The Role of Phospholipase C Isozymes in Cardiac Hypertrophy and Failure Due to Volume Overload

By

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LIST OF ABBREVIATIONS

ACE.....Angiotensin converting enzyme

ANG I.....Angiotensin I

ANG II.....Angiotensin II

ANF.....Atrial natriuretic factor

AT_1.....Angiotensin II type 1 receptor

AT_2.....Angiotensin II type 2 receptor

ATN.....Angiotensinogen

ATP.....Adenosine triphosphate

ATPase.....Adenosine triphosphatase

AV.....Aortocaval shunt

BW.....Body weight

cAMP.....Cyclic adenosine monophosphate

CHF.....Congestive heart failure

CO.....Cardiac output

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

+d*P*/dt.....Rate of pressure development

-d*P*/dt.....Rate of pressure decay

ERK.....Extracellular signal regulated protein kinase

ET-1.....Endothelin-1

ET_A-receptor.....Endothelin type A receptors

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

GAPDH.....Glyceraldehyde-3-phosphate dehydrogenase

GDP.....Guanine diphosphate

GTP.....Guanine triphosphate

GTPase.....Guanine triphosphatase

H_2O_2.....Hydrogen peroxide

HW.....Heart weight

IgG.....Immunoglobulin G

IP_4.....Inositol 1,3,4,5-tetrakisphosphate

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

KDa.....Kilodaltons

LV.....Left ventricle

LVEDP.....Left ventricular end diastolic pressure

LVSP.....Left ventricular systolic pressure

LVH.....Left ventricular hypertrophy

LVW.....Left ventricular weight

MAPK.....Mitogen activated protein kinase

MHC.....Myosin heavy chain

MI.....Myocardial infarction

NE.....Norepinephrine

PA.....Phosphatidic acid

PC.....Phosphatidylcholine

PH.....Pleckstrin homology

PI.....Phosphatidylinositol

PI-4 kinase.....Phosphatidyinositol 4 kinase

PI4-P5 kinase.....Phosphatidylinositol phosphate 5 kinase

PIP_2.....Phosphatidylinositol 4,5-bisphosphate

PKA.....Protein kinase A

PKC.....Protein kinase C

PLC.....Phospholipase C

RAS.....Renin angiotensin system

SHR.....Spontaneous hypertensive rat

SL.....Sarcolemma

SR.....Sarcoplasmic reticulum

SNS.....Sympathetic nervous system

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ABSTRACT

Volume overload due to AV shunt results in cardiac hypertrophy followed by the progression to heart failure. The PLC converts PIP_2 to DAG and IP_3, which are known to influence cardiac function. Therefore, we examined the time course of changes in DAG and IP_3 as well as PLC isozyme gene expression, protein content and activities in cardiac hypertrophy and heart failure induced by AV shunt in Sprague-Dawley rats. An increase in the LV to body weight ratio demonstrated that LV hypertrophy was established at 4 weeks after the induction of the shunt. PLC β_1 activity was increased two-fold at 3 days and 1 week and an increase by seven-fold was seen at 2 weeks after the induction of volume overload, respectively. These changes were associated with increases in the mRNA and SL protein content; however, no changes in PLC β_1 were detected at 4 weeks. On the other hand, a significant increase in PLC γ_1 activity as well as mRNA and SL protein was seen at 3 days and 4 weeks. A progressive decrease in PLC δ_1 activity with concomitant reductions in the gene expression and SL protein abundance was detected during 1 to 4 weeks. Activity of γ_1, and δ_1 isozymes was significantly depressed during the 8 and 16-week time points, whereas β_1 isozyme was increased significantly during these time points. A progressive decrease in the SL PIP_2 content was observed during cardiac hypertrophy and heart failure. Our findings indicate that PLC isozyme signaling processes are increased in hypertrophy and decreased in heart failure due to volume overload.

I. INTRODUCTION

PLC isozymes play a central role in activating intracellular signal transduction pathways during early key events in the regulation of various cell functions (40, 106, 110, 171). Its most common physiological substrate, PIP_2 is converted into two messenger molecules, IP_3 and DAG. These two products participate in many different physiological processes (77, 99, 116, 117, 168, 169) including myocyte hypertrophy via downstream signaling mechanisms and Ca^{2+} movements within the cardiomyocyte. In addition, PIP_2 also serves as an anchor point for proteins containing pleckstrin-homology (PH) domains. (236)

The role of PLC in the development of some types of cardiac hypertrophy has been documented; for example, in stroke-prone spontaneously hypertensive rats, the development of cardiac hypertrophy has been suggested to involve an increase in the PLC signaling pathway (114, 193). Other studies with the cardiomyopathic hamster (BIO 14.6) have shown that cardiac hypertrophy is associated with an increase in PLC activity as a consequence of an enhanced responsiveness to angiotensin II (ANG II) (181). Lamers et al. have reported that stimulation of cultured rat neonatal cardiomyocytes by endothelin-1 (ET-1) induces a rapid activation of PLC β isozymes and is accompanied by characteristic phenotypic changes related to the development of cardiac hypertrophy (125). In

addition, recent studies in neonatal rat cardiomyocytes stimulated with different hypertrophic stimuli, have shown an increased mRNA expression of PLC β isozymes (185). On the other hand, reductions in PLC isozyme activities and protein abundance at the cardiac SL membrane have been reported in congestive heart failure (CHF) due to myocardial infarction (209) as well as in the failing heart of the cardiomyopathic hamster (UM-X7.1) (236).

Although these lines of evidence demonstrate an increase in PLC isozyme activity during the development of hypertrophy and specific decreases in PLC isozyme activities in the failing heart, no information is available in the literature regarding the status of the PLC signaling pathway in cardiac hypertrophy and heart failure due to volume overload. We have recently reported and perfected the needle technique to create an AV shunt or fistula for inducing volume overload that results in consistent hypertrophic growth, which develops within the first 2 weeks after the induction of the AV shunt (223), followed by progression to moderate and chronic CHF at 8 and 16 weeks, respectively after the surgical procedure.

Given the role of PLC and its products in influencing cardiac function, the present study was therefore undertaken to investigate the time course of changes in the level of cytosolic IP_3 and SL DAG and how these relate to the PLC isozyme status during the development of cardiac hypertrophy and subsequent progression

to heart failure due to volume overload. In addition, the SL amount of PIP_2 was also assessed during this time course.

II. LITERATURE REVIEW

A. Cardiac Hypertrophy and Heart Failure

Cardiovascular disease remains the major cause of death in Western countries and CHF represents an enormous clinical problem. It is estimated that about 1.5% of the total North American population suffers from CHF (50). In the United States alone, there are 400,000 new cases of CHF each year and more than 4.7 million people are currently suffering from this condition. Furthermore, the five-year survival rate is about 25% in men and about 38% in women (137). Due to the high incidence rate of CHF in United States, it has become a major social and economic burden, with the management of CHF costing approximately $15 billion per annum (137). Therefore, extensive efforts are made to develop promising therapeutic procedures/protocols to alleviate some of these clinical heart problems.

Cardiac hypertrophy may be defined as the change in phenotype, which includes the process of enlargement of ventricular cells (66). When an excessive workload or hemodynamic overload on the heart is sustained, ventricular myocytes grow in response to a complex series of events; this cardiac hypertrophy is an adaptive mechanism by which the heart compensates for a sustained increase in hemodynamic loading. This adaptation is brought about by hypertrophy of the

cardiomyocytes, a process that is accompanied by the early induction of transcription factors and the later reactivation of the fetal gene program (179), together with an isozyme switch in myosin heavy chain (MHC) expression and downregulation of proteins of the Ca^{2+} handling system; the latter maybe the cause of the systolic and diastolic dysfunction of the hypertrophied heart (218). Initially, the resultant increased workload is compensatory to normalize wall stress and maintain normal cardiac function. Once hypertrophied, the initially excessive mechanical stress on the myocardium is corrected toward normal by operation of the Laplace law, whereby an increased wall thickness decreases wall stress (67, 155, 188).

Cardiac hypertrophy can be induced by a variety of cardiovascular diseases, myocardial infarction, cardiomyopathy and endocrine disorders. It is well known that a variety of stimuli including mechanical stress, ischemia, and neurohormonal activate multiple intracellular signaling pathways leading to the development of cardiac hypertrophy and subsequent heart failure. Cardiac hypertrophy is a very important issue for cardiologists, not only because it is one of the most critical risk factors for many cardiac diseases such as ischemic heart disease, arrhythmia and sudden death but also because it shows diastolic dysfunction and often leads to CHF (131).

Left ventricular hypertrophy occurs as an adaptation prior to chronic CHF caused by excessive workload (pressure or volume overload), heart dysfunction and/or genetic mutation. (182) The remodeling of the myocardium during hypertrophy leads to an increased risk of ultimately developing cardiac failure. (215) Although cardiac hypertrophy may be initially a beneficial response that normalizes wall stress and maintains normal cardiac function, prolonged hypertrophy becomes a leading cause of heart failure and sudden death (201). The transition to heart failure is triggered by marked ventricular dilatation occurring once the myocardial hypertrophic response is exhausted (26).

CHF represents an enormous clinical problem and is a syndrome in which cardiac output is inadequate to meet the metabolic needs of the human body (48). It is of utmost importance for diagnostic, preventative and therapeutic purposes to understand the cellular events that trigger the cascade of functional and structural changes that result in the development and progression of heart failure.

CHF is a medical condition in which the heart cannot pump enough oxygenated blood to meet the needs of the body's other organs. The loss in the hearts pumping action is a result of an underlying heart problem (6, 155). Heart failure is the final clinical presentation of a variety of cardiovascular diseases. The progressive deterioration of left ventricular function is a characteristic feature of the heart failure state, however the mechanisms responsible for this myocardial

deterioration are unknown but have been attributed to a so-called vicious circle of compensatory mechanisms intended to maintain homeostasis. Ventricular remodeling (hypertrophy) and enhanced activity of the sympathetic nervous system (SNS) and renin-angiotension system (RAS) have proven to be factors that accelerate the process of left ventricular dysfunction (123).

As CHF has become one of the most common serious disorders and consequently a common cause of death, many different experimental models of cardiac hypertrophy and heart failure have been used to aid in the assessment of biochemical changes that occur during the development of cardiac hypertrophy and heart failure. Such models have also permitted the examination of various modes of treatment of the hypertrophied and failing heart. The molecular mechanisms that are responsible for compensated and decompensated hypertrophy are being elucidated by a number of studies that employ conventional experimental animal models, transgenics and clinical investigation. However, the exact mechanism accounting for the transition from compensated to decompensated hypertrophy remains to be determined (221).

1. Models used to study cardiac hypertrophy and heart failure

In its initial stages, the hypertrophied ventricle is able to compensate for an imposing increased workload, however in the later stages, the diastolic and

eventually the systolic function of the left ventricle become progressively impaired causing decompensation, and this leads to heart failure (54). Heart failure in humans; in some cases may be acute, for example immediately after a myocardial infarction or in hypertensive crises (54), but heart failure in humans generally refers to a chronic condition that follows years of hypertension or coronary heart disease. There are several models designed for experimental cardiology research that are used to study cardiac hypertrophy and heart failure, however no one animal model can mimic entirely any one pattern of human heart failure. These models can provide us with means to evaluate particular aspects of hypertrophy and failure such as pathogenesis and drug interventions. A few of the methods researcher use to study cardiac hypertrophy and heart failure are briefly described below:

(a) The spontaneously hypertensive rat (SHR) is a commonly used model of chronic hypertension which progresses to heart failure (54, 96). The SHR represents an animal model of essential hypertension with hypertensive heart disease characterized by concentric hypertrophy and dysfunction of the left ventricle and potential arrythmias that may develop with increasing age (150). This model is widely used, not only because it allows studies in chronic, stable disease; but also because it produces symptoms, which are predictable and controllable, and thus allows measurement of relevant cardiac, biochemical and hemodynamic parameters (54). The SHR is therefore a model that is generally

used to study the mechanisms of hypertension-induced hypertrophy as it progresses to heart failure.

Recent studies have shown that hypertensive heart disease in the SHR demonstrates excessive contraction of their cardiac fibroblasts and defective control of fibroblast contraction by angiotensin II (ANG II) (136). The SHR has displayed increased levels of cardiac angiotensinogen (ATN) mRNA and ANG II tissue levels (94). Also, scientists have shown that a part of the phosphatidylinositol (PI)-turnover pathway, such as the PIP_2-IP_3-Ca^{2+} pathway or the DAG-PKC pathway, may play an important role in the development of hypertrophy in the SHR (114). In accordance with this the activity of PLC increased with age in the SHR heart cells also suggesting that the PIP_2 or DAG pathways may play a role in the development of hypertrophy (113) Interestingly, Makita et al. demonstrated that PKC and membrane-bound PIP_2-PLC are altered during the period of hypertension development (135). This model has also been used extensively to study the relationship between apoptosis and spontaneous hypertension. Peng et al. have shown that there is significant increase in the apoptosis of SHR cardiac tissues with increasing age (163), therefore suggesting that apoptosis may be involved in the pathogenesis of genetic hypertension, as well as providing a contributory role in the remodeling that occurs in the transition from compensatory hypertrophy to decompensated heart failure (81, 101). Furthermore, it is also shown that in the SHR heart the action potentials are

prolonged (25, 149) as well as there is an impaired function of inward rectifier K^+ channels without changes in transient or delayed outward rectifying K^+ channels or L-type Ca^{2+} channels (25).

(b) Renal artery occlusion results in renovascular hypertension due to renal ischemia. This leads progressively to left ventricular concentric hypertrophy and failure (54). Approximately 12 weeks post-surgery, the rats develop a significant increase in left ventricular weight (LVW), increased left ventricular end-diastolic pressure (LVEDP) and wall stress with reduced stroke volume (SV) and cardiac output (CO) together with marked ventricular fibrosis, which are clear indicators of hypertrophy with signs of subsequent heart failure (54).

(c) Pressure overload involves an increase in systolic tension, resulting in myocardial fiber thickening and concentric hypertrophy (229). Pressure overload is induced by ascending aortic constriction in rats, which leads to the rapid increase in cardiac load and therefore causes compensated concentric hypertrophy at 6 to 8 weeks, followed by the onset of LV failure. The pressure overload model has been used in several species; heart failure due to pressure overload has been induced in guinea pigs, (118, 144) felines, (15), ferrets, (222) and rats (73, 175). Pressure overload is most commonly performed in rats; these rats exhibit increased systolic LV pressure and LV hypertrophy (153) as well as reduced mRNA levels for SR Ca^{2+}-regulating proteins (10).

The pressure overload model has been used extensively to define changes that occur during the development of concentric hypertrophy, for example changes have been defined in cell size, myofibrils and myosin isoforms, incidence of apoptosis and altered adrenoceptor responsiveness (54). This model is not a very good model for studying chronic heart failure because liver enlargement, pleural or peritoneal effusions are not observed following short term banding. However, heart failure may be induced by superimposing streptozotocin-induced diabetes on the hypertension of abdominal aortic constriction, this led to the presence of ascites and liver and lung congestion (54). It has been suggested that impaired ventricular relaxation was associated with decreased expression of phospholamban and SR Ca^{2+}-ATPase proteins (118). Recently, Bayer et al. (2003) have reported that PKC δ may be involved in the induction of pressure overload LVH, whereas both PKC δ and PKC α may be involved in the transition to heart failure (17). Recently, studies have shown that chronic ACE inhibition in this model decreased the extent of LV hypertrophy, attenuated cardiac dysfunction and LV dilatation (96). This model may mimic the heart failure that occurs in inadequately treated hypertension in humans.

