ischemia are potent modulators of enzyme activity (Gross and Sobel, 1983, Severson and Fletcher, 1985). The implication thus is that alterations in LPC may be attributed to changes in enzyme activity. The initial objective of this study is to determine whether LPC accumulation in the ischemic canine heart is associated with changes in the activity of the LPC-metabolizing enzymes. By necessity an *in vitro* approach will be

employed. Subcellular fractions will be prepared from non-ischemic and ischemic tissues and assayed for the LPC-producing enzyme phospholipase A and the LPC-eliminating enzymes LPC:LPC transacylase, acyl CoA:LPC acyl transferase and lysophospholipase. This approach will address the question as to whether increased production of decreased elimination is the fundamental biochemical cause for the altered LPC homeostasis in the ischemic canine heart.

One aspect of LPC catabolism in the heart which has so far eluded a satisfactory explanation is the relative importance of the catabolic enzymes to LPC clearance. Such information may be useful in designing pharmacological interventions to militate against alterations in LPC levels due to modulation of catabolic enzymes. The second principal objective of this study is to delineate the relative importance of catabolic enzymes in microsomal catabolism of exogenous LPC by a comparative analysis of the isolated intact rat and guinea pig heart. Assessment of enzyme participation will be based on the amount of radioactivity in the products of LPC catabolism. The isolated perfused intact heart allows for simultaneous assessment of several enzyme activities under identical conditions. Thus this model can offer insight into the dynamics of LPC clearance upon various experimental interventions.

MATERIALS AND METHODS

I. MATERIALS

a) Chemicals and buffers

Acetyl CoA, 1,1'-azobis(N,N-dimethyl-formamide)(diamide), butylated hydroxytoluene, DL-carnitine, carnitine acetyltransferase, catalase, Evan's Blue dye, glutathione (reduced form), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), palmitoyl-*l*-carnitine, para hydroxymercuribenzoate sodium (pHMB), phospholipase A2 (Naja Naja), sodium lactate, and sodium tetrathionate were purchased from Sigma Chemical Company (St. Louis, Missouri). Dowex 2-X8 anion exchange resin was obtained from Bio Rad Laboratories (Richmond, California). LPC and PC standards (pig liver) were the products of Serdary Research Laboratories (London, Ontario). Thin layer chromatography plates (SIL G-25) were from Brinkman Instruments (Rexdale, Ontario). Ecolume and [^{14}C]-acetyl CoA (46 mCi/mmol) were the products of ICN Biomedicals (Irvine, California). [1-^{14}C]linoleoyl CoA (57.9 mCi/mmol) and [^{3}H] methyl choline (80 Ci/mmol) were purchased from NEN (Lachine, Quebec). [1-^{14}C] palmitoyl LPC (54-58 mCi/mmol) was routinely purchased from both Amersham (Oakville, Ontario) and NEN (Lachine, Quebec). Lidocaine hydrochloride was provided by Astra Pharmaceuticals (Mississauga, Ontario). All other chemicals were of reagent grade and were purchased from Fisher Scientific (Winnipeg, Manitoba).

The standard Kreb's-Henseleit buffer used had the following composition: NaCl 120 mM, $NaHCO_3$ 25 mM, dextrose 5.5 mM, KCl 4.76 mM, $MgSO_4$ 1.19 mM, NaH_2PO_4 1.18 mM and $CaCl_2$ 1.27 mM. All solutions were maintained at 37°C and were gassed continuously with 95% O_2/ 5% CO_2. A pO_2 of > 500 mm Hg was typically achieved. Where required the composition of the buffer was modified in order to mimic an "ischemic" solution where: NaCl 123 mM, $NaHCO_3$ 6.0 mM, sodium lactate 20 mM, KCl 4.0 mM,

$MgSO_4$ 0.5 mM, NaH_2PO_4 0.9 mM and $CaCl_2$ 2.5 mM. In those solutions a pO_2 of <50 mm Hg was routinely measured when bubbled continuously with a 95% N_2/ 5% CO_2 gas mixture.

b) Experimental animals

Mongrel dogs (8-15 kg) of either sex were made available from the City Pound in Winnipeg. Male Sprague-Dawley rats (250-350 g) were obtained from Central Animal Care Services, University of Manitoba. Male guinea pigs (250-400 g) were purchased from Charles River Laboratories (St. Constant, Quebec). All animals were housed at the Central Animal Care holding facility, University of Manitoba in a light and temperature controlled environment. Tap water and chow were available *ad libitum*.

II. METHODS

A. LPC METABOLISM IN THE ISCHEMIC HEART

a) Surgical production of regional myocardial ischemia in the dog

Dogs were anesthesized with 30 mg/kg sodium pentobarbital and ventilated with room air using a Harvard respirator via a cuffed endotracheal tube. The heart was exposed by a left thoracotomy and the left anterior descending coronary artery was dissected free 1-2 cm from the origin. Coronary artery ligation was performed by a two-stage procedure as described by Harris (1950). In some dogs it was necessary to administer lidocaine (5 mg/kg i.v.) to manage arrhythmias which developed within 30 min after the second stage of ligation. The dogs were then maintained for up to 5 hr during which sufficient sodium pentobarbital was administered i.v. to maintain anesthesia. In order to aid in the

identification of the ischemic area, particularly in the early stages of this study, a modification of the procedure described by Romson et al. (1982) was used. A cannula was inserted into the aorta of the excised heart above the coronary ostia and securely fastened with braided umbilical tape. After clearing the coronary vasculature with saline, sufficient Evan's blue dye (0.5% in saline) was injected to provide a clear demarcation of the ischemic zone of the heart.

b) LPC extraction and determination

Sham-operated hearts and heart rendered regionally ischemic for 1, 3 and 5 hr were excised and the non-ischemic and ischemic zones identified. Transmural pieces (approximately 3 g) were removed and quickly homogenized with an Ultra Turrax homogenizer in 15 ml of chloroform/methanol 1:2 (v/v) for 30 sec (Mock *et al.*, 1984). The homogenate was transferred equally to 2 screw-capped test tubes. The homgenizing probe was then rinsed with an additional 5 ml of chloroform/methanol 1:2 (v/v) using the original tube. This was then combined with the homogenate. The tubes were then centrifuged in a table top centrifuge at full speed (approximately 2300 rpm) for 10 min. The supernatants were poured off and saved. The pellets were re-extracted with 5 ml of chloroform/methanol 1:2 (v/v). Centrifugation was repeated and the supernatants pooled in a separatory funnel. To the combined extracts were added 30 ml chloroform and 16 ml of 0.12 M KCl to effect a 2:1:0.8 chloroform/methanol/KCl volume ratio. The mixture was mixed vigorously and allowed to separate into an aqueous upper phase and a organic lipid-containing lower phase. When separation did not occur within a few minutes a small volume of 1 M KCl was added dropwise. The lower phase was drawn off into a round bottom flask. 10 ml of theoretical lower phase chloroform/methanol/H_2O 86:14:1 (v/v) (Folch et al., 1957) were added to the upper phase and the process was repeated. The combined lower phases were brought to dryness in a rotary evaporator. The lipid residue

was transferred to a capped vial with 3 successive rinses with chloroform/methanol 2:1 (v/v). The solvent was subsequently removed under a stream of nitrogen sealed with teflon tape and capped. The extract was then stored overnight at -20°C. Immediately before analysis the lipid residue was dissolved in 1 ml of chloroform/methanol 2:1 (v/v).

LPC was separated from other lipids by thin-layer chromatography. 50 μl of the lipid extract were applied to plates on a 3 cm wide lane. The plates were developed in chloroform/methanol/acetic acid/H_2O 75:60:10:8 (v/v) which provided good, reproducible separation of LPC from other lipids (R_f approximately 0.2). After staining in iodine vapor the bands corresponding to LPC were scraped off the plate into test tubes. Two 3 cm lanes were combined for each individual assay. Assays were performed in triplicate. LPC standards (0-50 nmol) were run simultaneously for calibration purposes. Lipid phosphate was measured by the method of Bartlett (1958). 250 μl H_2SO_4 were added to each tube. The silica gel-H_2SO_4 mixture was then incubated overnight at 160°C in a heating block. The next day 1.5 ml of H_2O_2 were added and the tubes were returned to the heating block for 3-4 hr. After cooling 4.55 ml of 0.26% ammonium molybdate and 200 μl of 1-amino-2-naphthol-4-sulfonic acid (1.537 g in 10 ml H_2O) were added. The tubes were vortexed well and placed in a boiling water bath for 10 min; the tubes were subsequently vortexed and centrifuged in a table top centrifuge. The absorbance of the resulting blue product was measured at 820 nM against a water blank.

c) Subcellular fractionation, protein determination and marker enzymes analysis

Transmural pieces of non-ischemic and ischemic myocardium (each 5 g) were finely minced with scissors and homogenized in 4 volumes 0.25 M sucrose/ 10 mM Tris-HCl/ 1 mM Na_2EDTA (pH 7.4). The homogenate was centrifuged at 20,000 x *g* for 10 min. The supernatant was poured off into another tube and centrifuged at 20,000 x *g* for 10

min. To prepare washed microsomes the supernatant of the latter spin was centrifuged at 100,000 x *g* for 1 hr. The 100,000 x *g* pellet was resuspended in 0.15 M Tris-HCl (pH 8.0) and again centrifuged at 100,000 x *g* for 1 hr. The pellet of this spin was resuspended in 0.25 M sucrose in 10 mM Tris-HCl (pH 7.4) and centrifuged at 100,000 x *g* for 1 hr. The final pellet, designated washed microsomes, was resuspended in an appropriate volume of 0.25 M sucrose in 10 mM Tris-HCl (pH 7.4). The supernatant fraction of the first 100,000 x *g* spin represented the cytosolic fraction. In both cases the final volume was noted in order to determine the recovery of protein. The combined pellets from the two 20,000 x *g* spins were used to isolate the mitochondrial fraction. After resuspension of the pellets in 0.25 M sucrose/ 10 mM Tris-HCl/ 1 mM Na_2EDTA (pH 7.4) nucleii and cell debris were removed by centrifugation at 1000 x *g* for 10 min. Mitochondria were isolated by centrifugation of the supernatant at 15,000 x *g* for 10 min. Two washes in the same buffer resulted in washed mitochondria. All centrifugations were carried out at 4°C. Each fraction was stored at -80°C with no apparent loss of activity for up to 1 year. Protein content in all fractions was measured by the method of Lowry et al. (1951).

Each fraction obtained was assayed for glucose-6-phosphatase activity, a microsomal marker, by the method of Bers (1979). The mitochondrial fraction was found to be contaminated with 11% microsomal material. No detectable activity was found in the cytosolic fraction indicating no microsomal contamination. Similarly, each fraction was assayed for succinate dehydrogenase, which is a mitochondrial marker (Pennington, 1961). The microsomal fraction was found to be contaminated with 7 % mitochondrial material; whereas the cytosolic fraction was devoid of any activity. Noteworthy was the finding that cross contamination of fractions prepared from ischemic tissue was not significantly different than non-ischemic tissue.

d) *In vitro* analysis of LPC-metabolizing enzymes

i) Phospholipase A

Labeled acyl PC substrate was prepared biosynthetically using the procedure of Cao et al. (1987). Isolated guinea pig hearts were perfused with 10 μM [^{3}H] methyl choline (800 μCi/ μmol) for up to 4 hr (see *Langendorff perfusion of the isolated rat and guinea pig heart* in section B, LPC metabolism in the isolated heart for method). The hearts were subsequently homogenized in chloroform/methanol 1:2 (v/v) (20% homogenate) containing 0.01% BHT. Extraction of lipid was performed as detailed in a previous section. The final lipid residue was dissolved in 1 ml of chloroform/methanol 2:1 (v/v). A 250 μl aliquot of this solution was applied to a 4.5 cm wide lane for thin layer chromatography. The plates were developed in chloroform/methanol/H_2O/acetic acid 70:30:4:2 (v/v). The PC band was visualized under U.V. light after spraying with 2,7-dichlorofluorescein (0.025 % in ethanol). The lipid was eluted from the silica gel by three 4 ml washes with chloroform/methanol/H_2O/acetic acid 50:39:10:1 (v/v) The extracts were pooled and the volume measured. To this was added 4 M NH_4OH (1/3 by volume) in a separatory funnel. After separation of phases the lower phase was drawn off. The solvent was evaporated in a flash evaporator and the residue was dissolved in a small volume of chloroform and transferred to a vial and stored under N_2 at -20°C. To destroy the alkenyl component the purified PC was subjected to mild acid hydrolysis. Aliquots of lipid (approximately 4 μmol) were placed in test tubes. The solvent was removed with a stream of N_2 and the residue was dissolved in 1.6 ml chloroform/methanol 5:11 (v/v) after which 400 μl of 0.05 M $HgCl_2$ were added. The mixture was then incubated at 37°C for 30 min. The lipid was then extracted, subjected to thin-layer chromatography and extracted from the silica gel as described above. The PC fraction obtained was chromatographically pure when compared with a commcercially available standard and had a specific activity of 8,000-10,000 dpm/ nmol. The labeled PC was stored at -20°C in chloroform.

Phospholipase A activity was measured from the rate of production of [^{3}H] LPC which is the hydrolytic product of cleavage of the ester linkage at either the C-1 or C-2 position of the labeled PC substrate. The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 5 mM $CaCl_2$, 200 nmol LPC, 100 nmol labeled substrate (dispersed in water with sonication) and 250 μg of the required protein fraction. The inclusion of LPC was necessary to inhibit lysophospholipase activity and allow for accumulation of the product. Similar results were obtained with the use of 0.2% deoxycholate as a lysophospholipase inhibitor. Reaction blanks contained water instead of protein. All assays were performed in triplicate. The reaction mixtures were incubated at 37°C for 30 min upon which 1.5 mL of chloroform/methanol 2:1 (v/v) and 250 μl of water were added. The tubes were vortexed and spun in a table top centrifuge at full speed. Aliquots of the lower phase were drawn and the solvent removed under N_2. The residue was dissolved in a small amount of chloroform/methanol 2:1 (v/v) and transferred to thin-layer chromatography plates along with unlabeled carrier LPC standard (pig liver). The plates were developed in chloroform/methanol/H_2O/acetic acid 75:60:8:10 (v/v). The plates were removed and briefly dried in an oven set at 110°C. Bands were visualized in iodine vapor and the LPC bands were scraped into scintillation vials to which were added 100 μl acetic acid, 500 μl water and 5 ml Ecolume. Phospholipase A activity is expressed as nmol LPC formed/mg protein/hr.

ii) Acyl CoA:LPC acyl transferase

The cytosolic and mitochondrial activities were assayed as described by Severson and Fletcher (1985). The reaction mixture contained 100 μM linoleoyl-CoA, 100 μM [1-^{14}C] LPC (specific activity 2.27 μCi/μmol), 50 mM sodium phosphate buffer (pH 7.5), 2 mM $MgCl_2$ and 50 μg protein in a final volume of 200 μl. Reaction blanks

contained water instead of protein. All analyses were performed in triplicate. The reaction tubes were incubated for 15 min at room temperature (23°C) after which 1.5 ml chloroform/methanol 2:1 (v/v) and 550 μl water were added. The tubes were vortexed and centrifuged for 10 min. Aliquots were drawn from the lower phase and applied to thin-layer chromatography plates. The plates were developed in chloroform/methanol/H_2O/acetic acid 70:30:4:2 (v/v). After staining in iodine vapor the area corresponding to the PC fraction was scraped into scintillation vials and analyzed for radioactivity as described above. Microsomal activity was assayed using 5 μg protein with a 2.5 min incubation. Similar reaction velocities were obtained using [1-^{14}C] linoleoyl CoA as the labeled substrate. Acyl CoA:LPC acyl transferase activity is expressed as nmol PC formed/mg protein/hr except for microsomal activity which is expressed as μmol PC formed/mg protein/hr.

iii) LPC:LPC transacylase and lysophospholipase

These enzyme activities were measured in each fraction using the method outlined by Severson and Fletcher (1985). The reaction mixture (200 μl final volume) contained 50 mM sodium phosphate buffer (pH 7.5), 2 mM $MgCl_2$, 100 μM [1-^{14}C] LPC (specific activity 2.27 μCi/ μmol) and 50 μg protein. The reaction was carried out as described for the cytosolic and mitochondrial acyl CoA:LPC acyl transferase except the incubation temperature was 37°C. For the estimation of LPC:LPC transacylase activity the PC band was scraped and analyzed for radioactivity. Since PC synthesized by LPC:LPC transacylase would have twice the specific activity of the LPC substrate, the radioactivity in PC was divided by two to calculate reaction velocity. LPC:LPC transacylase activity is expressed as nmol PC formed/mg protein/hr. Lysophospholipase activity was calculated from the amount of radioactivity in the FFA fraction (neutral lipid) and is expressed as nmol FFA formed/mg protein/hr.

e) Long chain acyl carnitine extraction and determination

Sham-operated hearts and hearts rendered regionally ischemic for 0.5, 1, 3 and 5 hr were excised and non-ischemic and ischemic transmural pieces (approximately 150 mg) were collected and quickly frozen in liquid N_2. The tissue was stored at -80°C until required. The frozen tissue was weighed and immediately homogenized for 20 sec in 2 ml of ice cold 6% $HClO_4$. The homogenate was poured into a centrifuge tube and kept on ice. The homogenizer was rinsed with an additional 2 ml of ice cold 6% $HClO_4$ which was added to the original homogenate. The tubes were centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was discarded while the pellet was resuspended in 1 ml of H_2O. Sufficient 1 N KOH was then added to produce a pH of 12.5 to 13.0 (determined by pH paper). The tubes were sealed and heated at 70°C for 2 hr. After cooling the pH of the hydrolyzate was brought to 7 with the addition of 17.5% $HClO_4$.

Aliquots of 500 μl were drawn from the neutralized hydrolyzate and analyzed for free carnitine by the method of McGarry and Foster (1976). In a total volume of 1.2 ml were 120 μmol HEPES (pH 7.3), 2 μmol sodium tetrathionate, 1 Unit carnitine acetyltransferase, 25 nmol [1-^{14}C] acetyl CoA (50 nCi per assay) and 500 μl of the hydrolyzate. DL-carnitine standards (0-20 nmol) were incubated under identical conditions. The reaction tubes were shaken at room temperature (23°C) for 30 min. Upon completion the tubes were put on ice. Separation of radioactive product from radioactive reactant was achieved by passage of the reaction mixture through a column of Dowex 2-X8 anion exchange resin prepared by mixing 0.2 g anion exchange resin with 2 ml H_2O and loading in a Pasteur Pipette stoppered with glass wool. The column was equilibrated with further addition of 2 ml H_2O. The column was capped until just prior to application of the reaction mixture. 375 μl of the reaction mixture were carefully loaded onto the column and scintillation vials were positioned underneath the tip to collect the eluant. Three 375 μl

washes with H_2O completely removed the [^{14}C] acetyl carnitine product. Unreacted [^{14}C] acetyl CoA remained in the column.

Long chain acyl carnitine content in washed microsomes prepared from non-ischemic and ischemic myocardium was measured by mixing 500 μg microsomal protein with ice cold 6% $HClO_4$ (total volume 2 ml). The mixture was centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 700 μl H_2O. Sufficient 1 N KOH was added to attain a pH of 12.5 to 13.0 and the mixture was incubated at 70°C for 2 hr. After neutralization with 17.5% $HClO_4$ an aliquot (700 μl) was removed and assayed for free carnitine as described above.

f) *In vitro* enzyme activity: effects of palmitoyl-*l*-carnitine and reduced pH

i) Microsomal acyl CoA:LPC acyl transferase

This enzyme was assayed exactly as described earlier except that 25 to 400 μM palmitoyl-*l*-carnitine (representative of long chain acyl carnitine) was included in each assay. Stock solutions of palmitoyl-*l*-carnitine were prepared in water. The reaction conditions for the investigation of the effect of reduced pH on enzyme activity were identical except that 50 mM sodium phosphate buffer (pH 6.5) was employed. Moreover, only 100 μM palmitoyl-*l*-carnitine was studied at this reduced pH.

The kinetics of palmitoyl-*l*-carnitine-mediated inhibition of enzyme activity at pH 7.4 was studied in the absence and presence of 40 and 160 μM palmitoyl-*l*-carnitine. Under each experimental condition the concentration of LPC was varied between 4 and 40 μM. The experiments were performed in triplicate as described earlier.

ii) Cytosolic lysophospholipase

The effects of palmitoyl-*l*-carnitine and reduced pH on the activity of this enzyme were studied under the same conditions illustrated above except that 200 μg cytosolic protein were assayed instead of 50 μg protein.

The kinetic analysis of palmitoyl-*l*-carnitine-mediated inhibition of this activity at pH 7.4 was performed as outlined for the microsomal acyl CoA:LPC acyl transferase.

iii) Microsomal lysophospholipase

The effects of palmitoyl-*l*-carnitine and reduced pH on the activity of this enzyme were investigated in the same manner as described for the microsomal acyl CoA:LPC acyl transferase.

The kinetic analysis of palmitoyl-*l*-carnitine-mediated inhibition at pH 7.4 was attempted with 50 μg protein and 150 and 200 μM palmitoyl-*l*-carnitine. The concentration of LPC in the assay mixture was varied between 7.5 and 75 μM.

B. LPC METABOLISM IN THE ISOLATED HEART

I. LANGENDORFF PERFUSION OF THE ISOLATED RAT AND GUINEA HEART

Rats and guinea pigs were treated with heparin (600 Units i.p.) one hr before start of experiment. The animals were sacrificed by a blow to the neck and the hearts were quickly

removed and placed in ice-cold oxygenated Kreb's-Henseleit buffer. The aorta was cannulated for retrograde perfusion according to the method of Langendorff (1895). The pulmonary artery was incised to ensure adequate coronary drainage. The hearts were perfused at a flow rate of 10 ml/min with buffer solution warmed to 37°C. Throughout the course of the experiment the hearts were surrounded by a water-jacketed chamber also warmed to 37°C. Upon completion of perfusion the hearts were removed from the perfusion apparatus and purged with 10 ml of ice-cold buffer to clear the vascular space. The buffer was in turn purged with injection of air.

