Cardiac Phospholipase D: Characterization, Regulation and Involvement in Ischemia-Reperfusion Injury

By

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

DOCTOR OF PHILOSOPHY

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Winnipeg, Manitoba

ISBN 0-315-86021-9

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- Pathology 0571
- Pharmacology 0419
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BY

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

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DEDICATED TO YIJING

Acknowledgements

I am deeply indebted to my advisor Dr. V. Panagia for his continual guidance, critical judgement, enthusiastic encouragement and, most importantly, his friendship throughout the years. I would like to express my sincere thanks to Dr. N.S. Dhalla for his wise guidance, support and encouragement. I would also like to thank my advisory committee members: Drs. P. K. Singal, E. J. Scott and P. K. Ganguly, who have provided me with helpful suggestions and directions during my studies.

I am very much obliged to Dr. W. Shen, my cousin, Professor Z-J. Jin, Professor S. Shen and Professor E-Y. Yu for initiating my scientific career.

I am extremely grateful to my colleagues Dr. R. Vetter, Dr. C. Ou, Dr. J. T. A. Meij, N. Mesaeli and J. A. E. Hays for their helpful suggestions, criticisms, experimental assistance, and also for providing me with an enjoyable and stimulating environment. Special acknowledgement is also due to my colleague J. A. E. Hays for her professional editorial help in preparing this thesis.

Furthermore, I very much appreciate the help, suggestions and friendship of Drs. G. N. Pierce, W. C. Cole, E. Kardami, N. Leblanc, I. Dixon and the members of the Cardiovascular Division in St. Boniface General Hospital Research Centre.

I owe many special thanks to Yijing, my loving wife, for her continuous emotional support and for sharing with me both the excitement of success and risk of the trial. Without her strong support, I would not have achieved today's accomplishment. I am also very grateful to my loving daughter Chen-Xi for her cooperation and for being such a terrific child.

Finally, I would like to thank the St. Boniface General Hospital Research Foundation and the Manitoba Health Research Council for their financial support during this study.

List of Abbreviations

Phospholipase D — PLD

Phosphatidic acid — PtdOH

Phosphatidylcholine — PtdCho

Phosphatidic acid phosphohydrolase — PAP

Diacylglycerol — DAG

Protein kinase C — PKC

Phosphatidylethanol — PtdEt

Cyclic Adenosinemonophosphate — cAMP

Phosphatidylinositol 4,5-bisphosphate — PtdIns(4,5)P_2

Inositol-1,4,5-trisphosphate — Ins(1,4,5)P_3

Phosphatidylserine — PtdSer

Acyl-coenzyme A — AcylCoA

Adenosine triphosphate — ATP

Guanyl-5'-yl imidodiphosphate — GppNHp

p-CMPS — p-choloromercuriphenylsulfonic acid

DTNB — 5,5'-dithio-bis(2)-nitrobenzoic acid

NEM — N-ethylamaleimide

MMTS — methyl methanethiosulfonate

Dithiolthreitol — DTT

Hydrogen peroxide — H_2O_2

Hypochlorous acid — HOCl

SL — sarcolemma

SR — sarcoplasmic reticulum

Thiol — SH

Table of Contents

List of Tables

List of Figures

Abstract

Phospholipase D (PLD) catalyses the specific hydrolysis of phosphatidylcholine (PtdCho) to form phosphatidic acid (PtdOH) and the non-phosphorylated base, choline. Newly formed PtdOH may directly modulate cellular functions or be further dephosphorylated to 1,2-diacylglycerol (DAG) by a functionally coupled PtdOH phosphohydrolase (PAP). DAG is an important activator of protein kinase C which may phosphorylate a number of target proteins. PLD activities have been reported in many tissues with different subcellular localizations and biochemical properties. It was shown by others that the agonists can enhance the efflux of choline with parallel formation of PtdOH in perfused heart, indicating the presence of PLD in that tissue and its involvement in signal transduction. However, this approach did not specify the cellular localization of PLD in cardiac tissue; neither did it provide biochemical evidence for such an activity. The importance of PLD to the heart was also indicated by its potential to modulate Ca^{2+} handling systems (Na^{+}-Ca^{2+} exchange, Ca^{2+} binding, Ca^{2+} pump and Ca^{2+} dependent slow action potential) when exogenous PLD or PtdOH were used. These studies suggested that PLD activity and subsequent formation of PtdOH may be related to the Ca^{2+} movements within the cardiomyocyte and may influence the heart function in normal conditions as well as in disease states characterized by abnormal Ca^{2+} homeostasis. Therefore, it was necessary to provide detailed information about the presence and enzymology of PLD activity in myocardial cell. Furthermore, in brain microsomes, PLD was found to be sensitive to sulfhydryl group modifiers and activated by fatty acids. Considering the possible homology of PLDs, these two regulatory mechanisms were also examined for the cardiac PLD. Fatty acid accumulation as well as free radical generation which may modify functional protein thiols are important features in cardiac ischemia-reperfusion injury. Thus, it was conceivable that changes in PLD activity might occur in this disease condition, and this was therefore examined.

For this study, an *in vitro* assay system was employed using different crude subcellular fractions isolated from rat ventricular tissue. In addition, two purified

subcellular fractions, sarcolemma and sarcoplasmic reticulum, were used for detailed PLD activity investigation. Different assay conditions (pH, temperature, time and kinetics etc.) were tested. PLD was assessed by 1) its hydrolytic activity using exogenous radiolabelled substrate as well as endogenous substrate which was prepared by perfusing the heart with [^{3}H] labeled choline, 2) its transphosphatidylation activity using [^{3}H]PtdCho and ethanol as exogenous substrates. Several sulfhydryl group modifiers, free radical generating systems and scavengers were used. Different naturally occurring fatty acids as well as endogenous fatty acid releasing mechanism were also tested. PLD abnormalities were assessed by monitoring the above mentioned types of PLD activities in membrane preparations isolated from globally ischemic hearts at different ischemia and reperfusion periods. The potential of coupled PLD/PA phosphohydrolase activities was also studied in this pathological model.

The results of this study demonstrate that PLD activity localizes in different membrane fractions of the cardiac cell. The majority of the enzyme activity is present in the SL fraction. The properties of SL and SR PLD are similar to certain extent and are coupled with PAP activities for the dephosphorylation of PtdOH to DAG. This was indicated by the predominant DAG production upon depriving the PLD assay medium of KF, a PAP inhibitor. Both activities showed similar dependence on free sulfhydryl groups and a requirement of unsaturated fatty acid for activation. However, a significant difference between SL and SR PLD was observed in the Fe^{2+} dependence and ischemia-reperfusion injury. In particular, cellular thiol modulators and oxidants which may be generated in pathological conditions, were found to inhibit cardiac PLD activity presumably through sulfhydryl group modification. The most effective unsaturated FA were arachidonic and oleic acids which maximally activated PLD at 0.5 mM concentration with endogenous substrate and, respectively, at 4 and 5 mM with exogenous substrate. Furthermore, the release of endogenous fatty acids was also found to stimulate cardiac PLD activity. Thirty min of ischemia did not change SL and SR PLD activity. Reperfusion of such ischemic hearts resulted in an increase and then decrease of SL

PLD activity, in contrast to a decrease and then a recovery of SR PLD. On the contrary, 60 min of ischemia resulted in the depression of PLD activities in both SL and SR fractions, which were aggravated by reperfusion. These results suggest that SL PLD and SR PLD respond differently to the ischemia-reperfusion insult, and they may be under different predominant control mechanism(s) and may play different roles in this pathological process. The SL PtdOH phosphohydrolase activity was depressed at the end of 30 min of ischemia and recovered after 5 min of reperfusion. This suggests that in the initial reperfusion period, PtdOH is the predominant product and may be involved in Ca^{2+} flux modulation.

This study showed, for the first time, the presence of a phosphatidylcholine-specific phospholipase D activity in cardiomyocytes, and provided a detailed characterization of the enzyme. It also gave some insight into the mechanisms of the regulation of cardiac PLD, and into its involvement in ischemia- reperfusion damage. The results may also suggest a new mechanism for understanding of reperfusion-induced injury.

I. Introduction

Recently, phospholipases have been shown to be involved in mediating many cellular functions, including stimulus-response coupling. Second messengers produced by phospholipase-mediated pathways outnumber those generated by any other route. Phosphoinositide-PLC is the first enzyme which has drawn a great deal of attention as new signal transduction pathway for Ca^{2+} mobilizing agonists.

However, there is a growing appreciation that PtdIns(4,5)P_2 is not the only phospholipid hydrolysed in response to cellular stimulation and that DAG is not only generated from the hydrolysis of PtdIns(4,5)P_2. A number of studies have shown that PtdCho, a major component of the biomembranes, can be rapidly broken down in response to a range of agonists. Hydrolysis of PtdCho can directly generate DAG by a PtdCho-PLC; alternatively, DAG may also be produced indirectly via PLD-dependent formation of PtdOH, which is then dephosphorylated by a PLD coupled-PAP activity.

It has been found that a great diversity of acyl chain structures of PtdCho exist, e.g.,1,2-diacyl-,1-O-alkyl-2-acyl-and1-alk-1'-enyl-2-acyl-cn-glycero-3-phosphocholine (Billah, and Anthes, 1990). Therefore, hydrolysis of PtdCho could generate multiple species of DAG which may differ in terms of fatty acid composition. This raised an interesting perspective since different DAG species may activate different PKC isoforms, thus inducing PKC-dependent phosphorylation of different target proteins and thereby different physiological responses.

A large body of evidence within the past few years has indicated that PLD may play an important role in receptor signaling mechanisms in mammalian cells. A variety of hormones and neurotransmitters have been shown to stimulate PLD activity in different tissues. The mechanism for the agonist control of PtdOH hydrolysis by PLD seems to involve G-proteins, PKC and Ca^{2+} (Exton, 1990; Billah, and Anthes, 1990).

PLD was originally identified in plants. In mammalian tissues, its activity was first shown (Saito and Kanfer, 1975) and extensively studied (Kanfer, 1989) in rat brain. These studies identified mammalian PLD as a membrane-bound enzyme which could act upon phosphatidylethanolamine or phosphatidylinositol, but first and foremost preferred phosphatidylcholine (PtdCho) (Taki and Kanfer, 1979). Since then, PLD activity has been reported in many tissues showing different subcellular localizations as well as different biochemical properties (e.g., optimal pH, Ca^{2+} or Mg^{2+} dependence, and substrate specificity etc.) (Kanfer, 1989; Billah and Anthes, 1990; Shukla and Halenda, 1990). Therefore, the occurrence of multiple enzyme isoforms seems likely.

The first detection of PLD activity in the heart occurred while testing crude microsomal preparations obtained from a variety of rat tissues (Chalifour and Kanfer, 1980). It was also shown that the agonist stimulation of the isolated hearts enhanced the efflux of choline (Lindmar, et al., $1986_{a,b}$) and the formation of PtdOH (Lindmar, et al., 1988), indicating the presence of PtdCho-hydrolysing PLD and its involvement in signal transduction. The potential importance of this enzyme for the heart is indicated by *in vitro* studies with exogenous PLD. Treatment with exogenous PLD or PtdOH increased the sarcolemmal Na^{+}-Ca^{2+} exchange activity (Philipson and Nishimoto, 1984), as well as the Ca^{2+} binding and force of contraction of the heart (Langer, et al., 1985). The observation that exogenous addition of PtdOH or PLD generates Ca^{2+}-dependent slow action potentials in depolarized rat atrium supports the role of PtdOH in mediating the increase of Ca^{2+} influx into the cardiac cells (Knabb, et al., 1984). PtdOH has also been reported to stimulate the SL Ca^{2+} pump (Carafoli, 1984) which is involved in the efflux of Ca^{2+} from the cell during the relaxation of the myocardium. These studies suggest that PLD hydrolytic activity and subsequent formation of PtdOH may be related to the Ca^{2+} movements within the cardiomyocyte and may influence the heart function in normal conditions as well as in disease states characterized by abnormal Ca^{2+} homeostasis.

In spite of the above findings, there are still serious uncertainties about the actual presence of PLD in the cardiomyocyte as well as a complete lack of information regarding the subcellular localization of this enzyme and its biochemical properties. The present study was therefore undertaken to examine in detail the PLD activity in cardiac cells by testing its subcellular distribution, pH dependence, temperature dependence, kinetic parameters and other biochemical properties.

Little is known about the regulation of PLD. In brain microsomes, an acidic PLD was found to be sensitive to sulfhydryl group modifiers (Kobayashi and Kanfer, 1987). In accordance with this, four cysteine residues have been found in a PLD gene from *Corynebacterium pseudotuberculosis* by a cloning study (Hodgson, et al., 1990). Being likely that cardiac PLD should share a certain homology with PLD from other cell types, we hypothesized that the sulfhydryl groups associated with cardiac PLD may be important for the structural and functional integrity of the enzyme. In such a case, we would expect that PLD activity in the heart may be controlled by the cellular glutathione/glutathione-disulfide redox state. Furthermore, free radicals, which are known to damage the enzymes by modifying functional protein thiols, may affect cardiac PLD. Therefore, PLD activity was assayed in the presence of different sulfhydryl group modifiers and after treating membranes with oxidants which occur in pathological states of the heart. The effect of biological SH-modifiers glutathione and glutathione-disulfide, on cardiac PLD was also tested.

Since in many cell free systems PLD activities can be detected only in the presence of detergents or oleate (Kobayashi and Kanfer, 1987; Martin, 1988; Qian and Drewes, 1989; Chalifa, et al., 1990; Siddiqui and Exton, 1992), we intended to explore if cardiac PLD shares this requirement. From initial experiments, we found that the *in vitro* expression of cardiac PLD activity was dependent on supraphysiological concentrations of sodium oleate. Oleic acid is one of the most widely distributed, naturally occuring unsaturated fatty acids. Its mechanism(s) of action in stimulating cardiac PLD and its minimal effective concentration were

unclear. It was also unknown if any other fatty acid may influence the enzyme activity. Therefore, we examined the effect of various long-chain fatty acid on cardiac PLD activity.

From our studies, fatty acid stimulation and sulfhydryl modification appeared to be important regulatory mechanisms of the cardiac PLD. Fatty acid accumulation (van Bilsen, et al., 1989) and free radical generation (Jennings and Reimer, 1991) which may modify functional protein thiols (Kaneko, et al., 1989) are important features in cardiac ischemia-reperfusion injury. Thus, it was conceivable that alterations in PLD activity might occur in this pathological condition. Therefore, changes in myocardial PLD activity were evaluated at different time-periods of ischemia-reperfusion injury. In particular, the following parameters were studied in SL and SR membranes: basal and fatty acid stimulated activities, kinetic parameters, and, importantly, PLD-PAP coupling mechanisms which generate DAG for PKC activation.

By completing the above studies, we hoped to reach a better understanding of the cardiac phospholipase D, its regulation and possible involvement in ischemia-reperfusion injury. We also hoped to provide foundations to further PLD research in the heart.

II. Review of Literature

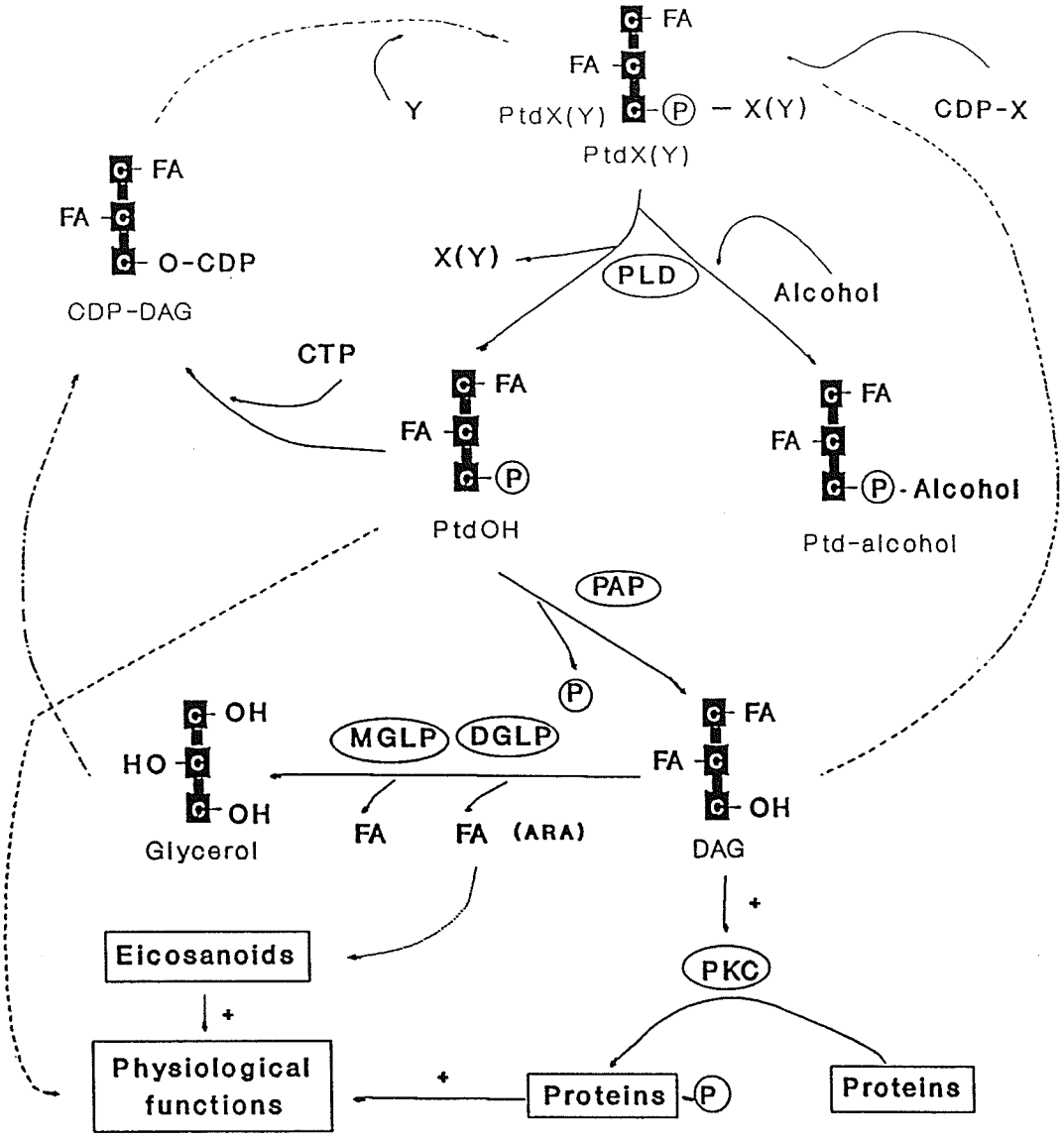
A. Mammalian Phospholipase D and Its Role in Cellular Signaling

Recently, there has been a growing interest in the possible involvement of PLD in cellular signal transduction. This concept arose from the accumulation of numerous reports in which PLD was shown to be activated by a variety of agonists including neurotransmitters, hormones, growth factors and tumor-promoting phorbol esters (Billah and Anthes, 1990). PtdOH, a product of phospholipid hydrolysis via PLD, has been shown to evoke various cellular responses (Exton, 1990; Billah and Anthes, 1990). In addition, PLD activity was found, in many cell types, to be associated with a phosphatidic acid phosphohydrolase that further hydrolyzes PtdOH to DAG (Martin, 1988). DAG can then activate PKC to regulate a host of cellular responses. Therefore, phospholipase D has become one of the main issues in the study of signaling processes.

1. PLD and related metabolic pathways as well as their physiological functions

Membrane phospholipid composition is important for maintaining the structural integrity of the cell and its physiological function. Phospholipids, like other cellular substances, are normally under metabolic dynamics. PLD (EC 3.1.4.4) is involved in such a metabolism. As depicted in figure 1, this enzyme catalyzes the hydrolysis of the ester bond between the phosphatidyl group and the polar head group of a phospholipid to form PtdOH with concomitant release of the non-phosphorylated base (Dennis, 1983). PtdOH has been shown to mediate a variety of physiological responses. For example, PtdOH has been found to inhibit adenylate cyclase activity (Moolenaar, et al., 1986), to generate Ca^{2+} dependent slow action potentials in atria (Knabb, et al., 1984), to stimulate cardiac Na^{+}-Ca^{2+} exchange (Philipson and Nishimoto, 1984), to increase Ca^{2+} binding, and to enhance cardiac contractility (Langer, et al., 1985), and to activate phosphoinositide synthesis (Moritz, A. et al., 1992) and degradation (Kurtz, et al., 1993). In addition, PtdOH can be

Figure 1. PLD-related metabolic pathways



Abbreviations:

ARA: arachidonic acid; CDP: cytidine diphosphate; CTP: cytidine triphosphate; DAG: diacylglycerol; DGLP: diacylglycerol lipase; FA: fatty acid; MGLP: monoglycerol lipase; P: phosphate; PAP: phosphatidic acid phosphohydrolase; PKC: protein kinase C; PLD:phospholipase D; Ptd: phosphatidyl; PtdOH: phosphatidic acid; X: choline or ethanolamine; Y: inositol

metabolized to form CDP-DAG which enters into resynthesis of phospholipid (Bosch, 1974). Alternatively, PtdOH may also be cleaved to DAG by a PAP activity (Martin, 1988). In the presence of Ca^{2+} and PtdSer, DAG can activate PKC which could then initiate a variety of physiological responses (Nishizuka, 1992). Also, DAG could enter 1) the sequential hydrolysis by intracellular lipases (Chuang and Severson, 1990) to yield fatty acids which could enter reacylation of phospholipids; if the fatty acid is arachidonic acid, then it could enter the eicosanoid synthesis pathway; 2) the resynthesis of PtdCho or phosphatidylethanolamine through CDP-choline (ethanolamine) pathways (Hatch, et al., 1988), and 3) the synthesis of triacylglycerol by accepting an acyl-group from acyl-CoA (van Golde and van den Bergh, 1977). In addition to the hydrolytic reaction, PLD may catalyze the transfer of a short-chain alcohol (e.g., ethanol or glycerol) to the phosphatidyl moiety of a phospholipid substrate (transphosphatidylation) to form phosphatidylethanol or phosphatidylglycerol (Kanfer, 1989). Because this capacity to catalyze transphosphatidylation is unique to PLD, it is often used to distinguish PLD from other hydrolytic activities.

2. Biochemical features of PLD

a. Occurrence in different mammalian tissues

PLD was first found in carrot roots and cabbage leaves by Hanahan and Chaikoff in 1947. Although Dils and Hubscher proposed the presence of PLD in the liver as an explanation of their results on base exchange reactions in late 50's (Dils and Hubscher, 1961), there was, for a long period, no conclusive report on the presence of PLD in mammalian tissue. Then, in 1975, Saito and Kanfer first demonstrated the presence of PLD activity in rat brain microsomes (Saito and Kanfer, 1975). Since then, PLD activity has been detected in a variety of mammalian tissues and cell types such as rat and canine brain (Chalifour and Kanfer, 1980; Qian and Drewes, $1990_{a,b}$), rat liver (Bocckino, S. B. et al., $1987_{a,b}$), rat embryonic neurons (Gustavsson and Hansson, 1990), rat aorta (Rapoport, et al., 1991), bovine retina (Mori, et al., 1989), bovine lung (Wang, et al., 1991), human

neutrophils (Balsinde, et al., 1989), human plasma (Davitz, et al., 1989), human platelets (Rubin, et al., 1988), HL-60 cells (Billah, et al., 1989), 3T3 cells (Cook, et al., 1989), ovarian granulosa cells (Liscovitch and Amsterdam, 1989), endothelial cells (Martin, 1988), etc. Regardless of the cell types where the PLD activities resided, most of ones found above were involved in signal transduction processes. Therefore, PLD activation seems to be one of the common signaling pathways in the mammalian tissues.

b. Detection of PLD activities

Most of the PLDs so far reported selectively utilize PtdCho as substrate. For the cell-free *in vitro* assay system, PLD hydrolytic activities are usually tested by measuring, in the assay medium, the amount of [^{14}C]PtdOH or [^{3}H]choline liberated from exogenous labeled PtdCho in the presence of PLD preparations (Bocckino, et al., 1987_a; Kanfer, 1989; Qian, et al., 1990_a). In addition, the unique transphosphatidylation activity of PLD can also be measured by detecting the formation of labeled phosphatidyl-alcohol, in the presence of labeled substrates, e.g., an alcohol and a labeled PtdCho or vice versa (Chalifour and Kanfer, 1980; Chalifa, et al., 1990). The biochemical characteristics of this activity have been shown to parallel those of the hydrolytic activity of PLD (Chalifour and Kanfer, 1980). Therefore, in many cases, PLD activity is tested only via transphosphatidylation. The advantage of the above *in vitro* assay for studying PLD is that 1) the optimal conditions for the maximal enzyme activity can be found (e.g., substrate type, pH, incubation time, best detergents, co-factors, ionic strength, etc.); 2) the complexity of different interactions concurrently taking place in intact systems can be reduced; 3) different PLDs from the different cellular fractions can be tested. However, in such a system, the physical status of the phospholipid substrate is quite different from that in intact systems. In order to overcome this point, several modified assay methods have been created.

The endogenous phospholipid pool can be labeled by radioactive molecules

which are parts of a phospholipid via a *de novo* phospholipid synthesis pathway. For assaying rat brain membrane-bound PLD, Witter and Kanfer (1985) used microsomal preparations containing [^{3}H] labeled PtdCho. This was obtained by *in vivo* intracerebral injection of the animals with either [^{3}H]oleic acid or [methyl-^{3}H]choline chloride. To measure PLD activity, Hurst (1990) employed labeled plasma membrane vesicles which were isolated from rats intraperitoneally administrated with [methyl-^{3}H] choline. Similarly, this labeling process can be achieved *in vitro*. For determining brain neutral PLD activity, synaptosomes containing [^{3}H]labeled endogenous phospholipids were used. Those phospholipids were labeled by preincubation of synaptosomes with a medium containing [^{3}H]fatty acid, coenzyme A, lyso-PtdCho and ATP through long-chain acyl-CoA synthetase and acyl-CoA: lysophospholipid acyltransferase pathways (Möhn, et al., 1992). Membranal [^{14}C]PtdCho was also obtained through phosphatidylethanolamine N-methylation pathway by incubating synaptosomes *in vitro* with S-adenosyl-[^{14}C] methionine and dimethylethanolamine (Hattori and Kanfer, 1985).

The physiological role of PLD is also evaluated in functionally intact systems. In intact cell preparations, PLD activities are tested upon stimulation by various agents. Usually, cellular membranal phospholipids undergo a radio-prelabeling procedure; e.g. membranal PtdCho could be labeled by incorporation of either [^{3}H]myristic acid (Cabot, et al., 1988), [^{32}P]lyso-PtdCho (Pai, et al., 1988), [^{3}H]glycerol (Gustavsson, et al., 1987), or [^{3}H]choline (Martinson, et al., 1989; Cook, et al., 1989) into PtdCho through *de novo* synthesis or reacylation pathways. In this system, increase of radiolabeled products in the cell medium due to PLD-dependent activities are taken as an indication of phospholipase activity. Experiments on PLD activation by different agonists as well as on G protein-PLD coupling mechanisms largely benefited from the intact cell preparations. However, the disadvantage is that the assay, in most cases, is not specific for a certain type of phospholipase. Some products measured can come from different phospholipid pathways. For example, for PtdOH and choline, the former can come either from PLD or from the phospholipase C/DAG kinase pathway; the latter can come either

from PLD or from the PLC / phosphocholine phosphatase pathway. Nonetheless, this flaw may be overcome by testing for transphosphatidylation activity which only measures a unique and stable product, a phosphatidylalcohol. In fact, employing [^{32}P] labeled PtdCho as substrate, Pai et al. (1988) have elegantly and conclusively demonstrated in cultured cells that [^{32}P]phosphatidylethanol is formed exclusively by PLD. The only drawback is that in some cases the measurement was established only on whole cell extracts. Therefore, it was impossible to clarify the loci of the reaction or where the PLD being investigated, resided. For such purposes, the *in vitro* assay is considered to be an advantageous method.

c. Subcellular distribution and purification of PLD

Most of the plant PLDs are soluble. Purified or partially purified PLD has been obtained from cabbage, cottonseeds and peanut seeds with a molecular weight of 22-200 kD (Heller, 1978). In mammalian tissue, most of the PLD activities are membrane-bound enzymes. Rat brain PLD was reported to be only present in the microsomal fraction but not in cytosol (Taki and Kanfer, 1979). In NIH 3T3 cells and bovine pulmonary artery endothelial cells, PLDs were also found to be localized in membrane fractions (Martin, 1988; Kiss, et al., 1990,). On the other hand, Billah 's group characterized a cytosolic PLD that was present in various bovine tissues (Wang, et al, 1991). This cytosolic PLD differed from membrane-bound PLD in chromatographic protein profiles, substrate specificities, kinetic parameters, and divalent cation dependencies. In a study in neutrophils, two forms of PLD were found (Balsinde, et al., 1989). One type was present in cytosol, having neutral pH optimum and requiring Ca^{2+}. The other, found in the azurophilic granules, had an acidic pH optimum and was insensitive to Ca^{2+} (Balsinde, et al., 1989).

Although a great deal of effort has been made, so far the only PLD purified to homogeneity in mammalian tissues is the soluble enzyme found in blood plasma which is specific for PtdIns-glycan (Davitz, et al., 1987). The success of this purification is due in part to the fact that this enzyme is in soluble form. However,

the majority of mammalian PLDs are in membrane-bound form. Thus, an effective detergent is essential for solubilization of PLD from membrane preparations without disturbing the enzyme activity in the first step of purification. Miranol H2M and Triton X-100 have been used to purify brain microsomal PLD (Taki and Kanfer, 1979; Kobayashi and Kanfer, 1991), but the enzyme could only be partially purified. Homogeneity of this enzyme will be achieved when a better detergent and optimal separation procedure are found. The molecular weight of plasma soluble PLD is 110 kD (Davits, et al., 1989), while partially purified rat brain microsomal PLD has a molecular weight of 200 kD (Taki and Kanfer, 1979). In contrast, in bovine lung cytosol preparation, two peaks (30 and 80 kD) of the chromatographic profile exhibited PLD activity (Wang, et al., 1991). The above differences may be due to the fact that the molecular weights of all PLDs reported were based on different separation procedures. Although the primary structure of PtdIns-glycan specific PLD was determined (Scallon, et al., 1991), until now no amino acid sequence is available for any mammalian PtdCho- specific PLD. This issue remains to be elucidated.

d. Substrate specificity, divalent cation dependence, and multiple forms of PLD

Different substrate specificity has been found in various PLD activities present in different mammalian tissues. Partially purified rat brain microsomal PLD favors PtdCho and phosphatidylethanolamine as substrates and is active in the absence of Ca^{2+}, but it responds to the addition of Ca^{2+} and is inhibited by Mg^{2+} (Taki and Kanfer, 1979). In contrast with the acidic PLD characterized by Kanfer (1989), a neutral PLD activity from rat brain synaptic membranes hydrolyzes only PtdCho, is inhibited by Ca^{2+} and is activated by Mg^{2+} (Möhn, et al., 1992). However, in the heart, a membrane-bound PLD activity was found which selectively hydrolyzes 1,2-diacyl-sn-glycero-3-phospho(N-acyl)ethanolamine and N-acylethanolamine lysophospholipids (Schmid, et al., 1983). Those molecules are abnormal phospholipids and accumulate in infarcted areas of the heart. This PLD activity is inhibited by Zn^{2+} (Schmid, et al., 1983). Neutrophils are reported to contain a

PtdIns-hydrolyzing, soluble PLD which requires Ca^{2+} for its activity (Balsinde, et al, 1989). In NIH 3T3 cells, phosphatidylethanolamine-preferring PLD activity has been described (Kiss, et al., 1990). The plasma PLD is specific for the glycosyl-phosphatidylinositol, an anchor for certain cell surface proteins, and is EGTA sensitive (Davitz, et al., 1987). Liver plasma membrane PLD is sensitive to Ca^{2+} (Bocckino, et al., 1987_a), while PLD present in pulmonary artery smooth muscle membrane shows no Ca^{2+} requirement for its optimal activity (Martin, 1988). Based on these studies, it is likely that these differences in substrate specificity and Ca^{2+}-dependence reflect the existence of different isoforms of PLD. However, it remains to be elucidated whether one or more enzymes are present in the same cell and are involved in transmembrane signaling, or perhaps have some other role such as phospholipid remodeling.

3. Modulation of PLD activity

a. Extracellular signals

An important step recognizing PLD's role in signal transduction came from a number of independent studies in which PLD of intact cultured cells was found to be activated by different hormones. Bocckino et al. (1987) reported that vasopressin as well as other Ca^{2+} -mobilizing hormones stimulated the formation of PtdOH in intact hepatocytes, and that PtdOH preceded DAG accumulation. In parallel, Pai et al. (1988) demonstrated that PLD activity in HL-60 cells was activated by a chemotactic peptide, N-formyl-Met-Leu-Phe (fMLP), by measuring both [^{32}P]PtdOH

and [^{32}P]phosphatidylethanol in [^{32}P]lyso-PtdCho prelabeled HL-60 cells. Liscovitch et al.(1989) have shown that gonadotropin-releasing hormone activated PLD in ovarian granulosa cells by using a gonadotropin-releasing hormone receptor agonist [D-Ala^{6},des-Gly^{10}]GnRH N-ethylamide (GnRH-A). During the past five years, numerous reports have emerged on the activation of PLD by different agonists. Listed in the next two pages are some reports in which the activation of PLD by agonists was demonstrated, either by an increase in PtdOH and choline or by formation of phosphatidylethanol. From this, it becomes obvious that agonist-activated PLD has been observed in a wide range of cells and mammalian tissues. This phenomenon indicates that the position of PLD in signal transduction is as important as that of phosphoinositide PLC.