(d) Catecholamines have been used to induce cardiac hypertrophy in animal models as well as cultured cardiomyocytes. Norepinephrine (NE) infusion leads to selective left ventricular hypertrophy due to the fact that cardiac hypertrophy and heart failure are characterized by the activation of the sympathetic nervous system

(SNS) leading to high concentrations of circulating NE. There is a strong correlation between the NE concentration and cardiovascular morbidity (54, 70).

(e) Transgenic rats and genetically engineered mice have become increasingly important for the investigation of the pathogenesis of various diseases. These models are now increasingly used since they offer the possibility of analyzing responses by selected genes (54). The murine Ren-2 gene was chosen to generate transgenic rats for studying the cardiovascular system because the RAS plays a pivotal role in controlling the cardiovascular system (130). Male rats have sustained an ANG II dependent increase in blood pressure with low circulating renin levels, at 12-14 weeks; male transgenic rats developed concentric hypertrophy but no signs of heart failure. They also observed a downregulation of the β_1-adrenoceptors (126). Female Ren-2 transgenics have been used to study the interplay between the RAS and estrogen in the pathogenesis of hypertension (190). This model may allow a clearer understanding of the role of local RAS in cardiovascular disease. There is also a transgenic line that expresses the human ATN gene and these studies have been used to test the functional importance of the local human RAS (54) These are just a few examples of some transgenic models that are currently being used to study the importance of certain genes in the pathogenesis of cardiovascular disease.

(f) CHF model induced by coronary artery ligation is the most commonly used model of heart failure. The procedure for the rat model of myocardial infarction (MI) with coronary artery ligation has improved gradually and has been applied extensively to study CHF with respect to morphological features, metabolic and mechanical adaptations and therapeutic evaluations. This model produces post-infarction heart failure that has long been used to study the effects of ACE inhibitors in CHF (53, 186), SL adrenoceptors (49) Ca^{2+} transport in SL and SR (53) and SL Na^{+}-K^{+}-ATPase activity (52). The rat MI results in LV remodeling causing myocardial hypertrophy, progressive chamber dilation and late failure of surviving myocardium. The rat infarction model is relatively simple to produce, is inexpensive, and has relevance to at least one form of clinical heart failure (96).

(g) Volume overload, such as aortic regurgitation, mitral regurgitation and AV shunt also induces cardiac hypertrophy with an increase in length and width (134, 223). Volume overload produces alterations that resemble human heart failure in that volume overload is tolerated better than pressure overload and the development of hypertrophy and heart failure occurs more gradually (30). There are also changes in peripheral arterial flow, high flow in the proximal part of the aorta, upstream of the shunt and low flow in the distal aorta, downstream of the shunt (12). Volume overload is accomplished by creating a surgical AV shunt between the abdominal aorta and the inferior vena cava; this clearly results in the development of compensated eccentric hypertrophy followed by LV dysfunction

and CHF (92, 167) The AV shunt produces a high CO CHF as seen in some congenital heart diseases. In volume overload LVEDP rapidly increases after the imposition of chronic volume overload, this rise in LVEDP peaks at an early phase, and declines thereafter (229).

This model has been used extensively in investigating the disturbances in fluid balances, electrolytes, and hormones commonly seen in heart failure. Also, this model has been used to study the cardiac effects of ANF (234) and ACE inhibitors (1, 76, 91). It has also been shown that Ca^{2+} uptake and release from SR isolated from volume overload heart are both decreased compared with those from the normal heart (92).

2. Pathophysiological Regulation of Cardiac Hypertrophy and CHF

Hypertrophy and progressive dilatation of the failing heart represent abnormal *proliferative* responses (111). In addition to mechanical factors, humoral actions play a role in the regulation of cardiac myocytes growth (34). Mechanical stimulation of hypertrophy may involve release of autocrine and paracrine neurohormonal factors from the mechanically overloaded heart, (217) and these stimuli that initiate hypertrophy transmit their signal to the nucleus of the cardiomyocytes by activation of intracellular signaling pathways (217). The biochemical changes of chronically overloaded cardiac cells are not well defined

however, it is thought that changes in the composition of the hypertrophied and dilated myocardial cell may consist of alterations of contractile proteins by modification of gene expression probably triggered by stretching of the myocytes. Regulating structures like local hormone systems or receptors, G proteins, or ion channels and pumps may also be altered (67).

The hemodynamic abnormalities in patients with heart failure can be viewed as *functional* responses that alter the behavior of existing structures. Additional functional responses, notably vasoconstriction, fluid retention and cardiac stimulation, are components of the neurohormonal response to reduced cardiac output (111). As mentioned above, there is activation of different neurohormonal systems at the onset of cardiac hypertrophy and subsequent heart failure, the activation of these systems such as the SNS, RAS and ANF occurs almost simultaneously during the development of cardiac hypertrophy and CHF (35, 176).

CHF is a syndrome that evolves with time as a consequence of initial damage and stress to the myocardium, such as increased systolic or diastolic ventricular load and/or the reduction in the number and contractile function of cardiac myocytes, which has led to cardiac hypertrophy. Heart failure is characterized by an inadequate CO, which is unable to provide the supply of oxygen and substrates to the periphery. In early stages of heart failure, several

compensatory mechanisms are activated in order to increase blood pressure and to maintain blood flow to vital organs (158). The inadequate cardiac performance associated with heart failure, has developed with time despite compensatory hypertrophy and dilation, and is progressively associated with activation of neurohormonal systems (72). The compensatory mechanisms involve an activation of the RAS (64), an increase release of vasopressin (18) and an activation of the SNS (36). All of these mechanisms have the potential to worsen the syndrome of heart failure when chronically activated.

Findings that inhibiting neurohormones reduces mortality and improves survival in patients with CHF, has led to the *neurohormonal hypothesis*. This hypothesis states that neurohormonal alterations are more important than hemodynamic abnormalities in CHF (158). Neurohormones can directly damage the heart; therefore it is now thought that the progression and subsequent death in CHF is primarily due to the effects of neurohormones, independent of hemodynamic influences (8). The activation of the neurohormonal system is associated with a poor prognosis in CHF (37, 38, 199).

a.) Sympathetic Nervous System

The SNS plays an important role in the regulation of heart function; its influence on the heart is mediated by the release of NE and subsequent activation

of the β-adrenergic and α-adrenergic receptors. The catecholamines were particularly regarded as "hypertrophy hormones" (124). However, excess adrenergic activity promotes failure by myocardial membrane damage, calcium overload, and by increasing the myocardial oxygen demand and afterload (156).

In the myocardium, sympathetic responses are mediated by both β and α-adrenoceptors. The β- adrenoceptors mediate their responses by increasing intracellular cAMP, while α_1 adrenoceptors activate PLC. In turn, the activated PLC hydrolyses PIP_2 and produces two important second messengers IP_3 and DAG. Although α_1 adrenoceptors mediate signal transduction in cardiac cells during normal conditions, their contribution appears to be more important in pathophysiological conditions. The α_1–adrenoceptor is composed of three, and possibly four subtypes; the α_{1A}, α_{1B} and α_{1C} subtypes. The α_{1A}–adrenoceptor has been linked both to the activation of PLC and of Ca^{2+} channel (127, 226); whereas the α_{1B} –adrenoceptor is known to activate PLC through a G protein (226). Upon the activation of G-proteins, either adenylate cyclase or PLC is activated, giving rise to the second messengers cAMP, IP_3, IP_4, and DAG, while receptor linked tyrosine phosphorylation may also play a role in the signal transduction (217). For example, IP_3 liberates Ca^{2+} ions from the SR and thus induces a positive inotropic effect while DAG stimulates protein kinase C (PKC) (237). It has been shown that α_1-adrenoceptor agonists increase tissue contents of

inositol phosphates and result in translocation of PKC to the particulate fraction, due to the activation of the PLC signaling pathway (2). Besides the positive chronotropic, inotropic, and lusitropic effects, stimulation of α-adrenoceptors is also known to shift isomyosin from the V3 to the V1 form and to induce cardiac hypertrophy (237).

NE, the physiological mediator substance of the SNS, stimulates both β and α - adrenoceptors. This important catecholamine has many effects on the heart and circulation. NE increases total peripheral resistance, and this increase may be involved in triggering cardiac hypertrophy. Specifically, β-receptor stimulation leads to an increase of adenylate cyclase activity and to the elevation of cAMP. Activation of cAMP-dependent protein kinase A (PKA) induces known metabolic effects, such as the increase in lipolysis and glycogenolysis. In addition, the activation of this PKA induces phosphorylation of several proteins; for instance, a channel protein involved in transsarcolemmal Ca^{2+} transport becomes phosphorylated, therefore this contributes to the positive inotropic effect. Also, phospholamban, a component of the SR, is phosphorylated subsequent to β-adrenergic stimulation. Consequently, the reuptake of Ca^{2+} into the SR is facilitated, thus resulting in increased relaxation velocity (237).

There are several functional effects of NE on the heart, such as an increase in heart rate, mean arterial pressure and total peripheral resistance. In some pathological conditions, the ratio of β - to α–adrenoceptors increases (95). Accordingly, it has been proposed that in pathological conditions, the α_1–adrenoceptor response may function to maintain the myocardial inotropic response to catecholamines. Moreover, activation of α_1–adrenoceptor PI pathway in some pathological conditions could also accelerate the damage by altering intracellular Ca^{2+} homeostasis. Activation of the α-adrenergic receptors and the Gq/PLC/PKC pathway has little acute effect on instantaneous myocardial contractility, but can be a potent stimulus for cardiac hypertrophy (55). Both the SHR (214) and the cardiomyopathic Syrian hamster (196) show an increase in myocardial catecholamines during the compensated stage of heart failure. Sympathetic nervous activity has also shown to be markedly increased in patients with chronic heart failure (34). The increased sympathetic activity along with other hormonal activation contributes to the elevated peripheral venous tone and systemic vascular resistance, which are characteristics of heart failure. These responses are elevated in order to maintain venous return, cardiac filling pressures and vital organ perfusion. A direct relation between plasma catecholamine level and mortality in patients with chronic, CHF has been demonstrated (34).

b. Renin-angiotensin system

It is well known that the RAS plays an important role in cardiac development and the process of cardiac hypertrophy and heart failure (16, 20, 191, 224). This important physiological system also plays an important role in fluid and electrolyte homeostasis and blood pressure regulation. However, the exact role of myocardial tissue RAS in hypertrophy and dilatation is not well defined, despite this, it is suggested by the effects of converting enzyme inhibitors independent of hemodynamic changes. Increased expression of important components of the RAS has been shown to occur in the failing as well as the hypertrophied heart, resulting in increased local action of ANG II. The increased cardiac ACE mRNA (90, 94) and expression of cardiac ATN (59, 94, 166) in rats with cardiac dysfunction support the potential importance of the cardiac tissue RAS (67).

ATN is a 452 amino acid globular glycoprotein of 55-56 KDa. The plasma is the major reservoir of ATN (78). ATN is mainly synthesized and secreted from the liver and released into the blood. It has been suggested to be an important determinant of both blood pressure and electrolyte homeostasis (107). The role of ATN in the pathogenesis of hypertension has been suggested by genetic approaches (32, 107). However, the exact role of ATN in cardiac remodeling has not been determined. ATN is cleaved by renin, which is produced by the kidneys to produce ANG I. Renin, is a glycoproteolytic single chain aspartyl protease of

37-40 KDa, it is highly specific for its substrate ATN. It is generated in the juxtaglomerular cells of the afferent arterioles of the kidney (78). Renin cleaves a leucine-valine bond in the N-terminal region of ATN for the generation of ANG I (63). ANG I is composed of 10 amino acids and does not have any significant effect on its own; ANG I is then converted to ANG II by ACE. ACE is a dipeptidyl carboxypeptidase, which is predominantly attached to endothelial cells (166). Cardiac ACE activity is upregulated and has been shown to correlate with the degree of hypertrophy in volume and pressure overload models of CHF in the rat (164). However, the influence of RAS on LVH seems to be more critical in volume than pressure overload (229). ANG II, the effector peptide acts as a growth-promoting factor in the cardiovascular system, (16, 187) while it also involved in increasing collagen synthesis in the interstitium of the heart (162). ANG II growth promoting capabilities are mediated via PKC and the expression of nuclear proto-oncogenes such as c-*fos*, c-*myc* and platelet derived growth factor (129).

The RAS plays an important role in cardiac development and the process of cardiac hypertrophy (16). Specific adverse consequences of excessive RAS activation, including (1) excessive peripheral vasoconstriction; (2) aldosterone-mediated sodium retention and myocardial fibrosis; (3) increased endothelial damage; and (4) excessive ANG II effects at intracellular sites (156).

The cardiac RAS may be involved in the development of cardiac hypertrophy caused by in vivo stretch of the heart muscle. It has been demonstrated that mechanical stretch induces secretion of ANG II from the cardiomyocytes and secreted ANG II evokes cardiac hypertrophy (20, 178, 232). The effects of ANG II are mediated by specific receptors, two ANG II receptors have been described, AT_1 and AT_2. Although the AT_1 receptor is abundantly expressed in the neonatal rat heart, the level of expression is quite low in the adult heart. AT_1 receptor is the major receptor in the adult ventricular myocytes as compared to the AT_2 receptor. The AT_1 receptor is a G-protein coupled membrane protein, the classic ANG II receptor whose gene and protein sequences have been determined; the structure of the AT_2 receptor is presently unknown (129). The AT_1 receptor is composed of 359 amino acids with AT_{1A} and AT_{1B} subtypes (74). Most of the ANG II effects are mediated by the AT_1-receptor only (213). In the cardiac RAS, ANG II stimulates the PI response in myocytes (112). The stimulation of AT_1-receptors activates PLC, which stimulates the breakdown of plasma membrane inositol phospholipids (27). PLC hydrolyzes PIP_2 to DAG and IP_3; IP_3 induces SR Ca^{2+} release and increases contractility (19, 147, 192) while DAG activates PKC and thus stimulates cardiac growth (65, 151). Therefore, ANG II accelerates PI turnover and activates PKC, which in turn increases the activity of MAP kinase, and these signals finally lead to enhanced protein synthesis and specific gene expression in cardiomyocytes (233). Since AT_1-receptor expression is increased again in the remodeling heart such as cardiac

hypertrophy and infarction, this suggests a key role for the ANG II/AT_1 signal in the hypertrophic response (93). ANG II also appears to make important contributions to the pathophysiology of heart failure.

An increasing number of studies suggested the existence of local RAS in several organs, among them the kidney, adrenal glands, brain, and the heart (29, 46, 129). Many studies have demonstrated local tissue synthesis of ATN, (29) renin (129) and ACE (129) indicating the presence of a physiologically active cardiac RAS. Research has revealed the importance of these local RAS in various tissues, the role of these systems is only beginning to be elucidated, and possible effects of ANG II as an autocrine or paracrine growth factor have been suggested (62). It has been suggested that locally produced ANG II has an influence on local tissue function and structure such as vascular tone, cardiac contractility and mass, renal hemodynamics and sodium handling (129). Cardiac ACE activity has shown to be upregulated and correlates with the degree of hypertrophy in volume and pressure overload models of CHF in the rat (163). This suggests that increases in ANG II may induce hypertrophy and related cardiac disorders. Interestingly, many clinical studies have shown that ACE inhibitors caused regression of cardiac hypertrophy (61). In support of this, it has been found that blockade of the RAS can prevent cardiac hypertrophy in response to increased volume load on the heart (176). ACE inhibitors can also lower the blood pressure in the SHR by reducing ANG II production and decreasing bradykinen degradation (80), but it is thought

that hypertensive and hypertrophic responses are caused by ANG II generation (187). ANG II appears to play some role in the production of new collagen formation by fibroblasts that occur during hypertrophy. This may provide an additional role for ACE inhibitors or ANG II receptor blockers in blunting the hypertrophy response to increased blood pressure and possibly heart failure (75).

