UNIVERSITY
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Function Of Phospholipase C Beta One In Diabetic Cardiomyopathy

By

NIDHI SAHI

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE
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Department of Physiology
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DEDICATED TO MY FAMILY

ACKNOWLEDGEMENTS

On an early November morning in 1998 I found myself in a car packed full of meager student belongings heading East from Edmonton. I was off to Winnipeg to pursue a degree of Master's of Science. My family was in Edmonton and most of my friends were scattered farther East and West of home. I had never before considered Winnipeg for school. Now it is the summer of 2003. I am a few paragraphs away from the completion of my Master of Science thesis, and two years away from graduating from medicine. Winnipeg has been an invaluable experience.

As I think back on the last few years here my thoughts turn constantly to the wonderful people I was fortunate enough to meet. I would like to first thank the members of my lab. Not only did they help guide my research, but they helped to shape me as an individual. I would like to thank Dr. Song-yan Liu, a person of foresight, a teacher of comedic Cantonese, and a source of sincere advice. Secondly, I would like to thank the famous Kaiser, Dr. Cappia. He is a true soccer fanatic who shared genuine enthusiasm for 80's British rock, Bob Marley, and Che Guevara. Pram helped to shape my research project. He taught me the specific techniques required and also was instrumental in data analysis and evaluation. My thesis would not be possible without his guidance. I would also like to thank his wife Sharon for sharing their home and family with me. She is someone I can always count on for support. Both their children, Soni and Sonil, are gems, bubbling with energy, smiles and hugs for me.

The members of my thesis committee are remarkable and unforgettable people. I thank them for their guidance, for their dedication to students, for their encouragement while I am in medical school and especially for their open door policy. From Dr. Chliveris I will remember *illigitamus non corborundum*. I thank Dr. Singal who generously provided careful giudance. His well timed words of advice and encouragement are greatly appreciated. He and his wife made sure I had enough to eat, enough sympathy mixed well with guidance to continue my work well. Dr. Singal is especially dear to me as he is the one who set in motion all the steps that eventually led me to meet my incredible professor.

The words "thank you" are both simple and inadequate to describe my gratitude to the head of my thesis committee and my co- supervisor Dr. Grant Pierce. Dr.

Pierce had the incredible task of taking over in the middle of my project and helping me to see it through. All the while he did this with the utmost patience and care, having promised my first supervisor Dr. Panagia, that he would ensure completion of my thesis. From Dr. Pierce I learned so much. He is an insightful and dedicated teacher and scientist, as well as a person of honor. I also thank his gracious wife Gail Pierce without whom I could not have succeeded the last major hurdle.

Above all my last heartfelt thank you is for Dr. Panagia and his incredible family. Dr. Panagia tragically lost his battle with cancer in 2001. Though at the time of his passing I knew him only for a couple of years it is an enormous and painful loss I have suffered. We have all suffered it- those who were ever fortunate enough to know him, even briefly, understand.

Reflecting back, as I contemplated whether or not to move to Winnipeg one event stands out as the deciding factor. It was a phone call I received from Dr. Vincenzo Panagia, my thesis supervisor. During that phone call we talked about several things; my undergraduate degree, my research experience, interests and hobbies. Yet, somehow, that phone call left me with a feeling that Winnipeg would be the best decision for me. There was something in his voice, a sincere dedication, a love of science and something more- a resonant, echoing wisdom, a clarity and a goodness. After working with Dr. Panagia just a short time, I immediately realized I was in the presence of an incredible person. Every morning he smiled and greeted me with "Good morning Nidhi. How is life?". Through his actions I learned what makes good science and I also learned how to be a better person, to help others unconditionally and to always strive to do what I think is good and right. He talked about art and music, about traveling and especially about his beloved Italy. He made the lab an incredibly uplifting place to work. He also shared his home and family with the entire lab and made me feel like I was the daughter he never had. He was a person for whom accolades meant nothing and the success and happiness of his students was his happiness also. I am honoured to be his last student. Dr. Panagia's values resound within his home. His wife, Mrs. Panagia, is similar to him; caring, affectionate, sincere, and good. They remain beautifully mirrored in each other. She is someone with whom I can share whatever is in my heart. It is always met with her unconditional love and support. I am so fortunate to have her a strong part of my

life. She is my mom away from home. Now that I am finished this thesis I can only hope to leave here with even a small fragment of all that they exemplify.

A master can tell you what he expects of you.
A teacher though, awakens your own expectations.
Patricia Neal

I am especially grateful to my aunt and uncle Ahuja for giving me a wonderful beginning. I also thank E for his encouraging words, unending support and for solving many computer crises. I thank my parents for instilling in me strong values and for always believing the best in me. I dedicate my thesis to you.

These are the teachers in my life. In them I found what is good and true. These are the people who have made this journey so worth the while.

Nidhi Pahi

LIST OF ABBREVIATIONS

A1-adrenoceptor.....α1-AR

Adenosine triphosphate.....ATP

Arachidonic Acid.....AA

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

Guanine nucleotide binding protein.....G- protein

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(1,3,4)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

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

Non-insulin dependent diabetes mellitus.....NIDDM

Phosphatidic Acid.....PtdOH

Phosphatidylcholine.....PtdCho

Phosphatidylethanol.....PtdEtOH

Phosphotidylinositol.....PtdIns

Phosphatidylinositol 3-kinase.....PtdIns 3-kinase

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

Phosphatidylinositol-4-phosphate.....PtdIns4*P*

Phosphatidylinositol-4-phosphate kinase.....PtdIns4*P*-kinase

Phosphatidylinositol-5-phosphate.....PtdIns5*P*

Phosphatidylinositol-4-phosphate 5 kinase.....PtdIns4*P,5*-kinase

Phosphatidylinositol transfer protein.....PITP

Phospholipase A_2.....PLA_2

Phosphalipase C.....PLC

Phosphalipase D.....PLD

Platelet-derived growth factor.....PDGF

Pleckstrin homology.....PH

Protein kinase A.....PKA

Protein kinase C.....PKC

Sarcolemma.....SL

Sarcoplasmic reticulum.....SR

syn 1,2- Diacylglycerol.....DAG

Sodium.....Na^+

Streptozotocin.....STZ

Tris-buffered saline-Tween 20.....TBST

Troponin I.....TnI

Troponin T.....TnT

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ABSTRACT

Diabetes mellitus is a disease characterized by a defect in carbohydrate metabolism and glucose tolerance. This disease can result due to either an insulin deficiency, as in insulin dependent diabetes mellitus (Type I) or due to insulin resistance, as in non-insulin dependent diabetes mellitus (Type II). As the disease progresses, several debilitating complications can result in addition to the metabolic defects. These complications include diabetic nephropathy, diabetic retinopathy, peripheral and central neuropathy as well as diabetic cardiomyopathy. Diabetic cardiomyopathy refers to the cardiac abnormalities secondary to clinical diabetes mellitus and has been demonstrated to exist independent of vascular abnormalities. Thus, it is considered to be an independent entity in this disease state. Characteristics of diabetic cardiomyopathy include impaired ventricular function prior to the development of clinical manifestations of cardiac failure. The diabetic cardiomyopathy is characterized by decreased contractility Ca^{2+} regulation and impaired intracellular. Previous studies have implicated abnormalities in the phospholipase C (PLC) signaling pathway in the development of insulin dependent cardiomyopathy.

In addition to PLC, phosphatidic acid (PtdOH) has also been implicated in the positive regulation of cardiac PLC isoenzyme activities. As well, a decrease in sarcolemmal PLD- derived PtdOH levels and a decrease in PtdOH- induced positive inotropic effects in the diabetic heart have been

demonstrated. Thus, it may be that a decreased stimulation by PtdOH may result in a decrease in PLC activity during diabetic cardiomyopathy.

In this study, the effect of diabetes on PLC and its substrate preference was examined. As well, because PtdOH is an important mediator of several cellular events, its effect on the total sarcolemmal PLC activity was studied. A single intravenous injection of streptozotocin (STZ, 65 mg/ kg body weight) was used to induce insulin dependent diabetes mellitus in male Sprague-Dawley rats. The following parameters were measured in both the diabetic and insulin treated diabetic states: First, we determined the different inositol phosphate species produced by PLC. We observed that in both the diabetic and insulin treated diabetic states, IP_3 seemed to remain the preferred product formed. However, this major product was significantly decreased when compared to the control hearts. Insulin treatment appeared to normalize IP_3 levels to control values. Second, the PtdOH stimulated total PLC activity and PLC β1 isoenzyme activity and protein determination were measured. We found that the basal activity of PtdOH stimulated total PLC was significantly depressed in the diabetic state. Insulin therapy resulted in an over correction of IP_3 levels when compared to control values. Immunofluorescence techniques were used to assess alterations in cardiac specific PLC β1 stimulated by PtdOH as well as the Gqα protein distribution and its expression quantified. Several studies have demonstrated that PtdOH activates PLC γ1 and PLC δ1. Our lab was the first to show the effect of PtdOH on PLC β1 using immunofluorescence. Gqα protein is an important transducer of the α1

adrenoreceptor signal. In the diabetic state Gqα staining appears intensified. Insulin treatment seems to have decreased the Gqα staining similar to control levels. Lastly, the effect of 2 week insulin treatment on PIP_2 was assessed. PIP_2 is synthesized via reactions with phosphatidylinositol (PtdIns) kinases and is the preferred substrate for PLC. Our study found that 14 days of treatment with insulin resulted in a partial correction of both the PtdIns 4 kinase and the PtdIns 4-*P* 5 kinase activities.

Our work has examined the status of the PLC β1 pathway in hearts from rats with insulin dependent diabetes mellitus. We have detected specific lesions in this pathway and conclude that these defects may play an important role in generating aspects of the cardiac contractile dysfunction exhibited by insulin dependent diabetic animals.

The heart has reasons that reason does not understand.

Blaise Pascal

The heart is forever making the head its fool.

François de La Rochefoucauld

I. INTRODUCTION

Diabetes mellitus is a disease that is increasing at alarming rates throughout the world. It is estimated that in Canada over 1 million people suffer from diabetes. It has been further estimated that 35- 45% of all diabetics remain undiagnosed. In the Canadian Aboriginal population, the incidence of diabetes has tripled that of the rest of the population. Health Canada reports that diabetes is ranked as the 7^{th} leading cause of premature death in Canada. According to comparative national health surveys, the prevalence of diabetes is expected to increase as the numbers of people with obesity increase. The Canadian health care system currently shoulders a tremendous financial burden of approximately $9 billion per year. Thus, it is of great importance to elucidate the disease mechanisms and target more effective treatment options.

Associated with diabetes are the severe complications that include entire biological systems. Diabetes is associated with retinal disease, neurological disease, renal dysfunction and cardiovascular disease. Diabetic cardiomyopathy remains a major cause of heart disease in Canada and throughout the world and research has suggested that it may be the primary cause of death among the diabetic population. Diabetic cardiomyopathy seems results from several subcellular defects such as structural abnormalities in the heart muscle, the contractile apparatus, and the cardiac membranous systems.

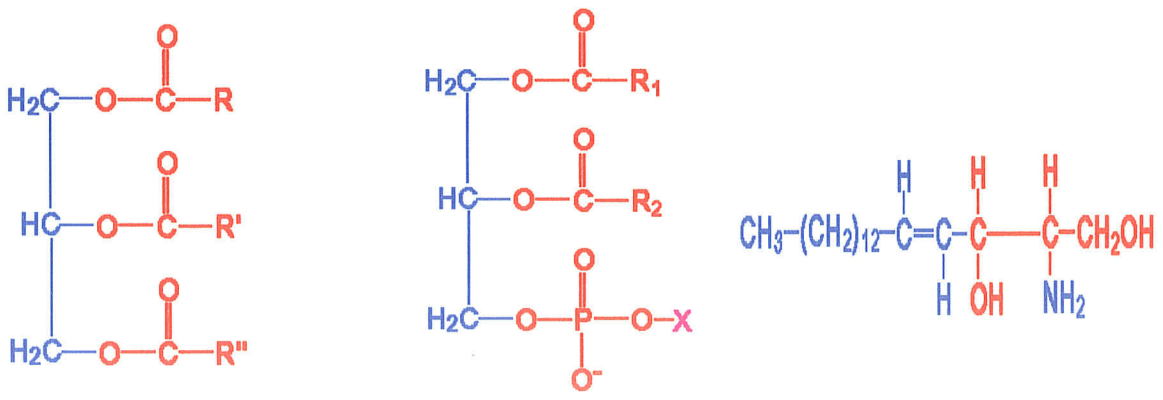
Defects in intracellular signaling and alterations in lipid membranes also have been suggested to have important consequences in terms of disease development. For example, phospholipase C is an important cell signaling

molecule that ultimately results in hydrolysis of phosphatidyl inositol 4, 5-bisphosphate into the lipid signaling molecules inositol 1, 4, 5- trisphosphate and 1, 2- diaglycerol. Inositol 1, 4, 5- trisphosphate induces the release of calcium from the sarcoplasmic reticulum and the second stimulates protein kinase C. Protein kinase C in turn activates several important cellular functions. Previous research has demonstrated that certain isoenzymes of PLC have altered activity and expression in the diabetic state. The purpose of our study was to examine the involvement of PLC β1, a specific PLC isoenzyme in diabetic cardiomyopathy, and to elucidate the mechanisms responsible for any defects in PLC β1 activity. Currently, there is little information available on this isoenzyme. The ultimate goal of our research is to clarify the evolution of diabetic cardiomyopathy with respect to the involvement of this specific intracellular lipid signaling pathway.

II. LITERATURE REVIEW

1. LIPIDS

The word lipid is derived from the Greek word, *lipos* meaning fat. Generally lipids are biological molecules, which are characterized by their relative insolubility in water or aqueous environments and solubility in organic solvents. Lipids compose several different components of biological significance such as fats, oils, vitamins, hormones and the majority of biological membranes. They can be categorized as fatty acids, triglycerides, phosphoglycerides, sphingolipids, and steroids. Examples of these are depicted below in Figure 1.



triglyceride basic phospholipid sphingosine

FIGURE 1. STRUCTURAL FORMULAE OF DIFFERENT LIPID MOLECULES
(www.rpi.edu/dept/MBWeb/Mb1/bcbp/molbiochem/)

Of particular interest in the study of membrane physiology are the phosphoglycerides, otherwise known as phospholipids. Phospholipids consist of a *syn*-glycerol-3-phosphate esterified at the C1 and C2 positions to fatty acids and at its phosphoryl group. They are amphiphilic molecules and therefore have a polar head group and non-polar aliphatic tail as illustrated in Figure 2.

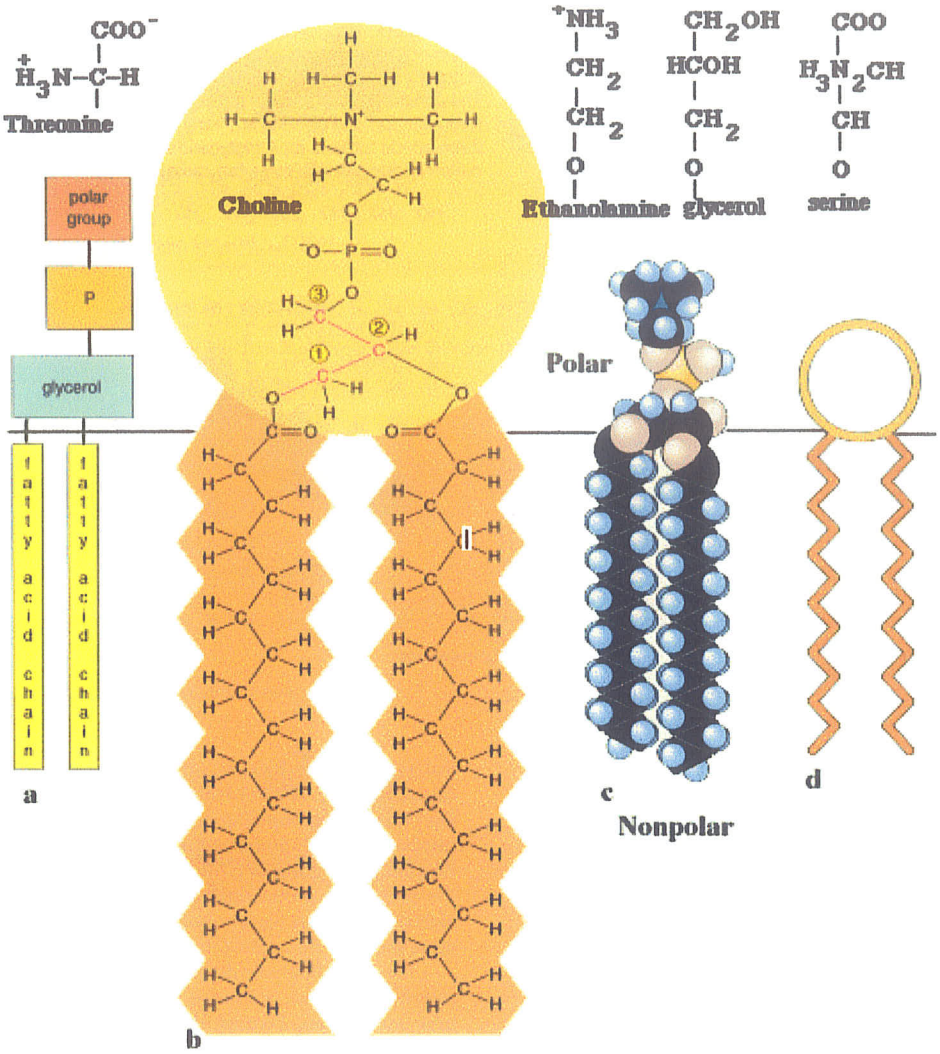


FIGURE 2. A PHOSPHOLIPID SHOWING THE POLAR HEAD REGION AND NON- POLAR TAIL (Wolfe, S.L., 1993)

The circle represents the polar end of the molecule and the zigzag lines the non-polar carbon chains of the fatty acid residues.

(a) The colored block represents the arrangement of subunits in phospholipids.
(b) Represents the formula for phosphatidyl choline, a common membrane phospholipid.
(c) The space-filling model of phosphatidyl choline.
(d) A diagram depicting a phopholipid molecule.

Because of the two hydrocarbon tails of phospholipids and the resulting steric requirements, these molecules tend to arrange themselves into bilayers. The idea of a lipid bilayer was first proposed by E. Gorter and F. Grendel in 1925 (Voet, D. and J. Voet, 1990) who observed that lipids extracted from erythrocytes covered twice the area when spread as a monolayer at the air-water interface than in the plasma membrane of the erythrocyte (the plasma membrane is the only membrane of the erythrocyte). The lipid bilayer serves many functions for the cell:

1. It provides a barrier for the cell to protect the cytoplasm and cellular contents from the external environment.
2. It allows the transport or diffusion of select molecules into the cell.
3. It provides structure and stability for the cell.
4. It serves as a site for second messenger systems, which relay messages from the external environment into the cell.

Other characteristics of the bilayer include its impermeability to most polar and ionic substances and its permeability to non-polar and non-ionic molecules. Perhaps one of the most interesting features of the lipid bilayer is its fluidity; they are a two dimensional arrangement of fluid molecules. Therefore, the phospholipids and other membrane associated substances such as proteins and carbohydrates can move within the membrane to different

locations. Lateral diffusion of molecules occurs with greater speed than transverse diffusion (Figure 3).

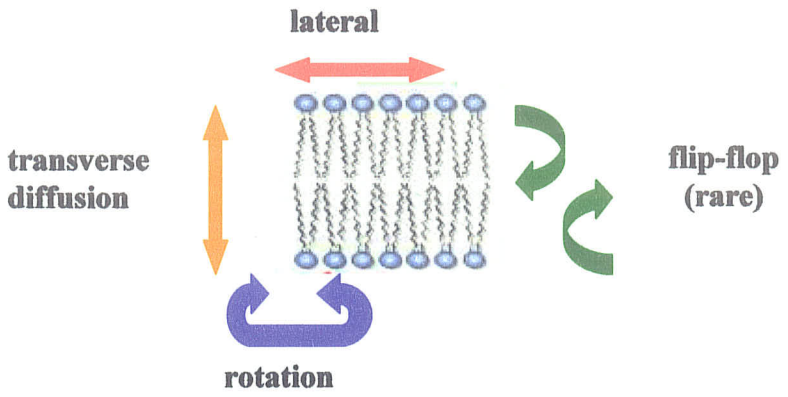


FIGURE 3. TRANSVERSE VERSUS LATERAL DIFFUSION WITHIN THE LIPID BILAYER

In addition, the fluidity of the bilayer is somewhat dependent on temperature. As the temperature cools, the bilayer tends to lose some of its fluidity and become solidified. As the temperature increases, the fluidity returns.

The Fluid Mosaic Model of the membrane structure was proposed by S.J. Singer and G. L. Nicholson in 1972 (Singer, S. J. and G. L. Nicholson, 1972). Their theory postulates that within the two-dimensional lipid float integral proteins which can freely diffuse laterally within the lipid matrix unless they are restricted by other cell components. In addition, both membrane proteins and lipids are asymmetrically distributed (Figure 4).