Agonists Induced PLD Activation in Different Cells/Tissues

Agonist	Cell or Tissue	References
Acetylcholine	Canine brain synaptosomes	Qian, et al.,1990_a
Angiotensin II	Rat hepatocytes	Bocckino, et al.,1987_a
	Bovine adrenal glomerulosa cells	Bollag, et al.,1990
	Rat renal mesangial cells	Pfeilschifter, et al.,1992
ATP	Rat hepatocytes	Bocckino, et al.,1987_a
	Bovine endothelial cells	Martin, et al.,1989
	NIH 3T3 mouse fibroblasts	Kiss, et al.,1990
Bradykinin	Bovine endothelial cells	Martin, et al.,1989
Carbachol	Chick heart	Lindmar, et al.,1988
	Canine brain synaptosomes	Qian, et al., 1990_a
ComplementC5a	Human neutrophils	Mullmann, et al.,1990
Endothelin-1	Rat aorta	Liu, et al.,1992
EGF	Rat hepatocytes	Bocckino, et al.,1987_a
Epinephrine	Rat hepatocytes	Bocckino, et al.,1987_b

Continue last page

Agonist	Cell type or Tissues	References
f-MLP	HL-60 granulocytes	Pai, et al.,1988
	Human neutrophils	Reinhold, et al.,1990 Billah, et al.,1989
GnRH-A	Rat ovarian granulosa cells	Liscovitch, et al., 1989
GM-CSF	Human neutrophils	Bourgoin, et al., 1990
Immunoglobulin E	Rat mast cells	Gruchalla, et al.,1990
Isoproterenol	Guinea-pig heart	Lindmar, et al., 1986_b
Norepinephrine	Rat aorta	Rapoport, et al.,1991
PAF	Rat mesangial cells	Kester, et al., 1990
	Human neutrophils	Reinhold, et al.,1990
PDGF	NIH 3T3 fibroblasts	Ben-Av, et al.,1989
Substance P	Parotid gland	Rollandy, et al.,1989
Thrombin	Human platelets	Rubin, 1988
	Human erythroleukemia cells	Helenda, et al.,1990
Vasopressin	Rat hepatocytes	Boccokino, et al., 1987_a
	REF52 rat embryo fibroblasts	Cabot, et al.,1988

Abbreviations: EGF, epidermal growth factor; fMLP, formyl-Met-Leu-Phe; GnRH-A, [D-Ala^6,des-Gly^{10}]gonadotropin-releasing hormone-N-ethylamide; GM-CSF, granulocyte-macrophage colony-stimulating factor; PAF,platelet activating factor; PDGF, platelet-derived growth factor.

b. GTP-binding regulatory protein

Activation of PLD may occur through more than one mechanism. One of the possible mechanisms may involve GTP-binding regulatory proteins. G proteins are composed of three distinct subunits, a guanine nucleotide binding α-subunit, a ß- and a γ-subunit. Upon activation by GDP-/GTP-exchange, the α-GTP-complex dissociates from the ßγ complex and then interacts with effector proteins. Upon hydrolysis of the GTP, inactivation occurs, and the heterotrimer is again formed. In many cases, GTP-gamma-S, a non-hydrolyzable analogue of GTP is used to stimulate G-protein instead of GTP, thus generating a long lasting signal to the effector proteins. G proteins have been shown to be implicated in the activation of PLC and PLA_2 by extracellular agonists (Birnbaumer, et al., 1990). Recent observations suggest that those proteins could also be the link coupling activated cell surface receptors and PLD. In fact, stimulation of PLD activity by GTP-gamma-S was observed when PtdEt and Ptd-glycerol were monitored as the products of PLD transphosphatidylation reaction in HL-60 cell lysates (Tettenborn, et al., 1988). Similarly, in permeabilized bovine pulmonary artery endothelial cells, GTP-gamma-S and ATP, a P_2 purinergic agonist, synergistically activated PLD-dependent hydrolysis of the prelabeled membranal PtdCho pool (Martin, et al., 1989). This suggests that activation of PLD by purinergic agonists and G-proteins may share the same pathway. Furthermore, pertussis toxin, which blocks certain G proteins by an ADP-ribosylation process, inhibited PLD activation in fMLP-stimulated granulocytes (Pai et al., 1988). In isolated rat and chick atria, incubation of intact tissue, in the presence of ethanol, with AlF^{-4}, an activator of G proteins, evoked the rise of

phosphatidylethanol, which indicated activation of PLD (Lindmar and L o ffelholz, , 1992). In membrane preparations, G protein coupling experiments for the activation of PLD were also successfully conducted. In hepatocyte membranes, GTP-gamma-S was shown to stimulate PLD activity synergistically with certain receptor agonists, such as ATP, vasopressin and angiotensin II (Bocckino, et al., 1987_a). In canine brain synaptosome preparations, GTP-gamma-S activated PLD in a dose dependent manner. Oppositely, GDP-beta-S inhibited such an activation, which confirmed the involvement of G proteins (Qian, et al., 1989_b). Finally, in isolated rabbit platelet membranes, GTP-gamma-S and phorbol 12-myristate 13-acetate (PMA) synergistically increased PLD activity (van der Meulen and Haslam, 1990).

In certain cases, the activation of PLD by GTP-gamma-S in cell membrane preparations seems to require cytosolic components. In HL-60 granulocytes, PLD was found to be activated by GTP-gamma-S only in the presence of both membrane and cytosolic fractions. Separation of these two fractions resulted in the complete loss of GTP-gamma-S stimulated PLD activity (Anthes, et al., 1991). It was also found that activation of PLD in the plasma membrane of human neutrophils by GTP-gamma-S, GppNHp and GTP required the presence of the cytosol fraction (Olson, et al., 1991). Therefore, this PLD pathway seems to be comprised of components located in two subcellular compartments (Olson, et al., 1991). In contrast, in NIH 3T3 cell membrane preparation, GTP-gamma-S stimulated PLD activity in a dose-dependent manner in the absence of cytosolic fraction. The pattern of the activation in control cells showed no difference from that of *ras*-

transformed cells, which meant that the participating G protein was not G_p, previously proposed as the *ras* gene product (Quilliam, et al., 1990).

c. Protein kinase C

Another mechanism for PLD activation may involve PKC, a ubiquitous protein kinase which is able to phosphorylate a host of intracellular proteins. This possibility arose from the findings that phorbol esters, powerful PKC activators, are capable of stimulating PLD activity and that their potencies and structure-activity relationships for PLD are similar to those for PKC activation (Billah and Anthes, 1990). Phorbol esters have been shown to stimulate PLD activity in a variety of tissues and cell lines, e.g., HL-60 cells (Billah, et al., 1989), rat pancreatic islet cells (Dunlop and Mets, 1989), neutrophils (Reinhold, et al., 1990; Mullmann, et al., 1990), REF52 cells (Cabot, et al., 1989), ovarian granulosa cells (Liscovitch and Amsterdam, 1989), Swiss 3T3 cells (Cook and Wakelam, 1989), NIH 3T3 cells (Ben-Av and Liscovitch, 1989), rat aortic smooth muscle cells (Huang and Cabot, 1990), MDCK cells (Huang and Cabot, 1990), rat embryonic neurons (Gustavasson and Hansson, 1990), canine synaptosomes (Qian and Drewes, 1989), and rat and chick atria (Lindmar and L ö ffelholz, 1992). Therefore, this seems to be a common stimulatory mechanism.

Apart from the use of active phorbol esters to stimulate PLD activity, the involvement of PKC in the activation of PLD is also supported by some additional findings in different cell lines and tissues. Firstly, some phorbol esters which are

inactive toward PKC, have no effect on PLD activity. Secondly, down-regulation of PKC by prolonged phorbol ester pre-treatment diminishes phorbol ester-induced PLD activation, e.g., in atria (Lindmar and L o ffelholz, 1992) and in endothelial cells (Martin, et al., 1990). Moreover, PKC inhibitors such as H-7 and K252 block phorbolester-induced PLD activation completely in some cells (Liscovitch, 1989; Martinson, et al., 1989), but only partially or ineffectively in others (Billah, et al., 1989; van der Meulen and Haslam, 1990; Cao, et al., 1990). Therefore, both PKC-dependent and -independent mechanisms for the activation of PLD by phorbol ester have been proposed (Billah and Anthes, 1990). Furthermore, the interaction between PKC and PLD may not involve ATP-dependent phosphorylation. This view is suggested by a direct effect of PKC on PLD, which was found in a cell-free system in the absence of ATP and in the presence of a high concentration of apyrase which eliminates any phosphorylation reactions (Conricode, et al., 1992). Therefore, PKC may directly activate PLD.

DAG and intracellular Ca^{2+} are the most important activators of PKC. Since DAG generation and Ca^{2+} elevation could be a result of the hydrolysis of PtdIns(4,5)P_2, the activation of PLD by PKC might be a step secondary to the activation of receptor-coupled phosphoinositide specific PLC. This is supported by the observation that many receptors which were found to stimulate PLD, had earlier been found to be coupled to phosphoinositide-PLC. However, dissociation of activation between phosphoinositide-PLC and PLD upon agonist stimulation has been found in certain cell types (Griendling, et al., 1986, Wright, et al., 1988, Sandmann, et al., 1991). Thus, PtdIns(4,5)P_2 hydrolysis may not be necessary but

only modulatory of PLD activity. Activation of PLD results in formation of PtdOH. In many cell types and tissues, PLD was found to couple with a PAP activity which further hydrolyzes PtdOH to DAG. DAG could stimulate PKC, resulting in further activation of PLD (Billah and Anthes, 1990). Therefore, such a circuit constitutes a positive feedback which may explain the long-lasting DAG signal upon PLD activation in certain cells (Liscovitch, 1992).

d. Other modulatory factors

Since the first mammalian PLD was reported (Saito and Kanfer, 1975), the basal activity of membrane-bound PLD toward exogenous substrate was not detectable in the absence of fatty acids or detergents. Chalifour and Kanfer (1982) first reported that sodium salts of unsaturated fatty acids effectively evoked PLD activity in cell free systems. Then, sodium oleate was shown to be the most effective stimulator of several membrane-bound PLD activities in cell free systems (Kobayashi and Kanfer, 1987; Gustavsson and Alling, 1987; Chalifa, et al., 1990; Siddiqui and Exton, 1992). Even in *ex vivo* conditions (isolated perfused heart preparations), PLD activity can be enhanced by sodium oleate (Lindmar, et al., 1988). Detergents are less effective agents in eliciting PLD activity. However, the type of effective detergent seems to be dependent on the source of the enzyme. Sodium taurodeoxycholate is the best activator for brain microsomal PLD (Chalifour and Kanfer, 1982) but is an inhibitor for cardiac N-acyl- ethanolamine-PLD (Schmid, et al., 1983), while Triton X-100 is most effective on PLDs from permeabilized pulmonary artery smooth muscle cells and from canine brain

synaptosomes (Martin, 1988; Qian, et al., 1991).

So far no specific PLD inhibitors are available. Wortmannin, a fungal metabolite, has been reported to inhibit PLD at a point between the activated receptor and the enzyme (Bonser, et al., 1991). Conversely, a recent study indicates that wortmannin enhances carbachol-induced PLD activity (Kanoh, et al., 1992). Apart form wortmannin, aminoglycoside antibiotics seem to inhibit PLD activity *in vitro*, but these drugs also inhibit phosphoinositide-PLC (Liscovitch, et al., 1991). In another report, PLD activity was depressed by lipocortin, a phospholipase A_2 modulator which, in the naturally occurring non-phosphorylated form, is known to inhibit phospholipase A_2 (Kobayashi, et al., 1988). Due to the fact that dexamethasone, a glucocorticoid, is able to induce lipocortin generation, *in vivo* treatment with dexamethasone also resulted in inhibition of PLD activity (Kobayashi, et al., 1988).

Similar to plant PLDs, some of the mammalian PLDs are inhibited by protein thiol modifiers such as p-CMPS, NEM, MMTS and DTNB, while DTT prevents the inhibition (Chalifour, et al., 1980; Kobayashi and Kanfer, 1987). These results suggest that free sulfhydryl group(s) may be essential for PLD activities. Reversible changes in cellular protein thiol-disulfide status are controlled by the redox status of glutathione. Therefore, in some pathological conditions, an abnormal redox status of glutathione may modify PLD activity.

e. Possible physiological role of PLD and its derivatives

It seems unlikely that the function of PLD in mammalian tissue is only to remove unwanted phospholipid molecules. As already mentioned, there is strong evidence for PLD activation by a variety of extracellular agonists. Therefore, the consequences of PLD-catalyzed phospholipid hydrolysis may be of particular importance in the biological systems.

Activation of PLD results in an accumulation of PtdOH in the plasma membrane. This negatively charged phospholipid has been shown to exhibit Ca^{2+} ionophore properties (Salmon and Honeyman, 1980; Serhan, et al., 1981). Although this effect has been questioned (Moolenaar, et al., 1986; Murayama and Ui, 1987; Exton, et al., 1992), in certain tissues the effect seems to hold true. Treatment with PtdOH increased the cardiac SL Na^{+}-Ca^{2+} exchange activity (Philipson and Nishimoto, 1984), Ca^{2+} binding and force of contraction of the heart (Langer, et al., 1985). The observation that exogenous addition of PtdOH generated Ca^{2+}-dependent slow action potentials in depolarized rat atrium supports a role for PtdOH in mediating an increase of Ca^{2+} influx into the cardiac cells (Knabb, et al., 1984).

PtdOH has also been shown to inhibit adenylate cyclase by interacting with a pertussis toxin sensitive G protein (Houslay, et al., 1986; Murayama and Ui, 1987). Moreover, PtdOH seems to have a growth factor-like action which stimulates the rise of cellular pH, induces the c-*fos* and c-*myc* proto-oncogenes and stimulates DNA synthesis in A431 carcinoma cells (Moolenaar, et al., 1986). Furthermore, a PtdOH-dependent protein phosphorylation has been observed, which does not

require Ca^{2+} and has a markedly different phosphorylated protein profile than those obtained from phosphatidylserine plus 1,2-diolein. This implies that a protein kinase other than PKC may be involved (Bocckino, et al., 1991). Another response to PtdOH is the activation of phosphoinositide-PLC (Jackowski and Rock, 1989). Interestingly, it has been also observed that PtdOH is a specific activator of phosphatidylinositol-4-phosphate kinase (Moritz, et al., 1992) by which the phosphoinositide cycle is accelerated. Furthermore, a recent study showed that PtdOH stimulates inositol 1,4,5-trisphosphate production in adult cardiac myocytes in the absence of extracellular Ca^{2+} (Kurz, et al., 1993). Taken together, a crosstalk between the PLD and phosphoinositide pathways presumably exists (Liscovitch, 1992; Lamers, et al., 1993).

PtdOH can be further hydrolyzed to DAG by a PAP activity. In some cell types, the agonist-induced formation of DAG is biphasic: it contains an early rapid and transient peak followed by a late phase which is slow in onset but sustained over many minutes. The former peak is generated directly from hydrolysis of PtdIns(4,5)P_2 and often parallels the increase in Ins(1,4,5)P_3. The latter is generated from hydrolysis of PtdCho via the PLD-PAP pathway (Exton, 1990; Billah and Anthes, 1990). The source of DAG was ascertained by extensive analysis of the DAG molecular species (Pessin and Raben, 1989; Augert, et al., 1989). PKC activation has been shown to inhibit the phosphoinositide pool signaling pathway (Orellana, et al., 1987; Meij, et al., 1991). In contrast to this, PKC activation may further stimulate PLD. Moreover, the cardiac membrane PtdCho pool is 12 fold greater than that of the phosphoinositide (Meij, et al., 1989). Prolonged hydrolysis

of PtdCho does not need to be compensated by a rapid recycling process like that for phosphoinositide (Meij and Panagia, 1991). Therefore, this might also explain the sustained DAG elevation from agonist stimulation of PLD.

One of the major functional roles of DAG is to activate PKC which, in turn, phosphorylates a series of cellular proteins. PKC activation results in a host of biological responses including metabolism, secretion, contraction, proliferation and differentiation (Nishizuka, 1988). Due to the fact that DAG derived from PtdCho via PLD/PAP lasts a relatively long period upon agonist stimulation (Billah and Anthes, 1990), it may stimulate PKC to execute long-term responses such as cell proliferation and differentiation, alteration of receptor functions (α_1 adrenergic receptors) and control of ion channel activity (Nishizuka, 1986). So far ten subtypes of PKC have been identified in mammalian tissues (Nishizuka, 1992). Although these members of the PKC family all depend on PtdSer, different subspecies of DAG may activate PKC subtypes to various degrees (Nishizuka, 1992). Therefore, it is conceivable that DAG which originates from PtdCho hydrolysis via PLD-PAP may produce different cellular effects than that derived from PtdIns(4,5)P_2 hydrolysis via phosphoinositide-PLC (Lamers, et al., 1993). Apart from activating PKC, DAG is also able to stimulate translocation of CTP: phosphocholine cytidylyltransferase from the cytosol to membrane (Pelech and Vance, 1989). This translocation results in activation of the enzyme. Thereby, DAG is then taken as a substrate entering PC resynthesis (Pelech and Vance, 1989). Such a process was proposed as the "termination" of the long-lasting DAG signal (Billah and Anthens, 1990).

Another product of hydrolysis of PtdCho via PLD activation is free choline. Acetylcholine synthesis requires free choline to be available in the vicinity of the presynaptic cleft. Hattori and Kanfer (1985) successfully demonstrated that newly released choline from PtdCho via PLD can enter acetylcholine synthesis. When incubating ([^{14}C]choline)-PtdCho with PLD-enriched brain synaptosome preparations in the presence of oleate and acetyl-CoA, they observed that [^{14}C]choline was incorporated into the acetylcholine fraction, and that this incorporation was barely detectable in the absence of oleate (Hattori and Kanfer, 1985). Phosphatidylethanol, the unique product of transphosphatidylation via PLD, is an abnormal metabolic substance. Transphosphatidylation does not occur under normal physiological conditions. Although phosphatidylethanol was found in tissues from alcohol-fed rats (Alling, et al., 1984), its physiological function is questionable. However, recent findings have shed light on this issue. It was found that phosphatidylethanol could replace PtdSer to stimulate PKCs of types I, II and III at high Ca^{2+} concentration (Asaoka, et al., 1989). At physiological Ca^{2+} concentration phosphatidylethanol was able to activate type I-PKC which is exclusively expressed in central nerval tissues (Asaoka, et al., 1989). On the other hand, ethanol has been reported to stimulate PLD activity (Kiss and Anderson, 1989). Therefore, in alcoholic subjects, it is possible that transphosphatidylation may participate in the signal transduction processes of the neuronal tissue, via changes in protein phosphorylation.

B. Cardiac Ischemia-Reperfusion Injury

Myocardial ischemia is present whenever the coronary flow is inadequate to provide enough oxygen to meet the energy demand of the tissue and to maintain heart function (Jennings and Reimer, 1981). Manifestations of altered function include cessation of contraction, alterations in membrane potential and a variety of metabolic changes, all of which develop when oxygen supply becomes limiting (Jennings and Reimer, 1981). As the period of ischemia is extended and ischemic injury becomes irreversible, ultrastructural changes such as mitochondrial swelling with amorphous matrix densities and discontinuities in the cell membrane occur (Jennings and Reimer, 1981). The development of membrane damage is related closely to the onset of irreversible injury *in vivo* and *in vitro*, suggesting that this may be the proximate cause of cell death during ischemia (Jennings and Reimer, 1981; Chien, et al., 1981). Both duration and severity of myocardial ischemia are important determinants of the transition from reversible to irreversible injury and consequent cell death; however, the duration of ischemia required to produce changes is considerable longer with *in vitro* ischemia models (Jenning, 1985).

Reperfusion refers to the recovery of blood perfusion to previously ischemic tissue. Despite the prospect for reperfusion procedure as an effective means of myocardial salvage, the evidence obtained under various experimental conditions has revealed a series undesirable effects due to reperfusion of the ischemic myocardium (Hearse and Bolli, 1992). For a short period of ischemia, both myocytes and the microvascular system are still in the reversible phase of injury. Therefore, the above

mentioned undesirable effects only appear as reperfusion arrhythmias and stunning. Stunning is characterized by a transient mechanical abnormality followed by a gradually slow recovery upon reperfusion (Braunwald and Kloner, 1982). If the period of ischemia is prolonged, the myocytes are irreversibly injured. Thus reperfusion will result in arrhythmias, poor force generation, enzyme leakage, accelerated cell necrosis, and microvascular damage. These phenomena are termed "reperfusion injury".

Up to now, many mechanisms have been proposed to interpret the cellular abnormalities in ischemia-reperfusion injury. Following are the major possible mechanisms underlying myocardial dysfunction in ischemia-reperfusion injury.

1. Abnormal Ca^{2+} movements

While ischemic-induced changes in myocardial metabolism and ultrastructure are well defined, comparatively less information is available on cellular Ca^{2+} movements. SL Na^{+}-Ca^{2+} exchange (Bersohn, et al., 1991), Na^{+},K^{+}-ATPase (Dhalla, et al., 1988; Bersohn, et al., 1991), Ca^{2+} pump (Dhalla, et al., 1988) and superficial Ca^{2+} stores (Nayler, et al., 1971) were found to be reduced during ischemia. A moderate degree of ischemia had a deleterious effect on the Ca^{2+} accumulating activities of both cardiac SR and mitochondria (Nayler, et al., 1971; Hess, et al., 1981; Dhalla, et al., 1988), and this may account for the observed rise in cytosolic free Ca^{2+} (Steenbergen, et al., 1987). Reperfusion of the myocardium irreversibly injured by ischemia is associated with a paradoxical extension of the

myocardial damage that is related to the inability of the ischemic tissue to maintain normal Ca^{2+} homeostasis during reperfusion (Jennings and Reimer, 1991). Such a dysfunction could occur when mechanisms for the entry as well as the removal of Ca^{2+} from the myocardial cell are defective (Tani, 1990). In this regard, SL Ca^{2+} pump, Na^{+} -pump and Na^{+}-Ca^{2+} exchange activities were found to be depressed (Dhalla, et al., 1988), while the Ca^{2+} channel seems to be inactive (Tani, 1990). Recent findings indicated that the combined action of Na^{+}-H^{+} and Na^{+}-Ca^{2+} exchangers is an important determinant of the massive Ca^{2+} influx upon reperfusion (Karmazyn, 1990).

2. Alterations of adrenergic signaling mechanisms

In ischemic-reperfused heart, there is an alteration of the adrenergic signaling mechanisms, which results in loss of the adrenergic control of the heart, genesis of malignant arrhythmias, the spreading of infarcted zone, Ca^{2+} overload and cell necrosis.

The elevated sympathetic tone caused by the ischemic stress increases the serum level of catecholamine released from the adrenal medulla. However, the response of the ischemic cardiac tissue to the adrenergic stimulation depends on the local concentration of norepinephrine. There is an increase of norepinephrine release from nerve ending in the first few minutes (< 10 minutes) of ischemia due to increased cardiac efferent sympathetic nerve activity (Schömig, et al., 1991). During that period, an accumulation of norepinephrine is prevented by active

neuronal uptake$_1$ which pumps back the catecholamine into the neuron terminals (Schömig, et al., 1988). Apart from that, locally accumulated metabolites such as extracellular K^+, H^+ and adenosine also contribute to an inhibition of the normal exocytotic release and thus prevent the accumulation of norepinephrine (Miyazaki and Zipes, 1990). In addition, ATP depletion will finally lead to a cessation of the energy-dependent norepinephrine exocytosis. However, after 15-40 minutes of ischemia, approximately one third of the cardiac norepinephrine content is released, thereby leading to an excessive accumulation of norepinephrine in the extracellular space of the ischemic myocardium (Schömig, et al., 1984). The catecholamine concentration can be 100-1000 times higher than that of the normal plasma (Schömig, et al., 1984). The mechanism of this release is not exocytosis. and has been proposed to occur through a two-step process (Schömig, et al., 1984, 1988). In the first step, norepinephrine escapes from the storage vesicles, resulting in increased axoplasmic norepinephrine concentrations. In normal conditions, recapture of norepinephrine into the neuronal storage vesicles is dependent on proton and norepinephrine exchange which is driven by a proton gradient resulting from H^+-ATPase activity localized in the vesicular membrane (Beers, et al., 1982). Neuronal energy depletion leads to loss of the proton gradient, thereby causing norepinephrine accumulation in the axoplasm. In the second step, norepinephrine is transported across the axolemma membrane into the synaptic cleft through the uptake$_1$ carrier operating in reverse of its normal transport direction. Apart from axoplasmic norepinephrine accumulation, this transport is Na_i dependent (Sammet, et al., 1979). Therefore in ischemic condition, cellular acidosis and failure of Na^+-K^+ ATPase will accelerate such a transport.

Excessive catecholamine can induce a depletion of energy stores and the development of myocardial necrosis in the non-ischemic heart (Opie, et al., 1979). Waldenstrom et al.(1978) observed that high dose of isoproterenol induced myocardial necrosis which is indicated by the histological evidence of hypercontracted myofibril, myofibrillar lysis, swollen mitochondria and derangement of the cell structure. High dose of norepinephrine also decreases both the resting membrane potential and action potential amplitude and generates a unidirectional block (Gilmour and Zipes, et al., 1980). These data suggest that the high local catecholamine concentration may play a role in the ischemic injury.

In physiological conditions, the ß-adrenergic receptor system takes main task of signal transduction from extracellular catecholamines to cardiomyocyte. Long term exposure of ß-receptors to adrenergic agonists results in desensitization of ß-receptors (Stiles, et al., 1984). However, in the ischemic condition, the increased presynaptic release of endogenous catecholamine does not promote the expected desensitization of ß-receptor (Strasser, et al., 1988). In contrast, acute ischemia leads to a hypersensitization of the ß-adrenergic system (Strasser and Marquetant, 1991). It has been suggested that such a hypersensitization of the ß-adrenergic system may be responsible for the malignant arrhythmias and spread of the infarcted zone in acute ischemia injury (Strasser and Marquetant, 1991). In the early stage of myocardial ischemia (15 minutes), the number of ß-receptors in the plasma membrane increases (Maisel, et al., 1985; 1990; Strasser, et al., 1990). ß-Receptor density further increases in prolonged ischemia (one hour or longer) (Mukherjee, et al., 1982; Maisel, et al., 1987; Strasser, 1990). Even after 15 min of reperfusion

following one hour of coronary occlusion, ß-receptor density in the plasma membrane remains elevated (Mukherjee, et al., 1982). The increase of ß-receptors in the plasma membrane due to acute ischemia is accompanied by an intact coupling with adenylate cyclase. Interestingly, coincident with an increase in ß-receptor density, the adenylate cyclase activity is also enhanced upon the onset of ischemia, and this is found to be independent of ß-receptors or G proteins (Strasser, et al., 1990). Unlike the ß-receptors, the increase in adenylate cyclase activity is a transient phenomenon (within 5-15 minutes upon onset of ischemia), and is probably due to covalent modification of the enzyme itself. This is suggested by the fact that partially purified adenylate cyclase retains ischemia-induced sensitization (Strasser, et al., 1990). Recent findings indicate that the ischemia-induced translocation of PKC from the cytosol to the membrane fraction might be responsible for the phosphorylation of adenylate cyclase (Strasser, et al., 1992). Distinctly, externalization of the ß-receptors from an intracellular pool (in which ß-receptors are functionally uncoupled to adenylate cyclase) to sarcolemmal membranes was found to be responsible for the increase of functionally coupled surface ß-receptors (Maisel, et al., 1985).

Under physiological conditions, α_1-adrenergic stimulation of myocytes elicits an inotropic effect which is generally less pronounced than that in response to ß-receptor activation (Nawzath, 1989). However, in both early ischemia and reperfusion there is a hyperresponsiveness to α_1-adrenergic stimulation (Butterfield, et al., 1990). Culling et al. (1987) demonstrated that methoxamine, a α_1-agonist, could induce ventricular tachycardia and fibrillation during myocardial ischemia and

reperfusion in isolated perfused guinea pig hearts pre-depleted of catecholamine(s). This effect was blocked by phentolamine. Similarly, it was observed that methoxamine elicited a rapid increase in the idioventricular rate during reperfusion of the ischemic myocardium (Sheridan, et al., 1980). These data suggest that α_1-adrenergic stimulation in ischemia and reperfusion is related to myocardial arrhythmogenesis. Concurrently with such a proposal, α blockade showed a strong antiarrhythmic effect on ischemic reperfused myocardium (Benfey, et al., 1984; Penny, et al., 1985; Culling, et al., 1987). The increased α_1 adrenergic receptor numbers may contribute to the enhanced α-adrenergic responsiveness during ischemia and the early reperfusion. Corr et al. (1981) demonstrated that in cat myocardium, within 30 minutes of ischemia *in vivo* α_1-receptor increased two-fold, persisted during early reperfusion, and returned to base-line after 5 minutes of reperfusion. A similar increase in α_1 receptor density in ischemia has been observed in cat (Dillon, et al., 1988), dog (Mukherjee, et al., 1980), guinea pig (Maisel, et al., 1987), and rat (Butterfield, et al., 1990) hearts. Maisel et al. (1987) observed that ischemia results in a marked increase in sarcolemmal α_1-adrenoceptors, without changes in the intracellular light vesicle fraction. These findings indicate that the increase in α_1-adrenoceptors in sarcolemma in response to ischemia is not mediated by the same mechanism as that of ß-adrenergic receptors in which the uncoupled receptor is translocated from an intracellular pool to the sarcolemmal membrane. The mechanism for increase in α_1 adrenoceptors is presently unclear. One proposal is that it may be attributed to membrane fluidity changes (Heathers, et al., 1987). This change can be caused by accumulation of long chain fatty acid derivatives in the sarcolemma, with subsequent unmasking of α

adrenoceptors already present in the sarcolemmal membranes (Heathers, et al., 1987). At present, only little and often superficial information is available about the status of the phosphoinositide pathway in the ischemic-reperfused heart. In isolated dog cardiomyocytes exposed to hypoxia as an *in vitro* model of myocardial ischemia, the increase in α_1 density led to an enhanced production of Ins(1,4,5)P_3 (Corr, et al., 1989). In another study, the suggestion that reperfusion stimulates the myocardial phosphoinositide PLC activity was inferred from assessing the phosphoinositide content of the whole heart homogenate (Otani, et al., 1988). Thus, the authors could not exclude the possibility of interferences by vascular smooth muscle cells or endothelial cells (Otani, et al., 1988). Similarly, the results of another study (Schwertz, et al., 1992), where the ischemia-induced changes of PLC activity were assessed in a total membrane fraction from heart homogenate, have no functional significance. Finally, a reduction in the SL phosphoinositide content as well as in Ins(1,4,5)P_3 levels was found during ischemia, which was followed by significant increases during reperfusion (Mouton, et al., 1991). At any rate, the α_1-adrenoceptor signaling pathway in ischemia-reperfusion may be relevant as an adaptive mechanism (Butterfield and Chess-Williams, 1990) which may also mediated the arrhythmogenic effect of catecholamines (Corr, et al., 1989).

3. Oxidant metabolites

Many recent studies have revealed that one of the mechanisms underlying the reperfusion injury is cellular damage caused by oxygen free radicals and other non-radical oxidants (Opie, 1989, Hearse, 1991). Oxygen-derived free radicals (ODFR)

are reactive intermediate metabolites containing unpaired electrons in their outside orbitals (Halliwell, 1991). The reactive oxygen metabolites which are putatively involved in ischemia-reperfusion injury are the superoxide radical ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Under normal physiological conditions, only a small amount of ODFR is produced from mitochondrial respiration and miscellaneous oxidative events within the cell (Singal, et al., 1988). However, the generation of these radicals is balanced by endogenous enzymatic antioxidant mechanisms. These antioxidant enzymes include superoxide dismutase (SOD), catalase(CAT) and glutathione peroxidase. Therefore, under physiological conditions, there is no cellular damage from ODFRs (Chance, et al., 1979).

It has been observed by the electron spin resonance spectroscopy technique that a burst of ODFRs occurs immediately after reperfusion of the ischemic myocardium in both intact and isolated hearts (Arroyo, et al., 1987; Garlick, et al., 1987; Bolli, et al., 1988). Theses ODFRs can originate from several possible sources. In the ischemic myocardium, hypoxanthine is accumulated from the degradation of ATP. Capillary endothelial cells of myocardium contain a xanthine dehydrogenase which converts hypoxanthine to xanthine and uric acid with the reduction of $NADP^+$ under physiological condition (Downey, et al., 1988). However, ischemia transforms xanthine dehydrogenase to xanthine oxidase (Parks and Granger, 1986). Once oxygen is available during reperfusion, the xanthine oxidase catalyzes the metabolism of hypoxanthine, taking O_2 as an electron acceptor instead of $NADP^+$, thereby generating superoxide radicals. Superoxide radicals can

dismutate to H_2O_2 by the SOD. In the presence of Fe^{2+}, superoxide radicals react with H_2O_2 to generate hydroxyl radicals which are the highest reactive oxygen species (Hammond and Hess, 1985). Apart from that, in ischemia-reperfusion injury, leaky mitochondrial respiration, catecholamine autoxidation and metabolism of arachidonic acid can also be the sources of ODFRs (Kukreja and Hess, 1992). The other major potential sources of ODFRs during reperfusion are activated neutrophils which produce the superoxide radical via the NADP oxidase system, H_2O_2 via dismutation of superoxide, and hypochlorous acid (HOCL) via the myeloperoxidase reaction (Fantone and Ward, 1982). The relevance of neutrophils to ischemia-reperfusion injury is supported by the evidence that neutrophils can be found in the tissue sections within 60 minutes of regional myocardial ischemia or shortly after the onset of reperfusion in irreversibly injured myocardium *in vivo*, (Engler, et al., 1986; Go, et al., 1988). The ODFRs can cause a series of damage to the living cells. ODFRs can initiate chain reactions of lipid peroxidation which damages the membrane structures of the cell, resulting in the impairment of cell integrity and cellular function (Freeman and Crapo, 1982; Weiss, 1986). In addition, ODFRs attack the sulfhydryl groups of protein molecules, causing alteration of the protein functions (Halliwell, 1991). Concurrently with the appearance of ODFRs, in ischemia-reperfusion injury, there is also a decrease in the activity and potency of cellular antioxidants (Meerson, et al., 1982; Julicher, et al., 1984; Ferrari, et al., 1985; Peterson, et al., 1985). Thus, the cellular damage caused by the ODFRs is aggravated. *In vitro* studies indicated that ODFRs alter many membrane bound activities in the myocytes such as the sarcolemmal Ca^{2+} pump (Kaneko, et al., 1989; Dixon, et al., 1990), the sarcoplasmic reticular Ca^{2+} pump (Kim, et al., 1988), SL

Na^+-Ca^{2+} exchange (Shi, et al., 1989) and the SL Na^+-K^+ ATPase (Kukreja, et al., 1990). ODFRs also depress the contractility of the perfused heart (Eley, et al., 1990). Application of antioxidants improved the cardiac function in ischemia-reperfusion injury, which further indicates the role of ODFRs in such an injury (Steward, et al., 1983; Myers, et al., 1985; Tamura, et al., 1988).

