Divergent Changes in Cardiac Phospholipase D-Phosphatidic Acid Phosphatase Activities during Insulin-Dependent Diabetes

By

Sean Angela Williams

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the requirements for the degree of

MASTER OF SCIENCE IN PHYSIOLOGY

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DEDICATED TO MY FAMILY

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FRIENDS

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LIST OF ABBREVIATIONS

12 O-tetradecanoyl-phorbol 13-acetate ..... TPA

Adenosine triphosphate ..... ATP

Aluminum fluoride ..... AlF_4^-

Alzheimer's disease ..... AD

Arachidonic acid ..... AA

Calcium ..... Ca^{2+}

Dithiothreitol ..... DTT

GTPase-activating protein ..... GAP

Guanine nucleotide binding protein ..... G protein

Guanine 5'[γ-thio] triphosphate ..... GTPγS

Inositol-1,4,5-triphosphate ..... Ins(1,4,5)P_3

Insulin-dependent diabetes mellitus ..... IDDM

Magnesium ..... Mg^{2+}

N-Ethylmaleimide ..... NEM

p-Choloromecuriphenylsulfonic acid ..... pCMP

Pertussin toxin ..... PTX

Phorbol 12-myristate 13-acetate ..... PMA

Phosphatidic acid ..... PtdOH

Phosphatidic acid phosphatase ..... PAP

Phosphatidylcholine ..... PtdCho

Phosphatidylethanol ..... PtdEtOH

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

Phosphatidylinositol-4-phosphate kinase ..... PIP

Phospholipase A_2 ..... PLA_2

Phospholipase C ..... PL C

Phospholipase D ..... PLD

Platelet-derived growth factor ..... PDGF

Potassium fluoride ..... KF

Propylthiouracil ..... PTU

Protein kinase A ..... PKA

Protein kinase C ..... PKC

Sarcolemma ..... SL

Sarcoplasmic reticulum ..... SR

sn 1,2-Diacylglycerol ..... DAG

Sodium ..... Na^+

Streptozotocin ..... STZ

Thyroxine ..... T_4

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ABSTRACT

Myocardial phospholipase D (PLD) is primarily localized at the sarcolemma level and selectively hydrolyzes phosphatidylcholine (PtdCho) to form phosphatidic acid (PtdOH), which is dephosphorylated by an associated phosphatidic acid phosphatase (PAP) to give PtdCho-derived *sn*-1,2-diacylglycerol (DAG). The products of PLD catalyzed PtdCho hydrolyses are believed to be involved in signal transduction pathways. PtdOH regulates the enzyme activity of other signal transduction proteins while PtdCho-derived DAG is an important activator of many protein kinase (PKC) isoforms. It has been shown that exogenous PLD is involved in regulating the Ca^{2+} handling processes of the cardiomyocyte due to its ability to alter the activities of Na^{+}-Ca^{2+} exchanger, the Ca^{2+} pump and Ca^{2+} channels. Although the studies with exogenous PLD identified a potential role for the myocardial PLD, they did not examine the enzyme activity located in the different subcellular components of the cardiac cell. Myocardial PLD is also located intracellularly at the sarcoplasmic reticulum (SR) and the mitochondrial level.

In disease states characterized by abnormal Ca^{2+} homeostasis, for example cardiac ischemic-reperfusion injury, PLD and PAP activities are differentially altered. Since abnormal intracellular Ca^{2+} handling is a major factor of myocardial dysfunction in insulin-dependent diabetes (IDDM), we hypothesize that changes in

the PLD/PAP pathway occur in diabetic hearts and constitute important mechanism underlying the defective cellular Ca^{2+} homeostasis.

In this study PLD and it's coupled PAP activities were examined in purified sarcolemma (SL) fractions that were isolated from rat ventricular tissues obtained from 8 weeks chronic insulin dependent diabetes which was induced by a single intravenous injection of streptozotocin (STZ, 65 mg/kg body weight). PLD and PAP activities were assayed *in vitro* by measuring the formation of labeled products from their respective substrates. The beneficial effect of insulin on the SL PLD and PAP activities was assessed by measuring the hydrolytic activity of these enzymes in membrane fractions prepared from rats that were diabetic for 6 weeks and then were treated for 2 weeks with insulin. The SL PLD was also examined by measuring: 1) the time course of its hydrolytic activity; 2) its transphosphatidylation activity. As the STZ-induced diabetes in rats is also characterized by depressed levels of thyroid hormones, the effect of hypothyroidism on PLD and PAP activities was examined. Finally, the effect of diabetes and insulin therapy on PLD and PAP activity in the sarcoplasmic reticulum and mitochondrial fractions were assessed.

The results of this study demonstrate that SL PLD and PAP activities are significantly altered in an opposing manner in hearts of chronic diabetic animals when compared to age-matched controls. The depression in SL PLD activity was reverted by two weeks of insulin therapy, whereas the increase in PAP activity was

significantly but not completely normalized by this treatment. The changes in SL PLD and PAP activities were specific to the diabetic condition since these enzymes' activities were unaltered in the hypothyroid condition. Furthermore, PLD and PAP activities in SR fractions from chronic diabetic animals were also decreased and increased, respectively, whereas only the mitochondrial PLD activity was depressed by diabetes. Insulin therapy was also found to be beneficial to the PLD and PAP activities in the tested membrane fractions.

This study showed for the first time that divergent alterations in the cardiac subcellular PLD/PAP activities occur during chronic IDDM. It also demonstrated the beneficial effect of insulin therapy at the subcellular level within of the myocardium. These results provide some insight into the alterations of the PLD/PAP signalling pathways of the myocardial cell during the insulin-dependent diabetic cardiomyopathy

I. INTRODUCTION

The movement of Ca^{2+} across the various cardiac subcellular membranes relates to the force and duration of the contractile cycle (Dhalla *et al.*, 1982; Pierce *et al.*, 1988). At the SL level many hormones, neurotransmitters and pharmacological agents bind to specific surface receptors, and contribute to the generation of second messenger molecules. These molecules influence the Ca^{2+} handling systems of the cardiomyocyte and thus modulate the contraction of the cardiac muscle (Meij and Panagia, 1993). In some cases, the signalling molecules originate from the hydrolysis of specific membrane phospholipids (Lamers *et al.*, 1993).

Agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate PtdIns(4,5)P_2 by phospholipase C (PLC) was the first phospholipase-mediated pathway to be implicated in cellular signal transduction (Steinberg *et al.*, 1989). Since then, the importance of another phospholipid, PtdCho, in the agonist-induced signaling in various cell types has also been reported.

A variety of cellular phospholipases hydrolyze PtdCho to produce many products that are mediators in transduction pathways (Exton, 1994). A very important PtdCho-derived second messenger, DAG, activates phospholipid-dependent PKC while another product, PtdOH modulates the activity of some signalling enzymes (Kurz *et al.*, 1993; Sato *et al.*, 1993).

Both DAG and PtdOH are generated either through the PtdCho-specific PLC pathway or through the action of a second phospholipase, PLD, on PtdCho. It seems likely that the existence of multiple phospholipase pathways within the mammalian cell types may be to generate multiple species of DAG that may activate different PKC isoforms (Dai, 1993).

Information on the localization, characterization and regulation of mammalian PLDs has increased over the last few years. PLD, which is important for receptor signaling (Billah *et al.,* 1991), is localized in membrane and cytosolic fractions from a variety of cell types (Wang *et al.,* 1991). The regulation of PLD involves many factors such as G-proteins, PKC, Ca^{2+}, and tyrosine kinase phosphorylation (Thompson *et al.,* 1993). The mechanisms by which these components exert their influence on PLD are not fully understood.

The first evidence that suggests that PLD activity may affect the functioning of the heart came in 1984. Exogenous PLD was responsible for the generation of Ca^{2+}-dependent slow action potential in the rat atrium (Knabb *et al.,* 1984). Alterations in other cardiac events have also been attributed to exogenous PLD or PtdOH (Philipson and Nishimoto, 1984; Langer and Rich, 1985). In spite of these findings, however, no information is currently available on the functional status of endogenous myocardial PLD. Recently, PtdCho-specific PLD activity was identified and characterized in the subcellular fractions of the cardiomyocyte (Panagia *et al.,* 1991), the cells that are predominantly responsible for cardiac

function. In each membrane fraction (SL, SR, and mitochondria), PLD activity is coupled to PAP (Dai, 1993), which generates DAG by hydrolyzing PtdOH (Billah *et al.*, 1989a).

Insulin-dependent diabetes is often associated with heart failure (Palumbo *et al.*, 1976). The diabetic cardiomyocyte is marked by irregular organelle structure, altered fatty acid metabolism and an imbalance in the Ca^{2+} handling processes (Pierce *et al.*, 1988). As mentioned before, exogenous PLD induces changes in Ca^{2+} homeostasis in the cardiac tissue (Knabb *et al.*, 1984; Philipson and Nishimoto, 1984; Langer and Rich, 1985). β-Adrenergic receptors are involved in SL PLD activation (Lindmar *et al.*, 1986) and it is known that they are marked by reduced density in the diabetic heart (Heyliger *et al.*, 1982). In addition, the imbalance of sympathetic circulating hormones (Black and McNeill, 1991) and in local neurotransmitters (Pierce *et al.*, 1988) may have functional consequences for the PLD pathway during IDDM.

Taken together, these finding suggest that alterations in myocardial PLD/PAP pathway in the diabetic heart may be an important mechanism underlying the defective Ca^{2+} homeostasis which is a major factor of cardiac dysfunction in insulin dependent diabetes. It is also important to examined PLD and PAP activities in purified membrane fractions since the enzymes may be differentially altered, as was observed in SL and SR fractions during cardiac ischemic-reperfusion injury (Dai, 1993).

A study of cardiac PLD/PAP pathways during IDDM will help in characterizing the subcellular defects responsible for the development of the diabetic cardiomyopathy.

II. LITERATURE REVIEW

A. Phosphatidylcholine Specific Phospholipases

Agonist-induced stimulation of PtdIns(4,5)P_2 in mammalian cells has been well characterized. However, it now appears that PtdIns(4,5)P_2 may not be the only substrate for cellular phospholipases. An increasing number of evidence suggests that PtdCho is also degraded in response to agonist stimulation in many mammalian cell types. The products of PtdCho hydrolysis possess either defined or putative second messenger properties.

PtdCho is the predominant phospholipid species in a variety of mammalian cells including the cardiomyocytes (Berridge and Irvine, 1989). Relatively high levels of PtdCho within the plasma membranes may be important in contributing to the structure of the membrane, and provide the cell with a large quantity of substrate that can be hydrolyzed by a variety of phospholipases. In addition, PtdCho is present in all cell membranes (Colbeau *et al.*, 1971), whereas other signalling phospholipid such as PtdIns(4,5)P_2 appears to be localized to the plasma membrane (Augert *et al.*, 1989). This implies that agonist-induced PtdCho hydrolysis could be occurring in other subcellular membrane compartments other than the plasma membrane, which may confer alternative mechanisms of cellular control (Exton, 1990a).

1. Metabolic fate of phosphatidylcholine hydrolysis

In mammalian cells, PtdCho species can be hydrolyze by either a A_2 (PLA_2), C (PL C) or D (PLD) type of phospholipase (Exton, 1994). Each phospholipase activity on PtdCho results in the generation of various lipid-derived second messengers.

1a. Phosphatidylcholine specific phospholipase A_2

PLA_2 hydrolyzes the fatty acid in the *sn*-2 position of the phospholipid substrate and generates lysophosphatidylcholine and a free fatty acid (Clark *et al.*, 1990; Diez and Mong, 1990). PtdCho species from mammalian tissue normally contain an unsaturated fatty acyl residue at the C-2 position of the glycerol moiety (Billah and Anthes, 1990) the fatty acid liberated by PLA_2 is often arachidonic acid (AA). In the brain, AA liberated by PLA_2 action on PtdCho is believed to be a second messenger (Naor, 1991). In various cell types, AA is metabolized through the cyclo-oxygenase and lipoxygenase pathways to prostaglandins, thromboxanes, leukotrienes or to other eicosanoids (Needleman *et al.*, 1986). The other product of PLA_2 action on PtdCho, lysophosphatidylcholine, is be acetylated to form platelet-activating factor in some cell types (Hanahan, 1986).

1b. Phosphatidylcholine specific phospholipase C

PtdCho cleaved by PLC yields water soluble phosphocholine and DAG (Irving and Exton, 1987). PtdCho specific PLC is present in many mammalian tissues. These include cytosolic fractions from rat brain cells (Edgard and Freysz, 1982) and canine myocardium (Wolf and Gross, 1985). It is also identified in membrane fraction from rat liver (Irving and Exton, 1987) and in sarcolemmal fractions isolated from rat cardiac muscle cells (Baldini *et al.*, 1994). The species of DAG produced from PtdCho hydrolysis are very effective activators of some PKC isoforms (Go *et al.*, 1987). DAG activates PKC by lowering its requirement for calcium (Steinberg *et al.*, 1995). PKC isoforms phosphorylate a variety of cellular proteins (Billah and Anthes, 1990). The proteins phosphorylated by PKC are involved in many biological processes including metabolism, secretion, contraction, proliferation and differentiation (Nishizuka, 1986).

Besides its primary role, that is, the activation of specific PKC isoforms, DAG produced through PLC pathway is involved in other cellular events. It can be phosphorylated by DAG kinase to give PtdOH (Tronchere *et al.*, 1994). DAG can also directly alter the activity of several other enzymes including that of cytidylyltransferases, PLD and PLA_2 (Billah and Anthes, 1990; Exton, 1990b). Another possible action of DAG within the cell involves its role as a precursor for PtdCho synthesis (Tronchere *et al.*, 1994).

Not all isoforms of PKC are activated by PtdCho-derived DAG. The translocation of PKC α isoform, for example, was insensitive to DAG produced from PtdCho hydrolysis (Brown *et al.*, 1990; Leach *et al.*, 1991). Furthermore, in thrombin-stimulated IIC-9 fibroblast, the inability of PKC α to translocate in response to molecular species of PtdCho-derived DAG was also associated with a decrease in "MARCKS" protein phosphorylation (Leach *et al.*, 1991). "MARCKS" protein is a 80-kDa endogenous substrate for PKC.

In explaining how the above mentioned observations are related, Leach *et al* (1991) has suggested the presence of a physical barrier. This barrier separates the site where DAG is produced from the site where the proteins with which it interacts are located. PtdCho-derived DAG is believed to be confined within a membrane pool, and is therefore physically unable to interact with both the α isoform of PKC and with the "MARCKS" protein. Other evidence that supports the idea that PLD and its products, namely DAG, may be located in a separate membrane pool, comes from studies using GH3 cells (Martin *et al.*, 1990). These investigators found that the translocation of PKC α isoform was associated with the first phase of the biphasic DAG response and was localized in the plasma membrane. During the second phase of the biphasic response which was localized to the intracellular membrane, the DAG produced was due primarily to PtdCho hydrolysis and was not associated with the translocation of PKC α isoform.

1c. Phosphatidylcholine specific phospholipase D

The hydrolysis of PtdCho by PLD releases choline and PtdOH (Billah *et al.*, 1989a). PtdOH can be dephosphorylated by PAP, thus giving rise to a second source of PtdCho-derived DAG within the cell.

PLD derived PtdOH has been proposed to be an intracellular mediator (Cook and Wakelam, 1992a). It induces both rapid DNA synthesis and cell proliferation when added to fibroblast in culture (Moolenaar *et al.*, 1986; Yu *et al.*, 1988), and is required for platelet-derived growth factor (PDGF) stimulation in Balb/c 3T3 cells (Fukami and Takenawa, 1992). The production of PtdOH in rat brain was also inhibited by an antitumor drug, suramin, which is an inhibitor of cell growth (Gratas an Powis, 1993).

PtdOH also regulates the guanine nucleotide state of $p21^{ras}$ by inhibiting the activity of GAP (GTPase-activating protein) and enhancing the activity of GAP inhibiting protein (Tsai *et al.*, 1988; Tsai *et al.*, 1990). The effect of PtdOH on $p21^{ras}$ is believed to be responsible for its mitogenetic properties (Tronchere *et al.*, 1994).

