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Expression of phospholipase D isozymes in scar and viable tissue in congestive heart failure due to myocardial infarction

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Abstract

The phospholipase D (PLD) associated with the cardiac sarcolemmal (SL) membrane hydrolyses phosphatidylcholine to produce phosphatidic acid, an important phospholipid signaling molecule known to influence cardiac function. The present study was undertaken to examine PLD isozyme mRNA expression, protein contents and activities in congestive heart failure (CHF) subsequent to myocardial infarction (MI). MI was induced in rats by occlusion of the left anterior descending coronary artery. At 8 weeks after the surgical procedure, hemodynamic assessment revealed that these experimental rats were at a moderate stage of CHF. Semi-quantitative reverse transcriptase-polymerase chain reaction revealed that PLD1 and PLD2 mRNA amounts were unchanged in viable left ventricular (LV) tissue of the failing heart. Furthermore, this technique demonstrated the presence of PLD1 and PLD2 mRNA in the scar tissue. While SL PLD1 and PLD2 protein contents were elevated in the viable LV tissue of the failing heart, SL PLD1 activity was significantly decreased, whereas SL PLD2 activity was significantly increased. On the other hand, although PLD1 protein was undetectable, PLD2 protein and activity were detected in the scar tissue. Our findings suggest that differential changes in PLD isozymes may contribute to the pathophysiology of CHF and may also be involved in the processes of scar remodeling.

Keywords: congestive heart failure • myocardial infarction • cardiac sarcolemma • scar tissue • phospholipase D isozymes

Introduction

The hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) is known to produce phosphatidic acid (PA) in the cardiac sarcolemmal (SL) membrane [1]. Agents such as norepinephrine, endothelin-1 and angiotensin-II exert positive inotropic actions in the heart through the PLD pathway, and thus result in an increase formation of PA in cardiomyocytes [2,3]. The importance of PA in heart function is evident from its ability to stimulate SL Ca^{2+} - related transport systems [4] as well as its potential involvement in the development of cardiac hypertrophy [5]. Furthermore, PA

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was reported to increase the intracellular concentration of free Ca^{2+} in adult cardiomyocytes and augment cardiac contractile activity of the normal heart [6]. In addition, PA has been implicated in cytoskeletal reorganization [7].

Two mammalian PLD isozymes, PLD1 and PLD2 have been identified and cloned [8]. PLD1 has low basal activity, requires phosphatidylinositol 4,5-bisphosphate (PIP_2) for its activation and is activated by protein kinase C (PKC), adenosine ribosylation factor (ARF) and Rho small G-protein family members via direct association [9-12]. PLD2 also requires PIP_2 for its enzymatic activity [13], but unlike PLD1, it can be activated by unsaturated fatty acids such as oleate [1,14-17]. PLD2 is now recognized as the major myocardial PLD isozyme specifically localized to the SL membrane [18]. It should be noted that PLD2 is constitutively active *in vitro* and thus its activity is relatively insensitive to the PLD1-activating factors PKCδ, ARF and Rho [7].

In view of the significance of defects in Ca^{2+}-mobilizing systems for the impairment of cardiac performance in the failing heart [19], and our recent finding that SL PLD2 activity is increased in congestive heart failure (CHF) subsequent to myocardial infarction (MI) [20,21], the present study was undertaken to examine the changes in the activity of another PLD isozyme, PLD1, as well as to investigate if the changes in PLD1 and PLD2 activities are due to alterations in their protein and mRNA levels in CHF subsequent to MI. For this purpose, we employed viable left ventricular (LV) and scar tissues taken from rats 8 weeks after ligation of the left anterior descending coronary artery when the animals were at moderate stage of CHF [21-23].

Materials and methods

Experimental model

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, Canada, following the guidelines established by the Canadian Council on Animal Care. MI was induced in male Sprague-Dawley (SD) rats (weighing 175-200 g) by surgical occlusion of the left anterior descending coronary artery [21-24]. The animals were anesthetized with 5% isoflurane in oxygen at a flow rate of 2 l/min. After shaving the thoracic fur, an incision was made along the left sternal border, the fourth rib was cut proximal to the sternum and retractors were inserted. The pericardial sac was pierced such that the heart could be exteriorized through the intercostal space. The left coronary artery was ligated 2-3 mm from its origin with a suture of 6-0 silk. The heart was repositioned in the chest and the incision was closed with a purse-string suture. Throughout the operative procedure, the rats were maintained on a positive-pressure ventilation system delivering 2.5% isoflurane in oxygen. Age-matched sham-operated animals served as controls and were treated similarly, except that the suture around the coronary artery was not tied. The animals were allowed to recover and were maintained on food and water ad libitum.

