Phosphoinositide-Phospholipase C in Diabetic Cardiomyopathy

By

Yun Tong

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

MASTER OF SCIENCE

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PHOSPHOINOSITIDE-PHOSPHOLIPASE C IN DIABETIC CARDIOMYOPATHY

BY

YUN TONG

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

MASTER OF SCIENCE

DEDICATED TO MY FAMILY

and

FRIENDS

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LIST OF ABBREVIATIONS

α_1-Adrenoceptor	α_1-AR
Angiotensin converting enzyme	ACE
Angiotensin I	Ang I
Angiotensin II	Ang II
Arachidonic acid	AA
Basic fibroblast growth factor	bFGF
Endothelin-1	ET-1
Epidermal growth factor	EGF
Fibroblast growth factor	FGF
G protein-coupled receptor	GPCR
G protein-coupled receptor kinases	GRK
GTPase activating protein	GAP
GTPase-activating protein for the small GTP-binding protein Rho	RhoGAP
Guanine nucleotide regulatory proteins	G proteins
Inositol 1,3,4,5-tetrakisphosphate	Ins(1,3,4,5)P_4
Inositol 1,3,4-trisphosphate	Ins(1,3,4)P_3
Inositol 1,3-bisphosphate	Ins(1,3)P_2
Inositol 1,4,5-trisphosphate	Ins(1,4,5)P_3
Inositol 1,4-bisphosphate	Ins(1,4)P_2
Inositol 1-phosphate	Ins1P
Inositol 3-phosphate	Ins3P
Inositol 4-phosphate	Ins4P
Insulin-dependent diabetes mellitus	IDDM
Nerve growth factor	NGF
Non-insulin dependent diabetes mellitus	NIDDM
Nonobese diabetic rats	NOD
Phophatidylinositol 3,4,5-trisphosphate	PtdIns(3,4,5)P_3
phosphatidic acid	PtdOH
phosphatidic acid phosphatase	PAP
Phosphatidylcholine	PtdCho
Phosphatidylinositol	PtdIns
Phosphatidylinositol 3-kinase	PtdIns 3-kinase
Phosphatidylinositol 4,5-bisphosphate	PtdIns(4,5)P_2
Phosphatidylinositol 4-phosphate	PtdIns4P
Phospholipase A_2	PLA_2
Phospholipase C	PLC
Phospholipas D	PLD
Phosphotidylinositol 5-phosphophate	PtdIns5P
Platelet derived growth factor	PDGF
Pleckstrin homology	PH

Polyunsaturated fatty acid ..... PUFA
Protein kinase C ..... PKC
PtdIns transfer protein ..... PtdIns-TP
Rat insulin promoter ..... RIP-Tag
Renin-angiotensin system ..... RAS
Sarcolemma ..... SL
Sarcoplasmic reticulum ..... SR
sn-1,2-Diacylglycerol ..... DAG
Streptozotocin ..... STZ
Transforming growth factor ..... TGF
Triose phosphate isomerase ..... TIM
Tris-buffered saline-Tween 20 ..... TBST
Troponin I ..... TnI
Troponin T ..... TnT

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ABSTRACT

In the heart, stimulation of many receptors, including those for angiotensin II (Ang II), endothelin-1 (ET-1), catecholamines and polypeptide growth factors, is associated with the activation of phospholipase C (PLC) and subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). The two second messengers produced in this reaction, namely *sn*-1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), are involved in the regulation of cardiac contractility and intracellular Ca^{2+} concentration.

Diabetic cardiomyopathy refers to the cardiac abnormalities secondary to diabetes mellitus. It is characterized by decreased cardiac contractility and impaired intracellular Ca^{2+} regulation. Several lines of evidence have shown that abnormalities in the phosphoinositide-PLC signaling pathway may be associated with the development of insulin-dependent cardiomyopathy, such as decreased phosphatidylinositol (PtdIns) 4-kinase and phosphatidylinositol 4-phosphate (PtdIns4*P*) 5-kinase activities, decreased α_1-adrenoceptor (α_1-AR) density and altered responsiveness to α_1-AR agonist stimulation, increased local renin angiotensin system activity and sustained elevation of DAG content and PKC activity. However, no information on the functional status of cardiac PLC and changes of its isoforms during diabetes is available in the literature.

Phosphatidic acid (PtdOH) has been demonstrated to stimulate cardiac PLCγ1 and PLCδ1 activities. In addition, decreased sarcolemma (SL) phospholipase D (PLD)-derived PtdOH levels and decreased PtdOH-induced positive inotropic effects in the diabetic heart

have also been reported. Therefore, it is hypothesized that a decreased stimulation by PtdOH might result in a decreased PLC activity during diabetic cardiomyopathy.

In this study, the effect of PtdOH on the phosphoinositide-PLC pathway were examined in isolated SL and cardiomyocytes from 8-week chronic insulin-dependent diabetic rats induced by a single intravenous injection of streptozotocin (STZ, 65 mg/kg body weight). The beneficial effect of insulin on this pathway was assessed in 6-week diabetic rats with 2-week insulin treatment. The activity and relative protein amount of PLCγ1, one of the prominent cardiac PLC isoforms, were also studied.

The findings of this study showed that cardiac total SL PLC activity and Ins(1,4,5)P_3 content in isolated cardiomyocytes were significantly decreased during diabetes, indicating that a depressed PLC activity may exist *in vivo*. The hyperresponsiveness of total SL PLC to PtdOH may act to compensate for the decreased PLD-derived PtdOH level and sustain insulin receptor tyrosine kinase activity during insulin-dependent diabetes mellitus (IDDM). Changes of SL PLCγ1 activity and its responsiveness to PtdOH were consistent with that of the total PLC, suggesting that alterations of this isoform contribute to that of the SL total PLC. Furthermore, decrease in PLCγ1 catalytic activity and the concomitant increase of SL protein content of this isozyme indicate that conformational changes in this isoform may occur in the chronic diabetic heart. Insulin treatment normalized the depressed SL PLC activities and the hyperresponsiveness of PLC to PtdOH, however, it did not fully correct the abnormal Ins(1,4,5)P_3 metabolism in the isolated cardiomyocytes and the decreased cytosolic PLCγ1 protein content.

This study is the first to report the functional status of cardiac phosphoinositide-PLC activities and, furthermore, changes in PLCγ1 and regulation of phosphoinositide pathway by PtdOH during chronic IDDM are shown. It also demonstrates the beneficial effect of insulin treatment on PLCs and Ins(1,4,5)P_3 levels. Results from this study suggest that the phosphoinositide-PLC pathway may play an important role in the regulation of heart function of IDDM subjects.

I. INTRODUCTION

The phosphoinositide-PLC pathway is involved in the long-term and short-term regulation of cellular responses (De Jonge *et al.*, 1995; van Heugten *et al.*, 1996; James *et al.*, 1997; Singer *et al.*, 1997; Rhee and Bae, 1997). Agonists such as Ang II, ET-1, catecholamines, and polypeptide growth factors can stimulate PLC activity and cause the hydrolysis of PtdIns(4,5)P_2 (De Jonge *et al.*, 1995; van Heugten *et al.*, 1996). The two hydrolytic products, namely DAG and Ins(1,4,5)P_3, have important effects on the function of cardiomyocytes. DAG has been shown to be involved in the regulation of cardiac contractility, myofibrillar Ca^{2+} sensitivity (Dosemeci *et al.* 1988; Venema and Kuo *et al.*, 1993; Schaffer and Mozaffari, 1996; Malhotra and Sanghi, 1997), and transsarcolemmal Ca^{2+} movement (Schruer and Liu, 1996; Conforti *et al.*, 1995), while Ins(1,4,5)P_3 is involved in intracellular Ca^{2+} regulation (Rhee and Bae, 1997; James *et al.*, 1997; Singer *et al.*, 1997; van Heugten *et al.*, 1996). The phosphoinositide-PLC pathway also plays an important role in heart disease, including hypertension, ischemia and reperfusion injury and heart failure (De Jonge *et al.*, 1995; Makita and Yasuda, 1990; Kawaguchi *et al.*, 1993; Meij *et al.*, 1997, Ju *et al.*, 1998). This pathway is regulated by receptors, G proteins, substrate availability and PtdOH (De Jonge *et al.*, 1995; van Heugten et *al.*, 1996; James *et al.*, 1997; Singer *et al.*, 1997; Rhee and Bae, 1997; Henry *et al.*, 1995).

Diabetic cardiomyopathy refers to the disease secondary to diabetes, and is associated with cardiac dysfunction and defective metabolism of glucose and lipid (Andreoli *et al.*, 1997; William *et al.*, 1997; Tomlinson *et al.*, 1992). The exact

mechanisms of the disease are still under investigation. Two important aspects of IDDM-associated cardiac dysfunction are diminished contractility and defective Ca^{2+} regulation (Schaffer and Mozaffari, 1996; Malhotra and Sanghi, 1997). As Ca^{2+} is closely related to a variety of cellular events (Schaffer and Mozaffari, 1996) and moreover, contractility is an important determinant of whole heart function, these defects would ultimately result in failure of the diabetic heart.

Two factors have been demonstrated to account for the decreased contractility under diabetic condition: 1) altered Ca^{2+} regulation due to a decreased sarcoplasmic reticular (SR) Ca^{2+} pump activity and defective transsarcolemmal Ca^{2+} fluxes (Pierce and Russell, 1997); 2) changes in the contractile proteins in the diabetic heart (Schaffer and Mozaffari, 1996; Malhotra and Sanghi, 1997), i.e. a myosin isoform shift from the fast V1 to the slower V3 (reviewed by Tomlison *et al.*, 1992), and a loss of Ca^{2+} sensitivity due to isoform shift of troponin T (TnT) from TnT1 to TnT3 and increased phosphorylation of troponin I (TnI) (Liu *et al.*, 1996; Akella *et al.*, 1995; Goodale and Hackel, 1953; Schaffer and Mozaffari, 1996; Malhotra and Sanghi, 1997). *In vitro* and *in vivo* studies have shown that phosphorylation by PKC decreases calcium sensitivity of contractile apparatus and calcium-stimulated myofibrillar MgATPase activity (Dosemerici *et al.* 1988; Venema and Kuo *et al.*, 1993). Thus, the sustained elevation of cardiac DAG content (Okumura *et al.*, 1991; Inoguchi *et al.*, 1992) and PKC activity in the diabetic heart (Inoguchi *et al.*, 1992; Malhotra and Sanghi, 1997) may contribute to the increased thin filament phosphorylation and the decreased contractile force.

Changes of the phosphoinositide-PLC pathway have also been found at receptor

and substrate levels during diabetes. 1) Receptor level. Several investigations have suggested the involvement of two G protein-coupled receptor signaling pathways in the diabetic heart. One is the α_1-AR pathway, as indicated by the decreased cell surface α_1-AR density and altered responsiveness to α_1-AR stimulation (Heyliger *et al.*, 1982; Wald *et al.*, 1988; Tanaka *et al.*, 1992; Heijnis and van Zwieten, 1992; Xiang *et al.*, 1991; Jackson *et al.*, 1986; Downing *et al.*, 1983). The other is the Ang II signaling pathway, as supported by the findings of an increased AT1 receptor density and mRNA level (Sechi *et al.*, 1994), prevention of the increase of TnI phosphorylation and translocation of PKCε in the diabetic heart by AT1 blockade (Malhotra *et al.*, 1997) and improvement of cardiac function in the diabetic rats by angiotensin converting enzyme inhibitor treatment (Given *et al.*, 1994). 2) Substrate level. A decrease in the levels of PtdIns(4,5)P_2 has also been indicated in the diabetic heart (Liu *et al.*, 1997).

Taken together, all these lines of evidence suggest that abnormalities in the phosphoinositide-PLC pathway may exist in the diabetic heart. The present study was therefore performed to ascertain the functional status and regulation of cardiac PLC and its isoforms during IDDM. It is envisaged that the findings will provide further insight into the contribution of phosphoinositide-PLC pathway to the development of diabetic cardiomyopathy.

II. REVIEW OF THE LITERATURE

1. Phosphoinositide-Phospholipase C Pathway in the Heart

The receptor-activated phosphoinositide cycle was first described by Hokin and Hokin in 1953 in pigeon pancreas slices (Hokin and Hokin, 1953). However, it was much later on when the involvement of phosphoinositide metabolism in the cardiac adrenergic system was identified and termed as the 'phosphoinositide effect' (Gaut and Huggins, 1966). Following on from this, studies conducted by Durell and Garland (1969) revealed that a specific 'phosphoinositidase C' catalysed the receptor-mediated inositol phospholipid hydrolysis. Subsequent studies on the phosphatidylinositol pathway were accelerated by three important findings in the early 1980's, which were: 1) that the preferred substrate for receptor activated PLC is PtdIns(4,5)P_2 (Michell *et al.*, 1981); 2) that the DAG derived from phospholipid hydrolysis is a potent activator of PKC (Takai *et al.*, 1979); and 3) that another product, Ins(1,4,5)P_3, could cause intracellular Ca^{2+} release from sarcoplasmic reticulum (SR) (Streb *et al.*, 1983). It is now known that the phosphoinositide-PLC pathway is a transmembrane signalling pathway and is involved in different cellular responses, including metabolism, contraction, growth, and proliferation. This pathway can be activated by many cardiac receptor types, including Ang II receptor, ET-1 receptor, α_1-AR, muscarinic receptor, and receptors with intrinsic or associated tyrosine kinase activity (Rhee and Bae, 1997; van Heugten *et al.*, 1996; Rhee and Choi, 1992; De Jonge *et al.*, 1995). Different PLC isoforms coupled to the receptors, their cellular localization, redistribution and possibly different DAG molecular species generated after stimulation

may account for the distinct final biological effects of these receptors.

1.1 Inositol Phospholipids

Phosphoinositides account for a minor component (approximately 8%) of the total cardiac SL phospholipid pool. Of the individual constituents, both the PtdIns(4,5)P_2 and PtdIns4P make up 0.2% of the pool respectively, whereas PtdIns constitutes approximately 7% of the total SL phospholipid pool (Lamers *et al.*, 1992a). PtdIns is the common precursor for all the inositol lipids (Hinchliffe and Irvine, 1997). Synthesis of PtdIns involves the conversion of PtdOH to CDP-diacylglycerol followed by incorporation of inositol (Choy *et al.*, 1997). The newly synthesised PtdIns is then transferred to the plasma membrane by PtdIns transfer protein (PtdIns-TP) (Wolf, 1990). In the plasma membrane, PtdIns undergoes sequential phosphorylation by PtdIns 4-kinase and PtdIns4P 5-kinase to form PtdIns4P and PtdIns(4,5)P_2, respectively (Quist *et al.*, 1989; Wolf, 1990). However, it should be noted that interconversion between PtdIns, PtdIns4P, and PtdIns(4,5)P_2 involves equilibrium reactions which occur through the action of specific kinases and phosphatases (Meldrum *et al.*, 1991; Hinchliffe and Irvine, 1997).

1.2 Phospholipase C Family

1.2.1 Phospholipase C in the heart

The PLC family can be subdivided into three distinct classes, namely β, γ, and δ. To date ten different mammalian isoforms have been identified; PLCβ1-4, PLCδ1-4, and PLCγ1 and 2 (James *et al.*, 1997; Rhee and Bae, 1997; Singer *et al.*, 1997).

All three types of PLCs are able to catalyze the hydrolysis of the three inositol phospholipids *in vitro*. Substrate specificity decreases in the order:

PtdIns(4,5)P_2≥PtdIns4P>PtdIns. Under physiological conditions, PtdIns(4,5)P_2 is the preferred substrate and the two hydrolytic products (DAG and Ins(1,4,5)P_3) play an important role in PLC signaling pathway (as reviewed by James *et al.*, 1997; Rhee and Bae, 1997; Singer *et al.*, 1997). No information on differences in isozyme substrate and product specificity is available at present.

The existence of myocardial endogenous PLC activities in both cytosolic and SL fractions has been demonstrated (Edes and Kranias, 1990; Schwertz and Halverson, 1989; Meij and Panagia, 1992; Wolf, 1992). Studies on the guinea pig myocardial PtdIns(4,5)P_2-PLC showed that 3-5% of the total activity is present in the SL, and over 80% is localized to the cytosolic fraction (Edes and Kranias, 1990). In contrast, in rat ventricle, 63% of PtdIns4P- and PtdIns(4,5)P_2-PLC activities have been found to be membrane-bound and only 33% located in the cytosol. The PtdIns$P$-specific PLC activity is mainly localized to the cytosolic fraction (Schwertz and Halverson, 1989; Meij and Panagia, 1992). Thus, it appears that the distribution of PLC activities is species-dependent.