It is important to note that there is an important interaction between the RAS and the sympathetic nervous system. Sympathetic neurons have presynaptic receptors, which are sensitive to ANG II. When ANG II binds these presynaptic receptors, there is facilitation of NE release.

c. Atrial Natriuretic Factor (ANF)

In the ventricle the potent vasodilator ANF is reexpressed in the compensatory phase of hypertrophy, which might be beneficial during hemodynamic overload (217). The plasma level of ANF is correlated with the degree of cardiac hypertrophy, suggesting that ANF is a good marker of cardiac volume overload in aortacaval fistula as it is in experimental myocardial infarction (12).

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

Ca^{2+} is an important intracellular second messenger in such processes as growth factor and hormone signaling, cell cycle regulation, gene expression, and apoptosis (97) Receptor tyrosine kinases and G-protein coupled receptors classically increase Ca^{2+} levels by producing IP_3, which induces Ca^{2+} release from the SR via the IP_3 receptors (97). Recent advances in research involving Ca^{2+} movements in the heart have been valuable for the formulation of new concepts with respect to the physiologic and pathologic aspects of Ca^{2+} metabolism in the myocardium during cardiac hypertrophy and CHF. It is well established that Ca^{2+} plays an important role in the excitation-contraction cycle of the cardiac cell, and it has been suggested that abnormalities in intracellular Ca^{2+} metabolism may be the basis of depressed inotropic effect seen in heart failure. Both intracellular Ca^{2+} overload and intracellular Ca^{2+} deficiency have been considered to be responsible for defective myocardial contractility. Interestingly, SL defects in Ca^{2+}-related transport processes have been identified in different models of heart failure (24, 85). Although still somewhat controversial, decreased abundance of cardiomyocyte Ca^{2+} cycling proteins may also play an important role in the cardiac dysfunction of heart failure (11, 205).

B. Characteristics and Properties of PLC

1. PLC Isoforms

PLC is a modular monofunctional enzyme, which is involved in numerous transmembranal signals (171). Its most common physiological substrate, PIP_2, is synthesized in the SL membrane by the coordinated and successive action of two key enzymes, PI 4 kinase and PI4-P5 kinase. PIP_2 is converted into two messenger molecules, IP_3 and DAG, which participate in many different physiological signaling processes (171). PLC enzymes have been characterized into four immunologically distinct PLC superfamilies, designated β, δ, γ and ε that are expressed in adult ventricular cardiomyocytes (173, 209). The β, γ and δ families have been well characterized but there is some controversy over ε families. PLCs of the β, γ, and δ classes display differences in terms of structure, activating mechanisms and functions (100). The diversity in primary structure together with different regional and cellular expression of the isozymes suggests that each isozyme has a defined function in processing the physiological response to different cell types to a variety of external stimuli (173). For example, the mechanisms of recruitment to the plasma membrane also differ among the different PLC classes (208).

PLC β family has four types (-β_1, -β_2, β_3 and β_4) with PLC β_1 being the major isoform expressed in the heart. PLC β_1 just like the other PLC isozymes, hydrolyzes the membrane lipid PIP_2 and the hydrolytic process generates two biologically active intracellular second messengers, DAG and IP_3. DAG activates the serine/threonine kinase, PKC, while IP_3 binds to its receptor on the endoplasmic reticulum and stimulates the release of calcium (203) PLC β is activated by receptors coupled to G proteins of the Gq family (71) Also, it has been shown that Gqα-coupled PLC β_1 has the distinct feature of being a GTPase activating protein for Gqα and it regulates the rate of termination of its signal (171, 209). ANG II, α_1-adrenergic agonists and ET-1 are relevant stimulators of PLC β isoenzymes via the α subunits of the heterotrimeric Gq subfamily (171, 227); to note, PLC β has also been shown to be activated by Gβq dimmer (128). Recently, by using the adenovirus infection, the α_1-adrenoceptor mediated IP_3 generation in rat neonatal cardiomyocytes has been shown to be mediated by PLC β_1 (13). Thus PLC β is regarded as a cardiac SL PLC along with PLC δ.

PLC γ-is cytosolic and is activated by growth factor receptor tyrosine kinases. Binding of polypeptide growth factors, (216) to their receptors with intrinsic or associated tyrosine kinase activates PLC γ and PLC β isoenzymes (171). Tyrosine kinases can activate a number of different intracellular signaling pathways including tyrosine phosphorylation in the case of PLC and PLD (41).

Binding of polypeptide growth factors to their receptors with intrinsic or associated tyrosine kinase activity activates PLC γ. A non-tyrosine kinase mediated activation as well as G protein coupled receptor via non-receptor tyrosine kinase activation of PLC γ isoenzymes has also been reported (189). PA was found to stimulate SL PLC γ_1 as well as PIP_3 however; PIP_3 cannot stimulate PLC β_1 and δ_1 isoenzymes (87, 171). Defects in the SH 2 and 3 domains of PLC γ1 may impair the enzymes association with, and phosphorylation by activated growth factor receptors and its subsequent localization to the cytoskeleton. This exemplifies the irreplaceable role of PLC γ_1 in mammalian growth and development.

It is pointed out that PLC δ_1 is the predominant SL PLC isozyme, (209) with its N-terminal part of the PH domain of PLC δ_1 possessing a critical region rich in basic amino acid residues, which bind with high affinity to the polar head of PIP_2 (206). This property confers on the δ_1 isoenzyme a unique capacity of association with the plasma membrane, which is lost with single basic amino acid replacement by a neutral or acidic amino acid (228). The receptor-initiated events for the activation of PLC δ isoenzymes are mediated via transglutaminase II, G_h; a new class of GTP binding protein.

2. *PLC activity*

Activation of PLC generates various lipid-derived second messengers such as IP_3 and DAG. IP_3 causes Ca^{2+} release from intracellular Ca^{2+} storage sites, and DAG activates PKC, which plays an important role in stretch-induced immediate early gene expression, such as c-*fos* and Erg-1 (179). Recently it has been reported that PI-PLC activity increases in cardiomyopathic hamster hearts (BIO 14.6) and SHR (114, 115, 181, 193). PLC-dependent PIP_2 breakdown in the SL of failing hearts is unknown. However, it is known that these two signaling molecules (IP_3 and DAG) produced by the activation of PLC are critical in increasing contractile force development, and it is likely that changes in PLC may be responsible in altering cardiac contractile force in CHF (211).

C. Regulatory mechanisms of the PLC pathway

Since the α_1-adrenoceptor agonist epinephrine and NE, as well as ANG II and ET-1 are able to activate the PI cycle in cultured neonatal rat myocytes through binding of Gqα (43). Van Heugton et al. (1993) compared the magnitude and duration of PI cycle activation in order to directly correlate PLC activation with the development of cardiac hypertrophy. They have previously shown that NE and ET-1 are equipotent with respect to PLC activation (43, 217) and ANG II was shown to be a much less potent activator of the PI cycle. However, DAG

production is much higher when ANG II is the activator rather than ET-1 and NE. On the whole they suggested that ET-1 is the most potent inducer of cardiac hypertrophy, suggesting that ET-1 holds a prominent role in the development of hypertrophy ultimately leading to heart failure. These three key regulators of PLC activity have shown to play a key role during normal conditions as well as pathophysiological conditions in the heart.

a. Angiotensin II

The local RAS is increasingly being regarded as playing a critical role in the development of hypertrophy (16). ANG II, the potent effector of the RAS, has multiple effects on the heart and periphery acting directly or indirectly on myocytes for the regulation of growth, vascular resistance and contractility. The effects of ANG II are mediated via its receptors, which are located on ventricular and smooth muscle cells and are linked to G proteins that control the generation of various downstream second messenger pathways (166). ANG II induces activation of extracellular-signal related kinase (ERK) and expression of the c-*fos* gene as well as an increase in protein synthesis in cardiomyocytes (16). It is believed that ANG II activates ERKs possibly through Gq-PLCγ-Ca^{2+}-Ca^{2+}-dependent tyrosine kinase Pyk2 and Src-Shc-Grb2-Sos-Ras-ERK pathways (238). Therefore, PLC, Ca^{2+} and the Src family are critical for ANG II-induced activation of ERKs (121). Binding of ANG II to its receptor in cardiac myocytes also increases the cellular

contents of inositol phosphates, the activity of PKC, and the cytosolic concentration of free Ca^{2+} (145). ANG II taken up from the circulation or generated in the heart may mediate the cardiac hypertrophic response to increased cardiac load (176). Therefore, the direct effects of ANG II include greater contractility and accelerated rates of protein synthesis (146).

There have been many studies looking at ANG II during different cardiovascular pathologies. As previously mentioned, ANG II is a key regulator of cardiovascular homeostasis and is thought to participate in cardiac hypertrophy and remodeling since inhibitors of RAS are highly cardioprotective (120). It has been shown that ANG II can cause hypertrophy of cultured cells (16) and this is associated with increased expression of contractile proteins and the reactivation of genes encoding fetal protein isoforms (180). It has been suggested that cardiac hypertrophy could be induced by pressure overload in the in vivo heart via AT_{1A} signaling (83). Pretreatment with an AT_1 receptor antagonist diminished an increase in protein synthesis, MAP kinase activity, and *c-fos* gene expression induced by the stretching of myocytes (180). Supporting this, cardiac ANG II is upregulated in both the canine mitral regurgitation and the rat aortocaval fistula models of volume overload in CHF (44). It is interesting to note that the AT_1 receptor overexpression has been reported to induce cardiac hypertrophy (160). Therefore we must acknowledge that it has also been shown that AT_1 antagonists have significantly suppressed mechanically stretched hypertrophy. These findings

are indicators supporting the fact that ANG II is involved in stimulating cardiac hypertrophy. Cardiac hypertrophy induced by volume overload after AV shunt can be prevented by AT_1 receptor blocker, losartan. This effect can only in part be related to the decrease in cardiac preload and afterload by losartan, since the ACE inhibitor enalapril showed similar hemodynamic effects but failed to prevent cardiac hypertrophy (176). However, there have been some studies that have shown prevention of hypertrophy after volume overload with an ACE inhibitor as a therapeutic treatment. ACE inhibitors have also been shown to have beneficial effects, with prolonged survival of rats having pressure overload (179) These studies clearly demonstrate why, after more than ten years of continuously increasing clinical use of ACE inhibitors, blockade of the RAS has become a well accepted treatment for hypertension, regression of hypertrophy and CHF due to the inhibitors ability to modify the process of adaptive myocardial growth (45, 164, 194)

Although both ACE inhibitors and ANG II antagonist aim at suppressing the activity of the RAS, there are interesting differences between the two therapeutic approaches. Briefly, ANG II antagonists are more specific, but as a higher specificity will reduce the incidence of side effects, these antagonists may conceivably have less therapeutic efficacy that ACE inhibitors. ACE inhibitors not only induce vasodilation in the peripheral arteries, but also inhibit progressive neuroendocrine stimulation (184).

b. Catecholamines

Cardiac insufficiency results in an increase in systemic and local myocardial sympathetic tone with release of the endogenous sympathetic hormones epinephrine and norepinephrine (55). These two hormones activate α and β adrenoceptors, which in turn activate signaling pathways in cardiomyocytes (55). The acute contractile function of the heart is controlled by the effects of released NE on cardiac adrenergic receptors (3). However, chronic NE release induces cardiomyocytes growth (hypertrophy), increased protein synthesis, alterations in gene expression and addition of sarcomeres. Due to the fact that catecholamine amounts are increased in CHF, the excess adrenergic activity may be promoting failure by myocardial membrane damage and calcium overload, and by increasing the myocardial oxygen demand and the afterload (156). Also it has been shown that the increase in NE seen in human heart correlates with prognosis (28). It is thought that alterations in immune function of patients with CHF is linked with increases in catecholamine levels seen in these patients, this is believed to play a large part in the pathogenesis and progression of the disease (84).

c. Endothelin-1

ET-1 has been a reported to be a strong inducer of cardiac hypertrophy (103). Since ET-1 induced activation of ERKS is also inhibited by downregulation of PKC, ET-1 activates ERKs possibly through the same pathway as ANG II. Stretch induced activation of ERKs is also suppressed by type A ET-1 receptor antagonists (231); this suggests that ET-1 is also involved in mechanical stress-induced hypertrophic responses. Also, the beneficial effects of ET-1 receptor antagonists on MI highlighted the importance of ET-1 on cardiovascular remodeling. Interestingly, the development of hypertrophy in cultured rat neonatal cardiomyocytes induced by ET-1 has been reported to be due to activation of PLC β isozymes (187). Similar to NE, ET-1 levels are increased in CHF, and the level of ET-1 is correlated to the mortality of the disease (148). Treatment with ET antagonist significantly increased survival in the rat model of CHF, suggesting that ET may play a negative role in development of CHF (148).

D. G proteins of the α_1-adrenoceptor and PLC pathway

Guanine nucleotide-binding regulatory proteins (G proteins) play a major role in the regulation of a variety of physiological processes (9). G proteins are a large family of GTP-binding proteins, which act as signal transducers across cell

membranes in all eukaryotic cells (21) G proteins are heterotrimeric proteins composed of an α (39- 46 kDa), a β (37 kDa) and a γ (8 kDa) subunit (88) and are involved in mediating the intracellular signal transduction between the superfamily and seven transmembrane spanning domain receptors, and their respective intracellular effectors. These essential proteins act as coupling proteins that become activated upon receptor stimulation (221) In its GDP-bound form, the protein forms an inactive heterotrimer composed of three subunits. Upon ligand binding, a conformational shift in the receptor is transmitted to the α-subunit, resulting in the release of GDP and its replacement by GTP. This can be considered the rate-limiting step in the activation process. It should be noted that GTP binding is Mg^{2+} dependent process. Subsequently, the α and βγ subunits become dissociated and are released from the receptor. This α-subunit stimulates the effector molecule by direct interaction. Deactivation of Gα occurs when GTP is hydrolyzed to GDP, a process that is mediated by the intrinsic GTPase activity of Gα and GTPase activity. The GTPase activity of the α-subunit then hydrolyzes GTP, releasing the γ phosphate group. This increases the affinity of β and γ for the α-subunit to reassociate and thus leaves the G-protein in the inactive form to enter a new activation cycle.

The purification of G-proteins was achieved by Godchaux and Zimmerman (79) who characterized GTPase as a heterodimer and were first to measure a single

cycle of the GTPase reaction. A total of eight G-proteins have been purified, but the cDNAs derived from a total of nine genes encoding Gα-subunits have been cloned, as well as at least four Gp genes and three Gγ genes.