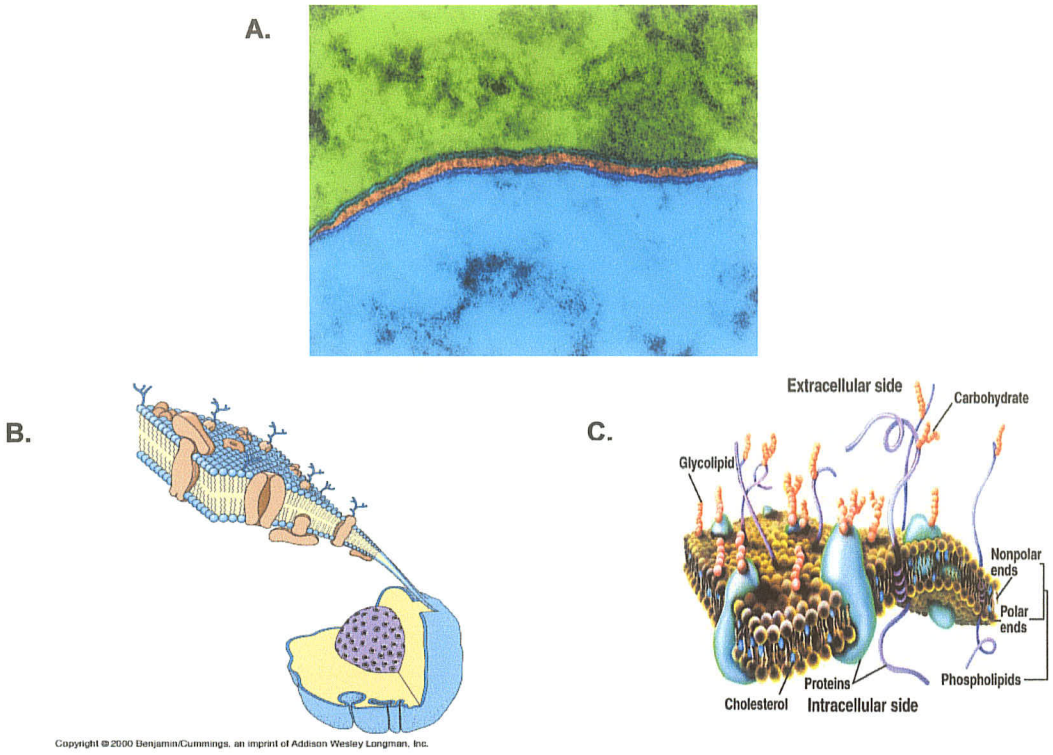


FIGURE 4. THREE DEPICTIONS OF THE PHOSPHOLIPID BILAYER

A. A scanning electron micrograph of a phospholipid bilayer (http://www.DennisKunkel.com).
B. A schematic showing the plasma membrane as it encompasses the cell.
C. A schematic diagram of the plasma membrane with asymmetrically distributed proteins and associated carbohydrate and cholesterol moieties (Fox, S.I., 1999).

Not only are lipids important to the plasma membrane itself but they also play a crucial role as potent intracellular mediators that regulate a variety of complex processes. Phospholipids are major components of the cell membrane and are involved in second messenger systems. These molecules are hydrolyzed by a class of enzymes known as phospholipases, which hydrolytically cleave the phospholipids at different sites to produce a variety of functionally and structurally different molecules. The cleavage of a typical phospholipid molecule by the different phospholipiases is diagramed in Figure 5 below.

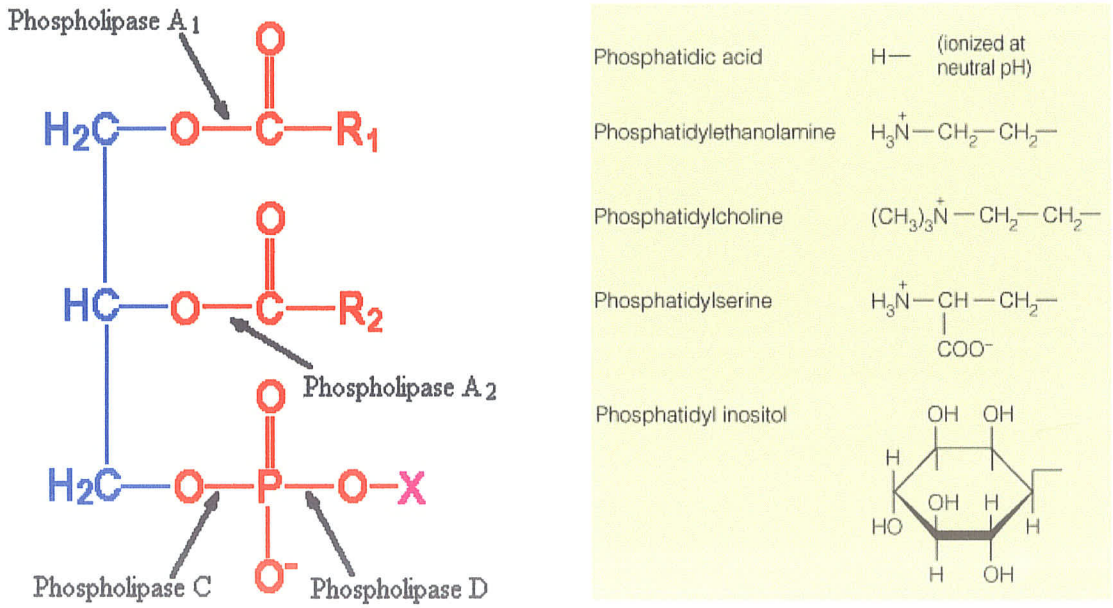


FIGURE 5. HYDROLYTIC CLEAVAGE OF A PHOSPHOLIPID BY DIFFERENT PHOSPHOLIPASES

R1 and R2 refer to any gylcerophospholipid group.
X refers to any group listed in the chart diagram.

As previously indicated, one of the most important functions of membrane lipids is the generation of second messengers, which convey information encoded by extracellular first messengers such as hormones, neurotransmitters and growth factors (Downes, C.P. and R. Currie, 1998, Williams, R. and M. Katan, 1996). The phosphoinositide specific phospholipase C, PLC, is an inositol phospholipid phosphodiesterase, which is involved in many transmembrane signals (Rhee, S.G. and Y.S. Bae, 1997). In eukaryotic systems, phospholipase C hydrolyzes the reaction of phosphatidyl inositol 4, 5- bisphosphate, a plasma membrane phospholipid, into the lipid signaling molecules 1,2 -diaglycerol (DAG) and inositol 1,4,5- trisphosphate (IP_3) (Michell, R.H., 1997, Wang, S., *et. al.,* 1998). Both of these second messengers have important physiological implications; IP_3 and its phosphorylated derivative, inositol 1, 3, 4, 5- tetrakisphosphate, induce the release and uptake respectively of sarcoplasmic reticular Ca^{2+}. DAG serves to activate protein kinase C (PKC), which in turn activates many other important cellular events (Michell, R.H., 1997). Ten distinct isoforms of this single polypeptide have been isolated from mammalian tissue and have been classified into three groups based on size and primary structures. They are identified as β, γ, and δ (Rhee, S.G. and Y.S. Bae, 1997, Williams, R. and M. Katan, 1996), and each class consists of a number of subtypes (Exton, J.H., 1996). Although, these three classes of isoenzymes display some similarities such as interaction with substrates, they have several differences in terms of structure, functional significance and activating mechanisms. These

differences are likely responsible for the specific cell type responses elicited. Thus understanding the distinct mechanisms of action of the isozymes may be crucial to elucidating their ultimate functional and biological significance.

2. *STRUCTURE AND FUNCTION OF PHOSPHOLIPASE C ISOENZYMES*

All mammalian PLC enzymes are organized into four primary domains: a catalytic domain and accessory units or modules, which include the pleckstrin homology (PH) domain, the EF hand domain and the C2 domain (Williams, R. and M. Katan, 1996). The enzyme core is formed from the EF domain, the C2 domain and the catalytic domain (Williams, R. and M. Katan, 1996). The accessory units are found in a variety of other proteins involved in signal transduction thus indicating that these domains have important roles in this process. Each domain has a certain specificity for ligand binding and the coordinate action of these domains regulates the isoenzyme role in signal transduction (Williams, R. and M. Katan, 1996). Significant similarities and differences between the isoforms exist, both of which are consistent with the similar catalytic activities and differing modes of regulation among them (James, S.R. and C.P. Downes, 1997). PLC β and γ have additional domains, which also help mediate the interaction with regulatory proteins. PLC β has an extension of 350- 400 amino acids, a region with a 40% enrichment in the C terminal, which is important for G protein α subunit interaction but not for the G protein β or γ subunit activation (Williams, R. and M. Katan, 1996). PLC γ has an additional module in the catalytic portion: 2 Src homology (SH2) domains and 1 SH3 domain split the PH domain (Rhee, S.G. and Y.S. Bae, 1997). The physical organization of the

enzyme is critical for the reactions it catalyzes. Figure 6 (Lee, S. and S.G. Rhee, 1995) is a linear representation of the domain structure of the three isoform families and Figure 7 (Williams, R. and M. Katan, 1996) illustrates the 3 dimensional structural model of PLC.

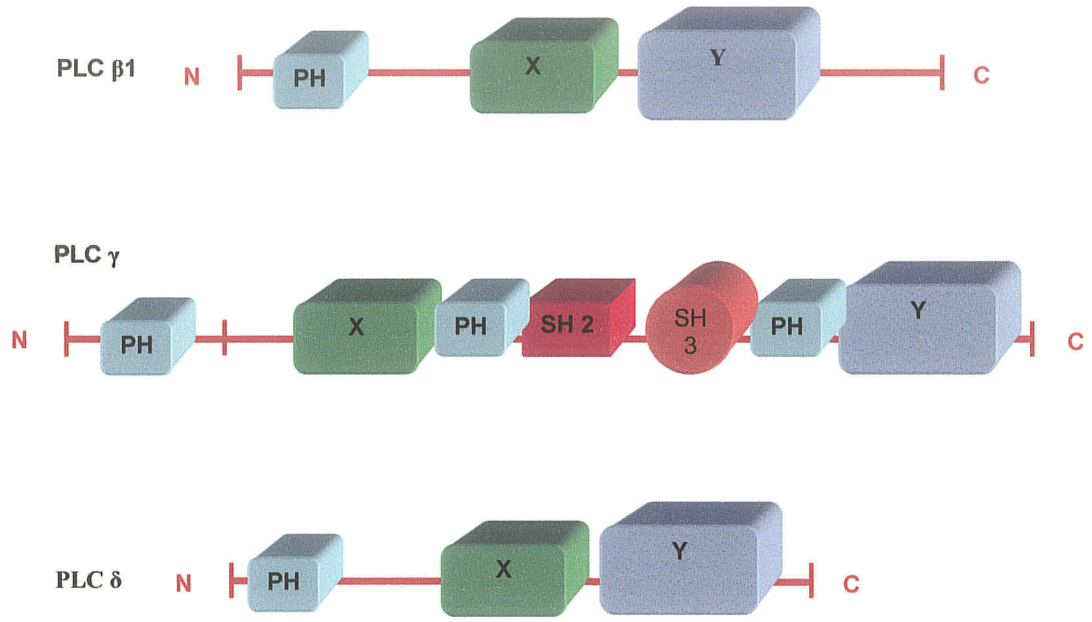
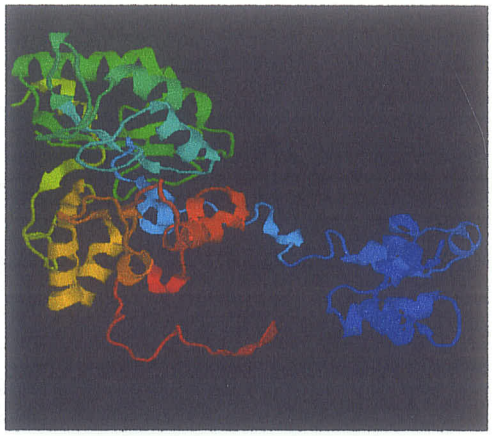


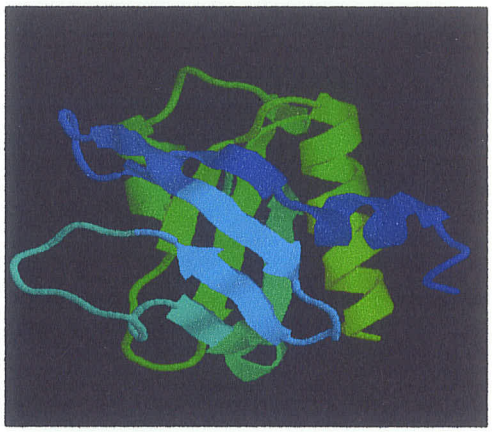
FIGURE 6. DOMAIN STRUCTURES OF THE PLC ISOFORMS

The letters in the boxes indicate the different domains of the PLC isoforms. For example, PH refers to the PH domains, X and Y boxes refer to the X and Y domains respectively. The N and C indicate the nitrogen and carbon terminals respectively, found inall isoforms of PLC.

A. PLC δ1



B. PLC δ1
PH domain



C. PLC δ1
catalytic domain

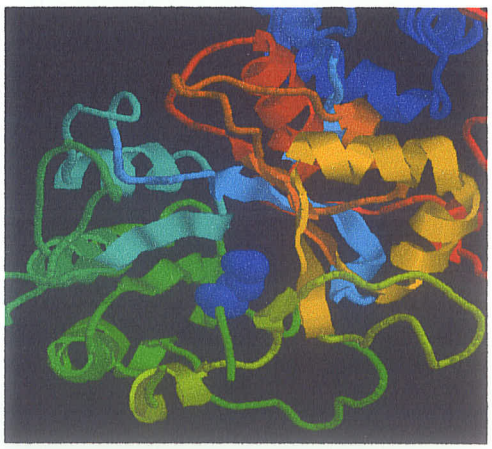


FIGURE 7. 3- DIMENSIONAL STRUCTURE OF PLC δ1 FROM RAT *Rattus norvegicus* IN COMPLEX WITH IP_3 (http://bssv01.lancs.ac.uk)

2.1. X Y Boxes

Overall amino acid similarity between the PLC isoforms is low except for two regions, which are labeled the X and Y boxes (Rhee, S.G. and Y.S. Bae, 1997). The X box consists of 170 amino acids and shows 60% homology and the Y box consists of 260 amino acids and shows 40% homology (Rhee, S.G. and K.D. Choi, 1992, Wang, S., *et. al.,* 1998). Deletions of either the X or Y boxes result in complete loss of the enzymatic activity, indicating that these regions constitute and are essential for the catalytic site of PLC (Noh, D.Y. *et. al.,* 1995). Specifically, mutations of either of the two histidine residues which are conserved in all the PLC isoenzymes were also shown to result in complete loss of catalytic activity (Noh, D.Y. *et. al.,* 1995). Both PLC β and δ contain a short amino acid sequence of 50-70 amino acids that separates the X and Y boxes and this region has up to ten negatively charged amino acids (Rhee, S.G. and Y.S. Bae, 1997). The Y region of PLC β is approximately 450 amino acids in length (Rhee, S.G. and Y.S. Bae, 1997). PLC γ contains a longer sequence of 400 amino acids between the X and Y boxes and this region also contains the Src homology domains (Rhee, S.G. and Y.S. Bae, 1997).

2.2. PH Domain

A 300 amino acid length amino terminus sequence precedes the X region, is found in all PLC's and it contains a PH domain. The PH domain is thought to be involved either in the binding of the enzyme to its substrate,

PIP_2, or to tether the enzyme to the membrane or the β or γ subunits of the G-proteins (James, S.R. and C.P. Downes, 1997). The minimum active site of PLC consists of the EF hand domain, the C2 domain and the TIM barrel (Williams, R. and M. Katan, 1996). The PH domain confers higher affinity membrane binding on the minimal functional unit and thus enables the enzyme to carry out many rounds of hydrolysis while remaining bound to the cell membrane (Williams, R. and M. Katan, 1996). Unlike PLC β and δ which have one PH domain, PLC γ contains one PH domain preceding the X box and another which is separated by the SH domains (Noh, D.Y. *et. al.,* 1995). The PH domain is an approximately 100-residue protein unit that is present in several signaling proteins (Rhee, S.G. and Y.S. Bae, 1997). The PH domains from different isoforms bind to inositol phosphates and PIP_2 with different affinities (Toker, A., 1998). To illustrate, the PH domain of PLC δ1 has a high affinity for PIP_2 and for its head group, IP_3 (James, S.R. and C.P. Downes, 1997). Although all the mammalian isoforms contain PH regions, these domains seem to have different functions depending on the isoform in which they are located. For example, the PH domain of δ1 is important because it seems to be the enzyme's point of anchorage at lipid interfaces. Residues in this domain are important for binding inositol trisphosphate (James, S., 1998). Furthermore, the PH domain of δ1 also seems to be a feedback inhibitory loop because competitive binding of IP_3 inhibits PLC δ1 activity (Cocco, L.M. *et. al.*, 1989). In PLC δ2 only two of the residues are conserved implying that the specific binding mechanism of this domain is not retained in other isoforms

(James, S., 1998). Little information is available on the PH domains of the β1 isoform. James has suggested that the non-catalytic anchoring points are found in the C2 domain. However, T. Wang. *et. al.* have shown that the C2 domain of β1 is not involved in membrane binding (1999). In PLC β1, only one residue is conserved (which is the lys-30 equivalent to PLC δ1) implying that inositol phosphate would only be weakly bound here and, therefore lipid binding is likely facilitated by another region. Williams and Katan (1996) have suggested that the EF hand domain functions in this capacity (see below). The function of these domains in the δ and γ forms has not been completely identified yet (Singer, W.D., *et. al.*, 1997).

2.3. EF Hand Domain

All PLC isoforms contain EF hand domains (James, S.R. and C.P. Downes, 1997). EF hand repeats consist of four helix-loop-helix motifs arranged pair wise in two lobes (Williams, R. and M. Katan, 1996). These two lobes have independent functions; the first lobe provides a flexible link to the PH domain and the second plays an important structural role in that it binds the C2 domain (Williams, R. and M. Katan, 1996). Although not much else is known about the four EF hand repeats, evidence suggests that they seem to be essential for enzymatic activity and may in fact be Ca^{2+} binding sites in the few forms of PLC that possess Ca^{2+} binding sites (as the majority do not) (James, S.R. and C.P. Downes, 1997, Williams, R. and M. Katan, 1996).

2.4. C2 Domain

Like the EF hand domains, the C2 domain is also common to all eukaryotic isoforms of PLC and is central to the interdomain interactions (Williams, R. and M. Katan, 1996). The C2 domain is approximately 103 residues long and is layered in a compact beta sandwich of 2 four- stranded beta sheets. Although the C2 domain is terminally located, this region forms an interface with the catalytic domain of the EF hand domain (James, S.R. and C.P. Downes, 1997, Williams, R. and M. Katan, 1996). It is likely that these interactions are essential for enzyme integrity (Williams, R. and M. Katan, 1996). All forms of the PLC isozyme require Ca^{2+} for enzyme activity. At first, C2 domains were thought to be Ca^{2+} binding domains. However, in contrast to S. R. James (1998), T. Wang, *et. al.* found that the C2 domain of $\beta 1$ does not bind with inositol phosphatases but rather the C2 domain serves as a docking site for the Gqα subunit (1999). Furthermore, the affinity of this domain for the Gqα is sensitive to the GTP/ GDP activation state. There is also evidence that these regions may serve as a third area of lipid binding, specifically Ca^{2+} dependent binding to lipid vesicles, again suggesting that they play a role in membrane interactions (Williams, R. and M. Katan, 1996).

2.5. Distinct Domains

Of the three classes, PLC γ and PLC β contain distinct protein domains that are unique to themselves. As mentioned earlier, the PLC γ has two PH domains, one preceding the X box and another which is separated by two SH

2 domains and one SH 3-domain (Rhee, S.G. and Y.S. Bae, 1997). These 2 domains are small protein structures of approximately 100 and 50 amino acids, respectively (Noh, D.Y. *et. al.*, 1995).

Furthermore, they are not unique to PLC but are found in a number of proteins of which fodrin is an example (Noh, D.Y. *et. al.,* 1995). SH domains are important because they mediate interactions between the enzyme and other protein molecules by binding to phosphorylated tyrosine residues (SH 2) or proline rich sequences. These sequences are found in several other proteins (SH 3), which are also present in cytoskeletal components (Lee, S. and S.G. Rhee, 1995, Rhee, S.G. and Y.S. Bae, 1997). These domains may also play a role in mitogenic signaling independently from that of the enzyme activity (Rhee, S.G. and Y.S. Bae, 1997). Furthermore, the domains suggest that PLC may be important for growth factor signaling (James, S.R. and C.P. Downes, 1997). PLC β also contains two distinct regions; the C-terminus terminal to the C2 domain, which is essential for α subunit interaction (discussed later), and another region, which seems to be essential for association with cell lysates (James, S.R. and C.P. Downes, 1997, Jenco, J.M., *et. al.*, 1997).

2.6. Localization and Translocation

Although most of the various isoenzymes are located primarily within the cytosol, a significant proportion of β1 is associated with the cell membrane. There is also evidence that the γ forms become membrane associated upon cell activation by growth factors (Exton, J.H., 1996).

Following stimulation by growth factors, cytosolic PLC γl is rapidly phosphorylated in vivo (Koblan, K.S. *et. al.,* 1995). This phosphorylation induces the translocation of γl from the cytoplasm to the membrane (Koblan, K.S. *et. al.,* 1995, Lee, S. and S.G. Rhee, 1995). As well, some association of PLC γl with the cytoskeleton has also been reported. Although PLC isoforms are found within the cell membrane, the majority of isoforms reside within the cytosol (Cockcroft, S., 1996). However, physiological concentrations of cytosolic PIP_2 are negligible, and in vivo, PIP_2 resides mainly in the membrane (James, S.R. and C.P. Downes, 1997, Tall, R.S., *et. al.*, 1997). Therefore, in vivo, the enzyme must migrate from the cytosol to the membrane where its substrates are found and consequently hydrolyzed (James, S.R. and C.P. Downes, 1997, Tall, R.S., *et. al.,* 1997). Although not elucidated in this paper, some forms of PLC are now known to exist within the nucleus and evidence seems to indicate that their mechanisms of action and regulation are different from cytosolic PLC isoforms (Martelli, A.M., *et. al.*, 1999).