II. EFFECTS OF pHMB ON MICROSOMAL LPC CATABOLISM

a) Intact heart preparation

i) Perfusion protocol

Hearts were stabilized for 10 min with oxygenated Kreb's-Henseleit buffer prior to the introduction of pHMB. Rat hearts were perfused with 0 to 15 μM pHMB for 30 min followed by perfusion with the same concentration of pHMB in buffer containing 2.5 μM [1-^{14}C] palmitoyl LPC (0.0075 μCi/ml) for 10 min. Guinea pig hearts were perfused with 0 to 7.5 μM pHMB under the same conditions. A higher concentration of pHMB led to visibly impaired contraction in the guinea pig heart and was not pursued. See Figure 3 for perfusion conditions.

ii) Preparation of microsomal fraction

The purged hearts were quickly trimmed of vessels and fat, cut open, blotted dry and weighed. Homogenization of the minced heart tissue was carried out

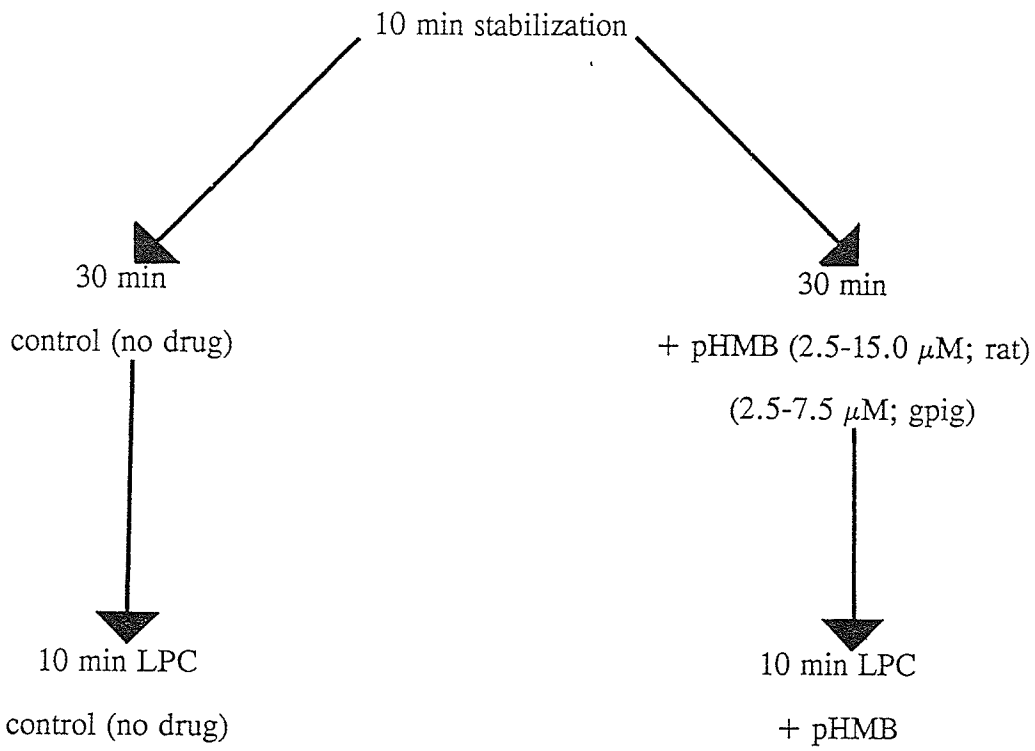


Figure 3. Scheme for the comparative study of the effects of pHMB on microsomal LPC catabolism in the isolated rat and guinea pig heart.

in 16 ml of ice-cold 0.25 M sucrose/ 10 mM Tris-HCl/ 1 mM Na_2EDTA (pH 7.4) in an Ultra Turrax homogenizer (approximately 10% homogenate) at a rheostat setting of 7 for 20 sec. The homogenate was centrifuged at 20,000 X *g* for 10 min at 4°C. The supernatant was decanted and centrifuged at 100,000 X *g* for 60 min at 4°C. The pellet obtained was termed the microsomal fraction and was resuspended in 1 ml of water and stored at -20°C.

iii) Lipid extraction and separation

Microsomal lipids were extracted using the method of Bligh and Dyer (1959). 800 μl of the thawed microsomal fraction were removed and placed in a test tube on ice. To this were added 2 ml of methanol and 1 ml of chloroform. This monophasic solution was vigorously vortexed and left on ice for at least 10 min. Addition of 1 ml of chloroform and 1 ml of water followed by vortexing and centrifugation for 10 min at full speed in a table top centrifuge produced two clear distinct phases. The organic lower phase was drawn and the solvent was removed under a stream of N_2. The lipid residue was dissolved in a small volume of chloroform/methanol 2:1 (v/v) and applied to a thin-layer chromatography plate along with unlabeled carrier LPC. Separation of LPC (R_f = 0.11), PC (R_f = 0.39) and FFA (R_f = 1) was achieved with development in chloroform/methanol/H_2O/acetic acid 70:30:4:2 (v/v). These lipid bands were visualized by iodine staining and scraped into scintillation vials. 100 μl acetic acid, 500 μl water and 5 ml Ecolume were added to each vial which was then subjected to scintillation counting.

iv) PLA_2 digestion of microsomal PC

In parallel series of experiments the PC band was extracted with chloroform/methanol/H_2O/acetic acid 50:39:10:1 (v/v) as described previously. The eluted PC was placed in a test tube and dissolved in 2 ml diethyl ether. 50 μl of 0.1 M Tris-

HCl/ 0.01 M $CaCl_2$ (pH 8.5) containing 0.2 mg phospholipase A_2 (approximately 40 to 50 units) were added to the dissolved lipid. This mixture was incubated at room temperature with frequent mixing for 1 hr. The solvent was then removed under a stream of N_2. The resulting residue was dissolved in a small amount of chloroform/methanol 2:1 (v/v) and applied to a thin-layer chromatography plate. Separation was achieved with the solvent mixture outlined earlier. The LPC, PC and FFA bands were visualized and analyzed for radioactivity.

b) *In vitro* analysis of microsomal acyl CoA:LPC acyl transferase and lysophospholipase activities

Enzyme activities in washed microsomes were assayed *in vitro* as described by Severson and Fletcher (1985). Acyl CoA:LPC acyl transferase activity was determined from the rate of production of labeled PC in a mixture containing 100 μM [1-^{14}C] palmitoyl LPC (2.27 μCi/umol), 100 μM linoleoyl CoA, 50 mM sodium phosphate buffer pH 7.4 and 5 μg protein in a volume of 200 μl. The reaction was initiated by the addition of labeled LPC and was carried out at room temperature (23°C) for 2.5 min. Lysophospholipase acitivity was measured by the release of labeled FFA in a mixture containing 50 μM [1-^{14}C] palmitoyl LPC (2.27 μCi/μmol), 4 mM $MgCl_2$, 50 mM sodium phosphate buffer pH 7.4 and 50 μg protein in a total volume of 200 μl. The tubes were incubated at 37°C for 15 min. Water was substituted for protein in the reaction blanks. The reaction was stopped by the addition of 1.5 ml chloroform/methanol 2:1 (v/v). The reaction products were extracted with the addition of 550 μl water. After vortexing and centrifugation an aliquot of the lower phase was drawn and dried under N_2. Lipids were separated and analyzed as described earlier. Unlabeled PC was spotted along with the lipid extract to aid in the identification of the PC band. In order to assess the effects of pHMB treatment on enzyme activities, reaction mixtures (minus LPC substrate) were incubated

with 3.61 to 36.1 ng pHMB/μg protein for 30 min at the desired temperature. The reaction was then initiated by the addition of LPC substrate as described above.

III. EFFECTS OF DIAMIDE ON MICROSOMAL LPC CATABOLISM

Hearts were stabilized for 10 min with oxygenated Kreb's-Henseleit buffer prior to perfusion with 0 (i.e. control), 0.25 or 2.5 mM diamide for 10 min. The hearts were subsequently perfused for 10 min with the same concentration of diamide in buffer containing 2.5 μM [1-^{14}C] LPC (0.0075 μCi/ml). In one series of experiments the buffer was switched to normal Kreb's-Henseleit buffer after the initial perfusion with diamide. This 10 min perfusion period was termed "wash" and was followed by a 10 min perfusion with normal Kreb's-Henseleit buffer containing radioactive LPC as above. In another series of experiments involving the isolated rat heart 2.5 mM diamide and 2.5 mM glutathione were perfused simultaneously for 10 min before switching to buffer containing both and radioactive LPC as above. See Figure 4 for summary of perfusion conditions. Microsomal fractions were prepared and lipids extracted, separated and analyzed for radioactivity in LPC, PC and FFA as described earlier.

IV. EFFECTS OF "ISCHEMIC" CONDITIONS ON MICROSOMAL LPC CATABOLISM

Hearts were stabilized for 10 min with oxygenated Kreb's-Henseleit buffer. Rat hearts were then perfused with "ischemic" buffer (see Materials section) containing 2.5 μM [1-^{14}C] LPC (0.0075 μCi/ml) for 10 min. A separate series of experiments involved 20 min perfusion with "ischemic" buffer after the stabilization period

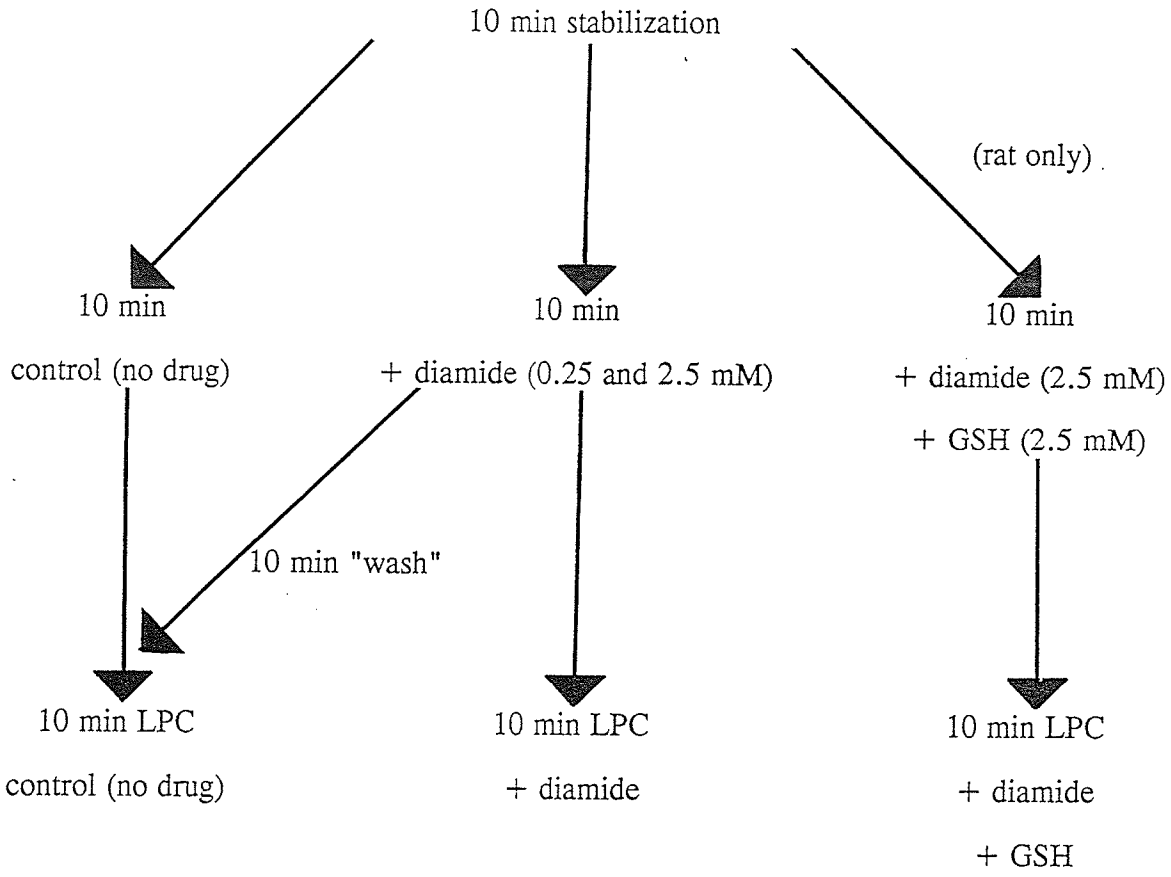


Figure 4. Scheme for the comparative study of the effects of diamide on microsomal LPC catabolism in the isolated rat and guinea pig heart.

and before perfusion for 10 min with "ischemic" buffer containing LPC. Guinea pig hearts were only subjected to the latter perfusion condition. See Figure 5 for summary of perfusion conditions. Microsomal fractions were collected and lipids extracted, separated and analyzed for radioactivity in the LPC, PC and FFA bands as before.

C. STATISTICAL ANALYSIS

To assess the effects of various treatments on experimental parameters such as : changes in enzyme activity, microsomal uptake of label, and amount of radioactivity in the required lipid fractions randomized analysis of variance (ANOVA) was performed. Where treatment effects were found to be statistically significant individual differences were analyzed for statistical significance by either a paired Student t-test, Newman-Keuls's test or Tukey's test as indicated. Where appropriate and specified in the text an unpaired Student's t-test was used. All data are expressed as mean $\pm$ S.E.

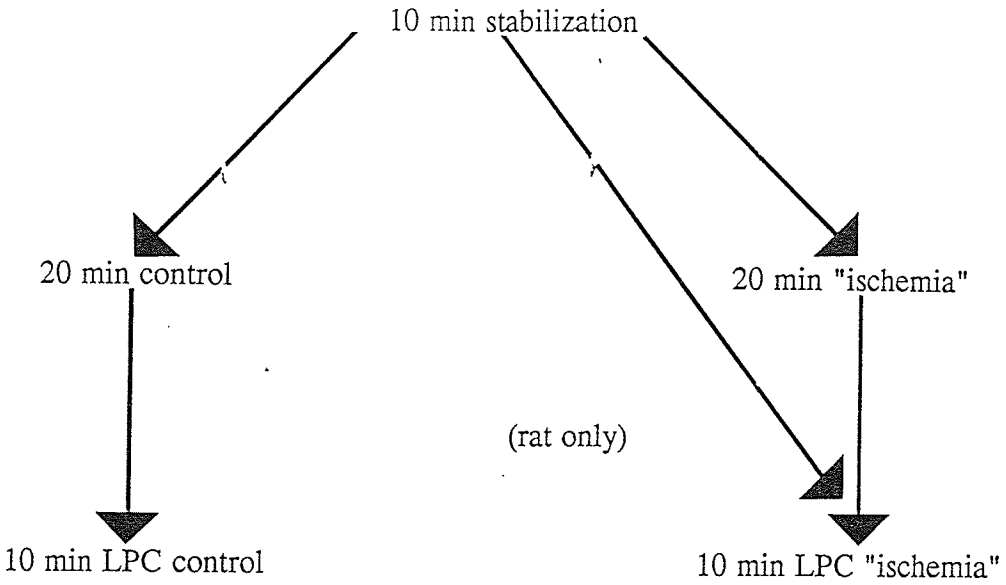


Figure 5. Scheme for the comparative study of the effects of "ischemic" conditions on microsomal LPC catabolism in the isolated rat and guinea pig heart.

EXPERIMENTAL RESULTS

A. LPC METABOLISM IN THE ISCHEMIC CANINE HEART

I. LPC ACCUMULATION IN THE ISCHEMIC MYOCARDIUM

In order to assess any meaningful difference in LPC content between the non-ischemic (area supplied by the circumflex artery) and ischemic region (portion of myocardium supplied by the left anterior descending coronary artery) the LPC content in these areas must first be established under control conditions. Transmural pieces of myocardium corresponding to the non-ischemic and ischemic areas were collected from three sham-operated hearts. The tissue was extracted and LPC content determined as described in Methods. No statistically significant difference was detected in myocardial LPC content between the designated non-ischemic and ischemic areas (by paired t-test). Pooled data from sham-operated hearts revealed LPC content to be 509 ± 35 nmol/g dry weight ($n=6$), based on tissue water content of 3.35 g H_2O/g dry weight.

Figure 6 depicts the measured changes in LPC content in ischemic canine heart over 5 hr. All data are corrected for tissue water content based on the following previously determined values: 3.335 g H_2O/g dry weight and 3.464 g H_2O/g dry weight for 1 hr non-ischemic and ischemic tissue, respectively; 3.367 g H_2O/g dry weight and 3.831 g H_2O/g dry weight for 3 hr non-ischemic and ischemic tissue, respectively and 3.405 g H_2O/g dry weight and 3.762 g H_2O/g dry weight for 5 hr non-ischemic and ischemic tissue, respectively. No significant changes in LPC content in non-ischemic myocardium over the course of 5 hr was found (ANOVA). Paired t-test analysis revealed significant increases in LPC content of ischemic myocardium compared with non-ischemic myocardium at both 3 hr (46%; $p<0.05$; $n=4$) and at 5 hr (114%; $p<0.01$; $n=4$).

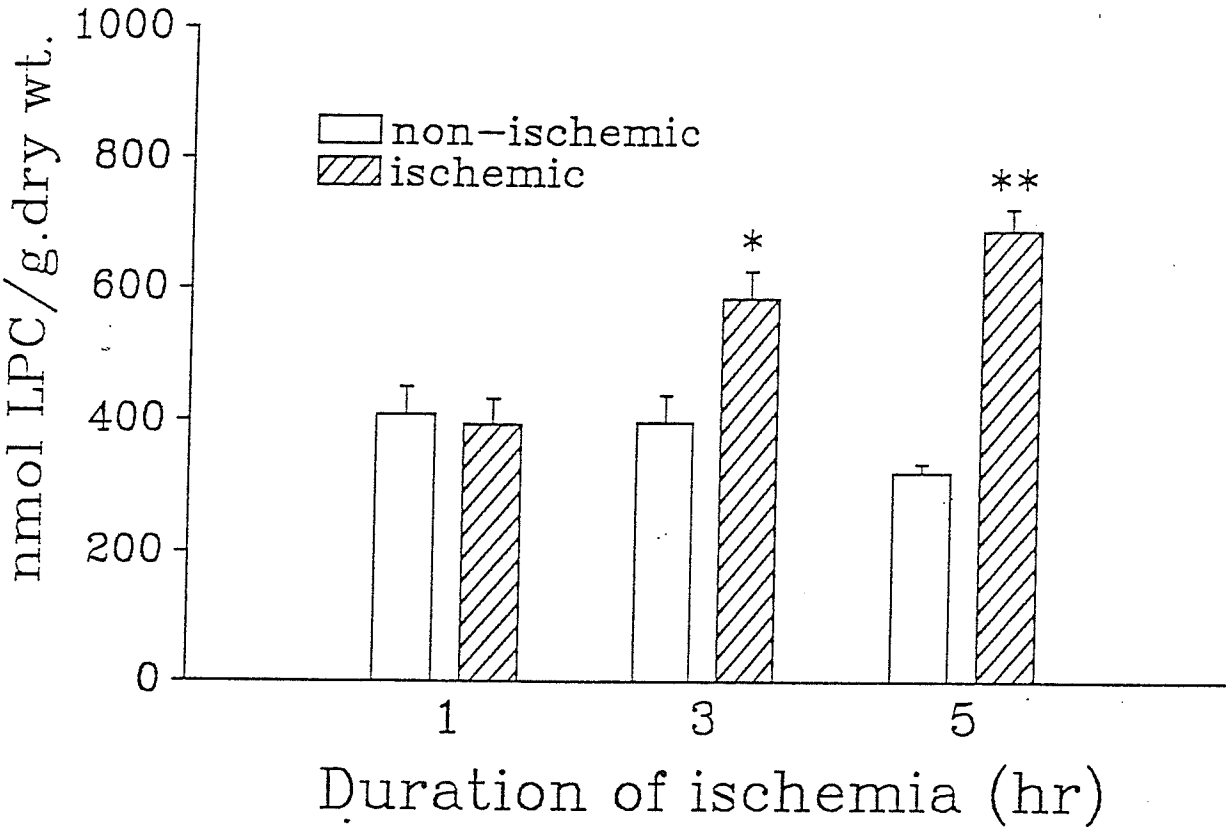


Figure 6. The accumulation of LPC in the ischemic canine heart. Regional myocardial ischemia of 1, 3 and 5 hr duration was produced in the dog by the method of Harris. LPC content was measured in transmural pieces of non-ischemic and ischemic myocardium as described in Methods. Values depicted are the mean$\pm$S.E. of 3 or 4 separate experiments. * $p<0.05$ and ** $p<0.01$ compared to corresponding non-ischemic control.

II. ISCHEMIA-INDUCED ALTERATIONS IN ACTIVITY OF LPC-METABOLIZING ENZYMES

Sham-operated hearts and non-ischemic and ischemic tissue from hearts rendered regionally ischemic were harvested and subcellular fractionation was carried out as detailed in the Methods section. The recovery of cytosolic and microsomal protein from ischemic myocardium is shown in Table 1. No differences were found in the recovery of cytosolic protein over the course of 5 hr. However, approximately 20% less microsomal protein was recovered in the microsomal fraction from 3 and 5 hr ischemic tissues when compared to their non-ischemic counterparts. All individual differences in this section were analyzed for statistical significance by paired t-test.

i) Phospholipase A

Phospholipase A activity of sham-operated hearts and the non-ischemic area of hearts subjected to 1 to 5 hr of regional ischemia did not differ among the respective cytosolic, mitochondrial and microsomal fractions (ANOVA). The pooled data are provided in Table 2. The highest specific activity was found associated with the microsomal fraction. Figure 7 depicts the % difference between the activity measured in the ischemic fraction *vs* the activity measured in the non-ischemic fraction . It is clear that no significant changes in enzyme activity occur over the course of 5 hr of ischemia. An apparent trend towards increased activity in the cytosolic fraction did not attain a level of statistical significance. Non-ischemic control activities at 1, 3 and 5 hr were 0.6 ± 0.2, 0.4 ± 0.1 and 0.4 ± 0.1 nmol LPC formed/mg protein/hr, respectively for the cytosolic enzyme (n=5); 0.8 ± 0.1, 0.9 ± 0.2 and 0.8 ± 0.1 nmol LPC formed/mg protein/hr, respectively for the mitochondrial enzyme (n=5; corrected for microsomal contamination) and 3.2 ± 0.5, 3.4 ± 1.0 and 2.8 ± 0.6 nmol LPC formed/mg protein/hr, respectively for the microsomal enzyme (n=5).

Table 1. Recovery of protein from subcellular fractions prepared from non-ischemic and ischemic tissues.