There are three major subfamilies of Gα protein; Gαs, Gαi, and Gαq, each of these subfamilies couple to different sets of receptors. Ang II, α1-adrenergic, and ET-1 receptors are coupled with Gαq, and the binding of ligands to their respective receptors subsequently activates PLC β (205). Activation of the Gαq signaling pathway, including downstream PKC, plays a crucial role in the development of cardiac hypertrophy and CHF due to different etiologies (205). The importance of Gq protein in the development of pressure-overload induced cardiac hypertrophy was demonstrated using transgenic mice (4). Class-specific inhibition of Gq-mediated signaling was produced in the hearts of transgenic mice by targeted expression of a carboxy-terminal peptide of the α-subunit of Gq. When pressure overload was induced, the transgenic mice developed significantly less ventricular hypertrophy than controls. This suggests that Gq-coupled receptors such as AT_1, ET_A receptors are important in the development of pressure-overload induced cardiac hypertrophy (121). Also, the targeted overexpression of α subunits of Gq in transgenic mice has been observed to evoke hypertrophy and failure, thus confirming the critical role Gq signaling plays in the development of cardiac hypertrophy and heart failure (39, 142). Transgenic cardiac specific Gαq

overexpression resulted in PKC ε activation, cardiac hypertrophy, and recapitulated of fetal gene program. Higher level of Gαq expression resulted in CHF (205). It has also been demonstrated that in the neonatal cardiomyocytes mechanical deformation activates the Gαq-PLC signaling pathway and reexpresses a number of genes, including ANF, skeletal α-actin, and β-MHC followed by an increase in new protein synthesis (121).

E. Protein Kinase C

PKC constitutes a family of related serine-threonine kinases that play fundamental roles in cell morphology, cell functions such as modulation of cardiomyocyte contraction and regulation of intracellular signaling pathways and cardiomyocyte growth (152). Currently, there are 11 PKC isoforms with the heart containing a minimum of 5-7 isozymes (55, 154) that have been identified and are believed to play different functional roles in cell signaling leading to alterations in cardiac contractility, hypertrophic response and tolerance to MI (203). Cardiomyocytes express several isoforms of PKC; the most widely detected are the Ca^{2+} sensitive PKC α, the novel PKC δ and PKC ε and the atypical PKC λ. (198). These isoforms can be classified into three major groups: 1) conventional isoforms which are responsive to calcium (α, β_1, β_2 and γ) 2) novel isoforms that lack the calcium binding site (δ, ε, η and θ) and finally 3) atypical isoforms that do

not respond to phorbol esters (ζ, λ and μ). Besides differing in tissue distribution and mode of activation, the various PKC isozymes also exhibit different substrate specificities, cellular distribution, and translocating properties.

The production of DAG by phosphatidyl hydrolysis causes activation of PKC, thereby promoting another series of phosphorylation reactions. Activation of PKC isoforms is accompanied by translocation of the enzyme from the cytosol fraction to the plasma membrane, (55) as well as activating different sets of downstream targets and modulates transcriptional factors, gene expression, ion channels, Na^+-H^+ exchangers, SR proteins and myofibrillar proteins (221). To note, it has been reported that translocation of PKC-α, ε, and γ from cytosolic to membranous fractions were significantly increased during POH and CHF. Jalili et al. concluded this differential PKC activation may be mediated by increases in Gαq and PLC β_1 activity rather than upregulation of expression (105). Specifically, Bayer et al. have reported that PKC δ may be involved in the induction of pressure overload LV hypertrophy, whereas PKC δ and PKC α may be involved in the transition to heart failure (17).

Clearly, PKC appears to play a key role in cell anabolism and growth (22). It has been shown that growth stimuli seem to act through the PKC pathway. Stretch of atria, ventricles and cultured neonatal cardiac myocytes increases tissue

contents of inositol phosphates, apparently by activation of membrane-bound PLC and subsequent activation of PKC (121). In adult guinea pig heart, stretch stimulates PI hydrolysis and translocation of PKC. Also cardiac specific overexpression in Gαq and PKC β2 in transgenic mice demonstrates gene dose dependent induction of cardiac hypertrophy and contractile depression. Furthermore, in failed human hearts the expression and activity of PKCα and PKCβ are elevated suggesting that the PLC-PKC signaling pathway plays a critical role in cardiac hypertrophy and heart failure (203) Wakasaki et al, generated transgenic mice with cardiac-specific overexpression of PKC β_2 (220). This overexpression caused left ventricular hypertrophy, cardiomyocytes necrosis and multifocal fibrosis and decreased in vivo left ventricular performance. β-MHC, ANF and c-*fos* genes were upregulated in hearts overexpressing PKC β_2. This data supports the idea that sustained activation of PKC can produce cardiac hypertrophy and contractile depression. PKC has been implicated in the modulation of cardiac contractile performance through phosphorylation of its substrate as well as in the control of cardiomyocyte hypertrophy (200). PKC isoform activation also initiates a phosphorylation cascade and may lead to changes in gene expression characteristics of the hypertrophic response (203).

F. α-adrenoceptors

The α-adrenoceptors are single, heavily glycolsylated peptides embedded as seven membrane-spanning domains in the lipid bilayer of cell membranes (102). Stimulation of the α_1-adrenoceptor, via G proteins leads to enhanced intracellular phosphatidylinositol metabolism. As previously stated the SNS is increased in CHF; due to this increase in the SNS there is elevated levels of catecholamines (NE). The elevated levels of catecholamines seem to downregulate the β-adrenoceptors in failing hearts; this leads to subsensitivity of the β-agonist-mediated biochemical and mechanical responses. In contrast, α-adrenoceptors are found to be upregulated in heart failure; these adrenoceptors are associated with the positive inotropic effect of catecholamines through the activation of membranal PLC. Therefore, the α_1 adrenoceptor has a greater proportion of the total adrenoceptor population in the failing ventricular, in view of the downregulation of the β-adrenoceptors. Thus, it appears that the α_1-adrenoceptors play a dominant role in eliciting the positive inotropic action of the catecholamines in the failing heart (161).

G. Role of PLC products in regulating cardiac function

a. DAG as a second messenger

DAG, through its association with PKC is now recognized as an important cellular messenger. In the presence of Ca^{2+}, membrane-inserted DAG binds and activates PKC (212). The activation of PKC by DAG, contributes to the elevation of intracellular calcium, perhaps by mobilizing intracellular stores and by acting on calcium channels of the cell membrane.

It is well accepted that DAG is one of the most important second messengers in the transmembranous cellular signal transduction system. An increase in DAG followed by activation of PKC is the initial event in PI hydrolysis. However, different DAG species may originate from different sources. Some DAG species originate from the hydrolysis of PIP_2 by PLC or some originate from that of PC by PLD (210). Accordingly, DAG species derived from PC or PIP_2 may activate different PKC isoforms, thus inducing PKC-dependent phosphorylation of different proteins and different physiological responses (210).

b. IP_3 as a second messenger

Generation of IP_3 has also proven to be an important second messenger in cellular signaling cascade. In myocytes, ET-1, ANG II and α_1 adrenergic agents

are all recognized as potent activators of IP_3 production through the activation of PLC (212).

Binding sites for IP_3 and its phosphorylated derivative IP_4 have been found at the cardiomyocyte SR level and may serve to enhance SR Ca^{2+} release and uptake. These messenger-mediated SR Ca^{2+} movements may modulate the inotropic response of the cardiac muscle to agonists (43). Immunolocalization of IP_3 receptors at the fascia adherens of the intercalated discs have also been found and may suggest a possible role of these receptors in local Ca^{2+} entry or in intracellular signaling between cardiomyocytes (117, 209).

H. PLC signaling in CHF due to different etiologies

A depressed total SL PLC activity has been reported in moderate and chronic stages of CHF due to MI. There is an overabundance and hyperactivity of the SL PLC β_1, isozyme; this finding is in direct contrast to a drastic reduction of PLC γ_1 and δ_1 activity and protein mass (209). Meij et al. (1997) also have shown a depression in the total PLC activity of the SL membranes from surviving LV tissue, as well as an elevation in PLC β_1-β_3 expression and increased PLC β_1 activity in crude membrane extracts (140). It is interesting to note that Ju and colleagues have reported that these changes were intensified in scar tissue and

remnant myocardium (108). From these studies it is clear that there is amplification of the PLC β-dependent function with almost complete loss of the PLC γ and δ functions at the cardiac SL level in post MI CHF (209).

It has been observed that there is an increase in SNS activity and circulating myocardial catecholamine levels (70, 174), a high density of α_1-adrenoceptors, normal Gqα level (108) and a high mass/activity of PLC β_1 in the surviving LV tissue of post MI failing hearts (108). ANG II and AT_1 receptor expression is also increased in post MI rat hearts (170). This may play a role in the increased activity of PLC β_1. In this context and from the observed increased activity of PLC β_1, the PLC β_1/α_1/Gqα pathway may contribute to the positive inotropy effect seen in the failing heart. The increase activation of the PLC β1/α1/Gqα pathway may explain the augmented responsiveness of the failing hearts to α_1-agonists in this model of CHF. It is also pointed out that signaling via Gqα/PLCβ when sustained results in apoptotic loss of cardiomyocytes, this change may be associated with decreased ventricular function in the failing heart (3).

A decreased number of SL PIP_2 molecules has been observed in CHF due to different etiologies such as post MI CHF, (209) diabetic cardiomyopathy (210) and in the cardiomyopathic hamster (236). The decrease in PIP_2 levels is suggested to compromise the contractile performance of the heart by causing

depression of the inward rectifier K^+ channel as well as the SL Ca^{2+} pump and Na^+/Ca^{2+} exchanger activities.

It is increasingly being suggested that the development of hypertrophy due to different etiologies involves an increase in the PLC signaling pathway for example, in stroke-prone SHR there is an increase in activity of the PLC signaling pathway (193). Studies with the cardiomyopathic hamster (BIO14.6) have shown that cardiac hypertrophy is due to an increase in PLC activity as a consequence of an enhanced responsiveness to ANG II (181). Also, Zieglehoffer et al. have found differential changes in SL PLC isozyme activities and their SL abundance in the failing heart of the cardiomyopathic hamster (UM X7.1) (236) Interestingly, the development of hypertrophy in cultured rat neonatal cardiomyocytes induced by ET-1 has been reported to be due to activation of PLC β isoenzymes. All of these studies confirm the suggestion that PLC signaling plays an important role in the development of cardiac hypertrophy and CHF.

III. STATEMENT OF THE PROBLEM

In view of the critical role played by different membrane phospholipids in maintaining cell structure and function, the objective of this research was to define phospholipid associated signal transduction mechanisms in cardiac hypertrophy and heart failure.

It is believed that changes in the PLC signaling transduction processes in cardiac hypertrophy and heart failure due to volume overload may be involved in the dynamic development of cardiac hypertrophy as well as its transition to heart failure.

This study was conducted to test the hypothesis that during cardiac hypertrophy (3 days-4 wks) there is an upregulation of the PLC isozymes and a downregulation of the PLC isozymes during heart failure (8 and 16 wks).

The proposed study will identify new mechanisms involved in cardiac hypertrophy and the dysfunction of the failing heart, the identification of these mechanisms will then become useful for designing novel therapeutic treatments for cardiac hypertrophy and CHF.

IV. MATERIALS AND METHODS

A. MATERIALS:

[γ-^{32}P] ATP (specific activity of 10 Ci/mmole) and ^{3}H-PIP_2 [inositol-2-^{3}H (N)]-(5.45 Ci/mmole) were purchased from DuPont Canada Inc./New England Nuclear (Mississauga, Ont., Canada). Sodium cholate and unlabelled ATP were purchased Sigma Chemical Co. (St. Louis, MO, USA) Nonlabelled PIP_2 was purchased from Calbiochem Co. (La Jolla, CA, USA). PLC β_1, γ_1 and δ_1 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Secondary antibodies: Goat anti-mouse and Goat anti-rabbit, IgG (H + L)–HRP Conjugate, blotting grade affinity purified, Dowex 1X8 (formate form, 100-200 mesh), TEMED-N,N,N,N'-tetramethylethylenediamine, ammonium persulfate, 30% acrylamide/bis solution were obtained from BioRad Labs. (Hercules, CA. USA). Benchmark prestained protein ladder was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Biosciences (Little Chalfont Buckinghamshire, England). Access RT-PCR kits and Trizol reagent were purchased from Promega Corp. (Madison, WI, USA). HP-KF silica gel high performance thin layer (200 μm) chromatography plates were purchased from Whatman International Ltd. (Clifton, NJ, USA) Kodak X-Omat-R X rays films were purchased from Picker International (Highland Hts., OH, USA)

Cytoscint^{TM}ES* is a product of ICN Biomedicals Inc. (Costa Mesa, CA., USA). Protein G sepharose 4 fast flow was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Instant skim milk powder was a product of Nestle Carnation (North York, ON, USA). All the solvents used were purchased from Fischer Biotech, (Fair Lawn, NJ, USA) and all the chemicals were purchased from SIGMA-ALDRICH Co. (St. Louis, MO, USA). All the reagents were of analytical grade or of the highest grade available.

B. METHODS

1. Experimental Model for Volume Overload

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following the guidelines established by the Canadian council on Animal Care. An AV shunt was performed in male Sprague-Dawley rats (weighing 150-200 g). A pre-operative dose of analgesia (Buprenorphine hydrochloride) was given one hour before surgery. The animals were anesthetized with 5% isoflurane with a flow rate of oxygen (2 l/min). Then after the abdominal fur was shaved, an abdominal laparotomy was performed. Following exposure of the abdominal aorta and inferior vena cava between the renal arteries and ileac bifurcation, the descending aorta and the ileac bifurcation will be temporarily occluded proximal to the intended puncture site.

An 18-gauge needle was inserted and withdrawn across the medial wall of the descending aorta three times to ensure the size and presence of the shunt and finally withdrawn. The puncture site was sealed immediately with a drop of isocynate (Krazy™ glue, USA). The creation of the shunt was visualized by the pulsatile flow of oxygenated blood into the vena cava from the abdominal aorta. Throughout the operative procedure, the rats were maintained on 2.5% isoflurane in 2 l/min of oxygen. Age-matched, sham operated animals served as controls and were treated similarly, except that the puncture into the descending aorta was not performed. The animals were allowed to recover and were maintained on food and water *ad libitum*. The circulation system was only occluded for 25 sec-1 min and the entire procedure was finished within 10 minutes. It is pointed out that the mortality rate of the control group was 0% and the mortality rate of the AV shunt animals operated on in this manner was less than 4% 6 hours following surgery. After this time point no mortality was seen in either group due to the surgical procedure. At each time point, rats were anesthetized by an injection of ketamine-xylazine (100:10 mg/kg ip) and sacrificed. The body weights were measured and the hearts were excised and divided into chambers of the atria, right ventricle and left ventricle. Weights were calculated for each chamber and the ratio of LV to BW, a common accepted marker of cardiac hypertrophy was measured in the sham control and AV shunt groups.

2. *Hemodynamic studies in vivo*

The LV function of animals from the each of the sham operated and AV shunt groups was assessed. Rats were anesthetized by an injection of ketamine-xylazine (100:10 mg/kg ip). The right carotid artery was exposed and a micromanometer-tipped catheter (2-0; Millar SPR-249) was inserted and advanced into the LV. The catheter was secured with a silk ligature around the artery, and after a 15-min stabilization of the heart function, LV pressures and maximum rates of isovolumic pressure development ($+dP/dt_{max}$) and decay ($-dP/dt_{max}$) were recorded. Hemodynamic data were computed instantaneously and displayed on a computer data acquisition workstation (Biopac, Harvard Apparatus).