Determination of left ventricular function

The LV function of the control and CHF groups was assessed according to the method described previously [21-24]. Rats were anesthetized by an injection of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). After intubation of the trachea to maintain adequate ventilation, the right carotid artery was exposed and a micromanometer-tipped catheter (2-0; Millar SPR-249) was inserted and advanced into the left ventricle. The catheter was secured with a silk ligature around the artery and, after 15 min stabilization of the heart function, LV pressures and maximum rates of pressure development ($+dP/dt_{max}$) and decay ($-dP/dt_{max}$) were recorded. Hemodynamic data were computed and displayed on a computer data acquisition workstation (Biopac Harvard Apparatus, Saint Laurent, Quebec, Canada). As in our previous studies [21-24], only animals with large transmural infarcts (> 40% of the LV free wall) were used, from which the viable LV and scar tissues were dissected for subsequent biochemical and molecular biology analyses.

Preparation of cardiac sarcolemmal membrane

The LV tissue from 4 to 5 hearts was pooled to prepare a SL membrane fraction. Briefly, the tissue was washed, and minced in 3.5 ml buffer/g tissue of a 0.6 M

sucrose, 10 mM imidazole, pH 7.0 solution. The solution was then aspirated to remove remaining blood cells and the pieces were resuspended in an equal volume of sucrose-imidazole (as above). The minced tissue was homogenized with a Polytron PT 3000 homogenizer (Kinematica AG, Switzerland) at 13,000 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 supernatant was collected. The supernatant was diluted with 300 mM KCl, 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS)/KOH, 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 $Na_4PO_4O_7$ and 0.1 M Tris-HCl, pH 8.3. After centrifugation at 100,000 g for 90 min in a Beckman swinging bucket rotor (SW-28), the 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 M sucrose, 10 mM histidine, pH 7.4 This SL enriched fraction was divided into aliquots, frozen in liquid N_2, and stored at -80°C until assayed. All the above steps were carried out at 0-4°C. Protein concentrations were determined by the Lowry method as described elsewhere [23].

Measurement of PLD isozyme activities

The guanosine triphosphate-γS (GTP-γS) and PKC-dependent PLD1 and the oleate-dependent PLD2 activities were determined as previously described [21]. The PLD isozyme activities were assayed by measuring the formation of labeled PA from 2.5 mM [^{14}C]-PC (0.167 μCi/μmol). The exogenous PC substrate (12.5 mM) was prepared by combining egg PC and tracer [^{14}C] PC. The assay was carried out at 25 ºC for 60 min in a final volume of 120 μl containing 50 mM 3,3-dimethylglutaric acid (DMGA), 10 mM EDTA (pH 6.5), 25 mM KF, SL membrane (50 μg) and either 100 μM GTP-γS or 10 μM PMA (for PLD1 activity) and sodium oleate 2.5 mM (for PLD2 activity). The reaction was terminated by the addition of 2 ml of $CHCl_3$/CH_3OH (2:1, vol/vol) and the separation of the two phases was facilitated by adding 0.5 ml of 0.1 M KCl. After centrifugation, the upper phase was discarded and the lower phase was washed to remove non-lipid contaminants. For the blanks, the same procedure was conducted except the reaction was prevented by adding SL after $CHCl_3$/CH_3OH. The final lipid extract was evaporated almost to dryness under N_2, and redissolved in $CHCl_3$ containing PA as a carrier, and was quantitatively applied to silica gel 60 A F-254 thin-layer chromatographic plates. These plates were developed in a solvent system containing $CHCl_3$/CH_3OH/acetone/acetic acid/H_2O (50:15:15:10:5, vol/vol). The lipid migration was monitored using authentic unlabeled lipid standards. The lipid spots were visualized by exposure to iodine vapors and scraped. The scrapings were extracted with cytoscint™ and counted for radioactivity in a Beckman LS 1701 liquid scintillation system. Since substantial phosphatidate phosphohydrolase (PAP) activity was present under assay conditions employed for PLD activity, it was completely inhibited by the addition of KF, a PAP inhibitor in the assay medium.

