Phospholipase D / Phosphatidic Acid Phosphatase Signal Transduction Pathway in Post Infarction Congestive Heart Failure

By

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of the University of Manitoba in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN PHYSIOLOGY

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PHOSPHOLIPASE D/PHOSPHATIDIC ACID PHOSPHATASE SIGNAL TRANSDUCTION PATHWAY IN POST INFARCTION CONGESTIVE HEART FAILURE

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CHANGHUA YU

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DEDICATION

To my wife, Bin Liu and my daughter , Xiang Yu

and my parents

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TABLE OF CONTENTS

Acknowledgment....i
List of Abbreviations....viii
List of Tables....x
List of Figures....xi
Abstract....xii

I. INTRODUCTION....1

II. LITERATURE REVIEW....8

A. Congestive heart failure....8
1. Animal models of CHF and their applications....9
2. Pathophysiological regulation of CHF....10
a) Sympathetic nervous system....11
b) Atrial natriuretic factor....12
c) Renin-angiotensin system....13
3. Ca^{2+} mobilization abnormalities in CHF....14
B. Characterization of the sarcolemmal phospholipase D....16
1. PLD Hydrolytic activity....16
2. PLD Transphosphatidylation....17
3. Other PLD Isotypes....19
a) Cytosolic PLD....19
b) Glycosylphosphatidylinositol-specific PLD....20
4. Purification and gene expression of PLD....21
C. The role of PLD / PAP signaling pathway in heart function....22
1. PA as a second messenger in the heart....24
2. Phosphatidic acid phosphatase....25

a) Tissue distribution of biochemical characteristics of PAP.....25
b) The role of DAG as a second messenger in heart function.....29
D. Regulatory mechanisms of the PLD / PAP coupling pathway..............30
1. ANF as a cofactor of PLD activity..............30
2. Angiotensin II activates PLD activity..............31
3. Role of G-protein..............33
a) Historical discovery of G-proteins..............33
b) G-proteins expressed in heart tissues and subcellular localization..............34
4. The role of PKC in the regulation of PLD..............36
a) Cardiac PKC isoforms..............36
b) PKC and myocyte hypertrophy..............37
c) PKC and myocyte contraction..............38
d) PKC regulation of PLD activity..............39
5. Effect of Ca^{2+} - mobilizing agents in the regulation of PLD..........41
a) Receptor orientated regulation of PLD activity..............41
(1) Adrenoceptors..............41
(2) P_2 - receptor..............42
(3) M - receptor..............43
b) Other Ca^{2+}- mobilizing agents..............45
6. Other regulatory factors..............46
E. Interaction of phospholipases..............47
1. Cross talk between PC- PLD and PC-PLA_2..............47
2. Cross talk between PC-PLD and PI-PLC..............48
F. Pathophysiological studies of the PLD/PAP pathway..............48

III. MATERIALS AND METHODS.....51
A. Materials.....51
B. Methods.....51
1. CHF model.....51
2. Isolation of sarcolemmal and cytosol fractions.....53
3. Phospholipase D assay.....54
4. Phosphatidic acid phosphatase assay.....56
C. Statistical analysis.....58
IV. RESULTS.....59
A. General characteristics of the CHF animals.....59
B. Sarcolemmal PLD activity in CHF.....61
1. Left ventricular sarcolemmal membrane PLD activity.....61
2. Right ventricular sarcolemmal PLD activity in 8 weeks CHF animals.....64
C. Examination of phosphatidic acid phosphatase activity in CHF rats.....66
1. Left ventricular sarcolemmal PAP activity.....66
2. Left ventricle cytosolic PAP activity.....67
3. Right ventricle sarcolemmal and cytosolic PAP activities in CHF.....69
D. PA and DAG formation from the PLD/PAP pathway in CHF in the absence of potassium fluoride.....69
E. Effect of Imidapril on sarcolemmal PLD and PAP and cytosolic PAP activities in CHF.....72
V. DISCUSSION.....75

A. The application of CHF model induced by coronary artery ligation....75
B. Possible mechanisms involved in the upregulation of sarcolemmal PLD activity in CHF....75
C. The status of cardiac ventricle PAP activities in CHF....79
D. The effect of Imidapril treatment on the PLD /PAP pathway in CHF and it's potential significance....82
E. The significance of altered PLD/PAP signalling pathway in CHF....83
VI. CONCLUSION....87
VII. REFERENCES....89

LIST OF ABBREVIATIONS

AA .... Arachidonic acid

AC .... Adenylyl cyclase

ACE .... Angiotensin converting enzyme

ADP .... Adenosine diphosphate

Ang-II .... Angiotensin II

ATP .... Adenosine triphosphate

Ca^{2+} .... Calcium

CHF .... Congestive heart failure

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

DTT .... Dithiothretol

EGF .... Epidermal growth factor

G protein .... Guanine nucleotide binding protein

GAP .... GTPase-activating protein

GPI .... Glycosylphosphatidylinositol

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

IP_3 .... Inositol-1,4,5-trisphosphate

KF .... Potassium fluoride

Mg^{2+} .... Magnesium

Na^+ .... Sodium

NEM .... N-ethylmaleimide

PA .... Phosphatidic acid

PAP .... Phosphatidic acid phosphatase

PC .... Phosphatidylcholine

PE .... Phosphatidylethanolamine

PEth .... Phosphatidylethanol

PKC.....Protein Kinase C

PLA_2.....Phospholipase A_2

PLC.....Phospholipase C

PLD.....Phospholipase D

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

PTX.....Pertussin toxin

RAS.....Renin angiotensin system

SL.....Sarcolemma

SR.....Sarcoplasmic reticulum

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

LIST OF TABLES

Table 1. General characteristics of the sham and CHF animals 8 weeks after ligation....................60

Table 2. Formation of phosphatidic acid by sarcolemmal phospholipase D in sham and CHF rats....................62

Table 3. Formation of diacylglycerol by sarcolemmal phosphatidic acid phosphohydrolase activity in sham and CHF rats....................68

Table 4. Formation of diacylglycerol by cytosolic PAP activity in sham and CHF rats....................70

Table 5. Effect of Imidapril on the cardiac SL PLD and PAP and cytosolic PAP activities in CHF....................74

LIST OF FIGURES

Figure 1. Comparison of cardiac sarcolemmal PLD hydrolytic activity and transphosphatidylation activity in 8 weeks sham and CHF rats due to coronary artery ligation..63

Figure 2. Right ventricular sarcolemmal PLD activity in sham and CHF 8 weeks after coronary artery ligation...65

Figure 3. Right ventricle sarcolemmal and cytosolic PAP activity in 8 weeks sham control and CHF rats after coronary artery ligation............71

Figure 4. PA and DAG formation in sham and CHF rats 8 weeks after coronary artery ligation in the absence of KF..............................73

ABSTRACT

Post infarct congestive heart failure (CHF) is a very common clinical syndrome with high morbidity and mortality. One of the important pathophysiological characteristics is abnormalities in the Ca^{2+} homeostasis which is, in part modulated by the phospholipase D / phosphatidic acid phosphatase (PLD / PAP) signaling pathway. Another pathophysiological characteristics is post infarction ventricle remodeling which is, in part, regulated by renin-angiotensin system (RAS). Angiotensin II (Ang-II) has been reported to be able to regulate phosphatidylcholine (PC)-specific PLD signal pathway. Therefore, it was hypothesized that, in congestive heart failure , one of the mechanisms by which the Ca^{2+}-handling abnormalities and ventricle remodeling in post infarct CHF occurs is the alteration of cardiac SL PLD / PAP signal transduction pathway . This study was designed to examine the status of the cardiac PLD / PAP signaling pathway, and observe the effect of the Angiotensin converting enzyme (ACE) inhibitor, Imidapril , on this pathway in CHF.

Post infarct CHF was induced by left anterior descending coronary artery ligation. Sarcolemmal (SL) and cytosolic membrane fraction was isolated from

sham control and CHF animals at 1, 2, 4, 8 and 16 weeks after surgery and from CHF rats treated with Imidapril at 8 weeks. PLD hydrolytic activity was detected by measuring the amount of phosphatidic acid (PA) with labeled ^{14}C-PC and also confirmed by the PLD transphosphatidylation in the presence of ethanol. The SL and cytosolic PAP activity was examined by measuring the amount of *sn*-diacylglycerol (DAG) with labeled PA as substrate. A time dependent increase in SL left ventricle PLD was observed which reached a maximum at 4 weeks after ligation of coronary artery compared to the corresponding sham control values. No significant differences were found in right ventricle SL PLD activity. Similarly, a time dependent increase in the LV SL PAP activity was observed . The SL PAP activity also peaked at 4 weeks post surgery. However, , in contrast, the cytosolic PAP activity peaked at 8 weeks post surgery. In the right ventricle neither SL nor cytosolic PAP activities showed significant changes in CHF. In the CHF rats treated with the ACE inhibitor, Imidapril, the upregulated SL PLD activity was normalized. Furthermore , the upregulated left ventricle SL and cytosolic activities were also normalized by the Imidapril treatment. In the absence of potassium fluoride (KF) , a potent PAP inhibitor, the net amount of PA was diminished and DAG was increased as compared to the corresponding sham control values. Although the exact mechanism by which PLD / PAP activation occurs in CHF has not

been established, a number of mechanisms have been suggested which include the fact the RAS is activated and Atrial natriuretic factor (ANF) level is elevated and that the sympathetic nervous tone is enhanced during the development of CHF, all these factors may work synergistically in PLD / PAP activation.

The pathophysiological significance of the activation of the PLD / PAP pathway in CHF is that initially it might be an adaptive response to cardiac dysfunction but ultimately it might contribute to the ventricular remodeling and Ca^{2+} -handling abnormalities. Therefore, it is suggested that the normalization of PLD / PAP activities upon Imidapril treatment may improve the symptoms and modify the progression of CHF.

I. INTRODUCTION

CHF is a very common clinical syndrome with high morbidity and mortality rates. More than 50% of the reported cases of CHF are due to ischemic disease. Several models of CHF have been designed for experimental cardiology and the one of choice depends on the research purpose. For example , CHF induced by coronary artery ligation in rats is an ideal model for studying subcellular changes because the pathophysiology is similar to clinical cardiac ischemic patients.

One of the most important pathophysiological characteristics of CHF is abnormal Ca^{2+} handling as a consequence of changes in for example SL and sarcoplasmic reticulum (SR) Ca^{2+} channels, SL Na^{+}- Ca^{2+} exchange, SL and SR Ca^{2+} pump. Several lines of evidence have indicated that the PLD / PAP pathway is regulated by the Ca^{2+} mobilizing agonists, Ang-II and ANF.

Intracellular calcium overload and deficiency have been postulated to contribute to heart failure and cell death. Ca^{2+} mobilization in the heart is regulated by several mechanisms, one of these mechanisms involves the

interaction of catecholamines with the cardiac membrane leading to SL defects including changes in Na^{+}-K^{+} ATPase and adenylyl cyclase activities, phospholipid N-methylation, β-adrenergic receptors, α-adrenergic receptors and guanine-nucleotide-binding (G-protein) mediated processes. All of these events are considered to be involved in the development of Ca^{2+} overload in CHF. Depression of SL Ca^{2+} channel has also been reported to occur during CHF. The intracellular Ca^{2+} overload may also occur when the Ca^{2+} handling abilities of both SR and mitochondria are impaired. In this regard, a defective SR Ca^{2+} pump activity has been shown to result in intracellular Ca^{2+} overload in CHF. In addition, the alteration of membrane fluidity, which is determined by the cholesterol / phospholipids levels may be associated with altered cellular Ca^{2+} - mobilization leading to intracellular Ca^{2+} overload (Dhalla et al., 1991).

It is well known that phospholipid signalling pathways are involved in Ca^{2+} handling, and regulation of the cardiac function. There are three major phospholipid signalling pathways including 1) phosphoinositide pathway which has been shown to modulate the movement of SR Ca^{2+} by producing inositol (1, 4, 5) - trisphosphate (IP_3), as well as DAG for activating protein kinase C (PKC) and subsequent membrane phosphorylation; 2)

phosphatidylethanolamine (PE) N-methylation which influences SL and SR Ca^{2}-pumps as well as SL Na^{+}-Ca^{2+} exchanger, and 3) SL PC - specific PLD which forms PA for regulating Ca^{2+} movements.

It is not unreasonable to assume, therefore, that the changes in the phospholipid signalling pathways may contribute to Ca^{2+} handling abnormalities in CHF.

It has been reported that PA accumulation in hormone-treated hepatocytes is via a G-protein coupled to PLD (Bocckino et al., 1987). Furthermore, isolated hepatocytes responded to a variety of Ca^{2+}- mobilizing agents such as vasopressin, Ang-II, epinephrine, epidermal growth factor (EGF), adenosine triphosphate (ATP) and adenosine diphosphate (ADP)with a rapid increase in PA mass (Thomas et al., 1983; Takemawa et al., 1982).

ANF is a polypeptide synthesized by mammalian atrial cardiomyocytes (De Bold, 1985) and is involved in cardiovascular homeostasis. It has been reported that in isolated rat cardiac sarcolemma, both phospholipase C (PLC) and PLD activities can be modulated by physiological concentrations of ANF. At low levels of ANF the degradation of PC occurs preferentially

through PLD with the formation of PA and choline, whereas at higher ANF concentrations, PLC activity is predominant suggesting that the products of PC hydrolysis are involved in the mechanism of action of this peptide, and also indicates that ANF may be an important regulator for phospholipid signalling (Baldini et al. , 1994).

Booz et al. (1994) have demonstrated that the PA formed in response to Ang-II in neonatal rat cardiac fibroblasts is likely due to PLD activation. Similar results have also been reported by Schorba et al.(1993). Taken together, we can assume that Ang-II plays an important role in the activation of PLD in CHF. It should be noted that the stimulation of PLD by Ang-II appears to involve PKC-ε isoform (Pfeilschifter and Huwiler, 1993).