3. *Preparation of cardiac SL membrane*

The LV tissue from 4 to 5 hearts was pooled to prepare a SL membrane fraction. Briefly, the tissue was washed, minced by scissors in 3.5 ml buffer/g tissue of a 0.6 mM sucrose, 10 mmM 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 x 10 seconds with 20 second intervals in between. The resulting homogenate was then centrifuged at 12 000 g for 30 min at

4°C in a Beckman centrifuge with a JA-20 rotor, this was done to remove large cellular particles. The pellet was discarded and the supernatent was collected. A 500 µl aliquot of the first supernatant was centrifuged at 110,000g for 60 min at 4°C in a Beckman TL-100 Ultracentrifuge to purify the cytosol, the resulting cytosolic fraction was divided, frozen in liquid nitrogen and stored at -80°C as the soluble cytosolic fraction. The remaining supernatant was diluted with 140 mM KCl, 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), pH 7.4 (5 mL buffer/g of tissue) and centrifuged at 100 000 g for 60 min. The resulting pellet was then resuspended in 140 mM KCl, 20 mM MOPS, pH 7.4, and layered over a 30 % sucrose solution containing 0.3 M KCl, 50 mM Na4PO4O7 and 0.1 M Tris-HCl, pH 8.3. After centrifugation at 100 000 g for 90 min in a Beckman swinging bucket rotor (SW-28), the layer at the sucrose-buffer interface was taken and diluted with 3 volumes of 140 mM KCl, 20 mM MOPS, pH 7.4. The final pellet was resuspended in 0.25 mM sucrose, 10 mmM histidine, pH 7.4 (225 µl/g tissue). This sarcolemmal enriched fraction was divided into aliquots, frozen in liquid N_2, and stored at -80°C until assayed. Protein concentrations were determined by the Lowry method as described elsewhere (209).

4. Determination of PLC isozyme activities

SL membrane proteins were solubilized with buffer containing 1 % (w/v) sodium cholate, 50 mmM HEPES (pH 7.2), 200 mmM NaCl, 2 mmM EDTA, 10 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin by rotation for 2 h at 4°C. The samples were then centrifuged (280, 000 *g* for 25 min); and the supernatent was recovered as the solubilized membrane fraction. The membrane extract was incubated overnight at 4°C (rotation) with monoclonal antibodies to PLCs [anti-bovine PLC β_1, mixed monoclonal antibodies (no. 05-164); anti-bovine PLC γ_1, mixed monoclonal IgG antibodies (no. 05-163); anti-bovine PLC δ_1, mouse monoclonal antibodies (no. 05-343); all from Upstate Biotechnology, Lake Placid, NY] (5 μg of antibody to 350 μg of membrane extract, ie. a ratio of 1:70 μg/μg). All the antibodies react with their corresponding PLC isozymes but not with the other two isozymes. The immunocomplex was captured by adding 100 μl of washed protein G sepharose bead slurry (50 μl packed beads) at 4°C by rotation for 2 h. The agarose beads were collected by pulse centrifugation (5 s) at 10, 000 *g* and assayed for the activity of PLC isozymes. The hydrolysis of [^{3}H] PIP_2 was measured basically according to the method described by Tappia et al., 1999. Briefly, the reaction was performed in the presence of 30 mmM HEPES (pH 6.8), 70 mmM KCl, 100 mmM NaCl, 0.8 mmM EGTA, 0.8 mmM $CaCl_2$ (free Ca^{2+}, 23.3 μmol/l), 20 μmol/l [^{3}H] PIP_2 (20-30 dpm/pmol) dissolved in 14 mmM sodium cholate overnight, and an aliquot (10 μl) of immunoprecipitate suspension. The

reaction was carried out at 37° C for 2.5 min and then stopped by trichloroacetic acid precipitation. Precipitates were removed by centrifugation at 10, 000 *g* for 5 min, and the supernatent was collected for quantification of inositol phosphates by liquid scintillation counting. The efficiency of the immunoprecipitation of each isoenzyme was ascertained by determining any residual PLC isozyme activity in the 10, 000 *g* supernatent after capturing the immunocomplex by protein G sepharose. The supernatent was concentrated to 100 µl by using microconcentrators (Centricon-3, Amicon Canada, Oakville, ON) and then tested for PLC isozyme activities. The immunoprecipitation was complete, as PLC-dependent [^{3}H] PIP_2 hydrolysis of any immunoprecipitated isoenzyme could not be detected in the supernatent as reported earlier (209, 236). For control experiments, immunoprecipitation and subsequent activity measurements were conducted with non-immune mouse IgG.

5. Western blot of PLC isozymes

High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 20 µg of sarcolemmal proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (14) Separated proteins were transferred onto 0.45-µm polyvinylidene difluoride (PVDF) membrane. PVDF membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% skim milk and probed with mouse monoclonal primary PLC

isozyme antibodies (Upstate Biotechnology, NY, USA). Primary antibodies were diluted in TBS-T (1:200 for PLC β_1, 1:2000 for PLC γ_1, and 1:10,000 for PLC δ_1, according to the manufacturer's instructions). It should be noted that all the antibodies react with their corresponding PLC isozyme, but do not cross-react with the other two isozymes (14). Horseradish peroxidase-labeled anti-mouse IgG (Bio-Rad, CA, USA) was diluted 1:3000 in TBS-T and used as secondary antibody. PLC β_1, γ_1 and δ_1 were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Boehringer Mannheim, Laval, PQ). Band intensities of the Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 800). The linearity of the Western blot procedure used for the quantification of PLC isozymes has been previously determined (209, 236) In subsequent blotting experiments 20 μg SL protein was used because it is in the linear range. Furthermore, the time of exposure used was 5 min. In some experiments, Western blotting with PLC γ_1 was performed with immunoprecipitated SL phosphotyrosyl proteins (immunoprecipitation was performed with anti phosphotyrosyl monoclonal antibodies (PY99, Santa Cruz Biotechnology, CA, USA); 5 μg of antibody to 855 μg membrane extract, solubilization procedures as described above).

6. RNA isolation and semi-quantitative PCR

Total RNA was isolated from left ventricular tissue using RNA isolation Kit (Life Technologies, ON, Canada) according to the manufacturer's procedures. Reverse transcription (RT) was conducted for 45 min at 48°C using the Superscript Preamplification System for First Strand cDNA Synthesis (Life Technology, ON, Canada) as previously described. (14, 195) Primers used for amplification were synthesized as follows: PLC β_1: 5'-AATAAGGAGACGGAGCTGTTAG-3' (forward) and 5'-ATGGAAGACAAGCCTCTAGCG-3'(reverse), PLC γ_1: 5'-CCTCTATGGAATGGAATTCCG-3' (forward) and 5'-CTAGGGAGGACTCGCTGGAGAACT-3' (reverse) and PLC δ_1: 5'-AGGATCGATGCTTCTCCATTGT-3' (forward), 5'-TTATCAGCCTTTCGCAAGCA -3' (reverse). Amplification of cDNAs of PLC isozyme genes was performed using specific primers and the Superscript Preamplification System (Life Technology, ON, Canada). Temperatures used for PCR were as follows: denaturation at 94°C for 30 s, annealing at 62°C for 60s, and extension at 68°C for 120s, with a final extension for 7 min; 25 amplification cycles for each individual primer sets was carried out. For the purpose of normalization of the data, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3' (forward) and 5'-GCATGTCAGATCCACAACGGATAC-3' (reverse) were used to amplify

GAPDH gene as a multiplex with the target genes. The PCR products were analyzed by electrophoresis in 2% agarose gels. The intensity of the bands was photographed and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) as a ratio of a target gene over GAPDH.

7. Determination of cytosolic IP_3 and SL DAG and PIP_2 contents

The cytosolic IP_3 concentration, SL DAG and PIP_2 amounts were measured using their respective Biotrak radioimmunoassay kit (Amersham Biosciences Corp., PQ, Canada) according to the manufacturer's instructions as previously described (209, 236). The D-myo-Inositol 1,4, 5-trisphosphate IP_3 Biotrak™ Assay System from Amersham Biosciences has been specifically designed to measure IP_3 at concentrations in the range 0.19-25 pmol (0.08-10.5 ng). The assay is based on competition between [^{3}H] IP_3 (the tracer) and unlabelled IP_3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP_3 is then separated from the free IP_3 by centrifugation, which brings the binding protein to the bottom of the tube. The free IP_3 in the supernatent can then be discarded by simple decantation, leaving the bound fraction adhering to the tube (159). Measurement of the radioactivity in the tube enables the amount of unlabelled IP_3 in the sample to be determined by interpolation from a standard curve. Each pack contains sufficient material for 100 assay tubes, 39 unknowns

can be measured in duplicate. This assay system has been adapted to allow for measurement of PIP_2 mass. The protocol is taken from Chilvers et al., 1991. Essentially the method involves the conversion of PIP_2 in crude lipid extracts into IP_3 by alkaline hydrolysis. Extracts are then neutralized and assayed for IP_3 in the usual way. The basis of the DAG assay procedure is a radioenzymatic assay employing the enzyme DAG kinase which quantitatively converts DAG to [^{32}P] PA in the presence of [^{32}P]-γ-ATP. Following a number of extraction steps to remove unreacted [^{32}P]-γ-ATP, separation of [^{32}P] PA is achieved by thin-layer chromatography. The [^{32}P] PA is quantitated by liquid scintillation counting. This kit allows for detection of DAG in the range of 31-1000 pmol per tube. It should be noted that the sensitivities are 0.1 pmol for IP_3 and PIP_2, and 10 pmol for DAG. The radioimmunoassay kits are reliable and generate accurate and reproducible values.

8. ***Statistical analysis***

All values are expressed as mean ± SEM. The differences between two groups were evaluated by Student's *t*-test. The data from more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests. A probability of 95% or more ($P<0.05$) was considered significant.

V. RESULTS

A. General characteristics and left ventricular function

The AV shunt resulted in a consistent and reproducible occurrence of cardiac hypertrophy and subsequent heart failure. The time course of changes in the general characteristics of the rats with and without AV shunt is shown in Table 1. Although there was no significant difference in body weight between the sham-operated and AV shunt groups at each time interval, heart weight of the experimental group increased progressively during the 3 day to 16 week time points. Accordingly, heart weight-to-body weight ratio was significantly increased at all time intervals in the AV shunt volume overload-induced groups.

Cardiac hypertrophy and failure lead to significant alterations in cardiac performance to compensate for the increased workload due to the volume overload placed on the heart. The time course changes in the *in vivo* cardiac performance due to volume overload are shown in Figure 1. A significant progressive elevation of LV end diastolic pressure (LVEDP) was detected throughout the 16-wk observation period (Figure 1A). On the other hand, the LV systolic pressure (LVSP) was decreased significantly throughout the 16-week observation period; however the results were biphasic in nature. The most significant decreases in LVSP occurred at 2, 8 and 16 wks, whereas the decrease in LVSP was reduced at

the 3-day, 1 and 4 week observation points. Also, no changes were detected for +dP/d*t* and –dP/d*t* at 1, 2 and 4 wk after the surgery, but progressive depressions were seen during the heart failure stages of 8 and 16 wk.

TABLE 1. General characteristics and LV function of rats with or without an aortocaval shunt for different time intervals.

	BW, g	HW, mg	LVW, mg	HW/BW mg/100g	LVW/BW mg/100g
3 day					
Sham	263 ± 5	904 ± 16	593 ± 1	440 ± 26	225 ± 3
AV	258 ± 4	1028 ± 22*	655 ± 14*	420 ± 9*	267 ± 5*
1wk					
Sham	267 ± 8	908 ± 3	581 ±2	340 ±20	218 ±10
AV	251 ± 10	1157 ± 4*	721± 3*	461 ±20*	287 ±10*
2 wk					
Sham	358 ± 12	1051 ± 3	694 ±2	293 ±2	194 ±10
AV	344 ± 20	1621 ± 4*	1027 ± 3*	471 ±1*	299 ±10*
4wk					
Sham	440 ± 21	1261 ± 13	875 ±13	287 ±20	199 ±10
AV	442 ± 11	2227± 35*	1350 ± 31*	504 ±16*	305 ±20*
8wk					
Sham	520 ± 27	1412 ± 20	905 ±26	272 ±5	174 ±10
AV	514 ± 16	2619 ± 24*	1493 ± 32*	509 ±4*	290 ±3*
16wk					
Sham	640 ± 22	1575 ± 24	1073 ±36	246 ±5	168 ±20
AV	638 ± 25	2962 ± 34*	1849 ± 22*	464±6*	290 ±5*

Data are mean ± S.E. of 12-14 animals for each group. BW, body weight; HW, heart weight. The viable left ventricular weight (LV wt) of the experimental animals refers to the weight of the LV plus septum. RV, right ventricle; *$P < 0.05$ *vs.* sham control values.

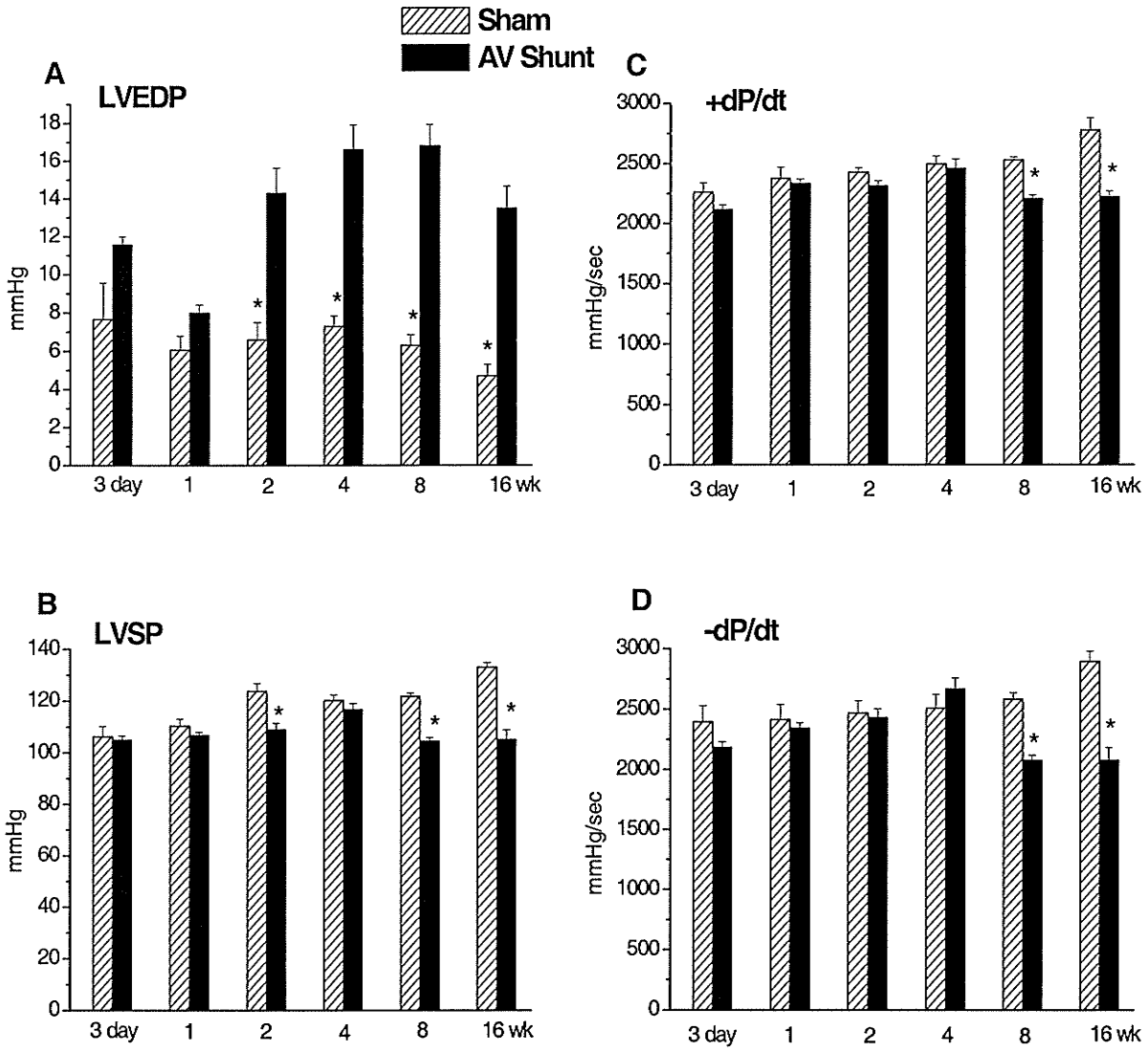


FIGURE 1. Time-dependent changes in left ventricular (LV) function of rats after AV shunt

Values are a means ± SE of 16-20 experimental animals in each group. Sham, age-matched sham-operated controls; LVEDP, LV end-diastolic pressure; LVSP, LV systolic pressure; +dP/d*t*, rate of contraction; -dP/d*t*, rate of relaxation. * $P < 0.05$ vs. sham-operated control values.