In terms of PLC isozymes, PLCγ1 and PLCδ1 are the most abundant in ventricular cardiomyocytes (Wolf, 1993). Existence of PLCβ family has also been identified (Hasen *et al.*, 1995; Ju *et al.*, 1998). PLCβ1 has been found to be barely detectable in adult rat cardiac tissue, while PLCβ3 is readily detected in neonatal cardiomyocytes and developmentally downregulated in adult atria, ventricle and whole heart (Hasen *et al.*, 1995; Ju *et al.*, 1998).

1.2.2 Structure and function relationship

Mammalian PLCs are modular proteins and consist of several domains, namely X,

Y boxes, pleckstrin homology (PH) domain, EF hand domain, and C2 domain (James *et al.*, 1997). The function of these domains is discussed below.

X, Y boxes

The two highly conserved regions, X (~170 amino acid residues, 60% identical) and Y (260 residues, 40% identical) boxes form a triose phosphate isomerase enzyme-like (TIM)-barrel motif and carry the catalytic activity of PLC isozymes. The region between the X and Y domains in PLCγ family is longer (~400 residues) compared to that of PLCβ and PLCδ isozymes and contains two SH2 and one SH3 domains (*src*-homology domains) which are also found in a variety of proteins such as GTPase activating protein (GAP), phosphatidylinositol 3-kinase (PtdIns 3-kinase), tyrosine phosphatase and tyrosine kinases (Koch *et al.*, 1991). The SH2 and SH3 domains play an important role in growth factor-mediated signaling (Rhee and Bae, 1997; Singer *et al.*, 1997; James *et al.*, 1997).

PH domain

The PH domain of PLC is typically located near the amino-terminus (Rhee and Bae, 1997; Singer *et al.*, 1997; James *et al.*, 1997). It also exists in G protein-coupled receptor kinase (GRK) and Bruton tyrosine kinase (Musacchio *et al.*, 1993).

NMR studies have revealed that the amino-terminal half of the PH domain is involved in the binding of the PLCs to PtdIns(4,5)P_2 molecules. Furthermore, the isolated PH domain of PLCδ1 also shows highest affinity for Ins(1,4,5)P_3 and PtdIns(4,5)P_2 (Lemmon *et al.*, 1995; Garcia *et al.*, 1995; Ferguson *et al.*, 1995). The low affinity interaction of some PH domains and PtdIns has also been observed (Harlen *et al.*, 1995; Garcia *et al.*, 1995). Such a role of the PH domain in PLCδ function may be viewed as the

effective membrane targeting to guarantee the efficient PtdIns(4,5)P_2 hydrolysis. Also this domain is involved in product inhibition by Ins(1,4,5)P_3 and in the determination of the substrate preference (James *et al.*, 1997; Cifuent *et al.*, 1993, 1994; Paterson *et al.*, 1995). The role of PH domains in the β and γ isozymes has not yet been fully established (Singer *et al.*, 1997).

The carboxyl-terminal half of the PH domain is able to bind to the G protein βγ complex as shown by studies performed with glutathione S-transferase fusion proteins contain PH domains from a variety of proteins including PLCγ1 (Touhara *et al.*, 1994).

EF hand domain

PLCs require Ca^{2+} for enzyme activity. The EF hand domain provides the Ca^{2+} binding site. Ca^{2+} binding has been suggested to be important in stabilizing the enzyme-substrate complex (Essen *et al.*, 1996). However, the exact role of this module in PLC function remains unclear (Singer *et al.*, 1997).

C2 domain

The C2 domain is located near the carboxy-terminus and has been proposed to serve as another possible lipid-binding region of PLCs (James *et al.*, 1997).

Distinct domain

PLCβ contains additional regulatory domains near the carboxy-terminus which might be involved in membrane association and interaction with Gqα subunit (Wu *et al.*, 1993; Park *et al.*, 1993; Kim *et al.*, 1996). The *src* homology domains in PLCγ are undoubtedly involved in the translocation of PLCγ from cytosol to the plasma membrane and cytoskeletal targets (James *et al.*, 1997; Singer *et al.*, 1997; Rhee and Bae, 1997).

Clearly, these peculiar regulatory domains are responsible for specific regulation of different families.

1.3 Functional Role of Phospholipase C-Derived Second Messengers

Two second messengers are generated when PtdIns(4,5)P_2 is used as the substrate, namely, DAG and Ins(1,4,5)P_3.

1.3.1 *sn*-1,2-Diacylglycerol

Intracellular pools of DAG exist in the plasma membrane, the nucleus as well as in the endoplasmic reticulum. DAGs generated in the plasma membrane are important for cell signaling (Quest *et al.*, 1996).

Multiple routes account for the generation of DAG. For example, agonist-stimulated DAG formation can be achieved through hydrolysis of phospholipids by the action of phospholipases or *de novo* synthesis (Quest *et al.*, 1996).

The kinetics of DAG production may be either monophasic or biphasic, depending on the agonist and cell-type involved. For example, monophasic DAG production, which is probably derived from phosphatidylcholine (PtdCho) hydrolysis (Exton, 1990), has been observed after stimulation of muscarinic receptors in PC12 cells (Altin and Bradshaw, 1990). On the other hand, biphasic generation of DAG in Swiss 3T3 fibroblasts in response to bombesin, as demonstrated by molecular species analysis, initially involves a transient increase in polyunsaturated DAG species, especially 18:0/20:3n-9, 18:0/20:4n-6, and 18:0/20:5n-3, as a result of PLC activation (Pettitt and Wakelam, 1993; Nishizuka, 1995). However, DAG produced during the more sustained phase is more likely derived from PtdCho hydrolysis, an observation that is supported by the analysis of fatty acid

composition (reviewed by Nishizuka, 1995) and by the finding that this phase is associated with an increase of choline (Pettitt and Wakelam, 1993). Agonists capable of inducing a two phase DAG elevation include growth factors, such as nerve growth factor (NGF) and basic fibroblast growth factor (bFGF), and cytokines (Altin and Bradshaw, 1990; Cockcroft, 1992). The sustained elevation of DAG species, in particular those derived from PLD-catalyzed PtdCho hydrolysis, is considered to be important in the long-term regulation of cell growth and differentiation (review by Nishizuka, 1995; Quest *et al.*, 1996). However, the physiological significance remains to be established.

The removal of DAG is also important in the regulation of the cellular response. In this regard, DAG can be metabolized in several ways: phosphorylation to PtdOH, breakdown by DAG lipase, and synthesis of other lipids (Quest *et al.*, 1996). However, it should be noted that DAG derived from phosphoinositide hydrolysis is preferentially used in the resynthesis of PtdIns(4,5)P_2 (Quest *et al.*, 1996).

Biological Effects of DAG

Okumura and co-workers in 1988 demonstrated an increased DAG production in homogenate from rat hearts following α_1-AR stimulation. This second messenger molecule is a potent activator of PKC (Nishizuka, 1995). Presently, at least 7 subtypes of PKC have been demonstrated in the heart and they fall into three classes: 1) conventional PKC, including PKCα, PKCβII and PKCγ which can be activated by Ca^{2+} and DAG; 2) novel PKC, including PKCδ, PKCε and PKCη that can be activated by DAG and some phospholipids; 3) atypical PKC, PKCζ that can be activated by phophatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (Steinberg *et al.*, 1995; Puceat and Vassort, 1996;

Nishizuka, 1995). The activation of PKC results in their subcellular redistribution and the phosphorylation of a number of intracellular target proteins (Lamers *et al.*, 1992a). PKC activation mediates many physiological responses including metabolic changes, secretion, contraction, cell proliferation, differentiation and gene expression (Lamers *et al.*, 1992a; Nishizuka, 1995). Alterations of PKC activity and translocation have also been shown in various conditions, such as agonist stimulation, hypertrophy, heart failure and ischemia/reperfusion injury (Steinberg *et al.*, 1995; Rouet-Benzineb *et al.*, 1996; Puceat and Vassort, 1996; Cohen and Downey, 1996).

Activation of PKC by DAG has been associated with the phosphorylation of a number of key regulatory proteins, including TnI and TnT (Noland and Kuo, 1991; Noland *et al.*, 1996; Jideama *et al.*, 1996), which results in a diminished Ca^{2+} sensitivity of the sarcomere (Dosemeci *et al.*, 1988) and a decreased Ca^{2+}-stimulated myofibrillar MgATPase activity (Venema and Kuo, 1993). Myosin light chain 2 is a poor substrate for PKC (Noland and Kuo, 1995), although its phosphorylation by PKC has been shown to increase Ca^{2+}-stimulated actomyosin MgATPase activity (Noland and Kuo, 1993); it is unclear whether this will play an important role *in vivo*.

Besides PKC activation, DAG has also been shown to exhibit other effects. For example, extracellular DAG treatment of cardiomyocytes inhibits the L-type Ca^{2+} channel independent of PKC (Schruer and Liu, 1996; Conforti *et al.*, 1995). Also, DAG has been reported to be involved in the regulation of insulin receptor activity (Arnold and Newton, 1996a). These workers demonstrated that DAG activates the insulin receptor tyrosine kinase by causing a marked increase in the affinity of the receptor for insulin. However,

DAG has no effect on the receptor's catalytic activity and its affinity for ATP. Also, activation of the receptor is not as a result of PKC activation by phorbol myristate acetate. This sensitization may occur by two mechanisms as proposed by these investigators: 1) DAG may alter the structure of the lipid environment of the insulin receptor, thus affecting receptor function; 2) DAG may interact directly with the receptor, acting as an allosteric activator by binding to the receptor *via* a hydrophobic interaction and increase the receptor's affinity for insulin by stabilizing the active conformation.

1.3.2 Inositol 1,4,5-trisphosphate

Ins(1,4,5)P_3, the other important product of this pathway, is metabolized in two ways: either by dephosphorylation to inositol 1,4-bisphosphate (Ins(1,4)P_2), inositol 4-phosphate (Ins4P) and further to inositol or by phosphorylation to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4), followed by the dephosphorylation to inositol 1,3,4-trisphosphate (Ins(1,3,4)P_3), inositol 1,3-bisphosphate (Ins(1,3)P_2) or Ins(1, 4)P_2, inositol 1-phosphate (Ins1P), inositol 3-phosphate (Ins3P), and Ins4P (reviewed by van Heugten *et al.*, 1996).

The heart maintains a relatively stable and high level of Ins(1,4,5)P_3 (Woodcock *et al.*, 1995, 1997; De Jonge *et al.*, 1994). Existence of the cardiac inositol tetrakisphosphate pathway has also been demonstrated. Studies performed in isolated perfused hearts demonstrated that norepinephrine causes a concentration-dependent production of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,3,4,6)P_4, which peaks between 2 and 5 minutes post α_1-AR stimulation (Scholz *et al.*, 1986; Heathers *et al.*, 1988). Further studies in myocytes demonstrate a rapid and transient increase of Ins(1,3,4,5)P_4 after 30 seconds

stimulation with norepinephrine (Heathers *et al.*, 1988). Also the formation of the isomers Ins(1,4)P_2, Ins(3,4)P_2, and Ins(1,3,4)P_3 after the addition of ET-1 or an α_1-adrenergic agonist to cultured neonatal rat ventricular myocytes has been observed (De Jonge *et al.*, 1994). However, in contrast, norepinephrine only induces rapid increases of Ins(1,4)P_2 and Ins(4)P in intact heart tissue, where Ins(1,4,5)P_3 kinase product is not detected (Woodcock *et al.*, 1995).

Several lines of evidence have shown that Ins(1,4,5)P_3 may bind to its SR Ins(1,4,5)P_3 receptor and cause Ca^{2+} release in the heart (reviewed by De Jonge *et al.*, 1995). Studies by Huisamen *et al* (1994) suggest that the cardiac Ins(1,4,5)P_3 receptor contains both a low affinity and a high affinity site, along with a putative Ins(1,3,4,5)P_4 binding site. A contradictory observation that Ins(1,4,5)P_3 receptors are mainly located around the intercalated disks rather than on the SR has also been reported (Kijima *et al.*, 1993). Ventricular myocytes have a lower concentration of Ins(1,4,5)P_3 receptors when compared to SR ryanodine receptors (Moschella and Marks, 1993). Although it has been reported that Ins(1,4,5)P_3 can increase intracellular Ca^{2+} release in cardiac muscle (Nosek *et al.*, 1986), the study of Zhu and Nosek (1991) has shown that instead of causing Ca^{2+}-induced Ca^{2+} release from SR, Ins(1,4,5)P_3 causes Ca^{2+} oscillations, which suggests that Ins(1,4,5)P_3 may not be involved in the beat-to-beat regulation of Ca^{2+} transients under physiological conditions.

Since the tissue Ins(1,4,5)P_3 content is relatively high and PLCδ1 has a high affinity for Ins(1,4,5)P_3 (Lemmon *et al.*, 1995; Garcia *et al.*, 1995; Ferguson *et al.*, 1995),

it has been proposed that cardiac PLCδ may be persistently inhibited under physiological conditions (Woodcock, 1997).

1.4 Phosphoinositide-PLC Pathway in Cardiac Pathologies

Chronic hemodynamic overload, either volume or pressure overload, will result in the compensational hypertrophy of the heart. The phosphoinositide cycle may contribute to the hypertrophic process in response to a variety of stimuli, including mechanical stress, α_1-AR agonists, ET-1, Ang II, and α-thrombin (reviewed by De Jonge *et al.*, 1995; Hefti *et al.*, 1997). A decrease in membrane-bound PtdIns(4,5)P_2-PLC activity is observed with the development of hypertrophy in SHRSP heart (reviewed by Berk and Corson, 1997; Unger *et al.*, 1996; Alexander and Griendling, 1996). In addition, results from our laboratory have demonstrated that abnormalities in the phosphoinositide cycle exist in moderate and chronic stages of congestive heart failure (Meij *et al.*, 1997) with selective downregulation or upregulation of certain PLC isozymes (Tappia *et al.*, unpublished observations). In particular, PLCβ1 activity has been shown to be elevated 2-3 fold in the left viable ventricular tissue and 5-fold in the scar tissue (Ju *et al.*, 1998). The changes in the phosphoinositide cycle are also related to cellular injury due to ischemia and reperfusion (De Jonge *et al.*, 1995). The alterations in phosphoinositide metabolism in diabetic cardiomyopathy will be discussed in detail in the last section.

2. Regulation of the Phosphoinositide-PLC Pathway

The following section reviews the regulation of the cardiac phosphoinositide-PLC pathway and some other possible regulatory mechanisms as suggested by studies

conducted on PLC derived from tissues and cells other than heart.

2.1 Receptor Level Regulation

Two classes of receptors have been identified as mediators of PLC activation. One is the G protein-coupled receptor (GPCR) class, the other comprises receptors with intrinsic tyrosine kinase activity or those are associated with tyrosine kinase activity (van Heugten *et al.*, 1996; Singer *et al.*, 1997; James *et al.*, 1997).

2.1.1 G protein-coupled receptors

GPCRs have seven transmembrane spanning domains. These seven α–helical transmembrane spanning domains form three extracellular and intracellular loops with an extracellular amino terminus and an intracellular carboxy-terminus (Benovic *et al.*, 1987; Lefkowitz *et al.*, 1988). Members in this family include α–AR, muscarinic receptors and Ang II receptors, which have been shown to be capable of activating PLC (reviewed by van Heugten, 1996; De Jonge, 1995).

Agonist stimulation of GPCR often results in rapid receptor desensitization (Ferguson *et al.*, 1996; Bohm *et al.*, 1997) and further limit of PtdIns(4,5)P_2 hydrolysis. Three events might contribute to this process: 1) receptor phosphorylation by either protein kinase A (PKA), PKC, and/or G protein-coupled receptor kinases (GRK) leads to the binding of the intracellular protein, arrestin to GPCR, thereby preventing G protein-receptor interaction; 2) internalization of cell-surface receptors; and 3) downregulation of receptor expression.

α_1-Adrenergic receptor

Three α_1-AR receptor subtypes, namely α_{1A}, α_{1B} and α_{1D} have been cloned and are

present in myocardium (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Lomasney *et al.*, 1991; Rokosh *et al.*, 1994). All three types are coupled to PLC activation (Schwinn *et al.*, 1995). In rat and rabbit hearts, the α_{1B}-AR is the predominant subtype expressed (Lazou *et al.*, 1994; Knowlton *et al.*, 1993).

Ang II receptor

Both AT1 and AT2 receptors are expressed in the rat heart. Expression of AT1 receptors is developmentally regulated (van Heugten *et al.*, 1996). In neonatal cardiomyocytes, $AT1_A$ as well as $AT1_B$ receptors are expressed, while $AT1_A$ is the predominant class expressed in adult myocardium (Gasc *et al.*, 1994; Matsubara *et al.*, 1994). Stimulation of cardiac AT1 receptor causes PtdIns(4,5)P_2 hydrolysis by activation of PLC (Baker *et al.*, 1988), however, the role of the AT2 receptor is unclear. AT1 receptors also mediate PLCγ1 phosphorylation (Harp *et al.*, 1997).