Duration of ischemia	Tissue	Cytosolic	Microsomal
1 hr	non-ischemic	23.6	1.24±0.10
	ischemic	23.6±2.4(100%)	1.26±0.11(102%)
3 hr	non-ischemic	22.6±0.8	1.06±0.08
	ischemic	23.1±1.1(102%)	0.88±0.12(80%)
5 hr	non-ischemic	25.0±0.4	1.25±0.09
	ischemic	24.9±0.5(99%)	0.97±0.09(78%)

All values are expressed as mg protein per g wet weight (mean±S.E.). n=3-5. Values in parentheses denote percent recovery of protein from ischemic tissue *vs* non-ischemic tissue.

Table 2. Activity of LPC-metabolizing enzymes in various subcellular fractions from normal and non-ischemic regions of canine heart.

Enzyme	Cytosolic	Mitochondrial[a]	Microsomal
Phospholipase A	0.5±0.01	0.8±0.1	3.2±0.3
(nmol LPC/mg protein/hr)	n=9	n=9	n=9
Acyl CoA:LPC acyl transferase	14±2	63±4	18.0±1.0[b]
(nmol PC/mg protein/hr)	n=12	n=12	n=13
LPC:LPC transacylase	[c]	[c]	25±2
(nmol PC/mg protein/hr)			n=18
Lysophospholipase	25±1	[c]	424±26
(nmol FFA/mg protein/hr)	n=32		n=33

Enzyme activities were assayed as described in Materials and Methods. No differences were detected among sham-operated (normal) and the non-ischemic regions of hearts rendered regionally ischemic for 1 to 5 hr. The pooled data represented are mean±S.E. [a] data are corrected for microsomal contamination [b] data expressed are μmol PC/mg protein/hr [c] no detectable activity

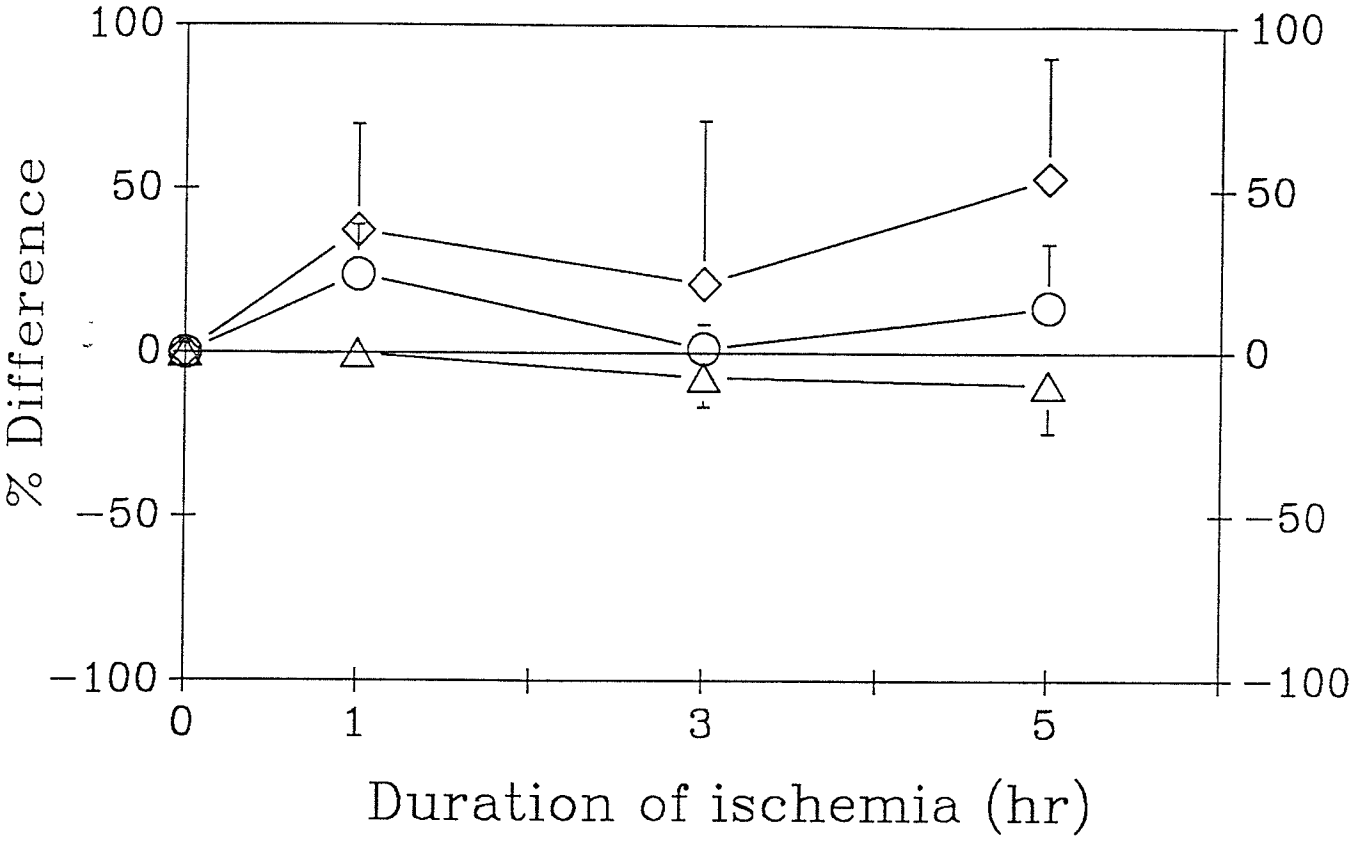


Figure 7. The effect of ischemia on canine myocardial phospholipase A activity. Cytosolic (open diamond), mitochondrial (open circle) and microsomal (open triangle) fractions prepared from the non-ischemic and ischemic regions of the heart subjected to 1 to 5 hr of regional ischemia were assayed for enzyme activity as detailed in Methods. Each data point depicts the percentage difference between activity measured in the ischemic fraction *vs* the corresponding non-ischemic fractions and represents the mean±S.E. of five separate experiments. Non-ischemic control activities are provided in Results.

ii) Acyl CoA:LPC acyl transferase

Acyl CoA:LPC acyl transferase activity in the respective cytosolic, mitochondrial and microsomal fractions of sham-operated hearts and the non-ischemic area of hearts subjected to 1 to 5 hr of regional ischemia showed no statistically significant differences (ANOVA). From the pooled data in Table 2 it is readily apparent that substantial activity exists in the canine heart with the highest specific activity residing in the microsomal fraction. Up to 5 hr of ischemia did not result in any changes in cytosolic activity (Figure 8) (data presentation as above). Non-ischemic control values were 13 ± 3, 14 ± 5 and 12 ± 3 nmol PC formed/mg protein/hr for 1, 3 and 5 hr, respectively ($n=4$). On the other hand a trend towards increased mitochondrial activity was seen which attained a level of statistical significance at 5 hr of ischemia ($p<0.01$) where a $64\pm12\%$ increase in activity compared with the non-ischemic control was obtained. The non-ischemic values measured for mitochondrial activity were 159 ± 55, 177 ± 38 and 135 ± 14 nmol PC formed/mg protein/hr for 1, 3 and 5 hr, respectively ($n=4$; corrected for microsomal contamination). Similarly there were significant changes in microsomal activity, however, in contrast to changes in mitochondrial activity there was a $23\pm8\%$ decrease in activity at 3 hr ($p<0.05$) and a $32\pm10\%$ decrease in activity at 5 hr ($p<0.05$). Non-ischemic control values were determined to be 16.7 ± 1.6 and 18.7 ± 1.5 μmol PC formed/mg protein/hr for 3 and 5 hr, respectively ($n=5$).

iii) LPC:LPC transacylase

The cytosolic and mitochondrial fractions were completely devoid of LPC:LPC transacylase activity under the experimental conditions used. No difference in the activity of the microsomal LPC:LPC transacylase among sham-operated

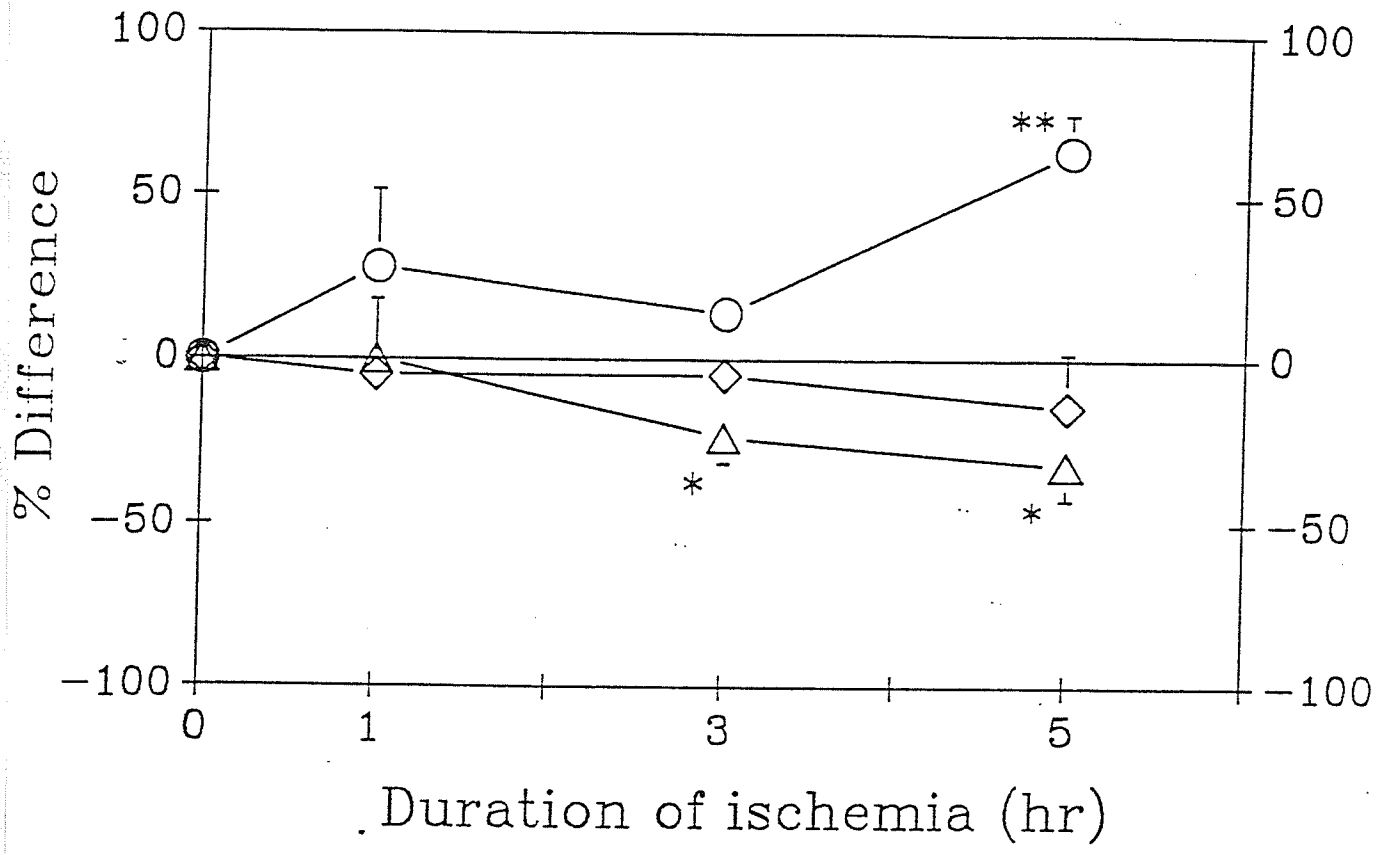


Figure 8. The effect of ischemia on canine myocardial acyl CoA:LPC acyl transferase activity. Data presentation and symbols as in Figure 7. Each point is the mean±S.E. of 4 or 5 separate experiments. Non-ischemic control data are provided in Results. * $p<0.05$ ** $p<0.01$ compared to corresponding non-ischemic control.

hearts and the non-ischemic region of hearts rendered regionally ischemic for 1 to 5 hr was detected (ANOVA). The pooled data are provided in Table 2. A time-dependent decrease in the activity of this enzyme from ischemic tissue was found (Figure 9; data presentation as before). At 3 hr there was a 39±14% decrease in activity ($p<0.01$) whereas a 54±16% decrease in activity at 5 hr was detected ($p<0.01$). Non-ischemic control values were determined to be 24.6±3.1, 22.6±2.0 and 27.8±4.7 nmol PC formed/mg protein/hr for 1, 3 and 5 hr, respectively (n=6).

iv) Lysophospholipase

Although lysophospholipase activity was detected in the mitochondrial fraction the amount corresponded with the extent of microsomal contamination of the fraction and therefore further analysis was not performed. No statistically significant difference among the sham-operated hearts and the non-ischemic region of hearts rendered regionally ischemic for 1 to 5 hr for the respective cytosolic and microsomal fractions was demonstrated (ANOVA). Table 2 contains the pooled data. Figure 10 depicts the ischemia-induced alterations in cytosolic and microsomal activity (data presentation as above). A time-dependent decrease in the activity of the cytosolic lysophospholipase was found. Statistically significant changes were found at 3 hr (36±6%; $p<0.001$) and 5 hr (46±8%; $p<0.001$). Similarly, alterations in microsomal activity attained a level of statistical significance at 3 hr (33±12%; $p<0.01$) and at 5 hr (55±8%; $p<0.001$). Non-ischemic control values were found to be 28±3, 26±2 and 25±3 nmol FFA formed/mg protein/hr for the cytosolic enzyme at 1, 3 and 5 hr, respectively (n=7-11), and 489±37, 352±69 and 436±50 nmol FFA/mg protein/hr for the microsomal enzyme at 1, 3 and 5 hr, respectively (n=7-11).

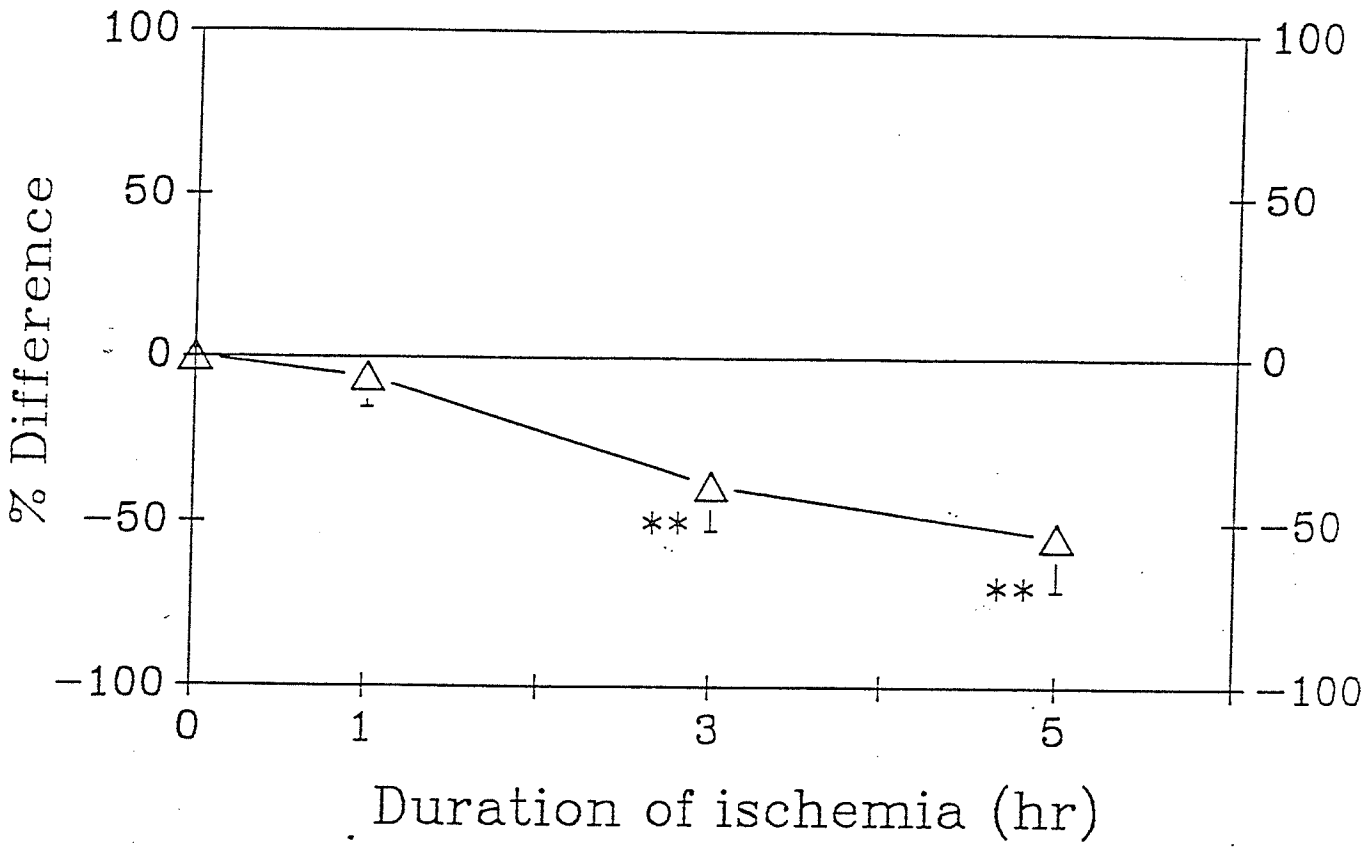


Figure 9. The effect of ischemia on canine myocardial LPC:LPC transacylase activity. Data presentation and symbols as in Figure 7. Each point is the mean±S.E. of six separate experiments. Non-ischemic control data are provided in Results. ** $p<0.01$ compared to corresponding non-ischemic control.

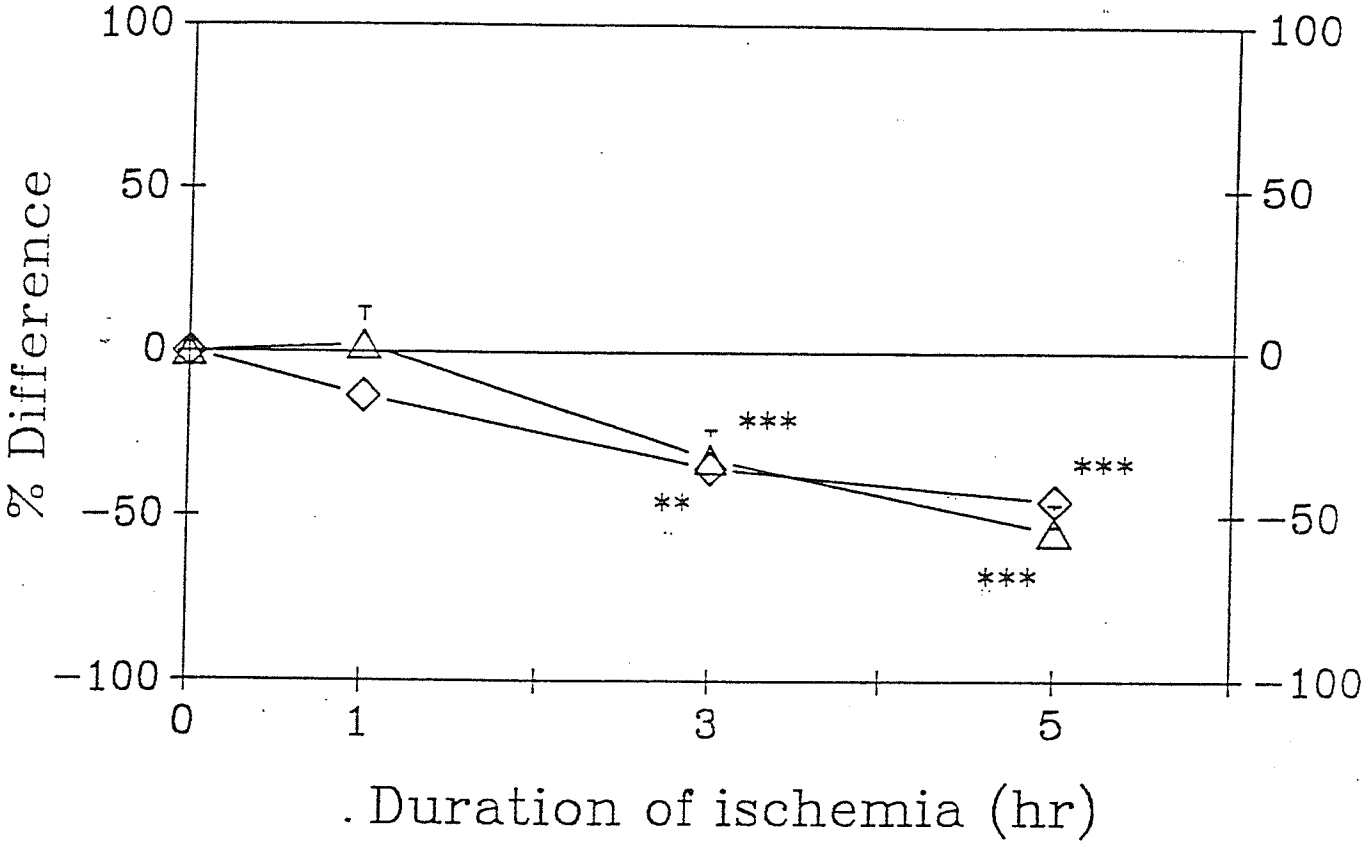


Figure 10. The effect of ischemia on canine myocardial lysophospholipase activity. Data presentation and symbols as in Figure 7. Each point is the mean±S.E. of 7-11 separate experiments. Non-ischemic control data are provided in Results. ** $p<0.01$ *** $p<0.001$ compared to corresponding non-ischemic control.