B. ***Cytosolic IP_3 and SL DAG content***

To examine the role of PLC activity in the development of cardiac hypertrophy and heart failure due to volume overload, we examined products of PLC activity, IP_3 mass, and the DAG mass in the cytosolic and SL membrane compartments respectively, of hypertrophied and failing hearts. As shown in Figure 2, increases in both IP_3 and DAG levels were seen during the development of hypertrophy (3 days to 2 weeks) that remained elevated at 4 weeks. Although at 8 weeks (moderate CHF) the IP_3 and DAG levels were significantly diminished, a further attenuation of the levels of these molecules was seen at 16 weeks (chronic CHF). Such a profile strongly indicated that PLC activities are increased during cardiac hypertrophy and decreased during CHF. It should be noted a 12 and 9% loss in SL DAG and cytosolic IP_3, respectively; due to two freeze-thaw cycles (LV tissue and SL membrane or cytosol) was measured (data not shown). These losses were taken into consideration when calculating the absolute SL DAG and cytosolic IP_3 values.

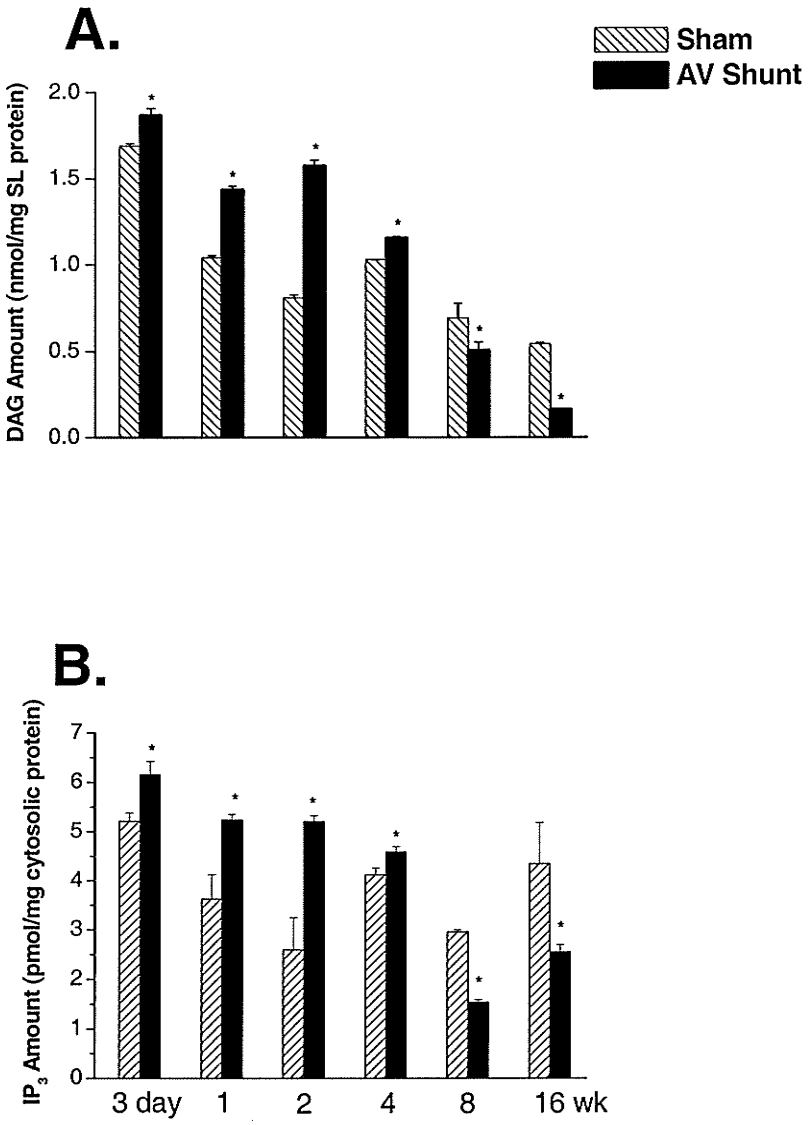


FIGURE 2. SL DAG and IP_3 levels in volume overload-induced hypertrophied and failing hearts.

Values are means ± SE of duplicate experiments performed with 4 different SL preparations. DAG and IP_3 levels were determined using Biotrak RIA kits as described in MATERIALS AND METHODS. Measurements were conducted at 3 days and 1, 2, 4, 8 and 16 wks after the AV shunt. * $P < 0.05$ vs. sham-operated control values.

C. SL PLC isozyme activities in hypertrophied and failing hearts

In order to understand if the changes in the IP_3 and DAG levels in hypertrophied and failing hearts are due to PLC, the activities of three predominant PLC isozymes (β_1, γ_1 and δ_1) were determined at 3 days, 1, 2, 4, 8 and 16 weeks after the induction of the AV shunt (Figure 3). It can be seen that both PLC β_1 and γ_1 activities were significantly increased during the hypertrophic stages (Figures 3A and 3B). While a six-fold increase in PLC β_1 activity was detected at 2 weeks after surgery, the peak activation of PLC γ_1 occurred at 3 days. Although PLC β_1 protein and mRNA levels were elevated at 2 weeks, a six-fold increase in PLC β_1 activity was seen at 2 weeks. In heart failure, although PLC γ_1 activity was significantly depressed at 8 and 16 weeks after the AV shunt, a second smaller peak of PLC β_1 activity was seen at 8 weeks, which was depressed at 16 weeks (Figures 3A and 3B). The profile of the PLC γ_1 activity at all the time points studied was consistent with the level of phosphorylation of its tyrosyl residues (Figure 4). In fact, the biphasic nature of the PLC γ_1 activity was correlated with the pattern of its phosphorylation. In contrast to the activity profiles of PLC β_1 and γ_1, a progressive decline in PLC δ_1 activity was observed throughout the time course, where heart failure was associated with approximately three-fold and five-fold decreases in PLC δ_1

activity at 8 and 16 weeks respectively, after the induction of the AV shunt (Figure 3C).

D. PLC isozyme protein abundance and mRNA levels in hypertrophied and failing hearts

To understand whether the changes in PLC isozyme activities are due to altered SL abundance and mRNA levels, Western blot analysis and RT-PCR were performed. Time dependent increases in the SL PLC β_1 protein content (Figures 5A and 6) and mRNA levels (Figure 7A and 8) were seen up to 8 weeks following the induction of the AV shunt. Whereas at 16 weeks the SL PLC β_1 protein abundance was markedly diminished, the mRNA level remained elevated. A significant increase in SL PLC γ_1 protein abundance was observed only at 4 weeks after the AV shunt, while a progressive decline in the SL PLC δ_1 protein content was observed (Figures 5 B and C). However a strict correlation (increased mRNA translates to increased protein) of these isozyme protein changes with their respective mRNA levels (Figures 7 and 8) was not revealed.

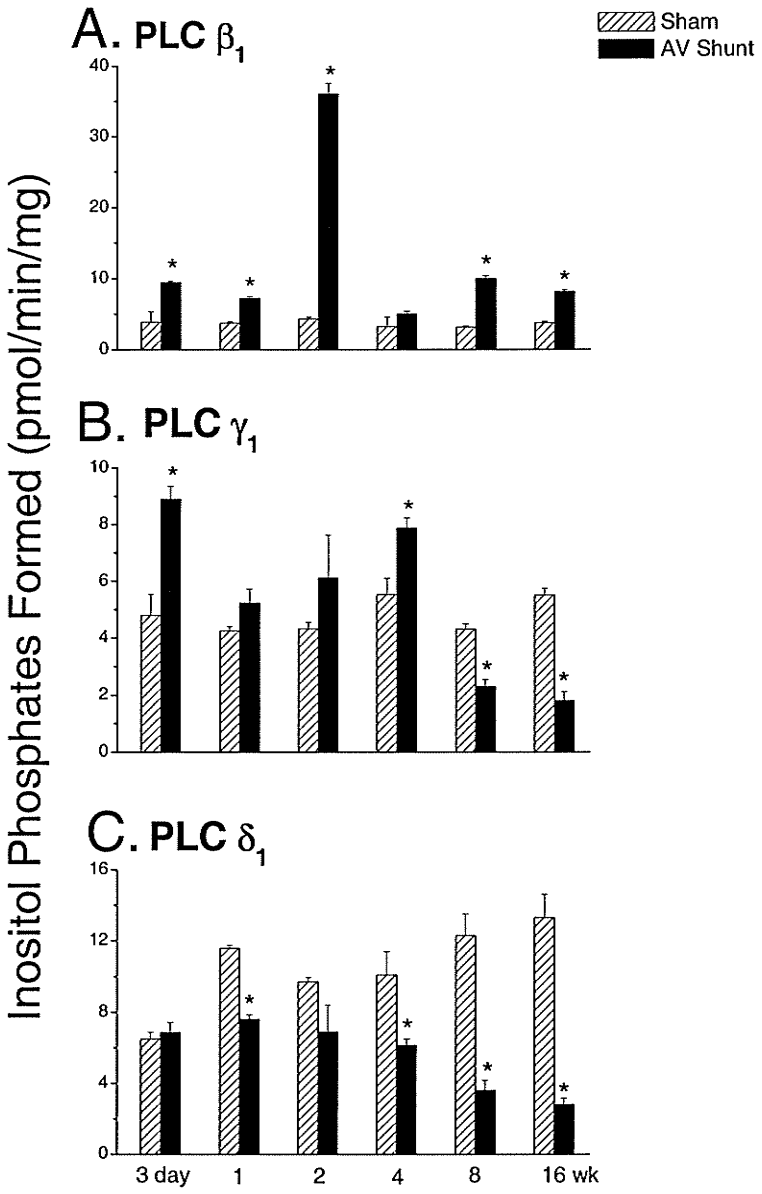


FIGURE 3. Changes in SL PLC isozyme activities in hearts of rats after the induction of volume overload.

PLC isozyme activities were measured after immunoprecipitation with specific monoclonal antibodies against PLC β_1 (A), -γ_1 (B), and δ_1 (C) as indicated in MATERIALS AND METHODS, at 3 days and 1, 2, 4, 8 and 16 wks after induction of volume overload. Inositol phosphates formed refers to the sum of the total inositol phosphates produced by PLC-dependent hydrolysis of PIP_2. Values are means ± SE of quadruplicate experiments performed using 4 different SL preparations. * $P < 0.05$ vs. sham-operated control values.

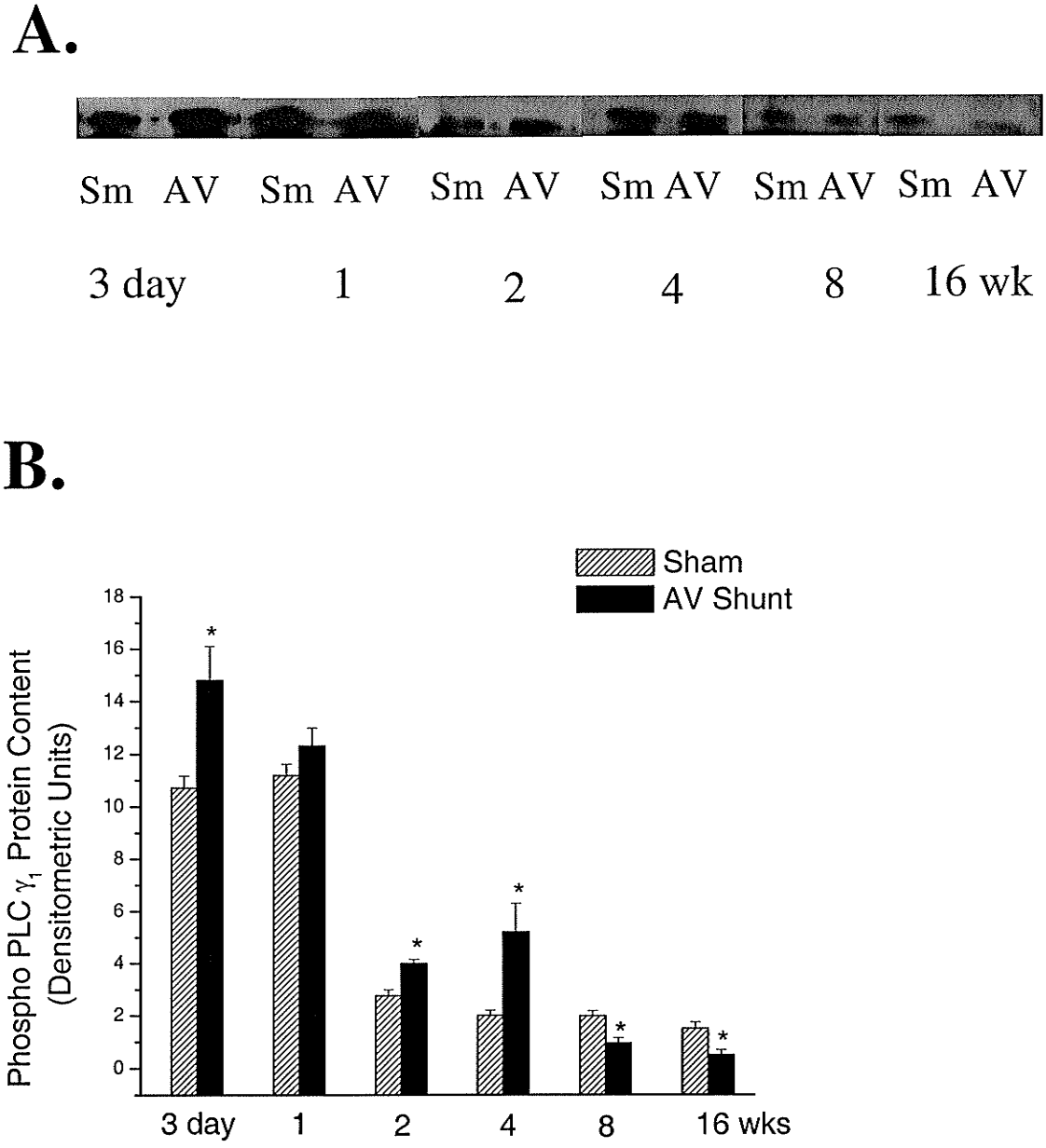


FIGURE 4. Representative Western blots (A) and quantified data (B) of the tyrosyl phosphorylation of SL PLC γ_1 in hearts of rats after induction of volume overload

Western blots show tyrosyl-phosphorylated PLC γ_1 at 3 days and 1, 2, 4, 8 and 16 wks after AV shunt. Values are means ± SE of 3 experiments performed with 3 different SL preparations. * $P < 0.05$ vs. sham-operated control values.