PtdOH produced from PtdCho by PLD action has also been implicated in the activity of other enzymes. This is evident in neutrophils, where NADPH oxidase activity is regulated by PtdCho-derived PtdOH (Agwu *et al.*, 1991b; Gélas *et al.*, 1992). In adult ventricular myocytes, PtdOH was found to be a potent activator of inositol phosphate production (Kurz *et al.*, 1993). The increase in the inositol

phosphate formation was associated with changes in PLC activity. Other enzymatic activities shown to be enhanced by PtdOH were that of phosphatidylinositol-4-phosphate 5-kinase (PIP) (Moritz *et al.*, 1992) and PLA_2 (Sato *et al.*, 1993). The mechanism (s) by which PtdOH regulates the activities of the above mentioned enzymes remains to be determined.

Furthermore, recent studies using rat tissue extract have suggested that PtdOH directly activates a protein kinase (Bocckino *et al.*, 1991). A DAG-independent isoform of PKC, ζ, which has been purified and characterized from bovine kidneys (Nakanishi and Exton, 1992), is among a newly identified class of PtdOH-dependent protein kinase.

The second product of PLD catalyzed PtdCho hydrolysis, choline, is not associated with signal transduction cascades (Billah and Anthes, 1990). However, in the brain where a rich source of PLD has been found (Chalifour and Kanfer, 1980a), it has been proposed that choline release from PLD action on PtdCho is a coproduct in the resynthesis of acetylcholine (Lee *et al.*, 1993).

Finally, it should be noted that the phospholipase that becomes activated upon agonist stimulation and hence is predominately responsible for PtdCho hydrolysis, depends both on the cell type and on the agonist involved. For example, some agonist, such as complement C5a action on human neutrophils (Mullmann *et al.*, 1990a) and fMet-Leu-Phe on promonocytic cell line (Anthes *et al.*, 1991) stimulate PtdCho hydrolysis exclusively through PLD, while others

utilize PLC as the primary route for PtdCho hydrolysis (Schutze *et al.*, 1991; Osada *et al.*, 1992). Other agonists, such as epidermal growth factor and vasopressin acting on human dermal fibroblast (Fisher *et al.*, 1991) and in mesangial cell line (Troyer *et al.*, 1992), respectively, activate both PLD and PLC hydrolysis of PtdCho. Thus, mammalian cells can produce a wide range of physiological effects in respond to agonists that induced PtdCho hydrolysis.

2. Cross-talk between phospholipids hydrolyzing phospholipases

In many cell types, agonists that induced PtdCho hydrolysis also cause phosphatidylinositol breakdown, particularly PtdIns(4,5)P_2. This is evident by a biphasic increase in DAG and by a prolonged activation of PKC when cells are stimulated (Exton, 1994). In explaining the biphasic response of DAG production, Exton and colleagues (1990b) have proposed the following general scheme. The first peak, is described as been rapid and transient, and is believed to be due to PLC-catalyzed hydrolysis of PtdIns(4,5)P_2 because it is associated with both an increases in inositol phosphates and in cytosolic Ca^{2+} concentration. The second phase is retarded and more prolonged and is associated with choline. It is believed to be the result of PLD-stimulated PtdCho hydrolysis. The two phospholipase pathways seem to be related because the initial DAG derived from PtdIns(4,5)P_2 hydrolysis starts PKC, while DAG that originates from PtdCho sustains the activation of PKC (Exton, 1990b).

Some experimental data support the above mentioned theory. For example, investigators have found that the metabolic turnover rate of DAG within the cell is extremely rapid (Asaoka *et al.*, 1991). In addition, in experiments using human neutrophils, a prolonged translocation of PKC was observed under conditions that allowed DAG to be produced from PtdCho (Nishihira *et al.*, 1986; Agwu *et al.*, 1989).

It has been proposed that in systems in which a sustained signaling occur, PKC may act as a switch by terminating inositol phospholipid hydrolysis and by activating the hydrolysis of PtdCho (Bishop *et al.*, 1992).

3. Evidence for receptor-coupled phospholipase D

The first evidence that hormones stimulated PtdCho breakdown came from a study on hepatocytes in which the fatty acid composition of DAG formed during agonist-induced stimulation were very different from that of inositol phospholipids (Bocckino *et al.*, 1985). The investigators suggested that the DAG may have been produced from another substrate, such as PtdCho. Since this early experiments, many agonists including neurotransmitters, hormones, growth factors, chemoattractants, interleukins, thrombin, platelet-activating factor, bradykinin, antigens, complement component, protaglandins, interferons, tumor necrosis factors, tumor-promoting esters and purinergic agonists, have all been shown to induce PtdCho breakdown via PLD activation in many cells and tissue types

(Exton, 1994). Recently, it was shown that at physiologically concentrations noradrenaline and endothelin-1 stimulates PtdOH production through PLD pathway in adult ventricular myocytes (Ye *et al.*, 1994).

Another supporting argument for the existence of receptor-coupled PLD activity surrounds the discrepancy between the amount of [^{32}P]PtdOH produced in stimulated cells as compared to the amount of [^{32}P] incorporated into the ATP pool. Cockcroft and colleague (1984) have found that the specific activity of [^{32}P]PtdOH produced in stimulated [^{32}P]orthophosphate labeled neutrophils was significantly less than that of the ATP pool. They concluded that if PL C/DAG kinase was the only pathway through which PtdOH was produced, then the specific activity of the two pools would be similar. The discrepancy between the two pools was explained by action of another major source of PtdOH production, the PLD pathway.

Another cellular event that has been also associated with PLD induced PtdCho breakdown is the transformation of cells by *ras* and *v-src* oncogenes (Price *et al.*, 1989; Song *et al.*, 1991). It is therefore unlikely that an enzyme such as PLD that is diversely localized and whose function is directly or indirectly altered by some many agonists, would not have a role in signal transduction.

4. Detecting the products of phospholipase D

It is now recognized that in intact cell systems, PtdCho-derived DAG is produced either through the direct action of PLC or by PLD coupled PAP pathway. Similarly, PtdOH is generated either by the direct activity of PLD on PtdCho or through an alternate route, the PLC/DAG kinase pathway. The initial task of identifying the pathway responsible for producing a particular product was achieved by using the following methods.

A useful technique for identifying PLD activity involves prelabeling the cell type with 1-O-alkyl-2-lyso[^{32}P]PtdCho (Thompson *et al.*, 1993). This method labels the PtdCho pool without incorporating ^{32}P into the cellular ATP pool. The lack of ^{32}P labeled ATP within the cell, imply that the amount of 1-O-alky [^{32}P]PtdOH detected when the cells are stimulated, is the result of agonist-dependent PLD hydrolysis of PtdCho. This technique has been successfully used in many studies including phorbol 12-myristate 13-acetate (PMA) and complement induced stimulations in human neutrophils (Mullmann *et al.*, 1990a,b) and in chemotactic peptide stimulated HL-60 granulocytes (Pai *et al.*, 1988).

Besides hydrolysis, PLD also catalyses transphosphatidylation (Dawson, 1967). This reaction is unique to PLD and involves the transfer of the phosphatidyl moiety of the phospholipid substrate to a primary alcohol to produce phosphatidylalcohol (Billah and Anthes, 1990). Since phosphatidylalcohols are poor substrate for PAP (Wakelam *et al.*, 1993), there is no rapid conversion of

these metabolites. Thus, in cell systems in which a sufficiently high concentration of an alcohol is added, the production of PtdOH by PLD is severely hindered (Bocckino *et al.*, 1987a; Cook and Wakelam, 1992a). In mammalian cell lines, several studies have demonstrated that transphosphatidylation is activated by a variety of agonists (Exton, 1994).

Another approach in which the formation of DAG and PtdOH can be identified involves the use of a PAP inhibitor, for example, propranolol (Koul and Hauser, 1987). The inhibitor causes a decrease in the conversion of PtdOH to DAG, as showed by a change in the ratio PtdOH/DAG. Although propranolol inhibits PAP activity, care must be taken in its use because studies have shown that at high concentrations it also inhibits PLD activity (Gay and Murray, 1991). Another often used inhibitor of PAP activity is potassium fluoride (KF) (English *et al.*, 1991).

5. Phosphatidic acid phosphatase

PAP activities have been characterized from different mammalian tissues including the liver (Pittner *et al.*, 1986), the lung (Walton and Possmayer, 1989) and in adipocytes (Taylor and Saggerson, 1986). Considering these and other studies, it has been proposed that at least two forms of the enzyme exist in mammalian cell types.

The form that is found in the cytosol and at the endoplasmic reticulum is sensitive to Mg^{2+}, N-ethyamaleimide (NEM), NaF and heat (Jamal *et al.,* 1991; Day and Yeaman, 1992). This form of the enzyme is believed to be involved in the synthesis of glycerol lipids (Höer and Oberdisse, 1994). The membrane-bound enzyme is insensitive to both Mg^{2+} and NEM, and is believed to be involved in signal transduction pathway through PLD (Day and Yeaman, 1992; Höer and Oberdisse, 1994).

Evidence in support of this claim comes from many studies in which the membrane-associated activity of PAP was altered by agonist (Höer and Oberdisse, 1994). For example, one such study using human leukocytes showed that fMet-Leu-Phe stimulated the membrane-bound form of PAP but not the cytosolic enzyme (Truett *et al.,* 1992). The effect of Mg^{2+} and NEM on the cardiac cell PAP activity is not known.

B. Mammalian Phospholipase D

Over the last few years, evidence has been accumulating to support the idea that PLD is involved in cellular signal transduction. An argument to support this claim comes from the fact that in numerous receptor-mediated systems the accumulation of PtdOH sometimes proceeded that of DAG (Exton, 1990b).

Furthermore, many agonists cause a rapid activation of transphosphatidylation. Thus PLD activity is believed to be major component in signal transduction cascade.

1. Localization and characterization

Though first identified in plants (Hanahan and Chaikoff, 1947), PLD activity has been found in many mammalian organs, cell types, and organelles. The first mammalian tissue in which PLD activity was identified, purified (240-fold) and characterized was the rat brain (Saito and Kanfer, 1975; Chalifour *et al.*, 1980a,b). Other organs in which PLD activity has been demonstrated include the lung (Wang *et al.*, 1991), the liver (Bocckino *et al.*, 1987b), the heart (Chalifour and Kanfer, 1980a; Corradetti *et al.*, 1983), the spleen (Chalifour and Kanfer, 1982), the kidney (Chalifour and Kanfer, 1980a) and the thymus (Wang *et al.*, 1991).

The variety of cells types in which PLD activity has been observed include cells of the inflammatory and immune systems (Truett *et al.*, 1989; Kennerly, 1987); endocrine cells (Peter-Riesch *et al.*, 1988); exocrine cells (Liscovitch and Amsterdam, 1989a); muscle cells (Farese, 1988); epithelial cells (Daniel *et al.*, 1986); adipocytes (Chalifour and Kanfer, 1982) and fibroblast (Fukami and Takenawa, 1989). PLD activity has been found in subcellular organelles. These include mitochondrial fraction from neurons (Chalifour and Kanfer, 1982), synaptic membrane fraction (Kobayashi and Kanfer, 1987), sarcolemmal (SL),

sarcoplasmic reticular (SR) and mitochondrial fractions from ventricular myocytes (Panagia *et al.*, 1991) and cytosolic fraction from bovine lung (Wang *et al.*, 1991). Thus mammalian PLD activity exists both in the particulate and in the cytosolic fractions.

Reports have suggested the existence of multiple PLD isoforms. This is based on the fact that PLD optimal pH, its requirement for divalent cations, fatty acids and detergents vary among cell and tissue type (Billah *et al.*, 1991). For example, PLD activity from rat brain synaptosome has an optimal pH of 7.2, is stimulated by oleate, Mg^{2+} and by low concentrations of Ca^{2+} (Chalifa *et al.*, 1990). This enzyme activity is inhibited by 2 mM Ca^{2+} and by the SH reagent p-choloromercuriphenylsulfonic acid (pCMPs) (Kobayashi and Kanfer, 1987). In comparison, PLD activity in plasma membrane from rat brain is stimulated by Ba^{2+} and Sr^{2+} and has shown no inhibition to millimolar concentrations of either Ca^{2+} or Mg^{2+} (Kanfer and McCartney, 1994). PLD activity from rat ventricular myocytes, has a pH optimal of 6.5 (Panagia *et al.*, 1991), is stimulated by long-chain unsaturated fatty acids (oleate and arachidonate were most effective) (Dai *et al.*, 1995), and is inhibited by thiol modifiers (Dai *et al.*, 1992).

The substrate specificity of PLD also depends on the cell type being tested. For example, in neutrophils and cardiomyocytes, PLD exclusively degrades PtdCho (Billah and Anthes, 1990; Thompson *et al.*, 1990), while in other cell systems, such as in fibroblast cell lines, HL-60 granulocytes, and HeLa cells, PLD

selectively hydrolysis phosphatidylethanolamine (Kiss and Anderson, 1989; Hii *et al.*, 1991). PLD hydrolyzing phosphatidylinositol has been reported in cell free preparations from neutrophils (Balsinde *et al.*, 1988), however, to date, the existence of agonist-induce PLD hydrolysis of phosphatidylinositol in intact cells, has not been demonstrated.

PLD also exhibits substrate specificity accordingly to its subcellular localization. In general, the membrane-associated activity of PLD shows a strict specificity for PtdCho whereas the cytosolic forms of the enzyme hydrolyze PtdCho as well as phosphatidylethanolamine and phosphatidylinositol (Billah, 1993). In SL and SR fractions purified from cardiac cells, PLD specifically hydrolyzes PtdCho (Panagia *et al.*, 1991).

Recently, the first membrane-bound isoform of PLD was purified from pig lung microsomes (Okamura and Yamashita, 1994). The enzyme has a molecular mass of 190-kDa and catalyzed both the hydrolysis and transphosphatidylation reactions. It preferred substrate is PtdCho. The enzyme is stimulated by unsaturated fatty acids, the most potent were oleate and arachidonate, whereas, N-ethylmaleimide (NEM) and detergents were inhibitory.

2. Regulation of phospholipase D

Studies to date indicate that in mammalian systems, receptor-mediated activation of PLD is regulated by several mechanisms. Various factors such as

PKC, GTP-binding proteins (G proteins), Ca^{2+}, and phosphorylation on tyrosine residue all seem to be involved in PLD regulation. In addition, studies also indicate that the regulation of PLD activity does not only vary with mechanism, but also depends on the cell type and on the agonist involved.

2a. Role of Protein Kinase C

Studies indicate that tumor-promoting phorbol esters such as PMA, causes both PtdCho-induced breakdown to choline (Hii *et al.,* 1989; Huang and Cabot, 1990a) and activation of PKC (Cabot *et al.,* 1989; Liscovitch, 1989). The activation of PLD by other agonists such as epidermal growth factor acting on Swiss 3T3 cell line, also required PKC activation (Yeo and Exton, 1995).

The activation of PLD by phorbol esters is sometimes sensitive to agents that inhibits PKC. These include staurosporine, H-7, R0-31 and sphingosine (Exton, 1994). In most cases, agonist-induced and phorbol esters-induced stimulations of PLD are blocked by these agents (Liscovitch, 1989; Gustavsson and Hansson, 1990). Inhibitors of PKC can also inhibit some receptor-dependent PLD responses (Huang and Cabot, 1990b; Cook and Wakelam, 1992b). Incomplete inhibition of PLD activity by staurosporine and sphingosine has also been reported (Lavie and Liscovitch, 1990; Kanaho *et al.,* 1992). In these studies, PKC inhibitors may stimulate PLD activity by activating pertussis toxin-sensitive G proteins.

However, it is recognized that in several studies, agonist-dependent and phorbol ester-dependent activation of PLD appears to be insensitive to PKC inhibitors (Billah *et al.*, 1989b; Liu *et al.*, 1992; Uing *et al.*, 1992). The discrepancy in the effect of PKC inhibitors on PLD activation may reflect the variations in cell types, agonists or may be due to the complex actions of some of the inhibitors (Exton, 1994).