III. LONG CHAIN ACYL CARNITINE ACCUMULATION IN THE ISCHEMIC MYOCARDIUM

LCAC content in normal myocardium from the designated non-ischemic region (area supplied by the circumflex artery bed) and the designated ischemic region (area supplied by the left anterior descending coronary artery) was obtained by analysis of frozen transmural pieces collected from five sham-operated dogs as described in Methods. Paired t-test analysis revealed equivalence in LCAC content between the two regions of the heart. When corrrected for control tissue water content (see LPC accumulation in the ischemic myocardium) the values obtained were 868±125 nmol LCAC/g dry weight (n=6) and 807±96 nmol LCAC/g dry weight (n=5) for the non-ischemic and ischemic regions, respectively. Since 94-97% of cellular LCAC is cytosolic in nature (Idell-Wenger et al., 1978) and assuming the cytosolic space occupies 2 ml/g dry weight as proposed by Morgan et al. (1964) the average content of LCAC (840±77; n=11) corresponds to a cytosolic contentration of 0.42 mM. Unless otherwise indicated individual differences were analyzed for statistical significance by paired t-test.

i) Whole tissue

The changes in LCAC content measured in myocardium rendered regionally ischemic for up to 5 hr are illustrated in Figure 11. The data are corrected for tissue water content as previously described and are thus expressed as nmol LCAC/g dry weight. There was no change in the LCAC content of non-ischemic myocardium over the course of 5 hr of ischemia (ANOVA). Although discernible differences in LCAC content between non-ischemic and ischemic were apparent as early as 30 min of ischemia, large variabilities in the data precluded assignment of statistical significance to data pairs collected up to and including 1 hr of ischemia. At 3 hr of ischemia there was a 75% increase in

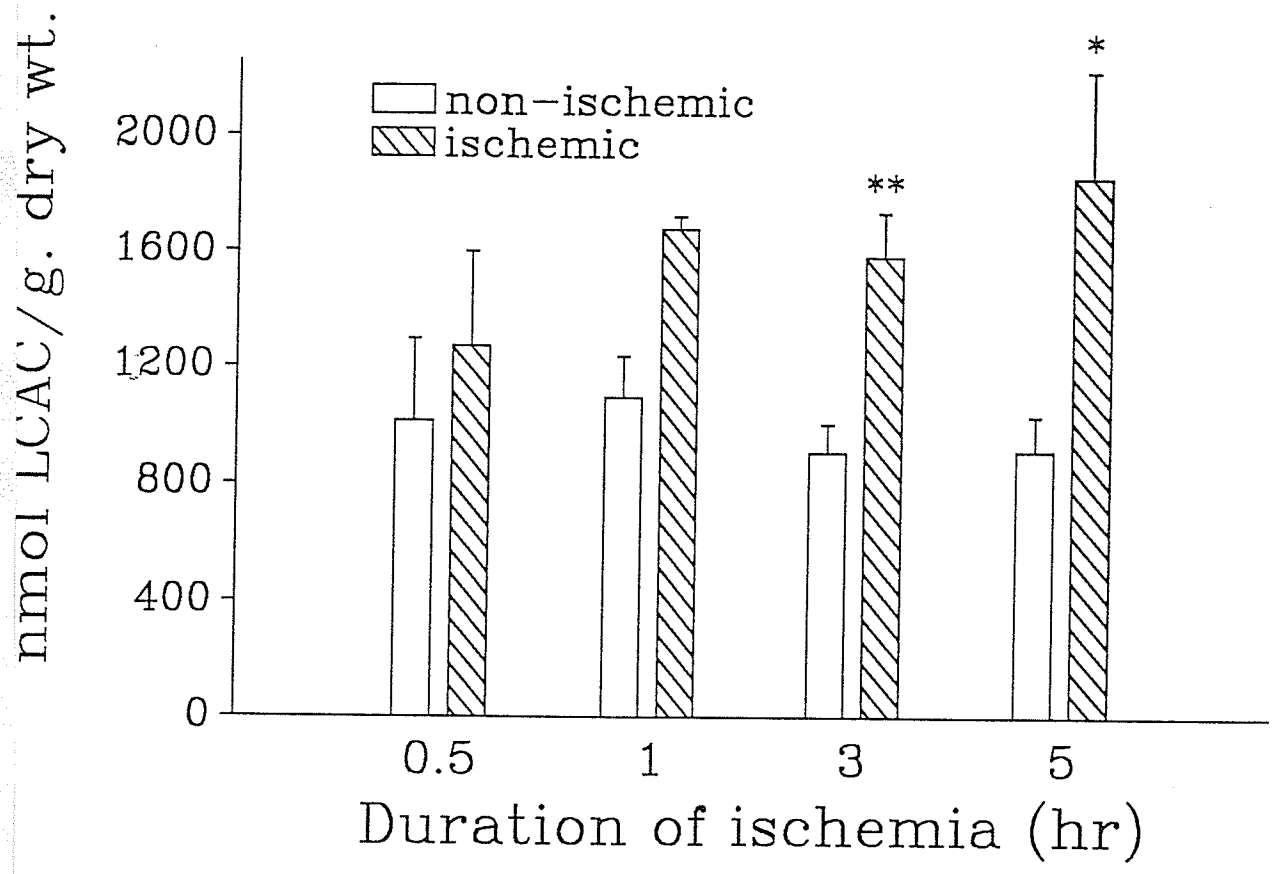


Figure 11. The accumulation of long chain acyl carnitine (LCAC) in the ischemic canine heart. Regional myocardial ischemia of 0.5, 1, 3 and 5 hr duration was produced in the dog. LCAC content was measured in transmural pieces of non-ischemic and ischemic myocardium as described in detail in Methods. Values depicted are the mean±S.E. of 5-8 separate determinations. * $p<0.05$ ** $p<0.01$ compared to corresponding non-ischemic control.

LCAC content in ischemic myocardium compared to non-ischemic tissue ($p<0.01$; $n=7$). Furthermore a 105% increase in LCAC content in tissue rendered ischemic for 5 hr was found ($p<0.05$; $n=8$). The calculated cytosolic concentration of LCAC in 5 hr ischemic myocardium is 0.93 mM.

ii) Microsomal fractions

Microsomal fractions from 3 and 5 hr tissue were prepared and assayed for LCAC as described in Methods. The data are listed in Table 3. No difference in LCAC content between microsomal fractions from non-ischemic tissue from 3 and 5 hr preparations was observed (unpaired t-test). At 3 hr of ischemia there was a greater than 5-fold increase in LCAC content while a 2-fold difference was found in microsomes from 5 hr tissue. In neither case, however, did the calculated p value indicate a level of statistical significance (i.e. p equal to or less than 0.05).

IV. MODULATION OF *IN VITRO* ENZYME ACTIVITY BY PALMITOYL-*l*-CARNITINE AND REDUCED pH

Cytosolic and microsomal fractions prepared from tissue collected from sham-operated dogs were assayed *in vitro* to assess the effects of palmitoyl-*l*-carnitine and reduced pH on activity. Statistical analyses were performed with an unpaired t-test.

i) Microsomal acyl CoA:LPC acyl transferase

Palmitoyl-*l*-carnitine produced a concentration-dependent inhibition of microsomal acyl CoA:LPC acyl transferase activity. (Figure 12). At 400 μM, which was the highest concentration of palmitoyl-*l*-carnitine studied, only 25% of control activity

Table 3. Long chain acyl carnitine content in microsomal fractions from canine heart.

duration of ischemia	non-ischemic	ischemic	
	nmol LCAC/mg protein		
3 hours	1.05±0.57 (n=6)	5.17±2.29 (n=6)	p=0.08
5 hours	0.78±0.25 (n=5)	1.58±0.35 (n=5)	p=0.10

Long chain acyl carnitine was assayed in microsomal fractions prepared from non-ischemic and ischemic myocardium as described in Methods. Values represent mean±S.E.

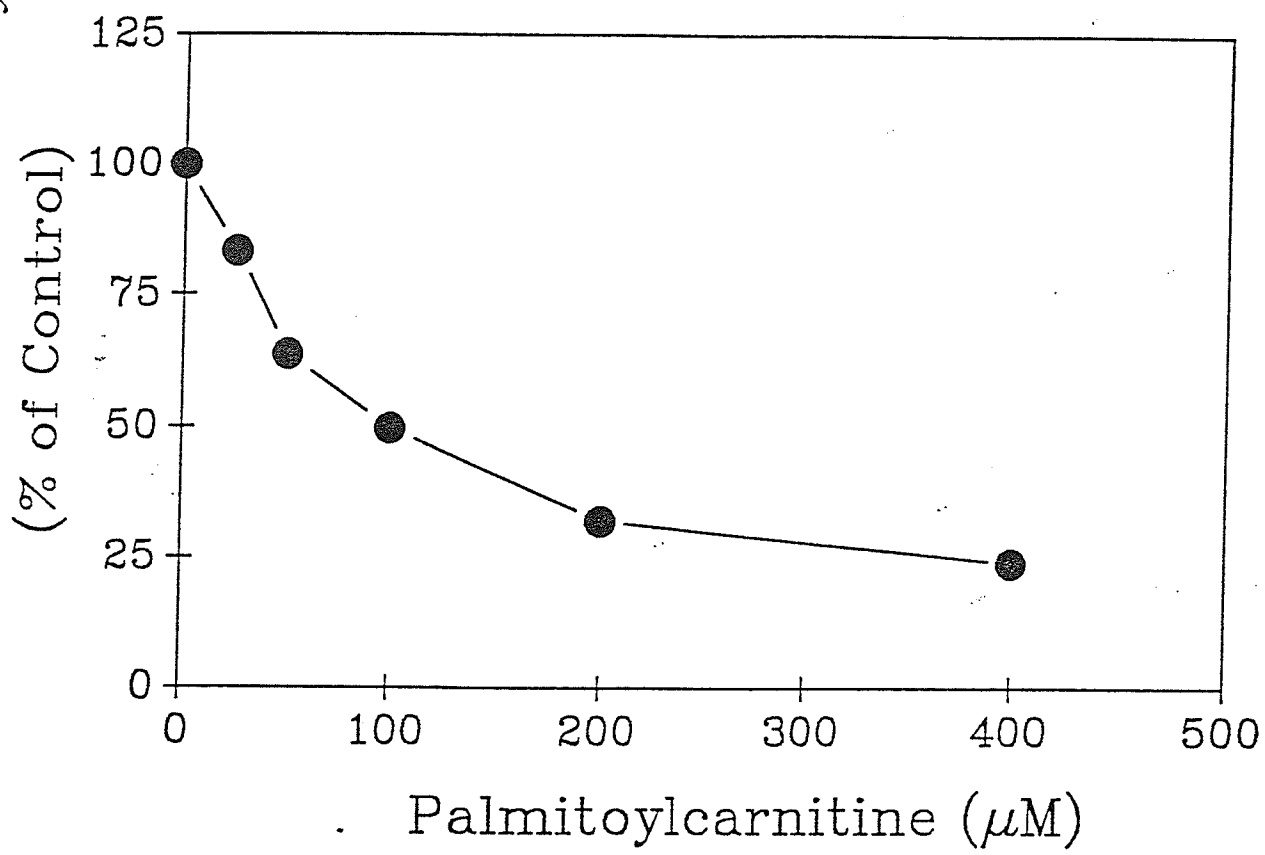


Figure 12. Inhibition of canine microsomal acyl CoA:LPC acyl transferase activity by palmitoyl-l-carnitine. Microsomal fractions were prepared from myocardial tissue and assayed for acyl CoA:LPC acyl transferase activity in the presence of 25 to 400 μM palmitoyl-*l*-carnitine as described in Methods. Control activity (measured in the absence of inhibitor) represents 100% activity. Each point is the mean of duplicate determinations from two separate experiments.

remained (measured in the absence of inhibitor). A concentration of 100 μM palmitoyl-*l*-carnitine produced about 50% inhibition of activity ($p<0.05$; $n=3$); this concentration was arbitrarily chosen in order to study the effects of reduced pH with and without inhibitor on enzyme activity. The results obtained are summarized in Table 4. A reduction in the assay pH to 6.5 did not lead to any significant change in activity. Palmitoyl-*l*-carnitine was also determined to be an effective inhibitor at pH 6.5 ($p<0.001$; $n=3$). Moreover, the extent of inhibition at pH 6.5 and pH 7.4 was identical (49% *vs* 51%).

The kinetics of palmitoyl-*l*-carnitine-mediated inhibition of activity were investigated. Lineweaver-Burk analysis produced a series of curves which intersected at a point left of the origin above the 1/LPC axis (Figure 13). According to Dixon and Webb (1964) this represents a form of mixed inhibition where the Km is increased and the Vmax simultaneously decreased. An apparent Ki of approximately 140 μM was determined. The control Km was determined to be 2.3 μM (for LPC) while control Vmax was determined to be 26.2 μmol PC formed/mg protein/hr.

ii) Cytosolic and microsomal lysophospholipase

Cytosolic lysophospholipase was inhibited by palmitoyl-*l*-carnitine in a concentration-dependent manner (Figure 14). Only 25% of control activity (i.e. no inhibitor) remained at an inhibitor concentration of 400 μM. Unlike cytosolic activity, microsomal activity was actually enhanced at low concentrations of palmitoyl-*l*-carnitine (Figure 14). At higher concentrations, however, there was a greater level of inhibition of microsomal activity compared to cytosolic activity. A concentration of 400 μM palmitoyl-*l*-carnitine resulted in only 10% of control microsomal activity (no inhibitor).

The effects of reduced pH on activity and the inhibitory potency of palmitoyl-*l*-carnitine are provided in Table 5. At pH 7.4 100 μM palmitoyl-*l*-carnitine produced 36% inhibition of cytosolic activity ($p<0.001$; $n=3$-6) while microsomal activity

Table 4. The effects of pH and palmitoyl-l-carnitine on canine myocardial acyl CoA:LPC acyl transferase activity.

	Acyl CoA:LPC acyl transferase activity (μmol PC/mg protein/hr)			
fraction	pH 7.4	pH 7.4+ palmitoyl-*l*-carnitine	pH 6.5	pH 6.5+ palmitoyl-*l*-carnitine
microsomal	19.4±2.3	9.6 ± 0.8[a]	14.4 ± 0.6	7.3 ± 0.5[b]
n	3	3	3	3

Microsomal fractions from canine myocardium were assayed for acyl CoA:LPC acyl transferase activity at pH 7.4 and pH 6.5 as described in Methods. The effect on enzyme activity of 100 μM palmitoyl-*l*-carnitine at these pH's was also studied. Values represent mean±S.E. [a] $p<0.05$ when compared to pH 7.4 without inhibitor

[b] $p<0.001$ when compared to pH 6.5 without inhibitor

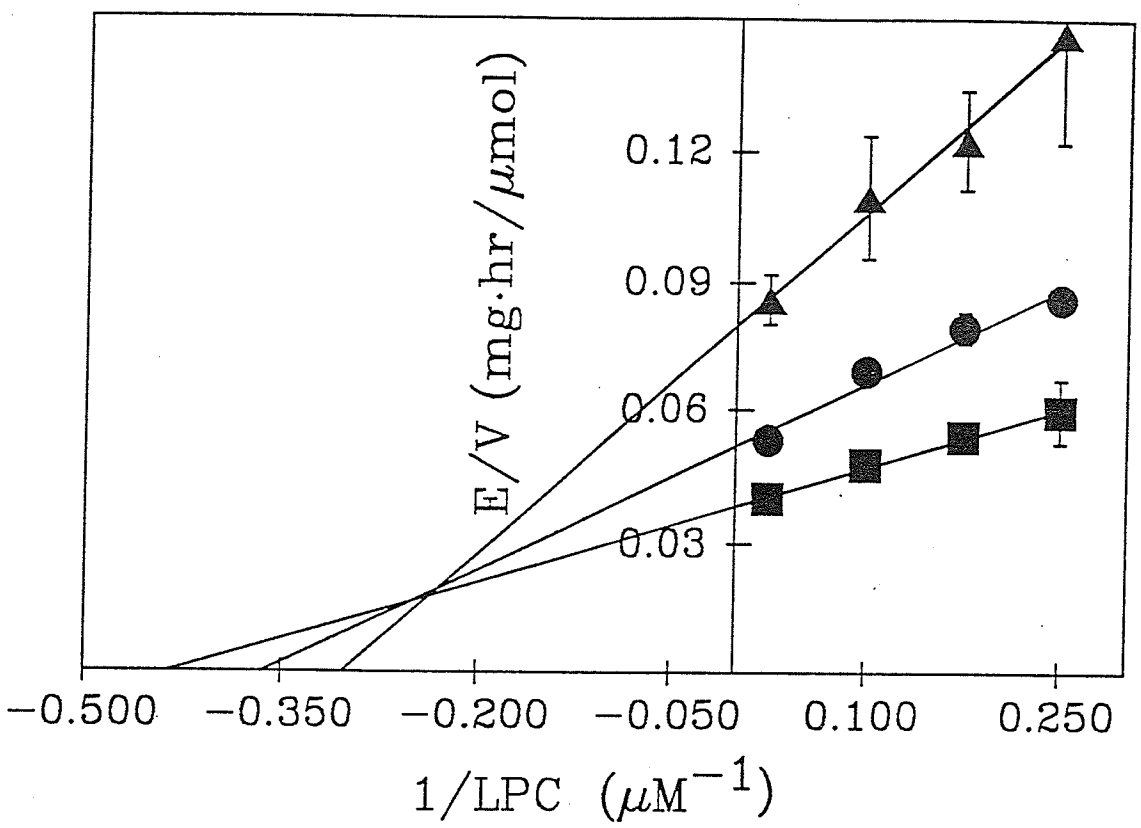


Figure 13. Lineweaver-Burk plot of palmitoyl-l-carnitine-mediated inhibition of canine microsomal acyl CoA:LPC acyl transferase activity. The effects of 40 μM (closed circles) and 160 μM (closed triangles) palmitoyl-*l*-carnitine on enzyme activity were investigated by double reciprocal plot analysis as described in Methods. Activity obtained in the absence of inhibitor is denoted by the closed squares. E refers to enzyme; V refers to velocity. Note the intersection of the lines occurs to the left of the origin and above the 1/LPC axis. Each point represents the mean±S.E. of four or five separate determinations.

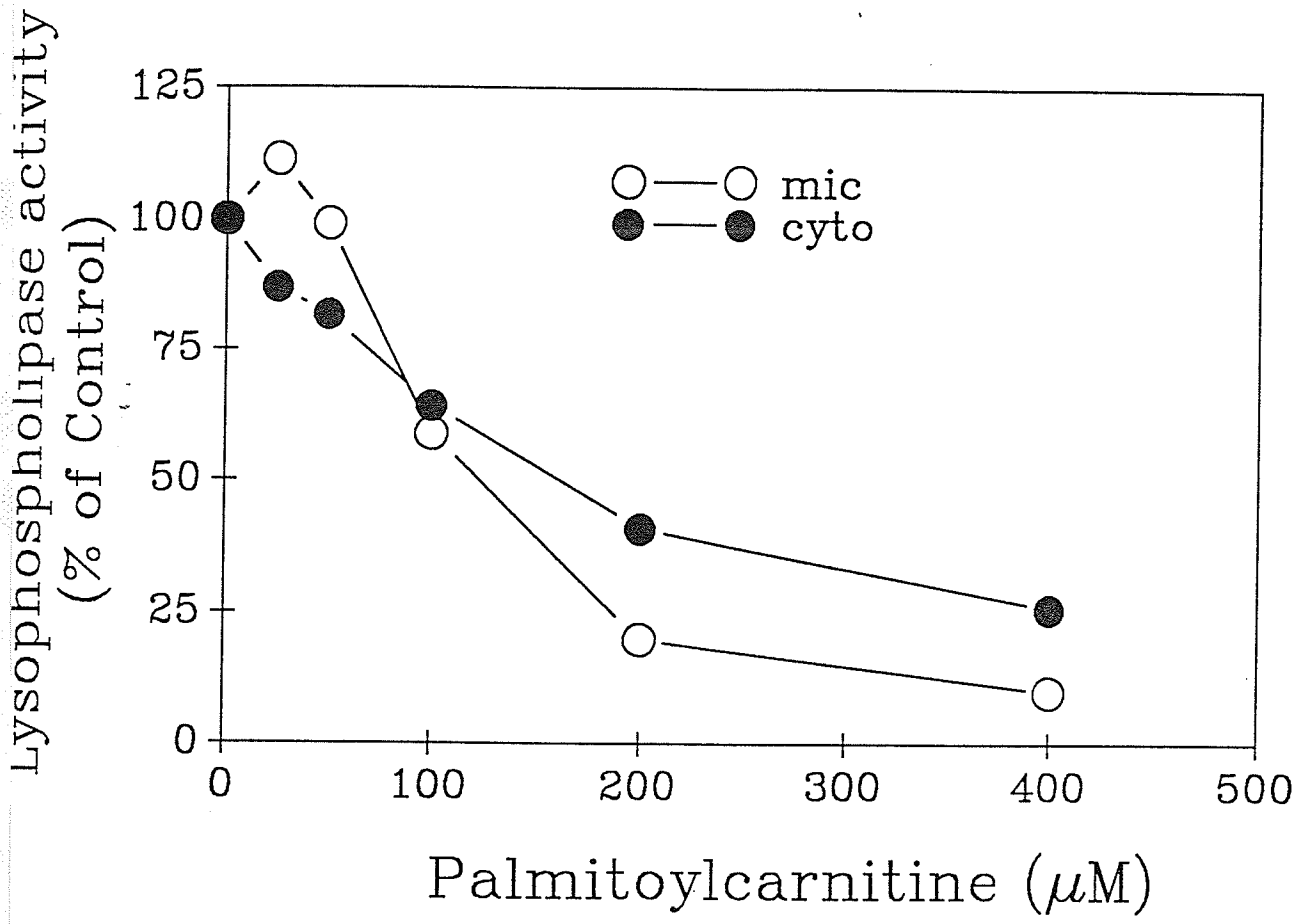


Figure 14. Inhibition of canine cytosolic and microsomal lysophospholipases by palmitoyl-l-carnitine. Data collection and presentation as in Figure 12. Each point is the mean of duplicate determinations from two separate experiments. mic = microsomal activity; and cyto = cytosolic activity.

Table 5. The effects of pH and palmitoyl-l-carnitine on canine myocardial lysophospholipase activity.

	Lysophospholipase Activity (nmol FFA/mg protein/hr)			
fraction	pH 7.4	pH 7.4+ palmitoyl-*l*-carnitine	pH 6.5	pH 6.5+ palmitoyl-*l*-carnitine
microsomal	424 ± 31	252 ± 28^{a}	210 ± 20^{a}	151 ± 18^{b}
cytosolic	19.6 ± 1.0	12.6 ± 0.9^{a}	18.5 ± 1.0	13.4 ± 1.2^{b}
n	6	3	6	3

Microsomal and cytosolic fractions from canine myocardium were assayed for lysophospholipase activity at pH 7.4 and pH 6.5 as detailed in Methods. Enzyme inhibition by 100 μM palmitoyl-*l*-carnitine at both pH's was also evaluated. Values represent mean$\pm$S.E. [a] $p<0.001$ when compared to pH 7.4 without inhibitor [b] $p<0.05$ when compared to pH 6.5 without inhibitor

was inhibited by 41% ($p<0.001$; n=3-6). A reduction in assay pH to 6.5 produced no effect on cytosolic activity. In contrast, there was a 50% reduction in microsomal activity ($p<0.001$; n=6). Unlike microsomal acyl CoA:LPC acyl transferase activity where the inhibitory potency of 100 μM palmitoyl-*l*-carnitine was unaffected by a reduction in pH, the level of inhibition of cytosolic and microsomal lysophospholipases was reduced in both cases. The reduction in inhibitory potency was determined to be statistically significant ($p<0.05$; n=3) for both the cytosolic (36% *vs* 28%) and microsomal lysophospholipases (41% *vs* 28%). Despite a lower inhibitory potency at pH 6.5 100 μM palmitoyl-*l*-carnitine produced significant inhibition ($p<0.05$; n=3) of both enzyme activities.