As already mentioned above, One of the important second messengers produced by the PLD / PAP pathway is PA which can increase Ca^{2+} influx into intact cells from several tissues (Salmom ad Honeyman, 1980). Recently, direct evidence that PA increases intracellular free Ca^{2+} and cardiac contractile force was reported by Xu et al. (1996). These investigators also found that PA in rat produced a significant increase in the left ventricular developed pressure , the maximal rates of cardiac contraction

and relaxation occurred within 5 Min. Taken together, it was suggested that PA may regulate $[Ca^{2+}]_i$ and contractile parameters in the heart. The possible mechanism by which PA mediates these effects on heart is that it might be acting as a Ca^{2+} ionophore or mobilizing cellular Ca^{2+} flux by activation of plasma membrane Na^{+}-Ca^{2+} exchanger (Grover et al., 1981; Gilbert and Meissner, 1982; Ghijsen et al., 1983), as well as inducing Ca^{2+} release from SR (Xu et al., 1996). It is interesting to note that the stimulation of protein synthesis by PA in rat cardiomyocytes has also been reported by Xu et al. (1996).

Another important second messenger from the PLD / PAP pathway is DAG. It is possible that the different molecular species of DAG may exert differential effects on the various forms of PKC. Therefore, DAG derived from PC- PLD hydrolysis could produce different cellular effects from that derived from PI -PLC.

It has been reported that ras (a small G-protein) rapidly activates PKC, which in turn activates a number of cellular signalling systems, leading to a sustained increase in DAG levels. It has been suggested that this elevation of

DAG could sustain PKC activation for the initiation of DNA synthesis (Price et al. 1989).

DAG has been shown to influence the cardiac Ca^{2+} transport system directly or indirectly via PKC phosphorylation (Gilbert et al., 1991; De Jonge et al., 1995; Lacerda et el., 1988). Alterations in this Ca^{2+} - mobilizing system are considered to result in the impairment of the cardiac performance during heart failure (Dhalla et al., 1991).

DAG from the PLD/PAP pathway is generally produced in much larger amounts and for longer periods than that from phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis. This may be due to the sustained activation of PLD in some cell types (Cook et al, 1991). Therefore, it is postulated that a long term upregulation of PLD / PAP pathway may involve the ventricle remodeling process in post-infarct CHF .

Overall, Ca^{2+} handling in heart is partially regulated by the PLD / PAP pathway, and Ang-II is involved in the ventricular remodeling after post infarction. As mentioned above, it is hypothesized that , the status of cardiac PLD / PAP pathway might be changed in CHF. Therefore, the present study

was designed to determine the status of PLD/PAP pathway in CHF and to provide a further understanding of mechanisms associated with the development of CHF and whether such changes can be normalized upon ACE inhibitor therapy. The effect of ACE inhibitors on the PLD / PAP pathway in CHF may provide the biochemical basis for understanding the mechanisms by which ACE inhibitor therapy can improve the symptoms and reduce the mortality due to CHF.

II. LITERATURE REVIEW

A. Congestive heart failure

CHF is a condition when the heart fails to pump enough blood to meet the needs of body (Opie LH et al., 1991). There are basically three mechanisms for myocardial failure: pressure overload, volume overload and primary myocardial disease such as cardiomyopathy, myocardial infarction (Guyton, 1991). It should be noted that the most common cause of CHF is ischemic heart disease (Litwin et al., 1991). It is estimated that about 1.5% of the total North American population suffers from CHF and that about 500,000 people develop CHF every year . The 5-year mortality from the time of diagnosis is about 60% in men and about 45% in women (Dhalla et al., 1991). As CHF has become one of the most common serious disorders and consequently a common cause of death, many different experimental models of heart failure have been used to aid in the assessment of the biochemical changes that occur during the development of cardiac dysfunction and such models have permitted the examination of various modes of treatment of the failing heart.

1. Animal models of CHF and their applications

Depending on the different research purpose, several CHF models have been designed for experimental cardiology research, and these are briefly described below: 1) CHF model induced by coronary artery ligation, which produces post -infarction heart failure, has been extensively used for the study of the effect of ACE inhibitors in CHF (Dixon et al., 1992; Himori and Matsura, 1989; Schoemaker et al., 1990), SL adrenoceptors (Dhalla et al., 1992; Dixon et al., 1991), Ca^{2+} transport in SL and SR (Dixon et al., 1992) and SL Na^{+}-K^{+} -ATPase activity (Dixon et al., 1992); 2) Adriamycin induced cardiomyopathy model has been used to study the harmful effects of oxygen free radicals in the heart and it's protection from damage by antioxidants (Singal et al., 1985; Singal and Pierce, 1986 ; Siveski et al., 1994; Earm et al., 1994). 3) Ventricular pacing induced heart failure model, which produces a pathological status similar to the heart failure caused by cardiac arrhythmia, has been used to study carotid sinus baroreceptor reflex (Wang, et al., 1992; 1990) and the ventricle mechanics, energetics and contractile reserve, electrophysiology (Wolff et al., 1992 ; Moe et al., 1991 ; Elsner et al, 1990) and endothelial function in heart (Drexler et al., 1992) . 4) Arterio-Venous fistula , which produces a high cardiac output CHF as seen in some congenital heart diseases,

has been used to study cardiac effects of ANF (Yechieli et al., 1993) and ACE inhibitors (Hirsch, 1992 ; Garcia et al., 1990; Abassi et al., 1990).

2. Pathophysiological regulation of CHF

Activation of different neurohormonal systems such as the sympathetic nervous system, RAS and ANF occurs almost simultaneously during the development of CHF (Cohn , 1990). Furthermore, activation of norepinephrine, Ang-II, vasopressin and ANF may be key factors in the vasoconstriction and increased impedance to left ventricular ejection seen in heart failure.

Clinical studies have shown that plasma levels of ANF, Ang-II, and catecholamines are enhanced in patients with acute myocardial infarction. Sustained neurohormonal activation after myocardial infarction mainly occurs in patients with clinical heart failure. Ang-II and norepinephrine remained elevated for 1 month whereas ANF remained elevated for up to 4 to 6 months. In this regard, a positive correlation has been found between infarct size and ANF, Ang-II, and norepinephrine on day 5 to 7 after the initial diagnosis of infarction (Sigurdsson, et al., 1993). Echocardiographic studies have indicated that in patients with high concentrations of neurohormones in the plasma a

week after their infarction, are highly likely to develop left ventricular dilatation and systolic dysfunction of the left ventricle. and therefore, prolonged neurohormonal activation, theoretically, may be harmful to the myocardial cell structure and function (Sigurdsson et al., 1996).

a). The sympathetic nervous system

Extensive studies has been shown that the first pathophysiological response in CHF is the activation of sympathetic nerve activity (Oren et al., 1991; DiBona and Sawin, 1994). It has been demonstrated that sympathetic nerve activity is significantly higher in CHF (induced by left coronary artery ligation) as compared with sham-operated rats (Feng et al., 1994). Also norepinephrine levels were elevated in CHF patients (295.7 ± 47.8 pg/ml), as compared to normal subjects (143.5 ± 33.3 pg/ml;) (Liguori et al., 1994).

Cardiac norepinephrine spillover was increased eightfold in CHF subjects (127 ng/min versus 14 ng/min in healthy subjects), and cardiac 3,4-dihydro-xyphenylalanine was increased twofold (Meredith et al., 1994). Increased concentrations of norepinephrine in coronary sinus plasma has also been reported in CHF resulting from either increased cardiac sympathetic nerve

firing and norepinephrine release or from failure of neuronal uptake mechanisms to recapture released norepinephrine (Eisenhofer , 1996). The baroreflex control of sympathetic activity is impaired in CHF leading to a marked sympathetic activation in CHF (Grassi et al., 1995).

b). Atrial natriuretic factor

ANF is produced by myocardial tissue, and participates in the homeostatic control of intravascular volume and vascular tone. It has been reported that the concentration of ANF in CHF patients is elevated from (35.91 ± 9.2 pg/ml in controls to 190.7 ± 34.2 pg/ml) (Liguori et al., 1994), which persists during CHF (Perrella et al., 1992; Cody et al., 1992). The increase in plasma ANF is regulated by release of stored peptide in acute CHF, and in chronic CHF the persistent elevation of plasma ANF is maintained by an increase in atrial synthesis of ANF (1992 Perrella et al.,). A positive correlation has been found between end-systolic ventricular volumes and plasma ANF (Donckier et al., 1991).

c). Renin Angiotensin system (RAS)

The RAS is now regarded as both a circulating and tissue hormonal system. All components of the RAS have been detected in the heart (Paul et al ., 1995). ACE is localized in cardiac valves, coronary vessels, atria, and myocardium. It has been demonstrated that ACE in the myocardium is markedly increased prior to plasma renin and aldosterone levels in experimental - induced myocardial infarction in rats. Treatment with ACE inhibitors suppresses cardiac ACE and produces hemodynamic improvement, reverses neurohumoral activation, prevents ventricular dilatation, and remodeling and reduces mortality rates (Johnston et al., 1993). The success of ACE inhibitors in reducing cardiovascular morbidity and mortality rates has led to the extensive study of role of the RAS in pathophysiology. Ventricular dysfunction leading to CHF is associated with sequential activation of the sympathetic system and increases in plasma ANF; however, increases in plasma renin and aldosterone do not occur until later on (Johnston et al., 1993). The local effects of Ang II on the heart may play an important role in cardiovascular physiology and pathophysiology (Paul et al ., 1995).

In CHF, hypertrophic growth of the myocardium includes the enlargement of cardiac myocytes, an adaptation governed by ventricular loading. Nonmyocyte

cell growth involving cardiac fibroblasts may also occur, but is not primarily regulated by the hemodynamic load. *In vivo* and *in vitro* studies suggest that the effector hormones, Ang-II is primarily involved in regulating the structural remodeling of the myocardial collagen matrix. In cultured adult cardiac fibroblasts, Ang-II and aldosterone have been shown to stimulate collagen synthesis while Ang-II additionally inhibits matrix metalloproteinase 1 activity, which is the key enzyme for interstitial collagen degradation in the myocardium (Brilla et al., 1994).

Activation of the intrarenal RAS may contribute to the pathophysiology of heart failure by accelerating the generation of Ang-II at local sites within the kidneys. Activation of the local intrarenal RAS has been observed in rats and with mild heart failure (Schunkert et al., 1993).

3. Ca^{2+} mobilization abnormalities in CHF

One of the most important pathophysiological characteristics of CHF is the abnormal Ca^{2+} homeostasis of cardiomyocytes (Dhalla et al., 1991). Intracellular calcium overload and deficiency has been postulated to contribute towards heart failure and cell death. In this regard, a number of studies have

indicated that the PLD/PAP pathway is regulated by Ca^{2+} mobilizing agonists, and that Ca^{2+} mobilization in the heart is regulated by several external factors. One of these regulatory mechanisms is the interaction of catecholamines with the cardiac membrane (Dhalla et al., 1982; Lee at al., 1976 ; Alto et al., 1981). A number of SL defects including changes is Na^{+}-K^{+} ATPase, adenylyl cyclase, phospholipid N-methylation. β-adrenergic receptors, α-adrenergic receptors and G-protein have been considered to be involved in the formation of Ca^{2+} overload in CHF (Dhalla et al., 1978; 1982; Panagia et al., 1984; Ganguly et al., 1984; Daly et al., 1987; Newman et al., 1988). Depression of SL Ca^{2+} channel has also been reported to occur during CHF (Wagner and Weisman, 1989; Dixon et al., 1990). The intracellular Ca^{2+} overload also occurs when the Ca^{2+} handling abilities of both SR and mitochondria are impaired (Ito et al., 1974; Dhalla et al., 1978; 1982). Defective Ca^{2+} pump activity of SR has been shown to result in intracellular Ca^{2+} overload in CHF (Gwathmay et al., 1985; 1987; 1990). In addition to this, altered membrane fluidity which is determined by the cholesterol / phospholipids level influences cellular Ca^{2+} mobilization systems , leading to the intracellular Ca^{2+} overload (Kutryk , 1991).

B. Characteristics and properties of Phospholipase D

1. Phospholipase D hydrolytic activity

In a wide variety of cells , PC hydrolysis, in response to diverse agents, is catalyzed by PLD activities that are believed to be membrane bound. SL-bound PLD activities have been detected in many tissues and cell free preparations using exogenous phospholipid as substrate (Panagia et al., 1991; Huang et al., 1992; Wang et al., 1991). These activities exhibited a near or absolute specificity for (PC). SL PLD has been shown to be stimulated by a variety of unsaturated fatty acids, in particular , oleic acid and arachidonic acid (AA) have been demonstrated to be the most potent activators of PLD activity (Dai et al., 1991).

PLD was first discovered in plants (Dils et al., 1961) and later identified in mammalian tissue by Satio & Kanfer (Satio and Kanfer, 1973). The subcellular distribution of PLD in the heart was first demonstrated by Panagia et al. (1991), in these studies, PLD activity was shown to be present in SL, SR

and mitochondria. PLD has been shown to catalyze the hydrolysis of the terminal diester bond of phospholipids with the formation of PA plus related bases; In this regard, PC, the optimal substrate of PLD, upon hydrolysis, produces PA and choline.

2. Phospholipase D transphosphatidylation activity

In addition to its hydrolytic activity, PLD also possesses a trans-phosphatidylation activity which catalyses the exchange of choline from PC with ethanol to produce phosphatidylethanol (PEth). PLD is a phosphodiesterase, and acts on phospholipid head to achieve its either hydrolytic or transphosphatidylation activities (Shukla and Halenda, 1991). Because PA and choline, the products of the PLD hydrolytic activity, may also be formed through other catalytic pathways (such as PLC) (Dils and Hubscher, 1961) and are metabolically unstable, these products of hydrolysis are far less specific and reliable indicators for the determination of PLD activity. Therefore, it is generally accepted that the transphosphatidylation activity, the unique reaction, is considered to be a specific measurement of PLD activity and (Shukla and Halenda , 1991; Xie and Dubyak, 1991; Liscovitch and Eli, 1991). This unique property of PLD has been widely used

to detect the PLD activity in intact cells and cell free systems (Exton, 1990; Liscovitch, 1991). It should be pointed out that the sum of transferase and hydrolytic activities is relatively constant (Kobayashi and Kanfer , 1987).