Evidence to support PKC involvement in PLD regulation also comes from studies in which the levels of PKC in the cell line were altered. In one such study on Rat-1 fibroblast in which PKC was down-regulated by prolonged exposure to phorbol esters, PMA induced stimulation of PLD activity was blocked (MacNulty *et al.*, 1990). Similar observations were made in other experiments in which PDGF induced-stimulation in Swiss 3T3 fibroblast (Plevin *et al.*, 1991) and angiotensin induced stimulation in rat renal mesangial (Pfeilschifter and Huwiler, 1993) were abolished by depleting the cell lines of PKC isoforms.

In addition, rat fibroblast over-expressing PKC β_1 isoform, showed enhanced PLD response to PMA (Pai *et al.*, 1991a), endothelin (Pai *et al.*, 1991b) and to α-thrombin (Pachter *et al.*, 1992) stimulations. The over-expression of another PKC isoform, α, in Swiss 3T3 cell line and in CCL39 fibroblast, also resulted in enhanced basal PLD activity (Eldar *et al.*, 1993; Conricode *et al.*, 1994). Thus, the basal level of PLD seems to be in a direct correlation with the levels of certain PKC isoforms in the cell.

Conricode and colleagues (1992) showed that phorbol ester-induced stimulation of PLD in CCL39 lung fibroblasts requires a cytosolic factor. They were able to show that a purified PKC from rat brain could substitute for the missing cytosolic factor. These investigators also found the PKC-dependent activation of PLD did not require ATP-dependent phosphorylation since the addition of ATPase, which inhibits the autophosphorylation of PKC, failed to block PLD activation. Their findings are supported by other experiments using permeabilized neutrophils in which agonist-induced stimulation of PLD required a cytosolic factor (Olson *et al.*, 1991). The PLD stimulated activity was also significantly inhibited by staurosporine, a known PKC inhibitor. Although not fully understood, the PKC regulation of PLD seems to involve both phosphorylated and nonphosphorylated mechanisms.

2b. Role of GTP-Binding Proteins

Many observations indicate the PLD-induce hydrolysis of PtdCho hydrolysis is regulated by G proteins. In permeabilized cells and in isolated plasma membrane, submicromolar concentrations of non-hydrolyzable GTP analogs such as guanine 5'[γ-thio] triphosphate (GTPγS) stimulate the production of choline, DAG and PtdOH and activate transphosphatidylation (Bocckino *et al.*, 1987a,b; Martin and Michaelis, 1989; Xie and Dubyak, 1991). In these experiments, there

was an associated decrease in PtdCho levels that is believed to be the result of PtdCho-specific PLD activation.

The role of G proteins in the activation of PLD was also been demonstrated in other experiments in which aluminum fluoride (AlF_4^-), GDP and its nonhydrolyzable analog, GDPβS, were able to mimic and/or inhibit the effect of GTP analogues in various cell types (Qian *et al.*, 1990; Kanaho *et al.*, 1991; Xie and Dubyak, 1991). Other studies have reported an enhanced PLD activity to AlF_4^- without inositol phosphate formation (Holler *et al.*, 1994). In this study it was proposed that the activation of PLD independent of inositol hydrolysis may be an important mechanism by which the cellular levels of DAG could activate PKC without mobilizing calcium.

GTPγS-mediated activation of PLD in permeabilized cells may sometimes require the presence of cations. In some studies, Mg^{2+} was needed in order to observe GTPγS-mediated activation of PLD (Xie and Dubyak, 1991; Liscovitch and Eli, 1991), while another required Ca^{2+} (Geny and Cockcroft, 1992). In a study on cell-free system derived from U937 leukocytes, GTPγS-stimulation of PLD was indifferent to ATP (Kusner *et al.*, 1993). This suggests that both ATP-dependent and ATP-independent pathways may be involve in regulating PLD activity.

In other experiments using pertussin toxin (PTX), an agent that prevents G protein stimulation, agonist-induced PLD stimulation was inhibited in neutrophils

(Kanaho *et al.*, 1991), HL-60 cells (Xie *et al.*, 1991) and in Rat-1 fibroblast (MacNulty *et al.*, 1992). In other experiments, lysophosphatidic acid-induced PLD stimulation was partially inhibited in IIC9 fibroblasts by PTX (Ha *et al.*, 1994). A G_i-like G protein is beleived to be involved in this system (Ha *et al.*, 1994). In addition, the G protein, RhoA, was shown to activate PLD activity in hepatocyte plasma membrane (Malcolm *et al.*, 1994). However, the mechanism by which the Rho G proteins interact with PLD is not known.

2c. Calcium Dependence

Several experiments have indicated that the activity of PtdCho-specific PLD is dependent on Ca^{2+} . For example, in many cell types such as hepatocytes (Bocckino *et al.*, 1987b), granulocytes (Billah *et al.*, 1989b), platelets (Huang *et al.*, 1991) and neurons (Llahi and Fain, 1992) calcium ionophores increase the effect of agonist-induced PtdCho-PLD stimulations.

PLD activity was affected in an opposing manner in experiments in which intracellular and/or extracellular calcium concentrations were changed (Bocckino *et al.*, 1987b; Billah *et al.*, 1989b). Using permeabilized neutrophils, the investigators found that the chemotactic peptide-dependent PLD response varied accordingly with the changes in free intracellular Ca^{2+} concentrations (Kessels *et al.*, 1991). The agonist-induce PLD activation was completely inhibited when intracellular Ca^{2+} concentrations were lower than 100 nm and reach a maximum at

around 1 μM. This is within the range of free intracellular Ca^{2+} concentrations (Thompson *et al.*, 1993).

Although the activity of PtdCho-specific PLD is dependent on free intracellular and/or extracellular Ca^{2+} concentrations, the exact mechanism by which Ca^{2+} regulated PLD activity is not known. The reason the mechanism has not been identified is due to the inability to correctly access and separate the many processes requiring Ca^{2+} during the signaling events. These include activation of both PtdIns(4,5)P_2-specific PL C (Exton, 1990b), and PKC isoforms (Steinberg *et al.*, 1995), and the action of G proteins on PtdCho-PLD (Geny and Cockcroft, 1992).

2d. Involvement of protein tyrosine kinases

Growth factors can induce PtdCho hydrolysis without causing PtdIns(4,5)P_2 breakdown (Cook and Wakelam, 1992b; Ha and Exton, 1993). The mechanism of PLD regulation, in the above mentioned experiments, are believed to be different from other agonist-induced cellular systems in which PtdIns(4,5)P_2 hydrolysis precedes PtdCho breakdown.

In cell systems in which growth factors induced PtdCho breakdown without causing PtdIns(4,5)P_2 hydrolysis, it has been proposed that PtdCho-PLD is regulated by PKC-independent mechanisms (Exton, 1994). Evidence in support of

this argument comes from many studies. For example, in experiments using permeabilized HL-60 cells vanadyl hydroperoxide inhibited P-tyrosine phosphatase activity and caused an increase in PLD activity (Bourgoin and Grinstein, 1992). Both enzymes' activities required ATP and Mg^{2+} and both activities were reduced by tyrosine kinase inhibitors. In another set of experiments on RBL cells, tyrosine kinase inhibitors blocked antigen-induced PLD activation without affecting PtdIns(4,5)P_2 metabolism (Kumada *et al.*, 1993).

Growth factors-induced PtdCho hydrolysis has also been observed in the presence of PtdIns(4,5)P_2 breakdown. For example, PtdIns(4,5)P_2 hydrolysis by PLC-γ1 and the activation of PKC were both necessary for PDGF stimulation of PLD in TRMP cell line (Yeo *et al.*, 1994). Furthermore, the activation of PLD in transfected NIH 3T3 cells was dependent on the level of PL C-γ1 (Lee *et al.*, 1994).

In summary, many experiments have indicated that changes in PtdCho-PLD activation are closely related an increase in tyrosine phosphorylation. However, the exact mechanism by which tyrosine kinases regulate PLD activity has not been fully established. It is speculated that such a mechanism may involve either the direct action of the kinase on PLD or may involve other phosphorylation events (Exton, 1994).

2e. Other modulatory factors

Studies have indicated that PtdIns(4,5)P_2 is a potent stimulator of brain membrane PLD activity (Liscovitch *et al.*, 1994). The investigators suggested that PtdIns(4,5)P_2 synthesis and hydrolysis may regulate PLD action independent of PKC.

Protein kinase A (PKA) is also implicated in the regulation of PLD. In one study, the activation of PKA enhanced vasopressin-induced phosphatidylethanol formation in rat hepatocytes (Gustavsson *et al.*, 1994). Since 12 O-tetradecanoyl-phorbol 13-acetate (TPA) stimulated and Ca^{2+} stimulated PtdEtOH formations were not affected by PKA activity, the investigators concluded that PKA was indirectly regulating PLD activity. PKA action, in this cell line, may involve shifting the balance of PLD activation between Ca^{2+}-dependent and PKC-dependent mechanisms.

PLD activity in plasma membrane from rat liver, is regulated by a PTX-insensitive G protein that is belived to be linked to P_{2y} purinergic receptors (Siddiqui and Exton, 1992)

3. Functional significance of cardiac phospholipase D

Many studies have indicated that PLD activity directly affects the functioning of the heart. In neonatal rat ventricular tissue, exogenous PLD caused an increase

in contractility (Burt *et al.*, 1984). A positive inotropic effect was observed in rabbit papillary muscle, in the presence of exogenous PLD (Langer and Rich, 1985)

Other changes in the cardiac tissue that have been attributed to PLD and PtdOH are: 1) the generation of Ca^{2+}-dependent slow action potential in the atria (Knabb *et al.*, 1984); 2) enhanced Ca^{2+} binding in papillary muscle (Langer and Rich, 1985); 3) stimulation of Na^{+}-Ca^{2+} exchanger in sarcolemma vesicles (Philipson and Nishimoto, 1984); 4) SR stimulated Ca^{2+} release (Limas, 1980). These findings strongly suggest that PLD may be involved in Ca^{2+} handling processes of the heart.

4. Alterations in PLD activity during pathophysiological conditions

Many pathophysiological conditions, such as myocardial ischemic-reperfusion injury, cardiac hypertrophy, and Alzheimer's disease, are associated with either alterations in PtdCho metabolism, abnormal Ca^{2+} homeostasis, and/or changes in the functioning of PKC or G proteins. Since the proper functioning PLD signalling pathway depends on the above mentioned cellular events, it seems reasonable to expect an abnormal PLD activity in these pathophysiological conditions.

4a. Myocardial ischemic-reperfused injury

One of the cellular abnormalities associated with myocardial ischemia-reperfusion injury is changes in Ca^{2+} homeostasis (Dhalla *et al.*, 1988; Jenning and Reimer, 1991). Studies have also indicated altered myocardial PLD activity upon ischemic-reperfused insults. Moraru *et al* (1992) demonstrated that PLD activity was significantly elevated in both membrane fractions and in intact cells isolated from rat heart that were subjected to 30 min global ischemia followed by 30 min reperfusion.

The opposite change in PLD activity upon global myocardial ischemia-reperfusion (30 min ischemia + 30 min reperfusion) has also been reported (Dai, 1993). In this study, PLD activity was assayed in purified SL and SR membrane fractions. Distinct changes were observed for the SL enzyme (its activity was significantly depressed by 30 min ischemia + 30 min reperfusion), and for the SR enzyme (its activity was unchanged during this time). The discrepancy in myocardial PLD activity upon ischemia-reperfusion injury is explained by the differences in the experimental protocol. In the study in which the elevated PLD activity was reported, the enzyme was tested in microsomal fractions (Moraru *et al.*, 1992). The other study examined the enzyme's activities in purified cardiac subcellular fractions (Dai, 1993).

4b. Cardiac hypertrophy

In a recent study using an *in vitro* model of load-induced cardiac hypertrophy, the investigators found that multiple signal transduction pathways, including PLD, were activated in stretched rat cardiomyocytes (Sadoshima and Izumo, 1993). In the same study, other proteins that are involved in the regulation of PLD, such as PKC and tyrosine kinases, were also activated in response to the stretch-induced condition. For this model of hypertrophy, it has been suggested that the signals generated from the activated pathways may be important for cellular growth, a process that is essential for hypertrophy.

In another form of load-induced cardiac hypertrophy, the thyroxine (T_4) model, the opposite change in SL PLD activity was observed (Williams *et al.*, 1995). The enzyme's hydrolytic activity was depressed by 57% in hypertrophied rat hearts as compared to age-matched control values. The deactivation of PLD signalling pathway, in this model, has been implicated in the imbalances of Ca^{2+} homeostasis that is associated with cardiac hypertrophy.

The above results suggest the occurrence of diverse changes in PLD signalling pathway in different types of cardiac hypertrophy, which may be related to the type of stimulus initiating the hypertrophied process. The status of PLD pathway in other forms of cardiac hypertrophy remains to be determined.

4c. Alzheimer's disease

Alzheimer's disease (AD) is a degenerative disorder that is characterised by the progressive loss of memory and mental function (Zeisel and Canty, 1993). Post-mortem studies of brain samples obtained from AD patients show massive degeneration of cholinergic nerve terminals (Wurtman *et al.*, 1990). Abnormal phospholipid metabolism including that of PtdCho, has also been reported in AD (Wurtman, 1992).

In cholinergic cells the coproduct for acetylcholine synthesis, choline, was shown to be produced through PLD-catalysed hydrolysis of PtdCho (Lee *et al.*, 1993). Given the above, it seems reasonable to peculate that PLD activity may be affected by the AD process. This was demonstrated in homogenates of AD brain tissue, in which PLD activity found to be significantly lower (by 63%) as compared to control values (Kanfer *et al.*, 1986). A significantly depression in choline acetyltransferase activity was also observed in the same study. Both observations imply that the metabolism of choline containing compounds are severely altered in AD pathology.

C. Cardiac Dysfunction During Diabetes

Insulin-dependent diabetes mellitus (IDDM) is a disease that is characterised by the lack of pancreatic insulin which results in an abnormal glucose homeostasis within the body. The glucose intolerance in IDDM can be controlled with daily injections of insulin. IDDM is not the only form of diabetes. Other diabetic classifications that also exhibit glucose intolerance include the non insulin-dependent type and various other types which are induced by either environmental factors or are result of genetic defects. Exception for IDDM, insulin therapy is not usually beneficial in treating most of the other forms of diabetes.

Some medical problems that are common among IDDM, and are believed to be secondary to the diabetic condition, are heart disease (Pierce *et al.*, 1988), blindness (Goldstein and Cogan, 1960; Appen *et al.*, 1980), renal disorders (Friedman, 1983) and neuropathy (Ellenberg, 1983). Of the above mentioned defects, studies have indicated that the cardiovascular failure is the leading cause of death in diabetics (Palumbo *et al.*, 1976). The elevated rate of cardiovascular failure among diabetics is due to a number of physiological abnormalities. These include an increase incidence of myocardial infarction, silent or painless infarction events, congestive heart failure, coronary vessel disease, strokes and possible hypertension (Pierce *et al.*, 1983). The pumping performance of the heart during IDDM is severely compromised. This is indicated by a disturbed mechanical

function (Shapiro *et al.*, 1981; Friedman *et al.*, 1982) and by abnormal electrophysiological readings (Blandford and Burden, 1984; Senges *et al.*, 1980).

1. Ultrastructural changes in the cardiomyocytes

Cardiomyopathy is a term often used regarding IDDM. By definition, cardiomyopathy refers to a disease of the myocardium that is manifested by changes in hemodynamic parameters, contractile abnormalities, and structural derangements (Olsen, 1979; Pierce *et al.*, 1988). The disease is not affected by external factors. Most studies on structural derangements of the heart during diabetes have concentrated on the cardiomyocytes. This is because the cardiomyocytes are primarily responsible for force generation, and enhancement of the cardiac performance.