In the case of PLC γl, the mechanism responsible for effecting its movement remains unclear. One hypothesis implicates the involvement of actin filaments in EGF- induced translocation (Rhee, S.G. and K.D. Choi, 1992). EGF (and PDGF) treated cells showed a translocation of PLC γl from the cytosol to membrane fractions (Rhee, S.G. and K.D. Choi, 1992). EGF contains actin-binding domains, which provide a structural basis for this association (Rhee, S.G. and K.D. Choi, 1992). The association of γl with the

cytoskeleton may be facilitated by phosphorylated PLC γl brought close to the membrane where it interacts with the Gα subunit for subsequent activation (Rhee, S.G. and K.D. Choi, 1992). This phenomenon seems to be unique for PLC γl and may in fact not be essential for enzyme activation in vivo.

3. *MEMBRANE ASSOCIATION AND HYDROLYSIS*

The reversible binding of the PLC enzyme to the membrane is mainly via an electrostatic event (James, S.R. and C.P. Downes, 1997). Subsequently, the PLC enzyme has been shown to penetrate the lipid monolayer at the hydrophobic part of the active site during the hydrolysis reaction (James, S.R. and C.P. Downes, 1997). In terms of PLC δ, the enzyme penetrates the surface of the membrane by approximately 1 nm^2/ surface area (Rebecchi, M., *et. al.*, 1992a). The hydrophobic section of the active site likely interacts with the hydrophobic part of PIP_2 in order to maintain PIP_2's active site stable and oriented for catalysis (James, S.R. and C.P. Downes, 1997). PLC δ has a high affinity for binding to PIP_2 (Rebecchi, M., *et. al.*, 1992b). This affect may also influence specificity of binding and may itself be influenced by G proteins, and tyrosine phosphorylation in the cases of PLC β and γ respectively (James, S.R. and C.P. Downes, 1997).

All three PLC isoforms catalyze the hydrolysis of three inositol-containing lipids:

1. phosphatidyl inositol (PI)
2. phosphatidyl inositol 4 monophosphate (PIP)
3. phosphatidyl inositol 4,5 bisphosphate (PIP_2) (Rhee, S.G. and K.D. Choi, 1992).

PIP and PIP_2 are the preferred substrates and the selectivity for PIP and PIP_2 over PI decreases in the order PLC β1> PLC δ1> PLC γ1 (Rhee, S.G. and

K.D. Choi, 1992). The hydrolytic reaction has three main requirements: Ca^{2+}, the PLC enzyme, and the PIP_2 molecule to be brought to the membrane in the correct orientation so that hydrolysis can occur. Once these conditions are met and PLC has penetrated the monolayer, the phosphodiester bond is cleaved as in a typical acid base reaction (Brady, L. *et. al.,* 1990). The reaction occurs in the 2 dimensional lipid- water interface formed at the membrane (James, S.R. 1998). The enzyme tethers itself to the lipid surface and subsequently a substrate molecule occupies the active site and is hydrolyzed (James, S.R. 1998). In the case of PLC δ, the residue at His 311, in PLC β2 in human at His 327 and the His 335 in human PLC γl deprotonates the 2-OH of the inositol ring, which attacks the phosphodiester bond (Essen, L.O., *et. al.,* 1997, James, S.R. and C.P. Downes, 1997). In PLC δ His 356 protonates DAG (Cifuentes, M.E., *et. al.,* 1994, Essen, L.O. *et. al.,* 1997). The reaction in another direction involves the His 356 as a base and His 311 as the acid (James, S.R. and C.P. Downes, 1997). The 1, 2- cyclic inositolphosphate intermediate is then linearized to form IP_3 (James, S.R. and C.P. Downes, 1997). In the time that the enzyme remains bound to the membrane, several rounds of hydrolysis of substrates may occur as the enzyme moves along the membrane (James, S.R. 1998).

4. RECEPTOR INTERACTION AND ENZYME ACTIVATION

Hormones, neurotransmitters, growth factors and other extracellular signalling molecules exert their physiological effects by binding to cell surface receptors, thereby activating PLC isoenzymes (Exton, J.H., 1996). This activation primarily occurs by one of two ways. In the case of PLC β, activation occurs when agonists for the seven transmembrane spanning receptors are stimulated via an intermediary activation of a heterotrimeric GTP protein (Essen, L.O. *et. al.,* 1997, James, S.R. and C.P. Downes, 1997). When the ligand binds to the receptor, dissociation of the heterotrimeric GDP bound Gq occurs to form GTP bound Gqα, which remains attached within the membrane (Rhee, S.G. and K.D. Choi, 1992). PLC β then binds the Gqα (still GTP bound) presumably at the carboxyl terminal region of both proteins and this results in enzyme activation (Rhee, S.G. and K.D. Choi, 1992). The sequence of these events is depicted below in Figure 8. All four members of the Gq receptor family can stimulate PLC *β*1with Gqα and Gq being most effective (Rhee, S.G. and K.D. Choi, 1992). G_{16} and Gqα primarily stimulate PLC β2 whereas Gnα and Gqα 14 are both less stimulated (Rhee, S.G. and K.D. Choi, 1992). PLC β2 and β3 can also be activated by the βγ subunit at the inter- X-Y region and at the NH_2 region. It can also be inhibited by protein kinase mediated phosphorylation (Jenco, J.M. *et. al.,* 1997, Lee, S. and S.G. Rhee, 1995, Rhee, S.G. and K.D. Choi, 1992). PLC β4 is refractory to the βγ subunit but has shown to be activated by ribonucleotides (James, S.R. and

C.P. Downes, 1997). Interestingly, PLC β1 acts as a GTPase activating protein or GAP protein for the Gα subunits of the Gq family, which activate it. Therefore, PLC β1 (and likely other δ forms) acts to terminate the very signal which stimulates it. Thus, overall enzymatic activity depends on a balance between receptor catalyzed GTP/GDP exchange on Gqα and a PLC β induced GTP hydrolysis by Gqα (James, S.R. and C.P. Downes, 1997). The apparent specificity of the Gqα subfamily for the different PLC β isoforms may be important in eliciting specific cellular responses. The receptors for the Gqα PLC β pathway include thromboxane A2, bradykinin, bombesin, angiotensin II, histamine, vasopressin, acetylcholine, (muscarinic ml and m3), alpha-adrenergic, thyroid stimulating hormone, and endothelin-1 receptors (Rhee, S.G. and Y.S. Bae, 1997).

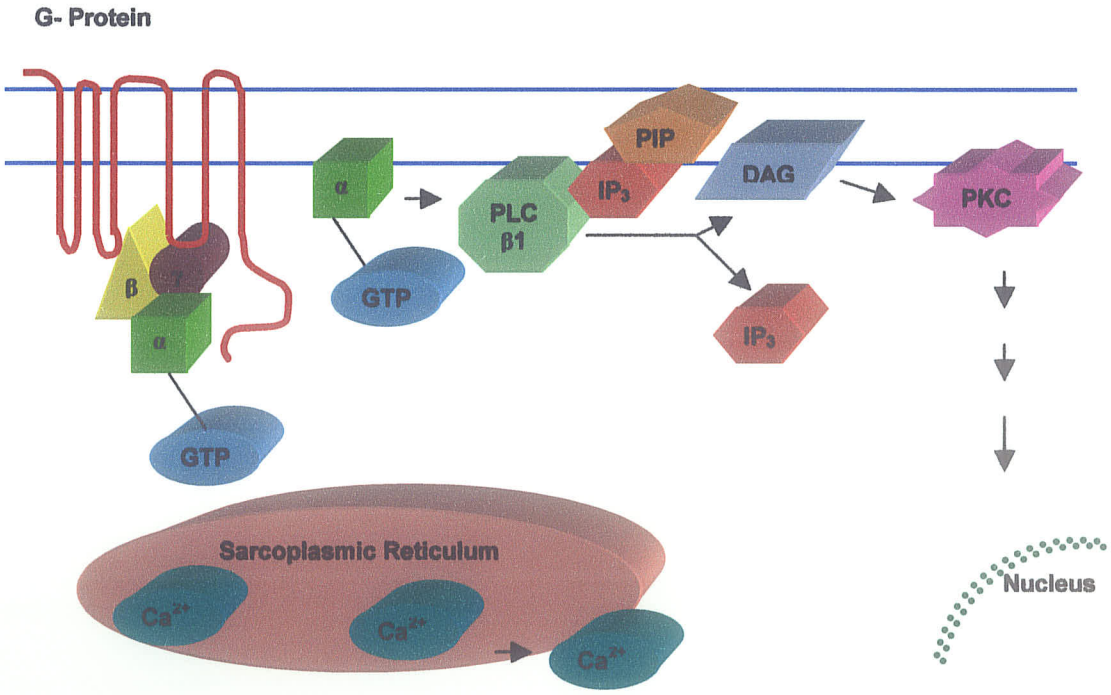


FIGURE 8. MECHANISM OF G- PROTEIN ACTIVATION

Polypeptide growth factors use a different mechanism to activate PLC. They activate γl, but not in the cases of δ1 or β1 (Rhee, S.G., and K.D. Choi, 1992). Many growth factor receptors possess tyrosine kinase activity (Lee, S. and S.G. Rhee, 1995, Meldrum, E. *et. al.,* 1991). Binding of the ligand causes receptor dimerization and activation of tyrosine kinase, which in turn causes the phosphorylation of tyrosine residues; tyr 771, tyr 783, and tyr 1254 in the cytoplasmic domains of the receptor. (Rhee, S.G., and K.D. Choi, 1992). These major sites of γl phosphorylation by the receptor seem to be identical for EGF, PDGF, and NGF (Rhee, S.G., and K.D. Choi, 1992). Tyr 783 and to a somewhat lesser degree, tyr 1254 are essential for PDGF- stimulated inositol phosphate formation in intact cells (Rhee, S.G., and K.D. Choi, 1992). Tyr 783 is essential for βl activation (Rhee, S.G., and K.D. Choi, 1992). Association of the receptor with PLC βγl and serine residue phosphorylation were not sufficient for PDGF induced γl activation (Rhee, S.G., and K.D. Choi, 1992). Phosphorylation may result in a conformational change that allows the SH3 domain to associate with the cytoskeleton, thereby bringing the enzyme into apposition with its substrate and move the catalytic X and Y domains to the cytoplasmic side of the cell membrane (Exton, J.H., 1997, Rhee, S.G., and K.D. Choi, 1992). Specific proteins then become associated to these domains and have an increased affinity for the receptor autophosphorylated sites. For example, PLC γl, GAP of p21 and others react in this manner (Exton, J.H., 1997). Activation of PLC γ occurs via tyrosine phosphorylation (Rhee, S.G., and Y.S. Bae, 1997). This process involves the

SH2 domain mediating binding of γl for example, to tyrosine autophosphorylated sites and the subsequent phosphorylation of γl itself (James, S.R. and C.P. Downes, 1997). Growth factor receptor phosphorylation and tyrosine phosphorylation requires the transient presence of H_2O_2 (Rhee, S.G., and Y.S. Bae, 1997). It is not yet clear however if γ forms are key factors in growth factor signalling and the subsequent cell response. For example, tyrosine phosphorylation seems to promote association of γl with the cytoskeleton; the SH3 domain targets the enzyme to the actin microfilament network (Rhee, S.G., and Y.S. Bae, 1997). It is not clear however, if the association serves to bring the enzyme into contact with its substrate or whether it promotes the interaction of other protein components essential for activation (Rhee, S.G., and Y.S. Bae, 1997). Tyrosine activation of γl can also be done via non-receptor protein tyrosine kinase (PTK) action. This is also the case for both γl and γ2 when certain membrane surface receptors are ligated in leukocytes (Rhee, S.G., and K. D. Choi, 1992). The sites for γ2 tyrosine phosphorylation are tyr 753, and tyr 759 (Rhee, S.G., and K. D. Choi, 1992). The sequence surrounding tyr 759 is similar to γl 's tyr 783 (Rhee, S.G., and K.D. Choi, 1992). However, unlike γl, tyrosine phosphorylation was not observed on the carboxyl terminal of γ2.

The activation mechanisms for PLC δ have not yet been elucidated (Rhee, S.G., and Y.S. Bae, 1997). However, another class of GTP binding protein, labeled Gh, contains a unit that can activate PLC δ1 by directly complexing in cells stimulated with the α1 adrenoreceptor (Rhee, S.G., and

Y.S. Bae, 1997). However, it is not yet known if the δ forms are also activated by Ghα and what other receptors complex with Ghα. PLC δ activation may occur via another mechanism. PLC δ is more sensitive to Ca^{2+} than other isoforms and can tether to membrane containing PIP_2 via the PH domain (Rhee, S.G., and Y.S. Bae, 1997). An increase in intracellular Ca^{2+} to a level sufficient to fix the C2 domain of PLC δ may trigger its activation (Rhee, S.G., and Y.S. Bae, 1997).

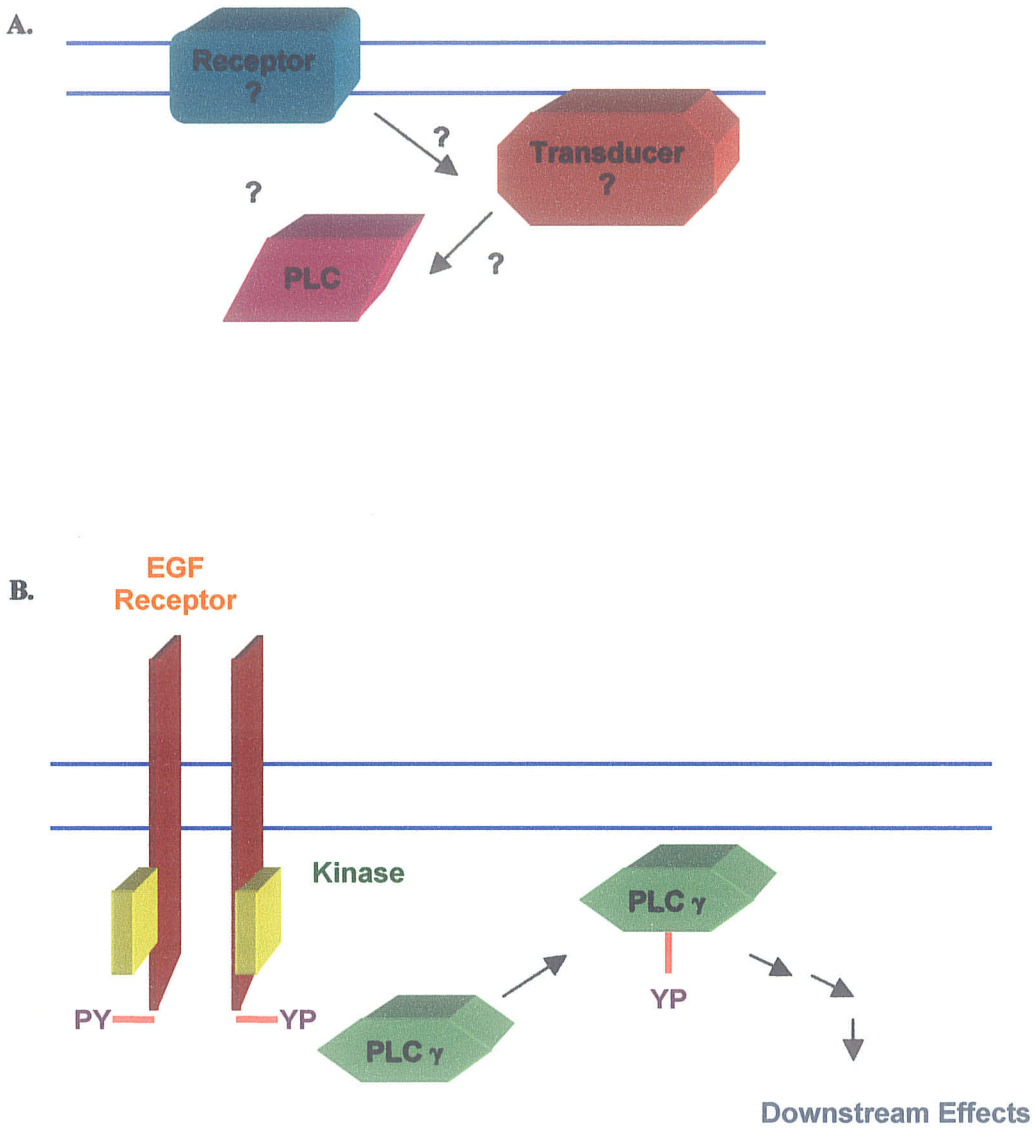


FIGURE 9. A SCHEMATIC REPRESENTATION OF THE ACTIVATION MECHANISM FOR PLC δ (A) and γ (B) ISOFORMS (Jones, G. and G. Carpenter, 1993)

5. PLC REGULATION

Regulation of PLC occurs via a number of different mechanisms including via G- protein subunits, tyrosine phosphorylation, phosphoinositide transfer protein, phosphatidic acid, arachadonic acid, PI 4-kinase, membrane properties and several other mechanisms.

5.1. Regulation Via G Protein Subunits

As previously mentioned, the G protein coupled receptors have seven transmembrane spanning domains (Dohiman, H.G., *et. al.,* 1991, Rhee, S.G., and K.D. Choi, 1992). These receptors are associated with G- proteins (Dohiman, H.G., *et. al.,* 1991, Rhee, S.G., and K.D. Choi, 1992). The G-proteins are heterotrimeric proteins composed of three units, which associate into two functional groups, an α and a $\beta\gamma$ subunit (Milligan, G. and M.A. Grassie, 1997). When a ligand binds at the receptor, the receptor induces the activation of the G-protein which in turn leads to the release of GDP from the α subunit (Dohiman, H.G., *et. al.,* 1991, Morris, A.J. and S. Scarlatta, 1997) (Figure 10). GDP is then replaced by GTP and which, results in a dissociation of the α subunit from the $\beta\gamma$ subunit (Morris, A.J. and S. Scarlatta, 1997). Approximately 20 α subunits and three $\beta\gamma$ subunits and 7 γ subunits have been identified (Simon, M.L., *et. al.,* 1991). Thus a large array of potential responses is possible. The individual and now dissociated subunits can elicit

responses in an independent, synergistic or antagonistic manner (Morris, A.J. and S. Scarlatta, 1997, Rhee, S.G., and K.D. Choi, 1992).

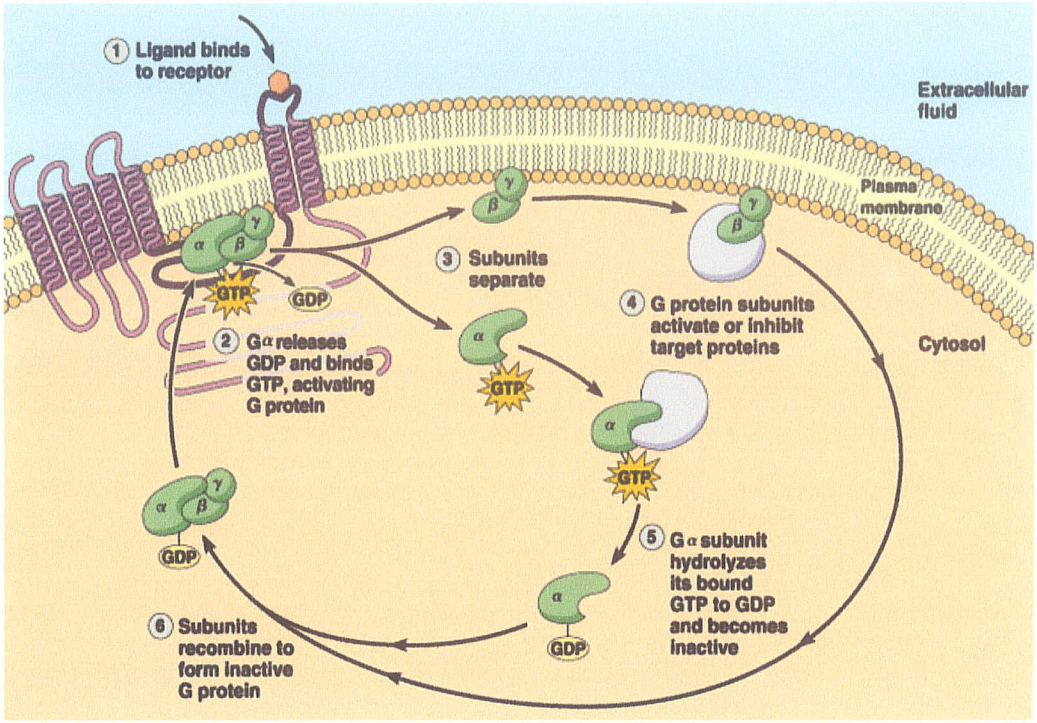


FIGURE 10. A SCHEMATIC OF THE MECHANISM OF ACTION OF THE G PROTEIN 2ND MESSENGER SYSTEM (http://desmid.biol.mun.ca/brian/BIOL2060/cellbio/CB10_4.html/)

The β isoforms are selectively regulated and activated by all four Gqα subunit types of the Gq subclass (Lee, S. and S.G. Rhee, 1995). As well, β isoform activation can result from βγ subunit interaction although α stimulation is 50- 100 times more potent in eliciting a response (Lee, S. and S.G. Rhee, 1995, Simon, M.L., *et. al.,* 1991). The sensitivity of PLC β

isoforms to Gqα is β1> β3> β4> β2, whereas the sensitivity for the βγ subunit is β3> β2> β1 and β4 is not activated by the βγ subunit at all (Lee, S. and S.G. Rhee, 1995). The specific interactions between the Gqα subtypes and certain isoforms may be responsible for the cell specific responses and may also contribute to a form of overlap to compensate for deficiencies or problems in cell biochemical pathways.