Endothelin receptor

ET receptors , ET_A and ET_B, are expressed in the atrial myocytes, stimulation of which induces phosphoinositide hydrolysis (Irons, 1993).

Muscarinic acetylcholine receptor

Five subtypes of the muscarinic receptor have been identified, and categorized as M1-5. M1 and M2 subtypes are suggested to mediate phosphoinositide hydrolysis. Interestingly, stimulation of M5 has been suggested to mediate tyrosine phosphorylation of PLCγ, which is indicative of crosstalk between GPCR and receptors with intrinsic tyrosine kinase activity (De Jonge *et al.*, 1995).

2.1.2 Receptors with intrinsic tyrosine kinase activity or associated with tyrosine

kinase activity

Upon binding of ligands, these receptors catalyse autophosphorylation and subsequent tyrosine phosphorylation of their target effector proteins such as PLCγ. Receptors in this family include those for cytokines, platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) (review by van Heugten, 1996; De Jonge, 1995; Rhee and Choi, 1992; Rhee and Bae, 1997).

2.2 G protein

G proteins (guanine nucleotide regulatory proteins) are heterotrimeric proteins that mediate the downstream events of GPCR stimulation. G proteins consist of three different polypeptide chains, designated α, β, and γ. The α subunit contains the GTP/GDP binding site. The βγ complex serves as a membrane anchor. Presently, 20α, 4β, and 5γ polypeptides have been identified (Simon *et al.*, 1991; Cali *et al.*, 1992). According to the amino acid homology, the 20 Gα are grouped into 5 classes, Gs, Gi, Gq, G12, and Go (reviewed by De Jonge *et al.*, 1995).

Upon ligand-receptor interaction, conformational changes in the GPCR activate the heterotrimeric G proteins. GTP is then exchanged for GDP and dissociation of α subunit from βγ complex occurs. The α subunit and βγ complex then exert regulatory actions on their specific cellular effectors. Hydrolysis of GTP to GDP by an intrinsic α subunit GTPase activity inactivates the G protein and is followed by the reassociation of the α and βγ subunits (Neer, 1995) and termination of extracellular stimulation.

The distribution of G protein subunits in the neonatal and adult heart has been identified recently. In neonatal and adult cardiac tissue the expression of αq family is

developmentally regulated. The αq/11, αq, and αz in neonatal tissues are the highest, and the adult atria contains a higher amount of these subunits relative to adult ventricles (Hasen *et al.*, 1995). These findings are consistent with the observation that high levels of the αq family are associated with the rapid growth phase of the heart (Morgan and Baker, 1991; Chien *et al.*, 1991). The same expression profiles of these G protein α-subunits are found in both the neonatal cardiomyocyte and fibroblast (Hasen *et al.*, 1995). The presence of α-subunits of Gh, a new class of G protein, has also been demonstrated in the heart (Das *et al.*, 1993).

The distribution patterns of β1, β2 are similar to those of αq/11, αq, while β3 subunit is not detectable in the heart. Examination of the distribution of γ subunits reveals the presence of γ3, γ5 and γ7 in both neonatal and adult cardiac tissue and among them, γ3 is only detectable in the neonatal cardiomyocyte. Antisera specific for γ2 failed to detect the presence of this protein in both tissue and cell. These studies also demonstrated that all the expressed β, γ subunits are also subjected to developmental regulation (Hansen *et al.*, 1995). The detailed coupling of these subunits and their downstream effectors will be discussed below.

2.3 Substrate Level Regulation

PtdIns(4,5)P_2 accounts for 0.2% of the cell membrane phospholipid pool (Lamers *et al.*, 1992a). During agonist stimulation, membrane PtdIns(4,5)P_2 levels fall rapidly and this may further limit the rate of PtdIns(4,5)P_2 hydrolysis and alters the time frame of DAG and Ins(1,4,5)P_3 production (Tobin *et al.*, 1996). Studies conducted in [^{3}H]inositol prelabelled cultured neonatal rat ventricular myocytes have demonstrated that

replenishment of the cardiac PtdIns(4,5)P_2 pool is fast, as after stimulation with ET-1 for 45 min, the PtdIns(4,5)P_2 level has been found to remain constant (van Heugten *et al.*, 1993).

2.3.1 PtdIns transfer protein

In order to replenish the membrane PtdIns(4,5)P_2 pool, the precursors for PtdIns(4,5)P_2, namely the membrane PtdIns*4P* and PtdIns, have to be maintained at a certain level. The cytosolic protein, PtdIns-TP is actively involved in the transfer of newly synthesized PtdIns from SR to the plasma membrane (Wolf, 1990). Evidence has shown that PtdIns-TP may also play an important role in maintaining a sustained cellular response, as indicated by the fact that cells depleted of cytosol show a reduced level of phosphoinositide hydrolysis in response to GTP-γS stimulation (Roberts, 1996). PtdIns-TP also enhances PtdIns 4-kinase activity (Roberts, 1996). Thus, it is suggested that PtdIns-TP may serve as another regulator of the phosphoinositide-PLC pathway (Tobin *et al.*, 1996).

2.3.2 PtdIns 4-kinase

PtdIns 4-kinase phosphorylates PtdIns at the fourth position on the inositol ring yielding PtdIns*4P* (Quist *et al.*, 1989). The majority of cardiac PtdIns 4-kinase activity has been detected in the SL. Enzyme activity has also been found in the intracellular membranes including SR and mitochondria (Quist *et al.*, 1989). Studies have shown that the activity of PtdIns 4-kinase is Mg^{2+}-dependent and sensitivity to Ca^{2+} appears to be related to the species. For example, in rat heart, the PtdIns*4P* formation is inhibited by micromolar concentrations of Ca^{2+}, whereas in canine ventricle, the kinase activity is

insensitive to Ca^{2+} ranging from 0.1-30 mM (Mesaeli *et al.*, 1992; Kasinathan *et al.*, 1989).

Evidence from other studies has also shown that PtdIns 4-kinase activity may be regulated by PtdIns-TP (Roberts, 1996), cAMP, phorbol and DAG (Pike, 1992).

2.3.3 PtdIns4*P* 5-kinase

Further phosphorylation of PtdIns4*P* by PtdIns4*P* 5-kinase gives rise to PtdIns(4,5)P_2. The subcellular distribution and regulation of cardiac PtdIns4*P* 5-kinase activity by Mg^{2+} and Ca^{2+} is similar to that of PtdIns 4-kinase (Mesaeli *et al.*, 1992; Kasinathan *et al.*, 1989; Quist *et al.*, 1989).

Two related subtypes have been identified (type I and type II) (Creba *et al.*, 1983). Reinvestigation of the type I enzyme has revealed that it catalyses phosphorylation of PtdIns4*P* at the D-5 position of the inositol ring. The type II enzyme is actually a PtdIns*P* 4-kinase. It phosphorylates phosphotidylinositol 5-phosphate (PtdIns5*P*) at the D-4 position, but when [^{32}P]PtdIns4*P* is used as the substrate, no production of [^{32}P]PtdIns(4,5)P_2 is detected (Rameh *et al.*, 1997). Also, in the same study, the existence of PtdIns5*P* is first suggested in NIH3T3 fibroblasts (Rameh *et al.*, 1997), and to be involved in an alternative pathway for PtdIns(4,5)P_2 synthesis (Rameh *et al.*, 1997; Hinchliffe and Irvine, 1997).

Regulation of PtdIns4*P* 5-kinase has been well reviewed by Loijens *et al* (1996). It has been reported that stimulation of the small molecular weight G protein RhoA and GTPγS treatment results in an increased kinase activity. PtdIns4*P* 5-kinase may also be subjected to regulation by PtdIns-TP, as studies have shown that a complex of PtdIns-TP

and PtdIns4*P* 5-kinase type I exists during the secretion of norepinephrine (Loijens *et al.*, 1996). Further, PtdOH has been shown to enhance PtdIns4*P* 5-kinase activity, which suggests that newly formed PtdOH may play a role in sustained cellular response by enhancing PtdIns(4,5)P_2 turnover rate (Moritz *et al.*, 1992).

2.4 Effects of Ca^{2+} on PLC Activity

All PLC isozymes require Ca^{2+} for catalytic activity (Rhee and Choi, 1992). *In vitro* studies have shown that agonist-induced PLC activation occurs at physiological Ca^{2+} concentrations (Schwertz and Halverson, 1989).

Changes in Ca^{2+} concentration also seem to affect the cardiac PLC substrate specificity. For example, Ca^{2+} concentrations higher than 1 mM shift the substrate selectivity from PtdIns(4,5)P_2 to PtdIns4*P* and PtdIns (Meij and Panagia, 1992).

2.5 Effects of Membrane Composition on PLC Activity

Factors which modulate substrate accessibility of PLC appear to be important in regulating Ins(1,4,5)P_3 and DAG production in stimulated cells (James *et al.*, 1997), and are discussed below.

2.5.1 Membrane chemical-physical properties

In vitro, PLC assays show that PtdIns(4,5)P_2 hydrolysis stops when less than 30% PtdIns(4,5)P_2 has been utilized and it is suggested that this might be due to the physical changes in the lipid vesicular micelle rather than being due to enzyme inactivation (James *et al.*, 1996). As DAG has been shown to destabilize the membrane and cause structural transition (Das and Rand, 1984, 1986), it has been suggested that it may have an inhibitory effect on PLC activity (James *et al.*, 1996).

2.5.2 PLC-membrane interaction

PLC activity associated with membrane fractions can generally be removed by high salt treatment, thus binding of PLCs to membrane is largely through electrostatic interplay (Cockcroft and Thomas, 1992). This is also confirmed by the fact that a reduction in the content of negatively charged phosphatidylserine in lipid vesicles significantly decreases the binding capacity and activity of PLCδ (Rebecchi *et al.*, 1992a,b).

2.5.3 Fatty acid profile of substrate

The fatty acid composition of the phosphoinositides may also affect the ability of PLC to hydrolyze PtdIns(4,5)P_2. Incorporation of high concentrations of polyunsaturated fatty acids, particularly of 18:2n-6 and 20:5n-3 into neonatal rat ventricular myocytes decreases both basal and phenylephrine stimulated PLC activities, while treatment with 18:0/18:1n-9 increases the basal PtdIns(4,5)P_2 hydrolysis (Lamers *et al.*, 1992b).

2.6 Other Mechanisms

Myocardial PLC activity is also subject to the regulation by oxidative stress. Exposure of SL membranes to superoxide dismutase, hypochlorous acid and H_2O_2 in concentrations similar to those generated during ischemia/reperfusion injury has been shown to suppress PLC activity (Meij *et al.*, 1994).

2.7 PLC Isoform-Specific Regulation

2.7.1 PLCβ

Gqα-PLC pathway

As mentioned before, Ang II, ET-1, α_1-AR agonists, and acetylcholine induce PtdIns(4,5)P_2 turnover through this pathway (De Jonge *et al.*, 1995). The α subunits (αq,

α11, α14, α16) of all four members of the Gq subfamily of heterotrimeric G proteins have been proven to be effective and selective regulators of PLCβ isozymes, but not PLCγ1 or PLCδ1 (Kozasa *et al.*, 1993). Sensitivities to Gαq and Gα11 decrease in the order: PLCβ1≥PLCβ3>PLCβ2. Together with the result that Gα16 is the most effective stimulator for PLCβ2, it has been suggested that the specific coupling of different Gq subfamily with different PLCβ isozymes might determine the specific response to certain stimuli in different cell types (Rhee and Choi, 1992). Interestingly, PLCβ can terminate the receptor-mediated activation of G proteins due to its GTPase activating function on Gqα subunits (Berstein *et al.*, 1992; Biddlecome *et al.*, 1996).

Structure-function studies reveal that the carboxy-terminal region is required for the stimulation of PLCβ2 and PLCβ1 by Gqα (Rhee and Bae, 1997; Singer *et al.*, 1997).

Gβγ dimer

PLCβ isozymes can also be activated by Gβγ dimer (Rhee and Bae, 1997; Singer *et al.*, 1997). Gβγ subunits stimulate PLCβ isozymes in the order: PLCβ3>PLCβ2>PLCβ1 (Noh *et al.*, 1995; Lee and Rhee, 1995). Prenylation of the γ subunit is essential for this process (Dietrich *et al.*, 1994). Furthermore, studies with PLCβ2 indicate that the first half of the Y domain may be also involved (Kuang *et al.*, 1996). It should be noted that no specificity of interaction of βγ subunits with their effectors has been reported.

Regulation of PLCβ by protein kinase C and protein kinase A

PKC has been shown to be capable of desensitizing GPCR, phosphorylating G proteins (Katada *et al.*, 1985) and PLCβ (Rhee and Choi, 1992). PKC-mediated phosphorylation of PLCβ1 does not change either the basal or G protein-stimulated

activity (Rhee and Choi, 1992). PKC has also been reported to phosphorylate α subunits of G12, Gz and Gi (Kozasa and Gilman, 1996; Lounsbury *et al.*, 1993; Katada *et al.*, 1985; Bushfield *et al.*, 1990; Daniel-Issakani *et al.*, 1989). Whether or not PKC phosphorylates any of the Gq's is still not clear. In COS cells transfected with cDNAs encoding PKA, G protein subunits and PLCβ2, PKA specifically inhibits Gβγ stimulated PLCβ2 activity (Liu and Simon, 1996).

2.7.2 PLCδ

Gh and RhoGAP

Gh, a new class of GTP-binding protein, was first identified by Im and Graham in 1990 (Im and Graham, 1990; Im *et al.*, 1990). Subsequent studies showed that this pertussis toxin-insensitive G protein contains a 74 kDa α and a ~50 kDa β subunit and mediates the signal from α_1-AR to PLC in rat myocardium (Baek *et al.*, 1993). Gh has been shown to couple to both α_{1B}-AR and α_{1D}-AR (reviewed by Im *et al.*, 1997), and the Ghα subunit specifically has been shown to stimulate PLCδ1 activity *in vitro* and during α_1-AR stimulation (Rhee and Bae, 1997; Feng *et al.*, 1996). It is possible that it may also couple to PLCδ1 in myocardium. The GTPase-activating protein for the small GTP-binding protein Rho (RhoGAP) also specifically stimulates PLCδ1, but not PLCγ1 or PLCβ1 (Homma and Emori, 1995).

Other regulators

Compared with other PLC isozymes, PLCδs are readily activated by Ca^{2+} *in vitro*. Thus, activation of PLCδ isozymes may occur secondarily to other factors which can increase intracellular Ca^{2+} concentration (Rhee and Bae, 1997). PtdOH has been

demonstrated to bind to cardiac PLCδ1 with high affinity and stimulate the enzyme activity, even in the presence of EGTA (Henry *et al.*, 1995).

2.7.3 PLCγ

Protein tyrosine kinase-dependent activation of PLCγ

Upon polypeptide growth factor binding to its receptor, the intrinsic protein tyrosine kinase activity is activated. The translocation of PLCγ from cytosol to plasma membrane and cytoskeleton occurs (review by Rhee and Bae, 1997). PLCγ interacts with the activated receptor with its SH2 domain and undergoes consequent tyrosine phosphorylation at tyrosine 771, 783, and 1254 (Kim *et al.*, 1990; Wahl *et al.*, 1990).

It should be noted that translocation itself to the particulate fraction cannot totally account for the increased activity (Singer *et al.*, 1997). As it is speculated that membrane penetration is required for PLCs to perform catalytic activity (James *et al.*, 1997), the translocation of the enzyme may function to bring the enzyme accessible to its substrate, PtdIns(4,5)P_2.

Studies have shown that association of PLCγ with receptors is necessary for growth factor-mediated activation, given the fact that PLCγ-mediated Ins(1,4,5)P_3 production is blocked when mutations are introduced to the PLCγ binding sites of receptors PDGF, EGF and NGF (Noh *et al.*, 1995).

Tyrosine phosphorylation may also be required for PLCγ's catalytic activity. Attenuation of PLCγ-mediated response can be achieved by the action of protein tyrosine phosphatases; inhibition of the phosphatase activity by H_2O_2 is also required for the tyrosine phosphorylation function of EGF (Bae *et al.*, 1997). The unphosphorylated PLCγ

exhibits some distinct characteristics: it is selectively inhibited by Triton X-100 and is unable to catalyze the hydrolysis of profilin-bound PtdIns(4,5)P_2 (Goldschmidt-Clermont *et al.*, 1991). As profilin is an actin-binding protein, this implies that tyrosine phosphorylation may be important for PLCγ to exert its receptor-stimulated regulation on cytoskeletal activity (Goldschmidt-Clermont *et al.*, 1991).