In a study on chronic diabetic animals, the investigators found an increase number of myocardial cell nuclei per examination area (Fluckiger *et al.*, 1984). They concluded that the diabetic myocardial cell was smaller as compared to the size of the cell in control hearts. A smaller cell size in the diabetic animal helps to explain the reduction in heart weight that is common among diabetics (Ganguly *et al.*, 1983; Makino *et al.*, 1987). Diabetics also exhibit significantly lower body weights and a higher heart to body weight ratios when compared to control values (Ganguly *et al.*, 1983; Makino *et al.*, 1987). The abnormally high heart to body

weight ratio in the diabetic animals is believed to indicate cardiac hypertrophy (Pierce *et al.*, 1983b; Seager *et al.*, 1984).

Other ultrastructural defects that are commonly associated with the diabetic cell are changes in the deposition of lipids and of carbohydrate (Giacomelli and Weiner, 1979; Jackson *et al.*, 1985). As compared to control hearts, the number of lipid droplets is significantly higher in the diabetic heart (Giacomelli and Weiner, 1979). Similarly, an increase number of glycogen particles is present during chronic diabetes (Jackson *et al.*, 1985). In electron microscopic studies, abnormal Ca^{2+} depositions were observed in the diabetic myocardium (Giacomelli and Weiner, 1979).

In addition, many studies using spontaneous and chemically induced diabetic animals reported significant derangement in contractile proteins. These alterations include loss of contractile proteins (Giacomelli and Weiner, 1979), alterations in the Z-band regions (Saito *et al.*, 1984), myofibrils' disorganisation (Saito *et al.*, 1984) and contracted sarcomere (Seager *et al.*, 1984). Studies have also correlated the depressed function of the diabetic heart with a decrease in myocardial myosin and actomyosin Ca^{2+}-ATPase activities (Malhorta *et al.*, 1981).

The above mentioned ultrastructural changes are not always present in every diabetic model. The development of abnormalities is affected by several factors including the duration and severity of diabetes, the type of diabetes and the presence of other accompanying diseases in the animal (Pierce *et al.*, 1988). In IDDM, for

example, animals exposed to short-term diabetes (a few days) exhibit no ultrastructural defects (Orth and Morgan, 1962). Changes in the mitochondria and at the SR tubules, however were observed as early as 7 days after treatment (Reinila and Akerblom, 1984). By 6 weeks after the induction of diabetes, IDDM animals show gross ultrastructural alterations (Seager *et al.*, 1984).

2. Subcellular abnormalities in the cardiomyocyte

The cardiac cell consists of many organelles. These include the SL, SR, mitochondria, contractile proteins and the nucleus. Each organelle performs a unique task that is important in maintaining the myocardial cell in a functional and viable state. If an organelle becomes impaired, as frequently occurs during many disease states, the functioning of the cardiac cell is compromised. In the case of IDDM, abnormalities in many organelles including the SL, SR and mitochondria have been demonstrated. These defects are believe to be responsible for the reduced performance of the diabetic heart.

2a. Sarcolemmal defects

The cardiac SL contains many functional proteins, including the Ca^{2+} pump, the Na^{+}-Ca^{2+} exchanger, the Na^{+}-K^{+} ATPase and the Ca^{2+} channels all of which are

directly involve in maintaining the myocardial Ca^{2+} homeostasis (Pierce *et al.*, 1988, 1991). Normal cardiac Ca^{2+} concentration is required for proper functioning.

A number of studies have indicated that the functioning of SL membrane proteins is severely disturbed during chronic diabetes. For example, the activities of the Ca^{2+} pump (Makino *et al.*, 1987; Heyliger *et al.*, 1987), and Na^{+}-Ca^{2+} exchanger (Makino *et al.*, 1987; Pierce *et al.*, 1990) were significantly depressed in membrane fractions prepared from IDDM rats. The depressed activities of these proteins were corrected by insulin therapy. A decrease SL Ca^{2+} binding has also been reported in diabetic membrane fractions (Pierce *et al.*, 1983). The impairment in the SL enzymes and in it's Ca^{2+} binding ability would result in abnormal Ca^{2+} homeostasis and may help to explain the reduced performance of the diabetic heart.

In addition, the diabetic SL exhibits abnormal receptor densities. α and β Adrenergic receptor densities were found to be significantly lower in the SL obtained from diabetic animal models, their affinities, however, were unchanged (Heyliger *et al.*, 1982). Since the depression in the adrenergic receptor densities correlated with the duration of the diabetes (Latifpour and McNeill, 1984) and were reversed by insulin therapy (Sundaresan *et al.*, 1984), it was proposed the receptors' defects were due to the diabetic condition of the animal. However, later studies showed that only the defect in the α receptor density is more likely to be due to the diabetic condition since the β receptor density is corrected by maintaining diabetic animals in a euthyroid state (Pierce *et al.*, 1988). Nevertheless, the significance of

having a reduced number of adrenergic receptors would mean that the diabetic heart would have a decrease capability in responding to adrenergic stimulation. This seems to be the case because the diabetic heart displaces a reduced heart rate (Pierce *et al.*, 1988).

The phospholipid composition of the diabetic SL is also altered. The concentrations of phosphatidylethanolamine, diphosphatidylglycerol and lysophosphatidylcholine are altered in the diabetic heart (Pierce *et al.*, 1983, 1988). These phospholipid alterations also help to explain the diabetic malfunction heart since the biophysical property of SL modulates the enzymatic activity of the proteins that are embedded or associated with this organelle (Paphadjopoulous *et al.*, 1973).

2b. Abnormal functioning of the Sarcoplasmic Reticulum

In the cardiac cell, the SR is the organelle that is primarily responsible for regulating cytoplasmic Ca^{2+} balance that is necessary for excitation-contraction coupling of the heart (Pierce *et al.*, 1988).

The first observation that suggests that the SR may be malfunctioned in the diabetic heart was due to changes in the diabetic relaxation rate (Fein *et al.*, 1980). The correlation between the SR Ca^{2+} accumulation and the relaxation rate of the heart is known (Dhalla *et al.*, 1982). This suggests that an abnormal SR Ca^{2+} reuptake may be responsible for the slower removal of cytoplasmic Ca^{2+} that would

reduce the rate of relaxation. Experiments on SR fractions obtained from chronic diabetic animals have shown that this was indeed the case. The diabetic SR exhibits depression in it's Ca^{2+} handing systems including the activity of Ca^{2+} pump and Ca^{2+}-stimulated ATPase activity (Ganguly *et al.*, 1983). A depression in SR Ca^{2+} binding was also observed (Lopaschuk *et al.*, 1983a).

The depression in the diabetic SR Ca^{2+} accumulating capacity is attributed to the diabetic condition since it was reversed by insulin therapy (Ganguly *et al.*, 1983). Aterations in the diabetic SR function is believed to be due a lesion in the SR Ca^{2+} transport (Pierce *et al.*, 1988). They could also have resulted from the accumulation of endogenous long-chain acyl-carnitines which inhibit the SR Ca^{2+} transport (Lopaschuk *et al.*, 1983b). Carnitine treatmentt to diabetic rats prevented the accumulation of long-chain acyl-carnitines in the myocardium and restored the SR Ca^{2+} transport function, however, cardiac function was still depressed (Lopaschuk *et al.*, 1983a). This suggests that defectives in the SR function contribute to the development of the diabetic cardiomyopathy. It is however not the only subcellular defect responsible for the cardiac pathology.

2c. Mitochondrial Changes

The function of the mitochondria in the cardiac cell includes its role in providing the energy needed for force generation (Pierce *et al.*, 1988) and its capacity to act as a Ca^{2+} sink under pathological conditions (Dhalla *et al.*, 1982).

Studies have shown that the mitochondrial function is abnormal during IDDM cardiac pathology. The oxidative phosphorylation capacity and Ca^{2+} uptake ability of the mitochondria were significantly depressed in isolated fractions from diabetic hearts (Pierce and Dhalla, 1985). Similar observations were also obtained from *in situ* studies on permeabilised diabetic cardiomyocytes (Tanaka *et al.*, 1992). The defect in mitochondria Ca^{2+} transport was dependent on high Ca^{2+} concentrations which imply that the diabetic myocardium is characterised by a disruption in its mitochondrial Ca^{2+} buffering capacity (Pierce *et al.*, 1988). The mitochondrial Ca^{2+} binding capacity was however unaltered by the diabetic condition (Pierce *et al.*, 1988). The cardiac mitochondrial changes that are associated with the diabetic state in the rat, were also corrected by insulin treatment (Pierce and Dhalla, 1985, Tanaka *et al.*, 1992).

Given the functional consequences of having unaltered Ca^{2+} homeostasis in the diabetic heart and the possibility that myocardial PLD is involved in maintaining the cardiomyocyte Ca^{2+} balance, it is possible that alterations in cardiac PLD and PAP activities may be a molecular mechanism underlying the cardiomyopathy.

III. MATERIALS AND METHODS

A. Materials

1-α-1-Palmitoyl-2[^{14}C]oleoylphosphatidylcholine (specific activity 58.0 mCi/mmole) and L-α-dipalmitoyl-[glycerol-^{14}C(U)]-phosphatidic acid (specific activity 144 mCi/mmol) were purchased from DuPont New England Nuclear, Mississauga, Ontario, Canada. Egg PtdCho, egg PtdOH and oleic acid (sodium salt) were obtained from Serdary Research Laboratories. Silica gel 60AF-254 thin-layer chromatography plates were purchased from Whatman International LTD, Maidson (U.K). Ultralente Insulin zinc suspension (beef and pork) was obtained from Connaught Novo Nordisk, Mississauga, Ontario, Canada. Solvents were purchased from Mallinckrodt, Canada. CytoScint^{TM} ES* is a product of ICN Biomedicals Inc. (Mississauga, Ontario., Canada). All other chemicals were bought from Sigma Chemical Company, St. Louis Missouri, USA. All reagents were of analytical grade or of the highest grade available.

B. Methods

1. Pathological Models

1a. Diabetes

Male Sprague-Dawley rats weighting approximately 200g were used in this study. Diabetes was induced by a single tail vein injection of streptozotocin (65mg/kg body weight) dissolved in a citrate-buffered vehicle (0.1 M citrate, pH 4.5) (Rakienten *et al.*, 1963). Age-matched control were injected with citrate buffer alone. All animals were housed in the Animal Holding at St. Boniface General Hospital Research Centre and were provided with food and water *ad libitum* during the entire study.

The diabetic animals were subdivided randomly into two groups 6 weeks after streptozotocin (STZ) injection. One group of animal received 3U Ultralente zinc insulin per day for 2 weeks (reversal) while the second group was given saline for the same time period (diabetic). All animals, including age-matched controls, were sacrificed by decapitation at end of 8 weeks. Blood samples were collected at the time the animals were sacrificed and were analyzed for insulin (by radioimmunoassay technique), glucose (by Sigma Diagnostics kit), and thyroxine (by time resolved Fluoroimmunoassay).

1b. Hypothyroidism

Hypothyroidism was induced in male Sprague Dawley rats weighting 175-200g. The animals received a 0.05% propylthiouracil (PTU) in their drinking water for 8 weeks. Age-matched controls were given water only for the same time period (Daly and Dhalla, 1985). Blood samples were collected when the animals were sacrificed and were used to assessed the thyroxine levels (by time resolved Fluoroimmunoassay).

2. Isolation of Cardiac Sarcolemmal Membranes

At the end of the treatment periods, all animals were sacrificed by decapitation, and the hearts were quickly excised and immersed in 0.6 M sucrose - 10 mM imidazole (pH 7.0). The ventricular tissues were washed and minced. The sarcolemmal isolation procedure was a modification of the Pitts method (1979). The tissues were homogenized in 0.6 M sucrose - 10 mM imidazole, pH 7.0 (3.5 ml/g tissue) with a polytron PT-3000 (6 x 15s, setting 13,000 rpms). The resulting homogenates were centrifuged at 12,000 g for 30 min. The supernatants were collected and diluted (5 ml/g) with 160 mM KCl - 20 mM 3-(N-morpholino)-propanesulphonic acid (MOPS), pH 7.4, and centrifuged at 100,000 g for 60 min. The resulting pellets were resuspended in 160 mM KCl - 20 mM MOPS buffer (pH 7.4) 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 Beckman SW28 swinging bucket rotor), the band at the sucrose-buffer interface was taken and diluted with 3 volumes of 160 mM KCl - 20 mM MOPS, pH 7.4. This mixture was again centrifuge at 100,000 g for 30 min. The resulting pellet which was rich in sarcolemma was suspended in 0.25 M sucrose - 10 mM histidine, pH 7.4 (approx. 3.5 mg/ ml) and the quickly frozen and stored at -70^{0}C.

3. Isolation of Sarcoplasmic Reticular Membranes

All animals were sacrificed by decapitation, hearts were immediately excised and atria and large vessels were carefully removed. The ventricular tissue was used to isolate sarcoplasmic reticular-enriched membranes according to the procedure of Harigaya and Schwarts (1969). The ventricular tissue was minced in 10 mM $NaHCO_3$, 5 mM NaN_3, 15 mM Tris-HCl, pH 6.8 (8 ml/g tissue), and then homogenized with polytron PT-3000 (3 x 10s, 10800 rpm). The homogenate was centrifuged at 10,000g for 20 min to remove the heavier subcellular organelles. The pellet was discarded and the supernatant was again centrifuged at 10,000g for 20 min. The resulting supernatant was furthered centrifuged at 40,000g for 45 min, after which the pellet obtained was suspended in 0.6 M KCl, 20 mM Tris-HCl, pH 6.8. This mixture was centrifuged at 40,000g for 45 min. The final pellet

obtained was suspended in a solution of 0.25 M sucrose, 10 mM histidine (pH 7.4) and then quickly frozen and stored at -70^{0}C.

4. Isolation of Cardiac Mitochondrial Membrane Fractions

Ventricular tissue was used to isolate mitochondria by method of Sordahl, *et al* (1971). Animals were sacrificed by decapitation and the hearts were removed and immediately suspended in a medium containing 0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl (pH 7.0). The ventricular tissue was homogenized in a Waring Blender for 20 sec in 10 volumes of a 0.18 M KCl, 10 mM EDTA, 0.5% bovine serum albumin solution (pH 7.4). The homogenate was centrifuged at 1,000g for 10 min to remove heavier subcellular organelles. The resultant supernatant was further centrifuged at 10,000g for 20 min. The pellet obtained was suspended in 0.18 M KCl, 10 mM EDTA, 0.5% bovine serum albumin solution (pH 7.4) and centrifuged two more times at 10,000g. The final pellet was suspended in 0.25 M sucrose - 10 mM histidine, pH 7.4 and then quickly frozen and stored at -70^{0}C. All isolation steps were carried out at 0-4 ^{0}C.

5. Phospholipase D Assays

Phospholipase D (PLD) hydrolytic activity in membrane fractions was assayed by measuring the formation of PtdOH from exogenous [^{14}C]PtdCho (Dai, 1993). PtdCho substrate was prepared by mixing aliquots of egg PtdCho and phosphatidylcholine-[oleoyl-^{14}C]. The organic solvent in the mixture was evaporated under a stream of N_2, and the lipids were resuspended in an aqueous solution of 25 mM sodium oleate by sonication for 30 min in a sonicator bath (model 1200, Branson Ultrasonics Corp., Danbury, Conn.). In the standard assay, incubations were carried out at 25°C for 60 min in a medium containing 35-50 μg membrane protein, 50 mM β,β-dimethylglutaric acid (DMGA) plus 10 mM EDTA (pH 6.5), 25 mM KF, 5 mM sodium oleate, and 2.5 mM [^{14}C] PtdCho (0.167 mCi /mmol) in a final volume of 120 μL. For measuring PLD transphosphatidylation activity, the reaction medium was similar to that used for the hydrolytic assay except that 400 mM of ethanol was also included. All reactions were terminated by addition of 2 ml chloroform: methanol (2:1, v/v), and the phases were separated by the adding 0.5 ml of 0.1 M KCl. The test tubes were vortexed vigorously for 2 min and then centrifuged. The upper phase was discarded, and the lower phase was washed with 1 ml of chloroform: methanol: 0.1 M KCl (2: 1: 0.5 v/v) to remove nonlipid contaminants. Blanks were performed by the same procedure, except that membranes were added after stopping the reaction. The final lipid

extracts were evaporated to almost dryness under a stream of N_2, and redissolved in chloroform containing PtdOH or PtdEtOH as a carrier. This mixture was quantitatively applied to silica gel 60A F-254 thin-layer (0.25 mM) plates. The plates were developed in chloroform: methanol: acetone: glacial acetic acid: water (50:15:15:10:5, v/v). The lipid spots were visualized with iodine vapor and scraped. Quantitation was done by liquid scintillation counting (model 1701, Beckman Instruments, Inc.).