The kinetics of palmitoyl-*l*-carnitine-mediated inhibition of the cytosolic and microsomal lysophospholipases were investigated by construction of Lineweaver-Burk plots. It is readily apparent from Figure 15 that palmitoyl-*l*-carnitine inhibited the cytosolic lysophospholipase in a purely competitive manner. The apparent Ki was determined to be 18 μM. The control Km was determined graphically to be 16.9 μM, similarly the control Vmax was found to be 23.7 nmol FFA released mg protein/hr. The construction of a Lineweaver-Burk plot for the microsomal lysophospholipase proved to be difficult because of several phenomena unique to this enzyme. Firstly the microsomal lysophospholipase, routinely measured at 50 μg protein, is subject to substrate inhibition at concentrations greater than 100 μM (Figure 16). Moreover, at concentrations lower than 7.5 μM the data obtained were highly variable. Since a minumum 10-fold difference in substrate concentration is required for analysis by double reciprocal plots the concentration range selected was 7.5 μM to 75 μM, which was different than both the cytosolic lysophospholipase (Figure 15) and microsomal acyl CoA:LPC acyl transferase (Figure 13). Secondly the concentrations of palmitoyl-*l*-carnitine studied (150 μM and 200 μM) were different than those for the other enzymes (40 μM and 160 μM). This was made necessary because of the facilitory effect of low concentrations of palmitoyl-*l*-carnitine on microsomal lysophospholipase activity (Figure 14). As depicted in Figure 17 the inhibition by 150 μM palmitoyl-*l*-carnitine was clearly uncompetitive in

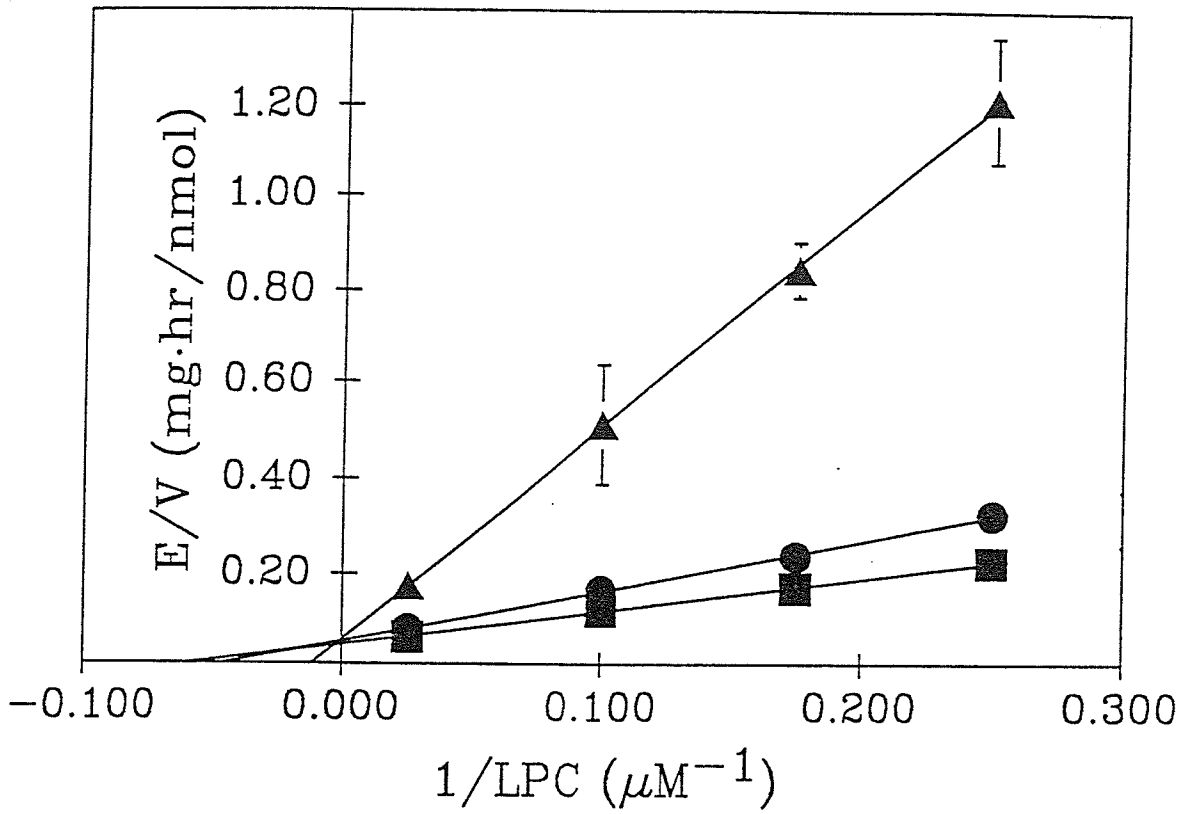


Figure 15. Lineweaver-Burk plot of palmitoyl-l-carnitine-mediated inhibition of canine cytosolic lysophospholipase activity. Data collection and presentation as in Figure 13. Intersection of the lines occurs on the E/V axis. Each point represents the mean±S.E. of 4 or 5 separate determinations.

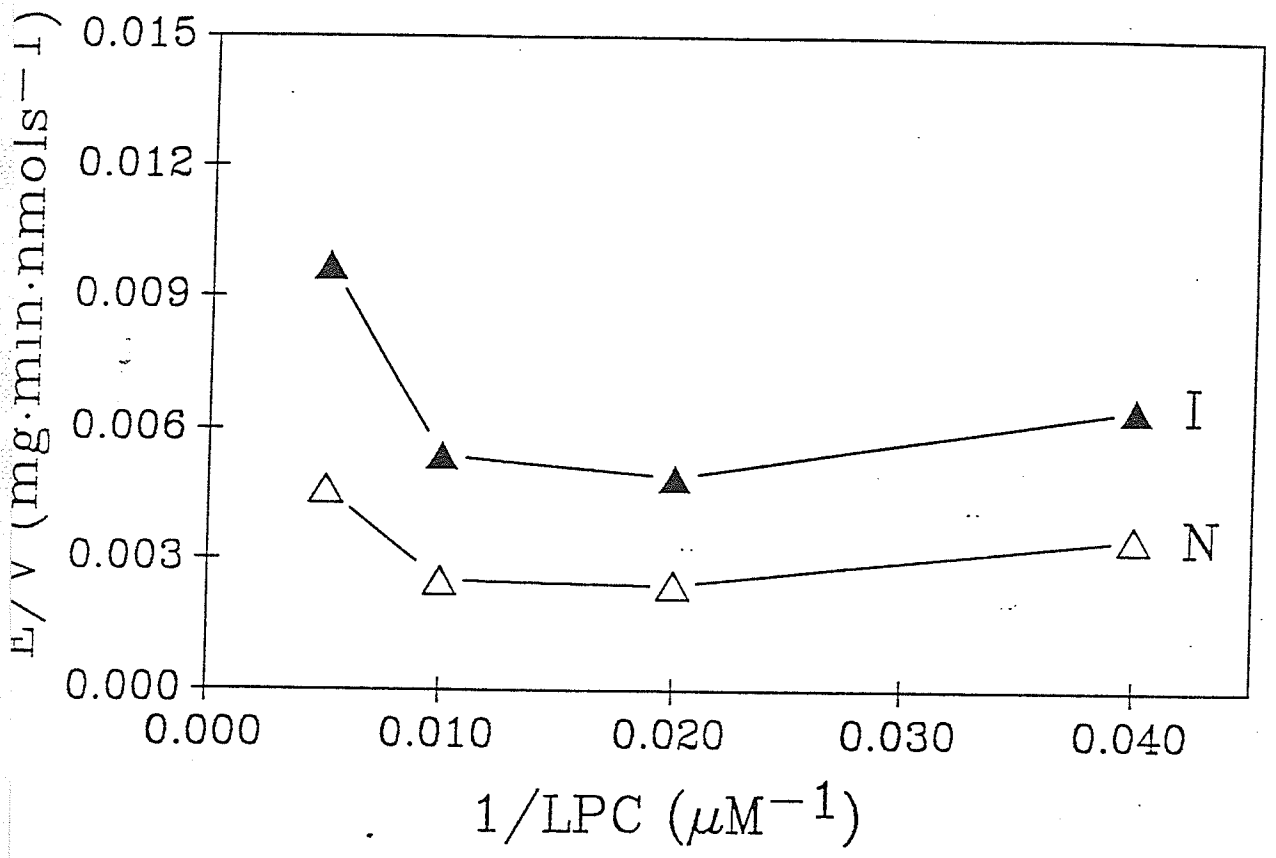


Figure 16. Substrate inhibition of microsomal lysophospholipase. 50 μg microsomal protein were assayed for lysophospholipase activity as described in Methods. The concentration of the labeled LPC substrate was varied between 25 and 200 μM. Note the marked inhibition of activity at substrate concentrations over 100 μM (i.e. <0.01 on 1/s axis). No appreciable qualitative difference between ischemic (I) or non-ischemic (N) fractions was observed. Each point is the mean of duplicate determinations from two separate experiments.

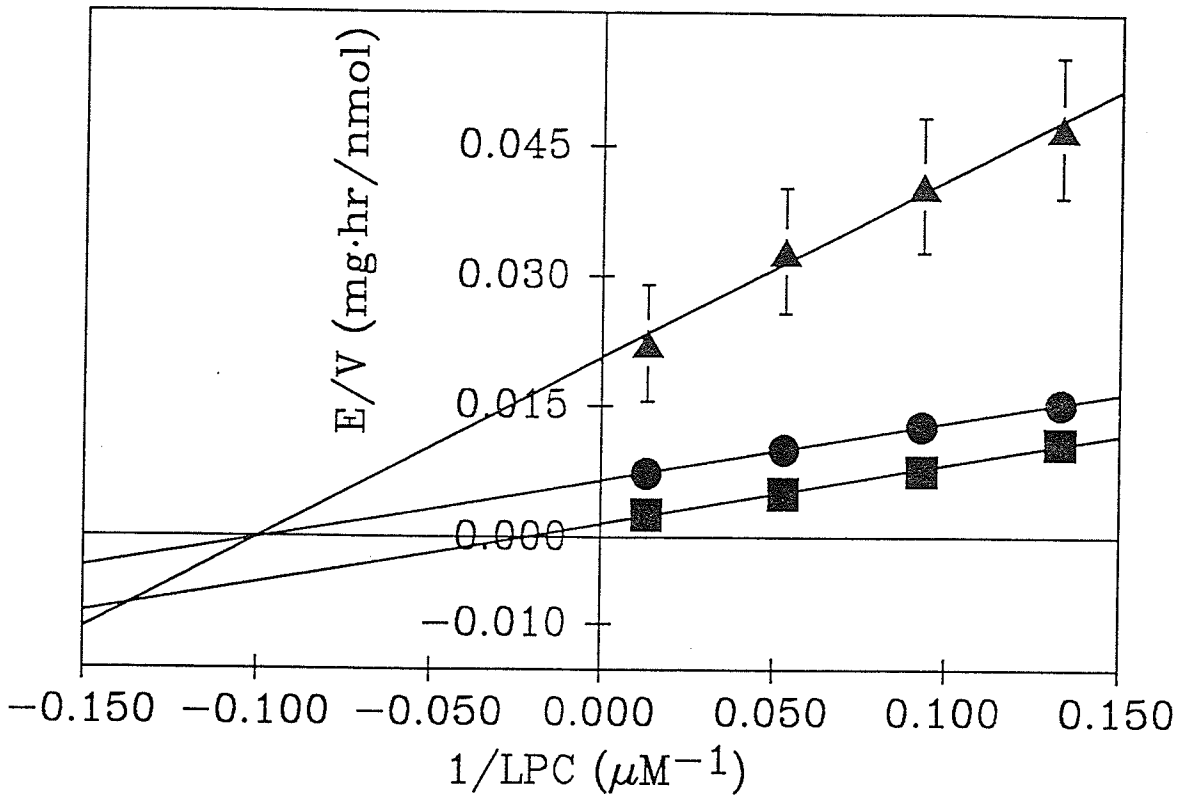


Figure 17. Lineweaver-Burk plot of palmitoyl-l-carnitine-mediated inhibition of canine microsomal lysophospholipase activity. The effects of 150 μM (filled circles) and 200 μM (filled triangles) palmitoyl-*l*-carnitine on enzyme activity were assessed by double reciprocal plot analysis as detailed in Methods. Activity obtained in the absence of inhibitor is depicted by the closed squares. Each point represents the mean$\pm$S.E. of 4 to 6 separate experiments.

nature. The slopes for control (no inhibitor) and 150 μM palmitoyl-*l*-carnitine were identical (.0670 and 0.0658, respectively). At the higher concentration (200 μM), however, the type of inhibition observed could best be described as a form of mixed inhibition where the Vmax is decreased but the Km is actually decreased. The disparate forms of palmitoyl-*l*-carnitine-mediated inhibition observed at the two concentrations studied precluded the determination of an inhibitory constant.

B. LPC CATABOLISM IN THE ISOLATED RAT AND GUINEA PIG HEART

The following studies were undertaken to gain insight into the relative roles of the recognized pathways for microsomal LPC catabolism in the isolated rat and guinea pig heart. These two species were selected because in a recent study by Giffin et al., (1988) susceptibility to LPC-induced arrhythmias in the isolated rat and guinea pig heart was determined to be a function of the catabolic fate of exogenously supplied LPC. The dynamics of LPC clearance in each species was then studied by perturbation of enzyme activity by various interventions.

Since interpretation of relative contribution is based on the amount of radioactivity associated with various lipids after perfusion with exogenous labeled LPC it must first be established that extraction of lipids is equal and complete. Extraction of microsomal lipids by the method described led to virtually 100% extraction of labeled lipid. Moreover, the radioactivity was confined exclusively to the LPC, PC and neutral lipid bands (henceforth referred to FFA). When labeled LPC was added to control homogenates over 92% of the label was recovered in LPC suggesting no appreciable intrapreparative conversion of LPC. The positional specificity of labeled PC was determined by extraction and separation of lipid as detailed in the Methods section. After elution from the silica gel the PC was hydrolyzed by PLA_2. No radioactivity was recovered in the FFA fraction. The radioactivity lost from the PC band was quantitatively recovered

in the LPC band. Unless otherwise indicated all individual differences in this section were analyzed for statistical significance by Tukey's test.

I EFFECTS OF pHMB OF MICROSOMAL LPC CATABOLISM

Figure 3 gives the experimental design under which the effects of pHMB on the microsomal catabolism of LPC by the isolated rat and guinea pig heart were studied and compared. pHMB is a sulfhydryl agent known to inhibit enzymes in other systems (Kröner *et al.*, 1981, Weller *et al.*, 1984). Uptake of labeled LPC by the microsomal fraction did not differ between control (i.e. untreated) rat and guinea pig hearts (unpaired t-test). The incorporation of labeled LPC amounted to 10120 ± 800 dpm/mg protein ($n=7$) for rat microsomes and 11230 ± 671 dpm/mg protein ($n=6$) for guinea pig microsomes. There was, however, a marked difference in the profile of radioactive products of LPC catabolism in these microsomal fractions (Figure 18). Table 6 lists in numerical form the data obtained. The amount of radioactivity associated with LPC was greater in the rat heart microsomal fraction than in guinea pig microsomes (20% difference). Similarly a 20% difference was found in the amount of radioactivity recovered in the PC fraction between rat and guinea pig microsomes. Most noteworthy, however, was a 47% greater amount of radioactivity associated with the FFA fraction of guinea pig microsomes compared with rat microsomes ($p<0.01$, unpaired t-test; $n=6$ and $n=7$, respectively). Unlike rat microsomes, where no significant difference in the amount of radioactivity in the PC and FFA fractions was detected, the amount of radioactivity associated with the FFA fraction of guinea pig microsomes was almost twice as much as that found with the PC fraction ($p<0.001$, unpaired t-test; both $n=6$).

Microsomal acyl CoA:LPC acyl transferase and lysophospholipase activities measured *in vitro* are provided in Table 7. It is clear that no apparent relationship exists between these activities obtained under optimal conditions *in vitro* and the relevant

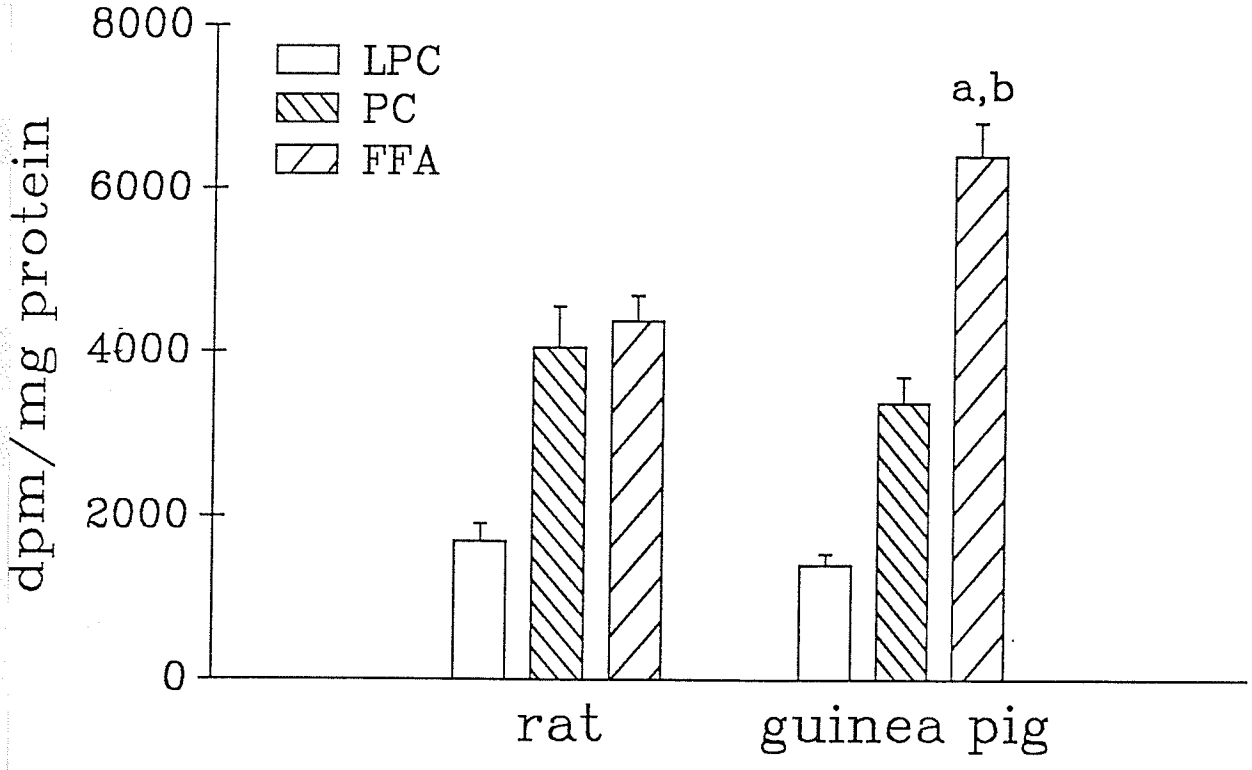


Figure 18. Profile of labeled LPC and products of its catabolism in the microsomal fraction from the isolated perfused rat and guinea pig heart. Isolated hearts were perfused with Kreb's-Henseleit buffer in the Langendorff Mode for 40 min (10 min stabilization + 30 min test period) followed by 10 min of perfusion with 2.5 μM [1-^{14}C] LPC (0.0075 μCi/ml). The hearts were homogenized in buffer and the microsomal fractions prepared as described in Methods. Lipids were extracted, separated and the LPC, PC and FFA bands analyzed. The data depicted for the rat heart microsomal lipid profile were collected from eight hearts and represent mean$\pm$S.E.; six hearts were used for the construction of the guinea pig microsomal lipid profile. [a] $p<0.01$ compared to rat FFA [b] $p<0.001$ compared to guinea pig PC.

Table 6. Profile of labeled LPC metabolites in rat and guinea pig microsomes.

	LPC (dpm/mg protein)	PC (dpm/mg protein)	FFA (dpm/mg protein)
Rat	1694±220	4059±494 (48%)	4370±317 (52%)
Guinea pig	1408±140	3391±309 (35%)	6423±418 [a,b] (65%)

Isolated rat and guinea pig hearts were perfused in the Langendorff Mode for 40 min with Kreb's-Henseleit buffer followed by perfusion with buffer containing 2.5 μM [1-^{14}C] LPC (0.0075 μCi/ml) for 10 min. Microsomes were prepared and lipids extracted, separated and analyzed for radioactivity as detailed in Methods. Values are expressed as mean±S.E., n=8 for rat, n=6 for guinea pig. [a] $p < 0.01$ compared to corresponding value in the rat, [b] $p < 0.001$ compared to guinea pig PC. Values in parantheses denote average weighted participation in clearance of LPC as determined from radioactivity in reaction products.

Table 7. Activity of microsomal LPC-catabolizing enzymes measured in vitro.

Enzyme	Rat	Guinea pig
Acyl CoA:LPC acyltransferase (μmol PC/mg protein/hr)	2.01 $\pm$ 0.65 n=3	1.69 $\pm$ 0.20 n=3
Lysophospholipase (nmol FFA/mg protein/hr)	44 $\pm$ 4 n=3	677 $\pm$ 70 n=3

Enzyme activities in rat and guinea pig heart microsomes were assayed as described in Methods. Values denote mean $\pm$ S.E. Note that units for enzyme activities differ.

participation in the intact isolated organ using the amount of radioactivity in the PC fraction as an index of acyl CoA:LPC acyl transferase activity and the amount of radioactivity in the FFA fraction as an index of lysophospholipase activity (data illustrated in Table 6). However, there appeared to be a qualitative relationship between lysophospholipase activity measured *in vitro* and the participation of lysophospholipase-mediated FFA release in the intact rat and guinea pig hearts.