Lindmar et al (1992) have reported PLD transphosphatidylation activity in the atria of rat and chicken. In contrast to the large amount of knowledge that exists concerning the PLD hydrolytic activity, very limited information about PLD transphosphatidylation activity is available(Yu et al., 1996). Studies in the rat have indicated that the greatest activity of PLD transphosphatidylation is in the lung , whereas in adipose tissue, heart , brain, pancreas and liver, very little activity is detected.

PEth biosynthesis in NG108-15 cells depends largely on the activation of PKC. This response is likely to reflect the stimulation of a PLD activity by this potent tumor promoter and activator of PKC (Liscovitch, 1989). Ethanol exposure causes several impairment of cell membrane function. The formation of an abnormal lipid, PEth, may be involved in such disturbances. However , conclusive evidence is lacking about the pathological effects of PEth and the simultaneous inhibition of PA synthesis. It has been suggested that effect of ethanol on protein phosphorylation and signal transduction could be due to the

presence of PEth (Gustavsson and Hansson, 1990). Furthermore, PEth has been demonstrated to exhibit different effects on membrane fusion as compared to other acidic phospholipids (Bondeson and Sundler, 1987).

3. Other Phospholipase D isotypes

PLDs from different sources have different characteristics such as differences in the preferred substrate, cofactor requirements, , activators and different inhibitors. Thus it has been suggested several isotypes of PLD enzymes exist. In addition to SL PLD , which has been the most extensively studied, there are two other isotypes: namely the cytosolic PLD (soluble PLD) and Glycosylphosphatidylinositol(GPI) - specific PLD.

a). Cytosolic PLD

The cytosolic PLD activity was first reported by Wang et al (1991) in a variety of bovine tissues including the lung, brain, spleen, heart, kidney, thumus, and liver. Studies in the rat have indicated that in the lung the majority of the detectable PLD activity is in the cytosol. This cytosolic PLD activity is different from a membrane-bound isozyme as demonstrated by

chromatographic mobilities on anion exchange and gel filtration columns, substrate specificity, substrate concentration dependence, and by divalent cation and detergent effects. For example, the preferred substrate for the cytosolic PLD is PE, whereas the SL PLD only hydrolyzes PC (Wang et al 1991) , also the cytosolic activity is strictly Ca^{2+} -dependent (Huang et al, 1992). Fractionation of the cytosol by anion exchange chromatography enhances PLD activity by up to 20-fold , suggesting the presence of a PLD inhibitory factor(s) in the cytosol (Balsinde et al., 1988). In view of this, PLD exists in multiple forms and that appropriate selection of assay conditions is critical for observing PLD activity in the cytosol. Using PI as a substrate, the cytosolic PLD activity in human neutrophils was stimulated by incubation of cells with the calcium ionophore A23187, and shown an optimal pH of 7.5 (Balsinde et al., 1988). It should be noted that PA and PEth were formed in a ratio of 1:3 (PEth / PA), and therefore, it was suggested that this cytosolic PLD can play a role in cell activating process.

b). Glycosylphosphatidylinositol-specific PLD

GPI-anchored protein is a key component for lipid biosynthesis and transport (Hielmstad and Bell, 1991) and was first observed as a result of its ability to

degrade the GPI anchor of alkaline phosphatase during extraction from mammalian tissue with butanol (Liscovitch, 1994). GPI-specific PLD has been purified to homogeneity from human serum by several investigators (Davitz et al., 1987; Low and Prasad , 1988; Cardoso et al., 1988). This enzyme is different from the membrane-bound and cytosolic PLD in the following aspects: i) the enzyme is present in plasma ; ii) the substrate is restricted to GPI-anchored protein in intact cell;, not PC, or PE; and iii) it can not cleave and release GPI-anchored protein from intact cell membrane (Low and Huang, 1991).

4. PLD purification and gene expression

The information about the molecular properties of PLD is very limited because the enzyme has never been highly purified. Taki and Kanfer (1979) purified PLD 240-fold from freeze dried rat brain. The specific activity of their preparation was very low 2 nmol / min/ mg protein . Wang et al (1991) have purified a cytosolic enzyme 20-fold and shown it to hydrolyze phospholipids in the order of $PE > PC > PI$ in bovine tissues. The enzyme exhibited a high Km whereas the membrane-bound enzyme specific to PC has a low Km. The cytosolic and membrane-bound PLDs are believed to be different isoforms.

Okamura and Yamashita (1994) purified and characterized PC-PLD from pig lung, the enzyme was solubilized with heptythioglucoside and purified 2,200-fold by successive chromatography on sulfate-cullulofine , ether-Toyopearl, chelate-Toyopearl , Q-Sepharose, heparin-Toyopearl, and hydroxyapatite. (Sheikhnejad and Srivastava, 1986; Wolf and Gross, 1985; Kater and Kanfer, 1980).

C. The role of phospholipase D / phosphatidic acid phosphatase pathways in heart function

There is growing evidence that cardiac function can be influenced by changes in the lipid composition of the cellular and subcellular membranes of myocardial cells (Gudbjarnason and Hallgrimsson, 1979) . Such changes can be induced by variations in the nature of dietary fats, which have been reported to influence heart rate, cardiac tolerance to catecholamines and myocardial contractility (Petersin et al., 1979), presumably by modifying the fatty acid composition of membrane phospholipids. In addition , the altered lipid metabolism that occurs in patients with ischemic heart disease appears to modify the structure of cardiac membranes leading to abnormal cardiac function (Katz and Messines, 1981).

Treatment of cultured neonatal rat myocardial cells with PLD results in an increase in total exchangeable Ca^{2+} (1.56 ± 0.27 mmol Ca^{2+} / kg dry weight) with a simultaneous increase in the contractility of neonatal rat ventricular tissue by 1.7-2.5 fold (Burt et al., 1984). A similar effect of PLD has also been observed on the SL Na^{+} - Ca^{2+} exchanger (Philipson and Nishimoto, 1984) . It was therefore suggested that Ca^{2+} bound to anionic SL phospholipids plays a major role in the transsarcolemmal Ca^{2+} flux and force development. Cardiac contractility and the regulation of Ca^{2+} is dependent on membrane integrity and on the quantity of Ca^{2+} available for influx (Bers and Langer, 1979 ; Frank et al., 1977; Langer et al., 1981), these conditions result in increased binding of Ca^{2+} to the SL and are associated with the stimulation of Na^{+}- Ca^{2+} exchanger (Burt and Langer 1982; Burt et al 1983; Philipson and Nishmoto , 1984) and increased contractility (Bers et al., 1981; Langer and Nudd, 1983).

The influence of PA on Ca^{2+} influx is also supported by the observation that PA and PLD generate Ca^{++} -independent slow AP in depolarized at atrium (Knabb et al., 1984).

PLD / PAP pathway produces two important signaling lipids, namely PA as a result of PC hydrolysis via PLD and DAG from PA by PAP activity. Their functional significance is summarized below:

1. Phosphatidic acid as a second messenger

The product of PLD hydrolytic activity , PA , is considered as a second messenger involved in transmembrane signal transduction process and is involved in the regulation of intracellular calcium levels (Freeman , 1994; 1995). PA can increase Ca^{2+} influx into intact cells in several tissues (Salmon and Honeyman, 1980; Putney et al., 1980; Harris et al., 1981; Ohsako and Deguchi , 1981). Recently, the direct evidence that PA increases the intracellular free Ca^{2+} and cardiac contractile force has been shown by Xu et al (1996b). These workers determined the effects of PA on intracellular Ca^{2+} level ($[Ca^{2+}]_i$) in freshly isolated adult rat cardiomyocytes by using fura-2 acetomethylester and free fura techniques, and it was found that PA at a concentration range of 1-200 μM produced a concentration dependent increase in $[Ca^{2+}]_i$, i.e. from basal level of 117 ± 8nM to a maximal increase to 233±50 nM. Furthermore, it was also found that PA administration to rat (0.34μg/100g body wt iv) produced a significant increase in the left ventricular developed pressure and the maximal

rates of cardiac contraction and relaxation occured within 5 Min. These data suggest that PA may regulate $[Ca^{2+}]_i$ and contractile parameters in the heart. The possible mechanism by which PA induces these effects on heart function may be due to PA acting as a Ca^{2+} ionophore or mobilizing cellular Ca^{2+} flux by activation of the Na^+-Ca^{2+} exchanger (Grover et al., 1981; Schellenberg and Swanson, 1981; Gilbert and Meissner, 1982; Ghijsen et al., 1983). Another possibility is that PA may induce Ca^{2+} release from SR (Xu et al., 1996a) . PA has also been reported to stimulate the SL Ca^{2+} pump (Carafoli, 1984). The stimulation of protein synthesis by PA in rat cardiomyocytes has also been reported by Xu et al (1996c).

It should be noted that PA can be dephosphorylated to DAG via PAP which is the second enzyme of PLD / PAP pathway, and thus PA may be considered as a regulator of DAG synthesis. This is discussed in further detail below:

2. Phosphatidic acid phosphatase

a). Tissue distribution and biochemical characterization of PAP

PAP, which was first identified in the yeast *Saccharomyces cerevisiae*

by HosaKa and Yamashita (1984), catalyzes the dephosphorylation of the PA and DAG . Jamal et al. (1991) characterized two distinct PAP activities in rat liver. One activity requires Mg^{2+} and is completely inhibited by NEM, and is located in the cytosol. Whereas, the other activity is Mg^{2+}-independent and is NEM -insensitive and is membrane associated. The NEM -sensitive PAP can translocate between the cytosol and membrane fractions in response to insulin, glucagon, cyclic AMP, and fatty acids (Brindley, 1987) . The regulation and subcellular localization of PAP indicate that the NEM-sensitive form is primarily involved in synthesis of triacylglycerol and phospholipids *de novo*. The role of NEM-insensitive PAP, an integral plasma membrane protein, in cell metabolism is not well defined. However, because of its cellular location (Jamel et al., 1991), it may regulate the relative concentrations of PA and DAG in the plasma membrane (Martin et al., 1993; Day et al., 1993) and thus participate in signal transduction processes.

The balance in the levels of membrane DAG and PA is important for the appropriate cellular response by cells to extracellular signals. PAP appears to be involved in controlling this balance. For example, in ras-transformed fibroblasts, the specific activity of PAP is decreased relative to nontransformed

cells , and also agonist-stimulated production of PA relative to DAG is increased (Martin et al., 1993).

NEM-insensitive PAP activity has been characterized in rat liver (Gomez et al., 1992; Day and Yeaman, 1992), heart , brain , and adipose tissue (Jamal et al., 1992; Jamdar et al., 1994; Fleming and Yeaman, 1995) and in fibroblasts (Martin et al., 1994), as well as in rabbit kidney cells (Swarts et al., 1992), human neutrophils (Perry et al., 1993; Taylor et al., 1993) and porcine thymus (Kanoh et al., 1992). The specific activity of PAP is the lowest in skeletal muscle and greatest in brain, kidney, and spleen (Jamal et al., 1991).

The NEM-sensitive , Mg^{2+}-dependent PAP has been found in the cytosol of many different tissues and cell lines. The classic function of PAP is the synthesis of triacylglycerols, PC, and PE (Jamal et al., 1990). It controls the conversion of PA to DAG rather than to other acidic phospholipids. The cytosolic form of PAP acts as a reservoir of activity which moves onto the endoplasmic reticulum to become metabolically functional, and this can be brought about by an accumulation of fatty acids, acyl-CoA esters, or phosphatidate within the membranes (Brindley, 1984;). Such an accumulation of metabolites acts as a feed forward signal to promote the production of

triacylglycerol when fatty acid availability increases (Buterwith et al., 1984). In addition, the liver can increase the total activity of PAP in the long term through the actions of glucocorticoids , glucagon (via cAMP), and growth hormone, whereas insulin antagonizes these effects (Brindley, 1987; Martin at al., 1987).

PAP is involved in signal transduction in many cells types, including hepatocytes, following the agonist stimulated breakdown of PC via PLD (Bocckino et al., 1987; Leffelholz et al., 1989; Billah and Anthes, 1990; Billah et al., 1989; Hoer and Oberdise, 1996; Kanoh, 1993b). Since PA itself is also a second messenger , PAP activity can be seen as a regulator of this signalling lipid , and at the same time producing DAG , which in turn would activate PKC. Therefore, it is assumed that the PAP involved in signal transduction should be situated in the plasma membrane, indeed such a location has been reported (Kent and Vagelos, 1976; Colemman, 1968).

In rat liver, the PAP activity has also been detected in SR and mitochondria , which share the same features as the cytosolic PAP (Jamal et al, 1991), whereas, in heart , the SR PAP exhibited the same characterization as the SL PAP (*Yu et al., 1996, unpublished data*). This indicates that tissue differences

exist in the biochemical characteristics of PAP. Recently, identification of 35 KDa cDNA clone of PAP from mouse H202 has been reported (Kay et al., 1996).

b). The role of diacylglycerol in the signal transduction

DAG , through its association with PKC , is now recognized as an important cellular messenger. It is interesting to speculate the indirect and direct pathways of DAG formation represent a biologically significant event, and that DAG levels are dependent on the interplay of these pathways (Huang and Cabot , 1990) . It is possible that different molecular species of DAG may exert differential effects on the various forms of PKC (Hunyadyl et al., 1990). If this were the case, DAG derived from PC hydrolysis could produce different cellular effects from the DAG resulting from PIP_2 breakdown. It has been reported that ras (a small G-protein) rapidly activates PKC , which in turn activates a number of cellular signalling systems, leading to a sustained increase in DAG levels (Hesketh et al., 1988) . This elevation of DAG could sustain PKC activation for initiation of DNA synthesis. (Price et al., 1989); and protein synthesis (Farese, 1988; Chu et al., 1986; Okumura et al., 1995)

DAG has been shown to influence the cardiac Ca^{2+} transport system directly (Gilbert et al., 1991; De et al., 1995) or indirectly via PKC phosphorylation (Lacerda et al., 1988) and therefore an alteration in this Ca^{2+} -mobilizing system is considered to contribute towards the impairment of the cardiac performance during heart failure (Dhalla et al., 1991). DAG from the PLD/PAP pathway is generally produced in much larger amounts and for longer periods than that from PIP_2 hydrolysis. This may be due to sustained activation of PLD in some cell types. It should be noted that in some cell types PLD activation is transient (Cook et al., 1991), which is presumably due to a slower metabolism of the PLD derived products (Martin and Michaelis, 1988; Thompson et al., 1990).