6. Phosphatidic Acid Phosphatase Assay

The phosphatidic acid phosphohydrolase (PAP) activity was assayed by measuring the formation of diacylglycerol (DAG) from exogenous labeled PtdOH. The assay procedure was basically according to Martin, *et al* (1991). The substrate was prepared by mixing aliquots of egg PtdOH, egg PtdCho and L-α-dipalmitoyl-[glycerol-^{14}C(U)]-phosphatidic acid (specific activity 144 mCi/mmol). The organic solvent was evaporated under a stream of N_2, and 5 mM EDTA plus 5 mM EGTA (pH 7.0) was added to give a final concentration of 3 mM PtdOH and 2 mM PtdCho. The lipids were dissolved by sonication for 5 min in a sonication bath (model 1200, Branson Ultrasonics, Corp.). In the standard assay PAP activity was measured in medium containing 100 mM Tris - maleate buffer (pH 6.5), 1 mM

dithiothreitol (DTT), 0.6 mM [^{14}C] PtdOH (1 Ci/mol), 0.4 mM PtdCho, 1 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, and 35-50 μg protein in a total volume of 100 μL. In some experiments 3 mM $MgCl_2$ was included and in others the membrane proteins were preincubated with 4.2 mM N-ethylmaleimide (NEM) at 37^{0}C for 10 min and then cooled on ice. Blanks contained the same assay medium as samples, but membrane were added after the reactions were stopped. The medium was incubated at 37^{0}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 KCl. The test tubes were vortexed vigorously for 2 min and the phases were separated by centrifugation. The upper phase was discarded. The lower phase was evaporated to almost complete dryness under a stream of N_2 and redissolved in chloroform containing DAG and monopalmitoylglycerol as carriers and quantitatively applied to silica gel 60A thin layer (0.25 mM) plates (Whatman Co.). The plates were developed in petroleum ether : ether: acetic acid (60:40:1 v/v) for 30 min. The lipid spot were visualized with iodine vapors and scraped. The R_f values were 0.34 and 0.06 for DAG and monoacylglycerol, respectively. The scrapings were solubilized with CytoScint^{TM} ES* (ICN Biochemical Canada Ltd.) and counted in a liquid scintillation system (model 1701, Beckman Instruments, Inc.).

7. Miscellaneous Assays

7a. Protein determinations

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

7b. Statistical analysis.

The differences between the means of two groups were evaluated for significance by Students' *t* test. For comparing more than two groups, multiple analysis of variance was carried out and Duncan's new multiple-range test was used to determined differences between the means within the populations. A probability of 95% or more was considered significant.

VI. RESULTS

A. The effect of diabetes on the sarcolemma PLD/PtdOH phosphatase signaling pathway.

1. General characteristics of the diabetic animals

Eight weeks after STZ injection, the body and ventricular weights of the diabetic animals were significantly lower than control values (Table 1). The ventricular-to-body weight ratios in the diabetic animals were significantly higher than those observed for the age-matched control group. Two weeks of insulin therapy resulted in higher body and ventricular weights in the insulin-treated diabetic group as compared to the untreated diabetics, however these parameters were not normalized to the control group.

Diabetes in the STZ-injected animals was confirmed by a significantly elevated plasma glucose level and by significantly depressed circulating levels of insulin and T_4 (Table 1). These parameters are similar to those reported in earlier studies in which the experimental protocol was the same as the one employed in this study (Pierce and Dhalla, 1983; Ganguly *et al.*, 1984; Makino *et al.*, 1987). It should be noted that the hypothyroid condition observed in the untreated diabetic animals is not usually present in all forms of diabetes but is associated with the STZ-induced rat model (Wilber *et al.*, 1981).

TABLE 1. General characteristics of the diabetic animals

	Age-matched controls	Diabetic	Insulin-treated Diabetic
Body weight, g	476.0 ± 4.6	264.1 ± 6.3*	335.2 ± 2.1*
Ventricular weight, g	1.22 ± 0.04	0.86 ± 0.01*	0.95 ± 0.01*
Ventricular/body weight ratios, mg/g	2.49 ± 0.04	3.26 ± 0.06*	2.82 ± 0.03*
Plasma Glucose, mg/dL	179.8 ± 1.7	532.1 ± 11.9*	180.7 ± 7.4
Serum Insulin, pmol/L	157.0 ± 5.2	44.67 ± 2.4*	166.7 ± 8.8
Serum T_4, nmol/L	136.7 ± 2.4	43.7 ± 1.8*	123.4 ± 2.1

Values are expressed as means ± SEM of 12-15 experiments. Animals were treated as described in Material and Methods. Serum levels of T_4 were determined by time-resolved fluoroimmunoassay technique (Wallac, Finland). Glucose levels were tested by Sigma Diagnostic kit and insulin levels by radioimmunoassay techniques.

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

2. Sarcolemmal PLD/PtdOH phosphatase activities

2a. PLD hydrolytic activity

Purified SL membrane obtained from the ventricles of control, diabetic, and insulin-treated diabetic animals were used to assess the hydrolytic activity of PLD. PtdOH formation from exogenous [^{14}C]PtdCho was significantly depressed by 30 ± 1.3% in untreated diabetic SL when compared to control values (Figure 1). No significant difference in PLD activity between the control and insulin-treated diabetic group was observed. When PLD activity was measured in the above mentioned groups, at various time points of incubation (from 15 to 90 min), the enzyme activity in the untreated diabetic group was significantly depressed at all time points tested (Figure 2). No significant difference was observed in PLD activity between control and insulin-treated diabetic groups for any of the time points tested. These results indicate that the specific hydrolytic activity of PLD, as measured by the production of PtdOH from PtdCho, is significantly depressed in chronic diabetes, but can be normalized by two weeks of insulin therapy.

2b. PLD transphosphatidylation activity

In order to verify if the changes in PtdOH production are due to depressed PLD activity, the transphosphatidylation mode of the enzyme was measured in the

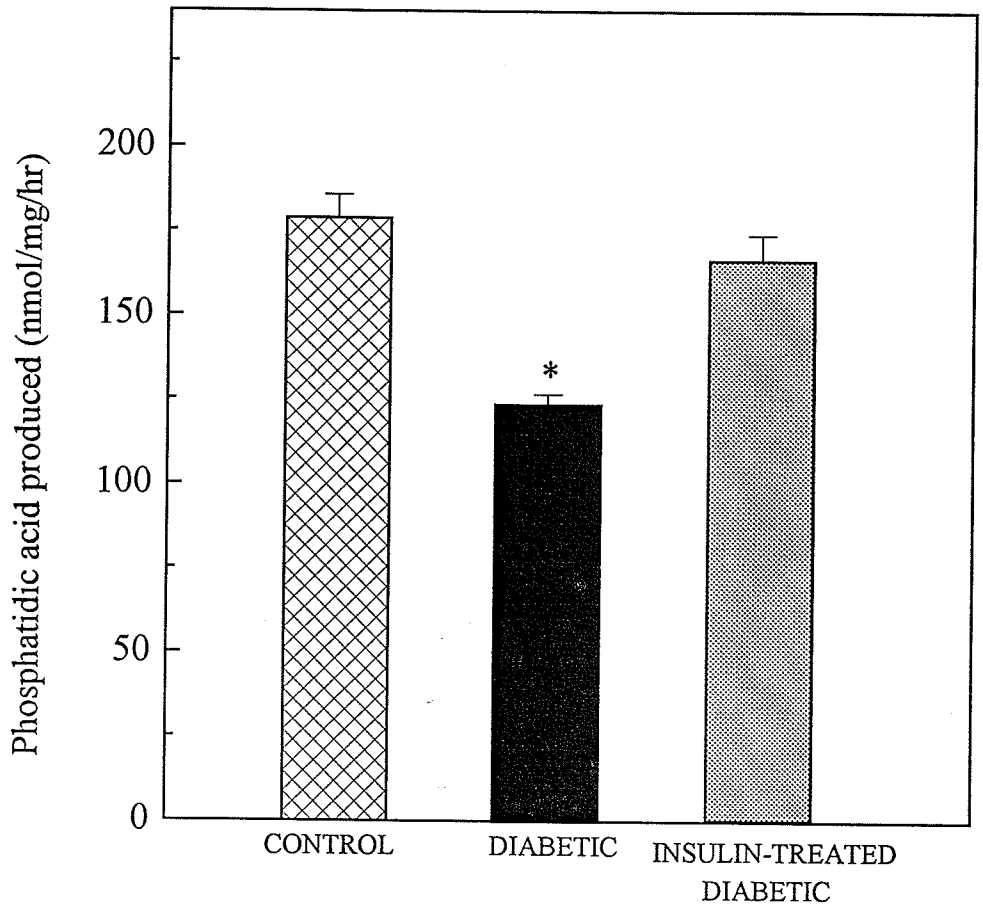

Figure 1. Phospholipase D hydrolytic activity in sarcolemma isolated from control, diabetic and insulin-treated diabetic rat heart.

The results represent the means ± SEM of four different membrane preparations that were tested in triplicate. SL membranes were incubated in 50 mM DMGA-10 mM EDTA (pH 6.5), 25 mM KF, 2.5 mM [^{14}C]PtdCho for 60 min at 25^{0}C and in the presence of 5 mM sodium oleate. Lipid extraction, separation and quantification were as described in Materials and Methods

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

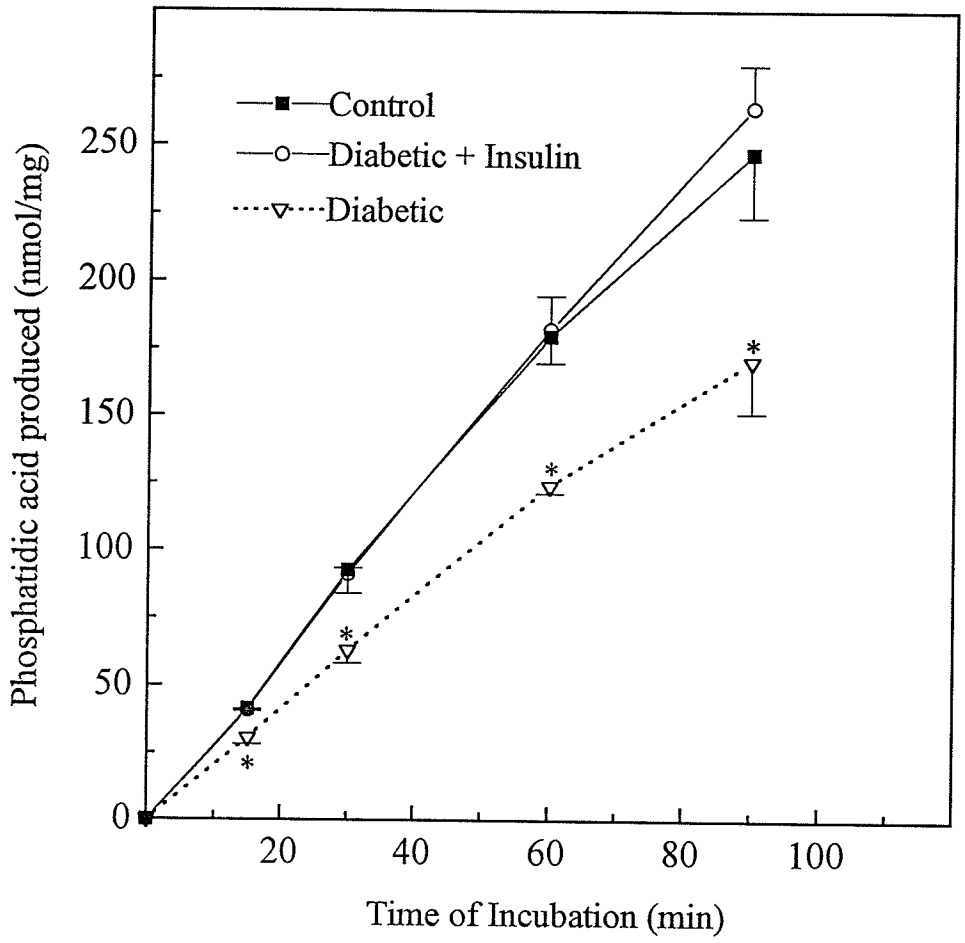

Figure 2. Time-course of phospholipase D hydrolytic activity

Values are mean ± SEM of four experiments done in triplicate. 50 μg of SL protein were incubated as described in Materials and Methods for the time points indicated time.

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

experimental groups. In the presence of 400 mM ethanol, the amount of phosphatidylethanol (PtdEtOH) produced in untreated diabetic SL was significantly depressed as compared to the control values (107.17 ± 3.6 and 79.35 ± 8.81 nmol/mg/h for control and untreated diabetic, respectively) (Figure 3). There was no observable difference in the amount of PtdEtOH produced in control and insulin-treated diabetic SL.

Figure 3 also indicates that even under this experimental condition in which minimal amounts of PtdOH are produced, there was still significantly less PtdOH generated in the untreated diabetic SL. These results indicated that the transphosphatidylation mode of PLD is also depressed in chronic diabetic rats.

2c. PtdOH phosphatase activity

Two forms of PAP are known to be present in mammalian tissue. One form, which is membrane-bound, is believed to be both NEM insensitive and Mg^{2+} independent. The other resides in the cytosol and in the endoplasmic reticulum, and is NEM sensitive - Mg^{2+} dependent (Martin *et al.*, 1991). Because the activity and localization of PAP were not previously established in cardiac cells, some initial experiments were performed to deduce the optimal assay conditions for SL PAP activity.

Table 2 shows that in the absence of 3 mM $MgCl_2$, cytosolic PAP activity from control hearts, was significantly lower (by 76.2%) than the enzyme activity

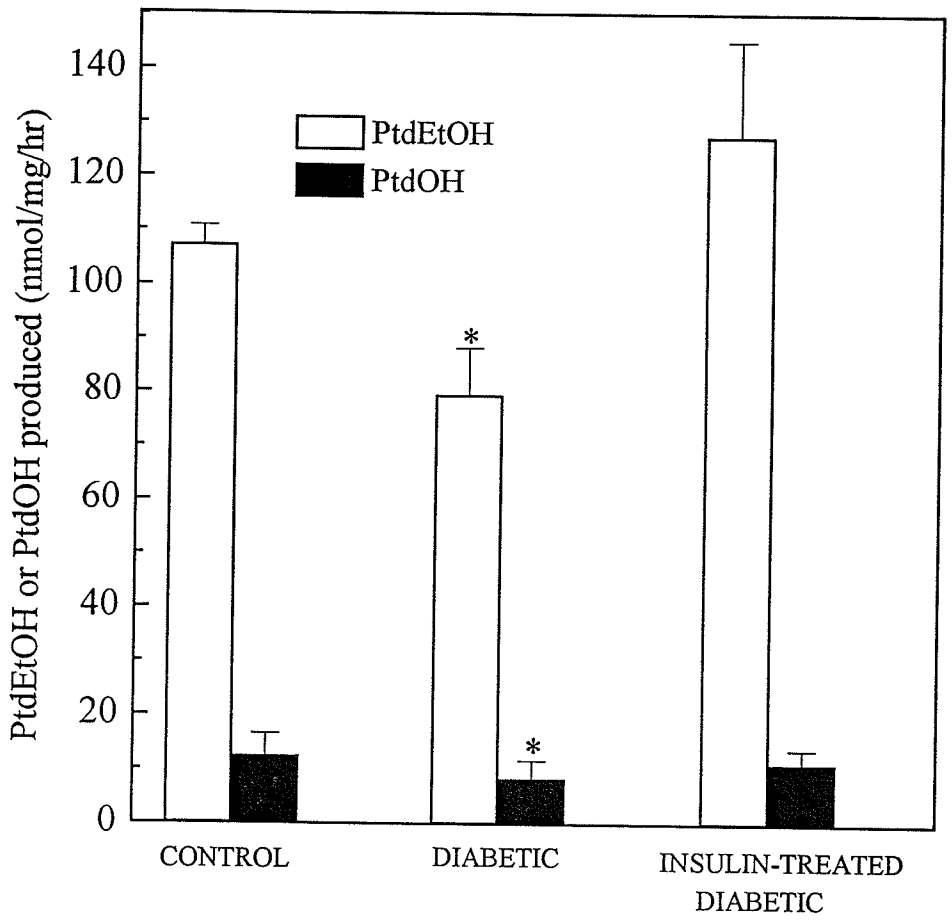

Figure 3. Transphosphatidylation activity of phospholipase D in sarcolemma membranes prepared from experimental rat hearts.