Uptake of LPC by the microsomal fraction did not differ among control and pHMB-treated groups with either the rat or guinea pig heart (ANOVA). pHMB pretreament of the isolated rat heart gave rise to a significant concentration-dependent increase in the amount of radioactivity in LPC in the microsomal fraction (ANOVA) as shown in Figure 19. The data shown represent % of total radioactivity. Control values differed significantly from those obtained at 7.5 μM pHMB (29% increase over control, $p<0.05$) and 15.0 μM pHMB (36% increase over control, $p<0.05$). Associated with this increase in the amount of radioactive LPC was corresponding decrease in the amount of radioactivity in FFA (ANOVA). A level of statistical significance was attained at both 7.5 μM pHMB (26% decrease compared to control, $p<0.05$,) and 15.0 μM pHMB (34% decrease compared to control, $p<0.05$). Although there appeared to be a trend towards increased amount of radioactivity in PC no statistical significance was detected. In contrast pretreament of the isolated guinea pig heart with up to 7.5μM pHMB did not result in any statistically significant change in the amount of radioactivity in LPC (ANOVA) as is evident in Figure 20. There was, however, a concentration-dependent decrease in the amount of radioactivity associated with PC (ANOVA). Values obtained at both 5.0 μM pHMB (30% decrease compared to control) and 7.5 μM pHMB (41% decrease compared to control) were significant different than control (both $p<0.05$). A corresponding increase in radioactivity in the FFA was observed over this concentration range (ANOVA). A level of statistical significance was achieved with 7.5 μM pHMB (26% increase over control, $p<0.05$).

i) Effect of pHMB pretreatment on microsomal lysophospholipase and acyl CoA:LPC acyl

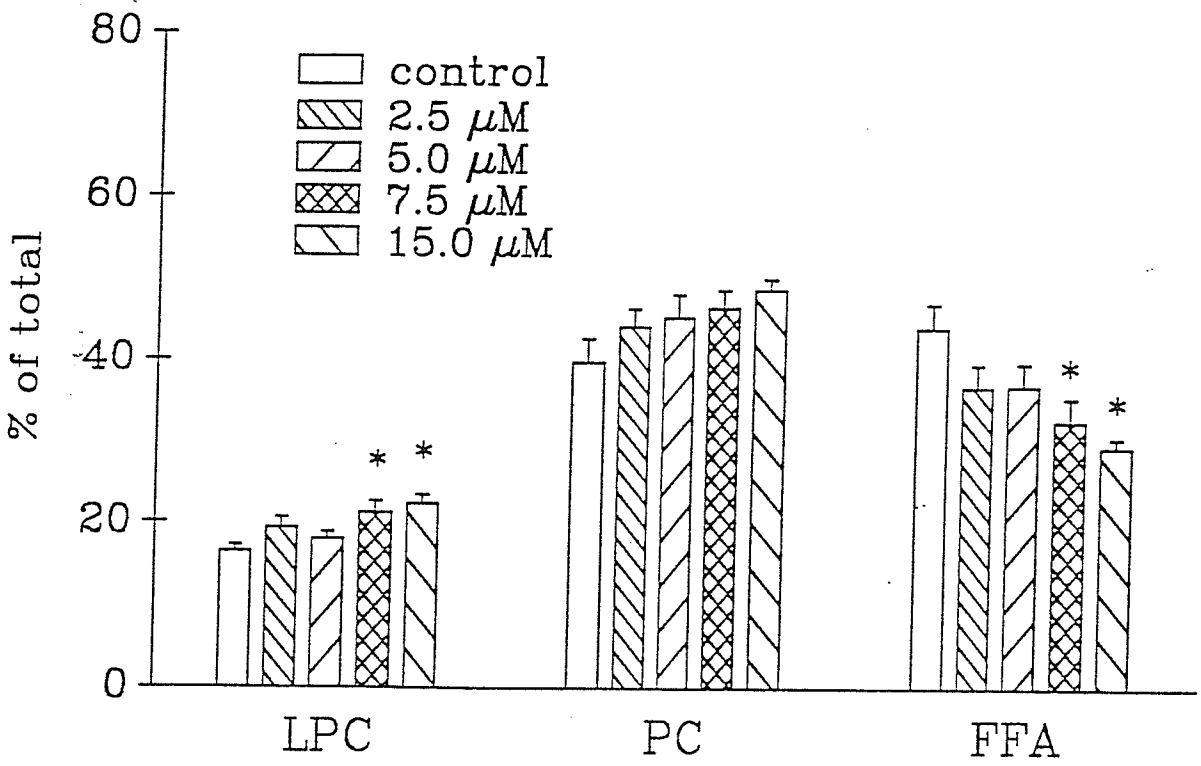


Figure 19. The effects of pHMB on microsomal LPC catabolism in the isolated rat heart. Isolated rat hearts were perfused with 0 to 15 μM pHMB in accordance with the experimental scheme given in Figure 3. Microsomal lipids were extracted, separated and analyzed as described in Methods. Data represent % of total radioactivity (i.e. LPC + PC + FFA) in each respective fraction and are the mean±S.E. of 5 to 8 separate experiments. * $p < 0.05$ compared to control.

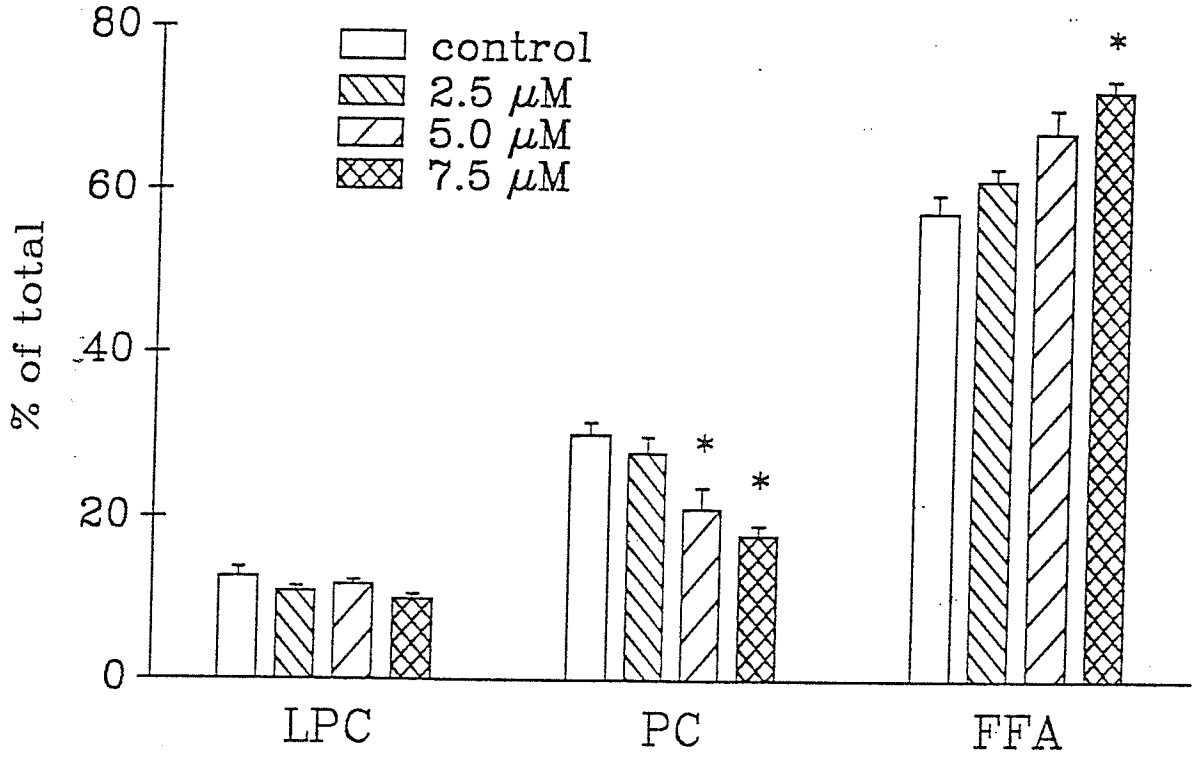


Figure 20. The effects of pHMB on microsomal LPC catabolism in the isolated guinea pig heart. Figure 3 provides a summary of the perfusion protocol. Data collection and presentation as in Figure 19. n=5 or 6. * $p<0.05$ compared to control.

transferase activity *in vitro*

As depicted in Figure 21 there appears to be no difference in the sensitivity of rat and guinea pig microsomal lysophospholipase and acyl CoA:LPC acyl transferase to pHMB-mediated inhibition *in vitro*. A slightly lesser amount of pHMB, on a per μg protein basis, was required to produce a level of moderate inhibition of lysophospholipase comparable to that of acyl CoA:LPC acyl transferase under these experimental conditions. However, the enzymes were equally inhibited at higher amounts of pHMB.

II. EFFECTS OF DIAMIDE ON MICROSOMAL LPC CATABOLISM

Figure 4 provides a summary of the various test conditions under which the effects of diamide on microsomal catabolism of LPC in the isolated rat and guinea pig heart were investigated. Diamide is widely used to deplete cellular glutathione. It is particularly useful as a tool to study the role of sulfhydryl modification in biological control since its effects are often rapidly reversible. Where the effects of diamide and other interventions on the amount of radioactivity in the lipid fractions were determined to be statistically significant by ANOVA individual differences were tested for statistical significance by Newman-Keul's test.

The presence of diamide in the perfusate did not result in any statistically significant difference in incorporation of labeled LPC into rat heart microsomes. Figure 22 depicts the changes in the pattern of labeling of the LPC, PC and FFA fractions produced by diamide under various conditions. A concentration of 0.25 mM did not result in any difference in the labeling profile compared to the non-drug control. However, at 2.5 mM there was a 3-fold increase in the level of unmetabolized labeled LPC ($p<0.01$; $n=3$). This

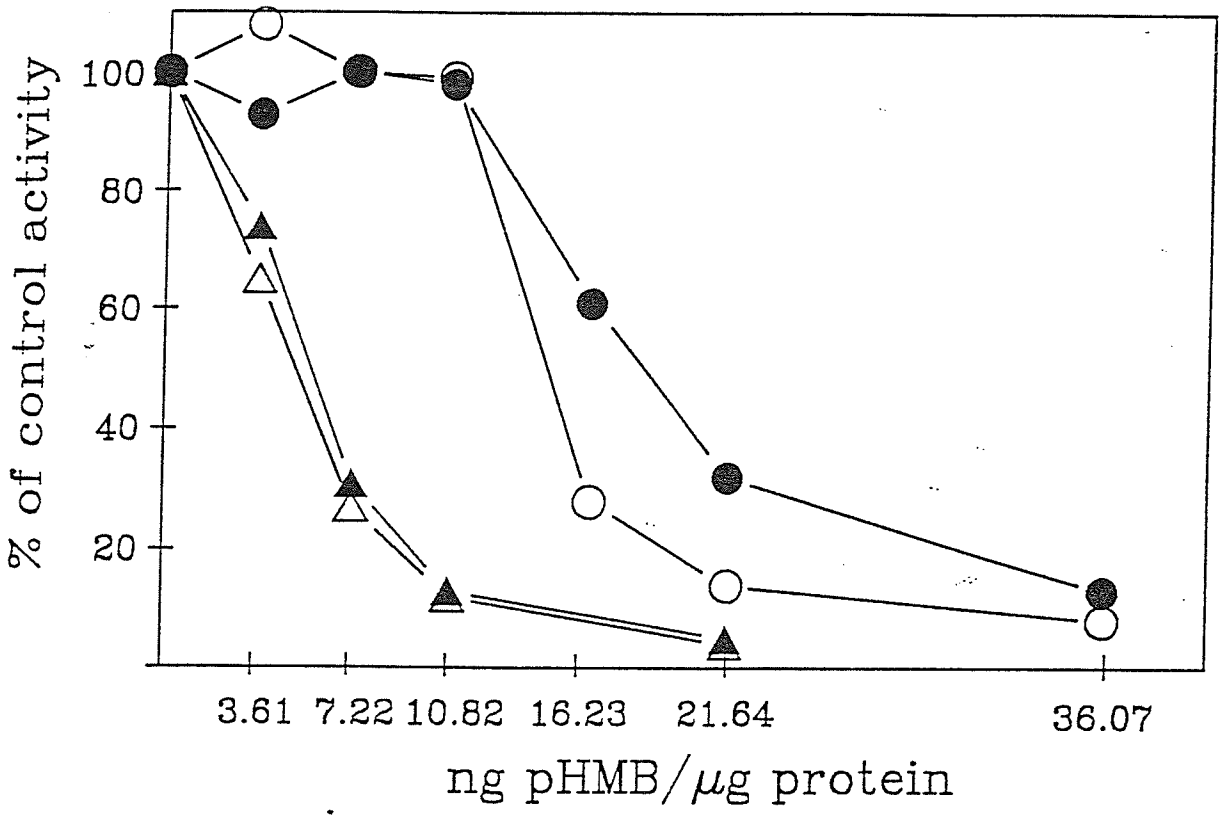


Figure 21. In vitro analysis of pHMB-mediated inhibition of acyl CoA:LPC acyl transferase and lysophospholipase in rat and guinea pig microsomes. Rat microsomal acyl CoA:LPC acyl transferase and lysophospholipase (open circles and triangles, respectively) and guinea pig microsomal acyl CoA:LPC acyl transferase and lysophospholipase (closed circles and triangles, resepectively) were assayed *in vitro* under optimal conditions in the presence of 0 to 36.07 ng pHMB/ug protein as described in Methods. Control activity (measured in the absence of inhibitor) is set at 100%. See Table 7 for control data. Each point is the mean of duplicate determinations from two separate experiments.

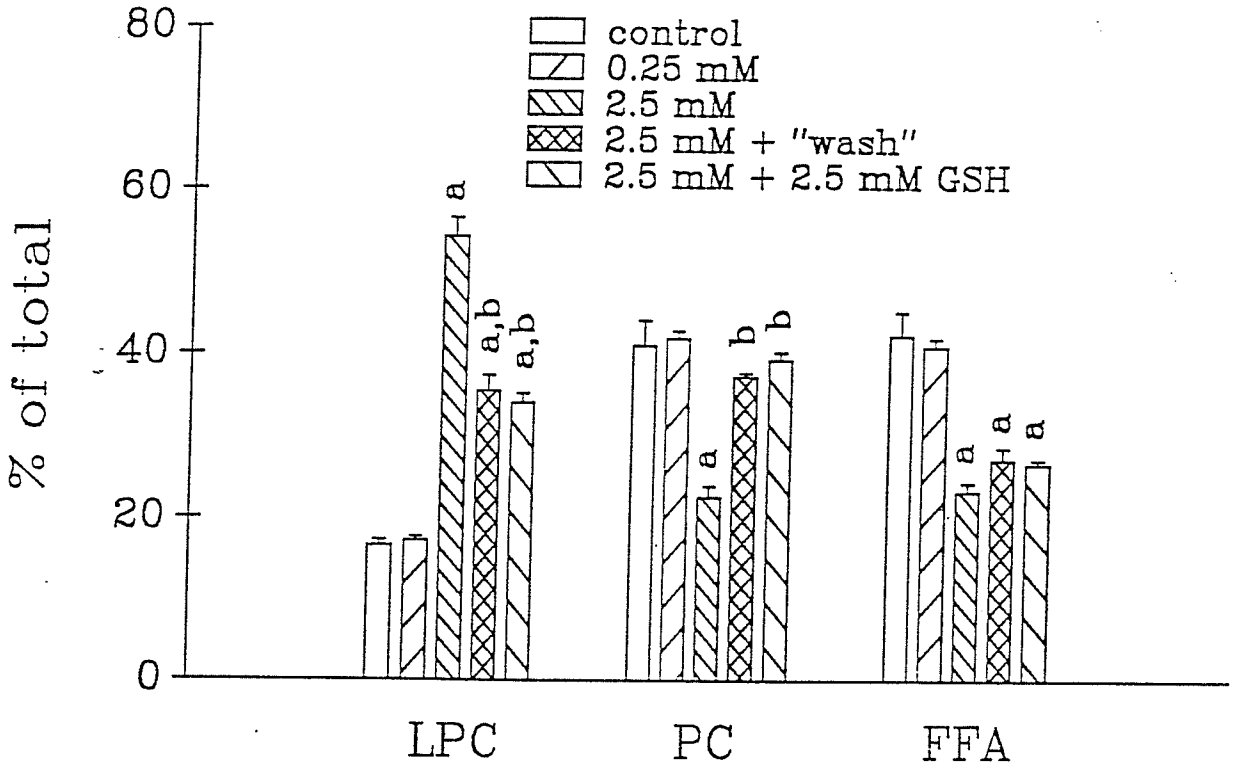


Figure 22. The effects of diamide on microsomal LPC catabolism in the isolated rat heart. Isolated rat hearts were perfused according to the experimental scheme given in Figure 4. Microsomal lipids were extracted, separated and the LPC, PC and FFA fractions analyzed for radioactivity. Data represent % of total radioactivity (i.e. LPC + PC + FFA) in each respective fraction and are the mean±S.E. of 3 to 7 separate experiments. [a] $p<0.01$ compared to control [b] $p<0.01$ compared to 2.5 mM diamide.

was accompanied by a parallel decrease in the amount of radioactivity both in the PC ($p<0.01$; $n=3$) and FFA fractions ($p<0.01$; $n=3$). The introduction of a "wash" period where diamide was absent from the perfusate resulted in a two-fold increase in the amount of label in the PC fraction compared to 2.5 mM diamide alone ($p<0.01$; $n=3$). This increase in the amount of label in PC to control levels was associated with a decrease in the amount of unmetabolized labeled LPC to a level significantly different than both 2.5 mM diamide and control (both $p<0.01$; $n=3$). No change was observed in the amount of label in the FFA fraction. Identical results were obtained with continuous simultaneous perfusion of diamide and glutathione.

In contrast to rat heart microsomes diamide did affect the incorporation of labeled LPC into guinea pig heart microsomes (ANOVA). Uptake of LPC under control conditions was 11230 ± 671 dpm/mg protein ($n=6$), whereas uptake in the presence of diamide was 5977 ± 533 dpm/mg protein ($n=3$) and 3227 ± 1241 dpm/mg protein ($n=3$) for 0.25 mM and 2.5 mM diamide, respectively (both $p<0.05$ compared to control). As with the rat heart the microsomal profile of radioactive LPC and the products of its catabolism did not change upon treatment of the isolated guinea pig heart with 0.25 mM diamide (Figure 23). At a concentration of 2.5 mM, however, the level of unmetabolized labeled LPC increased 3-fold compared to control ($p<0.01$; $n=3$). The amount of radioactivity consequently decreased in both the PC and FFA fractions ($p<0.01$; $n=3$), however, the extent of the changes was different both in relative and absolute terms. While the amount of radioactivity in PC decreased by 27% or 810 dpm (normalized) compared to control, the decrease in FFA amounted to 38% or 2200 dpm (normalized) compared to control. Removal of diamide from the perfusate resulted in a reciprocal increase in the amount of radioactivity in FFA and decrease in the level of unmetabolized labeled LPC to control values (both $p<0.01$ compared to 2.5 mM diamide). The amount of radioactivity

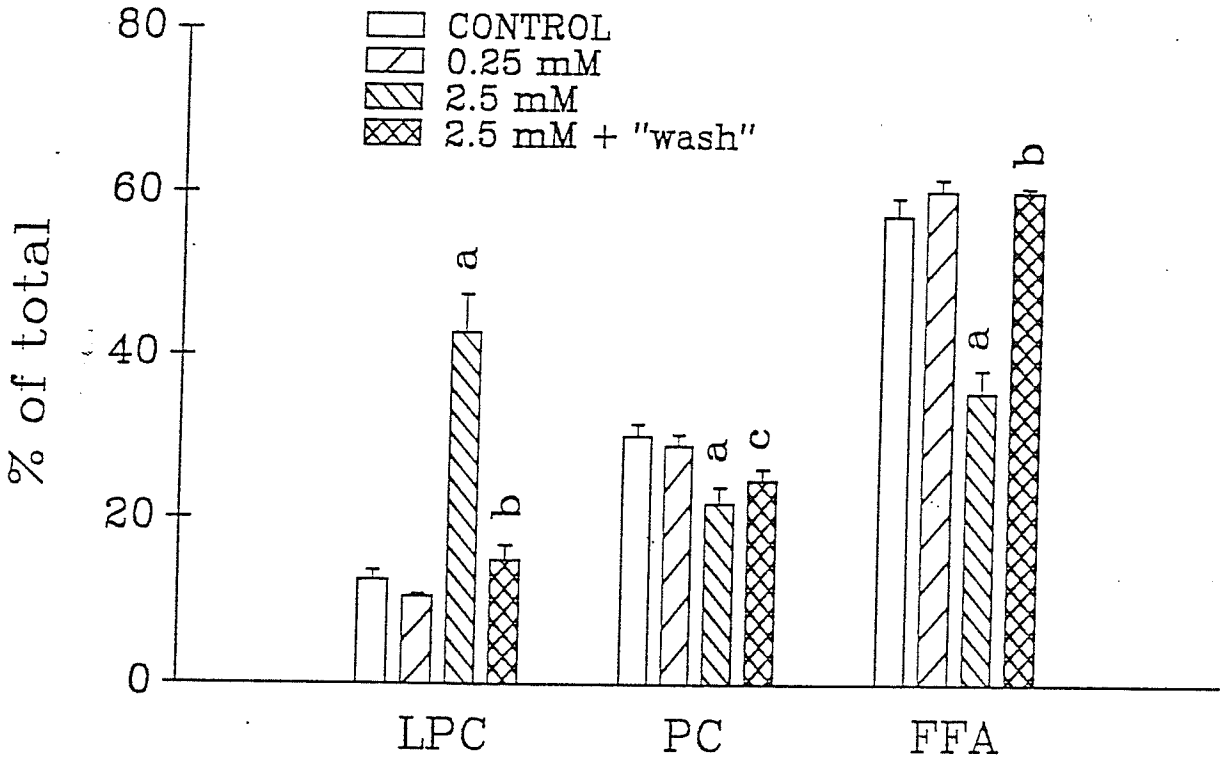


Figure 23. The effects of diamide on microsomal LPC catabolism in the isolated guinea pig heart. Data collection and representation as in Figure 22. n=3 to 6. [a] $p<0.01$ compared to control [b] $p<0.01$ compared to 2.5 mM diamide [c] $p<0.05$ compared to control.

associated with PC increased somewhat but was still significantly different than control ($p<0.05$; $n=3$).