D. Regulatory mechanisms of the PLD / PAP signalling pathway

1. Atrial natriuretic factor as a cofactor of phospholipase D activity

ANF is a polypeptide synthesized by mammalian atrial cardiomyocytes (De Bold, 1985) and is involved in cardiovascular homeostasis. It has been reported that in isolated rat cardiac SL, PLC and PLD activity can be modulated by physiological concentration of ANF. At low levels of ANF the

degradation of PC occurs preferentially through PLD with formation of PA and choline, whereas at higher ANF concentrations, it is predominantly via PLC activity, suggesting that the products of PC hydrolysis are not only involved in the mechanism of action of this peptide, but also indicates how a possible regulatory pathway of ANF production might be organized (Baldini et al. , 1994 Eskildson et al., 1996).

2. Angiotensin II activates the phospholipase D activity

Ang-II can elicit a significant increase PA via PLD (Booz et al., 1994), and has been demonstrated to be responsible for hypertrophy and activation of multiple phospholipid derived second messenger systems via the AT_1 receptor in cardiac myocytes (Rozengurt., 1991; Sadoshima and Izuma , 1993). PA formed in response to Ang-II in neonatal rat cardiac fibroblasts is likely to be due to PLD activation (Booz et al., 1994) , similar results have also been reported by Schorba et al. (1993). Taken together, we can assume that Ang-II play an important role in the activation of PLD in the CHF.

Ang-II activates PLD through AT_1 receptors in rat vascular smooth muscle cells (VSMC) and has been shown to cause a dose dependent increase in PLD

activity in VSMC (Freeman , 1994; 1995). The activation of PLD by Ang-II has been shown to be responsible for the increase in intracellular Ca^{2+} in response to Ang-II. The increased activation of PLD by Ang-II in genetically induced hypertension may reflect an additional mechanism linking enhanced contractile responses to enhanced growth (Freeman and Tallant, 1994; Lassegue , 1991). Similar results have been found in mesangial cells (Barnett, 1993; Pfeilschifter et al., 1992 ; Pfeilschifter, 1993), Chinese hamster ovary fibroblast line (Wen, 1995) and hepatocytes (Bocckino, 1987). These results have important implications for the role of these second messengers in growth and contraction (Lassegue , 1993).

Ang-II causes a rapid induction of immediate-early genes and hypertrophy in the cardiac myocyte. However, the signaling mechanism of Ang-II induced immediate-early gene expression in cardiac myocytes has not been characterized. Sadoshima (1993) examined signal transduction of Ang-II in neonatal rat cardiac myocytes, using c-fos gene expression as a model system. and it was found that Ang-II also caused a small increase in cAMP in cardiac myocytes. Measurements of phospholipid derived second messengers revealed that Ang-II increased production of IP_3, DAG, PA, and AA, resulting in a sustained increase in PKC activity. This and other evidence suggests that Ang-

II activates PLC (Baker et al., 1989), PLD, and possibly phospholipase A_2 (PLA_2) (Kojima et al., 1985). All of these second-messenger systems are activated through the AT_1 receptor.

3. Role of Guanine nucleotide binding protein

a). Historical dicovery of Guanine nucleotide binding proteins

G proteins were first purified by Gilman and his colleagues in 1981 (Sternweis et al., 1981). This 45 kDa protein , initially described as a GTP-binding regulatory component of adenylyl cyclase (AC) is now known as the stimulatory G protein α-subunit (Gilman, 1987). Shortly thereafter, another G protein, G_i, was described as the inhibitory regulatory component of AC (Bokoch et al., 1983).

G - Proteins are a large family of GTP-binding proteins which act as signal transducers across cell membranes in all eukaryotic cells (Bourne et al., 1990). In general, the term G protein refers to the heterotrimetric, plasma membrane-associated G proteins which transduce signals from receptors to effector enzymes or ion channels, in addition, there are several other G

proteins, such as the bacterial elongation factor Tu, the mammalian small-molecular weight ras proteins, yeast RAS_2 proteins, and mammalian and yeast ADP-ribosylation factors (Bourne et al., 1991). Although they share structural homology in regions of GTP-binding and GTPase activity , their role in cellular signal transduction differs substantially from that of the heterotrimertic G proteins

b). G-proteins expressed in heart tissues and subcellular localization

Most of the known G proteins have been detected in myocardial tissue, although $G_{11-\alpha}$ is not readily detectable by Northern blot in the heart. Expression of $G_{o\alpha}$ subtypes in the heart is controversial. Some authors regard $G_{o\alpha}$ as a mainly neuron specific G protein, but others have identified $G_{o\alpha}$ as a 39 kDa protein subjected to PTX-catalysed ADP-ribosylation in dog and as a 39 kDa band in rat cardiomyocyte immunoblots using a $G_{o\alpha}$ antiserum (Ishikawa et al .,1990). There is evidence that nonmyocytes contain considerably more Giα-2 than myocytes (Loe et al., 1992) and that distinct G protein α-subunits are localized not only at the plasma membrane, but at different subcellular location such as the Golgi (Ercolani et al., 1990), the intercalated discs, SR triads (

Nash, 1992), Low levels of $G_{o\alpha}$-mRNA have been found in human atrium and rat heart (Eschenhagen et al., 1992).

Although multiple signaling pathways , including G-proteins , tyrosine kinase , and PKC , have been implicated in the regulation of PLD in diverse cell types, the mechanism of enzyme activation is still largely unknown (Exton, 1990; Billah and Anthes, 1990; Cockcroft 1992; Ben-Av and Liscovitch, 1989; Kaszkin et al., 1992). G-protein and protein kinase mediated pathways have been shown to activate PLD following cell stimulation in many cell lines and also in a cell free system, but the relationship between these activation pathways remains to be further elucidated.

GTPγS-dependent activation of PLD has been also observed in both platelets (Van Dez Meulen and Haslam, 1990) and neutrophils (Barrowman et al., 1986). With regards to the SL PLD, the G-proteins involved in this regulation have yet to be identified.

4. The role of protein kinase C in the regulation of PLD

a). Cardiac PKC isoforms

The study of PKC isoform expression in heart has provided evidence that cardiac myocytes co-express several distinct isoforms of PKC (Strulovici et al., 1991; Baldassare et al., 1992; Heidenreich, 1988; Majumdar et al., 1991; Akita et al., 1990). Steinberg et al. (1995) reported that multiple PKC isoforms are expressed in the rat heart in an age dependent fashon. Four PKC isoforms (PKC α, PKCδ, PKCζ and PKCε) can be detected in whole extracts from the neonatal ventricle and cultured neonatal ventricular myocytes. In contrast, only two isoforms (PKCδ, PKCε) were detected in total protein extracts from isolated adult ventricular myocytes. In the adult ventricular preparations, immunoreactivity for PKCε PKCδ vastly exceeded that for PKCδ (Bogoyevitch et al1993), also , low level immunoreactivity for PKC α, and PKCζ was detected in whole tissue extracts from adult ventricle, but not in the preparation enriched with adult myocytes. The conventional Ca^{2+} sensitive isoforms of PKC (α and perhaps β) are confined to neonatal myocytes, whereas the novel Ca^{2+} insensitive isoforms exist predominantly in adult myocytes . It was therefore speculated that Ca^{2+} independent isoforms of PKC

may be advantageous in the heart function (Rybin and Steinber, 1994; Bogoyevitch et al., 1993; Osada et al., 1990.).

Although PKC enzyme activity has been reported to increase in certain pathophysiological states such as ischemia (Prasad and Jones, 1992), pressure overload hypertrophy (Kwiatkowska-Patzer and Domanska-Janik, 1992); the mechanisms that control PKC isoforms expression in the heart remain to be fully elucidated.

b). PKC and myocyte hypertrophy

Conventional PKC has been implicated as an important mediator of neurohumoral induction of myocardial cell hypertrophy. Virtually, every phenotype feature of the hypertrophic response has been reported to be induced by chronic stimulation by phorbol esters. These include an increase in cell size, an increased rate of transcription of rDNA which leads to increased RNA content and an transient induction of immediate early gene expression. It should be noted that neither of these studies explored the role of the novel calcium independent PKC isoforms as potential intracellular mediators of the response to hypertrophic growth stimuli (Steinberg et al., 1995).

c). PKC and myocyte contraction

There are major inconsistencies reported in the literature which preclude a precise understanding of the mechanisms underlying PKC dependent modulation of contractile function. On the one hand, several studies have indicated that phorbol esters modulate contractile function in cardiac myocytes and has been taken as evidence to implicate PKC in the regulation of myocyte contractile function. For example, phorbol esters have been shown to modulate the force of contraction of rat myocytes, and also to increase the contractility of isolated adult rat ventricular myocytes (Macleod and Harding, 1991). Whereas other investigators describe a pronounced negative inotropic effect of phorbol esters in the perfused beating adult rat heart and adult rat papillary muscles (Yuan et al., 1987; Capogrossi et al., 1990 ; Otani and Das, 1988); These seemingly paradoxical results have led to the speculation, that differences in experimental protocols may account for such contradictions (Macleod and Harding , 1991).

Although the evidence that PKC is involved in the regulation of contraction and the induction of hypertrophy in cardiac myocytes is compelling, there are many

inconsistences in the published literature that have not been resolved adequately.

d). PKC regulation of PLD activity

Protein phosphorylation is a basic mechanism by which many extracellular stimuli regulate cellular function. The modulation of PLD activity by PKC is an important mechanism in the PLD / PAP signal transduction cascade (Pai, 1981; Gustavsson and Hansson, 1990; Conricode et al., 1992; Hii et al., 1991; Gustavsson et al., 1994.)

PKC acts as a switch to up regulate PC-PLD and downregulate PLC activities. A working model for the regulation of PI-PLC and PC-PLD activities by PKC was suggested by Pachter et al. (1992). Stimulation of the thrombin receptor by α-thrombin activates PI-PLC via a regulatory G-protein, perhaps Gq (Taylor et al., 1991). PI-PLC hydrolyzes PIP_2 to yield IP_3 and DAG, resulting in PKC activation. This then feeds back to inhibit G-protein coupling to PI-PLC and to enhance G-protein coupling to PC-PLD activity. This shift from PI-PLC activity during the initial phase of the cellular response to PC-PLD activity during the later phase may serve to preserve cellular PIP_2, which is less

abundant than PC in the membrane (Fukami and Takenawa, 1989; Takamura et al., 1987). Alternatively , IP_3 and DAG , the products of PI-PLC activity , may be necessary for initiation of cellular responsiveness to hormones such as α-thrombin , while PA and its metabolites, which are derived from PC-PLD activity, may be necessary for sustenance of cellular responses (Cook and Wakelam, 1989; 1991).

It is worth noting that staurosporine, and sphingosine, which are PKC inhibitors , activate PLD via a pertussis toxin-sensitive G-protein in rabbit peritoneal neutrophils (Kiss and Anderson, 1990). Thus PKC-independent action of PLD activity may be involved in the direct activation of PLD. G-protein and PKC play an important role in regulation PLD activity. In this regard ,PKC has been shown to enhance that GTPγs-stimulated PLD activity in human platelet membrane (Van der and Haslam, 1990), canine brain synaptosomes (Qian and Drewes, 1989) and permeabilized HL60 cells (Geny and Cookcroft , 1992). In most of the these systems , PLD activity was significantly higher than that observed in response to either Ang-II or GTPγs alone when the cells were exposed to both GTPγs- and PKC (Olson et al ., 1991; Kanoh et al., 1993; Van et al., 1990; Coorsen and Haslam, 1993; Xie and Dubyak , 1991; Kanoh et al., 1993).

5. Effect of Ca^{2+} -mobilizing agents in the regulation of PLD

a). Receptors orientated regulation of PLD activity

In a variety of cells , PLD is activated by a number of agents to produce PA and subsequently DAG. Agonist control of PLD appears to occur by at least two distinct mechanisms characterized by the involvement of either G-protein or protein kinases. Interplay between these mechanisms and their activation patterns may lead to various complex activation patterns, depending on the cell types and the stimuli used (Billah et al., 1991).

(1). Adrenoceptors

α- adrenoceptors expressed in rat fibroblasts can regulate PLD-mediated hydrolysis of PC by interacting with pertussis toxin-sensitive G-proteins (MacNulty et al., 1992). The α2-C10 adrenergic receptor in human platelets have been demonstrated to interact directly with two distinct pertussis toxin-sensitive G-proteins, Gi2 and Gi3 (Milligan et al., 1991). High affinity GTPase activity in the membrane of cells from the various clones was

stimulated by the addition of the α2 adrenergic agonist UK 14304. Furthermore, the α2-C10 receptor in one clone(1c), but not in other clones promoted a marked stimulation in the generation of water-soluble products derived from PC via PLD which was confirmed by the transphosphatidylation activity (MacNulty et al., 1992). SL PLD is also indirectly activated under β-receptor stimulation (Lindmar et al., 1986).

(2). P_2 receptor

The effects of purinergic agonists on PC breakdown and prostacyclin synthesis were investigated in cultured bovine pulmonary artery endothelial cells (BPAEC) by Martin and Michaelis (1989). Cells incubated with [^{3}H]Choline and [^{14}C]myristic acid were selectively incorporated into PC. In BPAEC prelabeled with [^{3}H]choline, ATP stimulated a rapid 5-fold increase intracellular free [^{3}H]Choline. [^{3}H]Choline formation was associated with a concomitant loss of ^{3}H from PC, and it was not preceded by an increase in the ^{3}H content of other PC degradation products. In BPAEC prelabeled with [^{14}C]myristic acid, ATP stimulated a rapid increased in PA, and [^{14}C] DAG. These changes were associated with a loss of ^{14}C from PC, but not from PI. In permeabilized BPAEC prelabeled with [^{3}H]Choline but not [^{3}H]PC, the effect

of ATP and GTPγs were synergistic at low GTPγs concentration . Permeabilized BPAEC did not convert exogenous [^{3}H] PC into [^{3}H] choline. These data are consistent with the notion that the purinergic agonist stimulates PC breakdown by PLD mechanism. This line of evidence has suggested an important role for PC breakdown in the mechanism of signal transduction in endothelial cells (Irving and Exton, 1987; Bocckino et al., 1988) and in hepatocytes (Bocckino et al., 1987) stimulated with Ca^{2+}-mobilizing agonists.