Results represent the mean ± SEM of four experiments done in triplicate. SL membranes were incubated with 50 mM DMGA - 10 mM EDTA (pH 6.5), 25 mM KF, 400 mM ethanol, 2.5 mM [^{14}C]PtdCho for 60 min, 25^{0}C and in the presence of 5 mM sodium oleate. The presence of ethanol in the media was necessary to induce the PLD transphosphatidylation mode as opposed to the hydrolytic mode. This is indicated by an increase formation of PtdEtOH at the expense of PtdOH (Dai, 1993).
PtdEtOH = phosphatidylethanol; PtdOH = phosphatidic acid.

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

TABLE 2. The effect of magnesium and N-ethylmaleimide on sarcolemmal and cytosolic PtdOH phosphatase activities.

	Phosphatidic Acid phosphatase (nmol/mg/hr)	
	Sarcolemma	Cytosol
3 mM $MgCl_2$	501.02 ± 32.75 (100 ± 6.5%)	45.06 ± 4.60 (100 ± 10.2%)
Without $MgCl_2$ or NEM	490.36 ± 15.70 (97.9 ± 3.1%)	10.72 ± 0.80* (23.8 ± 1.8%)
4.2 mM NEM	438.50 ± 31.23 (87.5 ± 6.2%)	9.89 ± 0.29* (21.9 ± 0.6%)

Values represents the mean ± SEM of three experiments done in triplicate. 50μg of SL or cytosolic proteins were incubated with [^{14}C]PtdOH, 2.5% Triton X-100, 1 mM DTT, 50 mM Tris-maleate buffer (pH 6.5), at 37^{0}C for 10 min in the presence or absence of either 3 mM $MgCl_2$ or 4.2 mM NEM. Lipid extraction, separation and quantification were described in Materials and Methods. Data in parentheses indicate the percentage of the value obtained in the presence of 3 mM $MgCl_2$.
NEM = N-ethylmaleimide.

*Significantly different ($P < 0.05$) from respective value in the presence of $MgCl_2$.

of the same fraction with Mg^{2+}. The cytosolic enzyme activity was also significantly depressed by 4.2 mM NEM. In comparison to the cytosolic enzyme, the SL PAP activity was not dependent on Mg^{2+} as a co-factor and was not affected by NEM. Therefore, all subsequent experiments on the SL PAP were done without Mg^{2+}.

SL membranes isolated from control, diabetic and insulin-treated diabetic animals were used to assess the activity of PAP by measuring the formation of DAG from [^{14}C]PtdOH. Figure 4 shows that in untreated diabetic SL the amount of [^{14}C]DAG produced was significantly higher than that which was produced in the control group. There was also a significantly higher (20.9 ± 1.9%) PAP activity in the insulin-treated diabetic SL as compared to control values.

These results indicate that the SL PAP activity is altered during insulin dependent diabetes. PAP activity in diabetic SL was significantly reduced but was incompletely normalized by the insulin therapy (662.70 ± 18.93 and 593.64 ± 9.46 nmol/mg/h for untreated and insulin-treated diabetic, respectively).

2d. Effect of KF on PLD hydrolytic activity

Because the activities of PLD and PAP seem to be altered in an opposite way in response to STZ-induced diabetic condition, we examined the amounts of PtdOH and DAG which were produced in the assay media with and without 25

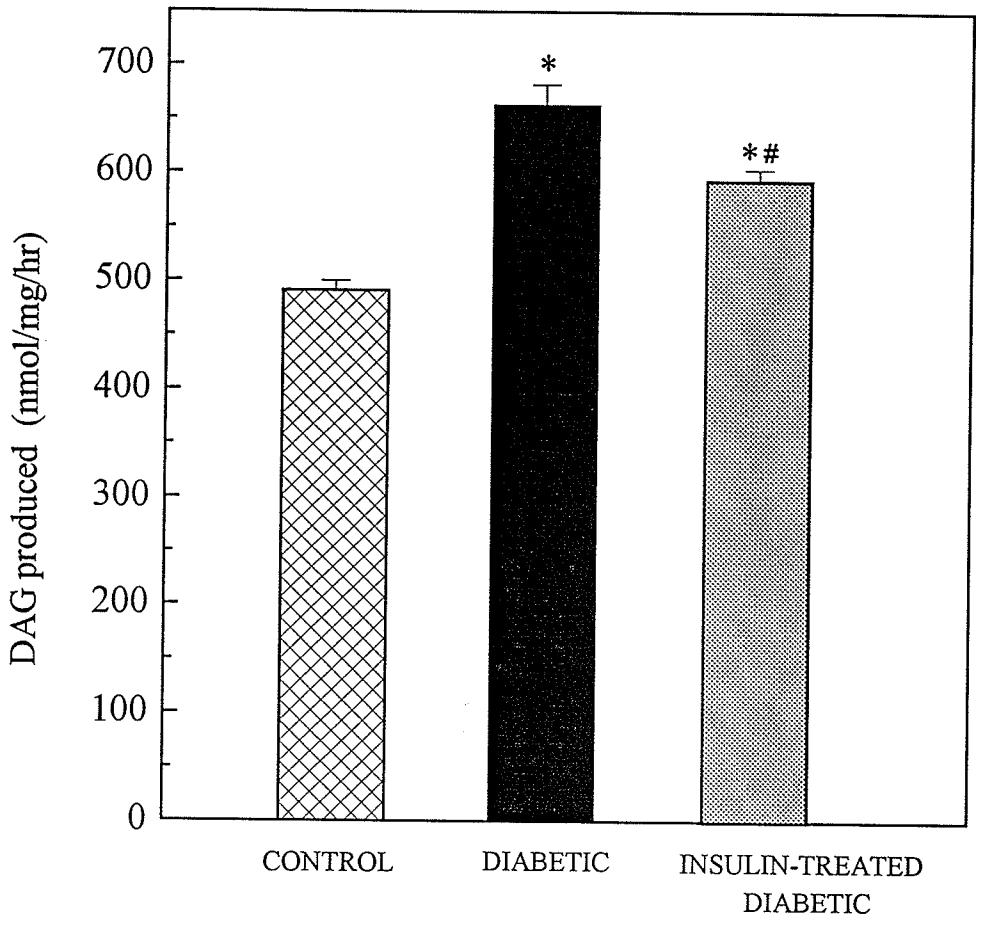

Figure 4. Phosphatidic acid phosphatase activity of sarcolemma isolated from experimental groups

The results represent the mean ± SEM of five to six different membrane preparations tested in triplicate. PtdOH phosphatase activity was assayed in the presence of 3 mM [^{14}C]-phosphatidic acid, 2.5% Triton X-100, 1mM DTT, 50 mM Tris-maleate buffer (pH 6.5) and 50μg of SL membrane protein at 37^{0}C for 10 min. DAG = Diacylglycerol

*Significantly different ($P < 0.05$) from the control value
#Significantly different ($P < 0.05$) from the diabetic value

mM KF, a partial inhibitor of PAP activity (English *et al.*, 1991). In the presence of 25 mM KF, the amount of PtdOH produced in the untreated diabetic SL was 29.1 ± 1.7% less than that obtained in the control group (Table 3). There was no difference in PtdOH found in insulin-treated group verses control values. There was no significance difference in the amount of DAG produced in all groups, in the presence of KF.

In comparison, the amount of PtdOH produced in the absence of KF was significantly decreased by 66.9 ± 0.6% and by 23.6 ± 0.6% in untreated and insulin-treated diabetic groups, respectively, as compared to the control values (Table 3). The amount of DAG produced without KF was also significantly lower (by 27.3%) in the untreated diabetic groups whereas for the insulin treated groups, the values were similar to those obtained in the control groups (Table 3).

TABLE 3. Effect of KF, an inhibitor of PtdOH dephosphorylation to DAG on PLD- and PAP-dependent sarcolemmal levels of PtdOH and DAG during diabetes.

	PtdOH (nmole/mg/hr)	DAG (nmole/mg/hr)
25 mM KF		
control	174.31 ± 5.90 (100 ± 3.38%)	11.12 ± 0.66 (100 ± 5.9%)
diabetic	123.42 ± 2.88* (70.8 ± 1.7%)	10.56 ± 0.66 (94.9 ± 5.9%)
diabetic + insulin	166.45 ± 7.48 (95.5 ± 4.3%)	12.46 ± 0.82 (112.0 ± 7.4%)
Without KF		
control	62.46 ± 4.61 (100± 7.4%)	151.31 ± 8.62 (100 ± 5.5%)
diabetic	20.69 ± 4.24* (33.1 ± 6.8%)	110.04 ± 12.73* (72.7 ± 8.4%)
diabetic + insulin	47.69 ± 4.29* (76.4 ± 6.8%)	174.12 ± 19.07 (115.0 ± 12.6%)

Values represent the mean ± SEM of four experiments done in triplicate. Sarcolemmal membranes were incubated with or without 25 mM KF as described in Materials and Methods. Data in parentheses are the percentage of respective controls.
DAG = diacylglycerol; PtdOH = phosphatidic acid; PLD = phospholipase D; PAP = phosphatidic acid phosphatase.

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

B. The effect of hypothyroidism on the SL PLD/PtdOH phosphatase signaling pathway

1. General characteristics of the hypothyroid animals

As shown in Table 1, the untreated diabetic animals exhibited both high glucose levels and low T_4 levels. This is consistent with the literature in that the hypothyroid condition as been shown to be associated with STZ-induced diabetic model, in rats (Wilber *et al.*, 1981). Because of this fact, it was necessary to examine a hypothyroid model to determine if the observed changes in PLD/PAP activities were due to the diabetic condition or were the result of the associated hypothyroid condition.

Table 4 shows that after 8 weeks of PTU-treatment, the animals were significantly smaller and had significantly lower circulating T_4 levels, as compared to age-matched control values. Similar observations were made in other studies that employed the same experimental protocol (Mesaeli, 1993).

TABLE 4. General characteristics of the hypothyroid animals

	Euthyroid	Hypothyroid
Body weight, g	457.8 ± 11.6	291.9 ± 6.3*
Ventricular weight, g	1.02 ± 0.04	0.56 ± 0.02*
Ventricular/body weight ratios, mg/g	2.24 ± 0.04	1.93 ± 0.03*
Serum T_4, nmol/L	147.5 ± 16.5	26.0 ± 2.1*

Values are expressed as means ± SEM of 6-12 experiments. Animals were treated as described in Material and Methods. Serum levels of T_4 were determined by time-resolved fluoroimmunoassay technique.

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

2. Sarcolemma PLD hydrolytic and PtdOH phosphatase activities

PLD hydrolytic and PAP activities were assessed in purified SL that was prepared from age-matched controls and 8 weeks PTU-treated hypothyroid animals. The assay conditions were identical to those used to assess the enzyme activities in the diabetic protocol.

Both PLD and PAP activities in PTU-treated group were similar to the respective activities in the control SL (Table 5). Thus, neither PLD nor PAP activity was significantly affected by a condition in which T_4 level alone was depressed. These results indicate that the observed changes in the SL PLD and PAP activities in STZ-induced diabetic rat hearts are due to the diabetic condition and are not the result of the associated hypothyroidism.

C. Effect of diabetes on phospholipase D and PtdOH phosphatase activities associated with the sarcoplasmic reticular and mitochondrial fractions

1a. PLD hydrolytic activity in the SR and the mitochondria

The SR and mitochondrial PLD activities have been found to share with the SL enzyme a number of properties such as optimal pH, temperature and KF concentrations as well as substrate specificity (Dai, 1993). Therefore, the SR and

TABLE 5. The effect of hypothyroidism on the sarcolemmal phospholipase D and PtdOH phosphatase activities.

	PLD activity	PAP activity
	nmole/mg/h	
Euthyroid	216.4 ± 19.2	798.88 ± 12.92
Hypothyroid	185.9 ± 8.6	828.51 ± 47.25

Values represents the mean ± SEM of three experiments done in triplicate. Both PLD and PAP activities were tested as described in Materials and Methods.

mitochondrial PLD activities were examined using experimental conditions identical to those used for the SL enzyme.

The SR and mitochondrial PLD hydrolytic activities in untreated diabetic membranes were significantly depressed by 31.5 ± 0.9% and by 50.4 ± 3.0%, respectively, as compared to the control values (Figure 5). In contrast to the SL enzyme, whose activity was brought back to control values by 14 days of insulin therapy, the SR and mitochondrial PLD activities were only partially corrected by the insulin treatment. This is evident by the 14.2 ± 3.7% and 28.9 ± 1.6% lower PtdOH produced in insulin-treated diabetic SR and mitochondria as compared to controls, respectively.

1b. PtdOH phosphatase activities in SR and mitochondria

As previously established assays for PAP did not assess the Mg^{2+} and NEM dependence of the cardiac SR and mitochondrial enzyme, these parameters were examined. The presence of 3 mM $MgCl_2$ or 4.2 mM NEM in the assay mixture did not significantly affect PAP activity, either in the SR or in the mitochondria (Table 6). It was therefore concluded that both the SR and mitochondrial PAP enzymes share similar characteristics with SL enzyme, both beings Mg^{2+} independent and NEM insensitive. Therefore, to measure the SR and mitochondrial PAP activities the assay condition that was identical to those used to examine the SL enzyme.

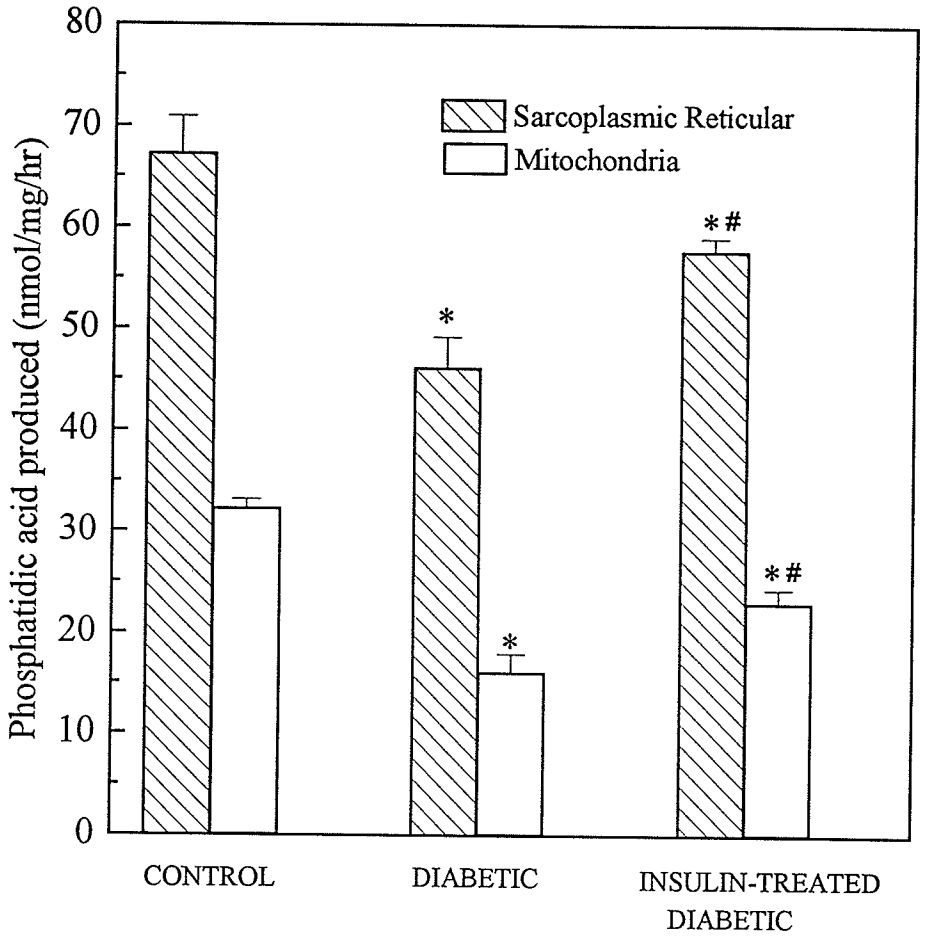

Figure 5. Phospholipase D hydrolytic activity in sarcoplasmic reticular and mitochondrial membrane preparations from diabetic animals.