5.2. Tyrosine Phosphorylation

As discussed above, PLC γ is activated by tyrosine phosphorylation of the enzyme itself, the receptor and other proteins (Rhee, S.G., and Y.S. Bae, 1997). Growth factors act on the receptors and cause a translocation of the enzyme from the cytosol to the membrane and can stimulate the phosphorylation of the lipase found on tyrosine residues (Singer, W.D., *et. al.*, 1997). Receptor autophosphorylation results in high affinity binding sites for SH2 domain- containing proteins (Rhee, S.G., and Y.S. Bae, 1997). This mechanism depends on the affinity of the tyrosine phosphorylated residues for the SH2 domains of γl and allows γl tyrosine residue phosphorylation (Cockcroft, S. and G.M. Thomas, 1992). However, the mechanism for increased PLC γ activity is unclear.

G- protein pathways can also regulate PLC γ in a more direct way (Singer, W.D., *et. al.*, 1997). Stimulation of G- protein linked receptors leads to an increase in phosphorylation of PLC γl in platelets and smooth muscle

cells (Singer, W.D., *et. al.*, 1997). The physiological significance of G- protein stimulation of γ isoforms remains to be established.

5.3. Substrate Level Regulation

Phosphoinositides are a family of phospholipids that have crucial roles in receptor mediated intracellular signaling cascades. As a result, they are essential for cell survival and adaptation (Weideman, C. and S. Cockcroft, 1998). Thus, these phosphoinositides require exact temporal and spatial regulation of both synthesis and degradation (Weideman, C. and S. Cockcroft, 1998). Multiple forms of PLC, which are differentially regulated, contribute to the complex second messenger system of PLC linked receptors. PIP_2 represents a very small pool of total cell membrane phospholipids (Tobin, A.B., *et. al.,* 1996). PIP_2 levels fall significantly during agonist stimulation (Tobin, A.B., *et. al.,* 1996). This depletion may be a heavy constraint on second messenger production. In order to maintain PIP_2 levels, its precursors, PIP and PI, have to be maintained. Therefore, Ptdins transfer protein, which is responsible for transferring the PtdIns to the plasma membrane, may be important in determining PLC second messenger responses (Tobin, A.B., *et. al.,* 1996).

a. Phosphatidylinositol Transfer Protein

Phosphatidylinositol transfer protein (PITP), a 35 kDa cytosolic factor, catalyses the exchange of phosphatidylinositol (PI), and phosphatidylcholine

(PtdCho) between mammalian membrane bilayers in vitro (Kauffmann-Zeh, A., *et. al.,* 1995, Kearns, B.G., *et. al.*, 1998, Westerman, J. *et. al.*, 1995). Two mammalian forms of PITP have been identified and are labeled α and β (Cockcroft, S. *et. al.,* 1997, Kearns, B.G., *et. al.*, 1998). PITP α is an essential requirement for the membrane receptor-G-protein coupled hydrolysis of PIP_2 by PLC-β2 and β3, γl and δ1 (Cockcroft, S., 1996, Cunningham, E.S.W., *et. al.,* 1996, Thomas, G.M.H., *et. al.,* 1993) and for the phosphorylation of PIP_2 to PI 3, 4, 5-trisphosphate by PI 3-kinase (Cockcroft, S. *et. al.,* 1997). It serves to bring the substrate, PIP_2, from the endoplasmic reticulum to the membrane for hydrolysis (Cockcroft, S. *et. al.,* 1997, Martelli, A.M. *et. al.,* 1999). In the case of PLC β, the reconstitution of PIP_2 at the membrane is dependent on MgATP (Thomas, G.M.H., *et. al.,* 1993). In addition, since the increase in PLC enzymatic activity occurrs without any effect on PIP_2 levels, it has been proposed that PITP, in addition to being a transfer protein, is also a cofactor (Weideman, C. and S. Cockcroft, 1998). However the exact molecular mechanisms by which PITP functions remain unresolved. Figure 11 (Cockcroft, S. *et. al.,* 1997) illustrates the participation of PITP in inositol lipid synthesis and also indicates its regulation by PI 4-kinase and PIP 5-kinase.

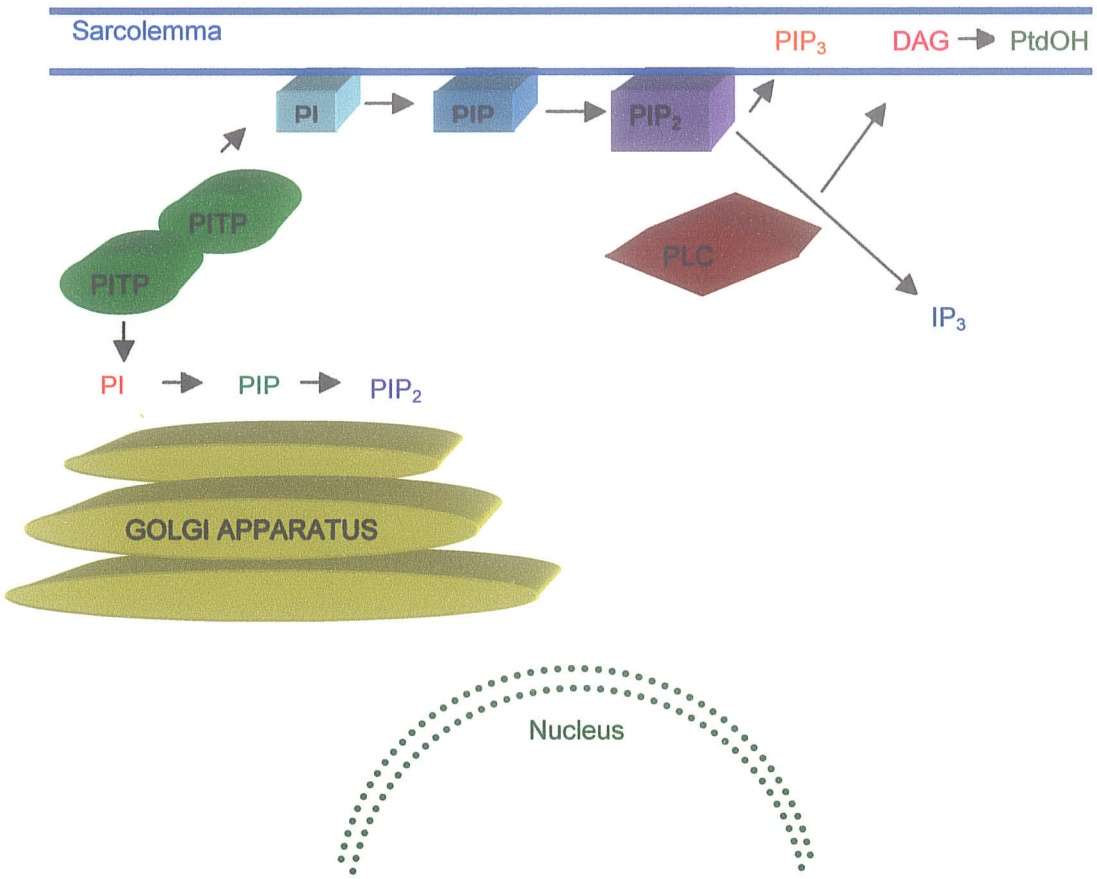


FIGURE 11. MECHANISM OF ACTION OF PHOSPHATIDYLINOSITOL TRANSFER PROTEIN (PITP)

b. Phosphatidic Acid

Phosphatidic acid (PtdOH) is an anionic phospholipid which is thought to be involved in many cell signaling systems as it has been shown to bind and regulate a number of intracellular signaling protein molecules (Henry, R.A., *et. al.*, 1995, Jones, G. and G. Carpenter, 1993). PLC hydrolysis of PIP_2 increases in the presence of phosphatidic acid and/ or lysophosphatidic acid in vitro (van Corven, E. *et. al.*, 1992). Phosphatidic acid acts as an allosteric modifier of enzyme activity (Jones, G. and G. Carpenter, 1993). In cardiac

cells for example, phosphatidic acid can stimulate both tyrosine phosphorylated and unphosphorylated PLC γl, with the unphosphorylated isoform having increased responsiveness (Henry, R.A., *et. al.,* 1995). However, despite this increase, both forms stimulated γl to the same levels, implying that this action may occur independently of phosphorylation and via an allosteric mechanism (Henry, R.A., *et. al.,* 1995). As phosphatidic acid is produced by cells in response to a number of stimuli (such as growth factors), it is likely that PLC γl may be regulated by this mechanism in addition to tyrosine phosphorylation (Hashizurne, T. *et. al.*, 1992). Calcium is also required for PtdOH to bind to PLC γl (Jones, G. and G. Carpenter, 1993). Tyrosine phosphorylation and PtdOH may act together to maximize PLC γl activation (Jones, G. and G. Carpenter, 1993). In terms of δ1, Rebecchi and co-workers (1992b) have shown that both PIP_2 and phosphatidic acid can increase the high affinity binding of the δ1 isoform to lipid bilayers. Regulation of PLC by phosphatidic acid has important physiological significance. The increase in intracellular $[Ca^{2+}]$ due to phosphatidic acid stimulation can alter the contractile performance of intact cells (Xu, Y. *et. al.*, 1994).

c. Arachidonic Acid

PLC γ isozymes can be stimulated by arachidonic acid (AA) when in the presence of the tau protein (a microtubule associated protein in neuronal cells) or tau like proteins (found in non-neuronal cells) (Hwang, S. C. *et. al.*,

cells) or tau like proteins (found in non-neuronal cells) (Hwang, S. C. *et. al.*, 1996). Both tau and AA effects were inhibited by phosphatidylcholine (PtdCho), suggesting that such activation may be increased by a simultaneous decrease in PtdCho and an increase in AA concentration, both of which occur when cells are activated by PLA_2 from the cytosol (Rhee, S.G. and Y.S. Bae, 1997).

d. PtdIns 4- Kinase

PtdIns 4-kinase phosphorylates the fourth position on the inositol ring of the PtdIns molecule to yield PtdIns-4*P*, which is a precursor to PIP_2 (Quist, E.E. *et. al.,* 1989, Toker, A., 1998). The activity of PtdIns 4-kinase is dependent on Mg^{2+} and sensitive to Ca^{2+}. This sensitivity to Ca^{2+} is species specific (Quist, E.E. *et. al.,* 1989). PtdIns-4*P* synthesis is inhibited by micromolar Ca^{2+} concentrations in rat heart, but in canine ventricle, the enzyme activity is insensitive to Ca^{2+} concentrations in the range 0.1- 30 mM (Mesaeli, N. *et. al.,* 1992).

e. PtdIns 4P, 5- Kinase

PtdIns-4*P,* 5-kinase can further phosphorylate PtdIns-4*P* to produce PtdIns (4,5)P_2 (Quist, E.E. *et. al.,* 1989, Toker, A., 1998). Magnesium ions and Ca^{2+} regulation of PtdIns-4*P,* 5-kinase activity is similar to that of PtdIns 4-kinase (Mesaeli, N. *et. al.,* 1992, Quist, E.E. *et. al.,* 1989). Two subtypes of this kinase have been identified and labeled as Type I and Type II (Toker, A.,

1998). Type I catalyzes the phosphorylation of PtdIns-4*P* at the D5 position of the inositol ring (Toker, A., 1998). The Type II kinase is a PtdIns 4-kinase as it phosphorylates the ring on the D4 position (Toker, A., 1998). As this enzyme serves to increase the levels of PtdIns (4, 5)P_2, it is evident that there are many sources of this substrate and they seem to involve the action of a number of different enzymes (Toker, A., 1998).

5.4. Membrane Level Regulation

The phospholipid membrane has many innate properties which may themselves act as regulators of hydrolysis. For example, membrane curvature, surface pressure, surface potential, degree of hydration and lipid composition may all influence the reaction (James, S.R. and C.P. Downes, 1997).

a. Membrane Curvature

Although no information is available on the effects of membrane curvature for PLC mediated hydrolysis, it is worthy to note that curvature has been shown to strongly influence PI 3- kinase activity (Hubner, S. *et. al.*, 1998). The PI 3- kinase activity is considerably greater when the substrate is in a convex surface than if the surface is a flat bilayer (James, S.R. and C.P. Downes, 1997). Furthermore, PI 3-kinase can also phosphorylate PtdIns (4, 5)P_2 (James, S.R. and C.P. Downes, 1997). Thus it is possible that a similar mechanism may be part of the regulatory processes for PLC hydrolysis.

b. Monolayer Surface Pressure

Hydrolysis measured in the monolayer seems to be reflective of the proportion of enzyme successfully penetrating the monolayer rather than the overall enzyme bound at the interface (James, S., 1998). It has been shown that the rate of PtdIns (4, 5)P_2 hydrolysis by the three PLC isoforms decreased as surface pressure of the monolayer containing PtdIns(4,5)P_2 increased (Boguslavsky, V. *et.al.*, 1994, Hubner, S. *et.al.,* 1998, James, S., 1998). For example, there was a significant decrease in PLC β1 and β2 catalysis due to the increasing inability of the enzyme to penetrate the monolayer (Boguslavsky, V. *et.al.*, 1994, James, S., 1998). As well, the activity of δ1 increased 100 fold when the surface pressure of the monolayer containing PtdIns (4,5)P_2 decreased from 40 to 20 mN/m (Rebecchi, M. *et. al.*, 1992a). Therefore, mechanical events may also be a critical part of PLC activation.

c. Membrane Electro- Chemical Properties

The electro- chemical composition of the phospholipid bilayer has been shown to affect the PLC mediated hydrolysis reaction. PLC binds at acidic phospholipids; specifically to the X box and PH domains (James, S.R. and C.P. Downes, 1997). This binding is stabilized by an electrostatic interaction between lysine and arginine residues and phosphate groups (James, S.R. and C.P. Downes, 1997). Furthermore, the activities of PLC β and PLC δ have been shown to significantly decrease when PtdSer content of the membrane has been decreased (Rebecchi, M. *et. al.,* 1992a, Rebecchi, M. *et.*

al., 1992b). Therefore, it seems evident that PLC hydrolysis decreases when the potential is reduced (James, S.R. and C.P. Downes, 1997). In addition, PLC molecules have an increased affinity for acidic molecules (Yang, L. and M. Glaser, 1994). Membrane regions abundant in acidic phospholipids may facilitate binding to substrate (James, S.R. and C.P. Downes, 1997). Lastly, PLC assays have revealed that when less than 30% of PtdIns (4,5)P_2 is hydrolyzed, the reaction halts (James, S.R. *et. al.*, 1996). James and Downes (James, S.R. and C.P. Downes, 1997) have suggested that this inhibition may be due to DAG induced effects as DAG has been shown to destabilize the membrane perhaps by changing surface properties at the lipid interface (James, S.R. and C.P. Downes, 1997).

5.5. Other Regulatory Mechanisms

a. Tubulin

Tubulin is a cytoskeletal protein that is involved in the regulation of adenylate cyclase activity through interactions with $G_\alpha S$ and $G_\alpha i1$ (Popova, J. *et. al.,* 1997). Tubulin has also been shown to bind to Gqα and activate it via the direct transfer of GTP from tubulin to Gqα (Popova, J. *et. al.,* 1997). PLCβ1 is activated by Gqα (Rhee, S.G. and K.D. Choi, 1992). Consequently, tubulin, at low concentrations has been shown to activate PLC β1 but at high concentrations to inhibit the enzyme (Popova, J. *et. al.,* 1997).

b. Regulation Via Ca^{2+}

Although the Ca^{2+} requirement for PLC activation is essential, very little is known about its mechanism of regulation. What is known is that Ca^{2+} affects PLC activity directly or indirectly via receptor modulation (Allen, V. *et. al.,* 1997, Cockcroft, S. and G.M. Thomas, 1992). All isoforms of PLC require Ca^{2+} for the hydrolytic reaction and the catalytic activity increases with a rise in calcium in the physiological range of 0.01-10 μM (Ellis, M. *et. al.*, 1998, Rhee, S.G. and K.D. Choi, 1992). Some cell types require only low concentrations of Ca^{2+} while in others such as pancreatic islet cells, spermatozoa and neuroblastoma cells, PLC activates at higher [Ca^{2+}] levels (Best, L., 1986, Smart, K.G. *et. al.,* 1995). Increases in Ca^{2+} concentration linked to inositol lipid hydrolysis may be due to different mechanisms such as the opening of receptor operated Ca^{2+} channels or voltage dependent Ca^{2+} channels (Allen, V. *et. al.,* 1997, Eberhard, D.A. and R.W. Holz, 1988). Low Ca^{2+} levels activate PLC γl whereas high Ca^{2+} concentrations inhibit the γl hydrolysis of phosphoinositides (Zhou, C. *et. al.,* 1999). The extent of the inhibition, however, depends on pH, the specific type and amount of substrate and the manner in which it is made available (Zhou, C. *et. al.,* 1999). The great amount of control that the many regulatory factors have on hydrolysis emphasizes the importance not only of enzyme regulation, but also the importance of signalling pathways to biological systems.

c. Regulation by PKC

Regulation of PLC β1 by PKC occurs in certain cell types where PKC and PKA have been shown to attenuate receptor coupled PLC activity in a negative feedback manner (Smart, K. *et. al.,* 1995). Specifically, PKC phosphorylates β1 *in vivo* and to a physiologically relevant level *in vitro* which results in a loss of overall catalytic activity rather than a specific modification of G-protein regulated activity (Henry, R.A. *et. al.*, 1995). Cunningham *et. al.* have shown that activation of PKC in turkey erythrocytes resulted in an attenuation of Gα11 stimulated PLC β1 activation possibly by promoting a change in Gα11 itself or in a protein which regulates Gα11 (Cunningham, E. *et. al.*, 1996, De Jonge, H.W., *et.al.*, 1995).

6. *BIOLOGICAL AND FUNCTIONAL SIGNIFICANCE OF PLC*

PLC isoenzymes are unequivocally distributed throughout biological tissues including brain, lungs, heart, ovary, and colon to name just a few examples (Watson, S. and S. Arkinstall, 1994). As previously mentioned, PLC is a key enzyme in the activation of cellular function. Alterations in the activity of PLC are also correlated and perhaps causative with certain pathophysiologies. However, before one can fully understand the implications of these alterations, it is important to understand the function of PLC in normal, healthy cells.

6.1. PLC Function in Normal Cells

a. Cellular Functions

PLC isoforms are involved in numerous cellular functions. Two important examples are cytoskeletal maintenance and intercellular communication. Phosphorylated PLC γ can catalyze the hydrolysis of prolifin bound PIP_2 and thereby release the prolifin molecule and alter actin polymerization (Goldschmidt- Clermont, P.J., *et. al.,* 1991). Prolifin has been implicated as a link between transmembrane signaling and cellular responses such as a change in structure, shape and increased cell motility (Goldschmidt-Clermont, P.J., *et. al.,* 1991). In many cell types, IP_3 receptors are coupled to Ca^{2+} release from intracellular stores (Van Heugten, H.A., *et. al.,* 1996).

Interestingly, heart and skeletal muscle cells control cytosolic Ca^{2+} via Ca^{2+} entry into cells and through ryanodine receptors, yet these cells do not respond strongly to IP_3 (Woodcock, E.A., 1997). In fact, IP_3 receptors are surprisingly absent from the sarcoplasmic reticulum but are present rather on the *fascia adherens* of the intercalated discs (Woodcock, E.A., 1997). Therefore, IP_3 and consequently, PLC may function in cell- to- cell communication (Woodcock, E.A., 1997). S. Shimohama *et. al.* showed that PLC β1 was undetected in fetal brains of rats but had increased at 4 weeks and not at 48 weeks implying that β1 may be important for maturation or aging (1998). PLC β1 seems to function in early synaptogenesis and establishment of the neural network (Dohiman, H.G., *et. al.,* 1991).

b. Ca^{2+} Flux

Calcium is involved in many cellular processes in eukaryotic cells. It is stored in and released from the sarcoplasmic reticulum in muscle cells and the endoplasmic reticulum in non-muscle cells (Somlyo, A., 1998). The products of PLC hydrolysis are DAG, a PKC activator, and IP_3 a Ca^{2+} mobilizing messenger (Murthy, K.S. and G.M. Malkouf, 1995). Thus it is evident that PLC plays a major role in cell functions which relate to Ca^{2+} influx and efflux such as: contraction of muscle cells, depolarizations in neurons, as a cofactor in certain stages of various biochemical pathways, protein synthesis and secretion, and cell division (Neylon, C.B. *et. al.,* 1998). In most cells, Ca^{2+} is released from stores upon IP_3 receptor activation (Neylon, C.B. *et. al.,* 1998).

When IP_3 binds with its receptor, a Ca^{2+} channel opens to allow Ca^{2+} release into the cytoplasm (Neylon, C.B. *et. al.,* 1998). However, IP_3 receptor regulation is a complex process and requires further study. Figure 12 illustrates the mechanism of the opening of the Ca^{2+} channel.