Xie et al. (1991) compared the ability of extracellular ATP to stimulate both PLD -based signal transduction and primary granule secretion in HL-60 cells via P_2-purinergic receptors. Their data showed that the receptor regulated PLD signaling pathway is induced during differentiation of myeloid progenitor cells ; and that the differential activation of this signaling system by various Ca^{2+} mobilizing receptor agonists may underlie the differential regulation of secretion and other phagocyte function by such agents.

(3). M_2-receptor

The stimulation of PLD activity by muscarinic receptor agonists has been reported in canine brain synaptosomes (Qian and Drewes, 1989);

Furthermore, muscarinic receptor stimulation of PLD in 1321N1 astrocytoma cells was inhibited after down regulation of PKC (Martinson et al., 1990) and significantly reduced by PKC inhibitors in LA-N-2 neuroblastoma cells (Sandmann and Wurtman, 1991) suggesting a role for PKC in receptor-mediated activation of PLD. However, the activation of PLD by receptor agonists has also been reported as a PKC - independent process in many cells (Reinhold et al., 1990 ; Lassegue et al., 1991).

Similar results about the role of muscarinic receptors in the stimulation of phospholipid metabolism have also been reported (Corradetti et al., 1983). Whatever the mechanism of the muscarnic receptor linked mobilization of cellular choline, these results indicate the important physiological , pathophysiological and pharmacological implication for the activity of cholinergic neurons , especially in the brain.

With regards to the heart it has not been determined whether M_2 receptors are involved the stimulation of PLD.

b).Other Ca^{2+} mobilizing agents

Bocckino et al. (1987) reported that PA accumulation in hormone-treated hepatocytes is via a PLD associated mechanism. Isolated hepatocytes responded to a variety of Ca^{2+} - mobilizing agents such as vasopressin , Ang-II, epinephrine, EGF, ATP and ADP with a rapid increase in PA mass. (Thomas et al., 1983; Takemawa et al., 1982) . Ca^{2+} - mobilizing hormones mainly increase PA levels in hepatocytes by a mechanism involving PC-PLD and not PI-PLC. That is, This process is involved in a G - protein coupled to PLD, not in phosphorylation of DAG to PA or *de novo* synthesis(Bocckino et al., 1987).

It should be noted that EGF induced hydrolysis of PC by PLD and PLC in human dermal fibroblasts has shown that PA formed by PLD catalyzed hydrolysis of PC is not the major precursor of the observed increased DAG, but it is via PLC-catalyzed hydrolysis of PC (Fisher et al. 1991); Taken together, it is suggested that EGF -induced signal transduction mechanism is dependent on the cell type.

6. Others regulatory factors

PLD activity is also activated by the PIP_2, *cis* unsaturated fatty acid (Dai et al, 1995) and tyrosine kinase (Bourgoin and Grinstein, 1992; Liscovitch et al., 1994; Chalifour and Kanfer, 1982; Kumada et al., 1993). The most suitable surfactants for this activation were oleate and palmitooleate. Complement of C5a activation of PLD in human neutrophils has also reported (Mullmann et al., 1990); Gonadotropin releasing hormone receptors can activate PLD in ovarian granulosa cells and results in an increase in endogenous PA levels has been reported (Liscovitch and Amsterdam, 1989) . It may be speculated that certain cellular constituents can be directly modulated by endogenous PA that is produced by a signal activated PLD.

It is well known that calcium is an important regulatory element for many cellular functions, and in this regard is involved in the regulation of PLD activity in a variety cells (Kessels et al., 1991; Huang et al., 1991; Billanh and Athes, 1990).

Gustavsson et al. (1994) investigated the Ca^{2+} - dependent and PKC - dependent mechanisms of PLD activation in rat heptocytes by measuring PEth

formation in the presence of ethanol. Stimulation of PEth formation by 12-O-tetradecanoyl-phorbol 13-acetate(TPA) and Vasopressin was observed indicating that the PKC affects PLD activity indirectly by enhancing the mobilization of intracellular Ca^{2+} stores.

It should be noted that the requirement of Ca^{2+}, detergents, and fatty acids for the activation of mammalian PLD seems to vary from cell to cell (Exton, 1990; Shukla and Halenda, 1991) .

E. Interaction of phospholipases

1. Cross-talk between PLD and PLA_2

PC-PLA_2 catalyzes the hydrolysis of PC to produce AA and lysophosphotidy-choline. PA formed from PLD can enhance the activation of PLA_2 (Saito et al., 1993); on the another hand, AA , which is the product of PLA_2 can stimulate the PLD activity (Dai et al., 1996).

2. Cross-talk between PLD and PLC

Many PLD regulatory factors such as G-protein, PKC and Ca^{2+} are closely associated to PI-PLC activation (Kanaho et al., 1992). In fact , PA can directly increase the activity of PI-PLC in cardiac ventricle myocytes, leading to an increase in IP_3 (Bocckino et al., 1991; and Kurz et al., 1993). PIP_2 , which is the substrate of PI-PLC, can act as a cofactor of PLD (Liscovitch, 1994). Activation of PLC-γ is necessary for stimulation of PLD by platelet-derived growth factor (Yeo et al., 1994; Lee et al., 1994). Therefore, it is conceivable that in some pathological situation, differential effects on these phospholipases may occur.

F. Pathophysiological significance studies of PLD / PAP

The pathophysiological significance of the PLD / PAP pathway has not been fully established , however, some studies have been performed in different pathological conditions. For example, a depressed SL PLD activity and an increased PAP activity has been observed in diabetic cardiomyopathy (Williams et al., 1995, 1996). The effect on SL PLD appears not to be specific since SR and mitochondrial PLD activities were also depressed. The elevated

SR PAP activity and unchanged mitochondrial PAP activity observed in the diabetic heart indicaes different regulation of these enzymes.

A depression of cardiac SL PLD activity by oxidant-induced thiol modification has been reported (Dai, 1993, Thesis). This may be due to the augmented plasma levels of catecholamine in CHF (Homcy. et al, 1991,), which may contribute to oxidative damage. In fact, recent studies have shown that oxidation of catecholamines results in the formation of partially reduced forms of oxygen, which impair the cardiac function (Dhalla et al., 1992, Kaul et al., 1993).

The study of phospholipid signalling pathways in thyroxine-induced cardiac hypertrophy has demonstrated that PLD activity was depressed in T4 - induced cardiac hypertrophy (Williams et al. , 1994). Other forms of hypertrophy (such as cardiomyocytes stretch model and strok-prone spontaneously hypertensive rat model) have been characterized by an activation of PLD. This implies that diverse changes in phospholipase signalling pathways may occur in different types of cardiac hypertrophy, which may be related to the type of stimulus initiating the hypertrophic process (Panagia et al., 1991).

A growing body of evidence has shown that membrane phospholipid degradation contributes to the pathophysiology of myocardial ischemic and reperfusion injury (Shaikh and Downar 1981; Chien et al., 1981; Otani et al., 1986; Das et al., 1986; Zalewski et al., 1988). Activation of the deacylation pathway catalyzed by PLA_2 as well as enhanced phosphodiesteratic breakdown catalyzed by PLC has been shown to contribute to the loss of myocardial phospholipids (Otani et al., 1989; Otani, 1988).

An enhanced PLD activity has been observed during myocardial ischemia /reperfusion (Moraru et al., 1992), resulting in an increase in PA and consequently an increase in DAG formation, which is suggested to protect against myocardial ischemia and reperfusion injury.

It has also been demonstrated that stimulation of PLD activity with sodium oleate (20μM) induced a significant improvement of functional recovery of ischemic hearts during reperfusion. These results suggest that a PLD-mediated signalling in the ischemia heart may aid in functional recovery during reperfusion. The status of the PLD / PAP pathway in the CHF has not been fully addressed.

III. MATERIALS AND METHODS

A. Materials:

^{14}C-PC[L-A-1-palmitoyl-2 oleoyl-(oleoyl-1-^{14}C)] and ^{14}C-PA[phosphatidic acid -dipalmitoyl-(glycerol-^{14}C)] were obtained from Dupout New England Nuclear, (Mississauga, Ontario, Canada). Egg PC, Sodium oleate and PA, were obtained from Serday Research Laboratory (London, Ontario, Canada). Silica Gel 60 AF- 254 thin-layer chromatography plates were purchased from Whatman International Ltd. (Madison, USA). Solvents were purchased from VWR Company (London, Ontario, Canada). Cytosint ™ ES* was obtained from ICN Biomedical INC (Mississaga, Ontario, Canada). All other chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri, USA) and were of analytical grade or of the highest grade available.

B. Methods:

1. CHF model

Post infarction CHF rats was induced in male Sprague Dawley rats of 175-200g

weight by coronary artery ligation (Dixon , 1990) . A pre operative dose of analgesia (Buprenorphine hydrochloride) was given 1 h before surgery. The animals were anesthetized with isofluora and an incision was made along the left sternal border. The third and fourth ribs were cut, and the retractor was inserted. Then the pericardial sac was perforated and the heart was exteriorized through the intercostal space. The left anterior descending aorta was ligated with a silk suture (6-0) about 2 mm from its origin. The heart was repositioned in the chest cavity and the incision was closed with 3-0 suture whilst simultaneously 5-10 ml air and exudate was suctioned with a syringe. During the whole surgical procedure, rats were maintained on a positive pressure ventilator delivering a mixture of 95% O_2 and 5% CO_2 mixed isofluora (2 liters/min). The mortality of the procedure was about 20 % within 48 h. The sham operated animals served as controls and the same procedure was employed except that the coronary artery was not ligated. The animals were allowed to recover and were maintained on food and water *ad libitum*. Imidapril (1 mg/kg of body weight , which was dissolved at a concentration of 1 mg/1 ml in the tap water) was orally administered to animals with a gavage tube at the end of the week 4 after the coronary artery ligation untill the end of week 8.

To evaluate the status of heart failure , the ratio of wet lung to dry lung, which reflects the pulmonary congestion and is considered to be an index of left ventricle function, was measured. The wet lung was measured just after removal from the chest cavity, after which, it was dried at the 95-100 °C until completely dry (The lung was weighed 3 times at 8 hours intervals, until the weight was constant). The ratio of LV to BW , a common accepted marker of cardiac hypertrophy was also measured in the sham control and CHF groups.

2. Isolation of sarcolemmal and cytosol fractions

The animal were decapitated (in accordance with the Animal Care Committee, University of Manitoba) at 1, 2, 4, 8 and 16 weeks after the surgical procedure. The isolation of SL was carried out at 4 ^{0}C according to the methods of Pitts (1979). Briefly, after decapitation, the hearts were quickly excised and (5ml buffer /g tissue) finely minced by hand in a 0.6 M sucrose 10 mM imidazole, pH 7.0, solution. The solution was then aspirated to remove remaining blood cells and the pieces were resuspended in an equal volume of sucrose-imidazole (as above). Then, the minced tissue was homogenized with a Polytron PT 3000 homogenizer (Kinematica AG, Switzerland) at 13000 RPM for 6 × 15 seconds with 20 second intervals in between. The resulting homogenate was

then centrifuged at 12 000 g for 30 min at Beckman centrifuge with a TL-20 rotor. The pellet was discarded and the supernatant was collected, a 0.5 ml aliquot of the supernatant was centrifuged at 100,000g for 60 min in a Beckman TL-100 Ultracentrifuge to purify the cytosol. The remaining supernatant was diluted with 160 mM KCL, 20 mM 3 - (N-morpholino)-propanesulfonic acid (MOPS), pH 7.4 (5 ml/ g tissue) and centrifuged at 100, 000 g for 60 min. The resulting pellet was resuspended in 160 mM KCL-MOPS buffer (pH 7.4) and layered over a 30% sucrose solution containing 0.3 M KCL-50mM $Na_4PO_4O_7$, and 0.1 M Tris-HCL, pH 8.3. and centrifuged at 100,000g for 90 min in a Beckman swinging bucket rotor (SW-28). The band at the sucrose-buffer interface was taken and diluted with 3 volumes of 160 mM KCL-20mM MOPS, pH 7.4. and centrifuged again at 100,000 g for 30 min, the resulting pellet was rich in SL membrane and was resuspended in 0.25 M sucrose, 10 mM histidine, pH 7.4 (225 μ /g tissue), and divided into aliquots, frozen in liquid nitrogen and stored at -70 ^{0}C for later use.

3. Phospholipase D assay

SL PC-specific PLD hydrolytic activity was determined by measuring the amount of PA , which is the direct product of PLD hydrolytic activity using

exogenous ^{14}C-PC as substrate (Dai et al. 1993). PC substrate was prepared by mixing aliquots of egg PC and PC-^{14}C. The organic solvents were evaporated under N_2 stream until completely dry. Then 25 mM sodium oleate solution was added to form a concentration of 12.5 mM PC-^{14}C-PC pool, which was sonicated for 30 min in a Branson 1200 sonicator bath , Ultrasonic Corp., Danbury, Conn.). The standard assay was carried out at 25 °C for 60 min in a final volume of 120 ul in an incubation medium containing 35- 60 μg membrane preparation, 50 mM 3,3-dimethylglutaric acid (DMGA)-10 mM EDTA (pH 6.5) , 25 mM KF, (a PAP inhibitor), 5 mM sodium oleate, 2.5 mM [14C] PtdCho (0.167 mCi / mmol). The reaction was stopped by adding 2ml of chloroform : methanol (2:1, v/v), and the organic and aqeous phases were separated by addition of 0.5 ml of 0.1M KCL, followed by vortexing vigorously for 2 min and then centrifuged at 3000 rpm for 5 min. The upper aqueous phase was discarded and the organic phase was washed with 1 ml of chloroform : methanol: 0.1 M KCL (2:1:0.5 v/v) to prevent contamination. The PLD transphosphatidylation activity was determined by the same incubation conditions except that it was performed in the presence of 0.4 M ethanol (final concentration). The blanks were treated in the same manner except that the membrane was added after addition of the stop solution. The final extracts were evaporated to almost dryness under a stream of N_2 and redissolved in

chloroform containing PA (for hydrolytic activity) or PEth (for transphosphatidylation) as a carrier and was quantitatively applied to silica gel 60A F-254 thin-layer (0.25 mm thick) plates.