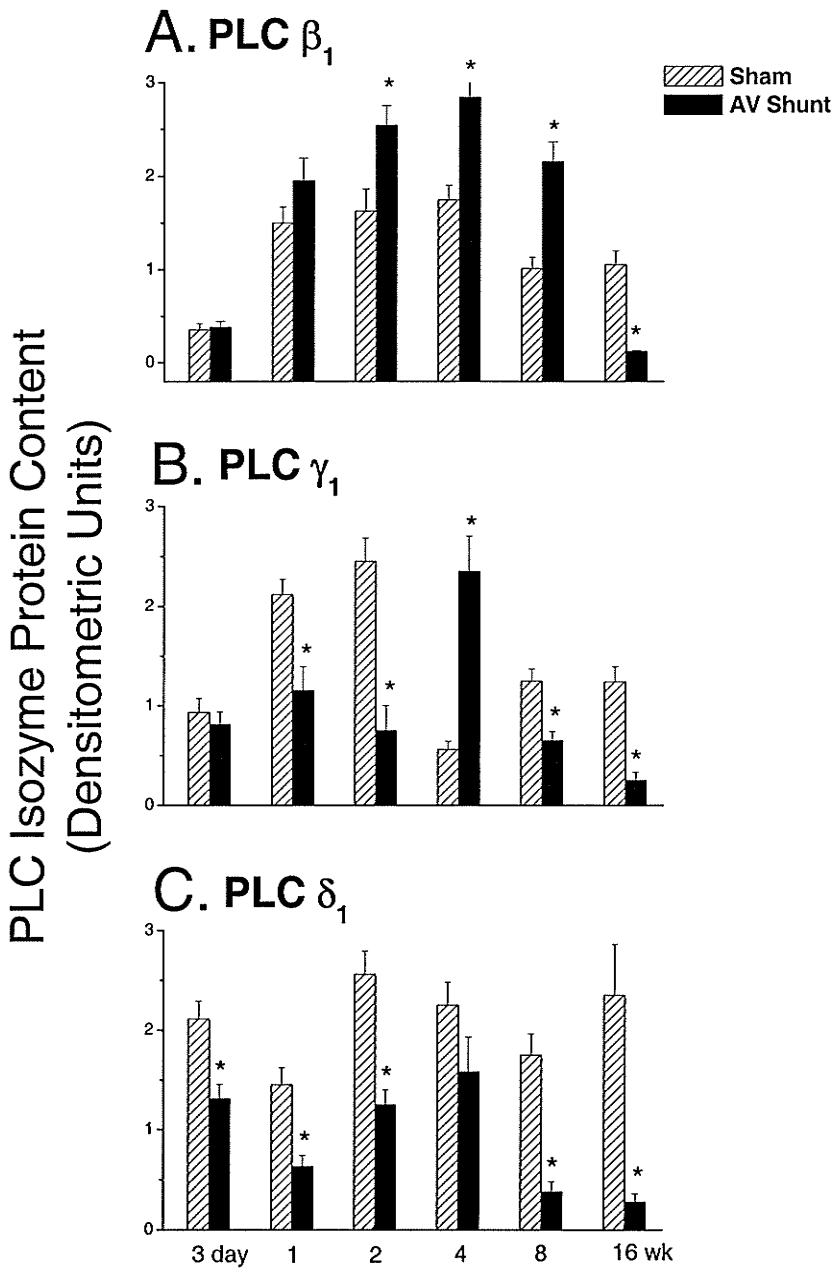
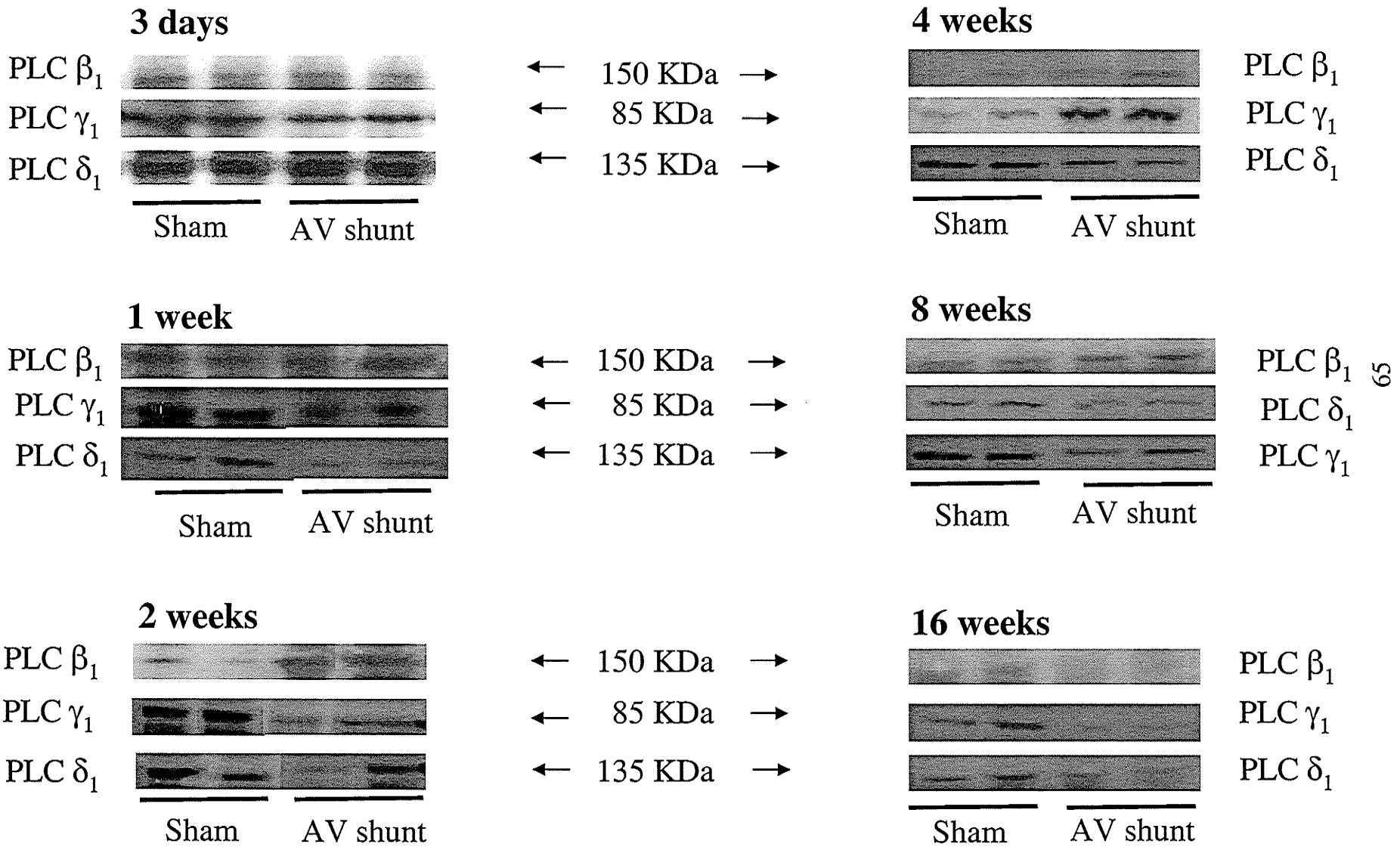


FIGURE 5. Quantified data of SL PLC isozyme protein content in hypertrophied and failing hearts due to volume overload assessed by Western blotting.

Values are means ± SE of 3 experiments performed with 3 different SL preparations. * $P < 0.05$ vs. sham-operated control values.

Figure 6. Representative Western blots of SL PLC isozymes in hearts of rats after induction of volume overload.



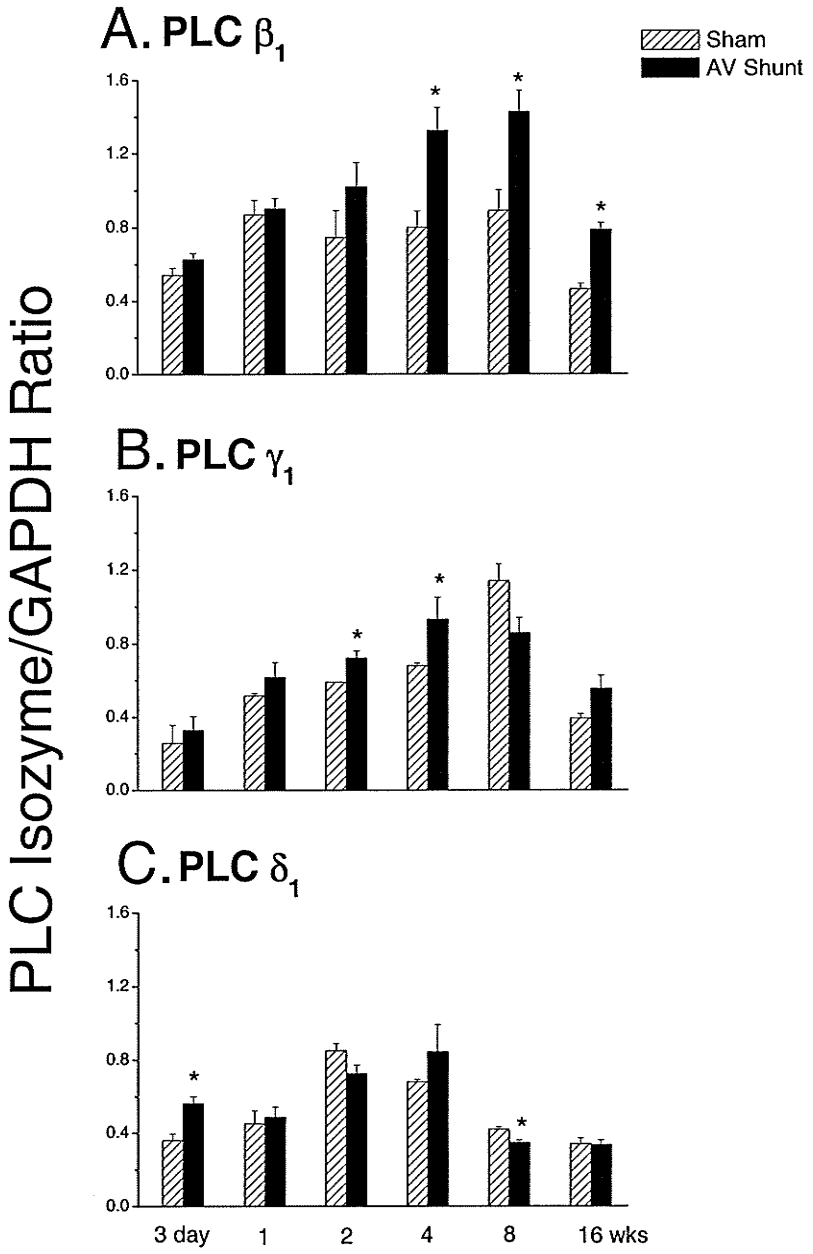
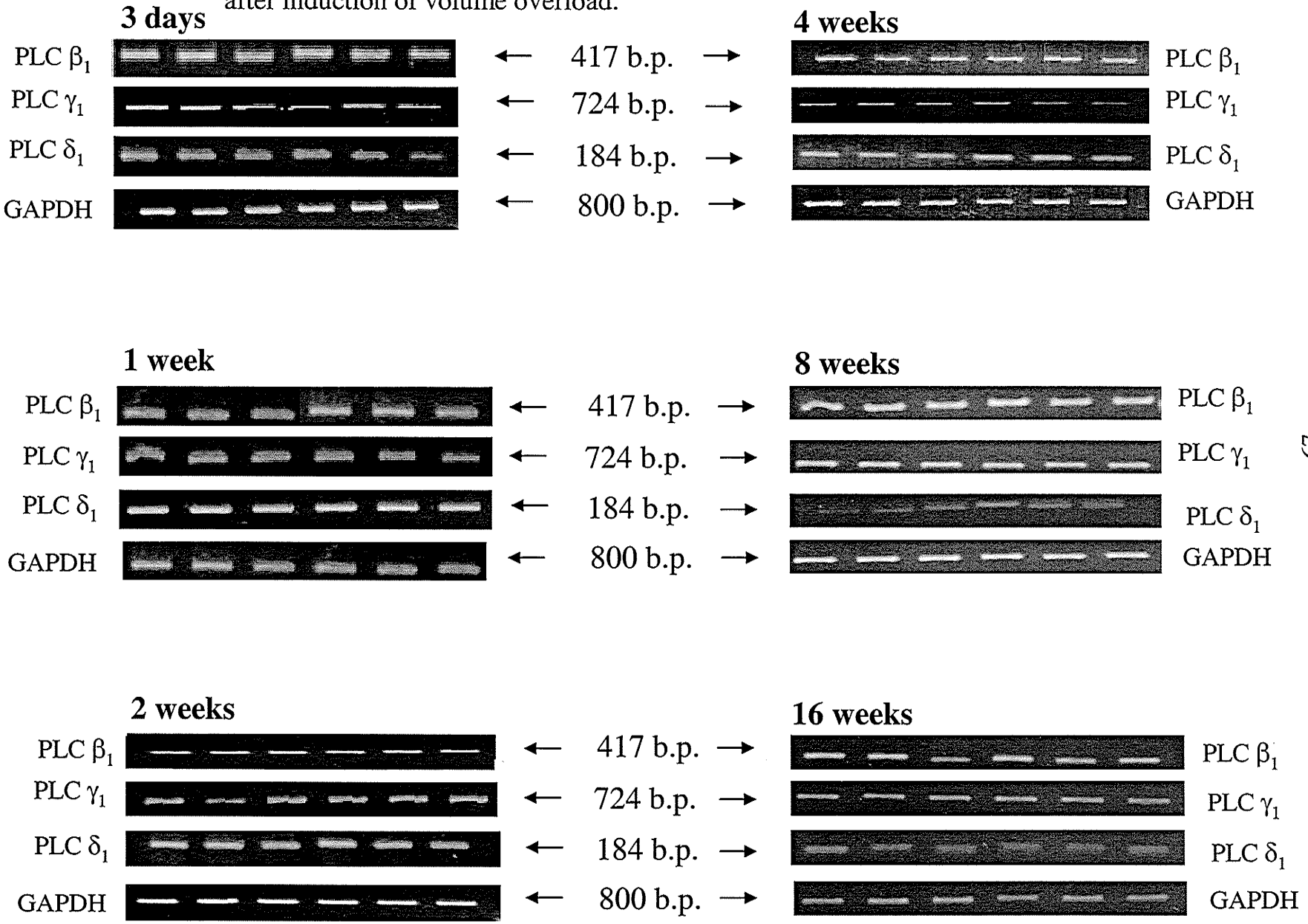


FIGURE 7. LV PLC isozyme mRNA levels in hypertrophied and failing hearts after the induction of volume overload.

PLC isozyme mRNA levels in left ventricular tissue were determined by RT-PCR using gene specific primers for PLC isozymes as described in MATERIALS AND METHODS. Values are a means ± SE of 3 experiments performed with 3 different SL preparations. * $P < 0.05$ vs. sham-operated control values.

Figure 8. Representive blots of LV PLC isozyme mRNA levels in hearts of rats after induction of volume overload.