The results represent the mean ± SEM of four SR or mitochondrial membrane preparations tested in triplicate. Membrane fractions were incubated using the standard assay as described in Materials and Methods.

*Significantly different ($P < 0.05$) from respective control.
#Significantly different ($P < 0.05$) from the diabetic value.

TABLE 6. The effect of magnesium and N-ethylmaleimide on PtdOH phosphatase activity of sarcoplasmic reticulum and mitochondria.

	Phosphatidic Acid phosphatase activity (nmol/mg/hr)	
	Sarcoplasmic reticulum	Mitochondria
3 mM $MgCl_2$	197.3 ± 26.3 (100 ± 13.3%)	93.1 ± 5.7 (100 ± 6.1%)
Without $MgCl_2$ or NEM	186.2 ± 21.2 (94.3 ± 10.7%)	95.0 ± 8.6 (102 ± 9.2%)
4.2 mM NEM	178.9 ± 23.4 (90.7 ± 11.8%)	84.4 ± 0.4 (90.7 ± 0.4%)

Values represent the mean ± SEM of three experiments done in triplicate. Membrane fractions from sarcoplasmic reticulum or mitochondria were incubated under standard conditions in the presence or absence of 3 mM $MgCl_2$ or 4.2 mM NEM. Data in parentheses are a percentage of the value obtained in presence of magnesium.

Figure 6 indicates that SR PAP activity was significantly elevated (by 30%) in the untreated diabetic membranes (215.32 ± 10.76 and 281.06 ± 20.64 nmol/mg/h for control and untreated diabetic groups, respectively). Two weeks of insulin treatment normalized the observed increase in the enzyme activities.

Unlike the SR enzyme, which had a higher activity in chronic untreated diabetic membranes, the mitochondrial PAP activity was not affected by the diabetic condition (Figure 6). There was no observable difference in the mitochondrial PAP activity in any of the three groups tested.

2a. Effect of KF on the SR PLD activity

Table 7 shows that the SR PLD hydrolytic activity in the untreated diabetic group was significantly inhibited (by 31.5 ± 0.9%) in the presence of 25 mM KF, as compared to control values. In the absence of KF, however, the inhibition of the enzyme activity was 61.0 ± 1.5%. For the insulin treated diabetic membranes, PLD activity was significantly lower (by 14.2 ± 3.7% and 63.1 ± 3.8%) when tested in the presence and absence of KF, respectively. A similar trend was also observed for DAG production (Table 7). These results confirmed that the SR PLD hydrolytic activity is depressed by chronic diabetes, and is not normalized by insulin therapy. Moreover, in the absence of KF or PAP inhibitor the intramembranal PtdOH level is further depressed.

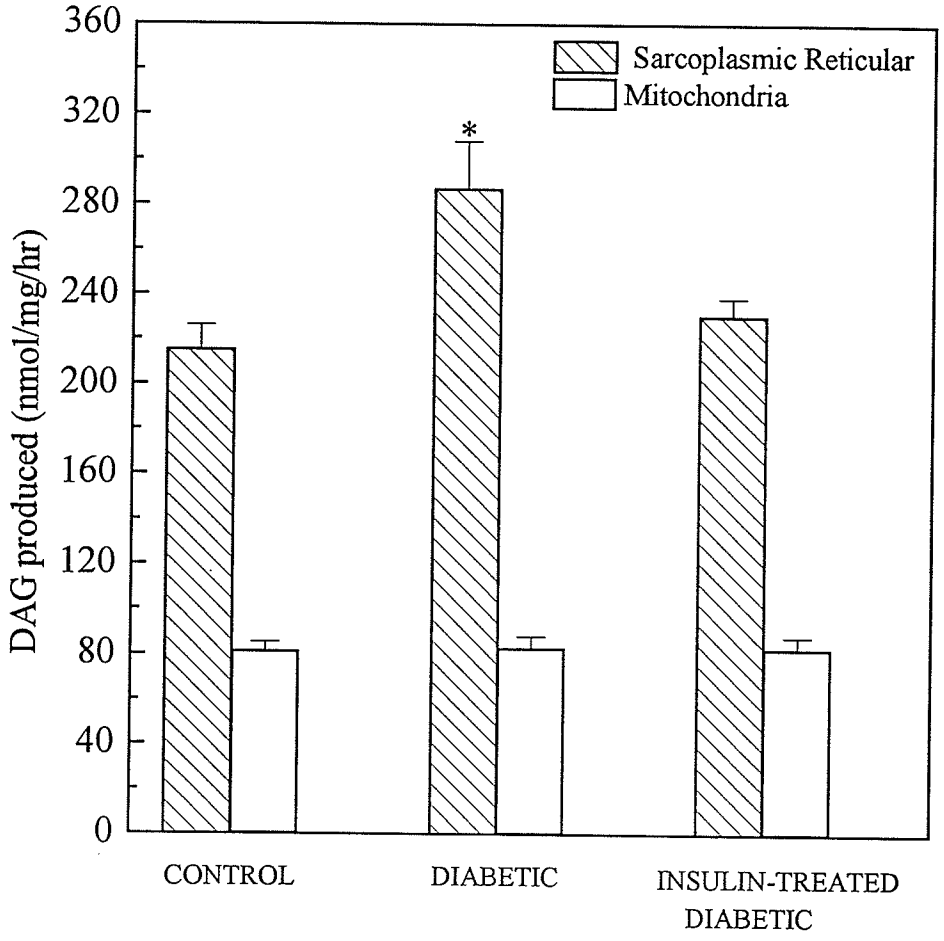

Figure 6. Phosphatidic acid phosphatase activity in sarcoplasmic reticular and mitochondrial fractions from experimental animals

The results represent the mean ± SEM of four SR or mitochondrial membrane preparations tested in triplicate. Membrane fractions were incubated using the standard assay as described in Materials and Methods. DAG = Diacylglycerol.

*Significantly different ($P < 0.05$) from respective control

TABLE 7. Rate of SR synthesis and dephosphorylation of phosphatidic acid in the presence and absence of KF, an inhibitor of phosphatidic acid phosphatase

	PtdOH produced (nmole/mg/hr)	DAG produced (nmole/mg/hr)
25 mM KF		
control	67.29 ± 3.70 (100 ± 5.5%)	2.73 ± 0.11 (100 ± 4.0%)
diabetic	46.11 ± 3.08* (68.5 ± 4.6%)	2.13 ± 0.32 (78.0 ± 11.7%)
diabetic + insulin	57.71 ± 1.24*# (85.8 ± 1.8%)	3.17 ± 0.16 (116.1 ± 5.8%)
Without KF		
control	35.97 ± 2.32 (100 ± 6.4%)	61.91 ± 3.35 (100 ± 5.4%)
diabetic	14.04 ± 1.78* (39.0 ± 4.9%)	41.60 ± 1.78* (67.2 ± 2.8%)
diabetic + insulin	13.26 ± 0.94* (36.9 ± 2.6%)	48.81 ± 2.04* (78.8 ±3.3%)

Results represents the mean ± SEM of four experiments done in triplicate. Sarcoplasmic reticular membranes were assayed with or without 25 mM KF as described in Materials and Methods. Data in parentheses are percentage of respective controls.

*Significantly different ($P < 0.05$) from respective control.
#Significantly different ($P < 0.05$) from respective diabetic value.

2b. Effect of KF on the mitochondrial PLD activity

In the presence of 25 mM KF, which partially inhibits PAP activity, the mitochondrial PLD hydrolytic activity in the diabetic and insulin-treated diabetic membrane fractions were depressed by 50.4 ± 3.0% and by 28.9 ± 1.4%, respectively, as compared to control values (Table 8). When the blockade of PtdOH dephosphorylation was removed from the assay system, i.e. in the absence of KF, the amount of PtdOH produced in the diabetic and insulin-treated diabetic mitochondrial fractions were 54.3 ± 5.4% and 78.7 ± 3.6%, respectively. Both values indicate that the decrease in PLD activity during chronic diabetic is further depressed without KF. These findings were similar to those observed for the SR enzyme. The amount of DAG produced in each group, however, was not significantly affected by either the absence or presence of KF.

TABLE 8. Effect of KF on the mitochondrial synthesis and dephosphorylation of phosphatidic acid.

	PtdOH produced (nmol/mg/hr)	DAG produced (nmol/mg/hr)
25 mM KF		
control	32.30 ± 0.89 (100 ± 2.8%)	13.51 ± 2.62 (100 ± 19.4%)
diabetic	16.02 ± 1.88* (49.6 ± 5.8%)	15.25 ± 1.57 (112.9 ± 11.6%)
diabetic + insulin	22.98 ± 1.35*# (71.1 ± 4.2%)	12.04 ± 1.91 (89.1 ± 14.1%)
Without KF		
control	19.00 ± 2.05 (100 ± 10.8%)	21.71 ± 2.69 (100 ± 12.4%)
diabetic	10.33 ± 1.03* (54.3 ± 5.4%)	25.96 ± 2.02 (119.6 ± 9.3%)
diabetic + insulin	14.95 ± 0.69 (78.7 ± 3.6%)	25.38 ± 1.18 (116.9 ± 5.4%)

Values represents the mean ± SEM of four experiments done in triplicate. Mitochondrial fractions were assayed with or without 25 mM KF as described in Materials and Methods. Data in parentheses are percentage of respective controls.

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

#Significantly different ($P < 0.05$) from respective diabetic value.

V. DISCUSSION

A. Status of Sarcolemmal Phospholipase D during Insulin-dependent Diabetes

This study was the first to examine the status of myocardial PLD activity in SL membranes that were purified from the hearts of chronic IDDM rats. STZ-induced diabetes caused a significant depression in the SL PtdCho-specific PLD hydrolytic activity. This was indicated by a lower production of PtdOH in the chronic diabetic membrane fractions. The change in PLD activity was also observed throughout the time course study of 15 to 90 min, and was confirmed by testing PLD in the transphosphatidylation mode in which was depressed by the same magnitude as the hydrolytic activity.

There are many ways in which the depression in PtdOH production through PtdCho-PLD pathway would contribute to the development of the diabetic cardiomyopathy. The addition of exogenous PtdOH to cardiac tissue caused the generation of Ca^{2+}-dependent slow action potentials in the atrium (Knabb *et al.*, 1984), increased the contractility in neonatal rat hearts (Burt *et al.*, 1984) and enhanced the activity of SL Ca^{2+} pump (Carafoli, 1984). Thus a reduction in PtdOH content due to the depression in PLD activity would suggest that these Ca^{2+}-dependent processes would be severely compromised during IDDM. Since the above mentioned processes contribute to the performance of the heart (Dhalla

et al., 1982), it is reasonable to conclude that the depressed level of PtdOH produced by PLD is one molecular mechanism which is responsible for the diminished contractility of the diabetic heart (Ku and Sellers, 1982).

PtdOH generated under normal physiological conditions may also stimulate the activity of other key functioning enzymes such as PLA_2 (Sato *et al.*, 1993), PIP (Kurz *et al.*, 1993), and PtdIns(4,5)P_2 specific PLC (Moritz *et al.*, 1992). A reduction in PLD generated PtdOH content during IDDM implies the activity of these enzymes would be significantly altered. In the heart, the depression in phosphoinositides breakdown during diabetes (Bergh *et al.*, 1988) may the consequence of having a decrease PtdOH stimulated PtdIns(4,5)P_2-PLC activity. PtdOH may also directly modulate the activity of PKC (Bocckino *et al.*, 1991). The significance of having an altered PKC activity will be discussed shortly.

PtdOH produced by the action of PLD on PtdCho, can also be converted to CDP-diacyglycerol for the resynthesis of phospholipids (Tronchere *et al.*, 1994). Since less PtdOH is generated in the diabetic membrane, the phospholipid synthesis pathways would be affected. The reduced phospholipids synthesis may help to explain the abnormally high circulating serum lipid contents seem in diabetics (Fein *et al.*, 1980). Lipid accumulation in the diabetic heart is prevented by treating the animals with either choline or methionine (Heyliger *et al.*, 1986). Since choline and methionine are involve in the synthesis of PtdCho (Kuksis and Mookerjea, 1978; Finkelstein and Marlin, 1984), these lipotropic agents (Young *et*

al., 1956) may be improving the diabetic cardiac performance by affecting PtdOH-related PtdCho synthesis.

In addition, the second messengers that are generated by PLC action on PtdIns(4,5)P_2, DAG and Ins(1,4,5)P_3 (Exton, 1994) would be produced in lower amounts in the diabetic heart due to reduced amounts of PtdOH and less stimulation of PLC. Both DAG and Ins(1,4,5)P_3 modulate myocardium cytosolic Ca^{2+} by activating PKC-mediated phosphorylation of sarcolemmal Ca^{2+} channels (Presti *et al.*, 1985) and by mobilizing SR Ca^{2+} stores (Nosek *et al.*, 1986), respectively. A disruption in these Ca^{2+} related processes would also contribute to the imbalances in the Ca^{2+} homeostasis observed during diabetes (Makino *et al.*, 1987, Pierce *et al.*, 1983, 1990).

PtdCho-specific PLC has been detected in rat cardiac tissue (Baldini *et al.*, 1994) and is coupled to DAG kinase to give PtdOH (Ye *et al.*, 1994). This pathway however, has no effect on the PtdOH produced under our experimental conditions since the system contained no ATP.

PtdEtOH production through PLD pathway was significantly lower in the diabetic SL. Although the physiological significance of having reduced PtdEtOH in the diabetic heart can not be discussed at this time, nevertheless, this phospholipid affects the physiochemical properties of rat brain membrane (Omodeo-Salé *et al.*, 1991) and may serve a similar role in the SL which would suggest an abnormal SL membranal structure in IDDM..

The depression in SL PLD hydrolytic and transphosphatidylation activities were reverted by two weeks of insulin therapy to chronic diabetic animals. Insulin-induced corrections of other sarcolemmal defects in diabetic animals have also been reported (Pierce and Dhalla, 1983; Ganguly *et al.,* 1984; Makino *et al.,* 1987).

When KF was excluded from the medium in which the SL PLD hydrolytic activity was assessed, the presence of a hyperactive PAP activity resulted in decreased levels of PtdOH with an associated increase in the amount of DAG generated. The amount of PtdOH produced in the diabetic membranes without a PAP inhibitor was significantly less due to a reduced synthesis by PLD and an enhanced dephosphorylation by it's coupled PAP. In the insulin-treated diabetic fractions, the decreased in PtdOH production is due solely to the higher PAP activity since the associated PLD activity was normalized by insulin therapy. These alterations in SL PtdOH synthesis and degradation would result in an imbalance in the intramembranal content of the phospholipid.