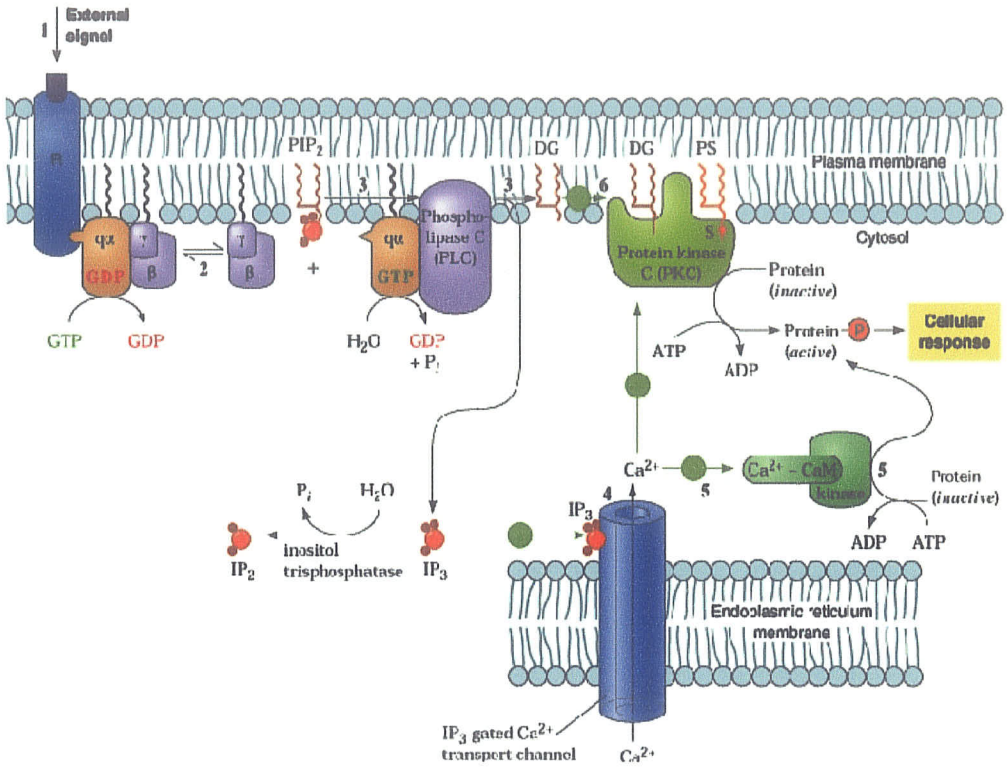


FIGURE 12. THE MECHANISM OF CALCIUM CHANNEL ACTIVATION VIA THE PLC SIGNALING PATHWAY (http://arethusa.unh.edu/bchm752/ppthtml/mar23/march23/sld020.html)

c. Parallel Signaling Networks

The products of one pathway can induce biological effects by having feedback on other signaling systems. For example, fMetLeuPhe stimulates phospholipase C, D, and A_2 (Cockcroft, S. and G.M. Thomas, 1997). PLC stimulation results in an increase in cytosolic Ca^{2+}, which in turn is an essential requirement for the G- protein coupled phospholipase A_2 pathway (Nielson, C.P., *et. al.,* 1991). Figure 13 identifies the relationship of the PLC pathway to the PLD and PLA_2 pathways in cardiac sarcolemma. Furthermore, many studies have revealed that the activation of one receptor can influence the response of other receptors (Cockcroft, S. and G.M. Thomas, 1997). To illustrate, in thyroid cells, TSH can stimulate both PLC and adenylate cyclase (Van Sande, J. *et. al.,* 1990). However cAMP formation occurs at a significantly lower TSH concentration that PLC activation does (Cockcroft, S. and G.M. Thomas, 1997).

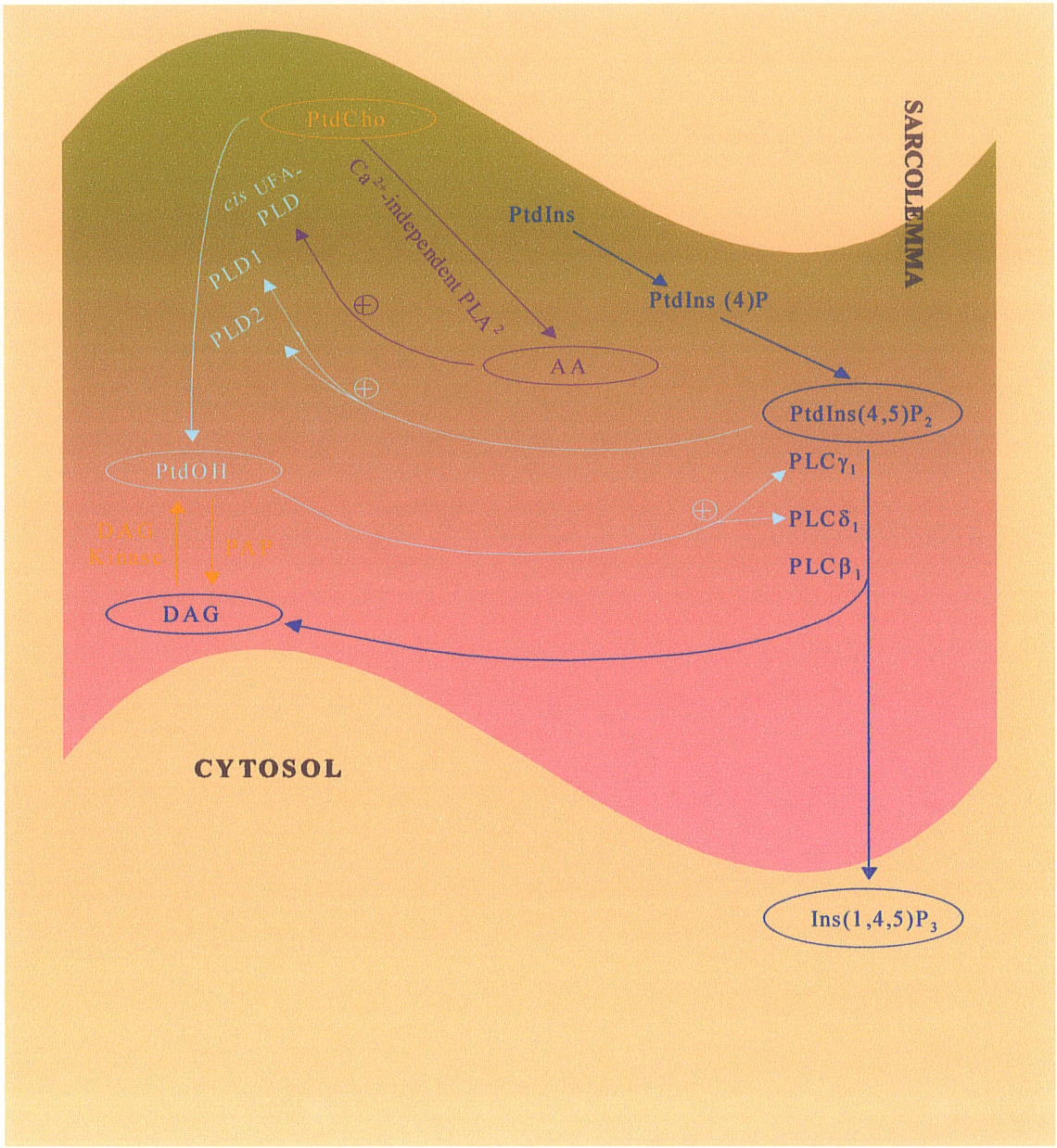


FIGURE 13. PLC AND PLD SIGNALING IN CARDIAC SARCOLEMMA

Phospholipase C, D and A_2 Signaling in Cardiac Sarcolemma.

Key: Phospholipase C Signaling Pathway

Phospholipase D Signaling Pathway

Phospholipase A_2 Signaling Pathway

6.2. PLC Function in Disease Conditions

a. Cardiac Preconditioning

In the heart PLC may be involved in the preconditioning mechanism via PKC (Van Heugten, H.A., *et. al.,* 1996). Inhibition of either the αl adrenoreceptor, which is thought to play a role in the protective effect, and inhibition of PKC, was shown to block the preconditioning effect of an ischemic period (Mitchell, M.B., *et. al.*, 1995).

b. Diabetic Cardiomyopathy

Cardiomyopathy is one of the complications of diabetes mellitus and is characterized by altered contractile properties, structure, and changes in haemodynamic parameters (Tomlinson, K.C., *et. al.*, 1992). In diabetic cardiomyopathy, PLC γ activity has been shown to significantly decrease (Yun, T., *et. al.*, 1998). However, T. Yun, *et. al.* reported that there was a concomitant increase in protein mass (1998). *In vitro* stimulation of γl with phosphatidic acid was greater in diabetic cardiomyopathy, suggesting that an abnormal sarcolemmal PLC γl isoenzyme is present (Yun, T., *et. al.,* 1998). Furthermore, membrane signaling levels of phosphatidic acid are decreased in failing hearts and since phosphatidic acid regulates PLC activity, this mechanism may have a profound role in diabetic cardiomyopathy.

c. Congestive Heart Failure Post Myocardial Infarction

Cardiac myocytes are a potential target for many growth factors and hormones. As previously mentioned, the PLC pathway results in the production of DAG and IP_3 (Rhee, S.G. and K.D. Choi, 1992). DAG in turn serves to activate PKC, which in turn has been shown to phosphorylate cardiac ion channels and thus is involved in cardiomyocyte hypertrophy (De Jonge, H.W., *et. al.*, 1995, Puceat, M and G. Vassort, 1996). However little is known about the PLC isoforms in cardiomyocytes. The dominant isozymes found in the heart are PLC γl, δ1, β1, β3 (Hansen, C.A., *et. al.,* 1995), with δ1 and γl being the major forms expressed in rat adult ventricular myocytes (Hansen, C.A., *et. al.,* 1995). Furthermore, the sarcoplasmic reticulum contains several binding sites for both IP_3 and inositol 1, 3, 4, 5-tetrakisphosphate (Tappia, P.S., *et. al.,* 1999). These binding sites may serve to enhance SR Ca^{2+} release and uptake (Quist, E.E., *et. al.,* 1994). In pathological states, PLC expression and consequently Ca^{2+} levels have been shown to be altered (Tappia, P.S., *et. al.*, 1999). For example, Tappia, P.S. *et. al.* have shown that despite the overabundance and hyperactivity of PLC β1 in the cardiac sarcolemma and the increase in the protein masses of β1 and β3, the total PLC activity of sarcolemmal PLC was decreased (1999). In contrast, PLC γl and δ1 both had decreased activities (Tappia, P.S., *et. al.*, 1999). It has been established that PLC β1 can regulate its own activation and termination and this phenomenon may be anticipated in failing hearts by the increase in β1 activity (Tappia, P.S., *et. al.,* 1999). The resulting alterations in Ca^{2+}

mobilization impair cardiac performance and ultimately lead to hypertrophy and heart failure (Meij, J.T.A., *et. al.*, 1997). The significance of PLC in the proposed pathway leading to cardiac dysfunction is depicted in Figure 14 (Dhalla, N.S., *et. al.*, 1997). In addition, increases in the release of other phosphatidyl cycle coupled agonists during ischemia can also increase cell damage during reoxygenation of previously ischemic tissue (Van Heugten, H.A., *et. al.*, 1996). Furthermore, one of the responses of surviving cells to myocardial infarction is hypertrophic growth (Meij, J.T.A., *et. al.*, 1997). The role of PLC in proliferation and cell growth has been discussed previously. Thus, the role of PLC is multiple and complex. It is an important molecule implicated in proliferation, cell growth, hypertrophy, and ischemia.

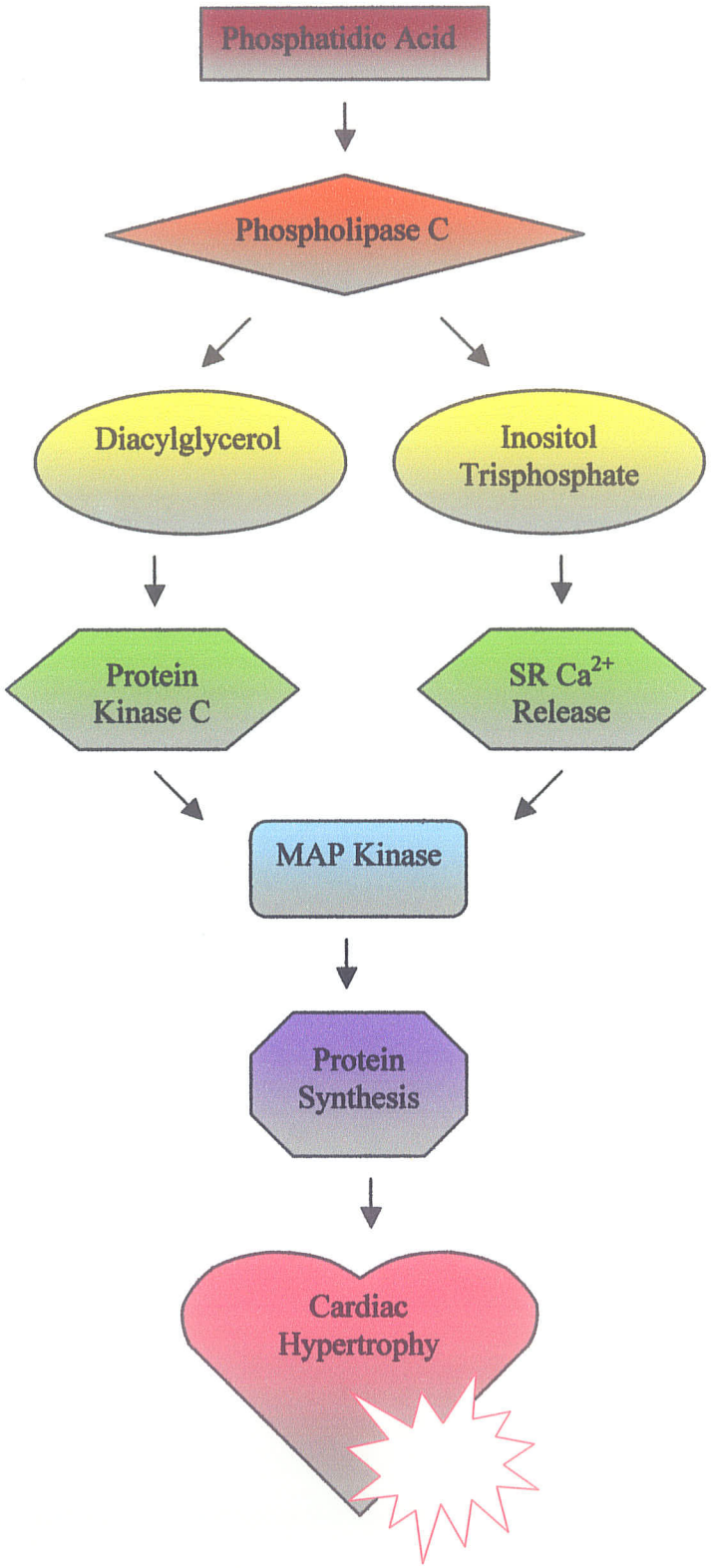


FIGURE 14. THE ROLE OF PLC IN CARDIAC HYPERTROPHY

7. DIABETIC CARDIOMYOPATHY

One of the most prevalent chronic conditions plaguing both the eastern and western worlds is diabetes mellitus. Diabetes is complex disease process, involving the whole body. The result is often large and small vessel disease and organ dysfunction, hyperglycemia, insufficient insulin and an inclination towards neuropathy and cardiovascular disease (Malhotra, A. and V. Sanghi, 1997). While the discovery of insulin helped to minimize deaths due to diabetic comas and infections, the death rate of the diabetic population has continued to increase. Research findings have implicated heart failure as the primary cause of deaths among diabetic patients (Palumbo, P.J. *et. al.*,1976). An important factor that may contribute to the increasing cardiovascular complications and death associated with diabetes is the phenomenon of the "silent" heart attack (Pierce, G.N. *et. al.,* 1988). Diabetic patients who suffer myocardial infarctions are often unaware of it because the infarct is painless. The implications of this silent killer (as diabetes is often referred to) are extremely serious. With the increasing incidence of cardiovascular complications and death it has become imperative to elucidate the underlying mechanisms involved.

A primary cardiomopathy is a disease of the muscles of the heart, existing independent of other external factors (Olsen, E.G., 1979, Pierce, G.N. *et. al.*, 1988). In 1972, the idea of a cardiomyopathy specific to diabetes mellitus was proposed by S. Rubler *et. al.*. By definition, this particular

cardiomyopathy was unrelated to the presence of macrovascular coronary artery disease and could in fact exist in the presence or absence of coronary disease, with or without diabetic nephropathy (LeWinter, M.M., 1996). Why does the heart fail in the diabetic patients? Lesions in the cardiovascular system have been implicated and include: major vessel disease (primarily as atherosclerosis), microvascular heart disease, a primary pathological disorder of the myocardium itself, and a neuropathic lesion (within the autonomic system) (Pierce, G.N., *et. al.*, 1988). The exact mechanisms of the subcellular changes underlying diabetic cardiomyopathy are not fully understood.

Research findings have suggested that structural abnormalities associated with diabetic cardiomyopathy include alterations in the vessels, interstitial tissue and as a result, the cardiomyocytes (LeWinter, M.M., 1996). Cardiomyocyte cellular and subcellular alterations include structural changes, myofibrillar changes resulting in alterations of the contractile apparatus within the heart cells, and perhaps changes in the cardiac sarcolemma and intracellular signalling. While all these factors are important to ascertaining the complete picture, this thesis will focus on the alterations of the cardiomyocytes and specifically at the membrane level.

Small and medium size vessel alterations can result in ischemic damage to myocardial cells. However, these types of lesions are absent in diabetics. This fact suggests that ischemia is not the only cause of alterations and or damage to the cardiac cells. Some other mechanism alone or in conjunction with other factors may be involved.

Alterations in the membrane lipids and intracellular signaling may have important consequences for the diabetic cardiomyocyte. A few of the implications have been described below.

7.1. Insulin Deficiency

Perhaps the most defining characteristic of chronic diabetes is resistance to insulin or insulin deficiency (Singer, W.D., *et. al.,* 1997, Smart, K., *et. al.*, 1995, Somlyo, A., 1998). In diabetic animal models both basal and insulin stimulated glucose uptake is greatly depressed. When the deficiency is severe, there is a concomitant decrease in the activation of cardiac glycogen synthase and phosphatase activity (van Corven, E., *et. al.*, 1992). Such metabolic changes in the diabetic heart become evident from pharmacological interventions such as carnitine and etomoxir both of which were found to improve cardiac function in chronic diabetes. These agents act by depressing the oxidation of free fatty acids and promote the utilization of glucose. Thus, excessive use of free fatty acids and the decreased use of plasma glucose play an important role in diabetic cardiomyopathy.

7.2. Alterations in Calcium Homeostasis

Calcium flux across the sarcolemmal membrane will determine force generation, alter metabolism, affect electrical activity and may cause necrotic developments if the calcium is in excess. Because in diabetes the normal flux of calcium is disturbed, cardiac performance is impaired. Several investigators

have reported changes in the ultrastructure in the heart in a wide variety of animal models including abnormalities in the sarcoplasmic reticulum (SR) and the SR Ca^{2+} transport system, which is the primary controller of intracellular calcium levels. As a result there is a depression of the pump activities. The inability of the heart to relax is due partly to the decrease in SR Ca^{2+} uptake. Other Ca^{2+} related abnormalities may include:

1. defects in the Na^{+}/ Ca^{2+} exchanger and Ca^{2+} pump, resulting in a intrcellular calcium overload (Ganguly, P.K. *et. al.*, 1983, Pierce, G.N. *et. al.*, 1983, Makino, N. *et. al.*, 1987)
2. a decrease in the sarcolemmal Na^{+}/ K^{+} ATPase and Ca^{2+} binding but an increase in SL Ca^{2+}/ Mg^{2+} ecto-ATPase

These changes, taken together, may have a great impact on the systolic and diastolic dysfunction associated with diabetic cardiomyopathy. Furthermore, studies where normalization of the SR Ca^{2+} pump activities resulted in partial improvement of contractility in the diabetic heart (Rodrigues, B. and J.H. McNeill, 1992).

7.3. Alterations in Contractile Apparatus

Chronic diabetes is associated with depressed activities of Ca^{2+} ATPase of the myofibrils, actomyosin and myosin. The force of contraction generated by the heart depends on the degree of myofibrillar ATPase activation by Ca^{2+} and depressions in these activities play a significant role in

the development of diabetic cardiomyopathy. Since depressed contractility cannot be completely corrected by normalization of the Ca^{2+} regulatory defects, it may be that contractile apparatus abnormalities such as the changes in the myosin heavy chain and/ or altered troponin Ca^{2+} sensitivity may also be involved (Schaffer, S.W. and M. Mozaffari, 1996). The decrease in activity may be due to alterations in myosin isoform composition and regulatory proteins and their phosphorylation (Henry, R.A., *et. al.*, 1995, Hemandez-Sotomayor, G and G. Carpenter, 1992, Hubner, S., *et. al.*, 1998, Hwang, S.C., *et. al.*, 1996). Animal models have shown a shift in myosin isozyme content from V1 to V3 (James, S., 1997, James, S.R. and C.P. Downes, 1997, James, S.R., *et. al.*, 1996). Furthermore, in terms of the myosin heavy chain, analysis has indicated a predominance of α- myosin heavy chain in controls but β-myosin heavy chain in the diabetic heart. In addition, myosin light chain, myosin light chain kinase and myosin light chain kinase phosphorylation (all of which are important for vertebrate striated muscle contraction) were all depressed in the diabetic rat model and were partially corrected by insulin.

Another important part of muscle contraction involves the troponin-tropomyosin complex (TnTm). The complex consists of 3 main troponin units; the TnC which binds Ca^{2+}, TnI which inhibits the ATPase and TnT which is the Tm binding unit. Tm and Tn are important to diabetes for two main reasons:

1. Phosphorylation of TnI and TnT by PKC results in a decrease in the actin-myosin interaction. The

sustained PKC activity in the diabetic heart may ultimately be responsible for the decrease in contractile force. In addition, similar phosphorylation by PKA may be associated with a decreased sensitivity of the myofibrillar Mg^{2+} ATPase to calcium. However this remains a controversial area (Pierce, G.N. and N.S. Dhalla, 1981).

2. Studies of the IDDM heart have demonstrated a significant shift from TnT1 to TnT2 and TnT3 isoforms (Goodale, W. T. and D. B. Hackel, 1983, Lee, S. and S.G. Rhee, 1995). This shift was later shown to coincide with a decrease in calcium sensitivity (Akella, A.B. *et. al.*, 1995) As a result there is an accompanied alteration in contractility. TnT and TnI are responsible for the Ca^{2+} sensitivity of the myofillaments.