4. Alteration of lipid metabolites

Several studies have suggested that alteration in lipid metabolism may be related to membrane abnormalities during ischemia (Katz and Messineo, 1981). In rat heart, 30-40 minutes of global ischemia results in a 2-3 folds increase of the total fatty acids. Reperfusion of prolonged ischemic hearts further enhances the rise of long chain fatty acids (Burton, et al., 1986, van Bilsen, et al., 1989). Perfusion of these hearts with a medium devoid of lipids suggests that these fatty acids originate from endogenous sources (van Bilsen, et al., 1989). In the ischemic myocardium, glycerol production, presumably originating exclusively from triacylglycerol pool, was found to be increased (van Bilsen, et al., 1989; Trach, et al., 1986). This suggests that triacylglycerols contribute to the elevated fatty acid level in the ischemic myocardium. It has been suggested that in ischemic condition, the degradation of triacylglycerols could be enhanced by the activation of triacylglycerol lipase which could be stimulated by locally released catecholamines (Heathers and Brunt, 1985). In fact, increased triacylglycerol lipase activity has been observed within 10 minutes after coronary occlusion (Heathers and Brunt, 1985). On the other hand, the appearance of significant amounts of arachidonic acid indicates that degradation of

phospholipids is taking place in ischemia-reperfusion injury, since the phospholipid pool is the only source of unesterified arachidonic acid in the cardiac tissue (Chien, et al., 1984). Indeed, degradation of myocardial phospholipid pool in prolonged ischemia or reflow conditions has been found in different animal models. In rat heart, Chiariello et al.(1987) observed that the total phospholipid pool was decreased by 47% after 2 hours of ischemia. Burton et al.(1986) reported that nearly 50% of the total phospholipid was decreased following 30 minutes of reperfusion after 60 minute of ischemia. Chien and co-worker (1984) found that in dog hearts the total phospholipid pool decreased by 10 % and 33% following 3 and 12 hours of regional ischemia, respectively. In pig heart, a decrease of phospholipid content by 8% with 15 minutes of reperfusion after 60 minutes of ischemia was also observed (Das, et al., 1986). However, in shorter period of ischemia (<45 minutes), a total phospholipid loss cannot be observed, although there is a significant increase of arachidonic acid content in the myocardium (Otani, et al., 1988; van Bilsen, et al., 1989). This may be explained by the fact that the quantity of fatty acids accumulating in post-ischemic tissue is quantitatively small compared to the total phospholipid amount (van der Vusse, et al., 1992).

The mechanisms underlying the enhanced degradation of phospholipids during ischemia-reperfusion injury is not well defined. One of the possibilities is that the enhancement of phospholipase A activity in ischemia-reperfusion may contribute to the accelerated phospholipid degradation. It has been shown that cardiac phospholipase A is stimulated by Ca^{2+} and has an acidic pH optimum (Franson, et al., 1978, 1989). Thus acidosis of the ischemic myocardium due to anaerobic

metabolism and enhanced cytosolic Ca^{2+} in the ischemic and reperfusion periods may favor phospholipase A activity. A recent study demonstrated that pre-perfusion of the heart with phospholipase A_2 antibody reduced the degradation of phospholipids during ischemia-reperfusion injury, indicating an involvement of phospholipase A_2 activity in accelerated phospholipid degradation (Prasad, et al., 1991). A second possible mechanism is that changes in the bilayer structure of phospholipids in ischemic condition may favor the reactions catalyzed by phospholipases. Schrijvers et al.(1990) found that multilamellar structures of membrane were formed after 30 minutes of reperfusion following 90 minutes of ischemia. It was speculated that the multilamellar structures may be more liable to be attacked by the phospholipases (Schrijvers, et al., 1990). It is however unknown for the time being which mechanism causes such a membrane structural change. A third possible mechanism responsible for degradation of phospholipids in ischemia-reperfusion injury is lipid peroxidation caused by ODFRs (see section B.3.).

The consequences of phospholipid degradation include the loss of cell integrity and alteration of membrane-bound activities, thereby causing loss of cellular function. Accumulation of long-chain fatty acids has also been shown to correlate with many detrimental consequences of the ischemic-reperfusion injury, such as contractile dysfunction, the depletion of high energy phosphate stores, release of lactate dehydrogenase, and accumulation of Ca^{2+} in the heart (Prienzen, et al., 1984; Burton, et al., 1986; van Bilsen, et al., 1989). Long-chain fatty acids contain a hydrophilic carboxylate group and a nonpolar lipophilic hydrocarbon chain. Thus it is an amphiphilic substance (van der Vusse, et al., 1992). Incorporation of

excessive amounts of such amphiphiles into membrane leads to disruption of membrane structure (Katz and Messineo, 1981). Therefore, many cellular functions could be affected by such an intervention. The direct detrimental effects of long-chain fatty acids on cardiac function *in vivo, in ex vivo*, or in isolated neonatal myocytes have been reported (Severeid, L. et al., 1969; Janero, et al., 1988; Lo, et al., 1991; Huang, 1992). In *in vitro* assay conditions, long chain fatty acids were found to uncouple the gap junctions between the cardiomyocytes and to stimulate voltage dependent Ca^{2+} current, which may contribute to the mechanism of ischemia-induced arrhythmias and conduction disturbances (Janero, et al., 1988; Burt, et al., 1991).

Apart from long-chain fatty acids, intermediate metabolites of fatty acid oxidation such as long-chain-acyl-CoA and acylcarnitine also accumulated in myocardium that suffered from low flow ischemia due to cessation of ß-oxidation. These metabolites affect cardiac functions in a similar manner as the fatty acids. However, their detailed mechanisms of action are beyond the scope of this review and are excellently described elsewhere (van der Vusse, et al., 1992).

Although above mechanisms (Ca^{2+} overload, alteration of adrenergic signaling, generation of oxidant metabolites and accumulation of long chain fatty acids) have been proposed to interpret the cause of abnormal cellular function in ischemic-reperfused heart, evidence that these mechanisms are central to the pathogenesis of the disordered myocardial function and detailed process initiating these

mechanisms remain incomplete. Therefore, exploration of the status of cardiac PLD in ischemia-reperfusion injury will provide information on this issue.

III. Materials and Methods

A. Animals

Adult male Sprague-Dawley rats weighing 250 -300 grams were used. Animals were housed (2 to 3 in a cage) in the Animal Holding of St. Boniface General Hospital Research Centre and were provided with standard rat chow and water *ad libitum*.

B. Chemicals

1-α-1-Palmitoyl-2[^{14}C]oleoylphosphatidylcholine (specific activity 58.0 mCi/mmol), [methyl-^{3}H] choline chloride (specific activity 87.8 mCi/mmol), and L-α-dipalmitoyl-[glycerol-^{14}C(U)]-phosphatidic acid (specific activity 144 mCi/mmol) were purchased from New England Nuclear, Mississauga, Ontario. Egg PtdCho, egg PtdOH, myristoleic acid, vaccenic acid and oleic acid (sodium salt) were obtained from Serdary Research Laboratories, London, Ontario. Elaidic acid, palmitoleic acid and linoleic acid (sodium salts) were purchased from Matreya, Inc. Pleasant Gap, Pennsylvania (U.S.A.). Xanthine oxidase (from bovine milk) was from Calbiochem Corp., La Jolla, California (U.S.A.). Silica gel 60A F-254 thin-layer chromatography plates were purchased from Whatman International Ltd., Maidston (U.K.). AG50W-X8 ion-exchange resin was bought from Bio-Rad Laboratory, Inc., Mississauga, Ontario. All the solvents were bought from Mallinckrodt (Canada). All other chemicals were of standard reagent grade from Sigma Chemical Company, St. Louis, Missouri (U.S.A.).

C. Isolation of Subcellular Fractions by Differential Centrifugation

Animals were sacrificed by decapitation. Hearts were immediately excised, and atria and large vessels were carefully removed. Three or more hearts were pooled within maximum of 2 minutes for immediate isolation of subcellular fractions. Crude subcellular fractions were prepared by differential centrifugation as described by Sulakhe and Dhalla (1973). The ventricular tissues were washed, minced, and then homogenized in 0.25 M sucrose-10 mM Tris-HCl, pH 7.4 (solution A)(10 ml/g tissue) in a Waring blender (2 x 20 sec, set 2). The homogenate was filtered through 4 layers of gauze, and the filtrate was centrifuged at 1000 g for 20 min. The 1000 g pellet was suspended in solution A, and this fraction contained sarcolemma, myofibrils, nuclei and cell debris. The supernatant was centrifuged at 10,000 g for 20 min to obtain the mitochondrial fraction which was suspended in solution A. The resulting supernatant was centrifuged at 40,000 g for 45 min. This pellet was suspended (solution A), and this fraction contained crude heavy microsomes. The light microsomal fraction was obtained by further centrifugation of the 40,000 g supernatant at 105,000 g for 60 min, and the pellet was suspended in solution A. The 105,000 g supernatant was taken as the cytosolic fraction.

D. Isolation of Sarcolemmal-Enriched Membranes

Animals were sacrificed by decapitation. Hearts were immediately excised, and atria and large vessels were carefully removed. The ventricular tissue was washed

and minced or, in case of frozen samples, pulverized in an alloy mortar cooled in liquid nitrogen (N_2). The isolation procedure was basically according to Pitts (1979). The tissue was homogenized in 0.6 M sucrose-10 mM imidazole, pH 7.0 (3.5 ml/g tissue) with a Polytron PT-20 (6 x 15 s, setting 6). The resulting homogenate was centrifuged at 12,000 g for 30 min; and the pellet was discarded. After diluting (5 ml/g) with 140 mM KCl- 20 mM 3-(N-morpholino)-propanesulphonic acid (MOPS), pH 7.4, the supernatant was centrifuged at 100,000 g for 60 min. The resulting pellet was suspended in 140 mM KCl, 20 mM MOPS, pH 7.4 buffer and layered over a 30% sucrose solution containing 0.3 M KCl- 50 mM $Na_4PO_4O_7$, and 0.1 M Tris-HCl, pH 8.3. After centrifugation at 100,000 g for 90 min (using a Beckman swinging bucket rotor) the band at the sucrose-buffer interface was taken and diluted with 3 vol of 140 mM KCl, 20 mM MOPS, pH 7.4. A final centrifugation at 100,000 g for 30 min resulted in a pellet rich in sarcolemma. All isolation steps were carried out at 0-4 °C. The final pellet was suspended in 0.25 M sucrose - 10 mM histidine, pH 7.4 (approx. 3.5 mg/ml) and then quickly frozen and stored in liquid N_2. In these membranes, the ouabain sensitive K^+pNPPase activity, a well-known marker for SL was found to be 11-fold purified over homogenate. In addition, these sarcolemmal membranes was charactrized by normal values of maximum specific binding for the [^{3}H] labeled α- (0.35 ± 0.05 pmol/mg protein, n=18) and ß- (0.21 ± 0.03 pmol/mg protein, n=18) adrenergic antagonists, prazosin and dihydroalprenolol, respectively. As already reported (Mesaeli, et al., 1992), their angiotensin converting enzyme activity (endothelial plasmalemmal marker) is 0.3 fold that of the corresponding value in the heart homogenate.

E. Isolation of Sarcoplasmic-Reticular Enriched Membranes

The isolation procedure was basically according to Harigaya and Schwarts (1969). Animals were sacrificed by decapitation, hearts were immediately excised and atria and large vessels were carefully removed. The ventricular tissue was minced or pulverized in an alloy mortar cooled in the liquid N_2 for the frozen samples, and then was homogenized in 10 mM $NaHCO_3$, 5 mM NaN_3, 15 mM Tris-HCl, pH 6.8 (8 ml/g tissue) with a Polytron PT-20 (3 x 10 s, setting 2). The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was centrifuged at 10,000 g for 20 min. The resulting supernatant was carefully aspirated and further centrifuged at 40,000 g for 45 min. The resulting pellet was suspended in 0.6 M KCl, 20 mM Tris-HCl, pH 6.8 (8 ml /g tissue) to solubilize the contractile proteins and then recentrifuged at 40,000 g for 45 min. All isolation steps were carried out at 0-4 °C. The final pellet was suspended in 0.25 M sucrose - 10 mM histidine, pH 7.4 (approx. 3.5 mg/ml) and then quickly frozen and stored in liquid N_2.

F. Marker Enzyme Assays

Ouabain sensitive K^+-para-nitrophenyl phosphatase (pNPPase) is an accepted SL marker enzyme (Vetter, et al., 1991). The K^+-pNPPase was assayed by measuring the formation of para-nitrophenol (pNP) from paranitrophenyl phosphate (pNPP). The assay medium contained: 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 20 mM KCl, 1 mM ouabain (+ or -) and an adequate amount of alamethicin (1 μg/μg protein) in a total volume of 1 ml. The mixture was preincubated at 37 °C for 10 min. The reaction was started by the addition of 15 mM PNPP (final concentration) and further incubated for 20 min. The reaction was stopped by the 0.1 ml of ice-cold 50% tetrachloric acid followed by the addition of 2 ml 0.5 M Tris(base). The

absorbance of the final solution was measured at 410 nm by a spectrometer (Spectronic 601, Milton Roy Co., Rochester, N.Y.).

Rotenone-insensitive NADPH cytochrome c reductase is considered to be a marker enzyme for (endo)sarcoplasmic reticulum (Ragnotti, et al., 1968). The enzyme activity was assayed by measuring the rate of change of absorbance at 550 nm at 25°C, in the initial 3 min. The medium contained 44 mM potassium phosphate buffer (pH 7.6), 66 mM KCl, 0.1 mM NaCN, 1.5 μM rotenone, 0.05 mM oxidized cytochrome c and 50 μg membrane protein in a total volume of 2 ml. The reaction was initiated by adding of 0.1 mM nicotinamide-adenine dinucleotide phosphate (NADP). The blank cuvettes contained all the components except NADPH. The enzymatic activity was estimated from the difference between the initial rates of cytochrome c reduction in the complete reaction mixture and in the blank. The reduction of cytochrome c was calculated from the molar extinction coefficient of 27.7×10^6 cm^2/mole (Ragnotti, et al., 1969).

Cytochrome c oxidase activity, a mitochondria marker enzyme, was assayed according to Wharton and Tzagoloff (1967) by measuring the initial rate of change of absorbance at 550 nm. The medium contained 1 mM potassium phosphate buffer (pH 7.0), 20 μg reduced cytochrome c and 50 μg membrane protein in a total volume of 1 ml. The blank was oxidized with 10 mM potassium ferricyanide.

G. Phospholipase D Assay

1. Exogenous substrate

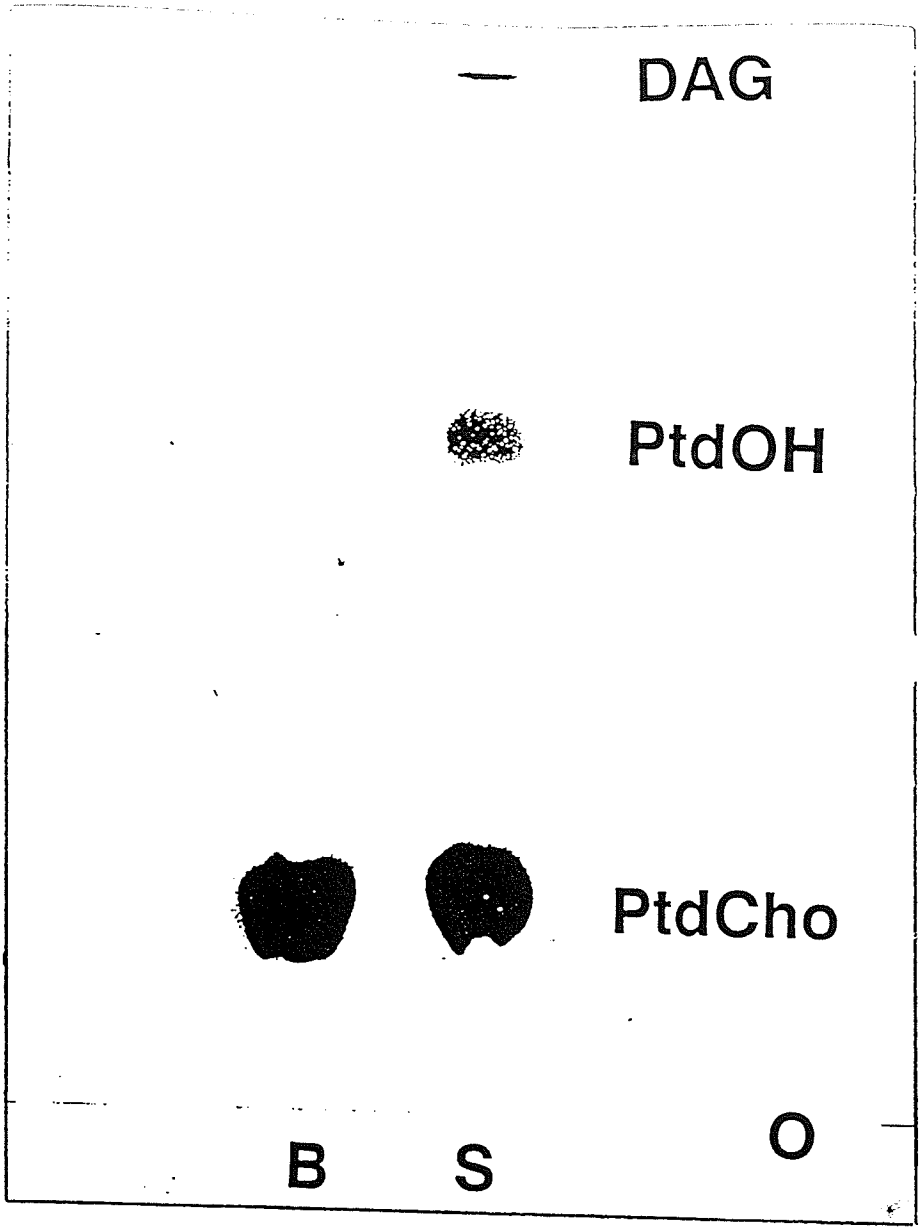


Figure 2. Autoradiograph of [^{14}C]labeled lipids after thin-layer chromatography fractionation of a chloroform extract from phospholipase D assay.

PLD hydrolytic activity was assayed as described in Materials and Methods. After thin-layer chromatography, the spots were visualized by autoradiography (Kodak X-OMAT AR) and identified by comparison with unlabeled lipid standards which were run in parallel and detected by iodine vapors. B:blank; S:sample; O:origin; PtdOH: phosphatidic acid; DAG:1,2-diacylglycerol; PtdCho: [^{14}C] labeled phosphatidylcholine.

PLD hydrolytic activity was assayed by measuring the formation of labelled PtdOH from 2.5 mM [^{14}C]-PtdCho (0.167 μCi/μmol). The transphosphatidylation activity was assayed by determining the formation of [^{14}C] phosphatidylethanol (PtdEth) in the same conditions as the hydrolytic activity except that 0.4 mM ethanolwas included. The exogenous PtdCho substrate was prepared by combining egg phosphatidylcholine and tracer [^{14}C]PtdCho; the solvents were evaporated under N_2 stream. Either water or 25 mM sodium oleate solution was added to give a concentration of 12.5 mM PtdCho. This mixture was sonicated approximately for 20 min (with negligible hydrolysis of phosphatidylcholine) in a Branson 1200 sonicator and appropriate aliquots were used for the assay. The assay was carried out at 25 °C for 60 min in a final volume of 120 μl containing 50 mM 3,3-dimethylglutaric acid(DMGA)-10 mM EDTA (pH 6.5), 25 mM KF, and SL membranes (25-50 μg). The reaction was terminated by the addition of 2 ml of chloroform: methanol (2:1,vol/vol) followed by 0.5 ml of 0.1 M KCl. The chloroform phase was washed according to Folch et al(1957). The lipid extract was evaporated almost to dryness under N_2, redissolved in 30 μl of chloroform containing PtdOH or PtdEth as a carrier, and was quantitatively applied to silica gel 60 A F-254 thin-layer chromatographic plates(0.25 mm thick). These plates were developed in a solvent system containing chloroform: methanol: acetone: acetic acid:H_2O (50:15:15:10:5, vol/vol) with authentic phosphatidic acid or phosphatidylethanol as a standard for hydrolytic and transphosphatidylation activity, respectively. After the solvent front had migrated approximately 14 cm, the plates were air dried at room temperature. The lipid spots were visualized by exposure to iodine vapors, scraped after disappearance of the iodine color, and radioactivity was counted by liquid

scintillation. In such an assay condition, less than 5 % of the precursor was actually converted to product. In the initial experiments, the areas corresponding to PtdOH and DAG were visualized autoradiographically (Figure 2) to confirm the separation of reaction products. It should be noted that DAG standard co-migrated with the solvent front (R_f= 1) which, in sample runs, contained radioactive DAG derived from PtdOH dephosphorylation. Free fatty acid standard (oleic acid) migrated distinctly below theDAG spot with a R_f value of 0.94, while PtdOH had a R_f value of 0.64. In transphosphatidylation case, the R_f value of PtdEth was 0.82.

2. Endogenous substrate

SL membranes in which PtdCho was prelabeled with [^{3} H]-choline (1802 dpm/nmol membranal PtdCho) were used and PLD activity was assayed by measuring the [^{3}H]choline release in the aqueous phase after incubation in a reaction medium at 25 °C for 60 min. The incubation medium was the same as for the exogenous assay, except that 5 mM of cold phosphorylcholine was included to avoid the possible interference from phospholipase C activity. The reaction was terminated by adding 2 ml of chloroform/methanol (2:1, by vol/vol) followed by 0.5 ml of 0.1 M KCl to favor phase separation. The lipids were extracted as indicated above. [^{3}H]-Choline present in the upper methanolic aqueous phase was separated from contaminants through a (cat)ion exchange column with AG50W-X8 (200-400 mesh, BioRad) resin (Cook, 1988). After preliminary and extensive washing of the resin in H_2O until the washing reached a constant pH (approx. 4.0), 2 ml of mixture (50% resin and 50% H_2O) were packed into a disposable quick snap column

(Mandel Co.). The upper methanolic aqueous phase was loaded on the resin bed, and then the column was washed free from possible glycerophosphocholine and phosphorylcholine contamination with 20 ml H_2O. The [^{3}H]-choline was eluted with 20 ml of 1 M HCl. Aliquots of each fraction were taken and the associated radioactivity was determined by scintillation counting (Cook and Wakelam, 1989). Elution profiles were checked by parallel running of a separate column loaded with radio-labelled standards. The radioactivity of the choline fraction was always greater than 95% of the total radioactivity recovered in the aqueous phase after lipid extraction. Blanks were zero time incubation samples containing all the components of the assay mixture (Chalifa, et al., 1990).

H. Phosphatidic Acid Phosphohydrolase Assay

The PtdOH phosphohydrolase activity was assayed by measuring the formation of DAG from exogenous labeled PtdOH. The assay procedure was basically according to Martin et al. (1991). The substrate was prepared as follows: aliquots of egg PtdOH, egg PtdCho and L-α-dipalmitoyl, [glycerol-^{14}C(U)]-PtdOH (144 mCi/mmol) solutions were mixed, the organic solvent was evaporated under a stream of N_2, and 5 mM EDTA plus 5mM EGTA, adjusted to pH 7 with KOH, were added to give a final concentration of 3mM PtdOH and 2 mM PtdCho. The lipids were dissolved by sonication for 5 min in a sonication bath (model 1200, Branson Ultrasonics Corp.). The PtdOH phosphohydrolase assay medium contained 100 mM Tris-maleate buffer (pH 6.5), 1 mM dithiothreitol, 0.6 mM [^{14}C] PtdOH (1 Ci/mol), 0.4 mM PtdCho , 1 mM EGTA, 1mM EDTA, 0.5% Triton-X100 and 50

μg membrane protein in a total volume of 100 μl. The medium was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 2 ml of chloroform :methanol (2:1,v/v) and 0.5 ml of 0.1 M of KCl. The test tubes were shaken vigorously for 2 min. The upper phase was discarded. The lower phase was evaporated to almost complete dryness under a stream of N_2, redissolved in chloroform containing DAG and monopalmitoylglycerol as carriers, and quantitatively applied to silica gel 60A K6F thin-layer (0.25-mm) plates (Whatman Co.). The plates were developed in petroleum ether:ether:acetic acid (60:40:1, vol/vol/vol) for 30 min. The lipid spots were visualized with iodine vapor and scraped. The R_f values were 0.34, 0.06, and 0 for DAG, monoacylglycerol and phospholipids, respectively. The scrapings were extracted with Cytoscint (ICN Biochemical Canada Ltd) and counted for radioactivity in a liquid scintillation system (model 1701, Beckman Instruments, Inc.).

I. Miscellaneous Assays

1. Labeling of the endogenous PtdCho pool

Rats were sacrificed by decapitation and the hearts were immediately excised and immersed in ice-cold saline. The aortic artery was cannulated and the atria and connective tissues were carefully removed. The heart was perfused retrogradely according to the Langendorff (1895) mode, at 37°C, with a modified Krebs-Henseleit buffer (same as that used in ischemia-reperfusion model) bubbled with 95% O_2 and 5%CO_2 (Gupta, et al., 1988). The hearts were electrically stimulated as indicated in section H. 6.. After 15 min stabilization, the heart was switched to

Krebs-Henseleit buffer containing 5 μM [methyl-^{3}H] choline chloride (6-8 μCi/ml) and perfused in a recirculating mode. After 60 min perfusion, the heart was freeze-clamped with a Wollemberger clamp preimmersed in liquid N_2. The frozen samples were used for SL isolation. SL phospholipids were extracted from an aliquot of membrane and separated through thin layer chromatography (Panagia, 1984). The PtdCho spot on the plate was identified, its radioactivity was counted and its phosphorous content was quantitated according Bartlett (1959). Approximately 90% of the radioactivity was found to be associated with the PtdCho component. The specific activity of the SL membranal [^{3}H] labeled PtdCho was found to be 1800 dpm/nmol.

2. Determination of sulfhydryl group content

The total sulfhydryl content of the SL membranes was determined with 5',5'-dithiobis(2-nitobenzoate (DTNB) according to the procedure described by Boyne and Ellman (1972). The assay medium contained 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM DTNB, 1% sodium dodecyl sulfate (SDS), 20 mM imidazole, pH 7.4 and 200 μg membrane protein. The medium was incubated for 3 min at room temperature and the absorbance at 412 nm was measured. A blank value was determined for each sample by the subsequent addition of 2 mg N-ethymaleimide (NEM). Calculation of the sulfhydryl group content was based on a molar extinction coefficient of 13,600/M/cm at 412 nm for the thiophenol reaction product. This was verified by using cysteine as a standard.

3. Determination of proteins

The membrane proteins were determined according to Lowry, et al. (1951), using bovine serum albumin (fraction V) as a standard.

4. Determination of phospholipid phosphorous

Phospholipid phosphorous was determined as described by Bartlett (1959) using sodium phosphate (Sigma Chem. Co.) as a standard. This procedure is based upon the release of inorganic phosphate from phospholipids by acid digestion followed by the colorimetric determination of this phosphate as its reduced phosphomolybdate complex.

5. Preparation of phosphatidylethanol

The preparation of phosphatidylethanol was basically according to Wang et al., (1988). Reaction medium included 50 mg egg phosphatidylcholine, 1.5 ml ethanol (anhydride), 200 μl Triton X-100, 2.5 ml ether, 1.4 mg cabbage phospholipase D (Sigma type I, 250 u.) and 2.5 ml sodium acetate/acetic acid buffer (pH 5.6). The medium was constantly kept shaking at room temperature over night (15 hours). The reaction was stopped by the addition of 20 ml of chloroform/methanol (2/1, v/v). After addition of 10 ml 0.1 M KCl, the mixture was vigorously shaken for 2 min. The organic phase was collected and the aqueous phase was washed once more with 20 ml chloroform:methanol (2:1). The volume of combined organic phase was

reduced to 10 ml under a stream of N_2. The residue was applied on heat-activated TLC plates (G-60). The plates were developed with chloroform:methanol:29% ammonia (65:30:3, vol/vol). The R_f values of phosphatidylethanol, phosphatidic acid and phosphatidylcholine were 0.8, 0.2 and 0.03 respectively. The spot containing phosphatidylethanol was scrapped and the lipid was extracted by 30 ml chloroform:methanol (2:1, vol/vol) twice. The combined extraction was concentrated under a stream of N_2 to a final volume of 4 ml.

6. Global Normothermic Ischemia-Reperfusion Model

Male Sprague-Dawley rats weighing 250-300 g were sacrificed by decapitation. The hearts were quickly excised and immersed in cold saline. The heart was immediately mounted onto an aortic cannula and cleared of atrial, fat and connective tissues. Perfusion was carried out retrogradely according to the Langendorff procedure (1895), in a closed chamber at 37°C, with modified Krebs-Henseleit buffer containing (mM): NaCl 120; $NaHCO_3$25; KCl 4.8; KH_2PO_4 1.2; $MgSO_4$ 1.25; $CaCl_2$ 1.25; and glucose 8.6, bubbled with mixed oxygen (95% O_2 and 5% CO_2)(pH7.4) (Gupta, et al., 1988). Coronary flow was maintained at 8 ml/min; and the heart was driven electrically using bipolar electrodes placed at or close to the AV node with 1 msec pulses at 4 Hz and a voltage of 10 % above threshold. After 15 min of stabilization, the hearts were subjected to one of the following protocols: (i) perfusion was terminated (pre-ischemic control); (ii) the heart was made globally ischemic by stopping the flow of perfusate for 30 min (ischemia), followed by 5, 10, or 30 min of reflow (reperfusion). After each of the above

protocols, the heart was immediately frozen by a Wollemberger clamp which was preimmersed in liquid nitrogen. The frozen samples were used for membrane isolation.

J. Statistics:

All of the experiments were carried out as triplicate or duplicate determinations. Results are presented as mean ± SE. The statistical differences between two groups were evaluated by Student's *t* test. The data from more than two groups were statistically evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. A value of $p < 0.05$ was considered to be significant.

IV. RESULTS

A. Characterization of Cardiac PLD

1. Subcellular distribution of phospholipase D

The subcellular distribution of PLD activity was monitored according to Kobayashi and Kanfer (1987) in crude fractions obtained from mild homogenization of myocardial tissue followed by differential centrifugation (Table 1). Although the specific activity of PLD in the 1000 g fraction (which is believed to contain sarcolemma, myofibrils, nuclei and cell debris) was low, this fraction, due to very high protein yield, accounted for the largest amount (54%) of total enzyme activity present in the homogenate. The 40 000 g fraction (mainly containing sarcoplasmic reticulum) showed the highest specific activity but, due to the low protein yield, the total enzyme activity in this fraction was about 15% of that in total homogenate. About 17% of enzyme activity was associated with the 10 000 fraction (primarily containing mitochondria). The enzyme activity was not detected in the soluble fraction (105 000 g supernatant).

2. PLD activities in purified sarcolemmal and sarcoplasmic reticular membrane preparations

Data from crude fractions indicated that an abundant amount of the total cellular PLD activity was associated with the 1000 g fraction which contains sarcolemmal membranes, while a quite active intracellular PLD was found in the 40 000 g microsomal fraction. Therefore, we furthered our study with purified sarcolemma and, to a lesser extent, with sarcoplasmic reticular preparations.

Table 1. Distribution of phospholipase D activity in fractions obtained by differential centrifugation of the homogenate from rat heart ventricular tissue

Fraction	Protein yield	Specific activity	Total activity	Recovery
	(mg/g tissue)	PtdOH formed (nmol/mg/h)	PtdOH formed (nmol/g tissue)	%
Homogenate	166.7	6.4	1066.9	100.0
1000 g (10min)	81.0	7.1	575.1	53.9
10 000 g(20min)	8.6	21.2	182.3	17.1
40 000 g(45min)	1.4	115.6	161.8	15.1
105 000 g(60min)	1.2	44.6	53.5	5.0
105 000g Supernatant	44.4	0.0	0.0	0.0

Values are average of two separate experiments done in triplicate (variation < 10%). Phospholipase D hydrolytic activity was assayed as described in Materials and Methods. Recovery (%) indicates the percentage distribution of total activity in various fractions, relative to homogenate activity (100%).

Sarcolemma-enriched and sarcoplasmic reticulum-enriched preparations were isolated according to Pitts (1979) and Harigaya and Schwartz (1969), respectively. To assess the purity of the membranes, both subcellular membrane preparations were examined for the marker enzyme activities (Table 2). After alamethicin treatment, the SL marker ouabain-sensitive K^+-pNPPase showed an 11-fold increase in SL in comparison to the homogenate value; the low activity detected in SR suggests a small degree of SL contamination in this fraction. The cytochrome-c oxidase (mitochondrial marker) and rotenone-insensitive NADPH-cytochrome-c reductase (sarcoplasmic reticular marker) activities in the SL fraction were 0.6- and 0.8-fold of the corresponding values in the heart homogenate, respectively. The relative specific activity of cytochrome-c oxidase and rotenone-insensitive NADP-cytochrome-c reductase in SR preparations was about 0.2- and 6.8-fold, respectively (table 2). These observations appear to indicate that above fractions were relatively pure and had minimal cross contamination. PLD activity was examined by quantitating the formation of PtdOH from exogenous [^{14}C]PtdCho in both SL and SR preparations. It was found that, in purified preparations, SL PLD specific activity was higher than that in SR (Table 2). To confirm the presence of PLD in SL and SR preparations, transphosphatidylation activity, which is unique to PLD, was examined. Figure 3 shows that formation of PtdEth is dependent on ethanol concentration. The apparent Vmax values for SL and SR are markedly different (427.2 ± 35.3 and 57.5 ± 6.4 nmol/mg/h for SL and SR, respectively). However, due to large standard errors, there was no significant difference between apparent Km values of SL and SR (244.0 ± 44.3 and 162.9 ± 45.8 mM for SL and SR, respectively). It can be noticed that the specific activity of PLD in purified SR is

Table 2. Phospholipase D hydrolytic activity and marker enzyme activities in cardiac sarcolemmal and sarcoplasmic reticular membranes

	Sarcolemma	Sarcoplasmic reticulum
A.		
Marker enzymes		
Ouabain-sensitive K^+-pNPPase (a)	1.39 ± 0.13 (11.0)	0.17 ± 0.01 (1.3)
Cytochrome c oxidase (b)	59.10 ± 7.40 (0.6)	13.60 ± 2.00 (0.2)
Rotenone-insensitive NADPH-cytochrome-c reductase (c)	1.34 ± 0.17 (0.8)	11.41 ± 1.02 (6.8)
B.		
PLD hydrolytic activity (d)	181.00 ± 11.54	63.22 ± 4.24

Values are means ± SE of three to four different membrane preparations and are expressed as (a) μmol pNPP/mg/h; (b) nmol cytochrome c/mg/min; (c) nmol cytochrome c reduced /mg/min; (d) nmolPtdOH formed/mg/h. Sarcolemmal and sarcoplasmic reticular fragments were isolated according to Pitts (1979) and Harigaya and Schwarts (1969), respectively. Assays were performed as indicated in Materials and Methods. Ouabain-sensitive K^+-pNPPase activity was determined in the presence of alamethicin (1 mg/mg membrane protein). Data in parentheses indicate the relative specific activity that is the specific activity in the fraction/the specific activity in the homogenate.