Receptors associated with tyrosine kinase activities, such as the cytokine receptors, integrin receptors and some Ig receptors, also phosphorylate and activate PLCγ isozymes (Rhee and Bae, 1997).

Activation of GPCR may also regulate PLCγ activity. In cultured vascular smooth muscle cells, Ang II causes a maximal increase of PLCγ1 phosphorylation after 30 sec followed by a temporally correlated increase in Ins(1,4,5)P_3 production, which is 75% inhibited by the tyrosine kinase inhibitor genistein (Marrero *et al.*, 1994). Further investigations find that Ca^{2+} antagonist, Ca^{2+} chelator and verapamil increase the basal level of PLCγ1 phosphorylation and inhibit the rapid dephosphorylation of PLCγ1, which is suggestive that the Ang II-mediated PLCγ1 phosphorylation antedates the increase of intracellular Ca^{2+} and is Ca^{2+}-independent (Harp *et al.*, 1997). Since the AT1 receptor has no intrinsic tyrosine kinase activity and PLCγ is typically linked to growth factor receptors with intrinsic tyrosine kinase activity or receptors associated with tyrosine kinase activity (Rhee and Bae, 1997), it is suggested that a calcium independent cytosolic tyrosine kinase is required to accomplish the AT1 receptor mediated PLCγ1 tyrosine phosphorylation (Marrero *et al.*, 1994). Furthermore, as Ang II has been shown to activate pp60-*src* in smooth muscle cells (Ishida *et al.*, 1995) and anti-*src* treatment partially prevents Ang II

mediated PLCγ phosphorylation (Schelling *et al.*, 1997), it is conceivable that *src* may be involved in this process. Additional studies by Sadoshima and Izumo suggest that the *src* kinase family member Fyn may mediate the Ang II-stimulated activation of $p21^{ras}$ in cultured myocytes (Sadoshima and Izumo, 1996), therefore, it is possible that PLCγ might also be involved in the Ang II-initiated downstream events in cardiomyocytes.

Protein tyrosine kinase-independent activation of PLCγ

Phosphatidylinositol 3,4,5-trisphosphate

A recent study (Bae *et al.*, 1998) has shown that PtdIns(3,4,5)P_3, the phosphorylation product of PtdIns(4,5)P_2 on the D-3 position by the activity of PtdIns 3-kinase, stimulates PLCγ purified from Hela cells. Incubation with SH2 domain-containing proteins did not affect basal PLCγ1 activity, while these SH2 proteins markedly suppressed the PtdIns(3,4,5)P_3-induced activation. Thus, it is suggested that the SH2 domain may play a role in the PtdIns(3,4,5)P_3-mediated activation of the enzyme. Transient expression of the p110 catalytic domain of PtdIns 3-kinase in COS-7 cells causes an increase in inositol phosphate production, an effect which is abolished by preincubation with a specific PtdIns 3-kinase inhibitor LY294002. However, the inhibitory effect of LY294002 on growth factor-activated PLC activity is cell-type dependent (Bae *et al.*, 1998). The exact mechanism involved is unclear.

Arachidonic acid

PLCγ is activated by several unsaturated fatty acids in the presence of tau or tau-like proteins. Such unsaturated fatty acids include arachidonic acid (AA), linolenic acid, oleic acid and palmitoleic acid (Hwang *et al.*, 1996b). Tau-like proteins are associated with

microtubules and widely expressed in many tissues. Studies by reverse transcriptase-coupled polymerase chain reaction and immunoblotting show that tau is also expressed in the heart (Gu *et al.*, 1996). Though tau alone cannot significantly activate phosphoinositide-PLC, the AA stimulated PtdIns4*P* or PtdIns(4,5)P_2 hydrolysis requires the presence of this protein. PtdCho specifically inhibits tau and AA-dependent activation PLCγ1. It has been speculated that the activation is achieved through an interaction with PLCγ1's SH3 binding motifs and the PH domains near the amino-terminus. This is suggestive that the generation of AA or other unsaturated fatty acids by phospholipase A_2 (PLA_2)-mediated hydrolysis of PtdCho may be involved in the crosstalk between PLCγ and PLA_2 (Hwang *et al.*, 1996b).

Phosphatidic acid

PtdIns(4,5)P_2 hydrolysis in adult cardiac myocytes is increased following treatment with PtdOH (Kurz *et al.*, 1993). Gel filtration analysis shows that PtdOH can bind to and stimulate cardiac PLCγ1 enzyme activity (Henry *et al.*, 1995). It has also been observed that PtdOH can stimulate both unphosphorylated (non-EGF stimulated) and tyrosine-phosphorylated (EGF-stimulated) PLCγ1 activities in A-431 cells, with the unphosphorylated PLCγ1 being more responsive. Interestingly, although the EGF-stimulated PLCγ1 exhibits higher basal catalytic activity, addition of PtdOH increases the activity of both non-EGF and EGF-stimulated PLCγ1 to the same level, suggesting that this action is independent of phosphorylation. Further experiments reveal that PtdOH does not affect the substrate binding capacity of PLCγ1. It is speculated that stimulation of the unphosphorylated PLCγ1 by PtdOH may be through an allosteric mechanism (Jones and

both a non-catalytic binding site and a catalytic binding site may exist in PLCγ1 (Jones and Carpenter, 1993). PtdOH may bind to the non-catalytic site in an allosteric manner and subsequently change the tertiary structure and facilitate the binding of PtdIns(4,5)P_2 to the catalytic site. The exact mechanism involved in stimulation of tyrosine-phosphorylated PLCγ1 remains unclear (Jones and Carpenter, 1993).

3. Diabetic Cardiomyopathy

Diabetes mellitus is one of the leading causes of death in the industrialized world. According to insulin dependence, the disease can be classified into two types; type I, insulin-dependent diabetes mellitus (IDDM) and type II, non-insulin dependent diabetes mellitus (NIDDM). IDDM is characterized by insufficient secretion of insulin by the β-cells of pancreas. Usually people are afflicted with IDDM before 30-year old, and need insulin support for a life-long time. Diabetic cardiomyopathy, one of the chronic complications of diabetes, was first described by Rubler and coworkers in 1972 (Rubler *et al.*, 1972). It refers to cardiovascular changes secondary to diabetes mellitus, and is characterized by alterations in hemodynamic parameters, contractile abnormalities, structural derangements (Olsen, 1979; Pierce *et al.*, 1988; Tomlinson *et al.*, 1992). Clinical studies have shown that cardiomyopathy may occur in patients even without evidence of vascular diseases (Andreoli *et al.*, 1997; William *et al.*, 1997; Fein and Scheuer, 1997).

3.1 Animal Models of Diabetes

As reviewed by Shafrir (1997), the commonly used animal models can be divided into several categories.

3.1.1 Diabetes induced by cytotoxins specific for pancreatic β-cells

STZ is the major chemical used to induce experimental diabetes. It is mainly composed of a deoxyglucose and a highly reactive nitrosourea side chain and selectively attacks pancreatic β-cells. The diabetogenic action might be due to the following events: free radical formation, suppression of enzymes of the tricarboxylic acid cycle and Ca^{2+}-dependent dehydrogenases, DNA strands breakage and finally, irreversible cessation of insulin production and β-cell necrosis. One single high dose of STZ treatment can induce IDDM in rodents.

3.1.2 Animals with diabetes autoimmune in origin

Diabetes in diabetes-prone BB rats (a colony isolated from Wistar rats at BioBreeding Laboratories in Ottawa) might be due to autosomal recessive transmission. The rats present the three typical symptoms of IDDM and require insulin support for survival.

Nonobese diabetic rats (NOD) are also insulin-dependent and do not become ketoacidotic. This model is especially useful for understanding the influence of environmental, nutritional, and hormonal factors in the onset of IDDM.

3.1.3 Transgenic animals

The most commonly used are the rat insulin promoter (RIP-Tag) lines.

3.1.4 Insulin-resistant diabetic rodents

Diabetes in these species is recessive and autosomal mutant in origin. db/db Mice is the commonly used model.

3.2 Abnormalities in Diabetic Cardiomyopathy

Cardiac dysfunction has been observed in both diabetic patients and animals (Tomlinson *et al.*, 1992; Schaffer and Mozaffari, 1996). The most prevalent defect in cardiomyopathy secondary to IDDM is depression of contractility and impairment of relaxation process. The possible mechanisms involved are discussed below.

3.2.1 Alterations in the Ca^{2+} transport pathways

Beat-to-beat intracellular Ca^{2+} regulation is due to the following process: a small amount of Ca^{2+} enters the cell *via* L-type Ca^{2+} channel and induces intracellular Ca^{2+} release from the SR Ca^{2+} release channel; the elevated $[Ca^{2+}]_i$ initiates crossbridge cycling; intracellular Ca^{2+} is then decreased mainly by sequestration to SR by the SR Ca^{2+} pump and, to a lesser extent by extrusion by the activities of SL Na^+/Ca^{2+} exchanger and SL Ca^{2+} pump (Opie, 1998).

Although the studies on the changes in $[Ca^{2+}]_i$ in diabetic cardiomyopathy are inconclusive (Yu *et al.*, 1997; Hayashi and Noda *et al.*, 1997), impaired Ca^{2+} homeostasis has been identified and may play a role in the pathogenesis of diabetic cardiomyopathy (Pierce and Russell, 1997).

Abnormalities in SR Ca^{2+} release and uptake

The diabetic heart is characterized by a depressed SR Ca^{2+} pump activity and reduced density due to the changes in intracellular lipid metabolism (Pierce and Russell, 1997; Penpargkul *et al.* 1981; Ganguly *et al.*, 1983; Lopaschuk *et al.*, 1983; Black *et al.*, 989; Rupp *et al.*, 1994). As a result, decreased SR Ca^{2+} uptake might occur and lead to a slow relaxation rate (diastolic dysfunction). This is also confirmed by the fact that normalization of SR Ca^{2+} pump activity results in partial improvement of contractility in

the diabetic heart (Rodrigues and McNeill, 1992).

Abnormalities in SR Ca^{2+} release and storage are indicated by decreased peak systolic $[Ca^{2+}]_i$, decreased high-affinity ryanodine binding sites (Lagadic-Gossmann *et al.*, 1996) and reduced caffeine-induced Ca^{2+} release from SR (Yu *et al*, 1997), and may contribute to the slow tension generation (systolic dysfunction) in diabetic heart.

Abnormalities in Ca^{2+} influx and efflux

Decreases in the sarcolemmal Ca^{2+} pump, Na^+/Ca^{2+} exchanger, the Na^+/K^+ ATPase and the Na^+/H^+ exchanger have also been reported in the diabetic heart (Makino *et al.*, 1987; Heyliger *et al.*, 1987; Pierce *et al.*, 1983, 1990), while the functional status of L-type Ca^{2+} channel is controversial (Pierce and Russell, 1997). It appears that these changes in Ca^{2+} fluxes may have more significant effect on systolic and diastolic dysfunction.

3.2.2 Abnormalities in the contractile apparatus

Since depressed contractility cannot be completely normalized by correcting the defects in Ca^{2+} regulation (Rodrigues and McNeill, 1992), it is conceivable that abnormalities in contractile apparatus, i.e. changes in myosin heavy chain and/or alteration in Ca^{2+} sensitivity of troponin may also be involved (Schaffer and Mozaffari, 1996).

Myosin isoform shift

The efficiency of crossbridge cycle is closely related to myosin-ATPase activity, therefore, the myosin isozyme shift from the fast V1 to the slower V3 isoform may partially contribute to the depressed cardiac contractility seen in STZ-treated diabetic rats (Tomlinson *et al.*, 1992; Schaffer and Mozaffari, 1996). As the shift can be prevented by either thyroxine (Malhotra *et al.*, 1981; Dillmann, 1982) or verapamil treatment (Afzal *et*

al., 1989), it is indicative that myosin-ATPase activity is under the regulation of thyroid status and intracellular Ca^{2+}.

Loss of calcium sensitivity

Loss of calcium sensitivity of the myofibril might also be involved. TnT and TnI, two important regulatory components of the thin filament, are responsible for Ca^{2+} sensitivity of the myofilaments (Schaffer and Mozaffari, 1996). In the IDDM heart, early studies have shown an isoform shift from TnT1 to TnT3 (Goodale and Hackel, 1953). Later on, it was demonstrated that this shift is coincident with a loss of calcium sensitivity (Akella *et al.*, 1995). An increased TnI phosphorylation has also been observed under *in vitro* conditions, while no changes have been found in either TnI protein content or mRNA level (Liu *et al.*, 1996). As phosphorylation of TnI and TnT by PKC leads to decreased contractile force (Noland and Kuo, 1991; Noland *et al.*, 1996; Jideama *et al.*, 1996; Dosemeci *et al.*, 1988; Venema and Kuo, 1993), the sustained increase of PKC activity in the diabetic heart (Inoguchi *et al.*, 1992; Giles *et al.*, 1998; Malhotra *et al.*, 1997) may play a role in the depressed contractility.

3.3 Phosphoinositide-PLC Pathway in Diabetic Cardiomyopathy

The precise mechanism associated with cardiac dysfunction in diabetes is still under investigation. However, abnormalities at receptor level and substrate level have been proposed and are discussed below.

3.3.1 Alterations of phosphoinositide-PLC pathway at receptor level

α_1-Adrenoceptor signaling pathway

As mentioned in section 2.1, stimulation of cardiac α-AR leads to phosphoinositide

hydrolysis *via* Ghα and Gqα (Schwinn *et al.*, 1995; Knowlton *et al.*, 1993; Baek *et al.*, 1993; Hwang *et al.*, 1996a; Im *et al.*, 1990; Im and Graham, 1990). The detailed coupling of α_1-AR subtypes to G proteins is well reviewed recently (Im *et al.*, 1997). α_{1A}-AR and α_{1D}-AR have been shown to couple to PLCβ1 *via* Gq and PLCδ1 *via* Gh, respectively, while α_{1B}-AR has been shown to couple to both PLCβ1 and PLCδ1 (Im *et al.*, 1997; Baek *et al.*, 1993; Feng *et al.*, 1996). Therefore, alterations in this signaling pathway will modify the function of PLCδ1 as well as PLCβ3 and PLCβ1 on the heart and consequently play a role in the development of cardiomyopathy.

Reduced numbers of α_1-AR have been reported in acute and chronic diabetic hearts by several investigators (Heyliger *et al.*, 1982; Wald, 1988; Tanaka *et al.*, 1992). For example, in the 6-week diabetic heart, a decrease by 45% without any change in the dissociation constant was detected by using a hydrophobic α_{1A}- and α_{1B}- antagonist [^{3}H]bunazosin (Tanaka *et al.*, 1992). However, no information on the changes of individual α_1-AR subtype is available in the literature.

Except decreased receptor number, hyperresponsiveness of the diabetic heart to α_1-AR agonist has also been observed in acute and chronic diabetes (Heijnis and van Zwieten, 1992; Wald *et al.*, 1988; Jackson *et al.*, 1986; Downing *et al.*, 1983). In acute diabetes (3 days), the enhanced inotropic response to methoxamine, an α_1-AR agonist, was ascribed to an increased PLC activity (Wald *et al.*, 1988). Since STZ-treated diabetic rats are associated with hypothyroidism (Tomlison *et al.*, 1992), which has been shown to increase (Panagia and Mesaeli, 1995) or decrease (Jakab *et al.*, 1993) membrane-bound PLC activity, further experiments will be required to identify whether the

hyperresponsiveness to α_1-AR is due to the decrease in thyroid hormone.

Contradictory results on α_1-AR responsiveness to agonists have also been reported (Irlbeck and Zimer, 1996; Tanaka *et al.*, 1992). As the cardiac response to α_1-AR stimulation is the combined effect of changes of receptor density, receptor subtype, functional status of G proteins and PLC isozymes and DAG molecular species, studies at these levels may help to elucidate the mechanisms involved.

Cardiac renin-angiotensin system

All the components of the renin-angiotensin system (RAS), such as renin, angiotensinogen, angiotensin converting enzyme (ACE), angiotensin I (Ang I) and Ang II, have been identified in the heart (Paul *et al.*, 1995; Baker *et al.*, 1990; Kawaguchi and Kitabatake, 1995; Stock. *et al.*, 1995), indicating that the heart is not only a target, but also a site of endocrine or paracrine Ang II formation (Muller *et al.*, 1998).