III. MATERIALS AND METHODS

1. MATERIALS

[^{3}H] (4,5)P_2 inositol-2-3H (N)- 5.45 Ci/ mmole was purchased from DuPont Canada Inc./ New England Nuclear (Mississaugua, ON, Canada). Non-labelled PtdIns (4,5)P_2 triammonium salt was obtained from Calbiochem- Novabiochem Corporation (La Jolla, CA, USA). Cholic acid disodium salt was purchased from Sigma Chemical Company (St. Louis, Missouri, USA).

Mixed monoclonal antibovine antibody against phosphololipase C β1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein G Sepharose 4 Fast Flow was purchased from Amersham Pharmacia Biotech (Baie d'Urfe, QB, Canada). Dowex 1X-8 (formate form, 100-200 mesh) and goat anti- mouse IgG was obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). Polyvinylidene difluoride Western Blotting hydrophobic membrane and chemiluminescence reagents were obtained from Boehringer Mannheim (Laval, QB, Canada). High molecular weight markers were purchased from Bio-Rad GS 67, Hercules, CA, USA.

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 histolyticum* was bought from Worthington Biochemical Corp., (Lakewood, NJ, USA).

Microconcentrators were purchased from Centicon-3, Amicon Canada Ltd., (Oakville, ON, Canada).

Humulin (insulin zinc suspension prolonged, human biosynthetic (rDNA only) Ultralente) was obtained from Eli Lily Canada Ltd., (Oakville, ON, Canada). Kodak X-Omat X-ray flims and DuPont Cronex intensifying screen were purchased from Picker International (Highland Heights, OH, USA). CytoScint ™ ES* was obtained from ICN Biomedicals Inc., (Mississauga, ON, Canada). All other reagents were of the highest grade available or of analytical grade.

CHEMICALS USED

PRODUCT	*SOURCE*
acetic acid	Fisher- Scientific (Nepean, ON)
acetone	Fisher- Scientific (Nepean, ON)
acrylamide	Bio- Rad Laboratories (Canada) Ltd. (Mississaugua, ON)
alchohol	Mallinckrodt Inc. (St. Louis, MO)
ammonium persulfate	Bio- Rad Laboratories (Canada) Ltd. (Mississaugua, ON)
aprotinin	Sigma- Aldrich Canada Ltd, (Oakville, ON)
benchmark prestained protein ladder	Gibco/ BRL (Burlington, ON)
bovine serum albumin	Sigma- Aldrich Canada Ltd, (Oakville, ON)
buffer solution, pH 7.0	Fisher- Scientific (Nepean, ON)
calcium chloride	BDH Inc. (Toronto, ON)
carbon dioxide	Medigas (Winnipeg, MB)
chloroform	Fisher- Scientific (Nepean, ON)
cholic acid disodium salt	Sigma- Aldrich Canada Ltd, (Oakville, ON)
collagensase	Worthington Biochemical Corporation (Freehold, NJ)
copper sulfate $CuSO_4$-5 H_20	Sigma- Aldrich Canada Ltd, (Oakville, ON)
cytosine- B- D- arabinofuranoside	Calbiochem- Novabiochem (La Jolla, CA)
dowex	Bio- Rad Laboratories (Canada) Ltd. (Mississaugua, ON)
ECL kit	Amersham Pharmacia Biotech
EDTA	Sigma- Aldrich Canada Ltd, (Oakville, ON)

PRODUCT	*SOURCE*
fetal calf serum	Gibco/ BRL (Burlington, ON)
folin reagent	Sigma- Aldrich Canada Ltd, (Oakville, ON)
formic acid	Fisher- Scientific (Nepean, ON)
glucose	BDH Inc. (Toronto, ON)
glucose HK kit	Sigma- Aldrich Canada Ltd, (Oakville, ON)
glycine (electrophoreisis grade)	Sigma- Aldrich Canada Ltd, (Oakville, ON)
Hepes	Sigma- Aldrich Canada Ltd, (Oakville, ON)
histidine	Sigma- Aldrich Canada Ltd, (Oakville, ON)
Humulin U (insulin)	Ultralente (Indianapolis, IN)
imidazole	Sigma- Aldrich Canada Ltd, (Oakville, ON)
lauryl sulfate	Sigma- Aldrich Canada Ltd, (Oakville, ON)
laminin	Sigma- Aldrich Canada Ltd, (Oakville, ON)
leupeptin	Sigma- Aldrich Canada Ltd, (Oakville, ON)
magnesium chloride $MgCl_2$ -6 H_20	BDH Inc. (Toronto, ON)
Medium 99	Gibco/ BRL (Burlington, ON)
methanol	Anachemia (Rousse Point, NY)
MOPS/ KOH	Sigma- Aldrich Canada Ltd, (Oakville, ON)
nitrogen gas	Medigas (Winnipeg, MB)
OCT Tissue- Tek	Sakura Finetechnical Co., Ltd. (Tokyo, Japan)
oxygen gas	Medigas (Winnipeg, MB)

PRODUCT	*SOURCE*
penicillin/ streptomycin	Gibco/ BRL (Burlington, ON)
phosphate buffered saline PBS	Sigma- Aldrich Canada Ltd, (Oakville, ON)
PMSF	Sigma- Aldrich Canada Ltd, (Oakville, ON)
potassium hydroxide KOH	Mallinckrodt Inc. (St. Louis, MO)
protein G- sepharose 4 fast flow	Amersham Pharmacia Biotech (Baie d'Urfe, QB)
sodium chloride	Fisher- Scientific (Nepean, ON)
sodium cholate	Sigma- Aldrich Canada Ltd, (Oakville, ON)
sodium carbonate Na_2CO_3	Mallinckrodt Inc. (St. Louis, MO)
sodium dodecyl sulfate SDS	Sigma- Aldrich Canada Ltd, (Oakville, ON)
sodium hydroxide NaOH	BDH Inc. (Toronto, ON)
sodium pyrophosphate	Sigma- Aldrich Canada Ltd, (Oakville, ON)
sodium tartarate	Fisher- Scientific (Nepean, ON)
soybean-trypsin inhibitor	Sigma- Aldrich Canada Ltd, (Oakville, ON)
sucrose	Sigma- Aldrich Canada Ltd, (Oakville, ON)
temed	Bio- Rad Laboratories (Canada) Ltd. (Mississaugua, ON)
TRIS/ HCl	Sigma- Aldrich Canada Ltd, (Oakville, ON)
Trypsin	Gibco/ BRL (Burlington, ON)

ANTIBODIES USED

PRODUCT	*SOURCE*
Gqα (rabbit polyclonal IgG)	Santa Cruz Biotechnology (Santa Cruz, CA)
phospholipase C β1 (rabbit polyclonal IgG)	Santa Cruz Biotechnology (Santa Cruz, CA)

RADIOACTIVE COMPOUNDS USED

PRODUCT	*SOURCE*
^{3}[H]- phosphatidyl inositol 4,5-bisphosphate	NEN Life Science Products, Inc. (Boston, MA)

2. METHODS

2.1. INSULIN DEPENDENT DIABETES MELLITUS (IDDM) EXPERIMENTAL MODEL

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, Canada, and followed the established guidelines of the Canadian Council on Animal Care. The animals were housed in the Animal Holding facility of the St. Boniface General Hospital Research Centre and were provided food and water *ad libitum* during the entire course of the study. Ninety male- Sprague-Dawley rats weighing approximately 125-145 grams each were used in these studies (30 control, 30 diabetic, 30 insulin- treated diabetic). Diabetes was induced by a single tail vein injection of streptozotocin (STZ, 65 mg/ kg body weight) dissolved in 0.1 mol/L citrate buffer, pH 4.5, while aged matched controls received citrate buffer only. At the end of the 6th week after the STZ treatment, the diabetic animals were subdivided randomly into two groups. One group received 3 units Humulin U insulin zinc per day subcutaneously and the other group received a similar injection of saline alone for 14 days. At the end of the two week period 6 different experimental animal hearts from each of the following groups: control, diabetic and insulin- treated diabetic respectively, were processed for the isolation of cardiomyocytes and the hearts of the remaining 24 experimental animals from each of the groups were taken for subcellular fractionation to isolate sarcolemma and cytosol.

2.2. *ISOLATION OF CARDIAC SARCOLEMMA AND CYTOSOLIC FRACTIONS*

All isolation procedures were carried out at 4 °C. Twenty- four control, 24 diabetic and 24 insulin- treated diabetic total experimental animals were sacrificed by decapitation and the hearts were quickly excised and immersed in ice cold 0.6 mol/L sucrose, 10 mmol/L imidazole, pH 7.0 buffer. Atrial, macrovascular and connective tissue were carefully removed and the right ventricle was separated. The viable left ventricle tissue, including the interventricular septum, from 3-5 hearts was pooled (5 ml buffer/g tissue) to prepare the sarcolemmal and cytosolic fractions. Briefly, the tissue was washed, minced by hand and homogenized in 3.5 ml of buffer with a Polytron PT 3000 homogenizer (Kinematica AG, Switzerland) at setting 5 (13 000 RPM) for 6 x 10 seconds. Large particles were removed by centrifugation at 12 000 x g for 60 minutes at 4 °C. The pellet was discarded. A 500 μl aliquot of the supernatant was centrifuged at 100 000 x g for 60 minutes at 4 °C in a Beckman TL-100 Ultracentrifuge to remove any membrane fragments. The resulting fraction was frozen in liquid nitrogen and stored at -80 °C as the soluable cytosolic fraction. The rest of the supernatant was diluted with 20 mM 3-(morpholino)-propanesulfonic acid (MOPS), 300 mmol/ L KCl buffer (5 ml buffer/g tissue), pH 7.4 and centrifuged at 100 000 x g for 60 minutes to solubilize myofibrillar proteins. The resulting pellet was re-suspended in 0.25 mol/L sucrose, 10 mmol/L histidine, pH 7.4, and layered over a 30% sucrose solution containing 0.3 M KCl, 50 mM $NaPO_4O_7$ and 0.1 M Tris-HCl, pH 8.3. The solution was centrifuged in a Beckman swinging bucket rotor (SW 28) at

100 000 x g for 90 minutes. The band at the interface of the sucrose and buffer was taken and diluted with 3 volumes of 140 mM KCl, 20 mM MOPS, pH 7.4. The final pellet was then centrifuged for 30 minutes at 100 000 x g. It was re-suspended in 0.25 M sucrose, 10 mM histidine, pH 7.4 (225 μl/g tissue). The resulting fraction was the sarcolemmal enriched fraction. It was divided into aliquots, frozen in liquid nitrogen and stored at -80 °C until needed.

2.3. PHOSPHOINOSITIDE SPECIFIC PHOSPHOLIPASE C ASSAY

PLC activity was assayed as described previously by Meij and Panagia (1992). To prepare the substrate, an aliquot of [^{3}H]-PtdIns (4,5)P_2 was mixed with an aliquot of stock solution, in chloroform, of the cold substrate. The mixture was evaporated under a continuous stream of N_2 and re-dissolved in 10% Na-cholate (w/v) (232 mM). The substrate solution was kept under the N_2 gas overnight at 0- 4 °C. Prior to using, it was diluted to 160 μM substrate/ 112 mM Na-cholate. 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 $CaCl_2$, 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. The samples were incubated at 37 °C for 2.5 minutes and the reaction was terminated by the addition of 144 μl of ice cold chloroform: methanol: HCl (1:2:0.2 v/v). Phases were separated by adding 48 μl of 2 M KCl and 48 μl of chloroform. After mixing for 30 seconds and a 5 minute centrifugation at 15 000 x g (Hereau Sepatech Contifuge 28 RS), the upper phase was aspirated

and applied to a 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 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 (Ins 1 P), 0.4 M sodium 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) with 10 volumes of CytoScint ™ ES*.

2.4. IMMUNOPRECIPITATION AND MEASUREMENT OF PLC β1

Sarcolemmal membrane proteins were extracted using buffer containing 1% w/v Na- cholate, 50 mmol.L HEPES (pH 7.3), 200 mmol/ L NaCl, 2 mmol/L EDTA, 10 μg/mL PMSF, 10 μg/mL leupeptin, by rotation for 2 hours and the supernatant recovered as the solubilized membrane fraction. The membrane fraction was incubated overnight at 4 °C (rotation) with monoclonal PLC β1 antibody (5ug of antibody: 350 μg membrane extract, i.e. a ratio of 1:70 μg/ μg). The immunocomplex was captured by adding 100 μl of washed Protein G Sepharose bead slurry (50 μL packed beads) at 4 °C by rotation for 2 hours. The agarose beads were collected by pulse centrifugation (5 seconds) at 10 000 x g and assayed for the activity of PLC β1 isoenzyme. The hydrolysis of [^{3}H]- PtdIns (4,5)P_2 was measured as described by Tappia *et. al.* (1999). The reaction was carried out in the presence of 30 mmol/l HEPES (pH 6.8), 70 mmol/L KCl, 100 mmol/L NaCl, 0.8 mmol/L EGTA, 0.8

mmol/L $CaCl_2$ (free Ca^{2+} 23.3 μmol/L), 20 μmol [^{3}H]- PtdIns (4,5)P_2 (400-500 dpm/μl) disolved in 14 mmol/L Na-cholate overnight and an aliquot (10μL) of immunoprecipitate suspension. The reaction was performed at 37 °C for 2.5 minutes, after which it was stopped by trichloroacetic acid precipitation. Precipitates were removed by centrifugation at 10 000 x g for 5 minutes, and the supernatant was collected for quantification of inositol phosphates by liquid scintillation counting. The efficacy of the immunoprecipitation of the isoenzymes was ascertained by determining any residual PLC β1 activity in the 10 000 x g supernatant after capturing the immunocomplex by protein G Sephrose. The supernatant was concentrated to 100 μL by using microconcentrators and then tested for PLC β1 activity. The immunoprecipitation was complete as PLC dependent [^{3}H]- PtdIns (4,5)P_2 hydrolysis of any precipitated isoenzyme could not be detected in the supernatant. For control experiments, immunoprecipitation and subsequent activity measurements were conducted with non-immune mouse IgG.

2.5. WESTERN BLOT ANALYSES

High molecular weight markers and 20 μg of SL or cytosolic proteins were separated on sodium dodecyl sulfate- polyacrylamide gel electrophoresis (10% gels) (SDS-PAGE). Separated proteins were transferred electrophoretically onto microporous (0.45 μm) polyvinylidene difluoride (PVDF) hydrophobic membranes that had been previously treated briefly by immersing them in methanol for a few minutes and then soaked with transfer

buffer for (20% v/v methanol, 0.192 M glycine and 25 mM tris, pH 8.0). The transfer was performed at 100 volts for the 1 hour using a mini trans blot cell. PDVF membrane was blocked overnight at 4 °C, with gentle agitation, in the 10 mL Tris- buffered saline with 0.1% Tween 20 (TBS-T) containing 5% skim milk and probed with primary PLC β1 isoenzyme antibody for 1 hour. Primary antibody was diluted in TBS-T (1:200). After washing with TBS-T for 15 minutes, followed by 3 fast washes at 5 minutes each, horseradish peroxidase (HRP)-labelled anti-mouse IgG was diluted 1:3000 in TBS-T and used as secondary antibody. After the 1 hour incubation with secondary antibody, the membrane was washed again for 15 minutes with TBS-T followed by 3 quick washes for 5 minutes each. PLC β1 was visualized by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Boehringer Mannaheim). Autoradiographs from the Western Blot were quantified using a CCD camera imaging densitometer (BioRad GS 67) (Bio-Rad, Hercules, CA, USA).

2.6. IMMUNOFLUORESCENCE

Five rats from each of the respective groups: control, diabetic and insulin- treated diabetic, were used for this assay. Rats were sacrificed and hearts were quickly excised and the right ventricle was removed. The whole heart with the viable left ventricle was immersed in OCT compound (Miles Inc.) and stored at -80 °C. Serial cryostat sections, 7 μm thick, were sliced and mounted onto gelatin coated slides, prefixed in 4 % paraformaldehyde and air

dried. Six to twelve sections were obtained from each group and the representative sections were chosen. Immunohistochemical staining was performed by the indirect immunofluorescence technique: Phospholipase C β1 antibody at 0.926 μg/ μl was diluted 1:100 with 1% BSA in PBS and applied as the primary antibody. Sections were incubated overnight at 4 °C. The sections were then fixed with 1% PBS for 20 minutes and incubated with primary antibody and then incubated with biotinylated anti-mouse IgG secondary antibody conjugated to the FITC, followed by incubation with FITC- labelled streptavidin (Amersham Life Sciences Inc., Canada) for 90 minutes. The fluorescent images of the FITC were obtained using a Nikon Diaphot 300 epifluorescence microscope connected to a Bio-Rad MRC-600 ultraviolet confocal system. The cells were excited with a 488 nm laser line and the emission was collected at 520 nm (the FITC fluorescence). The images were obtained using a Nikon Fluor X 40 (numerical aperature 1.3) oil immersion. For Gqα immunohistochemistry similar slices of heart (7μm thick) were obtained using a cryostat and mounted onto gelatin coated slides, prefixed in 4% paraformaldehyde and air dried. Six to twelve sections were obtained from each group and the representative sections were chosen. Immunohistochemical techniques were used to observe specific localization of Gqα. Primary antibodies were diluted (1: 500), applied to tissue and incubated at 4 °C overnight as previously described by H. Ju *et. al.*, (1998). Confocal microscopy was employed to visualize fluorescence.

2.7. ISOLATION OF CARDIOMYOCYTES

Isolation of adult cardiomyocytes was done as described by H. M. Piper *et. al.* (1988). Six male Sprague- Dawley rats were taken from each of the control, diabetic, and insulin- treated diabetic groups respectively, 8 weeks old after STZ injection, weighing 200- 250 grams each, were sacrificed. The atria were removed and the ventricle was mounted on the Langendorff apparatus. The heart was first perfused with calcium free Kreb's solution containing (mmol/ L) 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose, pH 7.4 and gassed with a 5% CO_2 and 95% O_2 mixture. After 10 minutes of perfusion, the perfusate was switched to 0.1% (w/v) collagenase solution containing 0.1% (w/v) bovine serum albumin, fraction V (BSA), and 25 μmol $CaCl_2$. After a 60 minute re-circulation period, the heart was removed from the canula and placed in a pre-warmed Kreb's 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 x g for 2 minutes. The supernatant was then removed and the cells were re-suspended in warm Kreb's containing 1% (w/v) BSA and 50 μmol $CaCl_2$. It was centrifuged again at 1.7 x g for 2 minutes. This procedure was repeated, re-suspending each in warm Kreb's containing 1% BSA (w/v) and increasing $CaCl_2$ concentrations (at 200 μM $CaCl_2$ and then at 500 μM $CaCl_2$). Finally, cells were re-suspended in warm Kreb's solution containing 4% (w/v) BSA and 1 mM $CaCl_2$ and then centrifuged at 6.8 x g for 2 minutes. The cell pellet was then re-suspended in Medium-199 (M199)

containing 0.2% (w/v) BSA, 4% (v/v) fetal calf serum (FCS), 1% penicillin and streptomycin and plated out onto laminin coated 100 mm petri dishes at approximately 1 x 10^6 cells/ plate. After a period of 3 hours, the cells were placed into a 5% CO_2 humidified incubator at 37 °C for 18- 20 hours.

2.8. MYOCYTE STIMULATION AND FRACTIONATION

After 18- 20 hours, cells were stimulated for 10 minutes with 30 μM PtdOH. The incubation period was terminated by removal of the medium by aspiration and placing the petri dishes immediately on ice. Cells were then scraped off the plates in 1 mL of 10 mM HEPES (pH 7.2) containing 2 mM EDTA and 10% sucrose. The cells were then collected by centrifugation at 27.2 x g for 1 minute and processed for isolation of the cytosol. Cells were homogenized manually (20 strokes) in 1 mL of the buffer indicated above using a glass homogenizer. The cells were centrifuged at 280 000 x g for 25 minutes. The supernatant was collected and assigned as the cytosolic fraction. The pellet was re-suspended and homogenized in the aforementioned buffer and designated as the particulate fraction.

2.9. PTDINS 4- KINASE AND PTDINS 4P, 5- KINASE ASSAY

PtdIns 4- kinase and PtdIns 4*P,* 5-kinase activities were assayed as described by X. Liu *et. al.*, (1997). Thirty μg of SL protein was pre-incubated for 30 minutes at 30 °C in 100 μl 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 PtdIns 4*P*, 5- kinase was started by the addition of [γ-^{32}P] ATP in a final concentration of 1 mM (0.16 Ci/ mmol). After one minute the reaction was terminated by the addition of 2 ml of ice cold methanol: 13 N HCl (100:1 v/v), and vortexing for 10 seconds. To extract the phosphoinositide, 1 ml of 2.5 N HCl and 2 ml of chloroform were added. The tubes were vortexed for 2 minutes and centrifuged at 1000 g for 10 minutes. The aqueous phase was discarded and the chloroform phase was washed with 2 ml of chloroform: methanol: 0.6 N HCl (3: 48: 47 v/v/v). A second vortex and centrifuge was done. The final chloroform phase was then removed and an aliquot was evaporated to dryness under a continuous nitrogen stream. The residue was immediately re-dissolved 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 ^{o}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 wash was applied again 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/v/v/v) as described by J. Jolles *et. al.*, (1981). After the solvent front had migrated for approximately 1-2 centimetres from the top, the pates were air dried at room temperature. The ^{32}P- labelled phospholipid spots were visualized by overnight autoradiography using X-Omat-R X- ray film and DuPont Cornex

intensifying screen. Phosphoinositide species were identified in accordance with the methods of previous workers (Liu, X., *et. al.*, 1997). PtdIns 4*P* and PtdIns (4, 5)P_2 were scraped off the plates. 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. To determine the PtdIns 4*P*, 5- kinase, exogenous PtdIns 4*P* was added to the assay system. The exogenous PtdIns 4*P* was prepared by ultrasonication in a water sonicator (Branson 1200 sonicator) for 30 minutes and then added to the assay mixture before the pre-incubation at a final concentration of 25 μM.