III. EFFECTS OF "ISCHEMIC" CONDITIONS OF MICROSOMAL LPC CATABOLISM

A summary of the perfusion conditions selected to study the effects of "ischemic" conditions on microsomal LPC catabolism in the isolated rat and guinea pig heart is provided in Figure 5. LPC catabolism was studied under conditions which mimicked the substrate deprivation, metabolic acidosis, lactate accumulation and lowered pO_2 typical of ischemia *in vivo*. Where the effects are deemed to statistically significant by ANOVA individual differences were analyzed for statistical significance by Newman-Keul's test.

The amount of labeled LPC incorporated into the microsomal fraction of rat heart was not significantly different from control under the conditions studied. The effects of 20 min of perfusion under "ischemic" conditions followed by 10 min of perfusion with labeled LPC also under "ischemic" conditions was initially studied. As seen in Figure 24 the level of unmetabolized labeled LPC increased 2-fold ($p<0.01$; $n=3$). There was a corresponding decrease in the amount of radioactivity recovered in the FFA fraction ($p<0.01$; $n=5$). No change was observed in the amount of label in the PC fraction. When the hearts were perfused with labeled LPC under "ischemic" conditions immediately after stabilization the level of unmetabolized labeled LPC was also significantly greater than control ($p<0.01$; $n=5$). As before this was matched by a parallel decrease in the amount of label in FFA ($p<0.01$; $n=5$). Statistical significance was also detected between LPC values obtained under both "ischemic" conditions ($p<0.01$). The amount of radioactivity associated with PC did not change.

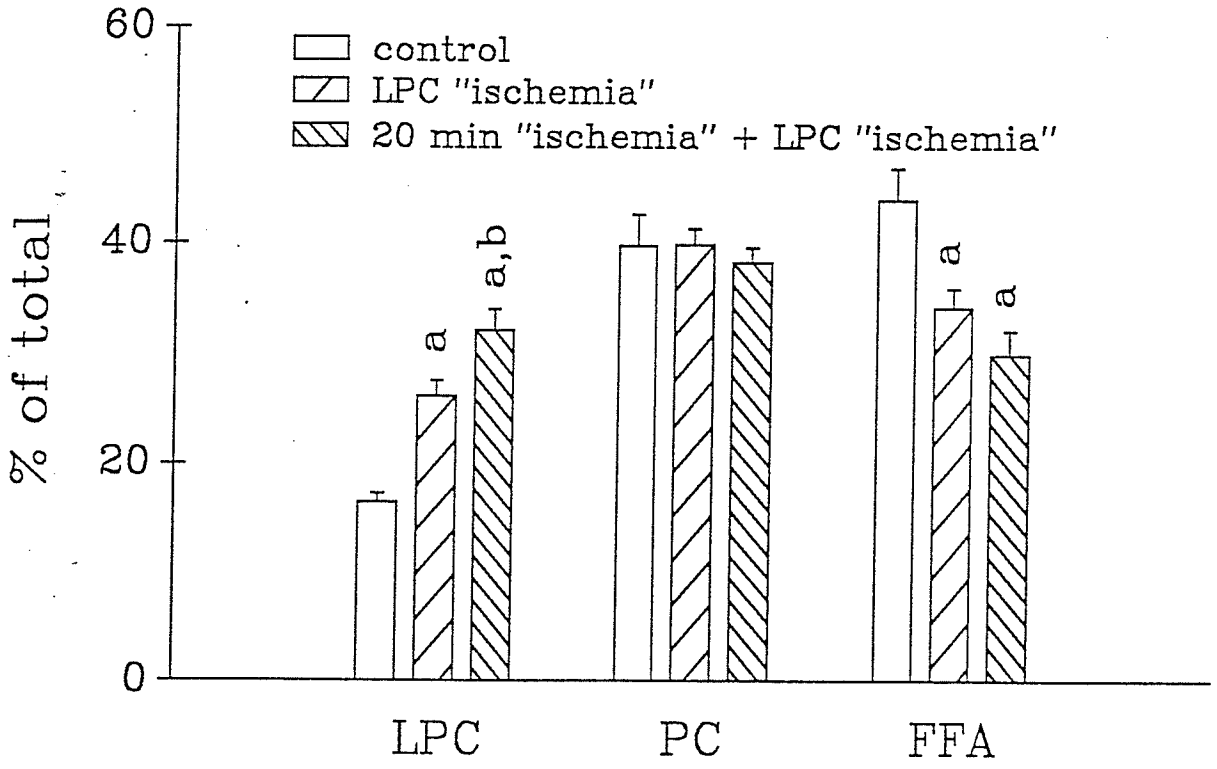


Figure 24. The effect of "ischemic" conditions on microsomal LPC catabolism in the isolated rat heart. Isolated rat hearts were perfused according to the experimental scheme shown in Figure 5. Microsomal lipids were extracted, separated and the LPC, PC and FFA fraction analyzed for radioactivity as described in Methods. Data represent % of total radioactivity (i.e. LPC + PC + FFA) in each respective fraction and are the mean±S.E. of 5 to 8 separate experiments. [a] $p<0.01$ compared to control [b] $p<0.01$ compared to 20 min "ischemia".

In contrast to rat heart perfusion under "ischemic" conditions (20 min "ischemia" + 10 min LPC "ischemia") did significantly alter the incorporation of radioactive label into guinea pig heart microsomes ($p<0.01$, unpaired t-test). Uptake under control conditions was 11230 ± 671 dpm/mg protein ($n=6$) while uptake under "ischemic" conditions was 6984 ± 973 dpm/mg protein ($n=5$). No alterations in the profile of radioactive LPC or the products of its catabolism were detected (Figure 25). Since no change was observed under these conditions the effects of 10 min of "ischemic" perfusion with LPC alone was not pursued.

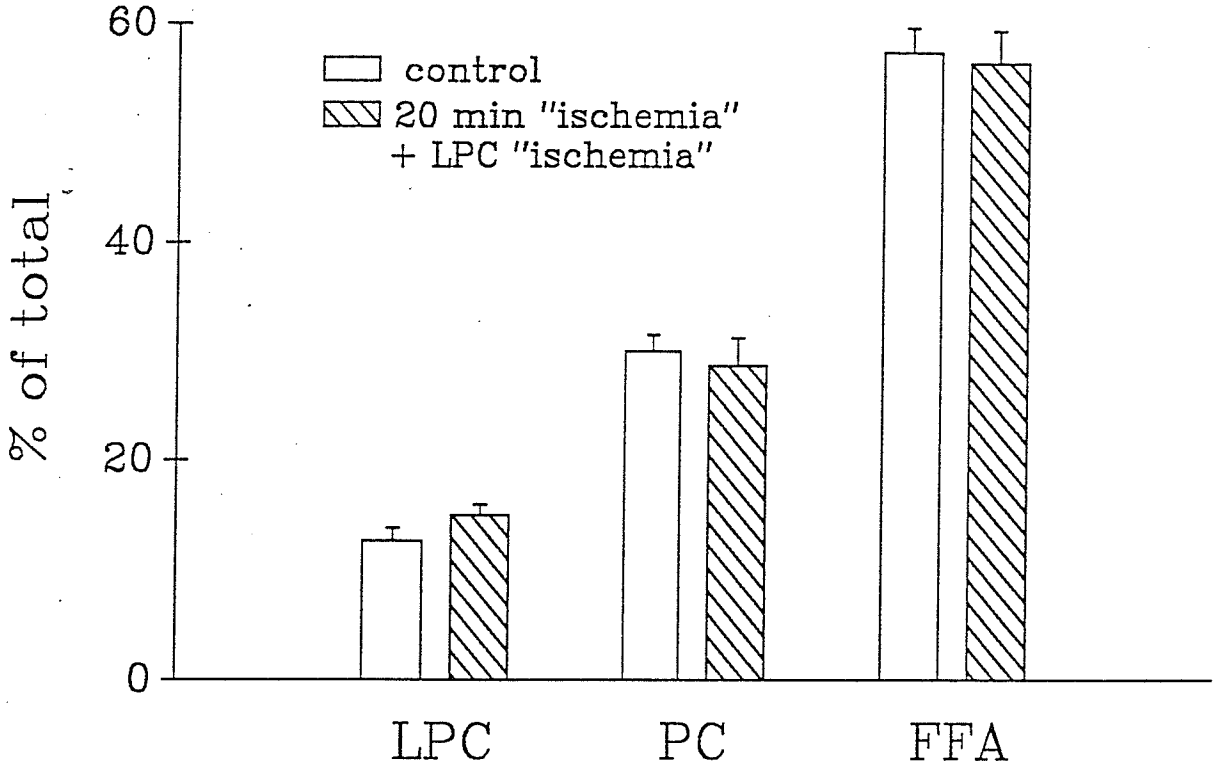


Figure 25. The effect of "ischemic" conditions on microsomal LPC catabolism in the isolated guinea pig heart. Data collection and presentation as in Figure 24. n=5 or 6.

DISCUSSION

The principal objective of this study was an understanding of LPC metabolism and its regulation in the mammalian heart with reference to the biochemical lesion(s) which underlie(s) the accumulation of LPC during ischemia. In the first stage of this study the hypothesis that alterations in LPC levels in the ischemic heart are associated with changes in the capacity for production and elimination of LPC was tested. A canine model of regional myocardial ischemia was selected because of ready availability of animals and the methodology required was developed and routinely used in the laboratory. Furthermore, the relatively large size of the canine heart enabled both non-ischemic and ischemic tissue to be collected from the same organ and thus direct comparison between tissues from a single source was possible. The approach used involved measurement of alterations in LPC content coupled with an *in vitro* assessment of changes in the activity of LPC-metabolizing enzymes.

The levels and time course of accumulation of LPC observed in this study offer some interesting similarities and contrasts with other models. The non-ischemic control values obtained in this study (approx. 400 nmol/g dry weight) compare favorably with previous studies in the dog heart (Steenbergen and Jennings 1984, Kinnaird *et al.*, 1988), pig heart (Das *et al.*, 1986) and the rat heart (Otani *et al.*, 1989). The time course for accumulation of LPC among different species, however, shows a marked variability. A significant accumulation of LPC in ischemic tissue *vs* non-ischemic control tissue after 3 hr of ischemia observed in the present study is in accordance with reports in the *in vivo* (Kinnaird *et al.*, 1988) and *in vitro* (Steenbergen and Jennings, 1984) ischemic canine heart. Whereas, significant increases in LPC were detected after only 1 hr of ischemia in the rat (Otani *et al.*, 1989) and pig heart (Das *et al.*, 1986). In an earlier study, Shaikh and Downar (1981) reported significant increases in LPC after only 8 min of ischemia in the porcine heart. The reason for the discrepant result between that study and the Das study is not known.

Relatively rapid accumulation of LPC has also been reported in the cat and rabbit myocardium (Sobel *et al.*, 1978, Corr *et al.*, 1982, Corr *et al.*, 1987) where significant increases were detected after only 3 to 5 min post-occlusion. Although methodological differences may be invoked to explain these apparent discrepancies it is likely that biological variability is a more important factor. The notion that differences in LPC metabolism among species and among subcellular fractions within a given species is an important determinant of this variability formed the basis for the present *in vitro* investigation of the LPC-metabolizing enzymes in the canine myocardium. In the second phase of this study a comparative analysis of microsomal LPC catabolism in the isolated rat and guinea pig heart was performed in an attempt to relate LPC levels to relative participation of key catabolic enzymes.

It is evident from Table 2 that the LPC-producing enzyme phospholipase A and the LPC-eliminating enzymes LPC:LPC transacylase, acyl CoA:LPC acyl transferase and lysophospholipase are all present in the canine heart albeit with varying specific activities and intracellular distribution. Phospholipase A activity was assayed using a biosynthetically prepared substrate which more closely mimics the endogenous substrate *in vivo* (Cao *et al.*, 1987). It was reasoned that this would provide a more accurate assessment of activity than commercially available synthetic substrate particularly since activity is critically dependent on the fatty acyl species at the C-2 position (Tam *et al.*, 1984). The phospholipase A activity measured in the cytosolic, mitochondrial and microsomal fractions in this study are in good agreement with those reported in the rat heart (Nalbone and Hostetler, 1985), hamster heart (Tam *et al.*, 1984) and canine heart (Wolf and Gross, 1985). Direct comparison with other preparations is hampered because of the routine use of phosphatidylethanolamine as substrate (Franson *et al.*, 1978, Franson *et al.*, 1979) which has been shown to be preferred over PC (Weglicki *et al.*, 1971, Tam *et al.*, 1984).

Unlike phospholipase A activity, where differences among species is minimal based on *in vitro* analysis, it is clear that the capacity for clearance by the LPC-metabolizing enzymes among species may vary greatly. The acyl CoA:LPC acyl transferase activity determined in the cytosolic fraction in the present study is in complete agreement with cytsolic activity in rat heart myocytes (Severson and Fletcher, 1985), while activity in rabbit heart cytosol is barely detectable (Gross and Sobel, 1982). Similarly mitochondrial activity in the rabbit heart (Gross and Sobel, 1982) is very low compared to the guinea pig heart (Arthur et al., 1987) and rat cardiac myocytes (Severson and Fletcher, 1985). The use of palmitoyl CoA as acyl donor in the former study may be at least partly responsible for the apparent discrepancy since only very low levels of saturated fatty acid residues at the C-2 position of cardiac phospholipids have been demonstrated (Weglicki *et al.*, 1970). The specific activity of the microsomal acyl CoA:LPC acyl transferase measured in the dog in the present study is 13-fold greater than both the rat and guinea pig heart (Giffin *et al.*, 1988) and 20-fold greater than the hamster (Savard and Choy, 1982) and rabbit heart (Giffin *et al.*, 1988). The complete absence of cytosolic LPC:LPC transacylase activity in the dog heart determined in this study contrasts sharply with the rabbit heart (Gross and Sobel, 1982). Indeed canine myocardial LPC:LPC transacylase activity is confined exclusively to the microsomal fraction unlike the rabbit heart where activity is present in both the cytosolic and microsomal fractions (Gross and Sobel, 1982). The specific activity of the canine cytosolic lysophospholipase found here agrees well with the cytosolic lysophospholipase from other sources including rabbit heart (Gross and Sobel, 1982) and rat heart (Severson and Fletcher, 1985, Giffin *et al.*, 1988) although it was much lower than the activity reported in the hamster heart cytosol (Savard and Choy, 1982). The specific activity of the canine microsomal lysophospholipase determined in this study is comparable to values reported in the rabbit (Gross and Sobel, 1982) but was almost 10-fold higher than the rat heart microsomal lysophospholipase (Severson and Fletcher, 1985, Giffin *et al.*, 1988).

From the foregoing discussion one may conclude then that species differences with respect to the presence, intracellular distribution and specific activity of enzymes are fundamental characteristics of LPC catabolism. Moreover the present study confirms earlier reports of a 10^3-fold to 10^4-fold greater capacity for elimination of LPC than for production of LPC based on *in vitro* analysis. Since LPC levels are rigidly controlled under normal conditions this implies that production and elimination are in equilibrium. Disruption of the equilibrium either by enhanced production or impaired elimination will favor the accumulation of LPC. The effects of ischemia on the activity of LPC-metabolizing enzymes were therefore determined.

The concept of increased production of LPC as a consequence of augmented PLA activity in ischemia is supported by a number of indirect observations. Depletion of cardiac PC (Chien *et al.*, 1981) and elevation of free arachidonic acid (Chien *et al.*, 1984) and various eicosanoids (Schrör, 1987) in ischemic myocardium are consistent with augmented PLA_2 activity. Moreover, chlorpromazine and mepacrine, known phospholipase A inhibitors, can to a large extent normalize phospholipid homeostasis (Okumura *et al.*, 1983, Das *et al.*, 1986), though the specificity of these drugs is suspect and effects on other cellular processes cannot be ruled out. Direct support for augmented PLA activity comes from studies in the *in situ* pig heart subjected to global hypothermic arrest (Das *et al.*, 1986) and in hypoxic rat myocardial slices (Kawaguchi and Yasuda, 1988). Hazen *et al.* (1991) have reported a pronounced increase in PLA_2 activity in ischemic rabbit heart microsomes directed toward PC plasmalogen. In the present study, however, ischemia of up to 5 hr duration had no effect on canine myocardial PLA activity measured *in vitro*. This suggests that canine myocardial PLA activity is not regulated by a mechanism(s) that is(are) relatively "permanent" which would allow detection in an *in vitro* assay. It is therefore possible to exclude changes in enzyme content, covalent modification and the presence of a tightly bound regulatory compound as potential regulators. One cannot rule out the participation of

possible regulatory mechanisms such as intracellular acidosis, availability of endogenous substrate and allosteric effectors which may be operative *in vivo*.

In the isolated rat heart, Bentham *et al.*, (1987) have actually measured a decrease in PLA_2 activity in tissue rendered ischemic by left anterior descending coronary artery ligation. This finding is particularly striking since the time of peak LPC accumulation corresponds with the greatest decrease in PLA_2 activity. It should be noted, however, incubations performed *in vitro* do not take into account changes in the intracellar compartment such as elevated intracellular Ca^{2+} which stimulates cardiac PLA_2 activity (Saxon *et al.*, 1984). Recently, however, the popular notion that increased intracellular Ca^{2+} during ischemia is inexorably linked to increased PLA_2 activity has been challenged. Poole-Wilson *et al.* (1984) have provided compelling evidence which questions whether free cytoplasmic Ca^{2+} increases to levels sufficient to stimulate activity. Moreover, it has recently been demonstrated that both LPC and long chain acyl carnitine inhibit PLA_2 activity *in vitro* at concentrations known to occur in the ischemic myocardium (Bentham *et al.*, 1987, Conricode and Ochs, 1989) which suggests product inhibition may serve to check any increase in activity. The lack of convergence of scientific opinion on the issue of enhanced production of LPC as the sole or even the predominant cause of elevated levels of LPC has prompted some investigators to suggest that other mechanisms must be operative in the accumulation of LPC in the ischemic heart (Otani *et al.*, 1989).

Indeed in the present study we have found ischemia-induced reductions in the activity of the LPC-catabolizing enzymes. Reductions in the cytsolic and microsomal lysophospholipases, microsomal acyl CoA:LPC acyl transferase and microsomal LPC:LPC transacylase achieved a level of statistical significance at 3 hr of ischemia which is in temporal agreement with a significant increase in LPC content determined earlier. The extent of reduction in activity in the microsomal enzymes was not uniform in that an

approximately 50% reduction in the activity of LPC:LPC transacylase and lysophospholipase was found but only a 30% reduction in acyl CoA:LPC acyl transferase activity after 5 hr of ischemia was demonstrated. It is unlikely, therefore, that a general phenomenon such as membrane damage is the sole cause of the alterations in activity since PLA activity was unaffected altogether. Membrane damage has been cited in the inhibition of glucose-6-phosphatase and Ca^{2+}-pump activities of microsomes prepared from ischemic liver (Chien *et al.*, 1978). By extension a similar mechanism is likely present in the ischemic heart, however, a quantitation of this effect was not pursued. The participation of inhibitory factors(s) which are retained in the microsomal and cytosolic fractions is thus a distinct possibility. Although statistically significant differences in long chain acylcarnitine content of ischemic microsomes were not observed (see page 87) it is possible that local accumulations were significant to lead to the observed alterations in activity.

In summary myocardial ischemia does result in measurable changes in the activity of LPC-metabolizing enzymes. Moreover the present results do not support the view that enhanced production by PLA is responsible for LPC accumulation in the ischemic canine heart. Rather the data show a temporal link between reductions in LPC catabolism and LPC accumulation.

It has thus been established that the catabolic enzymes are the likely targets of modulation during ischemia in the canine heart, therefore, an understanding of the regulation of these enzymes under conditions mimicking ischemia became necessary. Previous studies have demonstrated the inhibitory effect of reduced pH (Gross and Sobel, 1982) and palmitoyl-*l*-carnitine (Gross and Sobel, 1983, Severson and Fletcher 1985,) on the cytosolic and microsomal lysophospholipases and microsomal acyl CoA:LPC acyl transferase. Intracellular pH falls during ischemia as lactate and H^+ accumulate (Jennings *et al.*, 1986) as a result of altered glycolytic metabolism. Long chain acyl carnitine accumulates in the

cytosol during ischemia because of impaired fatty acid metabolism (Idell-Wenger *et al.*, 1978). The possible regulatory effects of these concomitants of ischemia on the canine catabolic enzymes *in vitro* were therefore studied.

The present results demonstrated that palmitoyl-*l*-carnitine, as a representative long chain acyl carnitine, inhibits canine heart cytosolic and microsomal lysophospholipase and microsomal acyl CoA:LPC acyl transferase. Similar findings have been reported in the rabbit heart (Gross and Sobel, 1983) and rat myocytes (Severson and Fletcher, 1985). The lack of uniformity in the type of inhibition produced by palmitoyl-*l*-carnitine among the three enzymes indicates complex interactions. Inhibition of the soluble cytosolic enzyme was purely competitive in nature likely reflecting competition for enzyme binding between palmitoyl LPC and palmitoyl-*l*-carnitine which are structural analogues (Figure 2). The form of mixed inhibition observed with the microsomal acyl CoA:LPC acyl transferase involves alterations both in the Km and Vmax. A increased Km reflects decreased affinity of the enzyme for the substrate likely due to competition for the binding site. The decreased Vmax may be ascribed to the membrane-perturbing properties of palmitoyl-*l*-carnitine (Fink and Gross, 1984) which can induce a non-catalytically active three-dimensional conformation of the enzyme molecule. More difficult is the characterization of the palmitoyl-*l*-carnitine-mediated inhibition of microsomal lysophospholipase. The non-Michaelis-Menten nature of lysophospholipase activity at high substrate/protein ratios is similar to the rabbit enzyme (Gross and Sobel, 1982) and likely reflects alterations in the biophysical characteristics of the microsomal membrane preparation. The effect would be to reduce the amount of LPC in the membrane that is potentially capable of forming a productive interaction with the enzyme. Given that palmitoyl-*l*-carnitine and palmitoyl LPC share similar amphiphilic properties (Fink and Gross, 1984) a similar mechanism of action by palmitoyl-*l*-carnitine must be given consideration. The fact that uncompetitive inhibition was clearly obtained at 150 μM palmitoyl-*l*-carnitine and a form of mixed inhibition at 200

μM palmitoyl-*l*-carnitine raises the possibility that a critical mole proportion of the amphiphile exists whereby the characteristics of the membrane and enzyme are modified resulting in altered interactions between enzyme, substrate and inhibitor.