In both cases, the plates were developed in chloroform: methanol: acetone: glacial acetic acid : water (50:15:15:10:5, v/v) with pure PA acid or PEth as a standard for hydrolytic and transphosphatidylation activity, respectively. After the solvent front had migrated to about 1 cm from the top edge of the plate, the plates were taken out and dried at room temperature. The lipid bands were visualized with iodine vapor and scraped. The scrapes were solubilized with CytoSint ™ ES, and radioactive activity was determined by a liquid scintillation counter (Model 1701, Beckman Instruments, Inc)

4. Phosphatidic acid phosphatase activity assay

PAP activity was assayed by measuring the formation of DAG from exogenous ^{14}C PA. The assay procedure was basically according to Martin, et al. (1991). Briefly, for the determination of sarcolemmal PAP activity, substrate was prepared by mixing the egg PA and L-α dipalmitoyl-[glycerol-^{14}C(U)] - phosphatidic acid (specific activity 144 mCi / mmol). The mixture was dried

under a stream of N_2, and 5 mM EGTA was added to a final concentration of 6 mM, and then was dissolved by sonication for 5-10 min (until clear) in a sonicator (Model 1200, Branson Ultrasonics, Corp.). The reaction was carried out in a incubation medium containing 100 mM Tris-maleate buffer (pH 6.5), 1 mM dithiothreitol (DTT) , 0.6 mM [^{14}C] PtdOH (1 Ci/ mol)1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, and 0.2% BSA and 30- 60mg membrane protein in a final volume of 100 ul. The reaction was incubated at 37 °C for 10 min and terminated by adding of 2 ml chloroform: methanol (2:1 v/v) and 0.5 ml of 0.1 M KCL. Blanks were performed in the same manner except the membrane was added after adding the stop mixture. The incubation medium was mixed completely by vortex for 2 min and the organic and aqueous phases were separated by centrifugation (3,000 rpm for 5min). The aqueous phase was discarded and the organic phase (lower phase) was dried under a stream of N_2 to almost dryness and redissolved in chloroform containing DAG and monopalmitolglycerol as carriers and quantitatively applied to solica gel 60 A thin layer (0.25 mm thick) plates . The plates were developed in a solvent mixture of petroleum ether : ether: acetic acid (60:40:1 v/v) for 45 min (until the solvent front reached 1 cm from the top edge of the plate). The phospholipid bands were visualized by exposure to iodine vapors and scraped . The radioactivity was determined by a liquid scintillation system (model 1701,

Beckman Instruments, Inc). The Rf values were 0.34 and 0.06 for DAG and monoacylglycerol, respectively.

Cytosolic PAP activity was determined as follow: the substrate for cytosolic PAP activity was a mixture of PA and PC (3:2) and labelled ^{14}C-PA (for 10 tubes , add 22.5 ul PA and 15.8 ul PA and 2 ul ^{14}C-PA) with PA concentration of 6 mM. The two sets of tubes (one containing cytosol and 4.2 mM NEM , the other containing cytosol and water) were preincubated at 37 °C for 10 min first. Then the reaction was carried out by same procedure as for the SL PAP activity except that it was carried out in the presence of 3mM $MgCl_2$. The net PAP activity was the difference between the activities from the two sets of tubes. i.e. the activity in the absence of NEM minus the activity in the presence of NEM.

C. Statistical analysis:

The Students t-test was used for the evaluation of the difference between the two groups and ANOVA was used to evaluate the difference among more than two groups . $P < 0.05$ was considered to be significant.

IV. RESULTS

A. General characteristics of the congestive heart failure animals 8 weeks after coronary artery ligation

The general characteristics of the sham control and CHF rats at 8 weeks after coronary artery ligation are shown in Table 1. CHF in rats was induced by the ligation of anterior descending coronary artery as described in the Methods section. This model has been extensively studied by Dixon et al (1992b). Furthermore, the existence of CHF in this model has been confirmed by the hemodynamic studies of Afzal and Dhalla (1992).

In the heart failure group, the scar weight was 0.32 ± 0.01 g and the transmural infarction was found to be greater than 30% , indicating that the coronary artery ligation procedure produced a severe infarction, which in turn caused CHF. The significant increase in the ratio of wet lung to dry lung, which reflects the pulmonary congestion, indicated that left ventricle function was compromised. Cardiac hypertrophy was indicated by the significant increase in the viable left ventricle (17%) ($P < 0.05$) and by the ratio of viable LV to body weight ratio. These results are similar to the previous studies conducted in our laboratory

Table 1. General characteristics of the sham and CHF animals 8 weeks after ligation

	Sham	CHF
Body weight (g)	523.9 ± 11.2	490.7 ± 11.1
LV weight (g)	0.85 ± 0.02	0.91 ± 0.01*
Viable LV/BW (mg/g)	1.61 ± 0.02	1.86 ± 0.04 *
RV weight (g)	0.26 ± 0.01	0.33 ± 0.01*
Lung wet wt/ lung dry wt ratio	4.80 ± 0.53	6.55 ± 0.36*
Scar weight (g)	ND	0.32 ± 0.01

Data are expressed as means ± SEM of 6 experiments with an average of 20 rats in each experiment . CHF animals were induced as described in the Methods section. CHF: Congestive heart failure; LV: left ventricle ; RV: right ventricle; ND: not detectable

* indicates significantly different ($P < 0.05$) from sham control values.

(Mesaeli, Ph. D. thesis, 1993) and form other investigators (Dixon et al., 1992b).

B. Sarcolemmal PLD activity in congestive heart failure

1. Left ventricle sarcolemmal membrane PLD activity

Cardiac SL membranes isolated from the left ventricle of sham control and CHF rats were used to detect the hydrolytic activity of PLD , using exogenous [^{14}C] PC as substrate. It was found that PA was significantly increased as early as the first week after ligation of the coronary artery, reaching a peak at 4 weeks. It should be stated that the increased PA levels were observed all the stages of post-infarction failure (Table 2). It is well known that the PA is not only the product of PC specific PLD activity, but also can be generated by PI-PLC activity . DAG , one of the direct products of PI - PLC, can be phosphorylated to PA by the DAG kinase in the presence of ATP. However, in the assay conditions employed in the present study, no ATP was present, therefore, PA

Table 2: Formation of phosphatidic acid by sarcolemmal phospholipase D in sham and CHF rats

	Phospholipase D activity (nmol/mg/h)		
Week	Sham	CHF	% Sham
1	269.5 ± 13.6	372.9 ± 7.5*	138
2	252.8 ± 17.6	371.5 ± 6.8 *	147
4	198.6 ± 8.3	318.6 ± 15.2*	160
8	187.8 ± 12.3	233.4 ± 13.2*	124
16	180.7 ± 4.41	230.7 ± 3.2*	128

Values are means ± SEM of 4-8 experiments done in triplicate and are expressed as nmol/mg/hr. The assay was performed as described in the Methods section. SL PLD: Sarcolemmal phospholipase D. CHF: Congestive heart failure

* indicates significantly different from corresponding sham value ($P<0.05$)

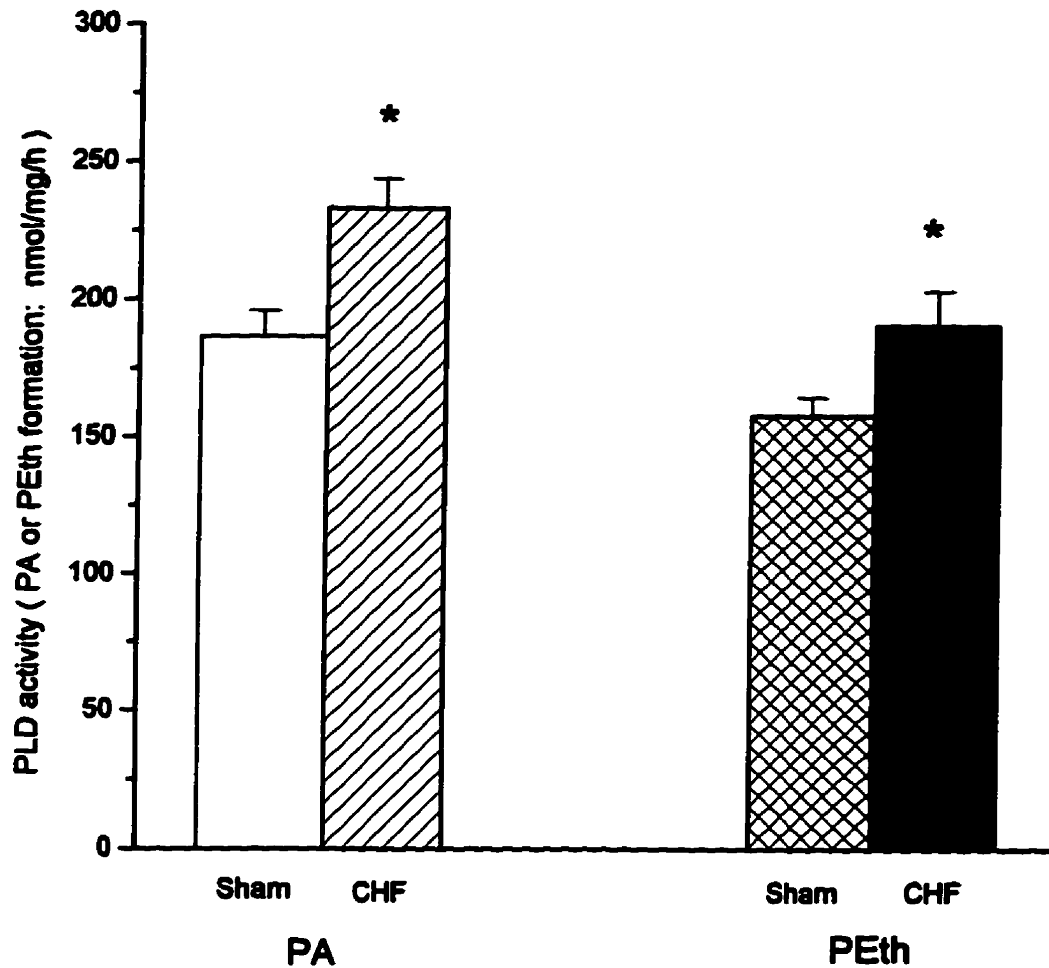

Figure 1. Comparision of cardiac sarcolemmal PLD hydrolytic activity and transphophatidylation activity in 8 weeks sham and CHF rats due to coronary artery ligation

Results are means ± SEM of 4 experiments in triplicate. The assay were done as described in the Methods section. PA: Phosphatidic acid; PEth: Phosphatidylethanol.

* $P<0.05$

was produced exclusively via PC-PLD activity. SL PLD transphosphatidylation activity was measured by the same procedure as the hydrolytic activity except in the presence of 0.4 M ethanol . In the 8 weeks CHF group, an increase in PEth levels (21% , $P < 0.05$) was observed as compared to the control sham group. This result was consistent with the PLD hydrolytic activity, which was elevated by 24% ($P<0.05$) (Fig. 1). The increase in both hydrolytic and transphosphatidylation confirmed that the PLD activity is significantly enhanced in CHF. It is interesting to note that an aging effect on SL PLD was observed in both experimental groups.

2. Right ventricle sarcolemmal PLD activity in 8 weeks CHF animals

Right ventricle SL was isolated from frozen right ventricular tissue by the usual Pitts isolation method. The determination of PLD activities demonstrated that no significant differences between sham control and CHF animals at 8 weeks (Fig 2) were observed. Also , it was found that the right ventricle PLD activity was much lower than the left ventricle SL PLD activity both in normal control and CHF group. This observation is understandable since in physiological terms, left ventricle plays a more important role than right ventricle in the heart

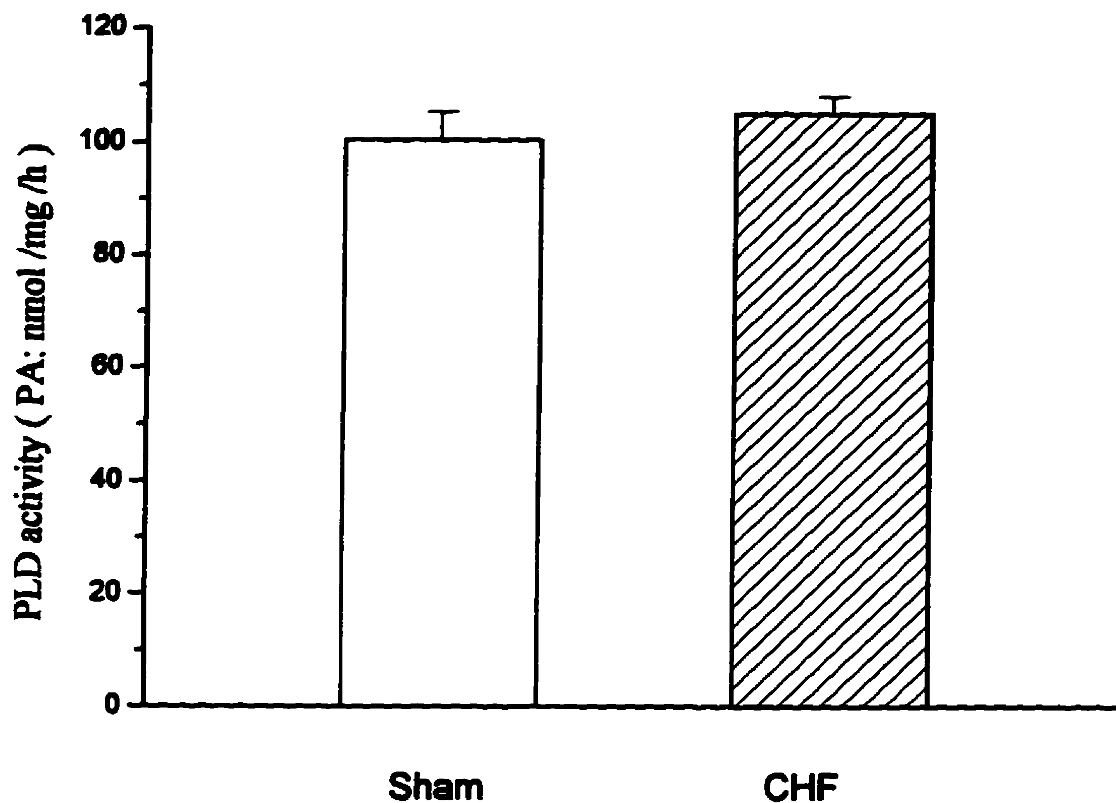

Figure 2. Right ventricular sarcolemmal PLD activity in sham and CHF rats 8 weeks after coronary artery ligation

Data are means ● SEM of 4 experiments in triplicate. The assay were done as described in the Methods section. CHF: Congestive heart failure; PA: Phosphatidic acid.