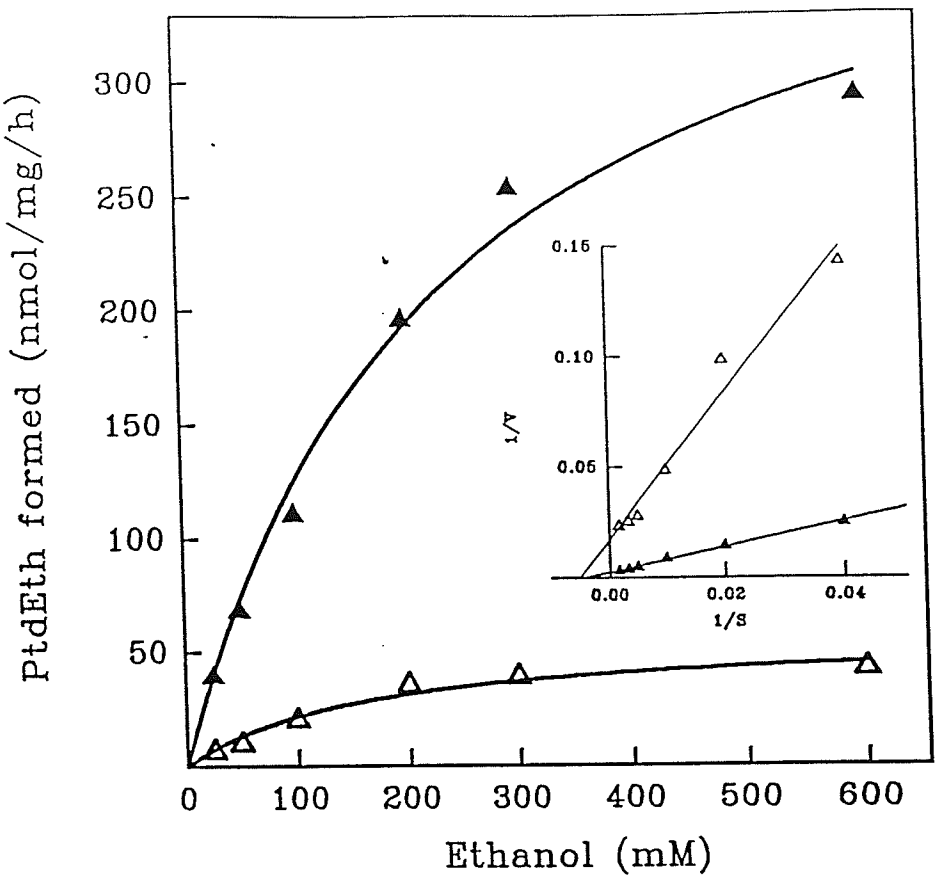


Figure 3. Effect of varying ethanol concentration on the PtdEth formation via PLD transphosphatidylation activity in sarcolemma and sarcoplasmic reticular membranes.

The results are from a typical experiment in triplicate determinations which differed less than 10%. SL or SR membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF, 25 mM [^{14}C], and 25 to 600 mM ethanol for 60 min at 25 °C, in the presence of 5 mM oleate. Lineweaver-Burk plot (inset) was constructed from the values of the enzyme specific activity at different concentrations of ethanol. SL (▲); SR (△).

lower than that in crude 40 000 g fraction. The exact reasons for this finding are not clear at present, but it is possible that the crude fraction may carry contamination from other non-myocardial cells of the heart. As could be expected, while PtdEth production is increased, there is a corresponding decrease in PtdOH production (Figure 4), which suggests that reactions are competitive. The results imply that PtdOH and PtdEth are produced by the same PLD enzyme and the enzyme activity that we are investigating is PLD.

3. Properties of sarcolemmal phospholipase D

Because PtdOH was found to be hydrolyzed to DAG by a membrane PtdOH phosphohydrolase (Martin, 1988) and because KF partially inhibits the PtdOH phosphohydrolase (Kanfer, 1989), different concentrations of this fluoride salt were tested to minimize the DAG formation. High levels of radioactive DAG and reduced PtdOH formation were found in the absence of KF (Figure 5). Inhibition of DAG and increase of PtdOH production were found to occur at 10 mM KF. The concentration of 25 mM KF, which was routinely employed in the initial sets of experiments (Kobayashi and Kanfer, 1987), proved to be optimal because the PtdOH formation was 4.4-fold higher than the DAG formation. Further increase in KF concentrations resulted in the PtdOH and DAG formation being progressively decreased and increased, respectively (Figure 5). At any rate, the partial inhibition of radioactive DAG formation by KF indicates the presence of substantial amount of PtdOH phosphohydrolase activity in cardiac SL.

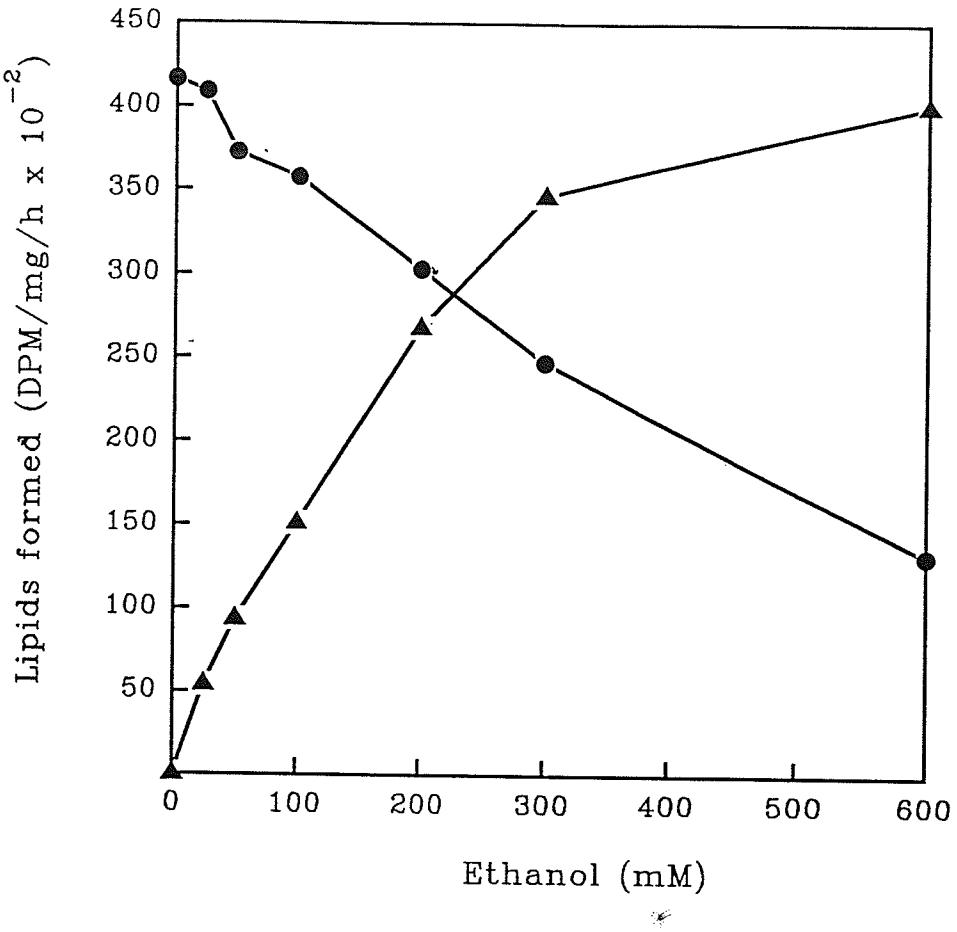


Figure 4. Dependence of PtdEth and PtdOH production on ethanol concentration.

SL membranes were incubated with 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF, 2.5 mM [^{14}C]PtdCho, and 25 to 600 mM ethanol for 60 min at 25 °C, in the presence of 5 mM oleate. Production of [^{14}C]PtdOH (●) and PtdEth (▲) was measured as detailed in Materials and Methods.

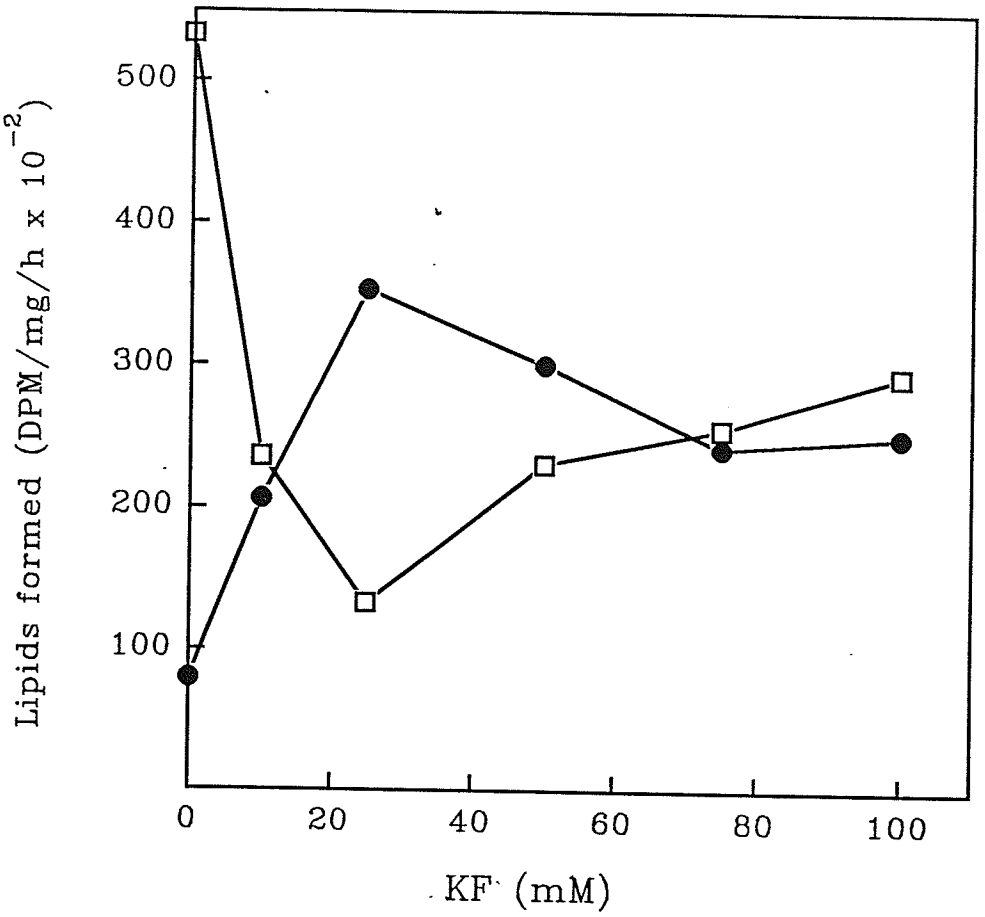


Figure 5. Effect of KF on phospholipase D activity and diacylglycerol level in heart sarcolemma.

Data are from a typical experiment; each point represents the average of triplicate determinations which differed less than 10%. PtdOH (●); DAG (□).

To determine the pH optimum for PLD, SL membranes were incubated at different pH values, from 5 to 9, using established buffer systems (Chalifour and Kanfer, 1980). The enzyme activity was observed over a narrow acid pH range with an optimum at pH 6.5 (Figure 6); this is in agreement with previous findings in other cell systems. In our assay condition (presence of 25 mM KF), formation of PtdOH was higher than that of DAG. The optimal temperature of incubation for PLD was 30°C (Figure 7), as reported for brain microsomes (Chalifour and Kanfer, 1982). However, shifting the temperature from 25°C to 30°C, a limited increase in PtdOH formation (1.12-fold) was noted while PtdOH to DAG ratio diminished significantly ($p < 0.05$, $n = 4$) from 8.4 ± 1.3 at 25 °C to 3.3 ± 0.2 at 30°C. Therefore, it was concluded that the temperature sensitivity of the enzymes responsible for the production of PtdOH (PLD) and DAG (likely PtdOH phosphohydrolase) was probably different, and that incubation at 25 °C would be favorable for minimizing PtdOH phosphohydrolase activity with only marginal reduction of PLD activity. Accordingly, studies were performed at 25 °C. Under optimal assay conditions, PtdOH formation was linear for a 90-min period of incubation (Figure 8) and the reaction rate was constant when 10 to 100 μg SL protein were employed for the assay (Figure 9). Different phospholipids, which have been found to be substrates for different forms of PLD in various cell types (Taki and Kanfer, 1979; Martin, 1988; Balsinde, et al., 1989), were examined as possible substrates for heart SL phospholipase D. Under our assay conditions, the enzyme showed a stringent specificity for PtdCho; phosphatidylethanolamine and phosphatidylinositol from various sources were hydrolyzed at a very low rate (Table 3).

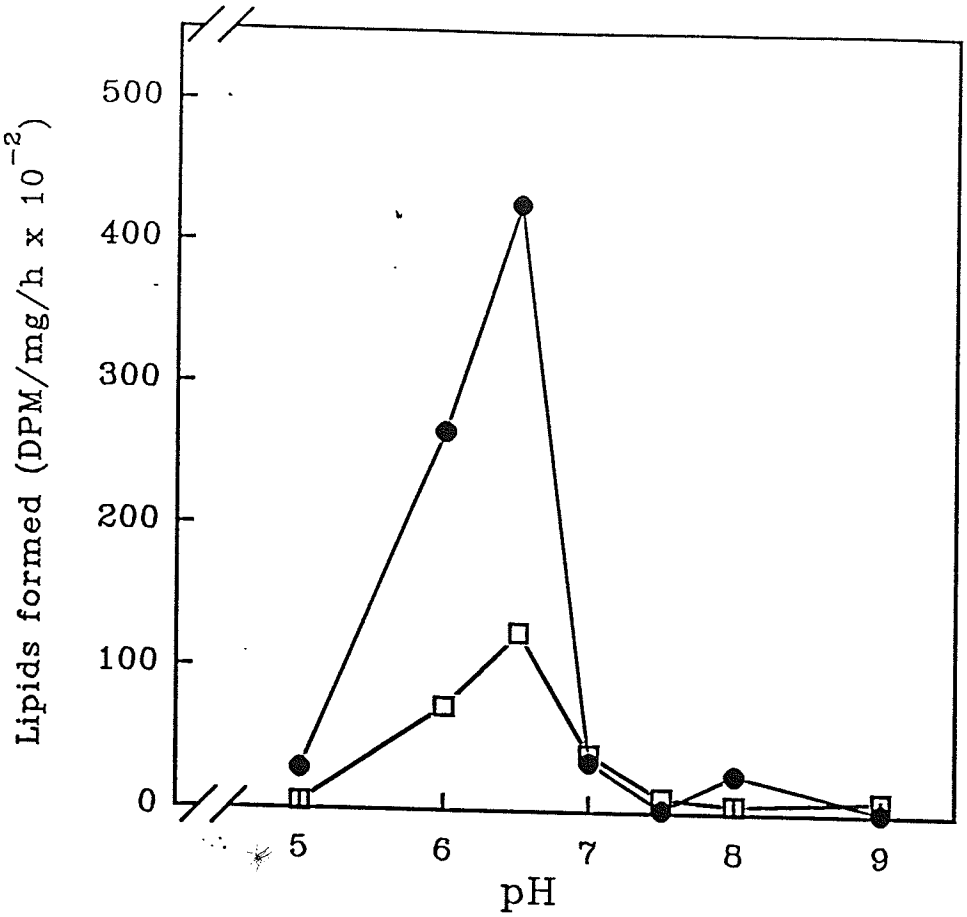


Figure 6. Effect of pH on phospholipase D activity of rat heart sarcolemma

SL membranes were isolated according to Pitts (1979). The buffers were: 50 mM DMGA-NaOH for pH 5.0 to 7.5, and 50 mM glycylglycine-HCL for pH 8.0 and 9.0. Assays were carried out as described in Materials and Methods. Data are from a typical experiment; each point represents the average of triplicate determinations which differed less than 10%. PtdOH (●); DAG (□).

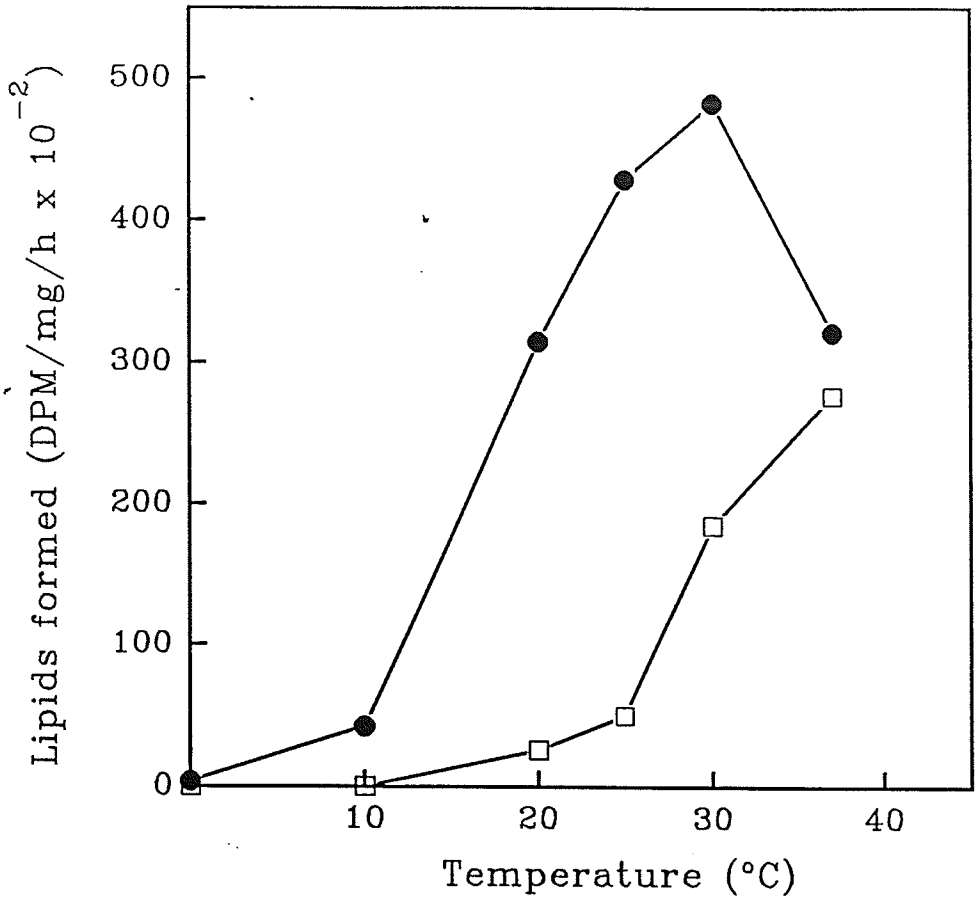


Figure 7. Temperature-dependence of sarcolemmal phospholipase D.

SL membranes were assayed at pH 6.5 and at the indicated temperatures. Data are from a typical experiment; each point represents the average of triplicated determinations which differed less than 10 %. PtdOH (●); DAG (□).

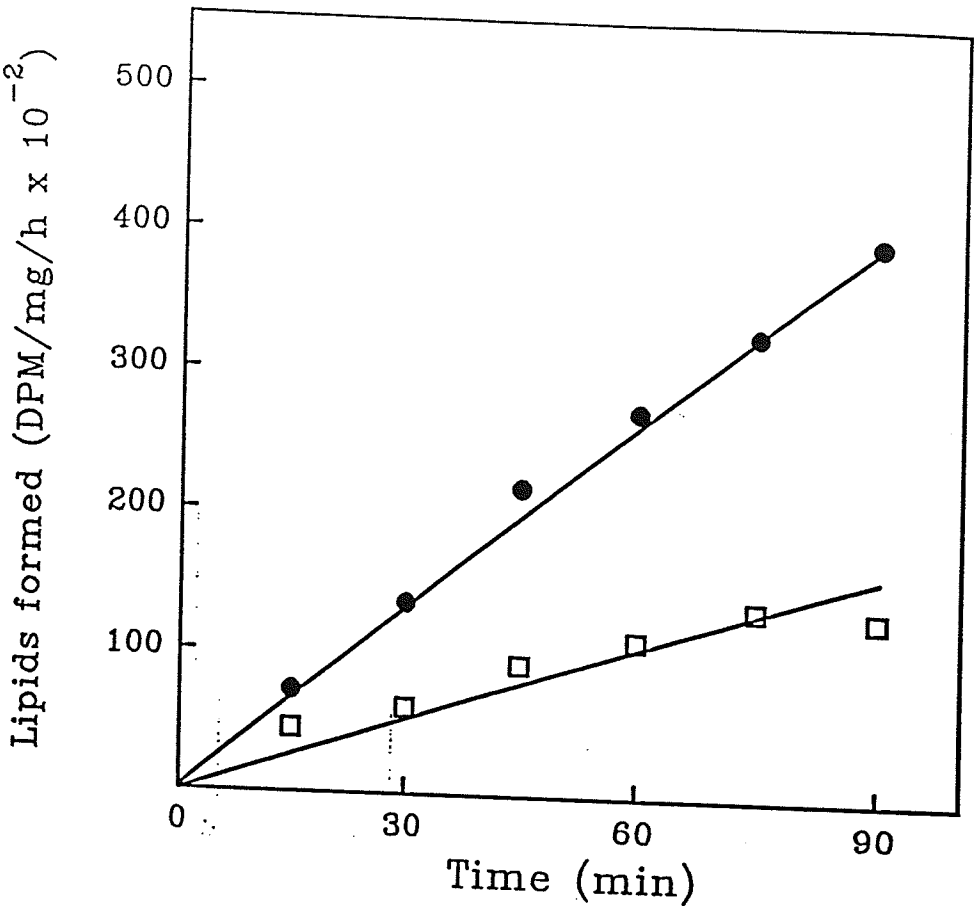


Figure 8. Time-course dependence of sarcolemmal PLD activity

PLD activity was assayed in the presence of SL membranes (50 μg protein) at pH 6.5, 25°C, and for the indicated times of incubation. Data are from a typical experiment; each point represents the average of triplicate determinations which differed less than 10%. PtdOH (●); DAG (□).

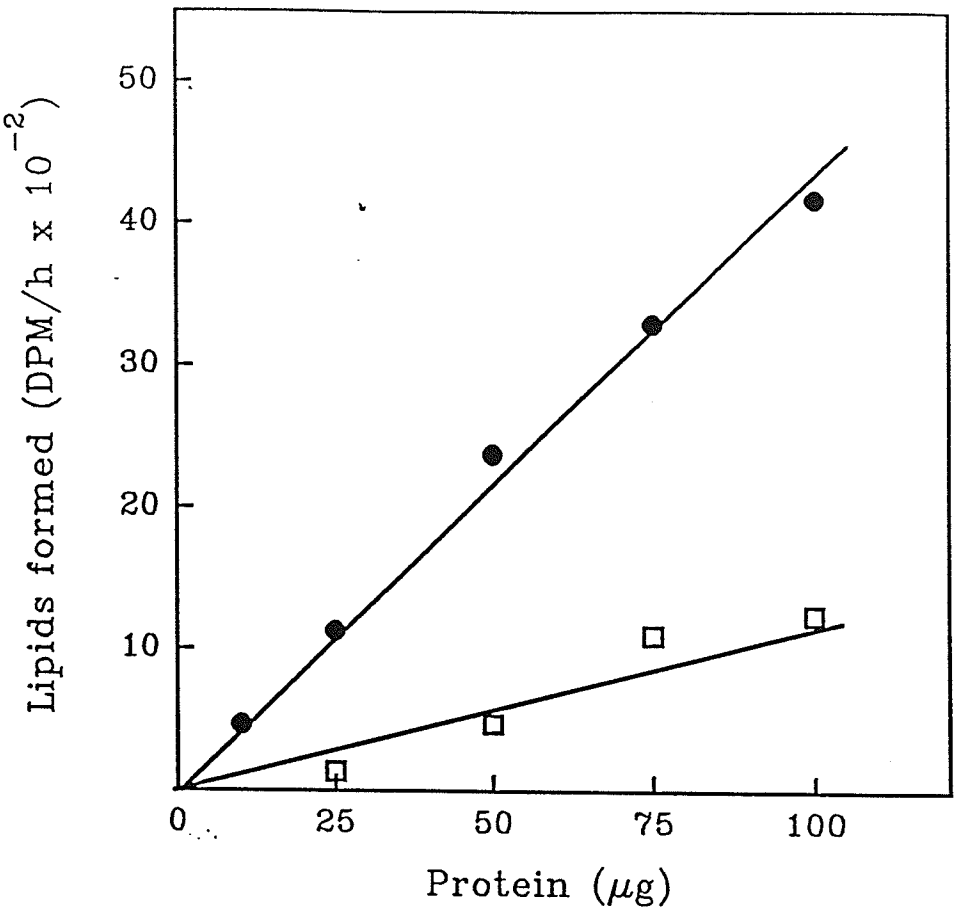


Figure 9. Protein-dependence of sarcolemmal PLD activity

The PLD activity of the indicated amounts of SL protein was estimated at pH 6.5, 25°C, and for a 90 min incubation period. Data are from a typical experiment; each point represents the average of triplicate determinations which differed less than 10%. PtdOH (●); DAG (□).

Table 3. Substrate specificity of sarcolemmal phospholipase D

Phospholipid substrate and source	Phospholipase D (relative activity)
A. Phosphatidylcholine	
egg	100
bovine heart	142.1
bovine liver	102.2
B. Phosphatidylethanolamine	
egg	6.7
bovine liver	6.5
C. Phosphatidylinositol	
soybean	2.8
bovine liver	4.5

Values are average of three experiments done in triplicate (variability < 10%).
SL membrane (50 μg) were assayed at pH 6.5, 25°C and for 90-min incubation periods. Relative activity is expressed as percent of the activity toward phosphatidylcholine from egg (100%). Various phospholipid substrates were prepared as indicated in Materials and Methods by combining: (A) phosphatidylcholine from egg, heart or bovine liver and tracer [^{14}C]phosphatidyl-choline; (B) phosphatidylethanolamine from egg or bovine liver and tracer [^{14}C] phosphatidyl-ethanolamine; (C) phosphatidylinositol from soybean or bovine liver and tracer [^{14}C]phosphatidylinositol.

4. Properties of the sarcoplasmic reticular phospholipase D

An active intracellular PLD was found at the SR level and shared a number of properties such as optimal pH, temperature and KF concentration as well as substrate specificity with the SL enzyme (Table 4). It should be noted that the change of incubation temperature from 25°C to 30°C induced a drop in the SR PtdOH to DAG ratio which was of the same magnitude of that observed with the SL. However, unlike SL, the PtdOH and DAG formation did not change by increasing KF concentration from 25 to 50 mM (Table 4).

B. Regulation of Cardiac PLD by Sulfhydryl Modification and Effects of Free Radicals

1. Effects of thiol group modification on myocardial PLD activity

Chemical modification of the thiol groups was used as an approach to study the importance of these groups for the function of myocardial PLD. To alkylate thiol groups, two reagents which differ in structure and mode of action (Smith, et al., 1975), NEM (5 mM) and methyl methanethiosulfonate (MMTS, 0.1 mM), were used; p-chloromercuriphenylsulfonic acid (pCMPS, 25 μM) was added to induce mercaptide formation, and DTNB (0.3 mM) was applied to elicit thiol/disulfide exchange (Strauss, 1984). In a parallel series of experiments, dithiothreitol (DTT, 1 mM), the threo-isomer of 2,3-dihydroxy-1,4-dithiolbuthane and synthetic reducing agent which protects against thiol group modification (Cleland, 1964), was included in the assay to check the specificity of the changes. As shown in Table 5, all three types of thiol modification, i.e. alkylation (NEM and MMTS),mercaptide formation (pCMPS) and (mixed) disulfide formation (DTNB), reduced the activity of PLD in

Table 4. Influence of various factors on the hydrolytic activity of sarcoplasmic reticular phospholipase D

Experimental conditions	Phosphatidic acid (nmol/mg/h)	1,2 Diacylglycerol (nmol/mg/h)
A. KF (mM)		
10	47.1 ± 4.3	26.2 ± 5.9
25	63.8 ± 1.5	15.0 ± 2.6
50	63.5 ± 0.7	17.2 ± 1.0
B. pH		
6.0	29.2 ± 3.8	5.5 ± 0.1
6.5	53.5 ± 1.3	14.0 ± 0.7
7.0	12.8 ± 1.2	13.6 ± 1.1
C. Temperature(°C)		
20	45.1 ± 3.2	5.3 ± 1.5
25	55.1 ± 2.1	5.6 ± 0.9
30	57.6 ± 3.3	18.2 ± 2.0
D. Phospholipid substrate		
phosphatidylcholine	63.3 ± 2.8	8.9 ± 1.3
phosphatidylethanolamine	1.8 ± 0.6	0.2 ± 0.1
phosphatidylinositol	1.2 ± 0.5	0.4 ± 0.2

Values are means ± SE of three experiments. SR fragments were isolated according to Harigaya and Schwartz (1969). PLD assays related to KF, pH, temperature and substrate were performed as indicated in the legends of Figs 5, 6 and 7 respectively, and as described in Materials and Methods.

cardiac SL as well as SR membranes. The inhibitory effects were prevented by the inclusion of DTT in the assay. At the concentrations used, the degree of inhibition by the alkylating agents NEM and MMTS differed considerably. As shown in Figure 10, both reagents were able to elicit full inhibition of PLD. However, PLD was more sensitive to MMTS (IC_{50}= 35.0 μM) than to NEM (IC_{50}= 2.4 mM). The effectiveness of the thiol group modification on myocardial PLD activity was tested by determining the total thiol groups in SL membranes after pretreatment with different concentrations of NEM, a highly specific thiol alkylating agent. At 0.1, 1 and 10 mM NEM, the number of SH-groups declined to 81.7%, 66.1% and 40.7% of control values, respectively. This relationship was similar to the NEM dependence of SL PLD activity (Figure 10). When thiol groups and PLD activity were examined in the same NEM-pretreated membranes and plotted against each other (Figure 11), a close correlation ($r=0.981$, $p<0.05$) was noted.

2. Effects of oxidants on SL PLD

Partially reduced forms of oxygen can oxidize protein thiol groups (Ferrari, et al., 1991). To examine whether SL PLD could be damaged by this type of oxidation, the activity of this enzyme was assayed in SL membranes that had been separately pre-incubated with each of the systems containing or producing a certain oxidant species. $O_2^{\cdot-}$ produced by xanthine plus xanthine oxidase did not affect SL PLD activity (Table 6). It should be noted that the same xanthine plus xanthine oxidase-treated membranes exhibited a concomitant marked decrease in phospho-inositide kinase activities (Mesaeli, et al., 1993). H_2O_2 (1 mM) induced 26% depression

Table 5. Effect of SH modifiers on phospholipase D activity of rat cardiac sarcolemma and sarcoplasmic reticulum

	Phospholipase D activity (% of control)	
Experimental Conditions	SL	SR
Control	100.0 ± 2.9	100.0 ± 0.9
NEM	65.3 ± 2.0 *	59.0 ± 2.6 *
NEM+DTT	99.3 ± 0.8	92.5 ± 2.7
MMTS	26.0 ± 4.0 *	38.6 ± 2.7 *
MMTS+DTT	98.0 ± 3.6	97.9 ± 6.3
pCMPS	24.3 ± 0.9 *	33.3 ± 1.0 *
pCMPS+DTT	100.0 ± 5.7	96.9 ± 5.1
DTNB	47.7 ± 0.3 *	52.3 ± 1.5 *
DTNB+DTT	102.0 ± 0.6	93.3 ± 7.0

Results are means ± SE of three to six experiments in triplicate. The control values were 165.1 ± 7.1 and 62.3 ± 5.9 nmol PtdOH/mg/h for SL and SR, respectively. The phospholipase D activity was assayed in the presence or absence of different SH modifiers. Final concentrations of N-ethylmaleimide (NEM), methylmethanethiosulfonate (MMTS), p-chloromercuriphenylsulfonic acid (pCMPS), 5,5'-dithio *bis*(2-nitrobenzoic acid) (DTNB), and dithiothreitol (DTT) were 5 mM , 0.1 mM, 25 μM, 0.3 mM and 1 mM, respectively.
* Significantly different ($P < 0.05$) from control values.