Cardiac effects of angiotensin II

Physiologically, direct stimulation of the cardiac angiotensin receptor induces both positive inotropic and chronotropic effects (Freer *et al.*, 1976; Kobayashi *et al.*, 1978). Growth effects of Ang II include induction of early gene expression, stimulation of protein synthesis and regulation of cell growth and differentiation (Baker *et al.*, 1990; Miwa *et al.*, 1984; Dostal *et al.*, 1996). Ang II has also been shown to be involved in structural remodeling of the myocardial collagen matrix. In isolated adult cardiac fibroblasts, Ang II stimulates collagen synthesis and inhibits matrix metalloproteinase 1 activity (Brilla *et al.*, 1994).

Angiotensin II signaling pathway

AT1 is the predominant subtype in adult rat myocytes (Sechi *et al.*, 1992), whereas AT2 is predominant in the human heart (Regitz Zagrosek *et al.*, 1996). It is well documented that stimulation of the AT1 receptor causes phosphatidylinositide hydrolysis by activation of PLC (Baker *et al.*, 1988). Since PLCβ isozymes are known to be regulated by the Gq class of heterotrimeric G protein (Smrcka *et al.*, 1991), and the AT1 receptor belongs to the G protein coupled receptor family, PLCβ is thought to be the most likely downstream effector in previous studies (reviewed by Dostal *et al.*, 1997; van Bilsen, 1997). However, recent findings strongly indicate that, besides PLCβ, PLCγ might also be involved in the Ang II-mediated downstream events in cardiomyocytes (Sadoshima and Izumo, 1996).

Renin-angiotensin system in diabetes

Under diabetic conditions, plasma Ang II levels are comparable with those of the control group (Makarious *et al.*, 1993; Drury *et al.*, 1984), while no information on cardiac renin, angiotensinogen, and Ang II levels during diabetes is available. It has been reported that there is a significant increase in myocardial AT1 receptor density and AT1 receptor mRNA level in 2-week STZ-induced diabetic rats. Furthermore, cardiac tissue renin or angiotensinogen mRNA levels are not altered by infusion of Ang II in either control or diabetic rats, indicating that changes in expression of these genes in the heart is attributed to the diabetic condition (Sechi *et al.*, 1994). Moreover, sustained elevation of PKC activity can increase the activity of tissue levels of ACE in the diabetic rat (Valentovic *et al.*, 1987) . In turn, elevated ACE activity can increase tissue levels of Ang II and cause a long-lasting cardiac vasoconstrictory effect (Muller *et al.*, 1998). Thus, the

increased stimulation of AT1 receptor may accelerate the development of diabetic cardiomyopathy, which is confirmed by the fact that ACE inhibition prevents the development of myocardial dysfunction in STZ-induced diabetic cardiomyopathy (Given *et al.*, 1994). Furthermore, treatment with a specific AT1 blocker, L-158,809 prevents the increase in TnI phosphorylation and translocation of PKCε in the diabetic heart (Malhotra *et al.*, 1997). All these above mentioned lines of evidence suggest that Ang II signaling pathway might be involved in the pathogenesis of diabetic cardiomyopathy.

3.3.2 Decreased substrate level

Depressed SL PtdIns 4-kinase and PtdIns4*P* 5-kinase activities has been reported by our laboratory (Liu *et al.*, 1997), indicating decreased SL PtdIns(4,5)P_2 levels may exist in the diabetic heart and limit the hydrolytic activity of PtdIns(4,5)P_2-PLC.

3.3.3 Second messengers in diabetic cardiomyopathy

Role of *sn*-1,2-diacylglycerol

The DAG content in the myocardium of diabetic rats has also been observed to be significantly elevated at 2, 4, and 8 weeks after STZ injection (Okumura *et al.*, 1991; Inoguchi *et al.*, 1992). Studies performed by Inoguchi and co-workers (1992) have demonstrated that early stage pancreatic islet cell transplantation in STZ-treated diabetes-resistant BB rats reverses the myocardial DAG content to normal level (Inoguchi *et al.*, 1992). In contrast, 2-week insulin treatment in the 6-week diabetic rats has no effect on the DAG content of the myocardium, however, the fatty acid composition of DAG revealed a different profile from both control and diabetic hearts (Okumura *et al.*, 1991).

DAG has been shown to sensitize the insulin receptor by acting as an allosteric

activator and/or by altering the structure of the lipids surrounding the receptor in a PKC-independent way (Arnold and Newton, 1996a). This suggests that the elevation of DAG content may be associated with the compensatory stage of diabetic cardiomyopathy, although the exact mechanism is unknown.

The possible sources of DAG has been reviewed by Quest and co-workers (1996). The increased DAG in diabetic heart may be due to: 1) increased phosphoinositide hydrolysis, as elevated plasma and cardiac catecholamine levels (Ganguly *et al.*, 1986) and increased of AT1 receptor density (Sechi *et al.*, 1994) have been suggested to occur in diabetic animals, the consequent stimulation of α_1-ARs and AT1 receptors may contribute to the increased DAG level; 2) decreased DAG degradation as a consequence of fatty acid accumulation in the diabetic heart (Farooqui *et al.*, 1989; Paulson *et al.*, 1991); 3) increased synthesis from PtdCho-PLC (Baldini *et al.*, 1994).

Insulin also has an effect on cellular DAG content. Insulin-stimulated increases in DAG levels from PtdOH have been reported in BCH-1 cultured myocytes (Farese *et al.*, 1984). In addition, insulin can activate PLCγ (Kellerer *et al.*, 1991), suggesting that the increases in DAG concentration might come from phosphoinositide hydrolysis as well. Furthermore, previous studies in our laboratory have demonstrated a decreased SL PLD activity and increased PAP activity in diabetic heart. Insulin treatment completely normalized SL PLD activity while PAP activity remained high, indicating that dephosphorylation of PLD-derived PtdOH by PAP may also contribute the elevated DAG level (Williams *et al.*, 1998). Although insulin has a positive effect on DAG accumulation, 2-week insulin treatment failed to change the increased DAG content observed in chronic

diabetic heart (Okumura *et al.*, 1991). The different DAG fatty acid profile (Okumura *et al.*, 1991) exhibited by the treated hearts may imply that this is due to insulin's effect on cellular fatty acid metabolism. The exact mechanism involved is still unknown.

Role of Protein Kinase C

Increases in membranous PKC activity in diabetic heart have been observed by several independent investigators (Tanaka *et al.*, 1992; Inoguchi *et al.*, 1992; Giles *et al.*, 1998). Protein levels of cardiac PKCβII have been observed to be preferentially increased in spontaneous diabetes-prone diabetic BB rats as well as STZ-treated Sprague-Dawley rats (Inoguchi *et al.*, 1992), however the findings on the subcellular redistribution of PKCα, PKCε, PKCδ are somewhat controversial since these studies have been conducted in different diabetic species and tissue preparations. In diabetes-prone BB/Wor diabetic rats, particulate PKCα and PKCδ in ventricular tissue are increased by 89% and 24%, respectively, whereas no change has been found in both soluble and particulate PKCε and PKCα (Giles *et al.*, 1998). In isolated myocytes from STZ-induced diabetic rats, translocation of PKCε from cytosol to membrane has been observed, whereas PKCδ did not change significantly. The same study also demonstrated that such alterations in PKC redistribution are reversed by either insulin treatment or AT1 receptor blockade (Dupont) (Malhotra *et al.*, 1997). The increase of membranous PKC content could be due to not only a redistribution by translocation, but also an increase in synthesis (Inoguchi *et al.*, 1992; Giles *et al.*, 1998; Malhotra *et al.*, 1997). As many regulatory proteins, such as TnI and TnT, are the targets of PKC (Noland and Kuo, 1991, 1993; Noland *et al.*, 1996), it is conceivable that PKC may be involved in the development of diabetic cardiomyopathy.

Taken together, evidence has shown that phosphoinositide-PLC pathway may be involved in the pathogenesis of diabetic cardiomyopathy; however, possible alterations in functional status as well as protein mass of PLC isoforms under this pathological condition remain to be clarified.

III. MATERIALS AND METHODS

1. MATERIALS

[γ-^{32}P]ATP (specific activity of 10 Ci/mmole) and [^{3}H]-PtdIns(4,5)P_2 [Inòsitol-2-^{3}H(N)]-(5.45 Ci/mmole) were purchased from DuPont Canada Inc./New England Nuclear (Mississauga, ON, Canada). Nonlabelled PtdIns(4,5)P_2, triammonium salt was obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). D-myo-inositol 1,4,5-trisphosphate [^{3}H]-assay kit was from Amersham International (Amersham, U.K.). L-α-phosphatidic acid was from Sigma Chemical Company (St. Louis, MO, USA).

Monoclonal antibody against phospholipase Cγ1 was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Protein G Sepharose 4 fast flow was purchased from Pharmacia Biotech (Baie d'Urfe, QB, Canada). Dowex 1X-8 (formate form, 100-200 mesh) and goat anti-mouse IgG were obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). Polyvinylidene difluoride Western blotting hydrophobic membrane and chemiluminescence reagents were from Boehringer Mannheim (Laval, QB, Canada).

Medium-199, trypsin (bovine pancreas), penicillin and streptomycin were obtained from Gibco BRL, Life Technologies Inc., (Grand Island, NY. USA). Collagenase (Type 2) from *Clostridium histoclyticum* was purchased from Worthington Biochemical Corporation (Lakewood, NJ, U.S.A.). Alamethicin, sodium cholate, laminin, unlabelled adenosine trisphosphate and all other reagents of analytical grade or of the highest grade possible were purchased from Sigma Chemical Company (St. Louis, MO, USA). HP-KF

silica gel high performance thin layer (200 μm) chromatography plates were purchased from Whatman International Ltd. (Clifton, NJ, USA).

Humulin (insulin zinc suspension prolonged, human biosynthetic (rDNA only) ULTRALENTE) was obtained from Eli Lilly Canada Inc.(ON., Canada). Kodak X-Omat-R X-ray films and Dupont Cronex intensifying screen were purchased from Picker International (Highland Hts., OH, USA). CytoScint™ES® was obtained from ICN Biomedicals Inc. (Mississauga, ON, Canada). All other reagents were of analytical grade or of the highest grade available.

2. METHODS

2.1. Insulin-dependent Diabetic Model

Male Sprague-Dawley rats weighing approximately 200g were used in this study. Diabetes was induced by a single tail vein injection of STZ (65 mg/kg body weight) dissolved in citrate-buffered vehicle (0.1 M citrate, pH 4.5) (Rakienten *et al.*, 1963). Age-matched controls were injected with citrate buffer alone. All animals were housed in the Animal Holding at St. Boniface General Hospital Reseach Center 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 STZ injection. One group of animals received 3 U Humulin per day for 2 weeks, while the second group was given saline for the same time period. At the end of 8th week, hearts were processed for the isolation of cardiomyocytes and SL and cytosol.

2.2. Isolation of Sarcolemmal Membranes and Cytosolic Fractions

All isolation procedures were carried out at 4°C. Ventricular tissue from 2 to 3

pooled hearts (5 ml buffer/g tissue) was finely minced by hand in 0.6 M sucrose, 10 mM imidazole, pH 7.0, solution. The original solution was aspirated to remove excess blood cells and the pieces were resuspended in an equal volume of the sucrose-imidazole buffer. The pieces were homogenized with a Polytron PT 3000 homogenizer (Kinematica AG, Switzerland) at 13,000 RPM for 6 x 15 seconds. The resulting homogenate was then centrifuged at 12,000 g for 30 min and the pellet was discarded. A 500 µl aliquot was centrifuged at 100,000 g for 60 min in a Beckman TL-100 Ultracentrifuge to remove any membrane fragments. The resulting supernatant was assigned as the cytosolic fraction and was frozen in liquid nitrogen and stored at -70°C until used. The remaining supernatant was diluted with 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 140 mM KCl (5 ml buffer/g tissue) pH 7.4, and centrifuged at 100,000 g for 60 min. The resulting pellet was then resuspended in 140 mM KCl, 20 mM MOPS, 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 in a Beckman swinging bucket rotor (SW-28), the band at the sucrose-buffer interface was taken and diluted with 3 volumes of 140 mM KCl, 20 mM MOPS, pH 7.4. The pellet from this final centrifugation at 100,000 g for 30 min was resuspended in 0.25 M sucrose, 10 mM histidine, pH 7.4 (225 µl/g tissue). This sarcolemmal enriched fraction was divided into aliquots, frozen in liquid nitrogen and stored at -70°C until used.

2.3. Phosphoinositide Specific Phospholipase C Assay

PLC activity was assayed as described previously by Meij and Panagia (1992). Substrate was prepared by mixing an aliquot of [^{3}H-] PtdIns(4,5)P_2, with an aliquot from

the stock solution (in chloroform) of the cold substrate. The mixture was evaporated to dryness under a stream of N_2 and redissolved in 10 % Na-cholate (w/v) (232 mM). The substrate solution was kept under N_2 gas overnight at 0-4°C and was diluted to 160 μM substrate/112 mM Na-cholate shortly before use. An aliquot was taken to determine the specific activity.

The PLC assay mixture contained 30 mM HEPES-Tris (pH 7.0), 100 mM NaCl, 2 mM EGTA, 3.13 mM $CaCl_2$ ([free Ca^{2+}]= 1 mM), 15 μg SL protein, 14 mM Na-cholate and 20 μM [^{3}H-]PtdIns(4,5)P_2 (400-500 dpm/μl) in a final volume of 40 μl in the absence or presence of 30 μM PtdOH. The samples were incubated at 37°C for 2.5 min and the reaction was terminated by the addition of 144 μl ice-cold chloroform: methanol: HCl (1: 2: 0.2 v/v) to each sample. Free calcium concentrations were determined with the computer program developed by Fabiato (1988). Blanks were carried out under identical conditions except that SL membranes were added after addition of the stopping mix. Phases were separated by adding 48 μl of 2 M KCl and 48 μl chloroform (Jackowski *et al.*, 1986). After mixing for 30 sec and 5 min centrifugation at 15,000 g (Hereaus Sepatech Contifuge 28 RS) the upper phase was aspirated and applied to 400 μl column of Dowex 1X8 (formate form, 100-200 mesh). The columns were rinsed with 0.75 ml of water, followed by the selective elution of inositol phosphates (Berridge *et al.*, 1985) in gradient steps consisting of 1 ml of 5 mM sodium tetraborate in 30 mM sodium formate (to elute Ins), 0.2 M ammonium formate in 0.1 M formic acid (Ins1*P*), 0.4 M ammonium formate in 0.1 M formic acid (Ins(1,4)P_2), and finally 1 M ammonium formate in 0.1 M formic acid (Ins(1,4,5)P_3). The radioactivity in each elutant was quantitated by liquid scintillation

(Beckman LS 1701) in 10 volumes of CytoScint™ES®.

2.4 Assay of Phosphoinositide-PLCγ Activity

Myocyte membrane protein was extracted as described by Ju *et al* (1998), using buffer containing 1% w/v Na-cholate, 50 mM HEPES (pH 7.2), 200 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml soya bean trypsin inhibitor and 10 μg/ml leupeptin and rotated for 2 hrs at 4°C. The sample was centrifuged (280,000 g for 25 min) and the supernatant recovered as the solubilized membrane fraction. This membrane extract was incubated overnight at 4°C with monoclonal antibody to $PLC\gamma_1$ (5 μg of antibodies to 350 μg membrane extract). The immunocomplex was captured with 100 μl (50 μl packed beads) of washed (3 times with 30 mM HEPES, pH 6.8) Protein G Sepharose at 4°C by rotation for 2 hrs. The agarose beads were collected by pulsing (5 seconds) at 10,000 g washed once with HEPES buffer and used for the determination of $PLC\gamma_1$ activity. For control experiments, immunoprecipitation and subsequent activity measurements were conducted after incubation of the membrane extract with non-immune mouse IgG.

The hydrolysis of [^{3}H]PtdIns(4,5)P_2 isoforms was measured by a modified procedure (Wahl *et al.*, 1992; Ju *et al.*, 1998). Briefly, the reaction was performed in the presence of 30 mM HEPES (pH 6.8), 70 mM KCl, 0.8 mM EGTA, 0.8 mM $CaCl_2$, and 20 μM [^{3}H]-PtdIns(4,5)P_2 dissolved in 14 mM Na-cholate overnight and an aliquot (10 μl) of immunoprecipitate suspension. The reaction was carried out at 37°C for 2.5 min in the absence or presence of 30 μM PtdOH, and was stopped by the addition of 100 μl of 1% w/v BSA followed by 250 μl of 10% w/v TCA. Precipitates were removed by

centrifugation (10,000 g for 5 min) and the supernatant collected for quantification of inositol phosphates by liquid scintillation.