$P > 0.05$

pump function.

C. Examination of phosphatidic acid phosphatase activity in sham control and CHF rats

There are two forms of PAP present in heart tissue. One is the membrane bound PAP, which is NEM insensitive and Mg^{++} independent, whereas the other form is located in the cytosol and SR, and is considered to be NEM sensitive and Mg^{2+} - dependent. Most of the studies on PAP have been performed in hepatocytes and brain tissue (Martin et al. 1991). Preliminary data from our laboratory has indicated that similar sensitivities to NEM and Mg^{2+} dependency occur in the myocardium (Williams. 1995, Thesis). In the present study, we examined both SL and cytosolic PAP activities in the failing heart.

1. Left ventricle sarcolemmal phosphatidic acid phosphatase activity

During the development of CHF, the SL PAP activity , which is measured by the amount of DAG formed in the assay, showed a time-dependent increase, peaking at 4 weeks (Table 3). The SL bound PAP activity was elevated at all stages after coronary artery ligation, which was parallel to the changes observed in SL PLD activity, but the percentage increase was much higher than PLD

activity at every stage. Unlike the PLD activity, an aging effect on the sham control PAP activity was not observed.

2. Left ventricle cytosolic PAP activity

The cytosolic PAP activity was expressed as the amount of DAG formed in the assay with the mixture of PA and PC (the ratio of PA to PC is 3:2) as substrate in the presence of 0.3 M $MgCl_2$, a parallel assay was also carried out by the same procedure except adding the 4.2 mM NEM (final concentration) into the assay medium. The difference between PAP activities measured in the untreated fraction, (which is cytosolic PAP activity plus the activity from the possible SL contamination) , and the activity obtained from the NEM treated group, (which is NEM-insensitive SL contamination activity). was considered as cytosolic PAP activity (Martin, 1991). The cytosolic PAP activity during the development of post-infarction CHF failure is shown in Table 4. It can be seen that the activity was increased at the week 1 after surgery, however, unlike the SL PAP, the cytosolic PAP activity reached a maximum at week 8, this maximal activity represented an increase of 130% ($P < 0.05$) as compared to sham control levels.

Table 3. Formation of diacylglycerol by sarcolemmal phosphatidic acid phosphatase activity in sham and CHF rats

	Phosphatidic acid phosphatase activity (nmol/mg/10min)		
Week	Sham	CHF	% Sham
1	74.6 ± 4.1	141.5 ± 16.2*	190
2	75.0 ± 2.9	172.7 ± 8.9*	230
4	76.2 ± 7.5	180.7 ± 5.3*	237
8	75.6 ± 2.1	101.6 ± 5.5*	135
16	70.2 ± 4.7	96.9 ± 5.9*	138

Results are means ± SEM of 8 experiments performed in triplicate and are expressed as nmol/mg/10 min. The assay was carried out as described in the Methods section. SL PAP: Sarcolemmal phosphatidic acid phosphatase; CHF: Congestive heart failure.

* indicates significantly different from corresponding sham control value ($P<0.05$)

Since both SL and cytosolic PAP activities were increased in CHF. it is unlikely that translocation of PAP from SL to cytosol or vice - versa, occurred in CHF.

3. Right ventricle SL and cytosolic PAP activities at 8 weeks after coronary artery ligation

Right ventricle SL and cytosolic PAP activities were examined under in the same assay conditions as described above. Unlike left ventricle PAP activity, no changes in either right SL or cytosolic PAP activities in the post-infarction CHF rats were observed, which is similar to the observation in PLD activity (Fig. 3).

D. PA and DAG formation from the PLD / PAP pathway in CHF in the absence of potassium fluoride

Detection of PLD activity is conducted in the presence of , potassium fluoride (KF), which is a potent PAP inhibitor, to prevent PA dephosphorylating to DAG. Since both PLD and PAP activities are significantly elevated in CHF rats, (the percentage increase of PAP activity is much higher than that of PLD), it is reasonable to suggest that the actual net amount of PA is decreased, whereas DAG is increased in CHF. In order to confirm this , SL PLD activity was examined in the absence of KF , the net PA formation decreased from 46.95 ±

Table 4. Formation of diacylglycerol by cytosolic PAP activity in sham and CHF rats

	Phosphatidic acid phosphatase activity (nmol/mg/10min)		
Week	Sham	CHF	% sham
1	10.55 ± 0.62	14.70 ± 1.32*	139
2	8.10 ± 0.17	15.82 ± 0.99*	195
4	8.89 ± 0.37	17.56 ± 0.49*	198
8	11.99 ± 1.98	25.58 ± 2.01*	213
16	7.39 ± 0.59	12.91 ± 0.93*	175

Values are means ± SEM of 4-8 experiments performed in triplicate and are expressed as nmol/mg/10min. The standard assay was carried out as described in the Methods section. PAP: Phosphatidic acid phosphatase; CHF: Congestive heart failure

* indicates significantly different from corresponding sham control value ($P<0.05$)

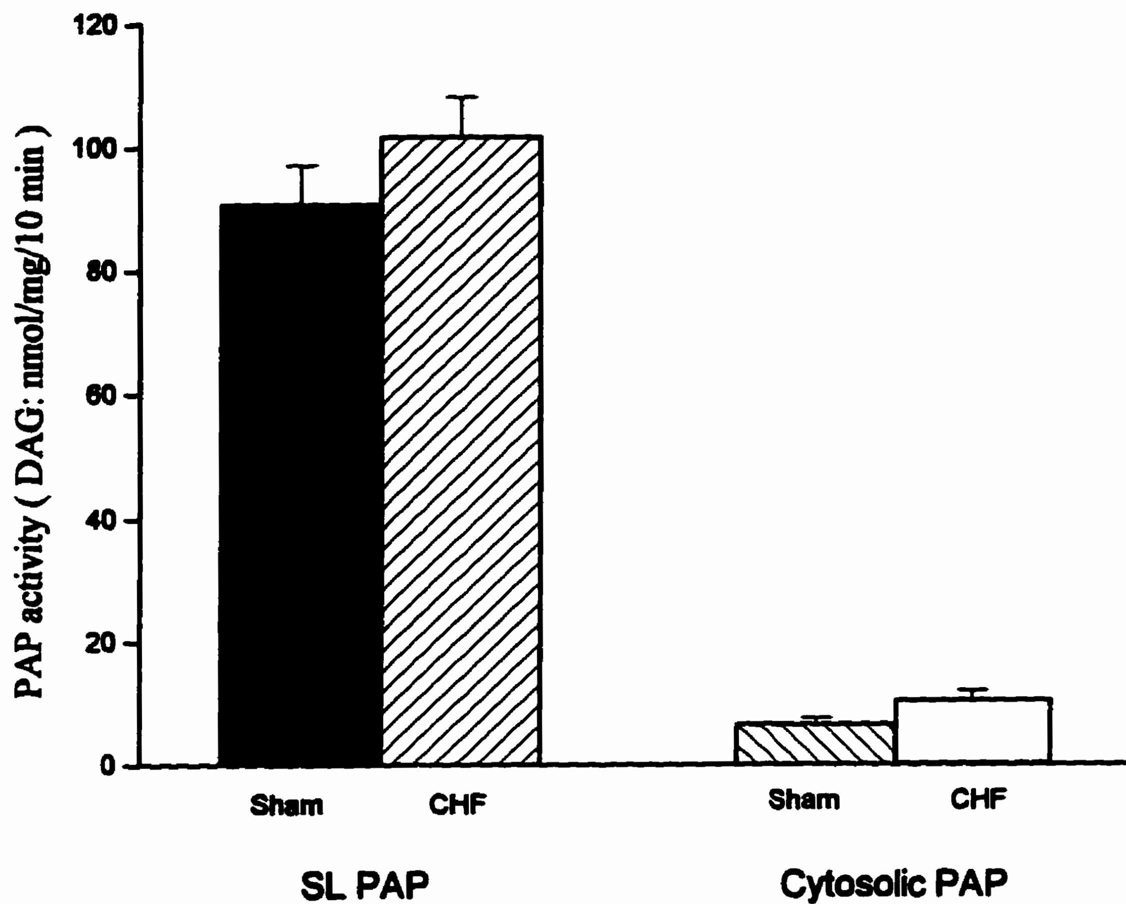

Figure 3. Right ventricle sarcolemmal and cytosolic PAP activity in 8 weeks sham control and CHF rats after coronary artery ligation

Data are means ± SEM of 4 experiments in triplicate. The assay was performed as described in the Methods section. DAG: diacylglycerol.

P>0.05

4.6 (nmol/mg/h) to 30.17 ± 4.97 (nmol/mg/h), i.e. 37 % ($P<0.05$), but the DAG formation , which reflects the activity of PAP activity, was increased from 171.32 ±18.8 to 230.33± 21.93 (nmol/mg/h), i.e. 36 % ($P<0.05$) compared to sham control values (Fig. 4).

E. Effect of Imidapril on SL PLD and PAP and cytosolic PAP activities

It is well known that the ACE inhibitors can improve the clinical symptoms and prognosis of CHF. In order to examine the effect of ACE inhibitors on the PLD / PAP signalling pathway in CHF, the effect of Imidapril , a new ACE inhibitor, on the PLD / PAP pathway in CHF was examined . Table 5 shows the effect of Imidapril treatment on SL PLD and PAP and cytosolic PAP activities. It can be seen that a normalization of the elevated activities of these enzymes was observed.

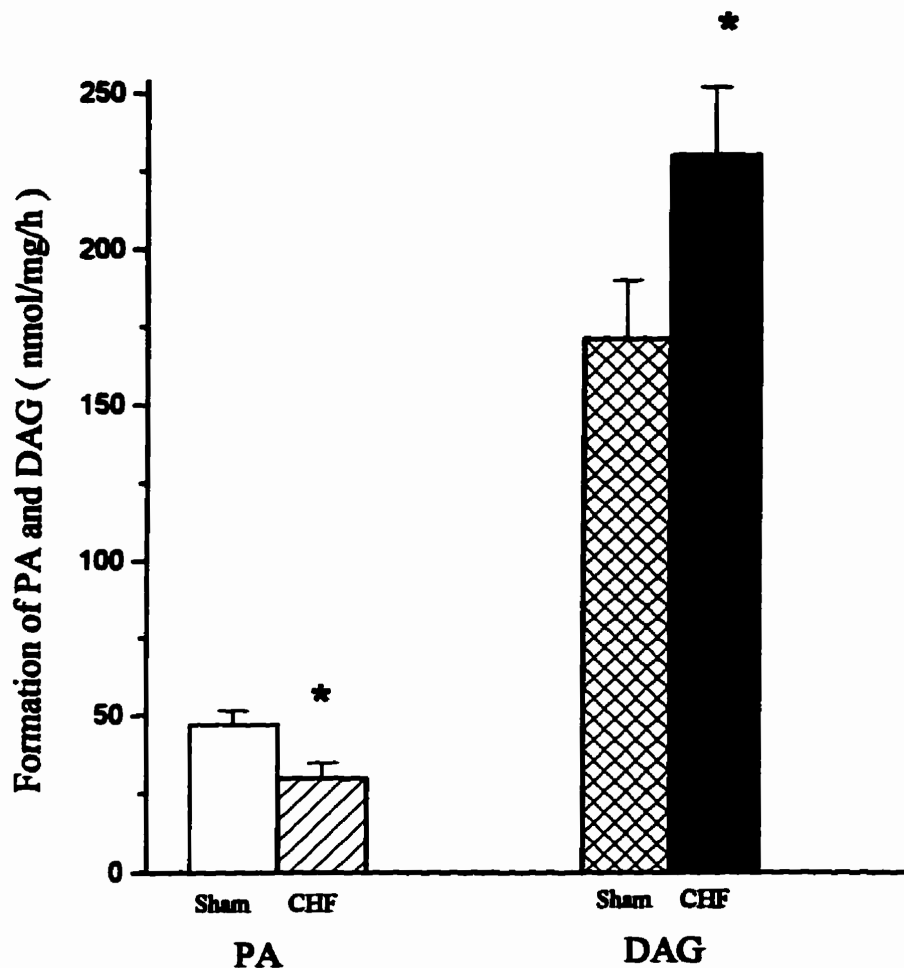

Figure 4. PLD -PAP avtivity from sham and CHF rats 8 wks after coronary artery ligation in the absence of KF

Results are means ± SEM of 4 experiments in triplicate . The assay were done in the absence of KF as described in Methods section. PA: phosphatidic acid; DAG: diacylglycerol. KF: potassium fluoride

* indicates significantly different from sham control value ($P < 0.05$)

Table 5 : Effect of Imidapril on the cardiac SL PLD and PAP and Cytosolic PAP activities in the CHF

	PLD activity (nmol/mg/h)	PAP activities (nmol/mg/10min)	
		SL	Cytosol
Sham	191.7 ± 9.30	79.4 ± 5.1	10.9 ± 2.5
CHF	235.3 ± 15.8*	105.4 ± 8.1*	23.9 ± 2.7*
CHF+Imidapril	203.6 ± 6.9#	80.4 ± 4.3 #	13.7 ± 2.2#

Data represent the means ± SEM of 4 experiments in triplicate and are expressed as nmol/mg/hr for phospholipase D and nmol/mg/10min for phosphatidic acid phosphatase. The assay was performed as described in the Methods section.