The data obtained for palmitoyl-*l*-carnitine-mediated inhibition of the cytosolic lysophospholipase and the microsomal acyl CoA:LPC acyl transferase revealed Ki's of 18 μM and 140 μM, respectively. Since long chain acyl carnitines are located primarily in the cytosol they would have access to both cytosolic and microsomal enzymes. Moreover, a calculated cytosolic concentration of 430 μM and the magnitude of the inhibitory constants obtained are compatible with a modulatory role in LPC clearance for long chain acyl carnitine *in vivo*. It could be argued, however, that protein binding decreases the amount of long chain acyl carnitine that is metabolically active *in vivo* i.e. the level of free long chain acyl carnitine. Bound long chain acyl carnitine would theoretically not be available to participate as a catabolic inhibitor. The capacity for protein binding of long chain acyl carnitine is often *presumed* because of the existence in cardiac cytosol of fatty acid binding protein (FABP) which binds avidly to free fatty acids (Fournier, 1987) and long chain acyl CoA's (Mishkin and Turcotte, 1974). However, in a recent study FABP from rat heart was found not to bind LPC (Burrier and Brecher, 1986). Positive controls were performed with the rat liver protein. The suggestion, therefore, that long chain acyl carnitine may indeed be metabolically active (i.e. free) has been confirmed in a recent study. Corr *et al.*, (1989) have shown that pretreatment of cats with the carnitine acetyl transferase-I inhibitor POCA (sodium 2-[5-(4-chlorophenyl)-pentyl]-oxirane-2-carboxylate abolishes the increase in LPC in ischemic myocardium. POCA specifically inhibits the formation of long chain acyl carnitine; the implication is therefore that the LPC-catabolizing enzymes are not subjected to long chain acyl carnitine-induced inhibition and act to maintain a basal level of LPC. If long chain acyl carnitine were protein bound and hence not capable of interacting with the enzymes of LPC catabolism then inhibition of formation of long chain acyl carnitine should

be inconsequential.

The inhibition of the LPC-catabolic enzymes would be exacerbated if the content of long chain acyl carnitine increases. In this study ischemia resulted in significant increases in long chain acyl carnitine content after 3 and 5 hr. In a rat whole heart preparation significant increases in long chain acyl carnitine have been detected after only 20 min of global ischemia (Idell-Wenger *et al.*, 1978). The different time courses may well be a function of the preparation studied since regional myocardial ischemia may result in a heterogenous preparation depending on the extent of perfusion by collateral vessels. It is possible that within the affected myocardium there exist pockets of tissue which received blood despite total occlusion of the left anterior descending coronary artery and were proportionally less ischemic. The use of radioactive microspheres to distinguish between areas of the myocardium which may or may not have received some blood for comparison purposes was not feasible. Thus the results obtained represent gross averages and a range of values likely exists within the affected area.

The amphiphilic nature of long chain acyl carnitine enables insertion and incorporation into the biological membrane. Elevated membrane levels may then exert a increased level of inhibition. Indeed increased sarcoplasmic reticulum content of long chain acyl carnitine has been implicated in decreased Ca^{2+} transport function in diabetic rat heart (Lopaschuk *et al.*, 1983). Ischemia-induced increases in long chain acyl carnitine have been described in sarcolemma-enriched membranes from porcine heart (Lamers *et al.*, 1987). Similarly in the present investigation increased levels of microsomal long chain acyl carnitine in 3 and 5 hr preparations were detected. The increases, however, did not attain a level of statistical significance ($p > 0.05$). The lower recovery of microsomal protein from 3 and 5 hr ischemic tissue suggests a more fragile membrane preparation. Although difficult to prove, it is possible that the long chain acyl carnitine content of ischemic microsomes is

an underestimation due to loss of amphiphile during homogenization and subcellular fractionation.

A reduction in assay pH to 6.5 inhibited only the microsomal lysophospholipase. Thus an ischemia-induced reduction in pH alone is insufficient to affect either the cytosolic lysophospholipase or the microsomal acyl CoA:LPC acyl transferase. The disparate regulation of the cytosolic and microsomal lysophospholipase and the microsomal acyl CoA:LPC acyl transferase with respect to pH and palmitoyl-*l*-carnitine suggest different rates of catalysis depending on metabolic status. Since no information is currently available concerning coordination of catabolism among cytosolic and microsomal enzymes the possibility of different, independently regulated pools of LPC exist (Wittels, 1970).

In summary reduced pH and palmitoyl-*l*-carnitine are two metabolic determinants which affect the catabolism of LPC by virtue of modulatory effects on the cytosolic and microsomal lysophospholipases and microsomal acyl CoA:LPC acyl transferase. The inhibitory constants obtained coupled with the calculated cytosolic concentration of long chain acyl carnitine suggest that these enzymes may be subject to tonic regulation by long chain acyl carnitine. This inhibition would be exacerbated under conditions where long chain acyl carnitine content increases. In this canine model of regional myocardial ischemia significant increases in long chain acyl carnitine content were detected after 3 hr of ischemia which is in temporal agreement with the decrease in activity of the catabolic enzymes and the increase in myocardial LPC. A trend towards increased microsomal long chain acyl carnitine content was also found which suggests that microsomal enzymes may be subject to inhibition from both cytosolic and membrane-associated long chain acyl carnitine.

From the foregoing discussion it is clear that there are several potential routes for LPC clearance in the heart based on *in vitro* analysis of subcellular fractions. Although

useful in defining certain characteristics of enzyme activity such as kinetic parameters and ion requirements etc. an *in vitro* analysis is of limited usefulness in describing how different enzymes may interact in the clearance of LPC and how the dynamics of this interaction may affect LPC catabolism. Also the frequent application of optimal reaction conditions for one enzyme will often mask or distort the normal contribution of other enzymes to LPC clearance. Moreover, specific activity measured *in vitro* under optimal conditions is often the only indicator of participation in the intact organ but may be misleading since conditions for optimal expression of activity may not be present.

In order to address these shortcomings microsomal LPC catabolism was studied in the isolated intact perfused heart which is more physiologically relevant. In order to differentiate between pathways the use of radioactive substrate was required. Since selective labeling of endogenous LPC in the heart is not feasible because of rapid and extensive catabolism an alternative approach became necessary. Perfusion of the isolated heart with exogenous labeled LPC followed by preparation of microsomes, extraction and separation of lipids offered a more facile means of measuring LPC catabolism. Moreover the isolated intact perfused heart model allowed for simultaneous evaluation of participation under identical conditions of acyl CoA:LPC acyl transferase and lysophospholipase in LPC clearance. The relative participation of these two enzyme activities in [1-^{14}C] palmitoyl LPC clearance was based on the amount of radioactivity in the products of LPC catabolism. Radioactivity in PC was used as an index of acyl CoA:LPC acyl transferase activity while radioactivity in FFA was used as an index of lysophospholipase activity. The participation of LPC:LPC transacylase in LPC clearance was ruled out by positional analysis of the labeled PC product. In the following experiments comparisons were made between the isolated rat heart and isolated guinea pig heart in an attempt to relate enzyme activity to extent of clearance upon various interventions.

In accordance with previous reports (Gross and Sobel, 1982, Severson and Fletcher 1985) the capacity for acylation of LPC by acyl CoA:LPC acyl transferase far exceeds that for deacylation by lysophospholipase in both rat and guinea pig heart microsomes based on *in vitro* analysis. The suggestion has therefore been made that deacylation by acyl CoA:LPC acyl transferase is the primary mechanism for LPC clearance in the heart. However, the profile of radioactive products of LPC catabolism in microsomes obtained from the intact heart in this study indicates that deacylation by lysophospholipase is of far greater relative importance than the *in vitro* data would suggest. In fact clearance by deacylation is the preferred pathway for catabolism in guinea pig heart microsomes. Deacylation by lysophospholipase and acylation by acyl CoA:LPC acyl transferase are of equal importance in LPC clearance in rat heart microsomes despite the fact that the specific activity of the acyl CoA:LPC acyl transferase is 46-fold greater than the lysophospholipase. Thus the present results suggest that specific activity measured *in vitro* shows no correlation with the relative participation of lysophospholipase and acyl CoA:LPC acyl transferase in the catabolism of LPC in the intact organ. With the assumption that the *in vitro* assay is valid it is clear that conditions necessary for optimal expression of activity are not present in the intact organ.

Both acyl CoA:LPC acyl transferase and lysophospholipase activities have previously been shown to be inhibited by sulfhydryl group reactive reagents (Kröner *et al.*, 1981, Weller *et al.*, 1984). One such agent, pHMB, has been used to promote Ca^{2+} mobilization and insulin release in rat islets of Langerhans as a direct result of LPC accumulation brought about by inhibition of these enzymes (Metz, 1988). This agent was therefore used as a tool to perturb enzyme activities in the isolated heart as a means of assessing the effects of changes in acylation and deacylation on LPC clearance. A fortuitous result was the finding that, within the concentration ranges of pHMB evaluated impairment of the microsomal acyl CoA:LPC acyl transferase in the guinea pig heart was manifest in a decrease in the amount

of radioactivity associated with PC, whereas in the rat heart impairment of microsomal lysophospholipase was reflected in a decrease in the amount of radioactivity in the FFA fraction. It was thus possible to measure the catabolic fate of exogenous LPC in two species with significantly altered capacities for clearance of LPC by these two major routes. Despite decreases in PC synthesis in microsomes from pHMB-treated guinea pig heart there were no differences in the amount of radioactivity in LPC because of compensatory increases in FFA release via lysophospholipase activity. It is probably that a degree of competition for LPC exists between the enzymes which enables the lysophospholipase to compensate for diminished acyl CoA:LPC acyl transferase-mediated clearance to maintain a constant extent of elimination. Thus it appears that there is an element of "crosstalk" between these enzymes and therefore they are not likely acting independently of each other. In contrast impairment of deacylation by lysophospholipase in rat microsomes was accompanied by an increase in the level of unmetabolized labeled LPC. Evidently rat microsomes are less able to maintain the same level of clearance once lysophospholipase activity has been compromised. Moreover, there appears to be no significant potential for compensatory elimination of LPC by acyl CoA:LPC acyl transferase. These findings provide evidence for a link between lysophospholipase activity and the extent of LPC catabolism. The differential inhibition of enzyme activity by pHMB in isolated intact rat and guinea pig hearts, as evidenced by the changes in the amount of label in the respective lipid fractions, cannot be satisfactorily explained by the sensitivity of the microsomal enzymes to pHMB inhibition measured *in vitro*. It is conceivable, however, that some as yet unknown characteristics of the intact heart are responsible for the apparent variance.

The demonstrated potential for sulfhydryl group modification in the regulation of LPC catabolism may be of relevance *in vivo*. The sulfhydryl status of cellular constituents is governed by the intracellular level of glutathione (δ-glutamylcysteinylglycine) (Kosower and Kosower, 1978). One of several actions of glutathione is participation in oxidation-

reduction reactions including a thiol-disulfide interchange (Fava *et al.*, 1957, Fava and Ileceto, 1958). These reactions play a prominent role in the three dimensional conformation of proteins containing multiple disulfide links and in the formation of glutathione-protein mixed disulfides. These thiol-disulfide exchanges are likely to be a significant element in biological control by regulating the concentration of proteins containing reactive thiol or disulfide groups. Glutathione levels have been directly linked to Ca^{2+} regulation in liver mitochondria (Beatrice *et al.*, 1984, Riley and Pfeiffer, 1986) and canine myocardial mitochondrial phospholipid homeostasis (Kajiyama *et al.*, 1987) through effects on lysophospholipid catabolism. Chemical depletion of glutathione has proved to be a useful tool in delineating those processes which are under control by glutathione by thiol-disulfide reactions (Plummer *et al.*, 1981). Blaustein *et al.*, (1989) have shown that administration of the weak electrophile diethylmaleimide rat heart impairs recovery of contractile function after short periods of ischemia. Diamide, a diazenecarboxylic acid derivative developed by Kosower and Kosower (1969) is a thiol oxidant which is reported to be specific for glutathione although oxidation of other thiol groups cannot be ruled out (Plummer *et al.*, 1981). The treatment of isolated rat heart cells with diamide results in the production of protein-mixed disulfides by depletion of cellular glutathione (Grimm *et al.*, 1985). Removal of diamide leads to a rapid reversal of effects. These properties make diamide an attractive tool in the study of LPC catabolism in the isolated rat and guinea pig heart.

A direct link between lysophospholipase activity and the extent of microsomal LPC catabolism in the intact rat heart previously indicated with studies involving pHMB were confirmed with the use of diamide. An additional level of control by acyl CoA:LPC acyl transferase was identified by the introduction of a "wash" period where diamide was absent from the perfusate. This maneuvre restored acyl CoA:LPC acyl transferase activity to control levels. The restoration of acyl CoA:LPC acyl transferase was coupled with enhanced elimination of LPC (i.e. decreased level of labeled unmetabolized LPC). Despite

a washout perfusion lysophospholipase activity was not restored to control levels and accordingly the level of labeled unmetabolized LPC remained elevated compared to control. The reason for the disparate responses of acyl CoA:LPC acyl transferase and lysophospholipase to this perfusion regimen is uncertain, however, different sensitivities to sulfhydryl modification is a possibility. Although identification of the mechanism of action of diamide was not a aim of this phase of the study the protective effect of simultaneous perfusion with glutathione suggests that cellular glutathione may indeed be a target for modulation. A similar link between lysophospholipase activity and extent of LPC catabolism was demonstrated in the isolated intact guinea pig heart. In contrast to rat heart microsomes the introduction of a "wash" period after perfusion with diamide resulted in a complete return of lysophospholipase activity to control levels. This recovery was accompanied by a return to control levels of unmetabolized labeled LPC. The discrepant results between rat and guinea pig heart with respect to restoration of lysophospholipase activity upon washout of diamide may be the result of species differences. No relationship between acyl CoA:LPC acyl transferase activity and the level of labeled unmetabolized LPC was apparent. It appears that sufficient lysophospholipase activity exists in guinea pig heart microsomes to compensate for impaired acylation by acyl CoA:LPC acyl transferase to maintain a constant level of labeled unmetabolized LPC.

Since diamide was used merely as a tool to impair LPC catabolism no attempt was made to relate changes in activity to glutathione depletion and production of mixed-disulfides. Although previous studies have strongly suggested such relationships may be operative in other systems (Hewitt *et al.*, 1974) proof of existence in the heart awaits a detailed study that would benefit greatly from the application of glutathione esters to replenish depleted glutathione i.e. reversal study (Anderson and Meister, 1989). Glutathione itself is not taken up to any significant extent by cells (Puri and Meister, 1983). These glutathione esters are not commercially available at present. The dramatic effects of

diamide on LPC catabolism observed in this study certainly warrant further study particularly since depletion of cellular glutathione occurs during hypoxia/ischemia (Guarnieri *et al.*, 1980, Kajiyama *et al.*, 1987) and is coupled to modification of protein thiols (Orrenius, 1988).

Several factors were considered when a study of the effects of ischemia on microsomal LPC catabolism in the isolated intact heart was attempted. Because experimentation was limited to delivery of exogenous labeled LPC, global ischemia, by stopping perfusion altogether, or regional ischemia, achieved by ligation of the left anterior descending coronary artery, would restrict delivery of the very substrate in question. Perfusion under "ischemic" conditions offered the only feasible approach. The compostion of the perfusate was altered to mimic the substrate deprivation, metabolic acidosis, lactate accumulation and reduced oxygen tension characteristic of ischemic tissue (Tsushima and Moffat, 1989). However, since continuous flow was required it was not possible to duplicate the reduced washout expected in ischemic zones. Nevertheless "ischemia" in the isolated rat heart did result in measurable changes in the profile of radioactive LPC and its metabolites. Under the conditions used it is readily apparent that the lysophospholipase is the target for modulation. This modulation appears to be quite rapid as perfusion of LPC under "ischemic" conditions without a preceding "ischemic" episode is sufficient to produce significant inhibition of lysophospholiase-mediated clearance of LPC. In both cases the reduction in lysophospholipase-mediated FFA release was accompanied by a corresponding increase in the level of labeled unmetabolized LPC. The link between lsyophospholipase activity and the extent of LPC clearance was thus clearly established under "ischemic" conditions. Unlike rat microsomes, "ischemic" conditions reduced the uptake of labeled lipid by guinea pig microsomes. This coupled with the fact that sulfhydryl modification induced by diamide also reduced the uptake of label suggests that LPC uptake by guinea pig heart microsomes is subject to regulation. Uptake is therefore not a function of simple

adsorbtion to the membrane followed by incorporation as would be anticipated from its amphiphilic nature. The existence of a tissue acceptor for LPC has already been proposed in the heart (Stein and Stein, 1966). No measurable change was observed in the lipid profile of guinea pig heart microsomes under "ischemic" conditions. It has previously been demonstrated that modest reductions in acyl CoA:LPC acyl transferase-mediated clearance of LPC in guinea pig heart microsomes are compensated for by corresponding increases in lysophospholipase-mediated FFA release. Since no change was observed in the PC fraction it is reasonable to assume that acyl CoA:LPC acyl transferase is not a target for modulation. It remains probable, therefore, that the relatively high lysophospholipase activity of guinea pig heart microsomes provides a sufficient buffer against changes.

In summary the present studies clearly indicate that specific activity measured *in vitro* under optimal conditions shows no correlation with relative participation in LPC clearance in the intact organ. The lysophospholipase activity may, however, be qualitatively related to the potential for clearance of deacylation in the intact organ. Inhibition of lysophospholipase activity in both rat and guinea pig heart microsomes and inhibition of acyl CoA:LPC acyl transferase activity in rat heart microsomes are linked to impairment of LPC clearance. However, inhibition of acyl CoA:LPC acyl transferase activity alone in guinea pig heart microsomes is not associated with a reduction in LPC clearance because of compensatory lysophospholipase activity. Thus the data suggest that in rat heart microsomes there is a rigid reciprocal relationship between enzyme activity and extent of LPC clearance, whereas in guinea pig heart microsomes this tight coupling extends only to lysophospholipase activity. The microsomal acyl CoA:LPC acyl transferase and lysophospholipase activities from both rat and guinea pig heart are subject to inhibition by sulfhydryl group modification. A reduction in the extent of LPC clearance under "ischemic" conditions in rat heart microsomes was linked exclusively to lysophospholipase inhibition. The absence of a similar effect in the guinea pig heart suggests that the lysophospholipase activity is either

high enough or the potential for lysophospholipase-mediated clearance sufficient enough to militate against increases in LPC under those experimental conditions.

Summary and future research directions

Regional myocardial ischemia in the dog produced significant alterations in LPC homestasis as evidenced by the increased levels in ischemic tissue *vs* non-ischemic control. The change in LPC content was temporally related to decreases in the activity of the enzymes of LPC elimination which suggests that impaired catabolism rather than increased production is the underlying biochemical lesion. Two concomitants of ischemia, reduced pH and long chain acyl carnitine, were found to be inhibitors of catabolic enzymes. Palmitoyl-*l*-carnitine, a representative long chain acyl carnitine, inhibited the cytosolic and microsomal lysophospholipase and microsomal acyl CoA:LPC acyl transferase while reduced pH produced significant inhibition of the microsomal lysophospholipase only. The complex kinetics of inhibition observed with palmitoyl-*l*-carnitine among these enzymes, however, suggest multiple actions. The calculated inhibitory constants for palmitoyl-*l*-carnitine-mediated inhibition of cytosolic lysophospholipase and microsomal acyl CoA:LPC acyl transferase are compatible with a modulatory role *in vivo* based on the estimated cytosolic concentration of long chain acyl carnitine. Long chain acyl carnitine content was found to increase in ischemic myocardium. Additionally a trend towards increased long chain acyl carnitine in ischemic microsomes was observed. The increases in long chain acyl carnitine and LPC content in ischemic canine myocardium were temporally linked, moreover the demonstrated potential for long chain acyl carnitine-mediated inhibition of LPC catabolism suggests interaction between long chain acyl carnitine and LPC.

Support for the suggested link between catabolic enzyme activity and the level of LPC was provided in studies in the isolated intact heart. The relative participation of

acylation by acyl CoA:LPC acyl transferase and deacylation by lysophospholipase was found to have a direct bearing on the extent of microsomal catabolism of exogenous labeled LPC. In rat heart microsomes where particpation of acylation and deacylation in LPC clearance were approximately equal, perturbation of either acyl CoA:LPC acyl transferase or lysophospholipase resulted in an increased level of unmetabolized LPC. In guinea pig heart microsomes where deacylation is clearly the preferred route of LPC elimination an increase in the level of unmetabolized LPC was effected only by inhibition of lysophospholipase activity. Modulation of acyl CoA:LPC acyl transferase activity was not associated with any change in the level of LPC. Moreover, perfusion under "ischemic" conditions was linked to changes in the level of LPC only in rat heart microsomes. These data suggest that inhibition of catabolic enzymes is one mechanism which governs the level of LPC in heart microsomal membranes. Moreover, it appears that the relative extent to which the microsomal lysophospholipase participates in LPC clearance is an important determinant of the variability in LPC catabolism between rat and guinea pig heart microsomes.

The association between catabolic enzyme inhibition and LPC level demonstrated in this study is an important first step in a greater understanding of LPC metabolism in the heart. It would be prejudicial to claim that metabolic determinants such as reduced pH and long chain acyl carnitine are unique in their capacity for modulation of enzyme activity. Several observations made in this study support this claim. The profound effects on enzyme activity by the thiol reagents pHMB and diamide warrant further investigation into the modulatory role of sulfhydryl modification. Sulfhydryl modification of cellular constituents has already been established to be a contributory mechanism to contractile dysfunction in cardiac muscle (Eley *et al.*, 1989). Cellular control of sulfhydryl status is one of the main functions of glutathione (Kosower and Kosower, 1978). This coupled with the fact that glutathione levels are decreased dramatically during hypoxia/ischemia (Guarnieri *et al.*, 1980, Kajiyama *et al.*, 1987) merits investigation into the role of glutathione in the

regulation of lysophospholipase and acyl CoA:LPC acyl transferase activity. Otani *et al.*, (1989) have reported that reperfusion of ischemic myocardium is a potent stimulus to additional accumulation of LPC. The involvement of free radicals in ischemic reperfusion injury in the heart is well documented (Hess *et al.*, 1982, Burton *et al.*, 1984, Kim and Akera, 1987, Tsushima and Moffat, 1989). Recent reports suggest that protein thiols are cellular targets of oxidative stress (Kako, 1987, Orrenius, 1988, Thomas and Park, 1988). The possible link between free radicals, protein thiol modification and reduced LPC catabolism offers another potential mechanism for LPC homoestasis.

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