Table 6. Effects of reactive oxygen species and hypochlorous acid on phospholipase D activity in rat cardiac sarcolemmal membranes

Treatment	Phospholipase D activity	
	nmol PtdOH/mg/h	% of control
Control	153.9 ± 6.0	100.0
(a)		
X	138.5 ± 5.3	90.0
XO	161.1 ± 12.0	104.7
X+XO	137.0 ± 9.2	89.0
X+XO+SOD	140.0 ± 11.4	91.0
SOD	163.1 ± 13.7	106.0
(b)		
H_2O_2	113.9 ± 5.2*	74.0
H_2O_2+CAT	158.5 ± 10.6	103.0
H_2O_2+MTOL	110.8 ± 11.1*	71.9
CAT	151.4 ± 10.1	98.4
MTOL	161.6 ± 6.3	105.0
(c)		
HClO	10.8 ± 0.3*	7.0
HClO+DTT	146.6 ± 2.9	95.2
DTT	158.4 ± 18.0	102.9

Results are means ± SE of three experiments in triplicate. Sarcolemmal membranes (0.2 mg/ml) were pre-incubated for 20 min at 37°C in 50 mM Tris-HCl (pH 7.4). Final concentrations of the chemicals were as follows: xanthine (X), 2 mM; xanthine oxidase (XO), 0.03 U/ml; superoxide dismutase (SOD), 260 U/ml; hydrogen peroxide (H_2O_2), 1 mM; catalase (CAT), 110 U/ml; D-mannitol (MTOL), 20 mM; hypochlorous acid (HClO), 0.3 mM; dithiothreitol (DTT), 1 mM (b) and 0.3 mM (c). After exposure to superoxide anion (a), hydrogen peroxide (b) or hypochlorous acid (c), membranes were ice-cooled and sedimented at 100,000 g in a Beckman type TLA rotor for 30 min. The pellets were resuspended in 50 mM DMGA-10 mM EDTA (pH 6.5) at a protein concentration of 1.5 mg/ml and assayed immediately for phospholipase D activity as described under Materials and Methods.

* Significantly different ($P < 0.05$) from control values.

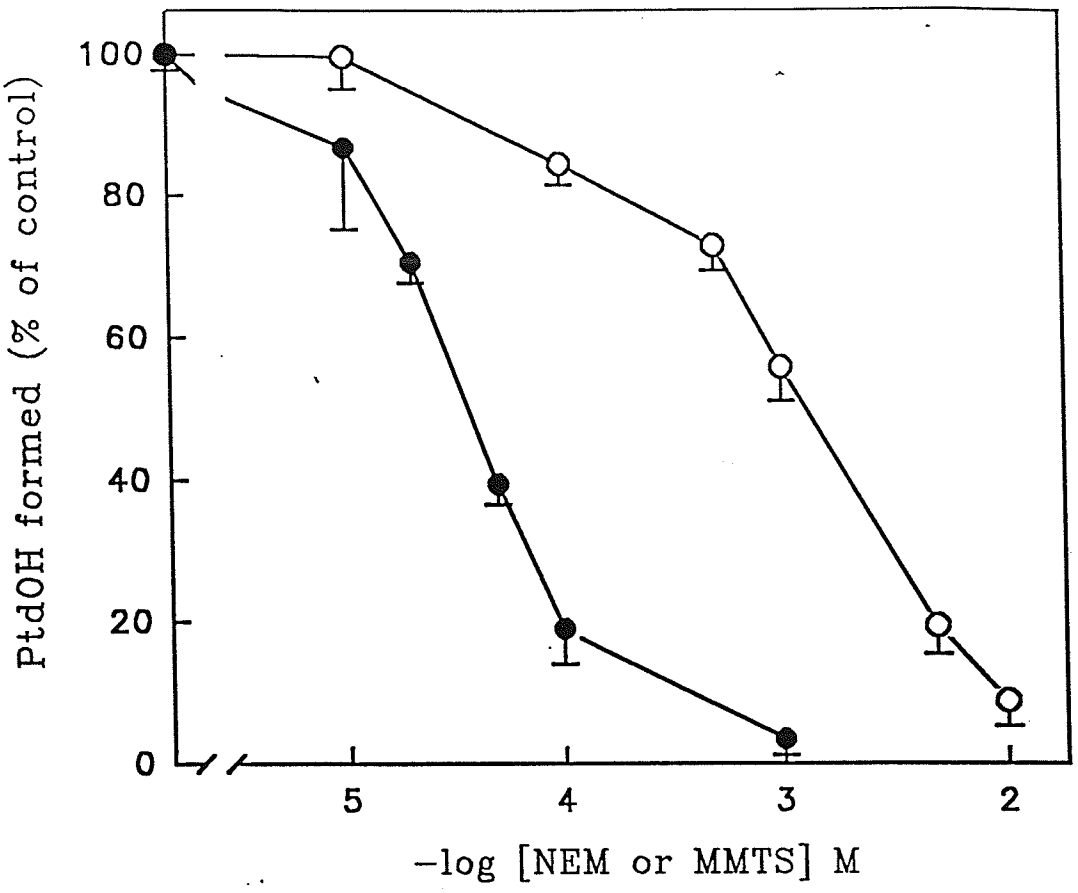


Figure 10. Inhibition of SL PLD activity by varying concentrations of the thiol reagents NEM and MMTS

Results are means ± SE of three different experiments. SL membranes were preincubated in 50 mM DMGA-10 mM EDTA (pH6.5) and 25 mM KF for 10 min in the presence of different concentrations of NEM (O) or MMTS (●). PLD assay was initiated in the same medium by the addition of 2.5 mM [^{14}C] PtdCho as described in Materials and Methods and terminated after 90 min. The PLD activity in the absence of NEM and MMTS was 148.1 ± 10.1 nmol/mg/h.

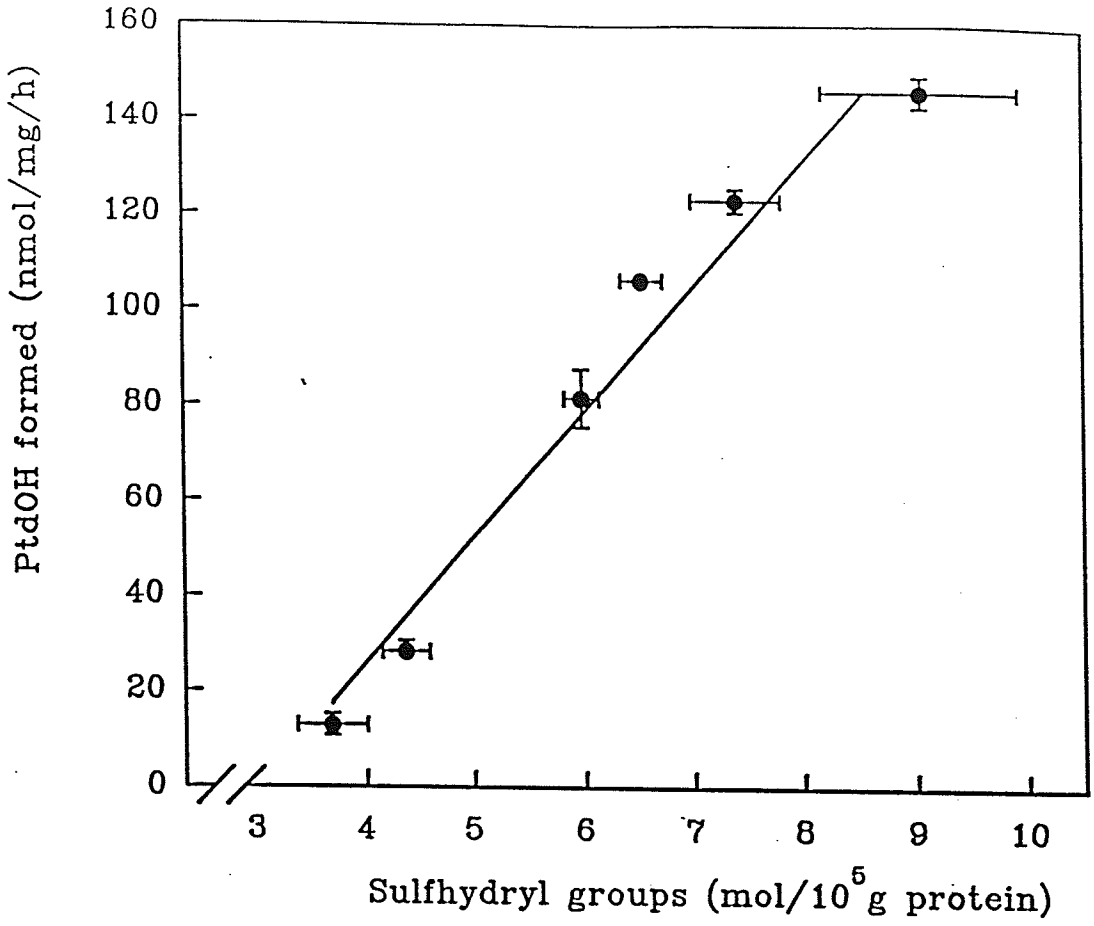


Figure 11. Relationship between the total thiol group content of SL membranes and PLD activity

Results are means ± SE of triplicate determinations in a typical experiment. The SL membranes were preincubated in 50 mM DMGA-10 mM EDTA (pH6.5) for 10 min at 30 °C in the presence of different concentrations of NEM (0, 0.1, 0.5, 1, 5, and 10 mM). The reaction was terminated by twofold dilution with DMGA-EDTA buffer. The final membrane suspension (2.5 mg/ml) was used for assaying the number of total thiol groups and PLD activity as described in Materials and Methods. The linear correlation coefficient ($r=0.981$) was calculated by the least-square method.

in PLD activity. This was prevented completely by catalase (110 U/ml), which had no effect on the enzyme by itself (Table 6). PLD was inhibited by H_2O_2 in a concentration-dependent manner (0.1-10 mM) and was protected by catalase over the entire range (Figure 12). In our experimental conditions, the inhibition of the enzyme could be ascribed to H_2O_2 only. Mannitol (MTOL, 20 mM), a scavenger for hydroxylradicals ($OH^{\cdot}$) (Kaneko, et al., 1989) that might have been formed because of trace amount of Fe^{2+} present as a contaminant (Bast, et al., 1991), showed no protective effect on the H_2O_2-induced PLD changes (Table 6). Fe^{2+} alone had a concentration-dependent inhibitory effect on PLD activity (Figure 13). Therefore, more direct test for the effect of $OH^{\cdot}$ on SL PLD by using H_2O_2 with Fe^{2+} according to the Fenton reaction (Bast, et al., 1991) could not be carried out.

The effects of HClO, which easily oxidizes thiol groups and is produced in neutrophils by the enzyme myeloperoxidase (Halliwell, 1991), was also studied. HClO (0.3 mM) inactivated SL PLD activity (Table 6), and the equimolar concentrations of DTT prevented its inhibitory action. This indicates that oxidation of protein thiol groups associated with PLD is responsible for the HClO effect.

3. Effects of oxidants on SR PLD

The same oxidative systems used to pretreat SL were also applied to SR membranes. The effects on SR PLD activity were similar to those observed for the

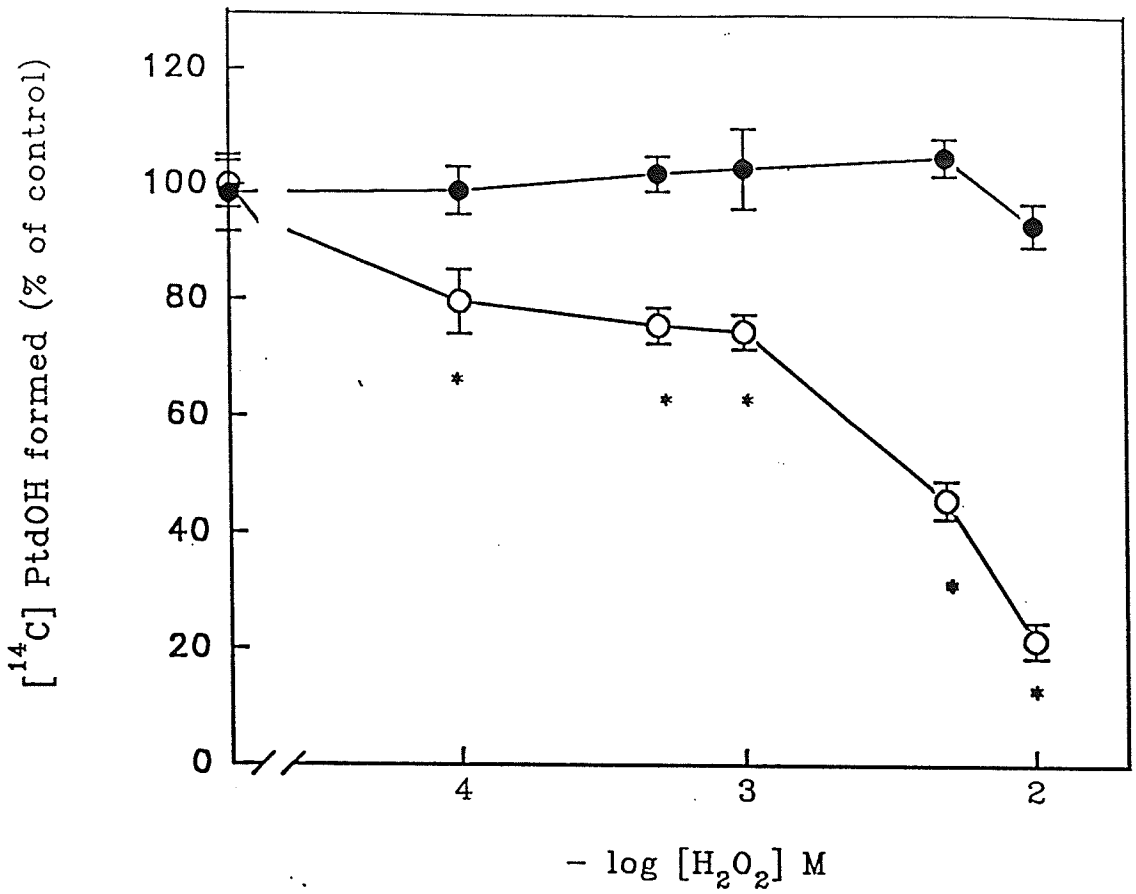


Figure 12. Concentration-dependence of the H_2O_2 effects on SL PLD in the absence or presence of catalase

Results are means ± SE of three experiments and are expressed as percent of the respective control values (154 ± 10.0 and 157 ± 3.6 nmol/mg/h in the absence (O) and presence (●) of catalase, respectively). SL membranes were preincubated at 37°C in 50 mM Tris-HCL (pH 7.4) in the presence of different concentrations of H_2O_2 with or without 110 units/ml catalase. After 20 min, the samples were ice-cooled and centrifuged for 30 min at 100,000 g. The pellets were resuspended in 50 mM DMGA-10 mM EDTA buffer (pH 6.5) and immediately assayed for PLD activity. * $p < 0.05$ vs. the presence of catalase.

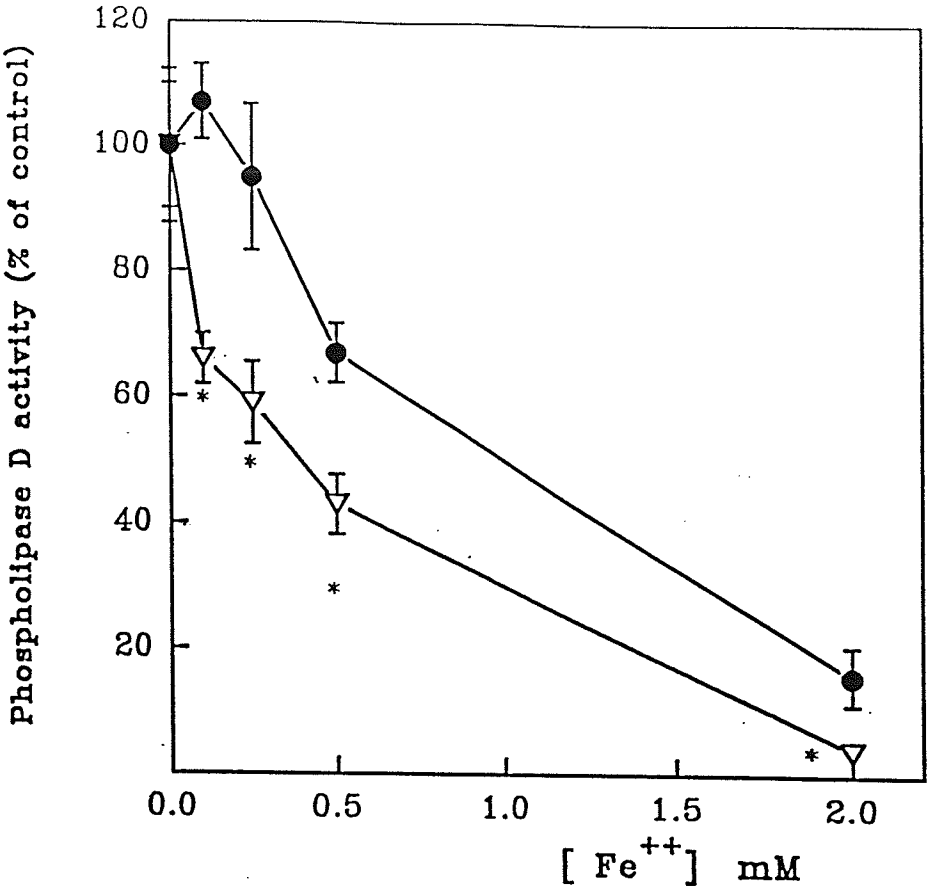


Figure 13. Direct inhibitory effect of Fe^{2+} on sarcolemmal and sarcoplasmic reticular phospholipase D.

Results are means ± SE of three experiments in triplicate. Control values were 166.0 ± 14.3 and 64.9 ± 4.4 nmol/mg/h for SL and SR, respectively. Membranes were pre-incubated with different concentrations of $FeSO_4$ in 50 mM Tris-HCl (pH 7.4) for 20 min at 37°C. The reactions were terminated by ice-cooling, and samples were centrifuged a 100,000 g for 30 min. The pellet was resuspended in DMGA-EDTA buffer and assayed for PLD activity as described in Materials and Methods. SL: (●); SR: (▽). * $p<0.05$ vs. correspondent value from SL.

SL enzyme. Unlike $O_2^{\cdot -}$, H_2O_2 depressed SR PLD by 33%, an effect that was prevented by the presence of catalase (Table 7 (a,b)). SR PLD activity was also severely diminished by 0.3 mM HClO, but only partially protected by an equimolar concentration of DTT. Finally, like in SL, in SR membranes the effect of hydroxyl radicals formed via the Fenton reaction (combination of H_2O_2 and Fe^{2+}) (Halliwell, 1991) could not be tested because Fe^{2+} severely damaged PLD. In this regard, it became apparent that the SR enzyme was significantly more depressed by Fe^{2+} than SL PLD (Figure 13), as also it was more sensitive to H_2O_2 (33% inhibition vs. 26%, Tables 6 and 7) and HClO (incomplete protection by DTT). This suggests that the SR-located enzyme is particularly susceptible to damage.

4. Effects of thiol-reducing reagents on the H_2O_2-induced depression of myocardial PLD

Since DTT is capable of maintaining protein thiol groups in a reduced state because of its low redox potential (Cleland, 1964), its protective action on the H_2O_2-induced depression of SL PLD was examined in detail. DTT, even at 1/10 the concentration of H_2O_2, prevented a significant inhibition of SL PLD, whereas at lower concentrations it showed a partial or no protective effect (Figure 14). DTT itself (10^{-5} to 10^{-3} M) did not modify the PLD activity, indicating the SH-integrity of the enzyme in the SL preparation employed for this study. Furthermore, it was examined whether DTT had merely a preventive effect or whether it was also able to restore H_2O_2-depressed SL PLD activity. Therefore, SL membranes were pretreated with 1 mM H_2O_2 alone, washed, and subsequently assayed for PLD

Table 7. Effects of various oxidant species on phospholipase D activity in rat cardiac sarcoplasmic reticular membranes

Treatment	Phospholipase D activity	
	nmol PtdOH/mg/h	% of control
Control	59.5 ± 3.0	100.0
(a)		
X	63.1 ± 4.4	106.1
XO	60.1 ± 4.2	101.0
X+XO	55.3 ± 4.2	92.9
X+XO+SOD	56.5 ± 3.6	95.0
SOD	64.9 ± 3.6	109.1
(b)		
H_2O_2	39.9 ± 3.0*	67.0
H_2O_2+CAT	53.6 ± 4.8	90.1
CAT	52.4 ± 5.5	88.1
(c)		
HClO	4.9 ± 1.0*	8.3
HClO+DTT	48.0 ± 1.3*	80.6
DTT	60.3 ± 1.3	101.3

Results are means ± SE of three experiments in triplicate. For final concentrations of reagents and details of method see legend to Table 6.

* Significantly different ($P < 0.05$) from control values.

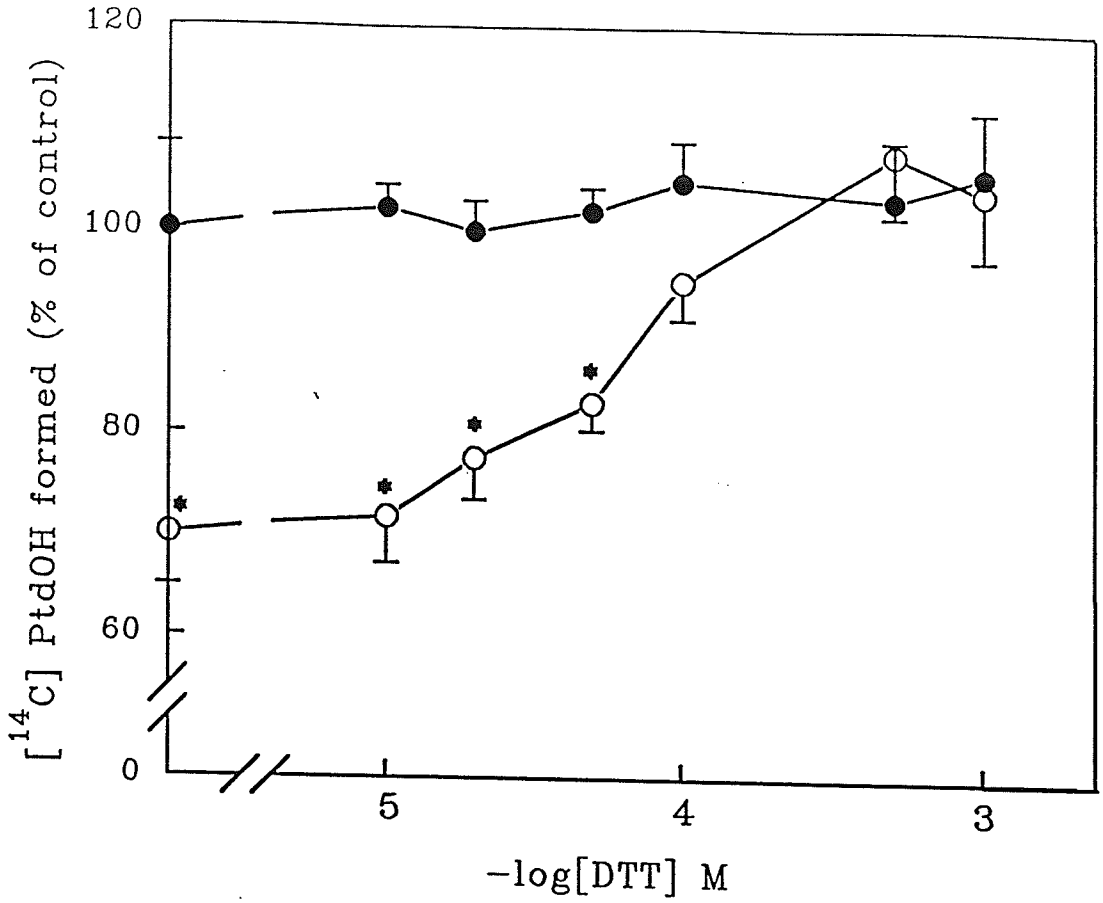


Figure 14. Protective effect of DTT against H_2O_2-induced depression of sarcolemmal PLD

Results are means ± SE of three experiments and are expressed as percent of the control value (no additions) (160.7 ± 14.6 nmol/mg/h). SL membranes were preincubated for 20 min in 50 mM DMGA-10mM EDTA in the presence of different concentrations of DTT with (O) or without (●) 1 mM H_2O_2. Membranes were centrifuged, resuspended, and assayed for PLD activity as described in legend to Figure 13. * $p<0.05$ vs. no H_2O_2.

activity in the presence or absence of 1 mM DTT. This concentration of DTT, that provided complete protection against 1 mM H_2O_2 (Figure 14), gave only a partial reversal of the H_2O_2-induced depression of SL PLD (Figure 15). A possible explanation of this finding is that some thiol residues might not have been accessible to DTT. At any rate, the concentration-effect relations for DTT-induced protection against, or restoration from H_2O_2 damage seem to differ.

To further support the contention that protein thiols are important for the manifestation of the cardiac PLD activity and to confer physiological significance to the present findings, the role of glutathione was investigated. Glutathione is the most abundant cellular thiol that serves as a biological antioxidant and maintains protein thiol groups in a reduced state (Shan, et al., 1990). In untreated SL and SR, PLD was unaffected by the presence of GSH in the assay medium (Table 8), confirming the integrity of PLD-associated thiols in the native membranes. However, the enzyme activity was significantly decreased by the oxidized form of glutathione, GSSG (Table 8 A). When SL and SR membranes were pre-incubated with H_2O_2, the simultaneous presence of GSH precluded the H_2O_2-induced depression of PLD activity (Table 8 B).

C. Regulation of Cardiac PLD by Free Fatty Acids

1. Effect of long-chain fatty acids on cardiac PLD

Long-chain saturated and unsaturated fatty acids (FA) were examined as possible activators of the PLD-dependent hydrolysis of exogenous [^{14}C]PtdCho

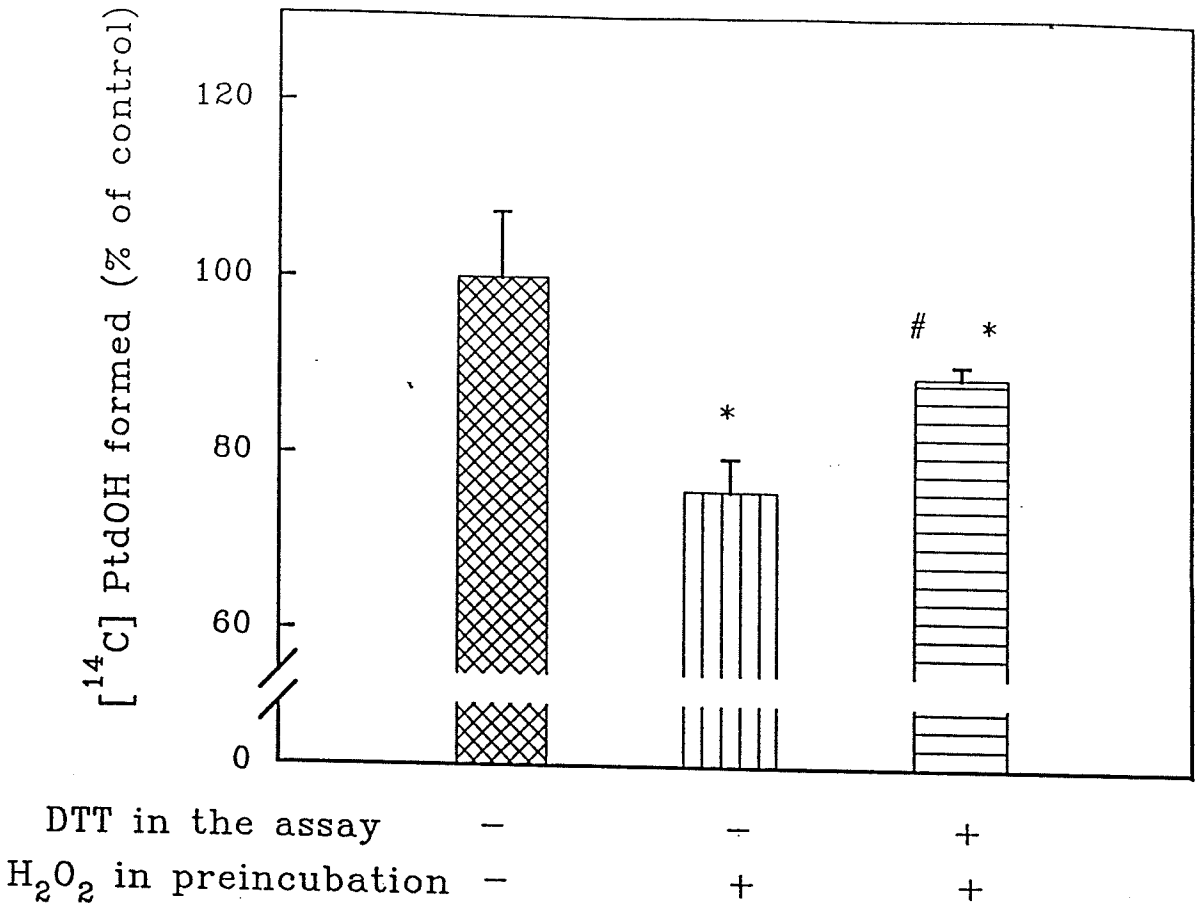


Figure 15. The partial restoration of the H_2O_2 impaired SL PLD activity by DTT

Results are mean ± SE of four experiments and are expressed as percent of the control value (145.9 ± 8.3 nmol/mg/h). SL membranes were preincubated with or without 1 mM H_2O_2 in the absence of DTT. After 20 min, the samples were ice-cooled and processed as indicated in the legend to Figure 13. PLD activity was assayed in the absence or presence of 1 mM DTT. * $p<0.05$ vs. the control value (cross hatched bar); # $p<0.05$ vs. H_2O_2 value (vertically hatched bar).

Table 8. Effect of glutathione on phospholipase D activity of rat heart sarcolemma and sarcoplasmic reticulum.

Treatment	Phospholipase D activity	
	nmol PtdOH/mg/h	
	SL	SR
A.		
Control	143.7 ± 8.0	61.6 ± 4.3
GSH	133.6 ± 12.9	56.7 ± 1.7
GSSG	89.1 ± 1.8 *	39.4 ± 2.5 *
B.		
Control	148.7 ± 3.0	55.5 ± 2.8
H_2O_2	110.0 ± 2.2 *	40.0 ± 1.2 *
H_2O_2+GSH	144.2 ± 4.3	50.0 ± 2.5

Results are means ± SE of three to four different experiments.
A. Phospholipase D was assayed in the absence or presence of 5 mM reduced glutathione (GSH) or 5 mM glutathione disulfide (GSSG).
B. SL or SR vesicles were pre-incubated in the presence of 1 mM H_2O_2 with or without 5 mM GSH. For preincubation, centrifugation and resuspension procedure see legend to Table 6. The phospholipase D assay was performed as described in Materials and Methods. * Significantly different ($P < 0.05$) from correspondent control.

(Table 9). SL proteins were exposed to different FA concentrations (3 and 5 mM). No PLD activity was observed where 3 or 5 mM Na^+ concentrations were present in the assay mixture deprived of FA. The enzyme activity was latent with saturated FA of increasing chain length, i.e., myristate (14:0), palmitate (16:0) and stearate (18:0). The presence of a single double bond was always stimulatory, and oleic acid (18:1, cis-9) at a concentration of 5 mM was the most potent of all the FA tested. The effect of cis-9 monounsaturated fatty acids was not dependent on the length of the acyl chain, as the efficacy sequence was: oleate (18:1) > myristoleate (14:1) > palmitoleate (16:1) (Table 9). Comparison between oleic and elaidic acids showed that the cis configuration of the FA was important in determining their effect on PLD. In the case of FA with equal acyl chain length (18 carbons), the efficacy of stimulation diminished at higher degrees of unsaturation, but was not related to the number of double bonds [oleate (18:1) > linolenate (18:3) > linoleate (18:2)]. Interestingly, arachidonate (20:4, cis-5, 8, 11, 14), an important polyunsaturated fatty acid and intermediate of lipid metabolism, induced 10 % less activation of PLD than oleate at 5 mM concentration, but displayed double potency at lower concentration (3 mM, Table 9). Both petroselenate (18:1, cis-6) and vaccenate (18:1, cis 11), that are analogues of oleate (18:1, cis 9) with different locations of the single double bond, activated PLD almost to the same extent as oleate (Table 9), suggesting that the position of the double bond is not a structural prerequisite for the fatty acids' effect. Methylesterification of the FA carboxyl group (e.g., methyloleate and methylarachidonate) abolished PLD stimulation, while carboxyl replacement with an alcohol group (e.g., oleyl and arachidonyl alcohols) resulted in a small activation of the enzyme. This suggests that the negatively charged carboxyl group of the FA

Table 9. Hydrolytic activity of heart sarcolemmal phospholipase D in the presence of different long-chain fatty acids and derivatives

	[^{14}C]PtdOH (% of maximal formation)	
	3 mM	5 mM
Myristate (14:0)	0.04 ± 0.04	1.30 ± 0.08
Palmitate (16:0)	0.20 ± 0.10	0.60 ± 0.10
Stearate (18:0)	0.50 ± 0.12	1.20 ± 0.04
Myristoleate (14:1, cis-9)	24.40 ± 7.10	71.80 ± 1.50
Palmitoleate (16:1, cis-9)	4.00 ± 0.53	20.50 ± 0.69
Oleate (18:1, cis-9)	32.50 ± 2.00	**100.00 ± 3.59**
Elaidate (18:1, trans-9)	0.70 ± 0.08	10.30 ± 0.69
Linoleate (18:2, cis-9,12)	20.00 ± 1.27	45.00 ± 2.53
Linolenate (18:3, cis-9,12,15)	26.80 ± 1.59	75.40 ± 1.55
Arachidonate (20:4, cis-5,8,11,14)	73.60 ± 2.45	91.70 ± 3.88
Petroselenate (18:1, cis-6)	28.70 ± 4.30	94.90 ± 4.40
Vaccenate (18:1, cis-11)	27.90 ± 7.80	91.40 ± 2.70
Methyl-Oleate	0.50 ± 0.08	0.90 ± 0.08
Methyl-Arachidonate	0.60 ± 0.08	1.40 ± 0.33
Oleyl alcohol	2.40 ± 0.20	2.90 ± 0.06
Arachidonyl alcohol	6.80 ± 3.80	10.20 ± 4.30

Values are means ± SE of three experiments in triplicate done in the absence or presence of various fatty acids (sodium salts) or derivatives. The hydrolytic activity of SL PLD was assayed with 2.5 mM [^{14}C]PtdCho as an exogenous substrate as described in Materials and Methods, and was maximal (287.71 ± 10.32 nmol PtdOH/mg/h) with 5 mM oleate. Various fatty acids, e.g. oleate, are characterized in sequence by their chain length (e.g.,18), number of double bonds (e.g.,1), cis vs. trans conformation of the double bond (e.g.,cis), and the locants of the double bonds (e.g.,9).

is required for stimulating PLD. Furthermore, the lack of effect by methyloleate and methylarachidonate excluded the possibility that PLD activation was due to oxidation products (Philipson and Ward, 1985).