2.10. PROTEIN DETERMINATION

Protein concentrations of the SL and myocyte cytosol fractions were determined according to O. H. Lowry *et. al.* (1951). Bovine serum albumin was used as the standard.

2.11. STATISTICAL ANALYSES

Experiments were carried out in triplicate or quadruplicate unless otherwise indicated. All values are expressed as mean $\pm$ SEM. The differences between the two groups were evaluated by one way analysis of variance (ANOVA) followed by Student- Newman- Keuls or the Duncan multiple comparison test. A probability of 95% or more ($P< 0.05$) was considered significant.

IV. RESULTS

1. GENERAL CHARACTERISTICS OF THE DIABETIC ANIMALS

Diabetes was induced in the animals by a single tail vein injection of streptozotocin (STZ) as described in the Materials and Methods section. Eight weeks after the injection the general characteristics of these animals were recorded and presented in Table 1. To confirm that diabetes had been successfully induced by the STZ- injection, plasma glucose levels were measured and found to be significantly elevated. In addition, to further confirm the diabetes, the levels of circulating insulin and thyroxine (T4) were both found to be significantly decreased. Similar values were obtained in previous studies employing the same animal protocol (Pierce, G.N. and N.S. Dhalla, 1983, Makino, N. *et. al.*, 1987). After treatment with insulin, these parameters were returned nearly to control levels.

After the eight weeks, the body weights and the left ventricular (LV) weights of the diabetic animals were significantly lower than the control values. The left ventricular to body weight ratios in the diabetic animals were significantly higher than those of the age matched control animals. After two weeks of insulin therapy (beginning at the end of the 6^{th} week to the end point of the study), the insulin treated diabetic group had higher body weights and ventricular weights than the untreated diabetic group. However, these parameters were only partially normalized with insulin therapy.

TABLE 1. GENERAL CHARACTERISTICS OF THE DIABETIC ANIMALS

Characteristics	Age Matched Controls	Diabetic	Insulin Treated Diabetic
Body Weight, g	428.8 ± 4.12	238.1 ± 5.67*	301.6 ± 1.9*
Ventricular Weight, g	1.34 ± 0.04	0.77 ± 0.01*	0.86 ± 0.01*
Ventricular/ Body Weight Ratios, mg/ g	2.74 ± 0.03	3.59 ± 0.07	3.10 ± 0.04*
Plasma Glucose, mg/ dL	162.2 ± 1.6	479.3 ± 10.7*	198.4 ± 6.6
Serum Insulin, pmol/	169.7 ± 5.7	41.2 ± 2.2*	150.1 ± 9.6
Serum T4, nmol/ L	150.3 ± 2.5	47.1 ± 1.9*	135.7 ± 2.3

Values are expressed as mean + SEM of 12- 15 experiments. Animals were treated as described in the Materials and Method section. Diabetes was induced by a single tail vein injection of streptozotocin. Blood samples were taken at the time of sacrifice and analyzed for insulin, glucose, and thyroxine as described in the Materials and Methods section.

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

2. *CHARACTERISTICS OF THE PURIFIED SARCOLEMMAL MEMBRANES*

The sarcolemmal membranes obtained in this study were highly enriched in marker enzyme activities typical for this membrane fraction (i.e. Na^+-K^+ ATPase, Na^+-Ca^{2+} exchange (Williams, S.A. *et. al.*, 1998). From earlier studies, it has been reported that the values of the relative specific activity (specific activity in the SL/ specific activity in the homogenate) for Na^+- K^+ ATPase (SL marker) and rotenone- insensitive NADPH- cytochrome *c* reductase (SR marker) indicated an equal amount of enrichment (15-fold) of the SL membrane in control and experimental SL preparations, with only minimal contamination (< 5%) by other subcellular fragments (Williams, S.A. *et. al.*, 1998).

3. *IN VITRO FORMATION OF DIFFERENT INOSITOL PHOSPHATE SPECIES BY SARCOLEMMAL PHOSPHOLIPASE C IN DIABETIC CARDIOMYOPATHY*

Phosphoinositide specific phospholipase C (PLC) can hydrolyze phosphoinositide 4,5 bisphosphate (PtdIns (4,5)P_2) to yield inositol 1, 4, 5 trisphosphate (Ins (1,4,5)P_3) and *syn*- 1,2 diacylglycerol (DAG) (Meij, J.T. and V. Panagia, 1992). While phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)P_2) is the preferred substrate, PLC also hydrolyzes phosphatidylinositol 4-phosphate (PIP) to give inositol 1,4-bisphosphate (IP_2) and PI to give IP_1. Thus a single reaction of PLC can potentially yield all three products. In this study, purified SL membranes obtained from the ventricles of control, diabetic

and insulin treated diabetic animals were used to assess the activity of PLC. Specifically, we wanted to determine if there was a shift in substrate preference in the diabetic disease state. In Table 2, the PLC derived formation of three different inositol phosphates in these experimental groups is recorded. The major product of this assay condition, Ins(1, 4, 5)P_3, was significantly decreased in the diabetic heart when compared to the control hearts. After insulin treatment for 14 days, the Ins(1, 4, 5)P_3 levels were normalized to control levels.

TABLE 2. *IN VITRO* FORMATION OF DIFFERENT INOSITOL PHOSPHATE SPECIES BY CARDIAC SARCOLEMMAL PHOSPHOLIPASE C IN DIABETES

EXPERIMENTAL GROUPS	Inositol Phosphate Species (nmol/min/ mg protein)		
	Ins(4)*P*	Ins(1,4)*P2*	Ins(1,4,5)*P3*
Control	0.18 + 0.00	1.15 + 0.02	7.32 + 0.08
Diabetic	0.18 + 0.03	0.54 + 0.02*	6.61 + 0.15*
Insulin Treated Diabetic	0.20 + 0.01	0.72 + 0.04**	8.11 + 0.48**

Sarcolemmal PLC activity was assayed under the standard conditions as described in the Materials and Methods Section. in the presence of 20 uM [^{3}H]-PtIns(4,5)*P2*. Values described are means + SEM of inositol phosphate formation in three experiments. Assays were performed in triplicate. Abbreviations: Ins(4)*P* corresponds to inositol 4-phosphate; Ins(1,4)*P2* corresponds to inositol 1,4-bisphosphate; Ins(1,4,5)*P3*corresponds to inositol 1,4,5-trisphosphate.

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

** Significantly different (P<0.05) from corresponding diabetic values.

4. 1. EFFECT OF PHOSPHATIDIC ACID ON THE TOTAL SARCOLEMMAL PHOSPHOLIPASE C ACTIVITY OF DIABETIC HEARTS

Phosphatidic acid (PtdOH) is an important mediator of many cellular functions. It can be synthesized by phosphorylation of DAG. Phosphatidic acid can increase via PLC stimulation, by *de novo* synthesis or through direct activation of the PLD pathway by several agonists (Shukla, S.D. and S.P. Halenda, 1991). Phosphatidic acid has been demonstrated to influence cardiac function in many ways. For example, phosphatidic acid can increase intracellular Ca^{2+} levels, thereby stimulating cardiac contractility. Phosphatidic acid can also stimulate the production of IP_3 via activation of the PLC pathway. Previously, it has been observed that intramembranal levels of phosphatidic acid in the diabetic heart are decreased (Williams, S.A., *et. al.*, 1998). In this experiment we wanted to assess if the sensitivity or responsiveness of PLC to phosphatidic acid was altered in the diabetic and insulin treated diabetic conditions in order to compensate for the decreased membrane levels of phosphatidic acid. Phosphatidic acid has been shown to activate PLC γ1 and PLC δ1 isoenzymes. However, no information is presently available in the literature in terms of PLC β1 activity in response to PtdOH.

In Table 3 the effect of PtdOH on total PLC activity is recorded. The basal activity of total phospholipase C in diabetes was significantly depressed when compared to the control samples. After insulin therapy for two weeks, the basal activity level of total PLC was normalized to

the control values. In the presence of 25 μM PtdOH, PLC hydrolysis of PtdIns(4,5)P_2 was significantly increased in the control, and diabetic groups when compared to basal levels. After insulin treatment for 14 days, the Ins(1,4,5)P_3 levels were corrected beyond control values.

TABLE 3. 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 (25 μM)	% of Basal
Control	8.01 ± 0.16	10.7 ± 0.31**	134 ± 3
Diabetic	5.93 ± 0.19*	10.4 ± 0.87**	175 ± 9*
Insulin- Treated Diabetic	8.14 ± 0.51***	11.96 ± 0.81**	146 ± 5***

Sarcolemmal PLC activity was assayed under standard conditions (as described in the Materials and Methods section) in the absence and presence of 25 μM phosphatidic acid. Values obtained are the means ± SEM of three experiments done in triplicate. Abbreviations: PtdOH corresponds to phosphatidic acid; Ins*P*s corresponds to total inositol phosphates (inositol 4-phosphate, inositol 1,4-biphosphate inositol 1,4,5-trisphosphate).

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

** Significantly different ($P<0.05$) from the corresponding basal values.

*** Significantly different ($P<0.05$) from the corresponding diabetic values.

4.2. ACTIVITY OF PLC β1 IN CARDIAC SARCOLEMMAL MEMBRANES FROM CONTROL, DIABETIC AND INSULIN TREATED DIABETIC RATS

Table 4 identifies the lesions in total PLC activity in diabetic rat cardiac sarcolemmal membranes. However it is not clear which one of the PLC isoenzymes is affected. We examined cardiac sarcolemmal PLC β1 because in the diabetic state it is not presently known whether the α1 adrenoreceptor activity is increased or decreased. The α1 adrenoreceptor is linked to PLC β1 via Gqα. We wanted to investigate if the status of the PLC β1 enzyme was altered in diabetes. Figure 15 depicts the quantified analysis of PLC β1 activity in control, diabetic and insulin- treated diabetic animals both in the presence and absence of phosphatidic acid. Figure 15 also illustrates that in the non- phosphatidic acid treated sarcolemma, the PLC β1 activity is significantly depressed in the diabetic condition when compared to the control sarcolemma. After two weeks of insulin treatment, the PLC β1 activity was partially corrected. When treated with phosphatidic acid, the lesion was further corrected. Phosphatidic acid significantly stimulated PLC β1 activity in the diabetic sarcolemma as well as in the insulin treated diabetic sarcolemma. Interestingly however, the phosphatidic acid did not produce any stimulation of PLC β1 in the control sarcolemma.

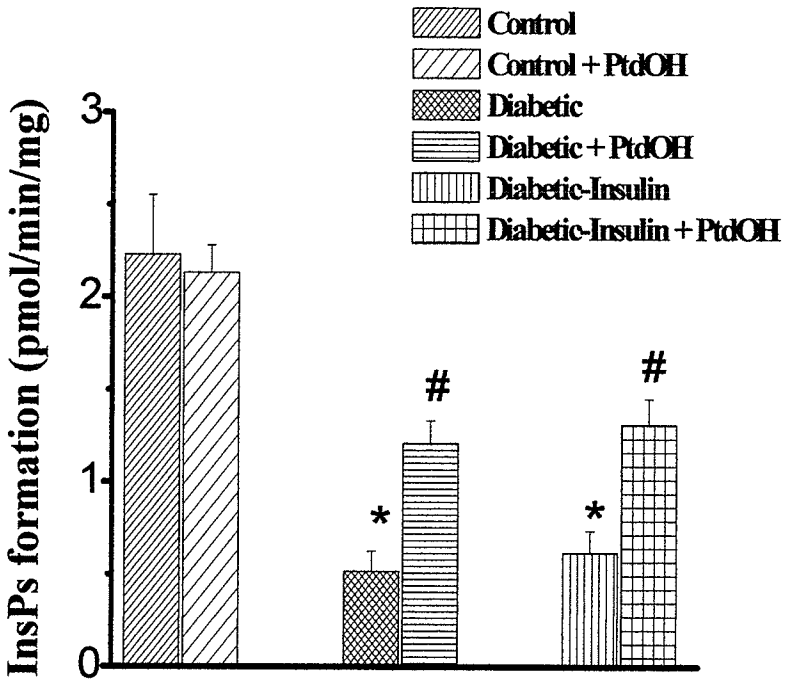


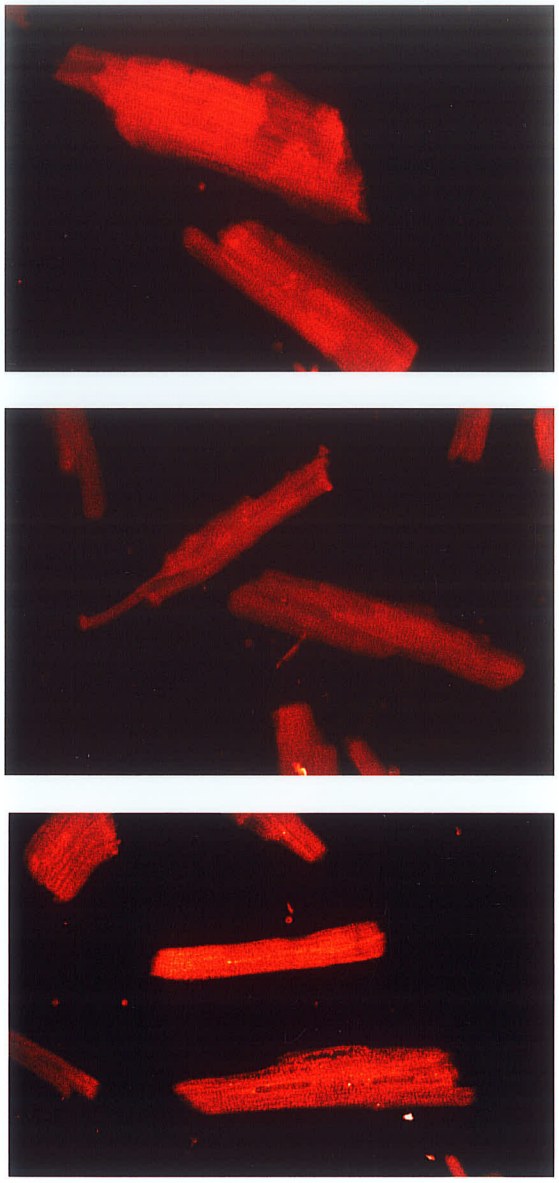
FIGURE 15. ALTERATIONS IN CARDIAC SARCOLEMMAL PLC β1 ACTIVITY IN THE PRESENCE AND ABSENCE OF PHOSPHATIDIC ACID

PLC β1 activity was assayed in the presence and absence of 25 μM phosphatidic acid (PtdOH) as described in the Materials and Methods section. Values are expressed as means ± SEM of 6 experiments. Assays were performed in triplicate.

* Significantly ($P < 0.05$) different from control value in the abscence of PtdOH.
\# Significantly ($P < 0.05$) different from control value in the prescence of PtdOH.

5. *ALTERATIONS IN CARDIAC PLC β1 IMMUNOPROTEIN EXPRESSION*

PLC isoenzymes are present in both the membrane and cytosol of cardiomyocytes. Figure 16 depicts photomicrographs of immunoreactive PLC β1 protein. In the control sample, the distribution of the PLC protein appeared as a diffuse staining pattern in the cytosol, when compared to the control cells. The intensity of the PLC β1 immunoprotein staining was profoundly diminished in the diabetic cardiomyocytes. After 14 days of insulin therapy, the staining intensity of total PLC β1 protein appears to have increased to levels similar to or perhaps slightly more than that of control cells. To confirm the observations made under the confocal microscope, Western Blot analysis was also done. Densitometric analysis of the immunoreactive PLC β1 protein bands is recorded in Figure 17. The PLC β1 Western blots reveal a significant decrease in protein levels within the diabetic cytosol when compared to the control sample. This decrease was normalized by insulin treatment. In contrast to the cytosol, in the diabetic sarcolemma, the protein level was significantly increased when compared to the control sarcolemma. After 14 days of insulin treatment, the sarcolemmal PLC β1 protein level was returned to that of the control value. It would appear, therefore, that the diabetic state induced a movement of PLC β1 from the cytosol to the sarcolemmal membrane.



A. Control

B. Diabetic

C. Insulin- Treated Diabetic

FIGURE 16. IMMUNOPROTEIN EXPRESSION OF PLC β1 IN CONTROL, DIABETIC AND INSULIN- TREATED DIABETIC CARDIOMYOCYTES.

The PLC β1 enzyme appears fluorescent against a red background to enhance the contrast. Panel A depicts the control cardiomyocytes. Panel B depicts the diabetic cardiomyocytes. Panel C depicts the insulin- treated diabetic cardiomyocytes.

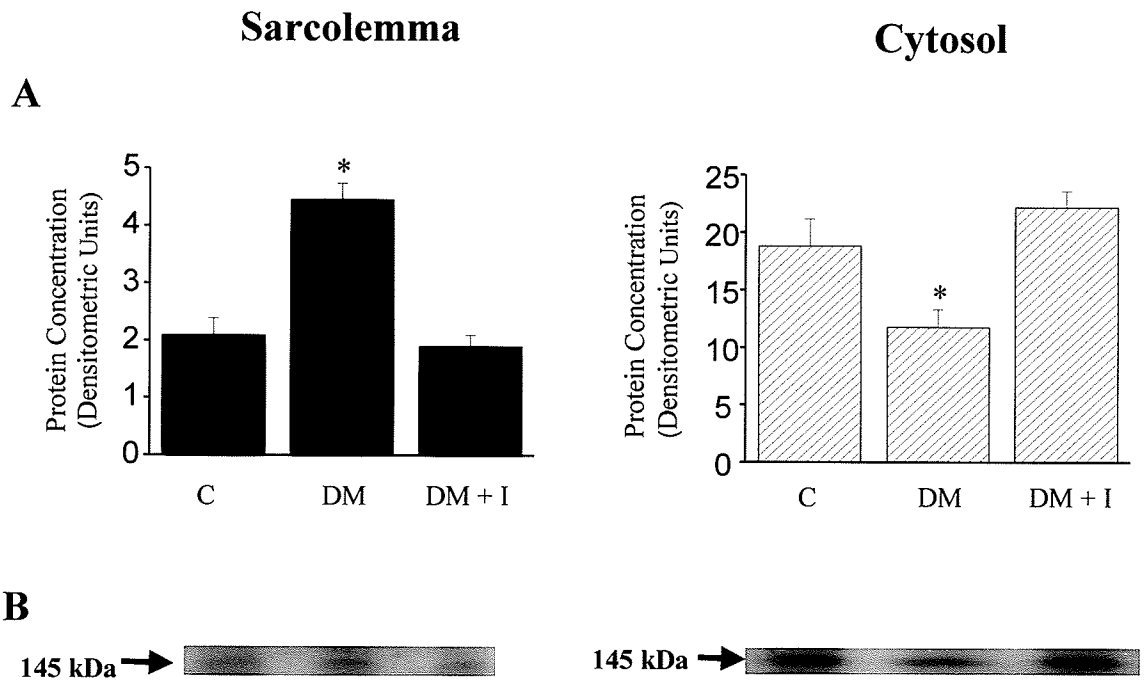


FIGURE 17. DENSIOMETRIC ANALYSIS OF PLC β1 IN THE SARCOLEMMA AND CYTOSOL OF CONTROL, DIABETIC AND INSULIN- TREATED CARDIOMYOCYTES.

A. Quantification of the protein bands obtained via Western Blot analysis
B. Representive blots are depicted in B. Quantification of the protein bands was carried out from a number of separate experiments (n= 3) and the results are presented in A.

C- control DM- diabetes mellitus DM+I- insulin-treated diabetes mellitus.
* Significantly ($P < 0.05$) different from control values.

6. *IMMUNOFLUORESCENCE OF PLC β1 IN THE PHOSPHATIDIC ACID STIMULATED CARDIOMYOCYTES*

Immunofluorescence techniques were used to localize the distribution of PLC β1 within isolated cardiomyocytes in the absence and presence of phosphatidic acid. Confocal images of this experiment are depicted in Figure 18. Panels A, C, and E depict photomicrographs of control, diabetic and insulin treated diabetic cardiomyocytes respectively, in the absence of phosphatidic acid. The PLC β1 enzyme (colored yellow for contrast) seems to be evenly distributed in the control panel. In the diabetic cells (panel C), the intensity of the enzyme staining appears to have decreased throughout the cytosol. After insulin treatment (panel E), the intensity of the PLC β1 staining appears to have increased and reached an even higher level than the control sample. Upon stimulation with 25 μM phosphatidic acid, the PLC β1 levels in control cardiomyocytes (panel B) appears to have intensified diffusely throughout the cytosol when compared to the non- phosphatidic acid stimulated control cells in panel A. In the diabetic cardiomyocytes stimulated with 25 μM phosphatidic acid (panel D), PLC β1 staining intensity appears to be similar to levels observed in non- phosphatidic acid diabetic cells (panel C). Finally, panel F shows that insulin treated diabetic cardiomyocytes appear to have no change in level of PLC β1 staining intensity at the level of the cytosol. Interestingly, the greatest change observed was seen at the periphery of these cardiomyocytes. The intensity of the PLC β1 staining was brightest at

the top and bottom ends of the cell. It may be that the PLC $\beta 1$ levels are localized at the intercalated discs.