2.5 Immunoblotting

The proteins present in sarcolemmal membranes and in the cytosolic fraction were separated by SDS-polyacrylamide gel electrophoresis (10% gels). The proteins were then transferred electrophoretically onto microporous polyvinylidene difluoride Western blotting hydrophobic membranes. Briefly, the membranes were pre-wetted prior to protein transfer by placing in methanol for a few seconds and then soaked with transfer buffer (20% v/v methanol, 0.192 M glycine and 25 mM Tris, pH 8.0). The transfer was performed at 100 V for 1 hr using a mini trans-blot cell. After blocking with 5% w/v milk solution, the polyvinylidene difluoride membrane was incubated with 10 ml Tris-buffered saline-Tween 20 (TBST) containing 5μg monoclonal antibody to PLCγ1, for 1 hr at room temperature with agitation. After washing with TBS-T for 1 hr (6 x 10 min washes), the polyvinylidene difluoride membrane was incubated with goat anti-mouse IgG linked to horseradish peroxidase; a 1:3000 dilution was used. After further washing with TBS-T for 1 hr (6 x 10 min washes), the PLC isoform was detected by BM chemiluminescence according to the manufacturers' instructions.

2.6 Isolation of Cardiomyocytes and Culture

The methodology for isolating adult rat cardiomyocytes was similar to that described by Piper *et al* (1988). 8-Week sham, diabetic, insulin-treated diabetic rats, were sacrificed by decapitation and the hearts excised, the atria removed, and mounted on the Lagendorff apparatus. The heart was initially perfused with calcium-free Krebs solution

containing (mM) 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, and 11 glucose (pH 7.4), and gassed with a mixture of 95% O_2 and 5% CO_2. After 10 min of perfusion, the perfusate was switched to 0.1% collagenase solution containing 0.1% BSA and 25 μM $CaCl_2$. After a 60 min recirculation period, the heart was removed from the canula and placed in pre-warmed Krebs solution containing 1% BSA and 25 μM $CaCl_2$ in a sterile Petri dish. Cells were liberated after gentle pipetting of the tissue. The cell suspension was collected by centrifugation at 6.8 g for 2 min, the supernatant was removed, the cells resuspended in pre-warmed Krebs buffer containing 1% BSA and 50 μM $CaCl_2$ and centrifuged again at 1.7 g for 2 min, the procedure was repeated, resuspending on each occasion in warm Krebs containing 1% BSA and 200 μM of $CaCl_2$, and then 500 μM $CaCl_2$ and finally the cells were resuspended in warm Krebs containing 4% BSA and 1 mM $CaCl_2$ and centrifuged at 6.8 g for 2 min. The cell pellet was then resuspended in medium-199 (M199) containing 0.5% FCS supplemented with 1% penicillin and streptomycin and incubated at 37°C in a 5% CO_2 humidified incubator for 24 hrs.

2.7 Myocyte Stimulation and Fractionation

After 24 hrs, cells were stimulated with 30 μM PtdOH for 10 min. Following termination of the incubation by removal of medium by aspiration and placing Petri dishes immediately on ice, cells were scraped off the plates in 1 ml 10 mM HEPES (pH 7.2) containing 2 mM EDTA and 10% sucrose and collected by centrifugation at 27.2 g for 1 min and processed for the isolation of the cytosol and particulate fractions. Briefly, cells were homogenized in 1 ml of the above mentioned buffer using a glass homogenizer,

followed by centrifugation at 280,000 g for 25 min. The supernatant was assigned as the cytosolic fraction, and the pellet was resuspended and homogenized in the aforementioned buffer and designated as the particulate fraction.

2.8 Determination of Inositol Trisphosphate Content

Ins(1,4,5)P_3 content of the cytosolic fraction (2.5 mg/ml) was measured using the Biotrak radioimmunoassay kit. The procedures involved were according to the manufacturer's instructions, based on the method described by Chilvers *et al* (1991). Unlabeled Ins(1,4,5)P_3 in the samples competes with a fixed amount of [^{3}H]-labeled Ins(1,4,5)P_3 for a limited number of sites on bovine adrenal Ins(1,4,5)P_3 binding protein. Bound is then separated from the free Ins(1,4,5)P_3 by centrifugation. D-myo-inositol 1,4,5-trisphosphate was used as standard.

2.9 PtdIns 4-Kinase and PtdIns4*P* 5-Kinase Assay

PtdIns 4-kinase and PtdIns4*P* 5-kinase activities were assayed as described (Liu *et al.*, 1997). The assay was initiated by preincubating 30 μg SL protein for 30 min at 30°C in 100 μl (final volume) of 40 mM HEPES-Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol and 30 μg alamethicin. The phosphorylation of endogenous PtdIns and PtdIns4*P* was started by the addition of [γ-^{32}P]-ATP in a final concentration of 1 mM (0.16 Ci/mmol). The reaction was terminated after 1 min by adding 2 ml of ice-cold methanol: 13 N HCl (100:1 v/v), and vortexing for 10 seconds. For polyphosphoinositide extraction, 1 ml of 2.5 N HCl and 2 ml of chloroform were added. The tubes were vortexed for 2 min and centrifuged at 1,000 g for 10 min. The aqueous phase was then discarded and the chloroform phase was washed with 2 ml of chloroform: methanol: 0.6 N

HCl (3: 48: 47 v/v/v). After a second vortexing and centrifugation step, the final chloroform phase was removed and an aliquot was evaporated to dryness under a nitrogen stream. The residue was immediately redissoved in 100 µl of chloroform: methanol: water (75: 25: 2 v/v/v) and quantitatively applied under a light nitrogen stream to high performance silica gel thin layer plates that had previously been impregnated with 1% potassium oxalate in methanol: water (2:3 v/v) and activated at 110°C for at least one hour. The test tubes were then washed once with 30 µl of chloroform: methanol: water (75: 25: 2 v/v/v) and this washing was again applied to the plate. The chromatogram was developed at room temperature in a solvent system containing chloroform: acetone: methanol: glacial acetic acid: water (40: 15: 13: 12: 8 v/v), as described by Jolles *et al.*, (1981). After the solvent front had migrated for approximately 1~2 cm from the top, the plates were air dried at room temperature. The ^{32}P-labeled phospholipid spots were visualized by overnight autoradiography using X-Omat-R X-ray films and Dupont Cornex intensifying screen. Phosphoinositide species were identified in accordance with the methods of previous workers (Liu *et al.*, 1997). PtdIns4*P* and PtdIns(4,5)P_2 were scraped from the plates, and the radioactivity associated with each spot was determined by scintillation counting. Blanks were carried out under identical conditions except that the membrane proteins were added after terminating the reaction.

For the determination of PtdIns4*P* 5-kinase, exogenous PtdIns4*P* was added into the assay system. The exogenous PtdIns4*P* was prepared by ultrasonication in a water sonicator (Branson 1200 sonicator) for 30 min and thereafter added to the assay mixture before the preincubation at a final concentration of 25 µM.

2.10 Protein Determination

Membrane and cytosolic proteins were determined according to Lowry *et al* (1951), using bovine serum albumin as a standard.

3. STATISTICS

All of the experiments were carried out by triplicate or quadruplicate determinations, unless otherwise indicated. Results are presented as means ± SEM. Statistical analysis was done using the One-way Analysis of Variance (ANOVA), followed by the Student-Newman-Keuls Multiple Comparisons Test if the variation among column means was significant according to ANOVA. A value of $P < 0.05$ was considered to be significant.

IV. RESULTS

1. Sarcolemmal phosphoinositide-phospholipase C activity in diabetic cardiomyopathy

PLC has been shown to hydrolyze the preferred substrate, PtdIns(4,5)P_2, to yield Ins(1,4,5)P_3 and DAG (Meij and Panagia, 1992). In this study, purified SL membranes obtained from the ventricles of sham, diabetic and insulin-treated diabetic animals were used to assess the status of PLC activity. Table 1 shows the PLC-derived formation of the different inositol phosphates in these three experimental groups. It can be seen that, Ins(1,4,5)P_3, the major product under the assay condition employed, was significantly depressed in the diabetic heart as compared to sham controls. Since no differences in Ins$P$ and InsP_2 were observed between the sham and diabetic groups, a redistribution of inositol phosphate formation can be discounted and therefore the diminished Ins(1,4,5)P_3 formation in diabetes is exclusively due to a depressed PLC activity. Treatment with insulin for 2 weeks completely reversed the depressed activity.

As well as being regulated at the receptor, G protein and substrate levels, certain cardiac PLC isozymes can also be activated by PtdOH, as already stated in the LITERATURE REVIEW. The effect of PtdOH on SL total PLC activity was examined. As shown in Table 2, a significant increase in PtdIns(4,5)P_2 hydrolysis was observed in all three experimental groups in the presence of 30 μM PtdOH. Of note, in terms of absolute activities, no differences in the PtdOH stimulated activity between the groups was observed, suggesting that stimulation occurs to the same level irrespective of the basal

activities. However, from the viewpoint of responsiveness to PtdOH, it can be seen that the diabetic cardiac PLC exhibited a significant hyperresponse (155%) to the stimulation. Two-week insulin treatment normalized the responsiveness to PtdOH in diabetic group.

2. Effect of phosphatidic acid on inositol 1,4,5-trisphosphate content in isolated cardiomyocytes

PtdOH has been shown to increase Ins(1,4,5)P_3 concentration in cardiomyocytes (Kurz *et al.*, 1993). Table 3 shows the effect of PtdOH on Ins(1,4,5)P_3 concentration in isolated cardiomyocytes of sham, diabetic and insulin-treated rats. In non-stimulated cardiomyocytes, a significantly decreased Ins(1,4,5)P_3 level in the diabetic group was found, which was only partially corrected by insulin treatment. Although the diminished levels are in agreement with the total SL PLC measurements *in vitro* (Table 2), a difference in the effect of insulin on PLC-derived Ins(1,4,5)P_3 was observed in cardiomyocytes. PtdOH induced a significant increase in Ins(1,4,5)P_3 formation in all the three groups, 175, 220, 303%, respectively. The Ins(1,4,5)P_3 concentration in diabetic myocytes after PtdOH stimulation was still lower ($P<0.05$) than that in sham and insulin-treated groups. Insulin treatment increased the responsiveness of diabetic myocytes to PtdOH, consequently, the Ins(1,4,5)P_3 concentration after stimulation was ~80% of the sham level.

3. Cytosolic phosphoinositide-phospholipase C activity in diabetic cardiomyopathy

The cytosolic PtdIns(4,5)P_2-PLC activity was also examined (Figure 1). While no significant difference between the sham and diabetic groups was observed, of interest was the significant decrease in PLC activity in the insulin-treated diabetic group as compared to sham and diabetic groups.

Table 1. *In vitro* formation of different inositol phosphate species by sarcolemmal phospholipase C in diabetic cardiomyopathy

	Inositol phosphate species (nmol/min/mg protein)		
	Ins(4)*P*	Ins(1,4)P_2	Ins(1,4,5)P_3
Sham	0.02 ± 0.00	1.18 ± 0.04	6.69 ± 0.09
Diabetes	0.02 ± 0.02	0.58 ± 0.04	5.64 ± 0.14 [a]
Insulin-treated	0.02 ± 0.02	0.80 ± 0.05	7.45 ± 0.44 [b]

Sarcolemmal PLC activity was assayed under standard conditions as described in "Materials and Methods" in the presence of 20 μM [^{3}H]-PtdIns(4,5)P_2. Values are means±SEM of inositol phosphate formation in three experiments. Assays were performed in triplicate. Abbreviations: Ins(4)*P*=inositol 4-phosphate, Ins(1,4)P_2=inositol 1,4-bisphosphate, Ins(1,4,5)P_3=inositol 1,4,5-trisphosphate.

[a] Significantly different ($p<0.05$) *vs.* corresponding sham values

[b] Significantly different ($p<0.05$) *vs.* corresponding diabetic values

Table 2. Effect of phosphatidic acid on the total sarcolemmal phospholipase C activity of diabetic hearts

	Total phospholipase C activity (nmol Ins*P*s formed/min/mg protein)		
	Basal	PtdOH (30 μM)	% of Basal
Sham	7.87 ± 0.18	9.69 ± 0.29 [b]	123 ± 4
Diabetes	6.27 ± 0.22 [a]	9.69 ± 0.94 [b]	155 ± 10 [a]
Insulin-treated	8.27 ± 0.70 [c]	10.92 ± 0.89 [b]	132 ± 4 [c]

Sarcolemmal PLC activity was assayed under standard conditions as described in "Materials and Methods" in the absence or presence of 30 μM PtdOH. Values are means±SEM of three experiments done in triplicate. Abbreviations: PtdOH=phosphatidic acid; Ins*P*s=total inositol phosphates (inositol 4-phosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate).

[a] Significantly different ($p<0.05$) *vs.* corresponding sham values

[b] Significantly different ($p<0.05$) *vs.* corresponding basal values

[c] Significantly different ($p<0.05$) *vs.* corresponding diabetic values

Table 3. Stimulation of Ins(1,4,5)P_3 formation by phosphatidic acid in isolated cardiomyocytes

	Ins(1,4,5)P_3 formation (pmol/mg protein)		
	Basal	PtdOH (30 μM)	% of Basal
Sham	6.53 ± 0.13	11.44 ± 0.04 [b]	176 ± 6
Diabetes	1.91 ± 0.01 [a]	4.21 ± 0.08 [b, a]	221 ± 8 [a]
Insulin-treated	3.00 ± 0.08 [a, c]	9.08 ± 0.05 [b, a, c]	331 ± 19 [a, c]

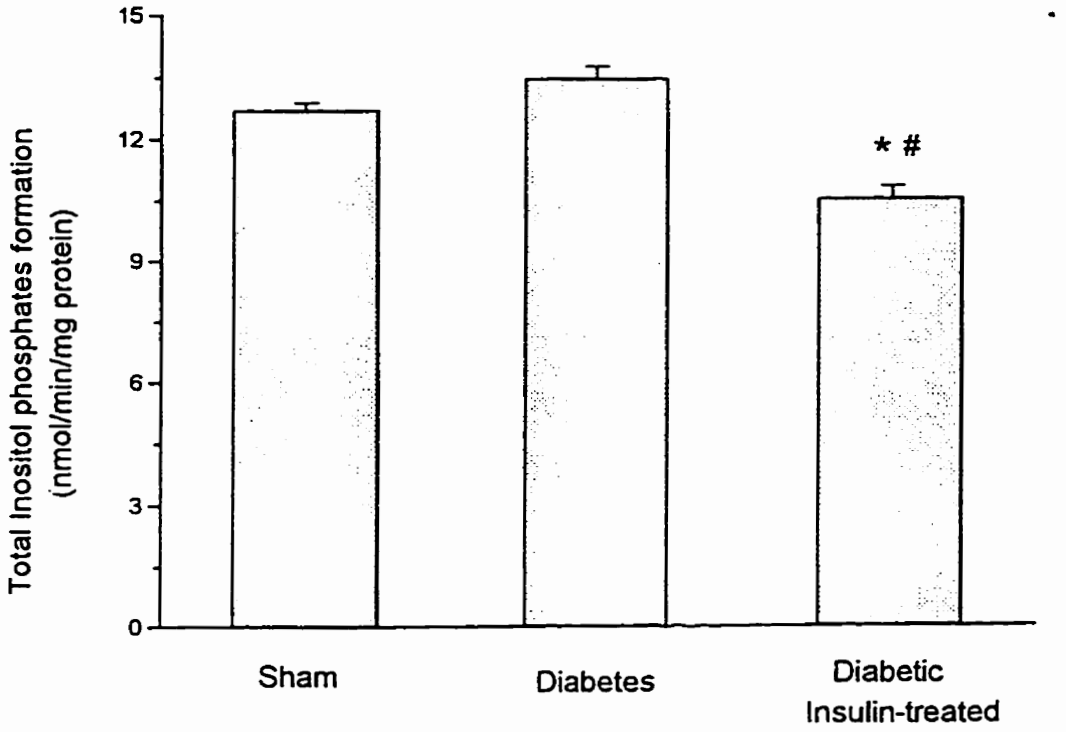
Cytosolic Ins(1,4,5)P_3 was assayed under standard conditions as described in "Materials and Methods", in the absence or presence of 30 μM PtdOH. Values are means ±SEM of three experiments done in triplicate. Abbreviations: Ins(1,4,5)P_3=Inositol 1,4,5-trisphosphate, PtdOH= phosphatidic acid.

[a] Significantly different ($p<0.05$) *vs.* corresponding sham values

[b] Significantly different ($p<0.05$) *vs.* corresponding basal values

[c] Significantly different ($p<0.05$) *vs.* corresponding diabetic values

Figure 1. Cytosolic phospholipase C activity in diabetic cardiomyopathy



Cytosolic PLC activity was assayed under standard conditions as described in "Materials and Methods". Values are means±SEM of three experiments done in triplicate.