* indicates significantly different ($P<0.05$) from respective control value
\# indicates significantly different ($P < 0.05$) from the corresponding CHF value

V. DISCUSSION

A. The CHF model induced by coronary artery ligation

In the present study, post infarction CHF in rats was induced by coronary artery ligation (Dixon, et al.,1990) with some slight modifications, i.e., the animals were given analgesia 1 h before surgery, and isoflura was used as the anesthetic instead of ether. Also 5-10 ml air and exudate suction from the chest cavity was performed while the chest was being closed. With these modifications , the mortality of the procedure was reduced from about 35 % to about 20%. Scar tissue was detected as early as one week. According to previous studies (Dixon et al., 1990; 1992), the CHF can be divided into 4 periods after the coronary artery ligation : prefailure stage (1-3 weeks) , early failure stage (4-7 weeks) , moderate stage (above 8 weeks), and severe stage (16 weeks).

B. Possible mechanisms involved in the upregulation of sarcolemmal PLD activity in congestive heart failure

The present study is the first to describe the status of cardiac ventricular PLD / PAP pathway in CHF induced by the coronary artery ligation. CHF caused a

significant enhancement of PLD activity, which was observed at the one week after the ligation of coronary artery, and was sustained at all stages of CHF. The maximal increase of 60 % in the activity as compared with sham controls occurred at 4 weeks after surgery. The mechanisms by which PLD activity is upregulated in CHF is still unknown, however, there are a number of possibilities which are discussed below.

In heart failure, a decreased cardiac output reflexively increased sympathetic tone, the activation of the RAS as well as increased circulatory atrial natriuretic factor are very important pathophysiological changes in CHF. These changes in initial stages are regarded at first as an adaptive response to heart failure in order to increase cardiac output to meet the requirements of the body, but later on these may contribute to heart dysfunction.

One of the possible mechanisms by which the PLD activity is altered in CHF could be due to the activation of RAS triggered by cardiac output decrease. In this regard, several studies have revealed that Ang-II can elicit a significant increase PA via PLD (Sadoshima and Isumo, 1993; Booz et al, 1994). Pfeilschifter and Huwiler (1993) reported that Ang-II seems to activate PKC-ε through DAG derived from stimulation of PLD hydrolytic activity. Therefore, it

is possible that the activation of the RAS may be responsible for the augmentation of PLD activity in CHF.

It should be noted that ANF is known as a PLD cofactor (Baldini et al., 1994). Furthermore, in isolated rat heart, the cardiac SL PLD and PLC activities can be modulated by physiological concentrations of ANF. Therefore, another possible mechanism may involve ANF, the secretion of which is increased because of the water and sodium retention in the body in CHF.

α-adrenergic receptor expressed in rat fibroblasts can regulate both adenylylcyclase and PC-specific PLD by interacting with pertussis toxin-sensitive G-protein (MacNulty et al., 1992), Also SL PLD activities can be stimulated indirectly by β-receptor activation in isolated hearts (Lindmar et al., 1986a;). Therefore, the upregulation of PLD activity may be due in part, to the activation of sympathetic nervous tone following CHF.

Recently, depressed PI-PLC activities have been demonstrated in CHF(Meij et al., 1996). PIP_2 acts as a substrate for PI-PLC and is also a potent cofactor for PC-PLD (Kumada et al., 1993; Liscovitch et al., 1994). Therefore, the enhanced PLD activity in CHF may be due to an increase in the availability of

the cofactor (PIP_2). DAG , one of the products of PI-PLC activity can activate PKC and consequently activate PLD via G-protein (Pachter et al., 1992). In view of this information , it is suggested that there might be a balance between PI-PLC and PC-PLD / PAP pathways, that is, when the PI-PLC is downregulated in CHF, the PC-PLD / PAP is upregulated to provide DAG to activate PKC and consequently PLD. This positive feed back cycle provides lasting production of DAG, leading to an increase in protein synthesis which finally results in cardiac hypertrophy and CHF. For confirmation of this assumption, it would be necessary to study the status of PKC activity in CHF. The activation of PLD in CHF may be a compensatory mechanism occurring to maintain adequate second messengers level. The right ventricle SL PLD activity did not show any significant changes as compared to corresponding sham control values. Similar results have also been found in PLC activities in CHF. Only left ventricular PI-PLC activity was significantly depressed, while no significant changes in right ventricular PI-PLC activity were observed (Meij et al., 1996).

In the present study, the sham control right ventricular PLD activity was much lower than that from left ventricle, obviously, in terms of pump function, the left ventricle plays a much more important role than the right ventricle. These

data may constitute an indirect evidence that the PLD / PAP pathway is involved the cardiac contractility, and might therefore contribute to establish the relationship between cardiac contractility and PLD activity. It has been reported that the treatment of cultured neonatal rat cardiac myocytes with PLD resulted in an increase in $[Ca^{2+}]_i$, and contractility of ventricular tissue (Burt et al., 1984). Also, PKC enzyme activity is increased in certain pathophysiological studies such as ischemia (Prasad and Jones, 1992) pressure overload hypertrophy (Kwiatkowska-Patzer and Domanska-Janik, 1992) and this may mediate the PLD activation.

C. The status of cardiac ventricle PAP activities in CHF

A proper balance of the amounts of DAG and PA is important for cellular responses to extracellular signals. PAPs play a key role for controlling this balance (Martin et al., 1993). However, little information about the pathophysiological significance of PAP in the CHF is available . The present study is the first report to describe the status of SL and cytosolic PAP activities in CHF due to the ligation of the left anterior descending coronary artery. Both the SL and cytosolic PAP activities increased 1 week after surgery. The upregulated activity was observed at all the stages of CHF with maximal

activity at 4 weeks (increased 137% as compared with the sham control). DAG has been shown to exert a direct effect on cardiac transport systems (Gilbert et al., 1991; De Jorge et al. , 1995) or indirectly via PKC activation (Lacerda et al., 1988). It should be pointed out that DAG can also be produced from the endogenous PI-PLC pathway, however, this can be discounted under our assay conditions because exogenous ^{14}C labeled PA was used as the substrate and , therefore, DAG generated from PI-PLC activity would not be radioactive and thus would not be detected by liquid scintillation counting. Another possible source of contamination is via PC-PLC activity, but under our experimental conditions, any contamination from PC-PLC is minimal, and can be ignored, because the myocardial PC-PLC requires a pH of 7.5 and the presence of cations for optimal activity (Baldimi et al., 1994). In our assay system, DAG was detected in the presence of EDTA and EGTA and performed at pH 6.5.

The cytosolic PAP activity, showed the same upregulation pattern as SL PAP activity, except that the peak activity (increased 113 % compared with the sham control) occurred at 8 weeks after ligation of the coronary artery. Since both SL and cytosolic PAP activities were enhanced, therefore, translocation of the enzyme between SL and cytosolic fraction in CHF is not likely.

Divergent changes in cardiac PLD / PAP coupling signal pathway has been demonstrated in streptozotocin - induced diabetes (Williams et al., 1996). These authors observed that PLD activities were donwregulated whereas PAP activities were upregulated. Interestingly, the results form the present study demonstrated that both PLD and PAP activities are upregulated. Upon further examination of the PA and DAG levels in the absence of KF, PA levels are decreased and DAG levels are increased as compared to the corresponding sham control values. However, in the diabetic rat heart , both PA and DAG levels were decreased as compared to the sham control values (Williams, 1995). In terms of the production of the two second messengers, the two different pathological conditions produced contrasting effects on the level of PA and DAG.

No significant difference in the SL and cytosolic PAP activities from the right ventricular tissue of both sham and CHF group were observed, which is consistent with PLD activities in the right ventricles.

D. The Effect of Imidapril treatment on the PLD / PAP pathway in CHF and it's potential significance.

Ventricular remodeling is one of the most important pathological processes in post infarction (Fara , 1993). Structural remodeling of the left ventricular myocardium develops in a time-dependent fashion following acute myocardial infarction and may be an integral component in the transition toward CHF. The ventricular remodeling process may be attenuated by ACE inhibitors (Cohn , 1993; Fornes, 1992; Goldstein, 1995).

Imidapril is a newly developed ACE inhibitor. It produces a longer and stronger effect than Captopril and Enalapril (Ogiku et al., 1994). It also reduces the mortality rates more significantly than Enalapril (Vandenburg et al., 1994). The data obtained in the present study shows that the upregulated PLD and PAP activities in CHF were normalized by Imidapril treatment. There are no significant differences between the sham and the CHF treated with imidapril ($P > 0.05$).

According to the information mentioned above, the PLD / PAP pathway might be involved in the ventricle remodeling in post infarction CHF. Therefore it is

suggested that the mechanism by which the ACE inhibitors inhibit the ventricular remodeling might be due in part to the normalization of the upregulated PLD / PLP activities.

E. The significance of altered PLD / PAP signalling pathway in CHF

Different signal transduction mechanisms including phospholipid signalling pathways are known to modulate heart function by altering Ca^{2+} handling properties of one or more membrane systems. Three phospholipid signaling pathways which produce messenger molecules in response to external stimuli include: (1) PI- PLC pathway , IP_3 and DAG are the product formed in this pathway; (2) PE N-methylation which has been shown to regulate SL and SR Ca^{2+} -pump as well as SL Na^{+}-Ca^{2+} exchanger and (3) SL PC -specific PLD which forms PA, the PA can be dephosphorylated by PAP to DAG, which is another important second messenger. Ca^{2+} paradox overload is an important pathological feature of cardiac myocytes in CHF (Dhalla et al., 1982, 1978). The potential action of PA and DAG in enhancing phosphoinositide synthesis (Moritz et al., 1992) and hydrolysis (Jackowski and Rock , 1989), stimulation of the phosphorylation of cardiac proteins (Bacckino et al., 1991,), and DNA and protein synthesis (Xu et al., 1996; Okumura et al., 1995) indicate that the

PLD / PAP signal pathway may be intimately involved in some cardiac pathophysiology such as cardiac Ca^{2+} homeostasis abnormality and ventricle remodeling in post infarction heart failure.

Since each phospholipid has its own characteristic fatty acid composition (Lamers et al., 1991; 1992) and that there are multiple forms of PKC with diverse biochemical and functional characteristics (Nishizuka, 1989,), the dependence of PKC on specific DAG molecular species may differ widely (Mori et al., 1982,). Accordingly, DAG species derived from PC or PIP_2 hydrolysis may activate different PKC isoforms , and induce PKC-dependent phosphorylation of different proteins and consequently different physiological responses. In this regard, future studies should be directed to examine individual phopholipid species and their functional significance.

Several hormones , neurotransmitters and pharmacological agents are considered to influence the rate and the extent of contraction of the cardiac muscle by binding to specific cell surface receptors . Agonist receptor interactions results in the generation of second messenger molecules in the cardiac cell (Meij et al., 1991; Lamers, 1987). Some of these messenger molecules originate from the PLD / PAP pathway and influence directly (

Panagia V. et al., 1991) or indirectly via PKC-dependent phosphorylation (Lancerda et al., 1988,) cellular function . A change in the status of cardiac Ca^{2+} transport systems is an important determinant of impaired cardiac performance during heart failure (Dhalla et al., 1991b, 1982b). It is noteworthy that an imbalance of sympathetic circulating hormones , local neurotransmitters and alterations in the characteristics of cardiac adrenoceptors has been shown to occur in heart disease (Schomig , 1990; Cohn , 1990; Corr et al., 1989).

Since no information about the cardiac PLD / PAP signalling pathway in CHF is available , the present study constitutes the first contribution in understanding the significance of this pathway in CHF. It is difficult at the present time to conclude if the changes in this pathway are a compensatory mechanism directed at maintaining the status quo of cardiac function or if it is a pathological injury after infarction , which facilitates the heart function defect and contributes to the development of heart failure. Based on the present study and evidence metioned above, it is suggested that derangements of phospholipid signalling pathways in particular the upregulation of PLD/PAP pathway in post infarction CHF seems to be an adaptive response to cardiac dysfunction at least in the initial stages but ultimately those may contribute to the cardiac remodeling and Ca^{2+} -handling abnomalities which have been considered to be the important

patholigical characteristics in CHF(Dhalla et al., 1991; Brillla et al., 1994). Normalization of PLD/PAP activities by Imidapril may modify the pathological process of the CHF.

VI. CONCLUSIONS

1. The upregulation of left ventricular sarcolemmal PC-PLD activities were found to be significantly enhanced during the development of postinfarction CHF due to coronary artery ligation.

2. The left ventricular sarcolemmal PAP activities were observed to be augmented during the development of postinfarction CHF.

3. An elevated left ventricular cytosolic PAP activity was also found in the CHF.

4. Sarcolemmal PLD and PAP and cytosolic PAP activities in the right ventricle did not change during the development of CHF.

5. Significantly lower sarcolemmal PLD activity was found in right ventricle as compared to left ventricle in normal control rats.

6. Treatment of the animals with Imidapril corrected the abnormal elevation of left ventricular sarcolemmal PLD and PAP and cytosolic PAP activities in CHF.

7. The finding that Imidapril can normalize the upregulated PLD / PAP signalling pathway might constitute one of the mechanisms by which ACE inhibitors prevent the ventricle remodeling and improve the Ca^{2+} handling ability. Finding in this study provide additional basis for treatment of post infarction CHF with ACE inhibitors.

8. Further studies on the expression of cardiac PLD / PAP and the regulatory pathways (i.e. PKC) would contribute to the precise understanding of the altered PLD / PAP activities observed in CHF. In addition, the analysis of PA and DAG levels in cardiac sarcolemmal membrane will provide more direct evidence to establish the relationship betewwn the altered PLD / PAP pathways and cardiac dysfunction, and thus permit the development of drugs which specifically alter the status of the PLD/PAP pathways in CHF.

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