E. SL PIP_2 content

PIP_2 serves as a substrate for PLC for the generation of second messengers. Therefore, assessment of the SL amounts of this phospholipid was conducted to understand if the observed changes in IP_3 and DAG concentrations levels as well as PLC isozymes were due to altered SL PIP_2 content. In addition, knowledge of the SL PIP_2 level would provide a mechanism to explain changes in the SL abundance of proteins containing PH domains such as PLC δ_1. (Ziegelhoffer A et al., 2001) The data presented in Figure 9 shows that a progressive decrease from 100% at 3-days to 19% (of 3 day value) at 16 weeks in the SL PIP_2 level occurred. Of note significant six- and four-fold decreases in PIP_2 levels were observed in the SL membrane fraction of the 8 and 16 week failing hearts, respectively. The PIP_2 loss during quantification due to the Dowex column was 13%, whereas in two freeze thaw cycles 8% (data not shown). Subsequently, the calculation for the absolute SL PIP_2 concentration took these losses into consideration.

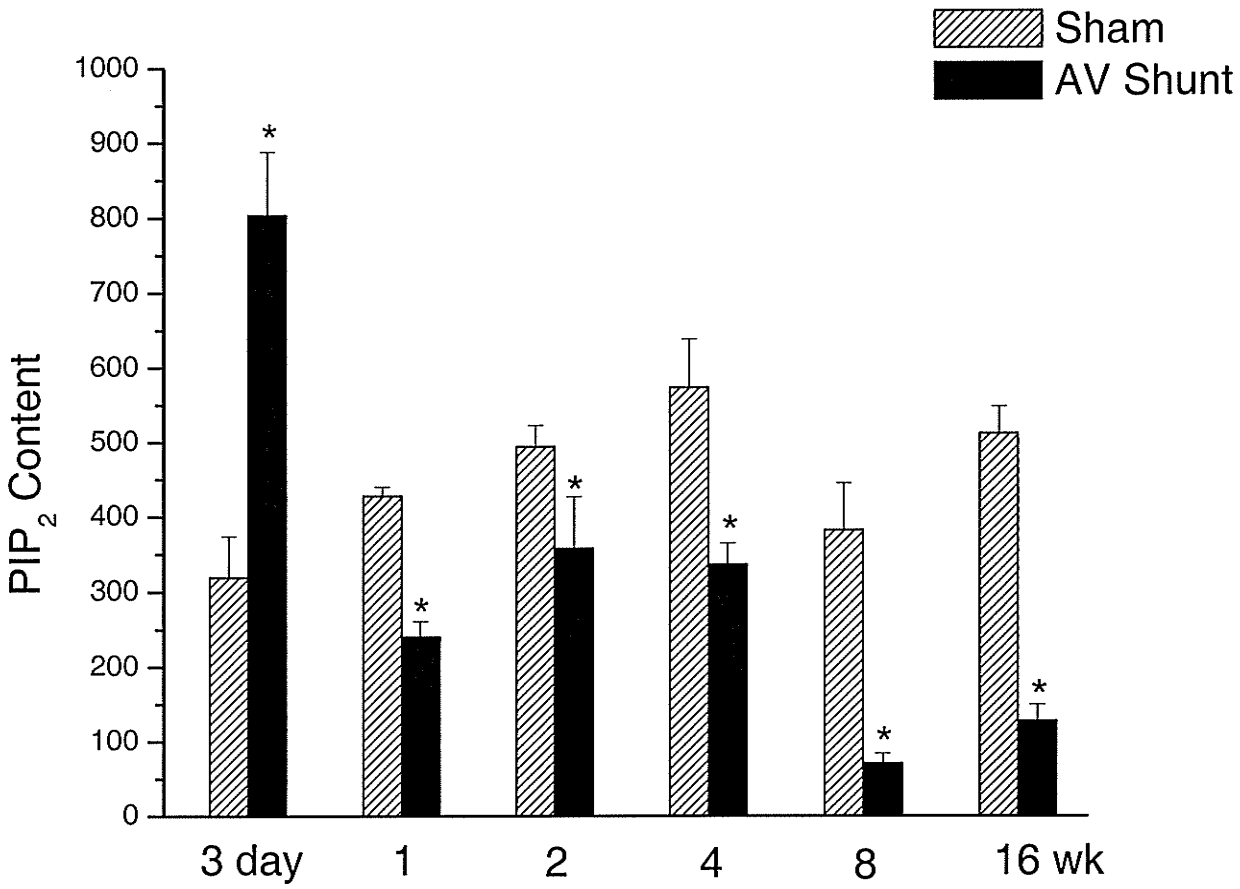


FIGURE 9. SL PIP_2 levels in hypertrophied and failing hearts due to volume overload.

Values are a means ± SE of 3 experiments performed with 3 different SL preparations. * $P < 0.05$ vs. sham-operated control values. The SL PIP_2 content was measured at 3 days, 1, 2, 4, 8 and 16 wks after the AV shunt using the Biotrak RIA kit as described in MATERIALS AND METHODS.

DISCUSSION

The needle technique used in this project is an established technique for inducing volume overload (76); this method provides reproducible animal models of cardiac hypertrophy and heart failure. The volume overload induced by this method results in cardiac hypertrophy and heart failure resembling that occurring in humans during hyperthyroidism, anemia, and bundle branch block (223). This project addressed the hypothesis that PLC mediated signal transduction processes are increased during cardiac hypertrophy and decreased during heart failure. The reported data is the first to show that the elevation of cytosolic IP_3 and SL DAG amount during cardiac hypertrophy is associated with specific increases in PLC β_1 and γ_1 isozyme activities, whereas the depressed production of IP_3 and DAG in the failing hearts is linked to marked reductions in PLC δ_1 and γ_1 isozyme activities.

Although PLC isozymes display differences in structure and activating enzymes (110, 171) the specific cardiac effects may depend on the type, quantity and activity of the PLC isozyme present at the SL membrane. The increase in PLC β_1 activity may be due to an enhanced SL compartmentalization as a result of elevated gene expression in combination

with agonist-evoked recruitment of PLC β_1 to the membrane. In fact, the high plasma level of norepinephrine reported in volume overload (225) may play a role. Because norepinephrine has been suggested to cause hypertrophic growth of cardiomyocytes via stimulation of α_1-adrenoceptors (7, 218), such a response may be related to an augmentation of the α_1/Gqα/PLC β_1 signal transduction mechanisms (68). In this regard, recent work from our laboratory has shown that the NE induced increases in ANF mRNA expression, protein synthesis and DAG formation in isolated adult rat cardiomyocytes is completely attenuated by prazosin, an α_1-adrenoceptor antagonist (195). In addition, because ANG II also acts through the PLC β class (172) via AT_1 receptors (216), which have been reported to be increased during cardiac hypertrophy (109), the increase in the responsiveness of hypertrophic myocardial cells to ANG II in volume overloaded hearts (132) could also be attributable to an increase in signaling via AT_1/Gqα/PLC β_1. Taken with the fact that ANG II may also mediate some of its cardiac effects by activation of PLC γ_1 (110, 171), it can be suggested that the activation of PLC β_1 and γ_1 isozymes may contribute to the hypertrophic response following the induction of the AV shunt. It is pointed out that selective β_1-adrenoceptor stimulation causes hypertrophic growth of ventricular cardiomyocytes by a mechanism that is cAMP independent but dependent on tyrosine kinase (183). In fact, since

PLC γ isoforms are activated by receptor (and non-receptor) tyrosine kinases (110, 171, 172) and that tyrosine kinase activation has been implicated in cardiac hypertrophy (5, 47, 82), the increase in PLC γ_1 activity seen during the hypertrophic stage may be due to an increase in the level of phosphorylation of the tyrosyl residues of PLC γ_1. In addition, the biphasic activation of PLC γ_1 (first peak at 3 days and a second peak at 4 wks) was consistent with its phosphorylation profile. The activation of PLC γ_1 may be related to the presence of growth factors.

Among potential mediators of hypertrophy, one upstream signaling protein of importance is Gqα, a heterotrimeric G protein to which are coupled the heptahelical, serpentine receptors for multiple growth factors including ANG II and norepinephrine (218). Stimulation of signaling pathways via Gqα provokes cardiac hypertrophy in cultured cardiomyocytes and transgenic mouse models overexpressing Gqα (56, 58, 142, 143). It is therefore conceivable that although the protein amount and mRNA expression of PLC β_1 were increased, an elevation in SL Gqα expression could have also occurred contributing toward the six-fold increase in PLC β_1 activity during hypertrophy, a possibility that warrants further investigation.

The primary step of the signal transduction pathway for the activation of PKC involves the stimulation of PLC. PKC isozymes, specifically PKC α (23) and ε (203) and DAG have been implicated in the regulation of hypertrophic growth of cardiomyocytes (23, 104, 203). Therefore, the observation of an increase in the total SL level of DAG during the hypertrophy in the present study is suggestive of a similar activation of PKC in cardiac hypertrophy due to volume overload and remains to be elucidated. Although DAG kinase is involved in the termination of PKC activation (202) and a decreased DAG kinase ε increases the PKC activity and may accelerate the cardiomyocyte hypertrophic response (165), a decrease in its activity, cannot be excluded. On the other hand, because the changes in DAG were not very large a compensatory increase in DAG kinase activity may have occurred. However, despite this possibility, the SL DAG levels were significantly elevated during cardiac hypertrophy. Similar changes in DAG lipase may also exist.

The progressive decrease in PLC δ_1 activity correlated to the reduction in the SL abundance of this isozyme. One of the reasons for this finding could be due to the NH_2-terminal part of the PH homology domain of PLC δ_1, which possesses a critical region rich in basic amino acid residues that bind with high affinity to the polar head of PIP_2 (228) that confers a unique capacity of PLC δ_1

to associate with the plasma membrane. The finding that a time dependent decrease in the SL PIP_2 content provides a mechanism whereby the attachment of PLC δ_1 to the SL membrane decreases during the cardiac hypertrophy and its transition to heart failure which could also account for the progressive decrease in SL PLC δ_1 activity. Also, the reduced PIP_2 could contribute to the depressed PLC γ_1 activity as a consequence of a decreased production of phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3), which is known to activate PLC γ_1 (171), via phosphatidylinositol 3-kinase phosphorylation of PIP_2 (60). Furthermore, it is pointed out that the depressed PLC γ_1 activity in the failing heart may also be related to the decreased level of phosphorylation of its tyrosyl residues. Therefore, taken together, it can be suggested that bioprocesses mediated by PLC γ_1 and δ_1 may be severely impaired during CHF due to volume overload. In this regard, it is interesting to note that it has been observed that there are similar reductions in PLC γ_1 and δ_1 activities in CHF subsequent to myocardial infarction, which were associated with a significant attenuation of the contractile responsiveness of the failing cardiomyocyte to phosphatidic acid (PA) (211), a known inotropic agent that mediates its effects via activation of PLC (51). Therefore, it is possible that such depressed responses to PA may also exist in cardiomyocytes of the failing heart due to volume overload. It is pointed that although a second smaller peak of PLC β_1

activity was seen at 8 weeks, the activity remained elevated at 16 weeks, but the extent was slightly reduced. It is possible that these elevations may be due to the fact that activation of the sympathetic and RASs are also seen in heart failure (70, 156). This response may be specific as the elevated AT_1 receptors during cardiac hypertrophy have been reported to be normalized in the failing heart (109). Interestingly, it has previously reported that inhibition of ACE by imidapril partially corrects the changes in SL PLC isozyme activity in the failing heart, indicating a pathophysiological significance of PLC isozymes (209). A similar elevation of PLC β_1 has also been seen in CHF due to MI. Although the time course of PLC β_1 activity was not determined, nonetheless the occurrence of an elevation in PLC β_1 activity at moderate heart failure seen in the volume overloaded hearts as well as in MI is suggestive of a pathophysiological role. Indeed, it has been reported that an increase in $G\alpha q$/PLC β_1 signaling may contribute to cardiac fibrosis. (108)

The diminished amount of PIP_2 in the SL membrane cannot be totally accounted for by the changes in PLC isozyme activities. Although the decreases detected at 1, 2 and 4 weeks could be attributed, in part, to the increased PLC isozyme activities and consequent increased PIP_2 hydrolysis, the diminished amount of SL PIP_2 during the heart failure stage may not be

explained on the basis of reduced PIP_2 hydrolysis by PLC activities (given that the total PLC activities ($\beta_1 + \gamma_1 + \delta_1$) were depressed), but perhaps could be explained on the basis of its reduced synthesis. In this regard, it has previously been reported that a reduced SL PIP_2 in CHF (subsequent to myocardial infarction) due to depressed phosphatidylinositol 4 and phosphatidylinositol 4-phosphate 5 kinases activities (209). Furthermore, the reduced SL PIP_2 level could also be an additional factor contributing to attenuation of the PLC dependent generation of IP_3 and DAG in the failing heart. It is interesting to note that a decrease in PIP_2 has also been seen in other cardiomyopathies and failing hearts and therefore conceivable that this may be a phenomena occurring in all cardiac pathologies. It is pointed out that independent of the effect of PIP_2 on SL PLC activities, there are a number of biochemical events that are sensitive to the membrane level of PIP_2 that could influence the contractile performance of the failing heart. For example, reduced PIP_2 can cause a depression of the inward rectifier K^+ channels (98), as well as SL Na^+-Ca^{2+} exchanger and Ca^{2+} pump activities (31, 89). Also of interest, the reduced SL PIP_2 levels in heart failure could affect the activities of other phospholipases known to modulate cardiac function and are sensitive to the membrane level of PIP_2. For example, it has recently been reported that the activity and SL binding of the type IV phospholipase A_2 as well as

phospholipase D_1 activity are markedly reduced in CHF subsequent to myocardial infarction (138, 228). It is possible that a similar situation may exist in heart failure due to volume overload.

CONCLUSIONS

1. HW: BW ratio increased in the AV shunt group during the 3 day- 16 week observation period. Also a significant progressive elevation of LVEDP, a decreased LVSP as well as a progression depression in +d*P*/dt and –d*P*/dt were detected throughout the 16 wk observational period. These results confirm the presence of cardiac hypertrophy (3 day – 4weeks) and heart failure (8 and 16 weeks).

2. Increases in IP_3 and DAG levels were seen during the hypertrophic stage (3 day-4 wk) and there was a significant depression of the levels of these two second messengers during the failing stage (8 and 16 weeks).

3. PLC β_1 and γ_1 activities were significantly increased during the hypertrophic stages. PLC γ_1 was significantly depressed at 8 and 16

weeks after the induction of the AV shunt, a second smaller peak of PLC β_1 activity was seen at 8 weeks, then depressed at 16 weeks. A progressive decline in PLC δ_1 activity was observed in the AV shunt hearts.

4. The activity of PLC γ_1 at all time points was in accordance with the level of phosphorylation of its tyrosyl residues.

5. SL PLC β_1 protein content and mRNA levels were increased up to 8 weeks following the induction of AV shunt and significantly decreased at 16 weeks. There was only an increase in PLC γ_1 protein content observed at 4 weeks and a progressive decline in PLC δ_1 protein content was observed. There was not a linear correlation between the activities, protein content and mRNA levels suggesting post-transcriptional or translational modification that would affect the enzyme activities.

6. PIP_2 content was progressively decreased over the 3 day to 16 week observational period.

7. Our findings of increased PLC β_1 and γ_1 activities during cardiac hypertrophy and decreased PLC γ_1 and δ_1 activities during heart failure suggests an important role of PLC isozymes in cardiac hypertrophy and heart failure induced by volume overload. In addition, the reduced PIP_2 level may also contribute to the depressed contractile performance of the failing heart. Therefore modulation of elements within the PLC signal transduction pathway may constitute potential therapeutic strategies for the prevention of cardiac hypertrophy and heart failure.

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