The different efficacy of the fatty acids at 3 and 5 mM (Table 9) implied a concentration-dependence of their effect on PLD. Figure 16 details this relationship for the most active mono- and polyunsaturated FA. Oleate, linolenate and linoleate had their peak activity at 5 mM as well as very close EC_{50} values (3.27, 3.22 and 3.07 mM, respectively). Arachidonate showed the lowest EC_{50} value (2.66 mM) and equalled the peak activity of oleate, but at lower concentration (4 mM). These data confirm that the potency of unsaturated FA is not related to the number of double bonds, and indicate arachidonate and oleate as major activators of SL PLD. The percent stimulation by oleate and arachidonate on the PLD activity of SL and SR membrane was similar (Figure 17, 18), which suggest that SL PLD and SR PLD share, at least to a certain extent, the same regulatory mechanism(s) of fatty acid activation.

Fatty acids are negatively charged amphophiles, and it could not be excluded that their PLD activation was due to a detergent-like action on the membrane structure (van der Vusse, 1982). However, this did not seem to be the case since all three types of detergents tested (anionic, zwitterionic and non-ionic) had very weak effects on PLD (Table 10). Among them, taurodeoxycholate and deoxycholate showed the highest effects which, at any rate, were marginal if compared to that of 5 mM oleate. With the exception of taurodeoxycholate, the peak activities of the

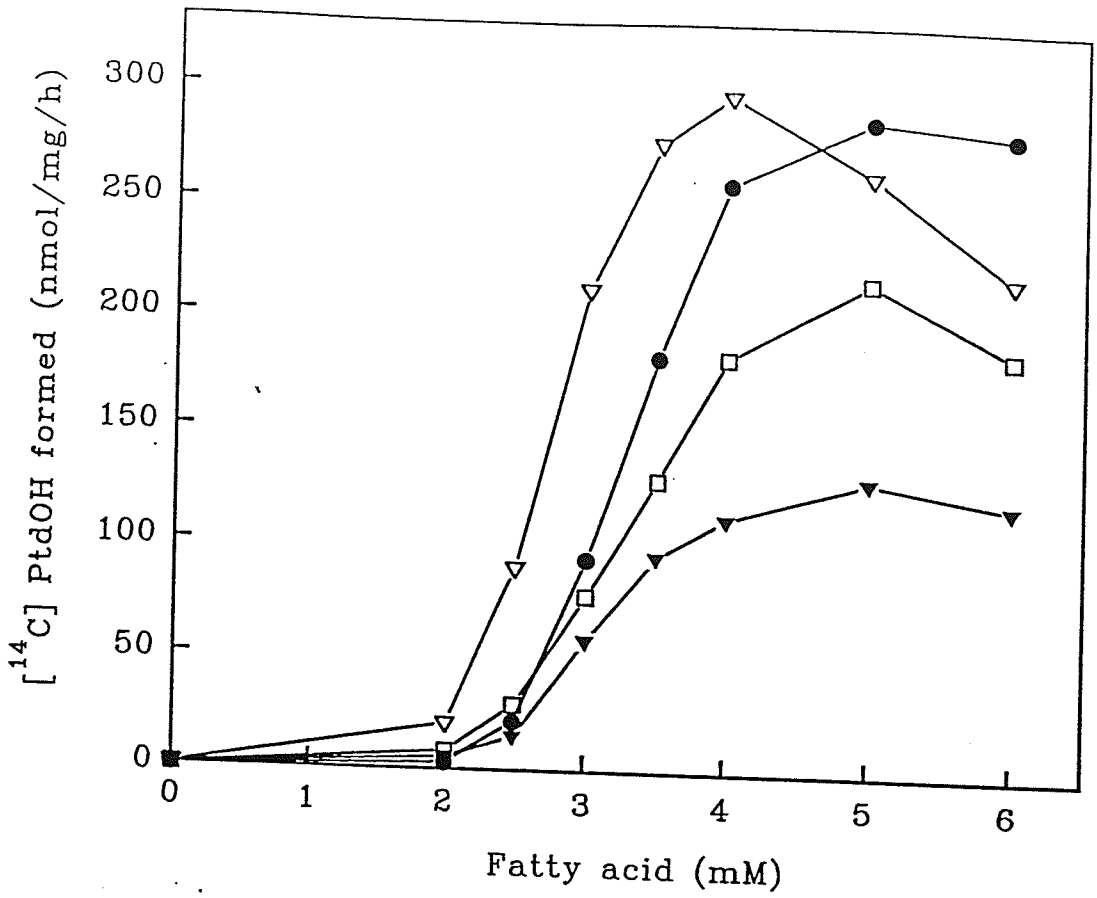


Figure 16. Concentration dependent effect of fatty acids on the activation of SL hydrolytic activity assayed with exogenous [^{14}C]PtdCho

SL membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5) for 60 min at 25 °C in the absence or presence of different concentrations of oleate (●), linolenate (□), linoleate (▼) or arachidonate (▽), respectively. Methodological details are described in Materials and Methods. Each data point is the average of three different experiments in triplicate (CV < 0.12).

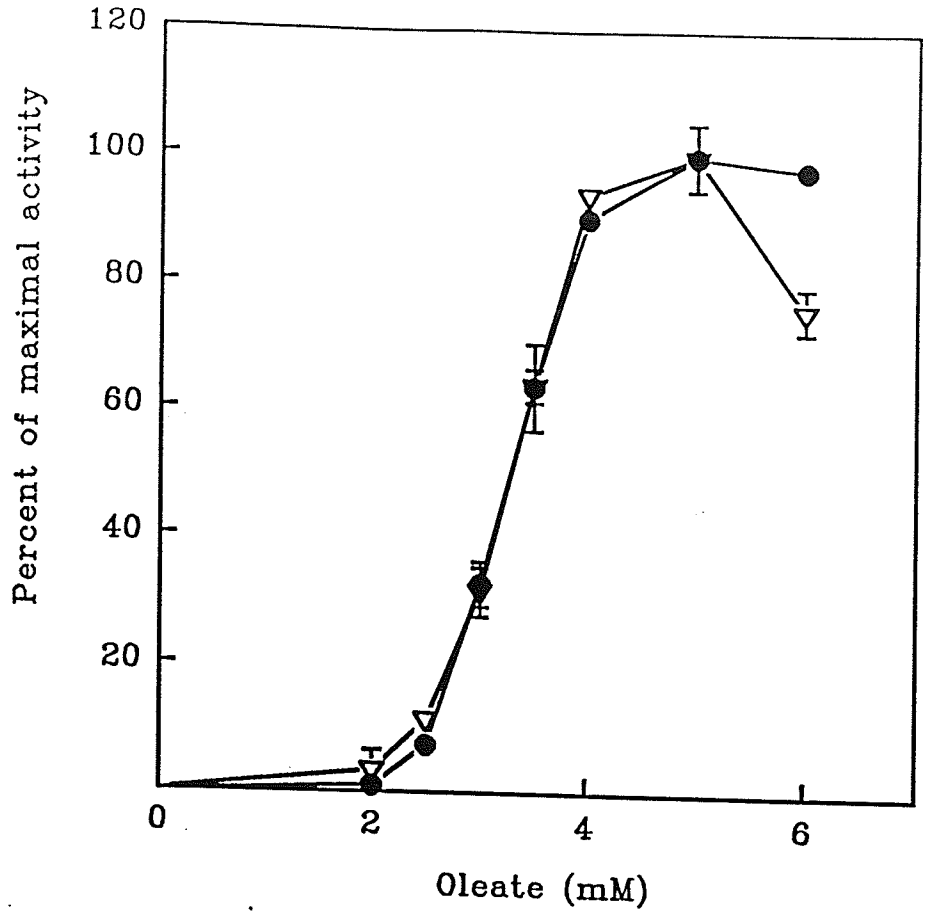


Figure 17. Concentration dependent effect of oleate on the activation of PLD activities of SL and SR

SL or SR membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5) for 60 min at 25 °C in the presence of different concentrations of oleate. Methodological details are described in Materials and Methods. Results are means ± SE of three experiments in duplicate. SL: (●); SR: (▽).

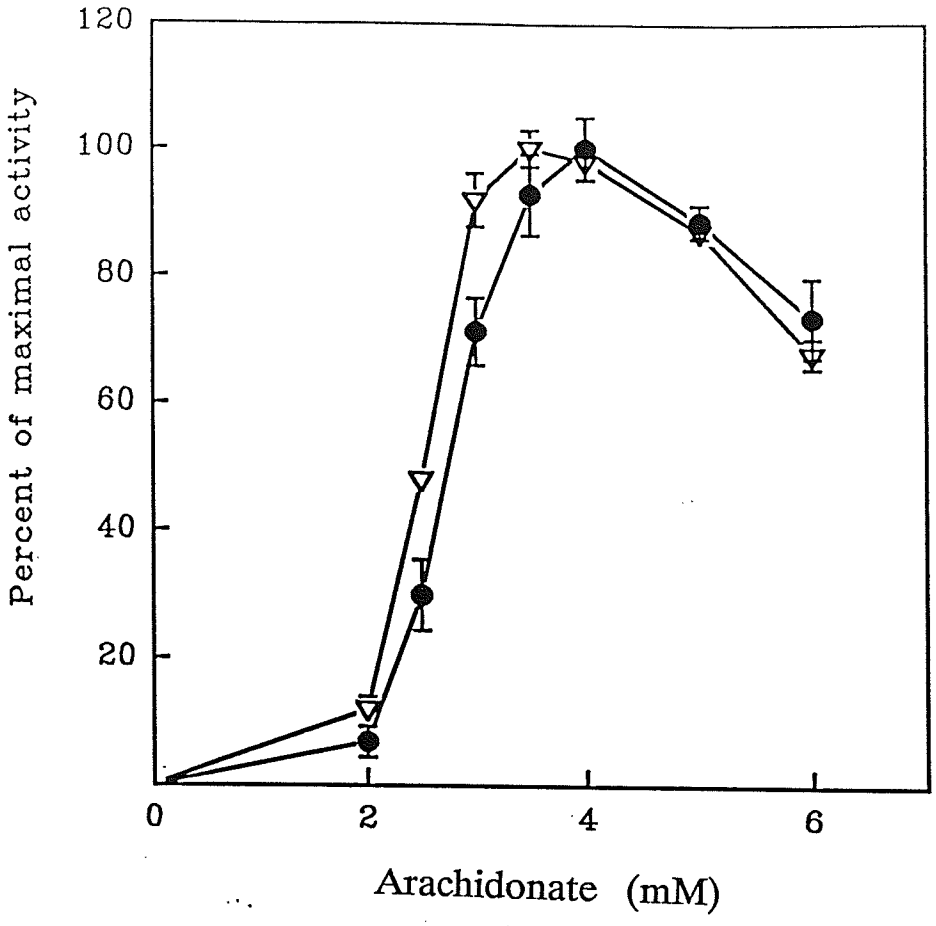


Figure 18. Concentration dependent effect of arachidonate on the activation of PLD activities of SL and SR

SL or SR membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5) for 60 min at 25 °C in the presence of different concentrations of arachidonate. Methodological details are described in Materials and Methods. Results are means ± SE of three experiments in duplicate. SL (●); SR (▽).

detergents were not observed at their critical micellar concentration (CMC) (Neugebauer, 1990) where the physical status of the lipid molecules changes. Even in the case of taurodeoxycholate, however, the detergents's maximal activity occurred at CMC value but differed little from the activities observed at concentrations below or above CMC (Table 10). In accordance with the results obtained by assessing PLD in the hydrolytic mode, 5 mM oleate maximally enhanced PLD transphosphatidylation activity while at 3 mM concentration arachidonate was markedly more potent than oleate (Table 11). Taurodeoxycholate, at equal molarities, had only marginal effects.

2. Stimulation of PLD-dependent hydrolysis of intraSL Ptd[^{3}H]Cho by arachidonate and oleate

The millimolar concentrations of FA needed to observe PLD activity in the presence of exogenous substrate were beyond the *in vivo* cardiac FA levels (Vork, et al., 1993). In our assay system, therefore, FA might have been merely required to solubilize the exogenous [^{14}C]PtdCho substrate, thus making it accessible to the PLD active site. To exclude this possibility, we examined the hydrolytic activity of SL PLD against [^{3}H] prelabeled endogenous PtdCho, in the presence of the most potent FA, arachidonate and oleate. Unlike the exogenous substrate, a basal rate of endogenous Ptd[^{3}H]Cho hydrolysis was detectable in the absence of FA (Figure 19). Both FA stimulated the PLD-dependent hydrolysis of intramembranal PtdCho in a concentration-dependent fashion. Their stimulatory effect was already

Table 10. Effect of detergents on the hydrolytic activity of sarcolemmal PLD

	[^{14}C]PtdOH (% of maximal oleate-stimulated formation)					
	Detergent (mM)					
	0.3	2	4	6.5	11	C.M.C
Taurocholate	0.71	0.49	0.69	1.63	0.78	11.0
Taurodeoxycholate	0.56	6.62	7.67	6.60	1.27	4.0
Deoxycholate	0.62	1.47	5.28	5.36	3.18	4.0
Triton X-100	0.55	0.38	0.55	0.25	1.20	0.3
CHAPS	0.26	0.13	0.38	0.26	0.39	6.5

Each value is the average of three experiments done in triplicate (CV <0.1) and is expressed as percent of the oleate (5mM)-stimulated PLD activity (276.1 ± 19.22 nmol PtdOH/mg/h, 100%). PLD hydrolytic activity was assayed with 2.5 mM [^{14}C]PtdCho exogenous substrate as described in Materials and Methods. Taurocholate, taurodeoxycholate, and deoxycholate sodium salts as well as Triton X-100 and CHAPS were added as fresh aqueous solutions to give the indicated final concentrations. Critical micelle concentration (CMC) values (mM) were from Neugebauer (1990). CHAPS: 3-[(3-cholamidopropyl)-dimethylammonio]-ropanesulfonate.

Table 11. Fatty acid and detergent effect on the transphosphatidylation activity of sarcolemmal PLD

	Phosphatidylethanol formation	
	nmol/mg/h	
(Sodium salts)	3 mM	5 mM
Oleate	36.27 ± 6.60 (12.5%)	288.95 ± 51.29 (100%)
Arachidonate	117.84 ± 23.50 (40.8%)	201.35 ± 39.18 (69.7%)
Taurodeoxycholate	5.26 ± 0.83 (1.8%)	7.50 ± 1.13 (2.6%)

Values are means ± SE of three experiments and are expressed as nmol phosphatidylethanol formed/mg/h. Data in parentheses are percentage of phosphatidylethanol formation in the presence of 5 mM oleate. The transphosphatidylation assay was performed with 400 mM ethanol and 2.5 mM [^{14}C]PtdCho as described in Materials and Methods. Fatty acid and detergent sodium salts were used as indicated in the footnotes of tables 9 and 10.

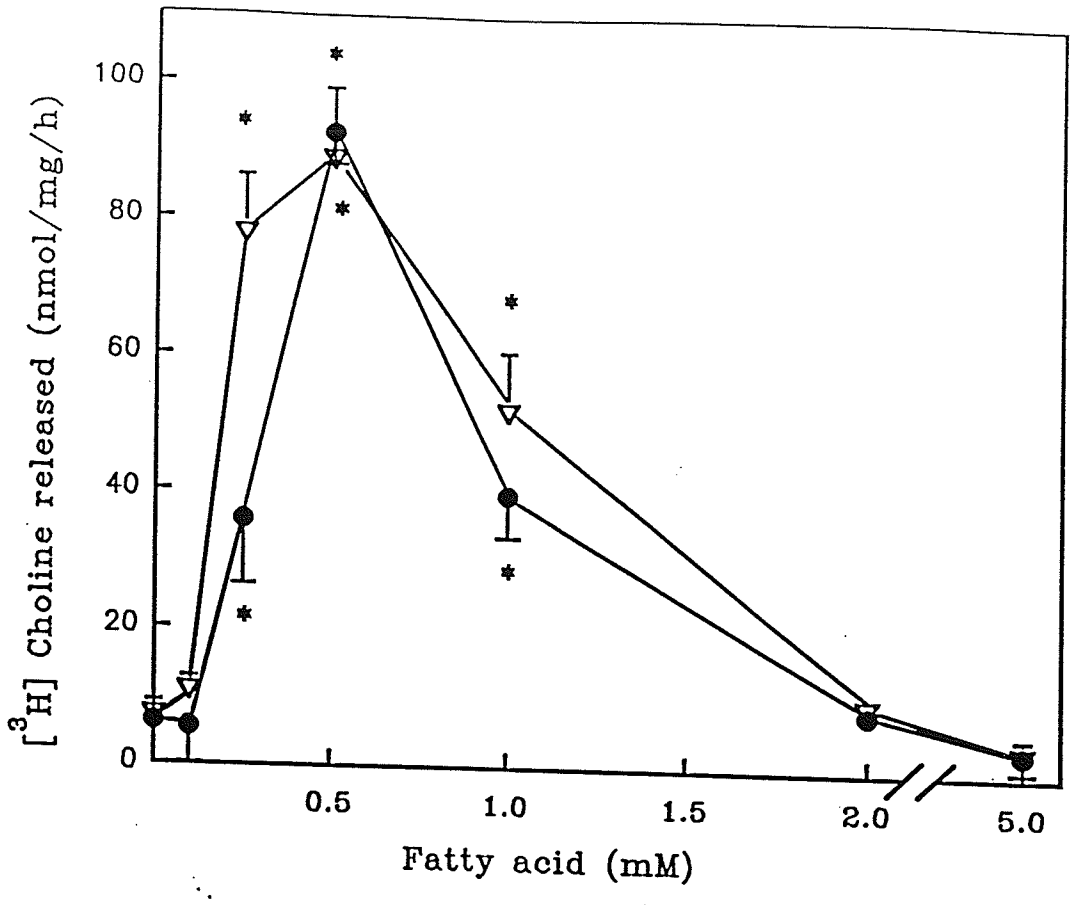


Figure 19. Concentration dependence of the most effective fatty acid activators on SL PLD activity assayed with endogenous Ptd[^{3}H]Cho.

The results are means ± SE of three experiments in triplicate. Prelabeled SL membranes containing Ptd[^{3}H]Cho ([choline-methyl-^{3}H]) were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 5 mM phosphorylcholine for 60 min at 25 °C in the absence or presence of different concentrations of oleate (●) or arachidonate (▽). Further details on the prelabeling of intraSL PtdCho and on extraction, separation and quantification of [^{3}H] choline are described in Materials and Methods. * Significantly different ($p < 0.05$) from control (no FA).

significant at 0.25 mM, maximal (13-fold enhancement over basal values) at 0.5 mM, and progressively declining at higher concentrations. However, like the exogenous substrate (Figure 16), arachidonic acid was more efficient in stimulating PLD because its calculated EC_{50} value (0.177 mM) was markedly lower than that of oleic acid (0.268 mM).

3. Effect of melittin and mepacrine on SL PLD

In a further series of experiments, a relationship was sought between phospholipase A_2 (PLA_2)-dependent release of FA and stimulation of SL PLD. PLA_2 is present in heart SL (Weigliki, 1980; Bentham, et al., 1987), and removes the fatty acid from the sn-2 position of the membrane phospholipids to form lyso-phospholipids. [^{14}C] FA release and [^{14}C]PtdOH formation were quantitated upon incubating SL in the presence of exogenous l-α-1-palmitoyl-2-oleoyl[oleoyl-1-^{14}C]PtdCho at different concentrations of melittin. This is a polypeptide from bee venom (Haberman and Jensch, 1967) that stimulates membrane-bound PLA_2 activity (Shier, 1979) with subsequent release of FA. Sodium taurodeoxycholate, that solubilizes unrelated membrane proteins rather than PLD (Kanfer, 1991), was also present in the assay to detect basal PLD activity. The release of [^{14}C] oleic acid from the sn-2 position of [^{14}C]PtdCho was taken as reflecting the total amount of FA formed by the melittin-induced PLA_2 activation. As illustrated in Figure 20, a direct and close correlation ($r = 0.99$, $p < 0.05$) was noted between melittin-related FA release and PtdOH formation. To exclude the possibility that part of PLA_2 activity was due to contamination of commercial melittin, PtdCho-hydrolysis was tested in

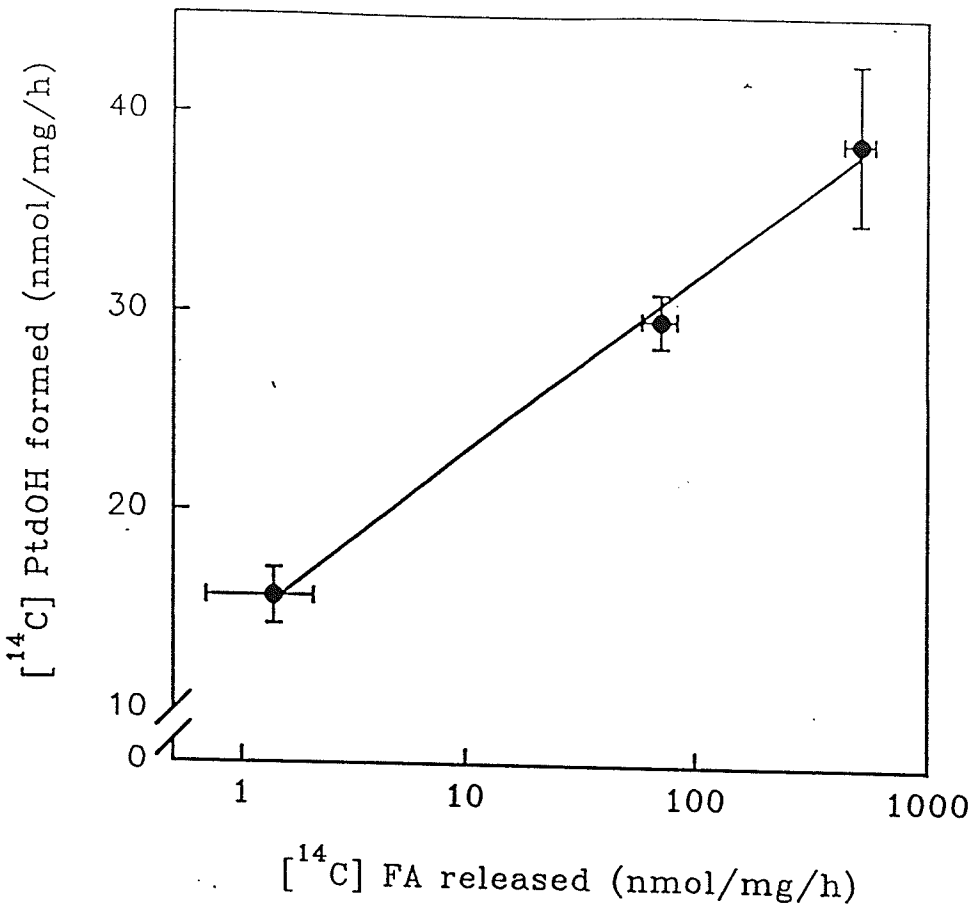


Figure 20. Relationship between fatty acid release and phosphatidic acid formation in the presence of melittin.

Results are means ± SE of three experiments in triplicate. SL membranes were assayed for PLD activity in a medium containing 50 mM DMGA-10mM EDTA, 3 mM taurodeoxycholate, and 2.5 mM L-α-1-palmitoyl-2-oleoyl[oleoyl-1-^{14}C]PtdCho (pH6.5) for 60 min at 25 °C, in the presence of different concentrations of melittin (0, 0.5, 5 μg/25 μg SL). Lipid extraction, separation and quantification were as described in Materials and Methods. After thin layer chromatography, FA and PtdOH spots were visualized by autoradiography (Kodak X-OMAT AR) and identified by comparison with unlabeled lipid standards (oleic acid, PtdOH) which were run in parallel and detected by iodine vapor. The correlation coefficient (r=0.998) was calculated by the least-square method.

the presence of melittin alone. The production of FA by 1 μg or 10 μg melittin in the absence of membranes was only 3% and 1% of that detected in the presence of SL membranes, respectively. This indicated that only SL-bound PLA_2 activity was responsible for FA release and subsequent PLD stimulation. Consistent with a previous report (Chalifour and Kanfer, 1982), lysophosphatidylcholine was found to have no effect on SL PLD.

One of the endogenous sources of fatty acids are the acyl chains of phospholipids (van der Vusse, et al., 1992). Myocardial phospholipids show positional specificity with preferential location of the unsaturated long chain FA at the sn-2 position of the glycerol moiety (Lamers, et al., 1987). We sought specific evidence for PLD activation by endogenous FA originating from intra-membranal phospholipid catabolism, e.g. via PLA_2. Therefore, SL membranes containing Ptd[^{3}H]Cho were incubated with increasing amounts of melittin to activate PLA_2 and liberate FA inside the membrane (Witter and Kanfer, 1985). Melittin in a range of 0.5 to 10 μg/25 μg SL enhanced PLD activity in a dose-dependent manner with attenuation of the response at the highest concentration of the drug (Figure 21). Micromolar concentrations of mepacrine inhibit myocardial PLA_2 activity and subsequent release of FA at the cell membrane (Sen, et al., 1988; Armstrong and Ganote, 1991). Figure 22 shows that the enhancement of PLD activity mediated by melittin via PLA_2 could be partially but significantly blocked by mepacrine in a concentration-dependent mode. Mepacrine alone did not affect the basal activity of PLD (Figure 22). One of the possible explanations for incomplete action of mepacrine is that some of the specific sites of the PLA_2 molecule and/or its

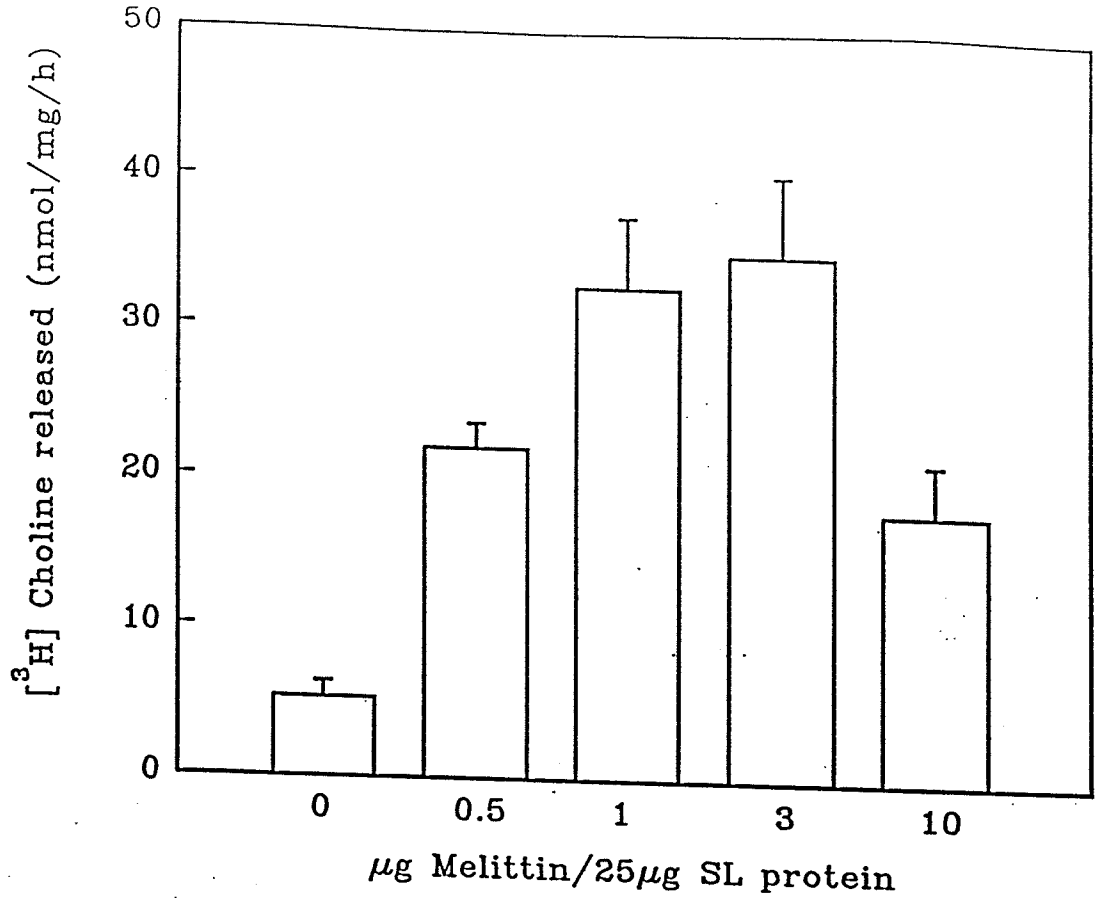


Figure 21. Effect of melittin on endogenous Ptd[^{3}H]Cho hydrolysis by sarcolemmal phospholipase D

The results are means ± SE of three experiments in triplicate. Prelabeled SL membranes containing Ptd[^{3}H]Cho were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5) and 5 mM phosphorylcholine for 60 min at 25 °C in the presence or absence of different concentrations of melittin.

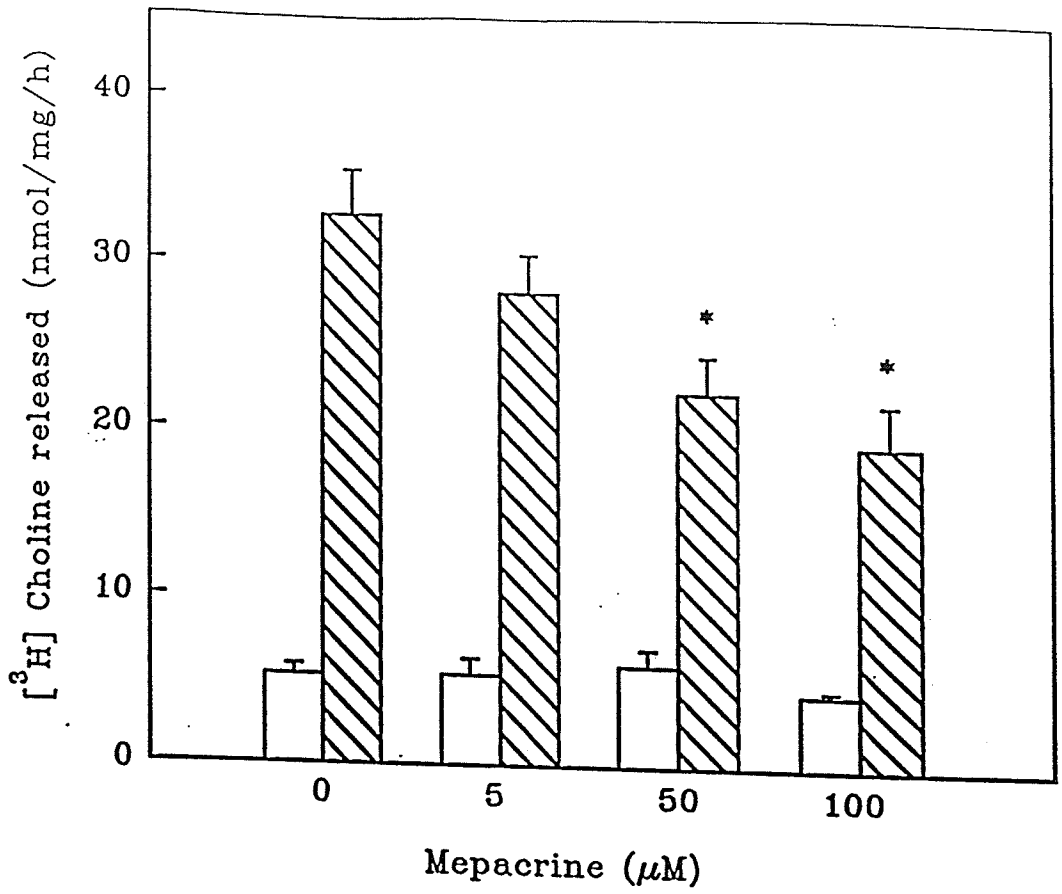


Figure 22. Inhibitory effect of mepacrine on the melittin-induced hydrolysis of endogenous Ptd[^{3}H]Cho by phospholipase D

The results are means ± SE of three experiments in triplicate. [^{3}H] Choline-prelabeled SL membranes were incubated with 50 mM DMGA-10 mM EDTA(pH 6.5), and 5 mM phosphorylcholine for 60 min at 25 °C with different concentrations of mepacrine in the absence (□) or presence (▨) of 1 μg melittin. * Significantly ($p < 0.05$) different from control (▨:25 μg SL/1 μg melittin, and 0 mepacrine).

environment that were accessible and responsive to melittin, might have not been accessible to mepacrine.

D. Cardiac PLD Activity in Ischemia-Reperfusion Injury

1. Effect of ischemia-reperfusion on sarcolemmal and sarcoplasmic reticular PLD activities

As already mentioned, the intramembranal formation of PtdOH via PLD is related to Ca^{2+} movement within the cardiomyocyte and may influence the heart function in normal conditions as well as in disease states characterized by abnormal Ca^{2+} homeostasis. One of the features of ischemia-reperfusion injury is the abnormal Ca^{2+} homeostasis. Therefore, changes in PLD activity might occur.

The above described results showed that while SL and SR PLD shared some features in their regulatory mechanisms, such as sulfhydryl group dependence and fatty acid activation, they displayed significantly different responses to Fe^{2+} ions. Therefore, in an attempt to detect possible changes of PLD activity in ischemia and reperfusion conditions, both SL and SR membrane preparations were used. PLD was assayed in the presence of sodium taurodeoxycholate, to express the basal activity of the enzyme, as already indicated (IV., C.,-1.). Initially, control hearts were perfused for 15 to 90 min to exclude any perfusion-related alterations in SL and SR PLD. Since no changes were detected after 15'(100 ± 8 %, n=4), 30'(103.5 ± 13.6 %, n=7), 60' (110 ± 5.3 %, n=9) and 90' (1.048 ± 12.1 %, n=3), the hearts that were perfused for 15 min stabilization period, were taken as a pre-ischemic control in all the experimental protocols.