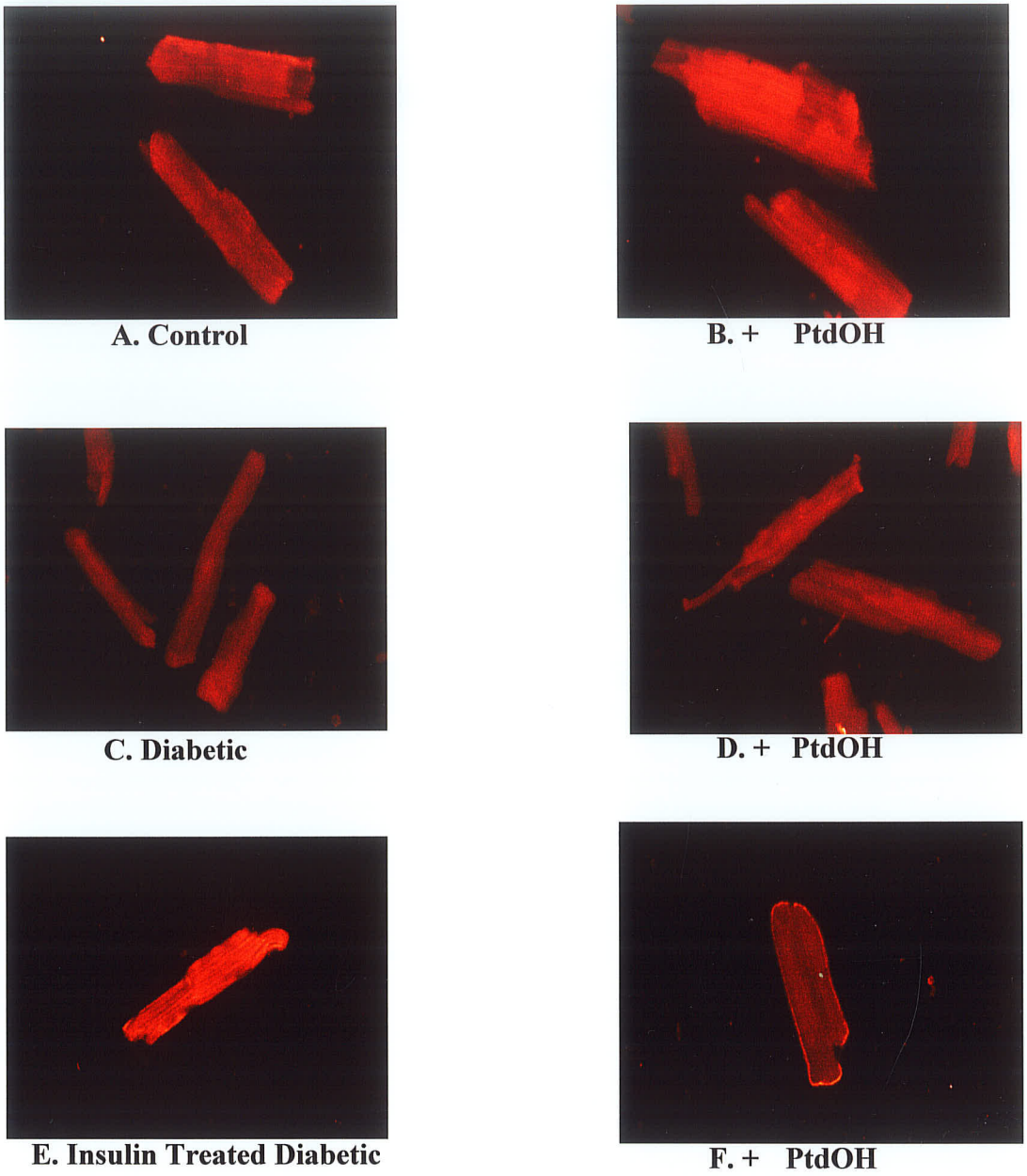


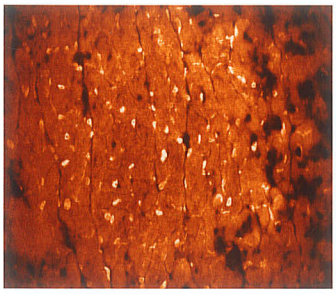
FIGURE 18. IMMUNOFLUORESCENCE OF PLC β1 IN THE PHOSPHATIDIC ACID STIMULATED CARDIOMYOCYTES.

The PLC β1 antibody is represented by the fluorescent yellow. A red background was used to enhance the contrast. Panels A, C, and E depict control, diabetic and insulin treated diabetic cardiomyocytes in the absence of phosphatidic acid. Panels B, D, and F depict control, diabetic and insulin treated diabetic cardiomyocytes in the presence of 25 μM phosphatidic acid respectively. Images were obtained as described in the Materials and Methods section. The fluorescent photomicrographs were obtaine using a Nikon Diaphot 300 epifluorescence microscope connected to a Bio- Rad MRC – 600 ultraviolet confocal system. The cells were excited with a 488 nm laser line and the emission was collected at 520nm. The images were obtained using a Nikon Fluor X 40 (numerical aperature 1.3) oil immersion.

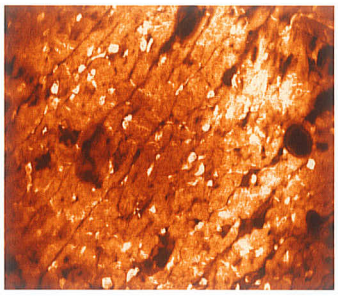
7. IMMUNOFLUORESCENCE OF Gqα

Earlier in the Review of Literature, it was noted that Gqα transduces the α 1 adrenoreceptor signal to PLC β1. In order to assess its influence on PLC β1 activity and localization, immunofluorescence techniques were used. These techniques helped to localize Gqα protein distribution and to quantify expression. Tissues samples were obtained from control, diabetic and insulin treated diabetic hearts in longitudinal and cross sections. As shown in Figure 19, the distribution of the immunoreactive Gqα protein is visualized. In both the longitudinal and cross- sections of control tissues, a punctate distribution of the Gqα protein was observed at the periphery of the cells. In the diabetic heart tissue, the Gqα staining appeared to be intensified in both the longitudinal and cross- sections. In samples taken from animals with diabetes who received 14 days of insulin treatment, staining of the Gqα protein appeared to have decreased, returning to levels similar to control levels.

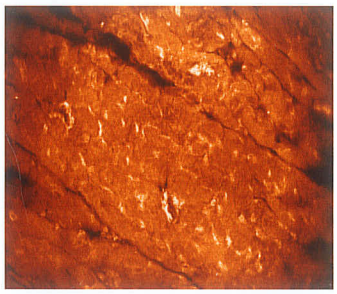
A. Longitudinal - section



Control

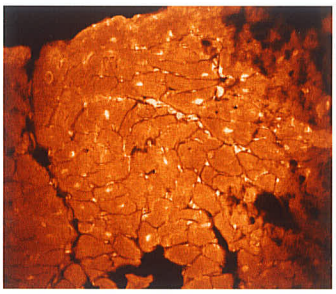


Diabetic

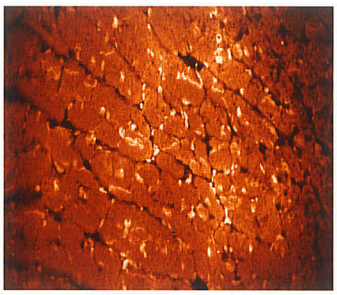


Diabetic - Insulin Treated

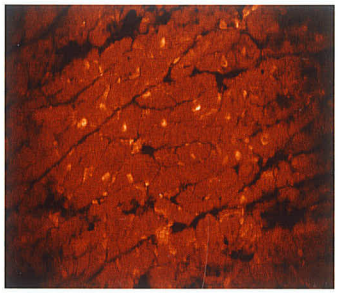
B. Cross - section



Control



Diabetic



Diabetic - Insulin Treated

FIGURE 19. IMMUNOFLUORESCENCE OF Gqα AS OBSERVED IN LONGITUDNAL AND CROSS SECTION.

The bright white dots represent the Gqα protein. An orange- brown background was used to enhance the contrast. Control, diabetic and insulin- treated diabetic hearts were frozen and sliced at 7 μm widths with a cryostst as described in the Materials and Methods section. Immunohistochemistry techniques were employed to obtain the pictorial distribution of the Gqα protein.

8. *SARCOLEMMAL PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE PHOSPHOINOSITIDE KINASES ACTIVITY IN DIABETIC HEARTS*

As discussed previously in the Review of Literature, the preferred physiological substrate for the phosphoinositide specific PLC is PIP_2. PIP_2 is synthesized via reactions involving PtdIns kinases. These kinases have both been reported to be localized primarily in the sarcolemma (Quist, E.E., *et. al.,* 1989). Sarcolemmal PtdIns 4-kinase and PtdIns 4*P,* 5- kinase activities were determined in the SL of control, diabetic, and insulin treated diabetic rats (Table 4). The activities of PtdIns 4- kinase and PtdIns 4*P*, 5-kinase were significantly ($P< 0.05$) depressed in the diabetic heart as compared to the control hearts. After insulin treatment for 14 days, the PtdIns 4- kinase and the PtdIns 4*P*, 5- kinase were partially corrected.

TABLE 4. SARCOLEMMAL PHOSPHOTIDE 4-KINASE AND PHOSPHOINOSITIDE 4*P*, 5- KINASE ACTIVITIES IN CONTROL, DIABETIC AND INSULIN- TREATED DIABETIC RAT HEARTS.

	PtdIns 4- Kinase (nmol PIP_4 phosphate/ mg SL protein/	PtdIns 4*P*, 5- Kinase (nmol PIP2/ mg SL protein/
Control	2113 ± 12	228 ± 19
Diabetic	1313 ± 67*	164 ± 10*
Insulin Treated Diabetic	1856 ± 70*	212 ± 10

PtdIns 4- kinase and PtdIns 4*P*, 5- kinase activities in control, diabetic, and insulin treated diabetic rats were assayed as described in the Materials and Method section. Values obtained are the means ± SEM of 5 experiments. Assays were performed in triplicate. Abrbreviations: PtdIns 4, 5-P_2 corresponds to phophatidyl inositol 4,5-bisphosphate; PtdIns corresponds to phosphatidylinositol; PtdIns 4-*P* corresponds to phosphatidyl inositol 4-phosphate.

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

V. DISCUSSION

Pathological and physiological studies have provided evidence for the existence of an independent cardiomyopathy specific to patients with clinical diabetes mellitus (Gargiulo, P. *et. al.*, 1998, Crepaldi, G. and R. Nosadini, 1988). Diabetic cardiomyopathy is characterized by impaired ventricular function, prior to signs of cardiac failure developing (Rubler, S., *et. al*, 1972). While some diabetic patients do suffer congestive heart failure (CHF), other important clinical consequences are the associated complications, including enhanced susceptibility to hypertension- mediated damage, increased mortality rate associated with acute myocardial infarction, blunted mechanical response to myocardial stress, and increased incidence of post- infarct congestive heart failure (Schaffer, S.W. *et. al.*, 1991). Diabetic cardiomyopathy is also present in experimental animal models of insulin dependent diabetes mellitus (IDDM), such as the one used in this study, streptozotocin (STZ) induced diabetic rats. Because it is quite difficult at the present time to fully examine the etiopathological changes that initiate diabetic cardiomyopathy in human patients, valuable information can be obtained employing the animal models.

Evidence presented from past research has implicated several possible mechanisms responsible for diabetic cardiomyopathy. One of the least studied is the phosphoinositide specific phospholipase C signaling pathway, specifically involving the β1 isoenzyme, and its contributions to this specific condition. Many studies have identified impairments in this pathway that may

contribute to the development of heart failure. The present study was undertaken therefore, to examine alterations in the phospholipase C $\beta 1$ signaling pathway and its regulation in insulin dependent diabetic heart disease.

In the present study, we showed that during the diabetic state, the cardiomyocytes exhibit significant alterations in the phospholipase C enzymes. A significant decrease in the total sarcolemmal (SL) phosphoinositide specific PLC activity was found in the STZ- induced diabetic rats under the *in vitro* assay conditions. This decrease was indicated by the lower production of total inositol phosphates (Table 2). As discussed in the Review of Literature, PLC has been shown to hydrolyze Ins (4, 5)P_2 to yield Ins(1, 4, 5)P_3 and DAG (Meij, J.T. and V. Panagia, 1992). Table 2 of the Results section illustrated that the major product of the assay condition, Ins (1, 4, 5)P_3 was significantly decreased in the diabetic heart when compared to the control hearts. This decrease could also be due to a dephosphorylation of IP_3 to IP_2 by an inositol phosphate phosphatase. However, one would then expect an increase in the level of IP_2. This increase was not observed (Table 2). Therefore, it is likely that the decrease in IP_3 is not due to a change in the inositol phosphate production profile. Thus, the decreased Ins (1,4,5) P_3 levels occurring in diabetes are likely due to a specific depression in PLC activity. However, insulin was able to normalize the Ins (1, 4, 5)P_3 levels to those of the control samples. A decrease, using other assay conditions, in the basal activity of PLC was identified and confirmed the existence of a defect during

diabetes (Table 3). We have demonstrated that the sarcolemmal phosphatidylinositol 4 kinase and the phosphatidyl 4-phosphate 5 kinase activities were down regulated in the diabetic heart. This indicates a potential for reduced sarcolemmal Ins (4, 5)P_2 content. This reduction of the preferred substrate for PLC could compromise the PLC activity *in vivo*.

It was also important to determine whether the PLC β1 isoenzyme was involved. The results for PLC β1 followed a similar pattern to that of total PLC. We found that in the diabetic state there was a significant decrease in the amount and activity of this isoenzyme. We investigated two of the many regulatory components of this signaling pathway: modulation of activity by PtdOH and Gqα- mediated regulation. In cardiac tissue, the formation of phosphatidic acid (PtdOH) derived from phosphatidylcholine (PtdCho) via the PLD pathway in SL membranes has been established by V. Panagia, *et. al.*, 1991. Phosphatidic acid can exhibit several important effects on the heart (Dhalla, N. S., *et. al.*, 1997). For example, PtdOH has been shown to increase the intracellular [Ca^{2+}] as a consequence of increasing both Ca^{2+} flux across the sarcolemma as well as Ca^{2+} release from the intracellular sarcoplasmic reticulum stores, thereby increasing the contractility (Xu, Y.J., *et. al.*, 1996a). PtdOH can also induce phosphorylation of a 14 kD cell surface protein, enhancing RNA and protein synthesis (Xu, Y.J., *et. al*, 1996b). In this study, in the presence of 25 μM phosphatidic acid (which is the physiologic range), the total PLC activity was significantly increased in control, diabetic and insulin treated diabetic samples (Table 3). Interestingly, phosphatidic acid (25

μM) (a known stimulator of PLC- see Review of Literature) only stimulated the β1 isoenzyme in the diabetic and insulin treated diabetic states (Figure 15). This would indicate that the PtdOH- mediated stimulation of the total PLC activity observed in control membranes was not achieved through the PLC β1 isoenzyme. In the control situation, phosphatidic acid (PtdOH) exerted no significant effect (Figure 15).

The Gqα regulatory mechanism was also examined. In the present study, we examined the transducing (Gqα) and effector (PLC β1) steps of the α1/ Gqα/ PLC β1 signaling pathway in the STZ- induced diabetic rats. The results indicate defects that may contribute in part to the cardiac dysfunction observed in the insulin- dependent diabetes mellitus. Under the *in vitro* assay conditions used in this study, a significant decrease in the activity of PLC β1 in the cardiac sarcolemma was detected (Figure 15). Preliminary studies in our laboratory (Tappia, P.S., *et. al.* unpublished data) have shown a decrease in the Ins(1, 4, 5)*P*3 levels in the cytosolic fraction of the diabetic cardiomyocytes. This observation confirms that in addition to the previously reported decrease in the α1 adrenoreceptor density (Black, S.C. *et. al.*, 1991), a decreased PLC β1 activity level may exist *in vivo*. The defective PLC β1 activity and the reduced levels of Ins(1, 4, 5)P_3 could also produce a diminished level of its phosphorylated derivative Ins(1, 3, 4, 5)P_4. This has an important biological significance. This depressed signaling may result in a down regulation of the Ins(1, 4, 5)P_3, Ins(1, 3, 4, 5)P_4 dependent force of contraction in response to the α1 adrenergic stimulation in the diabetic heart

(Heyliger, C.E. *et. al.*, 1982, Tanaka, Y. *et. al.*, 1992). The increase in the SL Gqα immunoprotein abundance may be viewed as a compensatory response to the defects in the α1 adrenoreceptor and PLC β1 and subsequent downstream signaling events. As previously mentioned, the phospholipase C signaling pathway has several implications for biological and physiological function, especially in stimulating Ca^{2+} induce Ca^{2+} release from the sarcoplasmic reticulum (SR) and effecting other downstream events such as activating protein kinase C (PKC). The decrease of PLC β1 activity, therefore, likely contributes to the total decrease observed in Figure 15. Insulin treatment was able to only partially normalize this decrease (Figure 15).

In diabetes contractile failure is due in part to abnormal intracellular Ca^{2+} concentrations (Teshima, Y. *et. al.*, 2000). These calcium abnormalities have been identified as the result of several mechanisms such as: defects in the sarcolemmal Ca^{2+} pump, defects in the Na^{+}- Ca^{2+} exchanger molecule and also due to the decrease in release of Ca^{2+} from the decreased levels of ryanodine receptors (Teshima, Y. *et. al.*, 2000). The decrease in PLC activity has been shown to result in a decrease in IP_3 levels in the diabetic state (Tanaka, Y. *et. al.*, 1992). If the IP_3 level has decreased, then Ca^{2+} release from the IP_3 channels may also be reduced. During the diabetic state, however, the IP_3 channels are up- regulated. This may in fact be a compensatory mechanism for the depressed function or down regulation of the ryanodine receptors. However, this still remains to be established. The direct

effect of IP_3 mediated Ca^{2+} release may therefore contribute to this contractile dysfunction and thus may be implicated in diabetic heart failure.

VI. CONCLUSION

In chronic insulin-dependent diabetes in rats, we have found that the preferred substrate for PLC in the heart remains as PIP_2. The major product of this assay was found to be IP_3 and in the diabetic state this product is significantly reduced. Insulin treatment of the diabetic rats resulted in normalized IP_3 levels.

Another important phospholipid that modulates cardiac function is phosphatidic acid (PtdOH). PtdOH has been shown to stimulate PLC and thereby stimulate production of IP_3. In the diabetic state, the total PLC activity was found to be significantly depressed. Insulin therapy served to normalize activity level to that of control values. Twenty- five μM PtdOH significantly increased PLC hydrolysis in the diabetic and control samples. However, total PLC activity even in the presence of PtdOH was significantly lower in the diabetic samples. After insulin treatment of the diabetic animals, PtdOH stimulated PLC activity above even control levels.

As little information is available regarding PLC β1 in diabetic cardiomyopathy, we examined the effect of this disease state on the activity of PLC β1. PLC β1 activity was significantly depressed in diabetes and treatment with insulin resulted in a partial correction of this defect. After stimulation with PtdOH, PLC β1 activity was significantly increased in both the diabetic and insulin treated diabetic samples. Interestingly, when PtdOH was introduced, it did not stimulate PLC β1 in the control state.

After determining the activity level of PLC β1, we examined the distribution of this enzyme within the cell by immunohistochemistry. We found that although PLC β1 activity was significantly reduced in the cytosol of diabetic cardiomyocytes, the levels were normalized with insulin treatment. In sharp contrast however, the PLC β1 protein level was significantly increased in the cardiac sarcolemmal membrane region in cells from diabetic rats. Insulin treatment resulted in a normalization of these levels. We may conclude that the diabetic state may induce a movement of PLC β1 from the cytosol to the sarcolemma. However, the mechanism responsible for this alteration in cellular distribution remains unclear.

When the cells were exposed to PtdOH, the staining of PLC β1 was qualitatively similar to that observed in assays of PLC activity. Stimulation with PtdOH resulted in a significant increase of the PLC β1 levels in the diabetic state. Interestingly, the insulin treated diabetic cells appeared to have no change in the cytosolic distribution of PLC β1. However, the intensity of the PLC β1 staining was greatest at the periphery of the cardiomyocytes, specifically at the region of the cell consistent with localization at the intercalated discs. The functional implications of this finding need to be elucidated with further experimentation.

Receptor/ PLC interactions in the membrane were also examined as a function of the diabetic state to determine if diabetes may induce an uncoupling of these two important processes. The Gqα protein transduces the α1 adrenoreceptor signal to PLC β1. Any change in Gqα expression or cellular

distribution may, therefore, affect PLC β1 activity and ultimately be responsible for the changes detected in PLC activity in cardiomyocytes during diabetes. Immunofluorescence analyses revealed that the Gqα protein in the tissue samples was increased in the diabetic state and seemed to return to control values in the insulin treated state. This altered cellular expression may be indicative of a change in coupling between this protein and PLC. Thus, a defect in the Gqα/ PLC β1 mediated signaling pathway may partly contribute to diabetic cardiac dysfunction.

In summary, we can conclude that PLC activity is significantly depressed in hearts from chronically diabetic rats. This may have important implications for signaling within the cardiomyocytes and may contribute to the cardiac contractile depression exhibited by diabetic rat hearts. Two mechanisms may be involved in the defect in PLC activity. First, PLC distribution in cardiomyocytes is altered during diabetes. This may influence enzyme activity. Second, the coupling of Gqα with PLC β1 may be altered. This would influence the signaling effects of any agonist that stimulates PLC β1 through an action with the α1 adrenoreceptor. We have also discovered that PtdOH may play an important adaptive role in the diabetic cardiomyocytes. PtdOH may partially compensate for the enzymatic deficiencies in PLC signaling by selectively stimulating PLC activity in cardiomyocytes from diabetic rats. In conclusion, our results demonstrate that phospholipid signaling through the PLC pathway is altered in hearts and cardiomyocytes from chronically diabetic rats. This may contribute to the diabetic

cardiomyopathy. Experiments examining the specific mechanisms involved will be required in the future.

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