* Significantly different ($p<0.05$) *vs.* sham values

Significantly different ($p<0.05$) *vs.* diabetic values

4. Sarcolemmal phosphoinositide-phospholipase Cγ1 activity in diabetic cardiomyopathy

Four PLC isoforms (PLCγ1, PLCδ1, PLCβ3, PLCβ1) have been demonstrated in the heart (Hasen *et al.*, 1995; Ju *et al.*, 1998; Wolf, 1993). All of them are capable of hydrolyzing PtdIns(4,5)P_2 under *in vitro* conditions (James *et al.*, 1997). The most abundant types are PLCγ1 and PLCδ1 (Wolf, 1993). PLCγ1 has been recently suggested to be a downstream effector of the Ang II-mediated signaling pathway in cardiomyocytes and abnormalities have been reported in the cardiac RAS in diabetic cardiomyopathy (see LITERATURE REVIEW). Therefore, PLCγ1 activity was assayed by immunoprecipitating this isozyme with a specific monoclonal antibody. It can be seen that PLCγ1 activity was significantly depressed in the diabetic group as compared to sham controls, and was completely restored following insulin treatment (Table 4). As in the case of the total PLC activity, the diabetic cardiac PLCγ1 exhibited a hyperresponsiveness to PtdOH stimulation, which upon insulin treatment was completely normalized.

5. Protein content of sarcolemmal and cytosolic PLCγ1

The relative protein contents of PLCγ1 in SL and cytosol were analyzed by Western blot. As shown in Figure 2, a significant increase in SL PLCγ1 content in the diabetic heart, coincident with a significant decrease in the cytosolic PLCγ1 content was observed. Insulin treatment completely normalized the PLCγ1 mass in the sarcolemma, whereas in cytosol, no significant effect of insulin was observed. In other words, the cytosolic PLCγ1 content in both diabetic and insulin-treated diabetic groups was similar.

Table 4. Effect of phosphatidic acid on sarcolemmal phospholipase Cγ1 activity in diabetic cardiomyopathy

	Phospholipase Cγ1 activity (pmol Ins*P*s formed/min/mg protein)		
	Basal	PtdOH (30 μM)	% of Basal
Sham	3.30 ± 0.21	5.65 ± 0.69 [b]	173 ± 22
Diabetes	2.23 ± 0.07 [a]	8.56 ± 0.41 [b, a]	387 ± 29 [a]
Insulin-treated	3.35 ± 0.14	7.37 ± 0.62 [b]	222 ± 23 [c]

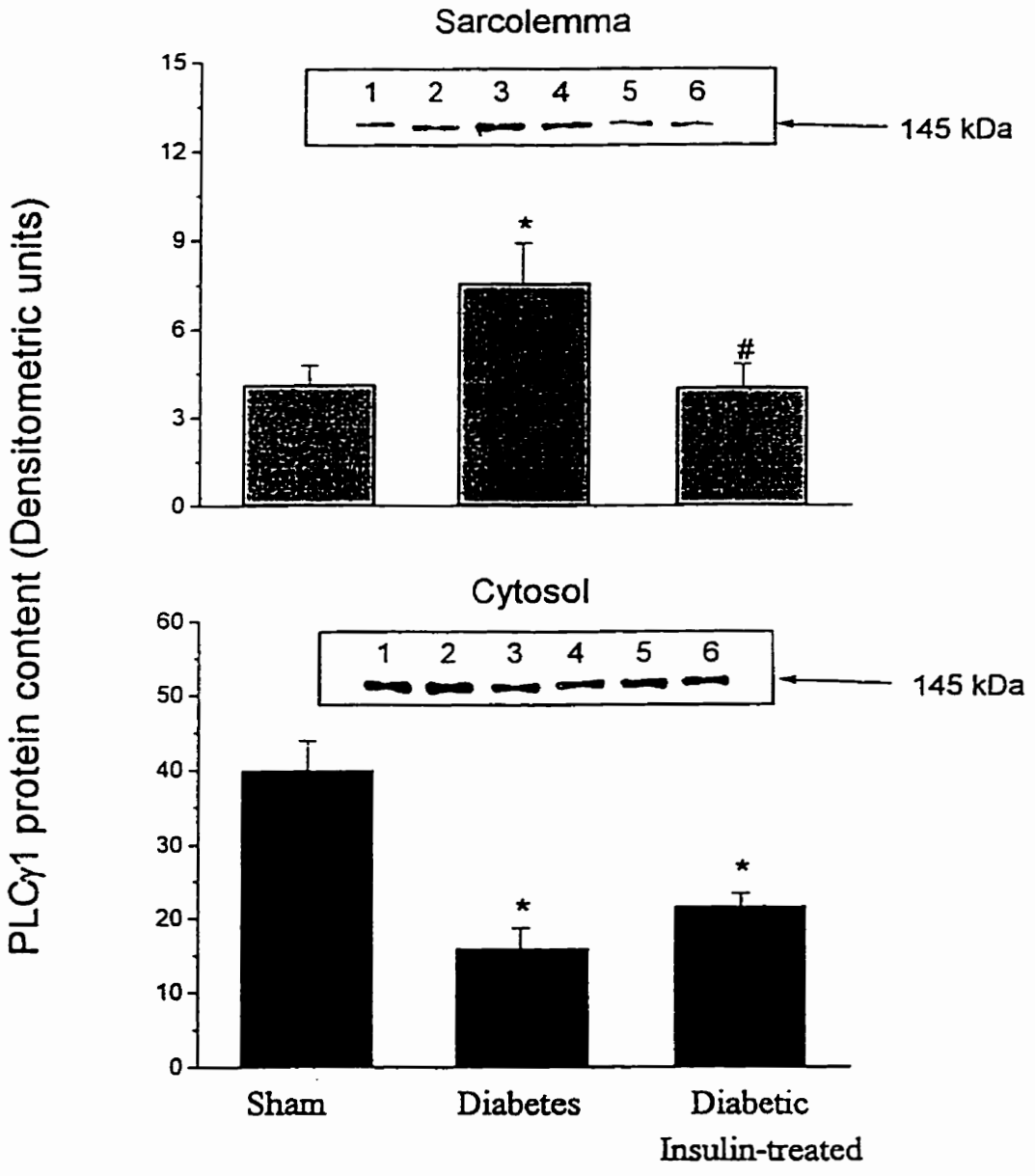
Sarcolemmal PLC activity was assayed under standard conditions as described in "Materials and Methods" in the absence or presence of 30 μM PtdOH. Values are means±SEM of three to four experiments performed in triplicate. Abbreviations: PtdOH= phosphatidic acid; Ins*P*s=total inositol phosphates.

[a] Significantly different ($p<0.05$) *vs.* corresponding sham values

[b] Significantly different ($p<0.05$) *vs.* corresponding basal values

[c] Significantly different ($p<0.05$) *vs.* corresponding diabetic values

Figure 2. Western blot analysis of the relative protein content of phospholipase Cγ1 in the sarcolemmal and cytosolic fractions isolated from hearts of sham, diabetic and insulin-treated diabetic rats



Values are means ± SEM of 4~6 samples

Lanes 1 and 2 represent sham group, lanes 3 and 4 represent diabetic group, and lanes 5 and 6 represent insulin-treated diabetic group.

* Significantly different ($p<0.05$) *vs.* sham values

Significantly different ($p<0.05$) *vs.* diabetic values

6. Sarcolemmal PtdIns 4-kinase and PtdIns4*P* 5-kinase activities in diabetic cardiomyopathy

Besides its stimulatory effect on the PtdIns(4,5)P_2 hydrolysis, we also test PtdOH's effect on phosphoinositide synthesis.

Both phosphoinositide kinases activities have been reported to be mainly located in the SL (Quist *et al.*, 1989). In this study, SL PtdIns 4-kinase and PtdIns4*P* 5-kinase activities were determined in the sarcolemma of sham, diabetic and insulin-treated rats. In Table 5, it can be seen that a significant depression ($P<0.05$) of SL PtdIns 4-kinase activity was observed in the diabetic hearts which was partially corrected by insulin treatment. PtdOH had no effect on kinase activity

A significant reduction of the SL PtdIns4*P* 5-kinase activity which was completely corrected by insulin treatment was also observed. Although PtdOH has been reported to stimulate bovine brain PtdIns4*P* 5-kinase activity *in vitro* (Moritz *et al.*, 1992), no effect of PtdOH on the cardiac kinase activity was observed (Table 6).

Table 5. Effect of phosphatidic acid on sarcolemmal phosphatidylinositol 4-kinase activity in diabetic cardiomyopathy

	PtdIns4*P* formation (pmol/min/mg protein)		
	Basal	PtdOH (30 μM)	% of Basal
Sham	2113 ± 12	2043 ± 131	97 ± 6
Diabetes	1313 ± 67[a]	1258 ± 66	96 ± 7
Insulin-treated	1856 ± 7[a, b]	1895 ± 45	102 ± 2

Sarcolemmal PtdIns 4-kinase activity was assayed under standard conditions as described in "Materials and Methods" in the absence or presence of 30 μM PtdOH. Values are means±SEM of three experiments conducted in triplicate. Abbreviations: PtdIns4*P*= phosphatidylinositol 4-phosphate; PtdOH=phosphatidic acid.

[a] Significantly different ($p<0.05$) *vs.* corresponding sham values

[b] Significantly different ($p<0.05$) *vs.* corresponding diabetic values

Table 6. Effect of phosphatidic acid on sarcolemmal phosphatidylinositol 4-phosphate 5-kinase activity in diabetic cardiomyopathy

	PtdIns(4,5)P_2 formation (pmol/min/mg protein)		
	Basal	PtdOH (30 μM)	% of Basal
Sham	224 ± 19	191 ± 11	87 ± 11
Diabetes	164 ± 10 [a]	152 ± 4	93 ± 4
Insulin-treated	211 ± 10 [b]	186 ± 4	89 ± 6

Sarcolemmal PtdIns4*P* 5-kinase activity was assayed under standard conditions as described in "Materials and Methods" in the absence or presence of 30 μM PtdOH. Values are means±SEM of three experiments done in triplicate. Abbreviations: PtdIns(4,5)P_2= phosphatidylinositol 4,5-bisphosphate; PtdOH=phosphatidic acid.

[a] Significantly different ($p<0.05$) *vs.* corresponding sham values

[b] Significantly different ($p<0.05$) *vs.* corresponding insulin-treated values

V. DISCUSSION

Diabetic cardiomyopathy refers to cardiac abnormalities secondary to diabetes mellitus (Rubler *et al.*, 1972; Andreoli *et al.*, 1997; William *et al.*, 1997). The mechanisms involved are unclear, however, from the evidence presented in the LITERATURE REVIEW, it seems that an impairment of phosphoinositide metabolism may contribute to the development of cardiomyopathy. Since no information is available on the functional status of PLC during insulin-dependent diabetic cardiomyopathy, the present study was therefore undertaken to examine the changes of PLC and its regulation under this condition.

1. Status of cardiac phospholipase C during insulin-dependent diabetes

In this study, a significant decrease in the cardiac total SL phosphoinositide-PLC activity was found in the STZ-induced diabetic rats under the *in vitro* assay conditions employed, as indicated by the lower production of total inositol phosphates (Table 2). It is therefore conceivable that a decreased basal PLC activity *in vivo* in diabetic cardiomyopathy may also exist. In support of this contention, additional experiments conducted on isolated cardiomyocytes showed a reduced basal Ins(1,4,5)P_3 concentration in diabetic rats (Table 3). In order to ascertain the relative contribution to the depressed total SL PLC activity by PLC isozymes, the status of one of the major PLC isozymes in the heart, PLCγ1 (Hasen *et al*, 1995, Wolf, 1993), was examined. Like the total SL PLC, PLCγ1 activity was found to be decreased in the diabetic heart and completely normalized by 2-week insulin treatment (Table 4).

As mentioned previously, the regulation of phosphoinositide-PLC activities occurs

at several levels, including receptor level, G protein level, substrate level and membrane physico-chemical properties (James *et al.*, 1997; Singer *et al.*, 1997; Rhee and Bae, 1997). In this regard, a number of possible mechanisms can be put forward to explain the depression in PLC activities observed in this model: **1)** Decreased PtdOH level. PtdOH has been shown to stimulate the two major cardiac PLC isoforms, PLCγ1 and PLCδ1, *in vitro* (Henry *et al.*, 1995). Since there is a 60% decrease of SL PLD-derived PtdOH level in the diabetic heart (Williams *et al.*, 1998), it is possible that the depressed PLC activity *in vivo* be partially due to the decreased PtdOH stimulation. **2)** Increased cellular DAG content, as DAG has been shown to destabilize the membrane and cause structural transition (Das and Rand, 1984, 1986), it has been suggested that it may have an inhibitory effect on PLC activity (James et al., 1996). **3)** A reduction in phosphoinositide turnover *in vivo*. The sequential phosphorylation of PtdIns on the 4th and 5th positions of inositol ring by PtdIns 4-kinase and PtdIns4*P* 5-kinase gives rise to the PLC substrate, PtdIns(4,5)P_2 (Quist *et al.*, 1989). Local substrate availability determines the hydrolytic activity of PLC (Tobin *et al.*, 1996). Although no change of free myoinositol levels has been reported in STZ-induced diabetic heart (Cameron *et al.*, 1989), reduced incorporation of radio-labeled myoinositol into phosphoinositides has been observed (Bergh *et al.*, 1988). Besides this, SL phosphoinositide kinases activities are significantly lower in insulin-dependent diabetic cardiomyopathy (Liu *et al.*, 1997), which was also confirmed by the present study. Although the membrane level of PtdIns(4,5)P_2 was not determined in this study, it is possible that a decreased synthesis of the substrate may partially contribute to a decreased basal PLC activity *in vivo*. **4)** Substrate shift from PtdIns(4,5)P_2 to PtdIns4*P* during

diabetes can result in the decreased SL PtdIns(4,5)P_2-PLC activity as PtdIns(4,5)P_2 was used as the substrate under our *in vitro* assay condition. 5) Structural modification on PLC enzymes, as indicated by decreased basal SL PLCγ1 activity and increased SL PLCγ1 protein amount (Table 4 and Figure 2). 6) Increased oxidative stress in diabetic cardiomyopathy (Kaul *et al.*, 1995, 1996; Mak *et al.*, 1996; Meij *et al.*, 1994).

It should be pointed out that STZ-treated rats are associated with hypothyroidism (Tomlinson *et al.*, 1992), which has been shown to increase SL PLC activity when assayed under physiological Ca^{2+} concentrations (Panagia and Mesaeli, 1995). However, in contradiction to this, a decrease in membrane-bound PLC activity has been reported by Jakab (1993). Despite this, it is clear that cardiac SL PLC activity is also related to the thyroid status. Although in the current study the depressed SL total PLC activity was completely normalized by 2-week insulin treatment, further experiments are needed to examine whether the reduced PLC activity is due to insulin deficiency or hypothyroidism.

In contrast to the findings of the SL PLC activities, *in vitro* measurements did not show significant change of cytosolic PLC activities in the diabetic heart (Figure 1). These differential changes are indicative of specific regulatory mechanisms that may influence the enzyme activity only when it is associated to the membrane. Interestingly, insulin treatment for 2 weeks resulted in a significant decrease in cytosolic PLC activity. It is possible that this reflects the effect of insulin on the regulation of PLC substrate specificity (Meij and Panagia, 1992). Furthermore, the uncorrected decrease of cytosolic PLCγ1 protein content (lower than sham values) in insulin-treated diabetic group also indicates that changes of this isoform may contribute to the decreased cytosolic PtdIns(4,5)P_2-PLC activity *in vitro*.

Taken together, it is clear that PLC is closely related to the cardiomyopathy due to insulin deficiency.

2. Effect of phosphatidic acid on phosphoinositide-phospholipase C pathway during insulin-dependent diabetes

In cardiac tissue, Panagia *et al* (1991) have characterized the PtdCho-derived formation of PtdOH by the action of PLD in SL membrane systems. PtdOH has been shown to exhibit several important effects on the heart (reviewed by Dhalla *et al.*, 1997), such as elevating $[Ca^{2+}]_i$ as a consequence of increasing both transsarcolemma Ca^{2+} influx and Ca^{2+} release from the intracellular SR stores, increasing cardiac contractility (Xu *et al.*, 1996b), inducing phosphorylation of a 14 kD cell surface protein and enhancing protein and RNA synthesis (Xu *et al.*, 1996a).

2.1 Effect of PtdOH on sarcolemmal phospholipase C

As shown by this study, although the basal total SL PLC activity was lower in the diabetic heart, no difference between the three groups was observed after PtdOH treatment (Table 2), indicating increased responsiveness of diabetic heart to PtdOH. This may serve as a compensatory mechanism involved in stimulating the decreased basal PLC activity towards normal *in vivo*. The hyperresponsiveness of diabetic PLC to PtdOH was normalized after 2-week insulin treatment.