Since hypothyroidism alters the heart contractility (Buccino *et al.,* 1967) by affecting cardiac membranal processes, including the SL function (Seppet *et al.,* 1991). It is therefore possible that the observed change in SL PLD activity in STZ-induced diabetic rats is due to the associated low thyroid state. SL PLD activity was unchanged in the PTU-induced hypothyroid fractions that suggest that hypothyroidism was not involved.

B. The significance of diabetic-induced change on the sarcolemmal PAP activity

In this study two forms of PAP were identified in the cardiac cell due to their differential sensitivity to Mg^{2+} and NEM. The cytosolic form was affected by both Mg^{2+} and NEM, while the other form was Mg^{2+}-independent and NEM-insensitive and was associated with the SL. These results agree with observations made in other cell types in which the behavior of the cytosolic PAP activity toward Mg^{2+} and NEM was notable different from that of the membrane-bound activity (Jamal *et al.*, 1991; Day and Yeaman, 1992; Höer and Oberdisse, 1994).

Severe IDDM in rats resulted in an enhanced SL PAP activity that was indicated by an elevated DAG production in the diabetic membrane fractions. Elevated PAP activity has been observed in cardiac microsomal fractions isolated from acute diabetic rats (Schoonderwoerd *et al.*, 1990) and an increase in DAG content in the myocardium from chornic diabetic rats has also been reported (Okumura *et al.*, 1988), however, our studies were the first to examined DAG production at the SL level during chronic diabetes. The main function of DAG within the cell is to activate some PKC isoforms, which in turn phosphorylate many cellular proteins (Exton, 1994). In the diabetic heart, an increase production of DAG would cause an enhanced PKC activity. The significantly high PKC activity that was reported by Xiang and McNeill (1992) in crude cardiac membrane

fraction may not only be due to PtdIns(4,5)P_2-derived DAG as proposed by these investigators, but is also the result on an altered PtdCho-PLD-DAG pathway.

Myocardial proteins that are phosphorylated by PKC include receptors, ion channels (Exton, 1994) and other cellular proteins which induce the phenotypic features of hypertrophy (Steinberg *et al.*, 1995). Since the diabetic heart exhibits hypertrophy (Pierce and Dhalla, 1983) molecular mechanism responsible for this defect is PtdCho-derived DAG induced activation of PKC pathway. PKC activation also modulates cardiac contractile function (Steinberg *et al.*, 1995), an another index of the heart that is severely compromised in the diabetic heart (Dhalla *et al.*, 1982).

Insulin treatment resulted in a significant but incomplete normalization of the diabetic SL PAP activity. This indicates that insulin therapy may not always correct all the cardiac subcellular defects that are associated with IDDM, and may also help to explain why the diabetic heart continues to degenerate despite insulin treatments (Kannel and McGee, 1979). Hypothyroid condition in the rat had no effect on the SL PAP activity that allows us to conclude that the observed alteration in the enzyme activity is due to the diabetic condition is not a consequence of the associated depressed thyroid state.

The presence of myocardial PtdCho-specific PLC (Baldini *et al.*, 1994) implies that DAG can also be produced through a route alternative to PLD/PAP pathway. We believe however that any contribution from this pathway under our

experimental conditions is minimal. The myocardial PtdCho-PLC had a pH dependence of 7.5 and required the presence of cations for optimal activity (Baldini *et al.*, 1994). In the assay medium in which we examined DAG production contained both EDTA and EGTA and had a pH buffering system of 6.5.

C. The effect of diabetes on sarcoplasmic reticular associated PLD and PAP activities.

The results obtained from this study indicate that the SR associated activities of both PLD and PAP were significantly altered by IDDM. The diabetic condition in rat induced a depression in SR PLD activity and an elevation in and SR PAP activity. These changes were similar to those that were observed for the SL enzyme. Two weeks of insulin therapy to diabetic animals was also beneficial at the level of the SR in that it resulted in a significant but incomplete recovery in PLD activity and completely normalized PAP activity.

Diabetes induced impairment in other SR enzyme activities has also been reported (Ganguly *et al.*, 1983; Panagia *et al.*, 1990). The ATP-dependent Ca^{2+} transport and the Ca^{2+}-stimulated ATPase activities were both significantly depressed in SR microsomal fractions from chronic diabetic animals (Ganguly *et al.*, 1983), which suggest that IDDM is characterized by an abnormal SR Ca^{2+}

accumulation. Since PtdOH has been implicated in cardiac Ca^{2+} homeostasis (Carafoli, 1984; Knabb *et al.*, 1984), it is possible that the phospholipid may be altering cardiac Ca^{2+} handling at multiple subcellular locations. Evidence in support of this claim goes from studies using endogenous PtdOH which cause Ca^{2+} to be released from the SR under *in vitro* conditions (Limas, 1980) and increase cardiomyocyte intracellular Ca^{2+} by mobilizing SR Ca^{2+} stores (Xu *et al.*, unpublished data). Thus a change in the relaxation rate of diabetic heart (Fein *et al.*, 1980) is due to a summation of many processes that are depressed by insufficiently intramembranal levels of PLD derived PtdOH. Other observations such as defective heart muscle contractility (Ku and Sellers, 1982) and a depression in force development (Bouchard and Bose, 1991), are also processes which dependent on precise Ca^{2+} flux across the cardiomyocyte (Dhalla *et al.*, 1982) and therefore would be affected by the depression in SR PLD activity.

The function of PtdOH on cardiac Ca^{2+} protein at the SL level has been demonstrated (Carafoli, 1984), however, information on the direct effect of PLD or PtdOH on SR located proteins are not available at this time and remain to demonstrated.

Without KF, an enhanced dephosphorylation of PtdOH and a correlated increase in DAG was also observed in the diabetic SR membranal fractions. This suggests that IDDM is associated with altered PtdCho metabolism at multiple subcellular locations. In the diabetic group, the amount of DAG produced in the

unblocked PLD/PAP pathway was significantly less from the amount that was expected when taking in account the 30% depression in PtdOH synthesis and 30% increased dephosphorylation. This may be due to other major undefined synthesis pathway present at the SR that may require PtdOH as a substrate.

Since STZ-induced diabetes in rats is associated with hypothyroidism (Wilber *et al.*, 1981), a condition that is known to depress SR Ca^{2+} handling (Suko, 1973; Seppet *et al.*, 1991), it is possible that the defect in SR PLD and PAP activities may be consequence of having low circulating T_4 levels. We believe that our observations however are diabetic-induced because other studies have shown that the administration of T_4 to diabetic animals to normalized serum T_3, did not correct the depressed enzymatic activities (Malhotra *et al.*, 1981; Ganguly *et al.*, 1983) nor did it increase cardiac mechanical function (Malhotra *et al.*, 1981). Both PLD and PAP activities were significantly altered towards control values by insulin therapy. Insulin also restored SR microsomal Ca^{2+} transport activities (Ganguly *et al.*, 1983; Lopaschuk *et al.*, 1983b, 1984) and directly activates the SR Ca^{2+} -stimulated ATPase activity presumably through regulating the phosphorylation/ dephosphorylation state of the enzyme (Gupta *et al.*, 1989).

D. Alterations in cardiac mitochondrial PLD and PAP activities during insulin-dependent diabetes.

The results of our experiments indicate that the myocardial mitochondrial PLD activity is significantly depressed in membrane fractions prepared from STZ-injected diabetic animals as compared to control values. The depression in the enzyme's activity was partially corrected by two weeks of insulin therapy. In contrast, the induced diabetic condition did not alter the mitochondrial PAP activity, which suggest that the alteration(s) in PAP activity may be secondary to that of PLD. The mitochondrial associated PAP enzyme is also less sensitive to the chronic diabetic state as compared to the enzymatic activity at the other subcellular locations examined, i.e. SL and SR.

No information regarding the roles served by PtdOH or DAG on the mitochondrial function is available in the literature. Nevertheless, the fact that PtdOH has been implicated in processes that required energy (i.e. action potential generation (Knabb *et al.*, 1984) and enzymes functioning (Carafoli, 1984; Moritz *et al.*, 1992)), which is supplied mainly by the mitochondria, suggest that the phospholipid may modulate mitochondrial function in the heart. The decrease in mitochondrial oxidative phosphorylation in the diabetic heart (Pierce and Dhalla, 1985; Tanaka *et al.*, 1992) may the a consequence of having depressed levels of PtdOH generated through PLD-PtdCho pathway.

Since PtdOH has direct effects on cardiomyocyte Ca^{2+} handling systems at other subcellular locations (Carafoli, 1984; Xu *et al.*, unpublished data), it effects may also be exerted at the level of the mitochondrial. This would suggest that a molecular mechanism that may be responsible for the reduced Ca^{2+} transport in the diabetic mitochondria (Pierce and Dhalla, 1985; Tanaka *et al.*, 1992) is the depression of PtdOH synthesis. The fact that insulin treatment reversed the alterations in mitochondrial function (Pierce and Dhalla, 1985; Tanaka *et al.*, 1992) and partially corrected the mitochondrial associated PLD activity further supports the notion that these events may be related.

E. The importance of diabetic associated membranal changes on the functioning of PLD and PAP.

Evidence suggests that modification of subcellular phospholipid components either through altered myocardial metabolism or by free radical-induced damages, can affect the functioning of the heart (Henderson *et al.*, 1970, Katz and Messineo, 1981). One mechanism by which these changes alter cardiac performance is due to subcellular phospholipid-induced conformational modification of enzyme functions that are associated with or embedded in the membranes (Ganguly *et al.*, 1983; Atkins *et al.*, 1985; Pierce and Dhalla, 1985).

Altered lipid metabolism is a characteristic of the diabetic heart. In particular, there is an enhanced oxidation of endogenous fatty acid (Rodrigues and McNeill, 1992), and increased in plasma levels of triacylglycerols (Chen *et al.*, 1984; Xiang *et al.*, 1988) and in cholesterol content (Heyliger *et al.*, 1986). These increases in myocardial oxidation and in its lipid content will affect the integrity of the membrane structures, which in turn affects the activity of associated enzymes. Thus the observed defects in PLD and PAP activities in the diabetic fractions may be due the alterations in phospholipid content of the SL (Makino *et al.*, 1987), SR (Ganguly *et al.*, 1983) and mitochondria (Pierce and Dhalla, 1985).

The induced functioning changes in these enzymes would result in an altered cardiac performance. This seems to be indeed the case because when myocardial lipid contents are low or corrected by various treatments (Heyliger *et al.*, 1986; Xiang *et al.*, 1988, Tada *et al.*, 1992), which presumably normalized subcellular phospholipid content and restores the function of key enzymes, cardiac performance is concomitantly increased.

Recently, work has focused on the role of fatty acids on the activity of heart sarcolemmal PLD (Dai *et al.*, 1995). The results suggest that the presence of *cis*-unsaturated fatty acids, in particularly, arachidonate and oleate, were stimulatory. A reduction in arachidonic acid (AA) content of STZ-induced diabetic rat hearts has been reported (Holman *et al.*, 1983; Huang *et al.*, 1984) which suggest that the observed depression in SL PLD activity could also be due to the deficiency of AA.

AA is also liberated through PLA_2 hydrolysis of phospholipid substrates (Clark *et al.*, 1990; Diez and Mong, 1990). PLA_2 is in turn activated by PtdOH (Sato *et al.*, 1993), the product of PLD action. Thus the depressed amounts of AA in the diabetic heart because of reduced quantity and depressed synthesis will interfere with the PLD loop by keeping the enzyme activity low. It is possible that a similar mechanism may also be present at the SR and mitochondrial locations.

An increase in plasma circulating levels of fatty acids during diabetes may contribute to ischemic injury (Lopaschuk, 1989). This is due the observations that the diabetic heart fails more rapidly than control hearts when subjected to hypoxic conditions (Feuvray *et al.*, 1979) and reduced coronary flow (Hearse *et al.*, 1975). Both conditions are known to increase myocardial fatty acid content (Lopaschuk, 1989). Ischemic causes damage to the cardiomyocyte membrane structures including the SL, SR and mitochondrial levels (Jennings and Reimer, 1981) which result in abnormal Ca^{2+} movement (Bersohn *et al.*, 1991; Dhalla *et al.*, 1988) and alterations in adrenergic signalling mechanisms (Schömig *et al.*, 1988).

Studies have revealed that ischemic induced membranal damages can be mediated by oxidant metabolites such as oxygen free radicals and other non-radical oxidants (Opie, 1989; Hearse, 1991). Thus the presence of these reactive intermediates in the diabetic heart (Pierce *et al.*, 1988), may also be responsible for the altered PLD and PAP activities, by inducing conformational changes. Evidence in support of claim comes from the fact that various systems involve in

cardiomyocyte Ca^{2+} homeostasis are affected by oxygen metabolites (Dixon *et al.*, 1990; Kaneko *et al.*, 1989). SL PLD damage by oxidant species has also been reported (Dai *et al.*, 1992), thus indicating that the enzyme contains functional groups that can be modified by free radicals.

In conclusion, the results of this study demonstrate divergent changes in cardiac subcellular PLD and PAP activities during insulin-dependent diabetes. A reduction in PtdOH production in the diabetic heart may contribute to the development of the diabetic cardiomyopathy by altering Ca^{2+}-dependent processes at the SL, SR, and mitochondrial levels. At the sarcolemmal level, the decrease production of PtdOH may directly reduce enzyme activities or indirectly, by affecting key transduction enzymes, some of which have known roles in Ca^{2+} homeostasis process of the cardiomyocyte. At the SR level, Ca^{2+} release and reuptake would be severely compromised which would affect the rate of contraction and relaxation of the heart. Finally, a disruption in cardiac PtdCho metabolism due to altered synthesis and degradation would affect the lipid environment which in turn would alter the activity of enzymes that are associated with membrane structure.

VI. CONCLUSION

1. SL PtdCho-specific PLD hydrolytic and transphosphatidylation activities were found to be significantly depressed during chronic insulin-dependent diabetes. Study of the enzyme activity at different time points of incubation (15 to 90 min) confirmed that the diabetic SL PLD activity was significantly depressed throughout this time course.

2. Two weeks of insulin treatment to chronic diabetic animals corrected the depression in SL PLD hydrolytic. The transphosphatidylation activity, a unique feature of PLD, was also normalized by insulin therapy. The results identify a subcellular basis of insulin beneficial effect in diabetes.

3. Preliminary characterization studies showed that the cardiac cytosolic PAP activity was inhibited by Mg^{2+} and NEM at concentrations of 3 mM and 4.2 mM, respectively, while the SL enzyme activity was found to be Mg^{2+}-independent and NEM-insensitive. The differences in PAP activity toward Mg^{2+} and NEM are attributed to its subcellular location and are similar to those observed in rat lung and liver. PAP activity in the SR and mitochondrial fractions insensitive to either Mg^{2+} or NEM, thus indicating

that the SR and mitochondrial forms of the enzyme share some characteristics with the SL enzyme.

4. SL PAP activity in chronic diabetic was significantly higher than that observed in controls. The administration of insulin to chronic diabetic animals caused a significant decrease in the enzyme's activity, as compared to untreated diabetic values. The enzyme activity, however, was not completely normalized by insulin therapy. The finding suggests that insulin does not correct all defects in the heart.

5. In contrast to the diabetic condition, the induction of hypothyroidism in the rats, did not significantly affect neither the SL PLD nor PAP activities. This indicates that the changes observed in these enzyme activities are characteristic of the diabetic state and are not due to the associated hypothyroidism.

6. PtdCho-specific PLD activity in the SR and mitochondrial fractions from chronic diabetic hearts was also significantly depressed. In contrast to the SL enzyme, however, the enzyme associated with these membrane fractions was not fully normalized by insulin therapy. The results suggest that

differential change in PLD activity occurs within multiple subcellular organelles during IDDM.

7. The diabetic SR PAP activity behaves similarly to the SL enzyme in that it was significantly higher than that of controls. Unlike the SL enzyme, however, the increased activity of SR PAP was normalized by two weeks of insulin treatment. In contrast to the enzyme associated with the SL and SR membranes, the mitochondrial PAP activity was not affected by the diabetic condition. The results suggest that the cardiac subcellular PAP activities are differentially altered during this pathological process.

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