Figure 23 shows that there were distinct changes in the basal PLD activities of SL and SR. The enzyme activity was unaffected after 30 min of ischemia in both SL and SR. Five min of reperfusion after 30 min of ischemia resulted in a significant increase of basal SL PLD activity (33%) followed by a temporary return to normal values (after 10 min) and a further decline (after 30 min) (Figure 23). In contrast to this, basal SR PLD activity was significantly depressed (44%) at the end of 5 min reperfusion after 30 min ischemia and gradually recovered to the pre-ischemic level during subsequent 25 min reperfusion (Figure 23). However, basal PLD activities in both SL and SR fractions were depressed after 60 min of ischemia and further depressed during the whole 30 min reperfusion period; this indicates that there was an irreversible damage in PLD enzymes (Figure 24). In the following experiments we focussed our attention on the distinct biphasic changes of SL and SR PLD activities occurring during the reperfusion of 30 min ischemic hearts.

To clarify whether the fatty acid potential for PLD activation remained after ischemia (30 min) and reperfusion (5-30 min), membrane-bound PLD from experimental hearts was assayed in the presence of 5 mM oleate. Both basal and oleate-stimulated activities from SL and SR displayed parallel changes in ischemic or reperfusion conditions (Figure 25 and 26), which suggests that the reperfusion-related activation and deactivation of PLD were tightly associated with the enzyme protein.

To confirm the observed changes of cardiac PLD hydrolytic activity, the

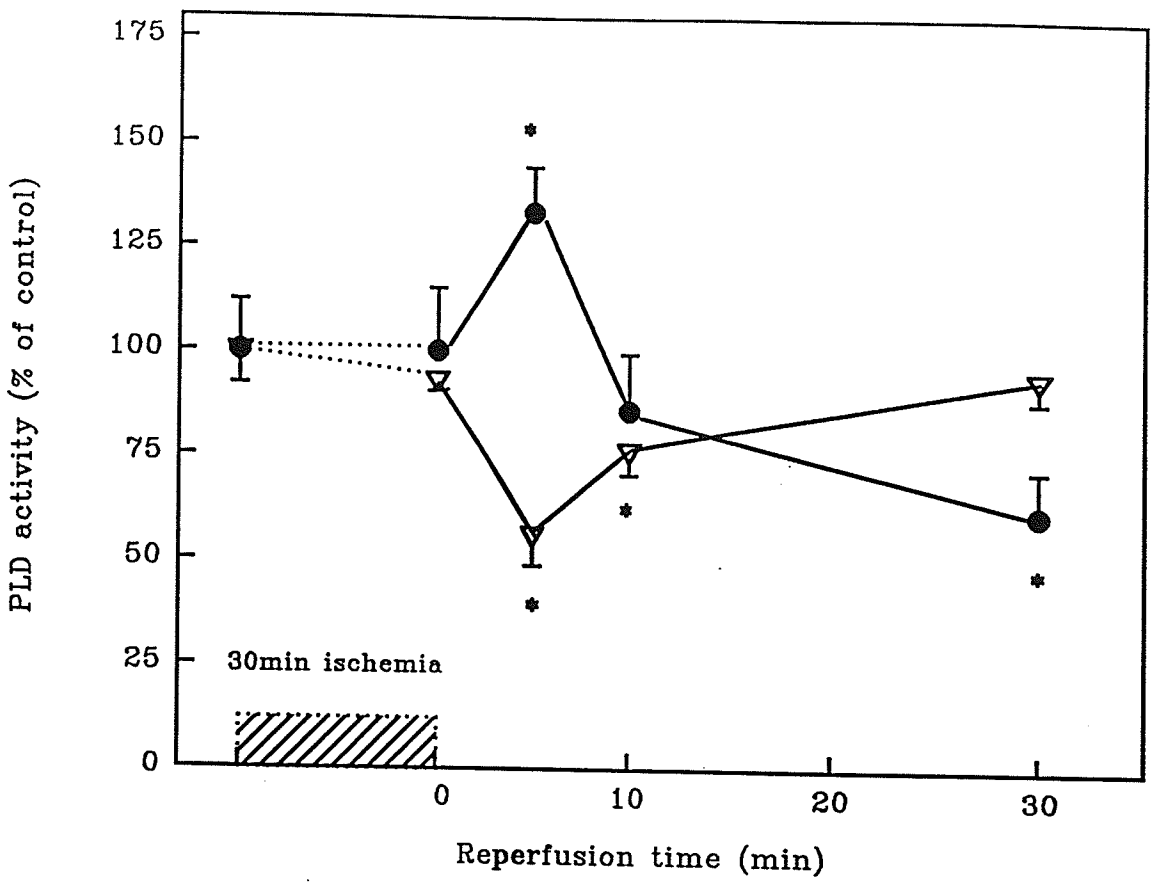


Figure 23. Biphasic changes of sarcolemmal and sarcoplasmic reticular phospholipase D hydrolytic activities during post-ischemic (30') reperfusion

The results are means ± SE of five to nine SL or SR membrane preparations isolated from Langendorff rat hearts without (pre-ischemia) or with 30 min global ischemia and different time-period of reperfusion. SL or SR membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF and 2.5 mM [^{14}C] PtdCho for 60 min at 25 °C in the presence of 3 mM taurodeoxycholate. The data are expressed as percent of pre-ischemic values, which were 11.2 ± 1.2 and 7.1 ± 0.6 nmol PtdOH/mg/h) for SL and SR, respectively. SL (●);SR (▽) * Significantly different ($p<0.05$) from pre-ischemic values.

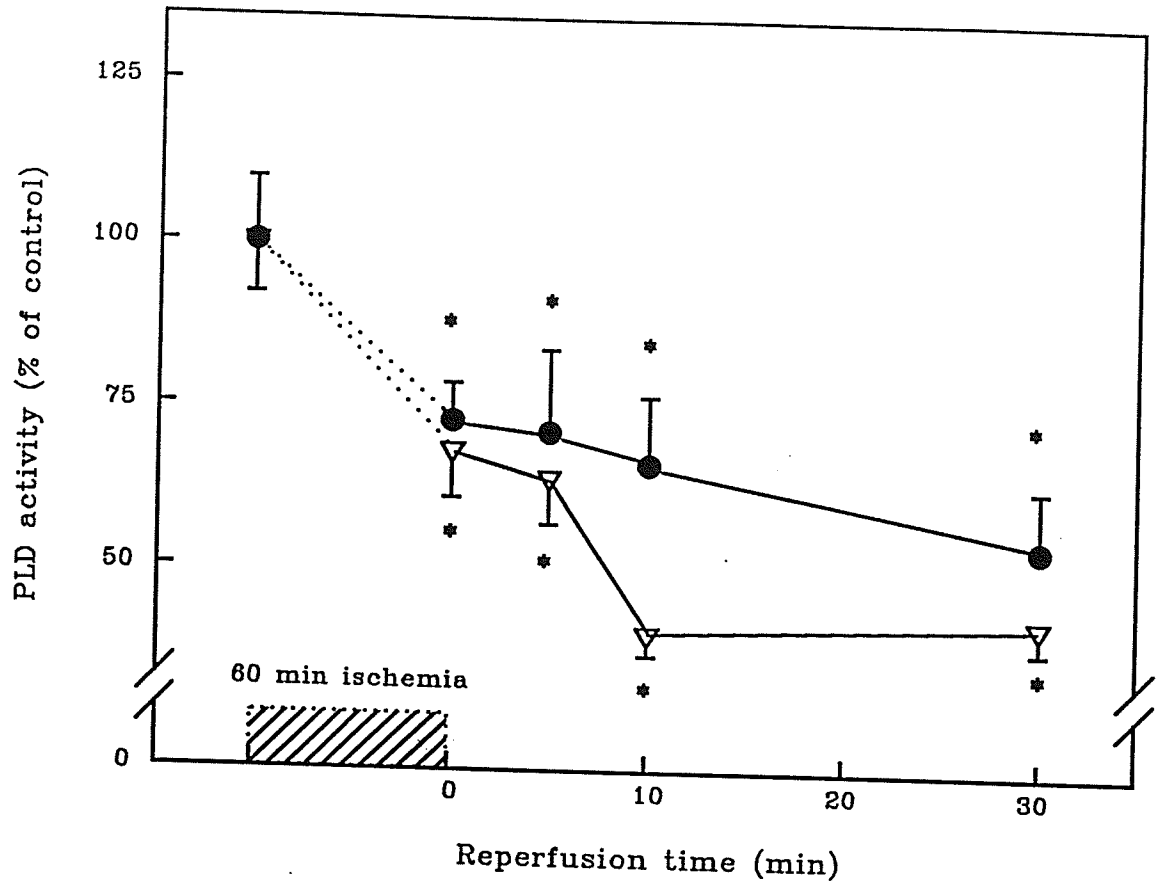


Figure 24. Effect of ischemia (60') and reperfusion on sarcolemmal and sarcoplasmic reticular phospholipase D hydrolytic activities

The results are means ± SE of five to nine SL or SR membrane preparations isolated from Langendorff rat hearts without (pre-ischemia) or with 60 min global ischemia and different time-period of reperfusion. SL or SR membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF and 2.5 mM [^{14}C] PtdCho at 25 °C for 60 min in the presence of 3 mM taurodeoxycholate. The data are expressed as percent of pre-ischemic values, which were 12.1 ± 1.5 and 6.3 ± 0.5 nmol PtdOH/mg/h for SL and SR, respectively. SL (●);SR (▽) * Significantly different ($p<0.05$) from pre-ischemic values.

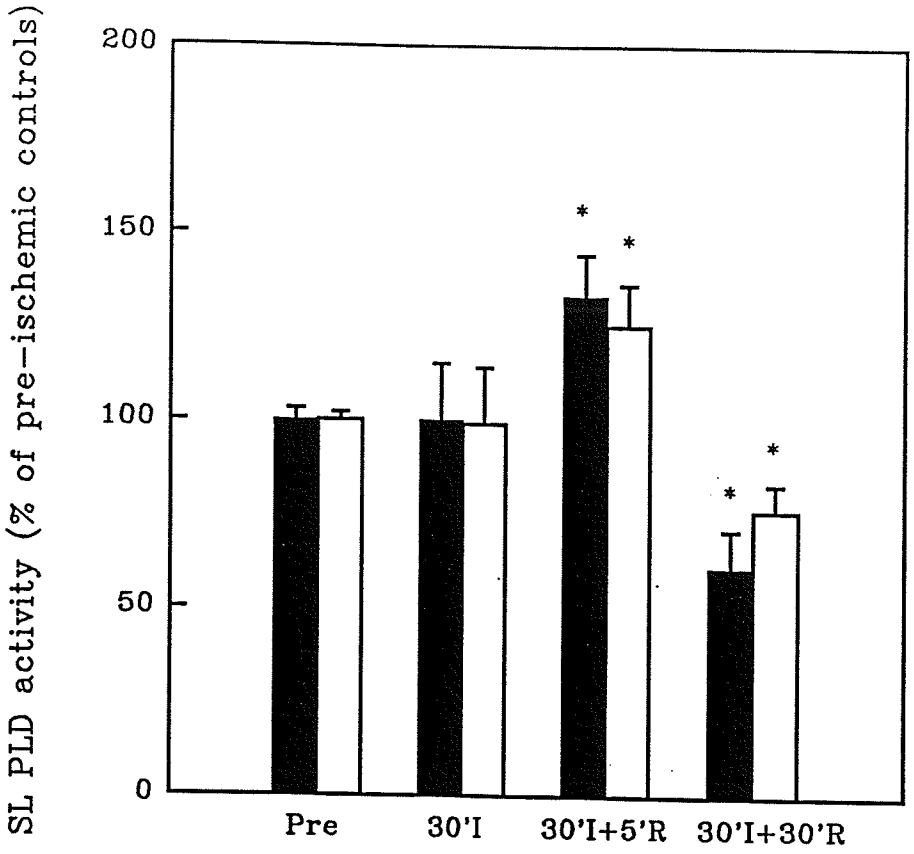


Figure 25. Comparison of basal and oleate-stimulated phospholipase D activity in sarcolemmal membranes from ischemic (30') and reperfused hearts

The results are means ± SE of five to nine SL membrane preparations isolated from Langendorff rat hearts without (pre-ischemia) or with 30 min global ischemia and different time-periods of reperfusion. SL membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF and 2.5 mM [^{14}C] PtdCho for 60 min at 25 °C in the presence of 3 mM taurodeoxycholate (basal activity) or 5 mM oleate (oleate-stimulated activity). The data are expressed as percent of pre-ischemic controls, which were 11.7 ± 1.5 nmol PtdOH/mg/h and 295.7 ± 41.9 nmol PtdOH/mg/h for basal (filled bar) and oleate-stimulated (open bar) PLD activity, respectively. Pre: pre-ischemia; 30'I: 30 min ischemia; 30'I+5'R: 30 min ischemia plus 5 min reperfusion; 30'I+30'R: 30 min ischemia plus 30 min reperfusion.

* Significantly different ($p < 0.05$) from pre-ischemic values.

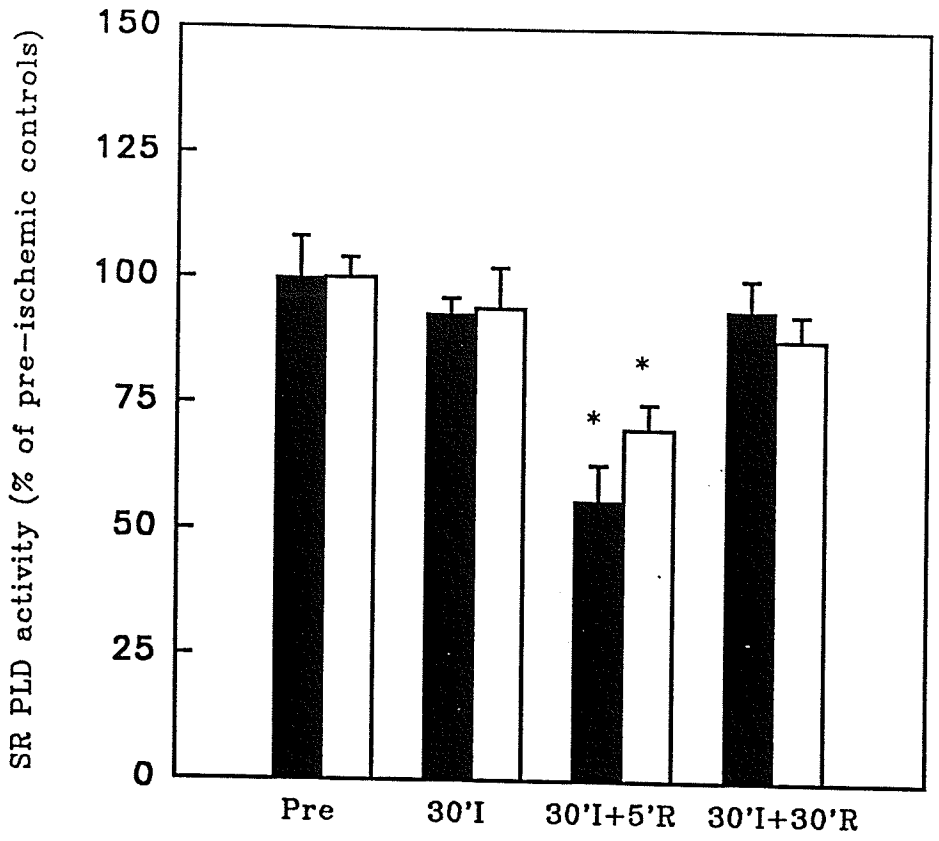


Figure 26. Comparison of basal and oleate-stimulated phospholipase D activity in sarcoplasmic reticular membranes from ischemic (30') and reperfused hearts

The results are means ± SE of five to six SR membrane preparations isolated from Langendorff rat hearts without (pre-ischemia) or with 30 min global ischemia and different time-period of reperfusion. SR membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF and 2.5 mM [^{14}C] PtdCho for 60 min at 25 °C in the presence of 3 mM taurodeoxycholate or 5 mM oleate. The data are expressed as percent of pre-ischemic values, which were 5.8 ± 0.5 and 60.9 ± 1.3 nmol PtdOH/mg/h for basal (filled bar) and oleate-stimulated (open bar) PLD activity, respectively. Pre: pre-ischemia; 30'I: 30 min ischemia; 30'I+5'R: 30 min ischemia plus 5 min reperfusion; 30'I+30'R: 30 min ischemia plus 30 min reperfusion.

* Significantly different ($p < 0.05$) from pre-ischemic values.

transphosphatidylation activity (oleate-stimulated) was assessed in membrane preparations isolated from ischemia (30 min) and reperfused hearts. Changes in SL and SR PLD transphosphatidylation activity (Table 12) were similar to those observed for the hydrolytic activity (Figure 25 and 26) under the same experimental conditions. The results from the SL and SR marker enzyme assays supported the specificity of PLD changes. There were significant decreases in both SL ouabain-sensitive K^+-pNPPase and SR rotenone-insensitive NADPH-cytochrome *c* reductase after ischemia and reperfusion (Table 13), but they were different from the PLD changes (Figure 23 and Table 12). Furthermore, because the enrichment factors for the marker enzymes of either SL or SR membrane preparations from control and ischemic-reperfused heart were similar, the possibility that the observed alterations of PLD activities might be due to the differential purification of SL or SR preparations in control and ischemia-reperfusion conditions is excluded (Table 13).

2. Changes in kinetic parameters of phospholipase D upon ischemia and reperfusion

The reperfusion of ischemic myocardium (30 min) resulted in distinct and significant changes in SL and SR PLD activities. To scrutinize the changes in enzyme kinetic properties, the substrate dependence of SL or SR PLD from control and ischemic-reperfused hearts was examined. No difference was found between basal and oleate-stimulated PLD activity during ischemia-reperfusion (Figure 25 and 26). Since the basal hydrolytic rate was low and hardly detectable at low substrate concentrations, the oleate-stimulated activity was analyzed in this set of experiments. Consistent with other PLD studies (Allgyer and Wells, 1979; Kobayashi and Kanfer,

Table 12. Phospholipase D - dependent transphosphatidylation activity in cardiac sarcolemmal and sarcoplasmic reticular membranes during ischemia (30') and reperfusion

	Oleate-stimulated transphosphatidylation nmol PtdEt/mg/h	
	SL	SR
Pre-ischemia	226.7 ± 14.8	59.0 ± 5.1
30 min ischemia	215.4 ± 15.0	52.1 ± 4.7
30 min ischemia plus 5 min reperfusion	271.4 ± 10.1 *	37.4 ± 1.3 *
30 min ischemia plus 30 min reperfusion	165.5 ± 15.7 *	49.7 ± 3.4

Values are means ± SE of five to seven membrane preparations. Transphosphatidylation activity was assayed in the presence of 5 mM oleate, 2.5 mM [^{14}C]-PtdCho, 0.4 mM Ethanol, 50 mM DMGA(pH 6.5), 10 mM EDTA and 50 μg membrane protein at 25 °C for 60 min. Lipids extraction, separation and quantification are described in Material and Methods.

Table 13. Ouabain-sensitive K^+-p-nitrophenylphosphatase activity and rotenone insensitive NADPH-cytochrome c reductase activity in sarcolemmal and sarcoplasmic reticular membranes from ischemic (30')-reperfused heart

	Pre-ischemia	30'I+5'R	30'I+30'R
A. SL membranes			
Ouabain-sensitive K^+-pNPPase (nmol/mg/min)	1.39± 0.11	1.12± 0.05 *	1.10± 0.01 *
Enrichment factor	10.9	10.5	9.8
B. SR membranes			
Rotenone-insensitive NADPH-cytochrome c reductase (nmol/mg/min)	12.2 ± 1.9	8.5 ± 0.2 *	7.1 ± 0.2 *
Enrichment factor	6.1	6.1	5.8

Values are means ± SE of four to five membrane preparations. Ouabain-sensitive K^+-p-nitrophenylphosphatase, a SL marker enzyme, was assayed in the presence of 1 mM ouabain, 15 mM pNPP, 1 mM EGTA, 20 mM KCl, 10 μg SL protein or 20 μg homogenate protein, alamethicin (1 μg/1μg protein), and 50 mM Tris-HCl at 37°C for 20 min. Rotenone insensitive NADPH cytochrome c, a SR marker enzyme, was assayed in the presence of 44 mM potassium phosphate buffer (pH 7.6), 66 mM KCl, 0.05 mM oxidized cytochrome c, 0.1 mM NaCN, 0.1 mM NADPH, 1.5 μM rotenone and 50 μg membrane protein. Enrichment factor is the ratio of SL or SR enzyme activity vs homogenate enzyme activity. 30'I+5'R: 30 min ischemia plus 5 min reperfusion; 30'I+30'R: 30 min ischemia plus 30 min reperfusion. * Significantly different ($p < 0.05$) from pre-ischemia value.

1987), PtdOH formation via cardiac PLD was increased as the exogenous [^{14}C]PtdCho increased. This would, however, not completely fit in the hyperbolic curve of the Michaelis-Menten equation if the substrate was below a critical concentration (Allgyer and Wells, 1979, Kobayashi and Kanfer, 1987). In this regard, a phase-separation model (Wells, 1974) was used. This model assumes that in an aqueous solution of phospholipid substrate under the "critical concentration", the physical status of phospholipid is a "monomer"; however, above the "critical concentration", two substrate species are present: monomers and aggregates. For kinetic calculation, only the contribution from the aggregated form was taken into consideration. This data could fit into a Lineweaver-Burk plot after substrate concentrations were corrected by subtracting a value known as "critical concentration" (Wells, 1974). The "critical concentration" was obtained by assuming various values to fit a Lineweaver-Burk plot with the experimental data at graphically feasible range of substrate concentration (Allgyer and Wells, 1979; Kobayashi and Kanfer, 1987). The best fit (correlation coefficient of 0.992 ± 0.002, n=30) was achieved with a critical concentration of 0.283 ± 0.008 mM. Figures 27 and 28 show that, compared to controls, the PLD substrate dependencies were changed in either SL or SR fractions from hearts subjected to 30 min ischemia plus 5 min reperfusion. The apparent V_{max} of SL PLD was significantly enhanced at the end of 5 min of reperfusion and depressed at 30 min of reperfusion without changes in apparent K_m values (Table 14). On the contrary, the apparent V_{max} of SR PLD was significantly depressed at 5 min of reperfusion and recovered at 30 min of reperfusion (Table 14). There was also a significant decrease in the apparent K_m of SR PLD at 5 min of reperfusion (Table 14). These results verified the distinct

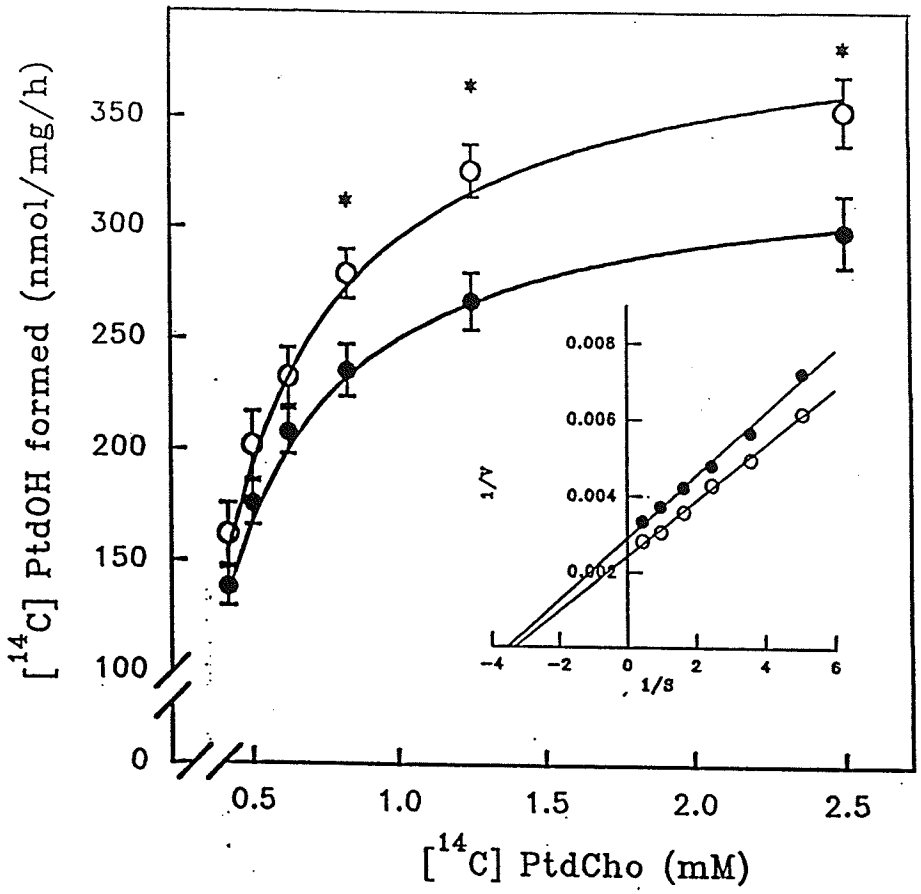


Figure 27. Effect of 30 min ischemia-plus 5 min reperfusion on SL PLD kinetics

The results are means ± SE of four to five SL membrane preparations isolated from Langendorff rat hearts without (pre-ischemia) or with 30 min of global ischemia and 5 min of reperfusion. SL membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF, and 0.47 to 2.5 mM [^{14}C] PtdCho for 60 min at 25 °C, in the presence of oleate (2:1, oleate/PtdCho) according to Chalifour et al.(1982). Lineweaver-Burk plot (inset) was constructed from the values of the enzyme specific activities corresponding to different substrate concentrations and of corrected substrate concentrations. Pre-ischemia (●); Ischemia plus reperfusion (O). * Significantly different ($p < 0.05$) from pre-ischemic value.

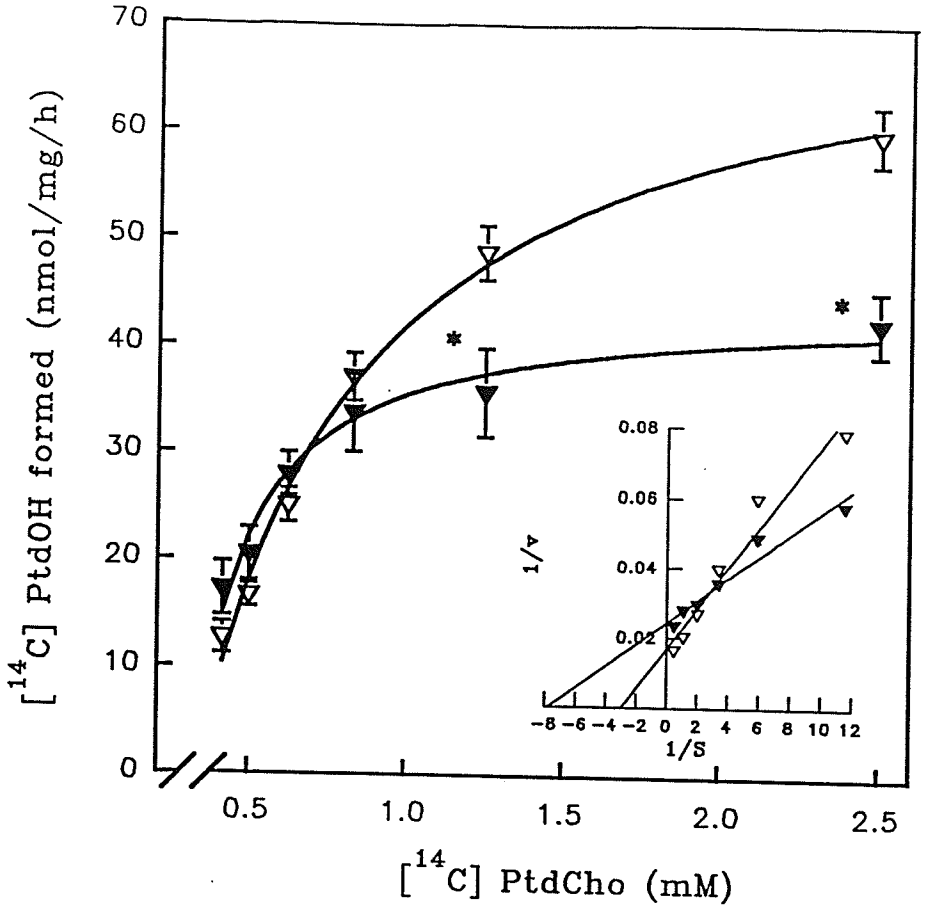


Figure 28. Effect of 30 min ischemia-plus 5 min reperfusion on SR PLD kinetics.

The results are means ± SE of four to five SR membrane preparations isolated from Langendorff rat hearts without (pre-ischemia) or with 30 min of global ischemia and 5 min of reperfusion. SL membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF and 0.47 to 2.5 mM [^{14}C] PtdCho for 60 min at 25 °C, in the presence of oleate (2:1, oleate/PtdCho) according to Chalifour et al.(1982). Lineweaver-Burk plot (inset) was constructed from the values of the enzyme specific activities corresponding to different substrate concentrations and of corrected substrate concentrations. Pre-ischemia (▽); Ischemia plus reperfusion (▼). * : Significantly different ($p < 0.05$) from pre-ischemia value.

Table 14. Kinetic parameters of sarcolemmal and sarcoplasmic reticular PLD after post-ischemic (30') reperfusion

	Pre-ischemia	30'I+5'	30'I+30'
SL			
Vmax (nmol/mg/h)	332.4 ± 10.5	405.8 ± 8.4*	256.5 ± 5.8*
Km (mM)	0.28 ± 0.03	0.29 ± 0.02	0.31 ± 0.02
SR			
Vmax (nmol/mg/h)	62.0 ± 3.4	44.2 ± 0.9*	55.1 ± 1.8
Km (mM)	0.30 ± 0.04	0.17 ± 0.01*	0.26 ± 0.03

Values are means ± SE of four to five membrane preparations. PLD hydrolytic activity was assayed in the presence of different concentration of [^{14}C]-PtdCho (0.416-2.5 mM), 50 mM DMGA(pH 6.5), 10 mM EDTA and 50 μg membrane protein at 25 °C for 60 min in the presence of 5 mM oleate. Kinetic data were analyzed according to the methods of Well et al.(1974). Apparent Vmax and Km values were calculated from non-linear curve fitting technique using corrected substrate concentrations. * Significantly different ($p < 0.05$) from pre-ischemic value.

changes in PLD activities of SL and SR upon the reperfusion of 30 min ischemic hearts.

3. Changes in PtdOH phosphohydrolase activity upon ischemia-reperfusion

In the characterization studies, it was noticed that the cardiac PLD activity was associated with a PtdOH phosphohydrolase (PAP) activity. The subsequent hydrolysis of the phosphate group of PtdOH results in the generation of DAG which is a signal molecule for the activation of PKC. Because PLD activity changed during ischemia and reperfusion, the activity of coupled PAP was also examined under the same conditions. Figure 29 shows that in both pre-ischemic control and ischemia-plus-reperfusion conditions, a shift of lipid product from [^{14}C]-labeled PtdOH to [^{14}C]-labeled DAG was observed if the PAP inhibitor, KF (see Figure 5), was removed from the PLD assay medium. There were significant increases in both PtdOH formation (with KF in the medium) and DAG formation (with and without KF in the medium) in the SL preparations from 5 min-reperfused-ischemic-hearts compared to that from the pre-ischemic hearts (Figure 29). In the presence of KF in the PLD assay medium, the DAG/PtdOH ratio was 0.10 and 0.12 for SL membranes from the pre-ischemic hearts and from 30 min ischemic-5 min reperfused-hearts, respectively. However, without KF in the PLD assay medium, the DAG/PtdOH ratio became 10.7 control hearts and 13.5 for 30 min ischemic-5 min reperfused-hearts (+26%). These results suggest that there was an enhanced coupling mechanism between PLD and PAP activities during 5 min reperfusion after

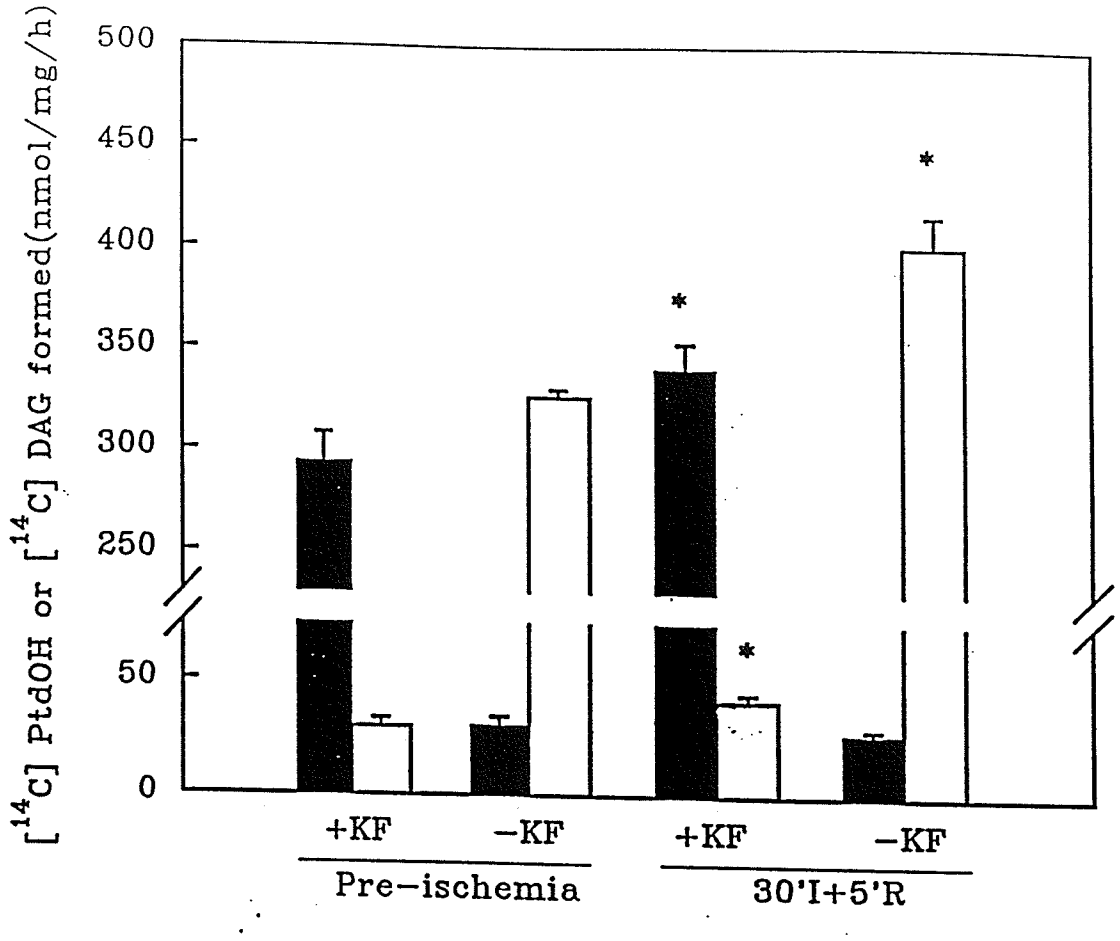


Figure 29. Effect of ischemia-reperfusion (30'I+5'R) on sarcolemmal phospholipase D hydrolytic activities with or without KF.