PtdOH also stimulated SL PLCγ1 activity (Table 4), which is in agreement with studies conducted on rabbit cardiac PLCγ1 (Henry *et al.*, 1995). The increased SL PLCγ1 responsiveness to PtdOH under diabetic condition may also act to compensate for the decreased PtdOH level. Two factors may account for this hyperresponsiveness: 1) Increased SL protein content as shown by the Western blot (Figure 2); 2) It is speculated

that PtdOH may interact with PLCγ1 and lower the binding constant for PtdIns(4,5)P_2, thus increasing its affinity for the catalytic site (Jones and Carpenter, 1993). Therefore, conformational changes in PLCγ1 may enhance the effect of PtdOH under the diabetic condition.

Also, as the responsiveness of SL PLCγ1 to PtdOH (387% increase in activity) is much higher than that of the total SL PLC (155% increase in activity), it is possible that differential changes of other PLC isoforms (PLCδ1, PLCβ1 and PLCβ3) may occur during insulin-dependent diabetes.

2.2 Effect of phosphatidic acid on phosphoinositide kinases

PtdOH had no effect on either of the cardiac phosphoinositide kinases (Table 5 and 6), indicating that PtdOH does not affect phosphoinositide synthesis in the normal or diabetic heart. It should be pointed out that this effect appears to be tissue specific as PtdOH is able to stimulate the brain PtdIns4*P* 5-kinase (Moritz *et al.*, 1992).

2.3 Effect of phosphatidic acid on inositol 1,4,5-trisphosphate formation in isolated cardiomyocytes

In order to confirm the functional status of SL PLC *in vivo*, the effect of PtdOH on isolated cardiomyocytes was examined. Preincubation with PtdOH resulted in an increase of Ins(1,4,5)P_3 content in the three experimental groups, which is in agreement with the studies conducted in isolated normal cardiomyocytes (Kurz *et al.*, 1993). The net increase of Ins(1,4,5)P_3 (2.30 pmol/mg protein) in the diabetic group after PtdOH stimulation was lower as compared to that of the other two groups (4.5-6 pmol/mg protein) (Table 3). As cellular Ins(1,4,5)P_3 levels reflect the balance of generation and removal, two possibilities may contribute to the decreased Ins(1,4,5)P_3 formation observed in the diabetic heart: 1)

Increased removal by either activation of Ins(1,4,5)P_3 phosphatase and/or increase of Ins(1,4,5)P_3 kinase activity. 2) Decreased generation due to depressed PLC activity, substrate shift from PtdIns(4,5)P_2 to PtdIns4P or PtdIns and decreased SL PtdIns(4,5)P_2 levels. PtdOH was seen to have no effect on the cardiac phosphoinositide kinase activities. Therefore, it is possible that decreased PtdIns(4,5)P_2 availability *in vivo* may partially explain why PtdOH was not able to increase Ins(1,4,5)P_3 content to normal levels in the diabetic myocytes even though *in vitro* studies showed that no differences were observed in stimulated SL PLC activities following PtdOH treatment in the diabetic heart.

3. The significance of altered phosphoinositide-phospholipase C pathway in insulin-dependent diabetes

3.1 Importance of two second messengers during diabetic cardiomyopathy

sn-1,2-Diacylglycerol

The increased DAG levels detected in the diabetic heart may be attributable to several reasons: 1) Reduced degradation due to a decreased DAG lipase activity due to an inhibition by an accumulation of intracellular lipids (Paulson *et al.*, 1991; Farooqui *et al.*, 1989). 2) Increased PAP activity in diabetic heart could cause an increased conversion of PLD hydrolytic product, PtdOH, to DAG, which may also contribute to the observed coincidental decrease in SL PtdOH level (Williams *et al.*, 1998). 3) Increased synthesis from PtdCho-PLC activity (Baldini *et al.*, 1994).

Since DAG can sensitize the insulin receptor tyrosine kinase (Arnold and Newton, 1996a), it is plausible that the increased responsiveness of SL PLC to PtdOH stimulation and consequently increased DAG level may act as a compensatory mechanism in the diabetic heart. Furthermore, as PtdOH has also been shown to inhibit insulin receptor

tyrosine kinase activity (Arnold and Newton, 1996b), decreased PtdOH in diabetes may work in concert with increased DAG to maintain the cellular response to decreased circulating insulin levels. On the other side, activation of PKC by DAG correlates with the phosphorylation TnI and TnT (Noland and Kuo, 1991; Noland *et al.*, 1996; Jideama *et al.*, 1996), which results in diminished calcium sensitivity of the sarcomere (Dosemeci *et al.*, 1988) and decreased calcium-stimulated myofibrillar MgATPase activity (Venema and Kuo, 1993). Thus the sustained elevation of PKC activity in diabetic heart may contribute to the depressed systolic function observed in cardiomyopathy. Insulin treatment does not lower the increase in DAG content in chronic diabetes (Okumura *et al.*, 1991), but has been shown to prevent the increased TnI phophorylation (Malhotra *et al.*, 1997). In addition, analysis of DAG species in these three experimental groups has shown different fatty acid composition profiles (Okumura *et al.*, 1991). Therefore more definitive approaches such as the identification of the targets of different DAG molecular species are needed to understand the signaling function of DAG species derived from different sources and DAG's effect on the development of diabetic cardiomyopathy.

Inositol 1,4,5-trisphosphate

The data obtained in the present study shows a significant decrease in Ins(1,4,5)P_3 levels in isolated diabetic cardiomyocytes which may be due to increased removal by phosphorylation/dephosphorylation processes and/or reduced generation as a result of decreased PtdIns(4,5)P_2-PLC activity.

Since the effect of hypothyroidism on the status of the phosphoinositide-PLC pathway of the heart is controversial (Jakab *et al.*, 1993; Panagia and Mesaeli, 1995), the

possibility that correction of the thyroid status in STZ-treated rats would normalize the decrease in PLC catalytic rate warrants further investigation. However, as 2-week insulin treatment of 6-week diabetic rats could normalize the plasma thyroxine level (Williams *et al.*, 1998), but not the Ins(1,4,5)P_3 content of diabetic cardiomyocytes (this study), it is conceivable that altered Ins(1,4,5)P_3 metabolism may be associated with the development of diabetic cardiomyopathy.

No evidence has shown that Ins(1,4,5)P_3 is involved in the cardiac beat-to-beat Ca^{2+} regulation, and more studies are needed to clarify its function in health and heart diseases. Since the tissue Ins(1,4,5)P_3 content is relatively high and PLCδ1 has a high affinity for Ins(1,4,5)P_3 (Lemmon *et al.*, 1995; Garcia *et al.*, 1995; Ferguson *et al.*, 1995), it has been proposed that cardiac PLCδ1 may be persistently inhibited under physiological conditions (Woodcock, 1997). Therefore, it is reasonable to assume that there is a decreased inhibition on PLCδ1 by Ins(1,4,5)P_3 in the diabetic heart, which may have some pathological relevance.

3.2 Importance of PtdIns(4,5)P_2 during diabetic cardiomyopathy

PtdIns(4,5)P_2 is not only the substrate for PLCs, but also the precursor for the putative second messenger PtdIns(3,4,5)P_3, and its exact role in the heart is still unknown. However, data on non-myocardial cells may suggest some functions. Besides limiting the substrate availability for PLCs, a decreased PtdIns(4,5)P_2 level in the diabetic heart itself may have some profound effect as it is able to provide a membrane docking site for proteins containing PH domains (reviewed by Lee and Rhee, 1995; James *et al.*, 1997), and is thought to play a key role in regulating cellular processes including actin assembly,

intracellular trafficking of vesicles, ARF-PLD activity and secretary mechanisms (reviewed by Lee and Rhee, 1995; James *et al.*, 1997). Specific experiments in the heart are required to elucidate its effect on the development of cardiomyopathy.

3.3 Importance of PLCγ1 in the development of diabetic cardiomyopathy

Although the depressed SL PLCγ1 activity, its hyperresponsiveness to PtdOH stimulation and its increased SL protein amount in the diabetic heart were completely corrected by insulin treatment, it should be noted that insulin had no effect on the observed decrease in cytosolic PLCγ1 protein content.

PLCγ1 has been shown to be closely related to cell differentiation (Bertagnolo *et al.*, 1997), maintenance of responsiveness of neurons (Grimes *et al.*, 1996), and embryonic growth and development (Ji *et al.*, 1997). Although the exact role of cardiac PLCγ1 is still unclear, function of PLCγ1 may not be substituted by other PLC isozymes, as translocation of PLCγ1 to cytoskeleton occurs following stimulation of receptors with or associated with tyrosine kinase activity (Singer *et al.*, 1997; James *et al.*, 1997), which has not been reported for PLCβs and PLCδ1.

Evidence has shown that activation of the AT1 receptor, a G protein-coupled receptor without intrinsic tyrosine kinase activity, induces Ca^{2+}-independent PLCγ1 phosphorylation and subsequent Ins(1,4,5)P_3 production in smooth muscle cells (Marrero *et al.*, 1994). Anti-*src* treatment partially prevents Ang II-stimulated PLCγ1 phosphorylation (Schelling *et al.*, 1997). On the other hand, studies conducted in cultured myocytes suggests that Fyn, a member of the *src* kinase family, may mediate the Ang II-induced activation of $p21^{ras}$ (Sadoshima *et al.*, 1996). It is reasonable to deduce that

PLCγ1 may also be activated by *src* kinase during Ang II stimulation in cardiomyocytes.

Under diabetic conditions, plasma Ang II levels are comparable with those of the control group (Makarious *et al.*, 1993; Drury *et al.*, 1984), but no information on cardiac renin, angiotensinogen, and Ang II levels during diabetes is available. Increased AT1 receptor density associated with an increase in AT1 receptor mRNA levels independent of changes in the circulating RAS has been observed in the diabetic heart (Sechi *et al.*, 1994), which may account for an increased AT1 receptor stimulation, as proved by prevention of both the translocation of PKCε from cytosol to membrane and the increase of TnI phosphorylation by AT1 blockade (Malhotra *et al.*, 1997) and beneficial effect of ACE inhibitor on cardiac function of the diabetic heart (Given *et al.*, 1994). It is possible that the increased Ang II stimulation might cause redistribution of PLCγ1 from cytosol to SL to compensate for the decreased basal activity under diabetic condition. However, further investigation on the response of PLCγ1 *in vivo* secondary to inhibition of cardiac RAS during diabetes is required to establish the effect of Ang II stimulation on cardiac PLCγ1. As PLCγ1 activation also occurs following activation of receptors with intrinsic or associated with tyrosine kinase activity, identification of changes in these pathways in the diabetic heart would also help to further understand the exact role that PLCγ1 will play during the development and pathogenesis of diabetic cardiomyopathy (review by Rhee and Bae, 1997).

VI. CONCLUSION

1. The significantly depressed SL total PLC activity *in vitro* and decreased basal Ins(1,4,5)P_3 content in the isolated cardiomyocytes indicate that a decreased basal PLC activity may exist in the diabetic heart *in vivo*.

2. Hyperresponsiveness of the SL PLC to PtdOH stimulation may act as a compensatory mechanism to maintain DAG levels and sensitize the insulin receptor to a reduced circulating insulin concentration during IDDM.

3. Depressed SL PLCγ1 activity and hyperresponsiveness of this isoform to PtdOH in the diabetic heart confirm that changes in these parameters associated with this isoform contribute to the observed alteration of total SL PLC. The decreased activity and coincidental increase of SL PLCγ1 protein content also implies that an abnormal SL PLCγ1 molecule may exist during diabetes.

4. Two-week insulin treatment not only normalized the depressed SL PLC activity and hyperresponsiveness to PtdOH stimulation, but also normalized the increased SL PLCγ1 protein content observed in the diabetic heart. However, it did not fully correct the abnormalities in Ins(1,4,5)P_3 metabolism and the decreased cytosolic PLCγ1 protein amount, indicating that either some irreversible changes have occurred secondary to insulin deficiency or longer treatment is required for recovery.

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IMAGE EVALUATION
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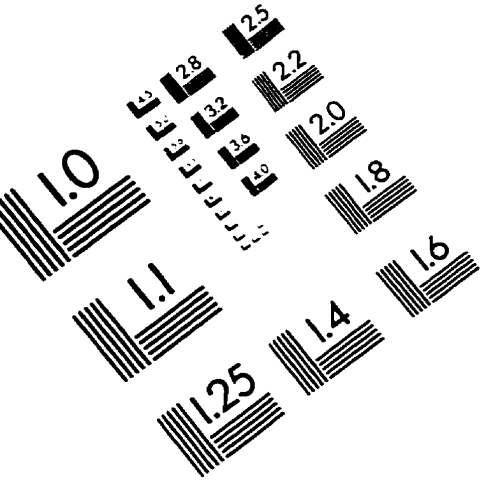

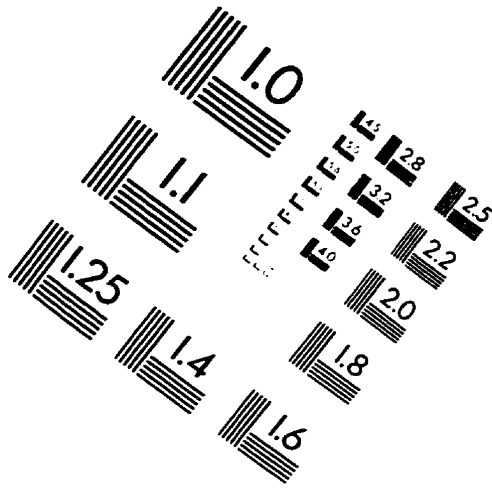

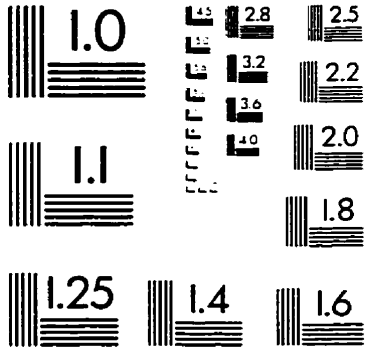

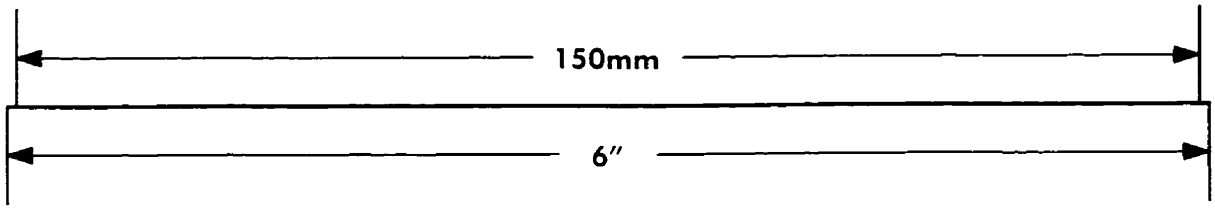

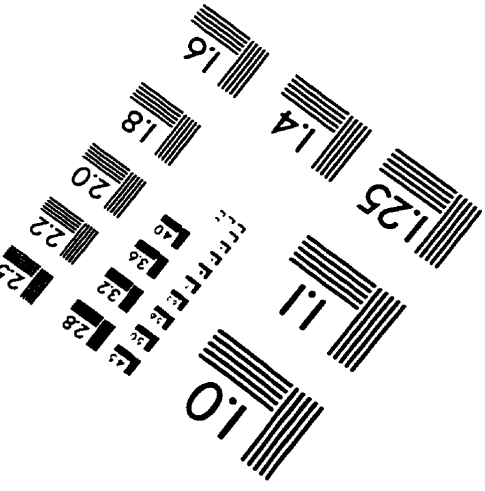

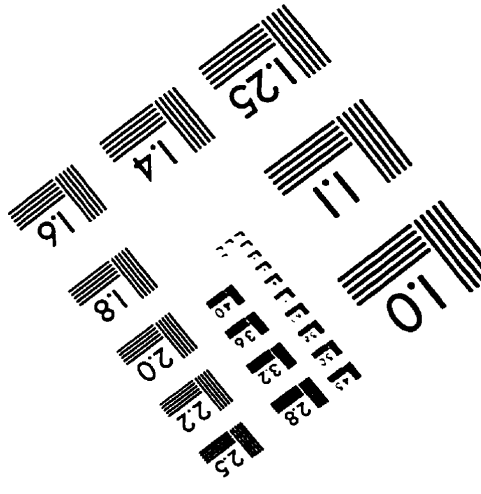

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