The results are means ± SE of four to five SL membrane preparations isolated from Langendorff rat hearts without (pre-ischemia) or with 30 min global ischemia and 5 min reperfusion. SL membranes were incubated with or without 25 mM KF in 50 mM DMGA-10 mM EDTA (pH 6.5), and 2.5 mM [^{14}C] PtdCho at 25 °C for 60 min. PtdOH (■); DAG (□). * Significantly different ($p<0.05$) from corresponding pre-ischemia values.

30 min of ischemia in SL fraction. The PAP activities of SL and SR were also independently examined in ischemia and reperfusion conditions. In all the conditions, the PAP activities were higher in the SL fraction than in the SR fraction (Table 15). The PAP activity of SL was significantly depressed at the end of the 30 min of ischemia, normalized at 5 min reperfusion, and significantly depressed again at 30 min of reperfusion (Table 15). However, in SR fraction, no changes were seen after 30 min ischemia period, whereas a 25% depression occurred at 5 min reperfusion, and an almost complete recovery was seen after 30 min of reperfusion (Table 15). These results indicate that 1) phosphohydrolase activities that hydrolyse PLD-derived PtdOH to form DAG are present in both SL and SR fractions; 2) after 30 min of ischemia, PtdOH might accumulate in SL membrane due to normal PLD activity (Figure 25) and the depressed PAP activity (Table 15), which might accelerate Ca^{2+} entry within the first minutes of the reperfusion period; 3) at the end of 5 min of reperfusion, DAG might be a major product in PLD pathway due to enhanced PLD activity (Figure 25), normalized PAP activity (Table 15), and enhanced coupling mechanism between PLD and PAP activities (see above); 4) DAG generation from SR through PLD-PAP pathway might be functionally less important during ischemia and reperfusion, since changes in the two enzyme activities seem to be similar (Figure 26 and Table 15).

Table 15. Phosphatidic acid phosphohydrolase activity in SL and SR membranes isolated from ischemic (30')-reperfused rat hearts

	pre-ischemia	30'I	30'I+5'R	30'I+30'R
			nmol/mg/h	
SL	330.1 ± 7.8 (100%)	226.1 ± 22.4 * (68.5%)	322.8 ± 44.1 (97.8%)	241.0 ± 27.8 * (73.0%)
SR	179.2 ± 10.2 (100%)	192.3 ± 13.3 (107.3%)	134.2 ± 5.7 * (74.9%)	159.0 ± 7.0 (88.7%)

Values are means ± SE of three to six different membrane preparations. PtdOH phosphohydrolase activity was assayed in the presence of 3mM [^{14}C]-phosphatidic acid, 2.5% of Triton-100X, 1mM DTT, 50 mM Tris-maleate (pH 6.5) and 50 μg membrane protein at 30°C for 10 min. Lipids extraction, separation and quantification were described in Material and Methods. Data in parentheses are percent of preischemic values. 30'I: 30 min ischemia; 30'I+5'R: 30 min ischemia plus 5 min reperfusion; 30'I+30'R: 30 min ischemia plus 30 min reperfusion. * Significantly different ($p<0.05$) from pre-ischemic value.

V. Discussion

A. Substantiation of Myocardial Phospholipase D

This study has provided , for the first time, direct biochemical evidence for the existence of PLD activity with stringent specificity for PtdCho in cardiomyocytes. The enzyme activity is localized in different membrane fractions of the cardiac cell. Subcellular distribution showed that a major proportion (50 to 55%) of the total activity was recovered in the 1000 g fraction containing sarcolemma, while no activity was detected in the cytosolic fraction. Although purified SL preparation with 11-fold enrichment of plasma marker enzyme activity revealed a high specific activity of PLD, the presence of PLD in SR as well as mitochondrial fractions suggests that a portion of the total cellular enzyme activity in the cardiomyocyte is associated to intracellular membranes. Since the SL and SR membrane preparations were well characterized by the positive and negative marker enzymes, the PLD activity detected in these two subcellular membranes seems not to be due to cross contamination. In addition, the different sensitivity of SL and SR PLD to Fe^{2+} and the distinct responses of SL and SR PLD to the ischemia-reperfusion insult suggest that the PLD activities detected in SL and SR fractions may be inherent to each membrane system. It is worth noting that, in this study, the lack of ATP in the assay medium excludes any contribution of the PLC/DAG kinase pathway for PtdOH production (Majerus, et al., 1986). This supports the view that the observed PtdOH generation could be accounted for only by a PLD-dependent cleavage. Furthermore, both purified fractions exhibited transphosphatidylation activity, a unique feature of PLD (Kanfer, 1989), and this can be taken to confirm the association of PLD to the

cardiac membranes. Apparently, this enzyme has no strict requirement for Ca^{2+} because in all the membranes it displayed a substantial activity in the presence of 10 mM EDTA. It should be noted that most of the mammalian membrane-bound PLD are Ca^{2+}-independent (Kanfer, 1989). However, the existence in human serum of a Ca^{2+}-dependent PLD with stringent substrate specificity for glycan-PtdIns and not for PtdIns has been reported (Davitz, et al., 1987), while two PLD activities with diverse subcellular localization and biochemical properties have been found in human neutrophils (Balsinde, et al., 1989). Both SL and SR PLD were found to display a high specificity for PtdCho as this was the only phospholipid hydrolyzed at significant rates. Therefore, The PLD being characterized here appears to be unrelated to the previously reported membrane-bound phosphodiesterase of the PLD type that hydrolyzes N-acyl-phosphatidylethanolamine-lyso-phospholipids and alkenyl-acyl-glycerophospho(N-acyl)ethanolamine, while it does not hydrolyse to any extent PtdCho (Schmid, et al., 1983).

The accumulation of radioactive DAG was noted in the assay extract after incubation of SL or SR membranes with exogenous radioactive PtdCho, as reported for different cell types (Martin, 1988; Billah, et al., 1989). Formation of DAG from PtdCho may be due to either a PtdCho-hydrolyzing phospholipase C (PC-PLC) activity or to the sequentially coupled PtdCho-PLD and PtdOH phosphohydrolase (PAP) activities (Martin, 1988; Billah, et al., 1989). The latter pathway seems to be responsible for the DAG formation observed in this study for following reasons: 1) the PtdOH to DAG ratio resulted to be 0.06 and 2.5 in the absence and presence, respectively, of an optimal concentration of KF (25 mM, Figure 3), which is known

to partially inhibit PAP activity (Kanfer, 1989); 2) PAP activity was found to be present in cardiac SL and microsomal vesicles (Philipson and Nishimoto, 1984; Schoonderwoerd, et al., 1990); 3) endogenous PtdCho can yield DAG upon PLD activation in various systems (Exton, 1990); 4) a soluble form of PLC specific for PtdCho and phosphatidylethanolamine has been found in dog heart cytosol (Wolf and Gross, 1985), but this cellular localization excludes any participation of PtdCho-PLC in forming DAG under our experimental conditions. Therefore, it can be proposed that in cardiac membranes a DAG pool is derived from PtdCho via a PtdCho-specific PLD/PtdOH phosphohydrolase pathway in addition to DAG formed by phosphoinositide hydrolysis (Majerus, et al., 1986; Exton, 1990; Lamers, et al., 1993).

The biochemical properties of the cell membrane PLD described here and the possibility that its activity is affected during hormone stimulation (Lindmar, et al., 1986; Lindmar, et al., 1988) suggests that the role of this enzyme may be that of actively generating important lipid molecules for the signaling processes. The functional significance of PLD-dependent increase of PtdOH in cardiac SL membranes is indicated by recent studies. For example, treatment with exogenous PLD increased the cardiac SL Na^+-Ca^{2+} exchange activity (Philipson and Nishimoto, 1984). The observation that exogenous addition of PtdOH and PLD generates Ca^{2+}-dependent slow action potentials in depolarized rat atrium supports a role for PtdOH in mediating an increase of Ca^{2+} influx into the cardiac cells (Knabb, et al., 1984). PtdOH has also been reported to stimulate SL Ca^{2+} pump (Carafoli, 1984) which is involved in the efflux of Ca^{2+} from cell during relaxation

of the myocardium (Dhalla, et al., 1982). These results suggest that SL PLD hydrolytic activity and subsequent formation of PtdOH may be related to Ca^{2+} movements within the cardiomyocyte and may influence the heart function in normal conditions as well as in disease states characterized by abnormal Ca^{2+} homeostasis. Furthermore, a rapid formation of PtdOH under agonist stimulation of the heart (Lindmar, et al., 1988) and its potential action in enhancing the phosphoinositide synthesis (Moritz, et al., 1992) and hydrolysis (Kurtz, et al., 1993) as well as inducing phosphorylation of cardiac proteins (Bocckino, et al., 1991) indicate that this phospholipid may have signaling functions.

The role of PLD-derived PtdOH in SR is unclear at present; however, it is worth noting that PtdOH has been reported to release Ca^{2+} from cardiac SR thus providing a new pathway for modulating SR Ca^{2+} transport (Limas, 1980). It is also possible that the PtdCho-hydrolyzing PLD activity alone or in association with PAP may contribute to synthesis of other phospholipids or triacylglycerol via PtdOH or DAG intermediates (Bosch, 1974; Hatch, et al., 1988). In this regard, increased cytosolic amounts of choline, another product of PtdCho degradation by PLD, may contribute to the final resynthesis of PtdCho at the SR level via the CDP-choline pathway (Zelinski, et al., 1980). These newly formed PtdCho molecules in SR membrane may then become available for inter-membrane translocation by phospholipid transfer proteins (Venuti and Helmkamp, 1988).

B. Importance of Thiol Modification in Myocardial PLD Activities

Our results show that functionally critical thiol groups are associated with SL and SR PLD and that changes of their redox state by biological oxidants can impair the enzyme activity. Thiol groups appear to be essential for SL and SR PLD activity because various sulfhydryl-modifying reactions (alkylation, mercaptide and mixed disulfide formation) inhibited the enzyme while DTT, which maintains thiols in the reduced state, (Cleland, 1964) had a protective effect in all the cases. In particular, treatment of SL membranes with increasing concentrations of NEM, a highly specific alkylating reagent (Ramani and Praissman, 1989), resulted in a PLD depression that correlated well with the decrease in thiol group content. The relevance of protein thiols for PLD activity is further supported by the finding of an inhibitory effect by MMTS. This chemical modifier acts via the introduction of methanethio groups to the protein thiol groups and leads to the formation of mixed disulfides on the proteins (Cleland, 1964). The steric effects, which are expected to be dissimilar for NEM and MMTS (Pierce, et al., 1986), may be the reason for the observed difference in their inhibitory potency. Inactivation of the enzyme also occurred when pCMPS and DTNB were used for thiol chemical modification, as well as in the presence of GSSG that inactivates cardiac proteins by reacting with cysteine residues to form glutathione-protein-mixed disulfides (Eley, et al., 1989). This confirms the importance of membrane thiol groups for PLD function. However, it remains to be defined if these essential residues are part of the enzyme molecule itself or of a regulatory component and, in the former case, where is their location in the spatial structure of the enzyme, i.e., within the catalytic domain or elsewhere.

In this regard, the activity of the solubilized and partially purified enzyme of rat brain microsomal membranes was completely abolished by p-chloromercuribenzoate, a commonly used thiol reagent (Taki and Kanfer, 1979). In addition, four cysteine residues have been found in the PLD amino acid sequence from a bacterial clone cDNA (Hodgson, et al., 1990). Since a certain homology among PLDs of various cell types seems likely, the enzyme molecule is the presumable location of reactive thiols in cardiomyocytes. At any rate, the intense inhibition exerted in our experiments by pCMPS, which has a modest lipid solubility (Hilden and Madias, 1991), suggests that the critical thiol groups are not located deep inside of the membrane.

When cardiac subcellular membranes were exposed to various oxidant species, it became apparent that the nonradicals H_2O_2 and HOCl depressed SL and SR PLD, while the effect of ·OH could not be assessed. The exact causes for the lack of responsiveness to $\cdot O_2^-$ under our experimental conditions are not known. However, a different sensitivity of an enzyme system to $\cdot O_2^-$ and H_2O_2 has previously been observed (Kaneko, et al., 1989). The mechanism(s) through which H_2O_2 and HOCl depressed SL and SR PLD activity may be two-fold. Peroxidation of membrane lipids in the micro-environment of the enzyme could be one mechanism. However, · OH formation from H_2O_2 was irrelevant in our experimental conditions because the · OH scavenger, D-mannitol, failed to modify the H_2O_2-induced inhibition of PLD. The other possible mechanism is the oxidation of functional thiol groups associated with the enzyme protein (Shan, et al., 1990; Ramasarma, 1990; Ferrari, et al., 1991; Bast, et al., 1991). Indeed, protection by DTT against oxidant-induced damage to the heart was shown to be related to the

preservation of tissue thiol groups (Eley, et al., 1989, 1991; Kako, et al., 1989). In our study, the protective effect of DTT on both thiol modifier- and oxidant-induced depression of PLD activity suggests that alteration of key thiol groups is the common mechanism of damage. It could be argued that the preventive action of DTT may not be related to thiol group protection, but that it may have resulted from a direct redox reaction of DTT with the thiol-modifiers or with the oxidants. However, incubation of SL with DTT after H_2O_2 pretreatment resulted in a partial but significant PLD reactivation, indicating that some of the enzyme-associated thiol groups had been reconverted to the reduced state. Furthermore, prevention of the H_2O_2 damage by GSH in the absence of glutathione peroxidase seems to exclude a direct detoxifying reaction between GSH and H_2O_2 and implies thiol protection as GSH mechanism of action (Shan, et al., 1990). Therefore, it can be concluded that oxidants, in particular H_2O_2 which was studied in detail, depressed the SL and SR PLD activity through oxidation of the enzyme-associated thiol groups. GSH protective and GSSG inhibitory effects also suggest that an adequate level of the glutathione redox status may play a major role in the physiological regulation of PLD through reversible conversion of the enzyme-associated thiols to disulfides.

The modification of PLD activity by thiol-dependent oxidation may have implications for pathophysiological conditions of oxidative stress characterized by the formation of H_2O_2 and HOCl and by alteration of the glutathione redox state, as is the case for clinical settings in which the heart is exposed to transient ischemia followed by coronary reflow (e.g. evolving myocardial infarction, vasospastic angina, coronary angioplasty or cardiopulmonary bypass). Recent studies showed

mitochondria-dependent production of H_2O_2 during ischemia (Shlafer, et al., 1990) and reperfusion (Turrens, et al., 1991). In the isolated perfused heart, an ischemic period induced a decline in the GSH over GSSG ratio (Ferrari, et al., 1991), whereas post-ischemic reperfusion led to the production of H_2O_2 (Repine, 1991) and other oxygen metabolites (Werns, 1990), and further decreased [GSH]/[GSSG] (Ferrari, et al., 1991). Indeed, results of the ischemia-reperfusion studies confirmed that PLD activities were depressed in isolated perfused rat heart in certain ischemia and reperfusion periods. Blood-borne cells which infiltrate the ischemic and reperfused myocardium *in vivo* , should also be taken into consideration as sources of oxidants causing stress to the cardiomyocytes (Ferrari, et al., 1991). Polymorphonuclear neutrophils, in particular, have been shown to produce not only $\cdot O_2^-$ and H_2O_2 but also to use these reactive species for HOCl synthesis in myeloperoxidase-catalyzed reactions (Halliwell, 1991). Hence, in addition to intracellular reactive oxygen species, H_2O_2 and HOCl generated by neutrophils seem to contribute to the tissue injury during reperfusion (Hess, et al., 1985; Eley, et al., 1989; Ferrari, et al., 1991). Since the oxidants' concentrations that significantly impaired PLD activity in this study are compatible with those occurring *in vivo* during ischemia-reperfusion (Turrens, et al., 1991; Cochrane, 1991), oxidative damage to SL PLD is likely to take place in ischemic reperfused hearts *in vivo*. This may destabilize the membrane lipid bilayer independently of peroxidative reactions, and may contribute to compromising the Ca^{2+} homeostasis and signal transduction processes of the cardiomyocyte.

C. Importance of Fatty Acid Activation of Myocardial PLD Activities

Our results demonstrated that cardiac PLD was intensely stimulated by cis-unsaturated fatty acids, especially by arachidonate and, less efficiently, by oleate. Both FA were active in the micromolar range, and the concentration required for 50% stimulation of endogenous Ptd[^{3}H]Cho hydrolysis by SL PLD was 177 μM and 268 μM for arachidonate and oleate, respectively. The results also showed that the endogenous mobilization of intramembranal FA enhanced PLD activity. It is noticed that in the absence of activator FA, the SL membrane displayed hydrolysis of endogenous Ptd[^{3}H]Cho with the formation of choline. Free choline may originate from PtdCho either via PLD or, as suggested for other cell-types (Witter and Kanfer, 1985), via a PLC-dependent formation of phosphorylcholine which is then cleaved to choline by a phosphatase. The latter pathway does not seem to be responsible for the choline formation observed in this study because cold phosphorylcholine was present in the assay system to avoid the possible interference by PtdCho-PLC activity (Witter and Kanfer, 1985; Qian and Drewes, 1989), and newly formed phosphoryl[^{3}H]choline was not detected. Therefore, a constitutively active PLD seems to be present under basal conditions in native SL membranes, as suggested by a study on isolated atria (Lindmar and L$\ddot{o}$ffelholz, 1992).

Different features of the FA stimulatory effect on PLD have been reported in cell-free system. For example, oleate has been proved to be the most potent FA in rat liver plasma membranes, while arachidonate was weakly effective (Siddiqui and Exton, 1992). Palmitoleate, that induced a limited PLD activation in this study, parallelled oleate in maximally stimulating the enzyme of rat brain microsomes (Chalifour and Kanfer, 1982). These different observations may be attributed either

to a tissue specificity of PLD with respect to its intramembranal organization as reported for other enzymes (Panagia, et al., 1987), or to the occurrence of distinct enzyme forms (Balsinde, et al., 1989). At any rate, our results demonstrate that the selectivity of the effect of fatty acids on PLD activity cannot be generalized and should be defined in any given cell type.

The mechanism(s) responsible for the differences in the ability of the various fatty acids to stimulated SL PLD is unclear. This study showed that cis-unsaturation was a minimal structural requirement, since saturated FA and elaidate, the trans-homologue of the potently active oleate, had very little influence on PLD. Cis-unsaturated FA are known to preferentially partition into the fluid domain disordering the membrane, while trans-unsaturated and saturated FA partition into the gel domain without causing disarray (Karnovsky, et al., 1982). In particular, rotation within the membrane of acyl chains containing cis double bonds disorders the membrane in regions distal to the double bond (Burt, et al., 1991). The magnitude of the disordering effect is a function of the length of the chain distal to the double bond (Burt, et al., 1991). However, our data do not fit in such a model because the length of acyl chains with the same double bond did not correlate with the PLD catalytic rate, suggesting that the enzyme activation may not be related to disordered membrane structure. This view is supported by the finding that PLD is not stimulated by FA methyl esters, which have double bonds and thus increase the disordered fluid state of the membranes. A detergent component in the FA action can be excluded because saturated fatty acids were ineffective, and anionic, zwitterionic and non-ionic detergents had very weak effects on PLD. In addition,

sodium dodecylsulfate, a detergent structurally similar to oleate with a negatively charged polar head and hydrophobic hydrocarbon chain, was tested at a concentration of 0.5 mM in the assay conditions described in Figure 19, and did not enhance the basal PLD activity. On the other hand, a special topological role or a direct interaction of the double bond with a specific amino acid residue of the enzyme molecule seems unlikely. In fact, cis-monounsaturated FA of equal chain length stimulated PLD to a similar extent, regardless of the double bond position. Finally, we cannot exclude the possibility that PLD activation may occur through accumulation of activator molecules forming a lipid boundary near the enzyme molecule (Cortese, et al.,1982; Nunez, 1993). As well, indirect PLD stimulation via PKC activation may be a possibility, since cis-unsaturated FA activate PKC (Sumida, et al., 1993) which , in turn, has been found to enhance PLD activity in a variety of tissues (Billah and Anthes, 1990; Exton, 1990) including the heart (Lindmar and L o ffelhoz, 1992). However, this alternative is precluded since FA preferentially activate soluble isozymes of PKC (Sumida, et al., 1993) and ATP was absent in our PLD assay medium. In any case, whatever additional mechanism(s) may occur, the negatively charged free carboxyl group is an essential functional moiety because its esterification abolished PLD stimulation, while its replacement with an alcohol group was minimally effective. This agrees with previous work on the effects of FA on cardiac Na^+-Ca^{2+} exchanger (Philipson and Ward, 1985) and gap junctions (Burt, et al., 1991).

The hydrolysis of intramembranal PtdCho by SL PLD was several fold stimulated by 10^{-4} M concentrations of exogenous arachidonate or oleate. Since FA

concentration in cardiomyocyte is about 20 μM (Vork, et al., 1993), the possibility of a PLD modulation by FA would seem unlikely under physiological conditions. However, our results have also shown that the stimulation SL PLA_2 by melittin released an endogenous amount of FA sufficient to activate SL PLD. A cellular protein, which has PLA_2 stimulatory properties and is antigenically and functionally related to melittin, has been identified in mammalian cells (Clark, et al., 1987). Furthermore, enhancement of membrane PLA_2 activity occurs under agonist-receptor interaction (Axelrod, 1990; Lindmar, et al., $1986_{a,b}$). Therefore, FA may constitute a physiological stimulus for SL PLD. On the other hand, an imbalance between uptake and oxidation of FA, disturbances in triglyceride accumulation or changes in PLA_2 activity will increase the cellular concentration of unsaturated FA (van der Vusse, et al., 1992), which may readily bind to the membranes due to their amphiphilic nature (Katz and Messineo, 1981). This may affect SL PLD in some pathological states of the heart. In essence, we have shown that cis-unsaturated FA are activators of the cardiac PLD and may mimic the response evoked by hormones known to stimulate this enzyme (Billah and Anthes, 1990; Exton, 1990; Lindmar, , 1988). Such an activation may occur in physiological conditions via hormonal stimulation of membranal PLA_2, as well as in heart diseases characterized by elevated levels of unsaturated fatty acids and / or by pathological hyperfunction of PLA_2.

D. Status of Myocardial PLD Activities in Ischemia-Reperfusion Injury

The present study, for the first time, explored the changes of PLD activity in

purified SL and SR fractions from the ischemic-reperfused hearts. Parallel monitoring of PLD activities in SL and SR fractions from reperfused ischemic heart is particularly important for the following reasons. As we observed in characterization studies, cardiac PLD is localized in different subcellular membrane fractions; their responses to the ischemia-reperfusion injury might be entirely different. SL and SR of the cardiomyocyte carry different functional proteins. In terms of Ca^{2+} handling, SL-associated Ca^{2+} channels, Na^{+}-Ca^{2+} exchange and Ca^{2+} pump are responsible for Ca^{2+} flux through the cell membrane, whereas the SR-associated Ca^{2+} channel and Ca^{2+} pump are responsible for release and uptake Ca^{2+} from or into the SR structures. Therefore, distinct modification of those proteins could generate different effects in the cardiomyocytes. In this regard, the previous studies indicated that exogenous PLD could affect the Ca^{2+} movement through modulation of SL- associated Na^{+}-Ca^{2+} exchanger, Ca^{2+} channels and Ca^{2+} pump as well as the SR- associated Ca^{2+} pump (Limas, 1980; Philipson and Nishimoto, 1984; Knabb, et al., 1984; Carafoli, 1984). These findings strongly suggested us to separately examine SL and SR fractions upon ischemia-reperfusion injury.

Thirty minutes of ischemia did not change PLD activities in either SL or SR. During reperfusion of 30 min ischemic heart, a rapid increase at 5 min, followed by a gradual decrease at 30 min in SL PLD activity was paralleled by a rapid decrease and a gradual normalization of SR PLD. These changes were confirmed by assessing the transphosphatidylation activity in the same membrane preparations. Moreover, these changes are not related to differential purification of membranes from control and experimental hearts, because the enrichment of the marker enzymes showed no

difference between the two types of preparations, and the direction of the marker enzyme changes was different from the PLD changes. Our kinetic data displayed decreased V_{max} and in increased affinity of SR PLD toward PtdCho at 5 min reperfusion following 30 min ischemia. Since similar changes were seen in cardiac Na^+-K^+ ATPase of the ischemic-reperfused heart (Vrbjar, et al., 1991), it is a matter of speculation that the augmented affinity to the substrate may be attributed to a self-adaptation mechanism in which the enzyme attempts to maintain its efficiency in the situation of reduced maximal velocity. On the contrary, the K_m of SL PLD kept constant although there was an increase in its V_{max} at 5 min reperfusion following 30 min ischemia. These results further supported the divergence in the changes of enzyme function between SL PLD and SR PLD. Our results also showed that prolonged ischemia (60 min) resulted in an irreversible damage of PLD activities in both SL and SR fractions, and that reperfusion further depressed PLD activity. The time schedule of the irreversible PLD damage upon ischemia seems to coincide with the development of irreversible ischemic injury (Opie, 1992).

The enhancement of heart PLD activity upon global ischemia-reperfusion (30 min ischemia + 30 min reperfusion) was also reported by other authors (Moraru, et al., 1992). However, that study only measured the transphosphatidylation activity in total cardiac tissue and the hydrolytic activity in a microsomal fraction. The purity of the microsomal fraction was not indicated. In our view, the experimental protocol of that study is questionable because: 1) PLD is localized at SL, SR and mitochondrial level, 2) different subcellular localization of an enzyme may undergo opposite changes in diseased hearts (Panagia, et al., 1990), and 3) arachidonic acid

(used in certain protocols of that study to preperfuse the heart) is a potent activator of cardiac PLD. Therefore, it is difficult to compare and evaluate those results with ours.

The mechanism(s) underlying the changes in PLD activities of SL and SR in the ischemic-reperfused heart could be multiple. One or more effecting factors could take place simultaneously, or one factor could play a dominant role. Our regulatory studies have indicated that thiol groups are critical for PLD activities, and these thiol groups are liable and can be modified by GSSG and H_2O_2. In the isolated perfused heart, Ferrari et al. (1991) showed a declined ratio of GSH over GSSG in ischemia, with further decrease in post-ischemic reperfusion. In addition, mitochondria-dependent production of H_2O_2 during reperfusion of the ischemic heart was also observed (Turrens, et al., 1991). Also, reperfusion of the ischemic heart results in multiple changes of lipid metabolism (van der Vusse, 1992). In particular, the myocardial content of oleic acid and arachidonic acid, which are among the most active stimulants of myocardial PLD, has been reported to increase 6 and 11 fold, respectively, during reperfusion (van der Vusse, et al., 1982). Under these conditions, relevant amounts of FA may readily bind to the membranes due to their amphiphilic nature (van der Vusse, et al., 1992). Furthermore, unsaturated FA can be released from the sn-2 position of intramembranal phospholipids by the action of PLA_2 (van der Vusse, et al., 1992). Our experiments with melittin showed that such kind of release resulted in 6-7 fold enhancement of SL PLD. Although we did not attempt to identify the FA liberated from intramembranal phospholipids under melittin treatment, arachidonic acid, which is the most abundant unsaturated

FA in SL phospholipids of the mammalian heart (Bester and Lochner, 1988), is a likely candidate. This may be viewed in light of recent findings that have shown an increase of membranal PLA_2 activity in ischemic-reperfused hearts (Prasad, et al., 1991), as well as selective catabolism of arachidonic acid-enriched SL phospholipids with local accumulation of arachidonic acid (Miyazaki, et al., 1990). The activatory effect of FA on PLD is more intense than the inhibitory effect of oxidants. Thus, we may speculate that the enhanced PLD activity of the SL membranes at 5 min reperfusion may be due to selective local accumulation of PLA_2-derived FA. Since such FA accumulation does not occur at the SR level (Miyazaki, et al., 1990), changes in cellular redox status may be responsible for the concomitant depression of SR PLD. Surprisingly, however, reperfusion of the myocardium induced similar changes in both basal and oleate-stimulated SL PLD activity, suggesting the occurrence of other mechanism(s). In isolated perfused heart, isoproterenol has been shown to stimulate free choline release, which implies the activation of PLD (Lindmar, et al., 1986). In ischemic conditions, there is hypersensitization of the ß-adrenergic system (Strasser and Marquetant, 1991) and an increased release of endogenous catecholamine (Schömig, 1984). Catecholamine release into the perfusate was observed after the onset of reperfusion (Mouton, et al., 1992). Therefore, it is also possible that SL PLD be activated by ß-stimulation in the first phase of reperfusion. This effect may be subsequently attenuated by a wash out of the catecholamines in an isolated perfused heart. Furthermore, the mitochondrial production of H_2O_2 as well as the increased GSSG/GSH ratio, both caused by the ischemia-reperfusion insult, could deactivate PLD. This may explain the depression of SL PLD at late reperfusion, but does not justify the return of SR PLD to normal

values.

The reperfusion-induced SL PLD enhancement that we have observed in the reversible phase of ischemia (Opie, 1991) might play a role in the pathophysiological process. Reperfusion of the heart after a short time of ischemia increased the incidence of arrhythmias (Manning and Hearse, 1984). Increased intracellular Ca^{2+} due to reperfusion of ischemic heart could be one of the mechanisms underlying the high incidence of arrhythmias (Opie, 1991). Ischemia results in an accumulation of intracellular protons due to the anaerobic metabolism (Opie, 1991). On reperfusion, Na^+ ions enter myocardial cells via a Na^+-H^+ exchanger (Meng, et al., 1991). Internal Na^+ accumulates, which favors Na^+-Ca^{2+} exchange (Meng, et al., 1991) and high levels of intracellular Ca^{2+} (Tani and Neely, 1989). Since exogenously applied PLD was shown to increase cardiac SL Na^+-Ca^{2+} exchange activity (Philipson and Nishimoto, 1984), it is conceivable that enhanced SL PLD activity during reperfusion could contribute to the increase in intracellular Ca^{2+}, thus resulting in an higher incidence of ventricular arrhythmias. Moreover, we found that the SL PLD is coupled to a PAP activity. This PAP activity was depressed after 30 min of ischemia and recovered during 5 min of reperfusion. Based on the fact that PLD activity was normal after 30 min of ischemia and increased at 5 min reperfusion, we may speculate that in the very initial reperfusion period, PtdOH would accumulate and contribute to the above mentioned arrhythmias. On the other hand, less Ca^{2+} may be concomitantly released from the SR due to the depressed SR PLD. Interestingly, once PAP activity had recovered, DAG might be the dominant final product of PLD activity. In fact, DAG lipase activity seems not to be present in sarcolemmal

membranes because, in assaying SL PAP, the ratio of monoacylglycerol + glycerol over DAG was found to be 0.066. DAG may stimulate PKC with subsequent activation of L-type cardiac Ca^{2+} channels (Bourinet, et al., 1992) and enhancement of myosin light chain kinase activity, thus intensifying the force development (Clement, et al., 1992). Reperfusion following 60 min of ischemia progressively aggravated the PLD depression observed after ischemia both at SL and SR level and , presently, it is unclear if this abnormality is a cause or a consequence of the irreversible ischemia-reperfusion damage. At any rate, the differential behavior of sarcolemmal and sarcoplasmic reticular PLD during post-ischemic (30 min) reperfusion leads to the suggestion that these activities may have a different role in the myocardial cell function.

VI. Conclusions

1. PtdCho-specific PLD activity localizes in different membrane fractions of the cardiac cell. The majority of the enzyme activity is present in the SL membrane, while, some enzyme activity is also present intracellularly.

2. Two purified subcellular fractions (SL and SR) were found to contain relatively high specific activity of PLD, when the enzyme was assayed in both the hydrolytic and transphosphatidylation modes. Kinetic studies showed that there is a significant difference between apparent V_{max} values of SL and SR PLD, while the apparent K_m values were similar. Both SL and SR PLD activities have a pH optimum at 6.5, which is similar to that of the PLD found in rat brain microsomes, and were found to couple with PAP activities. This was indicated by the predominant DAG production upon depriving the assay medium of KF. Both activities showed a similar dependence on free sulfhydryl groups and long-chain unsaturated fatty acids. However, a significant difference between the two activities was observed in the Fe^{2+} dependence and ischemia-reperfusion injury.

3. The basal PLD activity of the membranes was found to be stimulated by unsaturated fatty acids. The most effective compounds were arachidonate and oleate which maximally activate PLD at 0.5 mM concentration with endogenous substrate and, respectively, at 4 and 5 mM with exogenous substrate. Cellular thiol modifiers and biological oxidants such as reduced glutathione, H_2O_2 and HOCl were capable to inhibit cardiac PLD activity

glutathione, H_2O_2 and HOCl were capable to inhibit cardiac PLD activity presumably through sulfhydryl group modification. These results suggest that cardiac PLD is a modulated enzyme.

4. Thirty minutes of ischemia did not change SL and SR PLD activity. Reperfusion of such ischemic hearts resulted in an increase and then decrease of SL PLD activity, in contrast to a decrease and then normalization of SR PLD activity. On the contrary, 60 min of ischemia resulted in the depression of both SL and SR PLD activities, which were aggravated by reperfusion. These results suggest that SL PLD and SR PLD respond differently to the ischemia-reperfusion insult, and they may be under different predominant control mechanism(s) and play different roles in this pathological process. The SL PAP activity was depressed at the end of 30 min of ischemia and recovered after 5 min of reperfusion, which suggests that in the very initial reperfusion period, PtdOH is the predominant product and may be involved in Ca^{2+} flux modulation.

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