Western blot of PLD isozymes

High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 20 mg of SL proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto a 0.45-mm polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% skim milk and probed with PLD isozyme antibodies (gift from Dr. Sylvain Bourgoin) for 1h at room temperature with gentle agitation. Primary antibodies were diluted in TBS with 0.1% (vol/vol) Tween 20 (TBS-T) (1: 2000, for PLD1 and 1: 500, for PLD2). Horseradish peroxidase-labeled anti-rabbit IgG (Bio-Rad, CA, USA) was diluted (1:2000) in TBS-T and used as the secondary antibody. Subsequently PVDF membrane was incubated with this antibody for 1h at room temperature with gentle agitation. PLD isozymes 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 charge-coupled device (CCD) camera imaging densitometer (Bio-Rad GS 800).

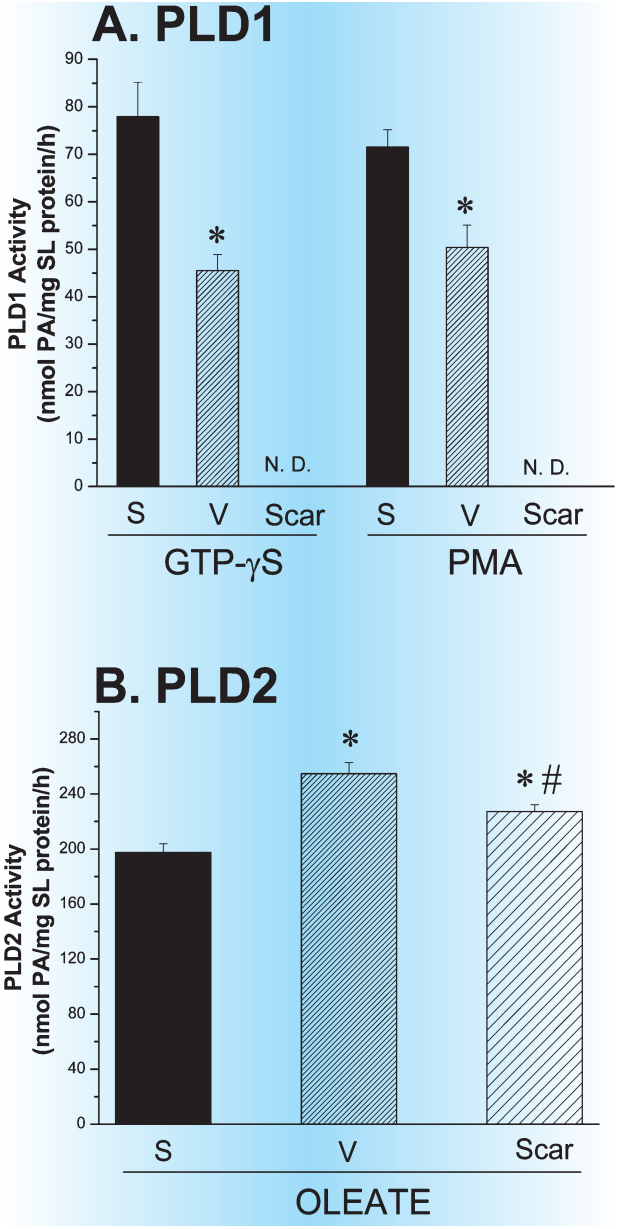

Fig. 1 Sarcolemmal PLD1 and PLD2 activities in the left ventricle and scar tissue of the failing heart. Values are the means ± S.E. of 4 different experiments. The PLD1 (A) and PLD2 (B) activities were determined in purified SL as well as in a crude scar tissue membrane preparations isolated from rats with congestive heart failure, as described in the Materials and Methods. N.D., not detectable; PA, phosphatidic acid, S, sham control; V, viable left ventricular tissue. *P<0.05 *vs.* sham control.

RNA isolation and semi-quantitative PCR

Total RNA was isolated from control, viable and scar LV tissue using a RNA Isolation Kit (Life Technologies) according to the manufacturer's procedures as described elsewhere [25]. Reverse transcription (RT) was conducted for 45 min at 48°C using the Superscript Preamplification System for First Strand cDNA Synthesis (Life Technology). Primers used for amplification were synthesized as follows: PLD1 (gift from Dr. John Exton): Primer A (Forward, 1216-1241) 5'-CTGATGCTGCTGTGAGAGAGCCCAG-3'; Primer B (Reverse, 1729-1705) 5'-GGCTCCTACGCTGCG-GTCCACGAG-3'; PLD2 (a gift from Dr. Satoshi Yamashita): Primer A (Forward, 2006-2028) 5'-TCAAGGCCAGATACAAGATACC-3'; Primer B (Reverse, 2341-2318) 5'-GAAGCACGTAGACTCG-GAAACAC-3'. Amplification of cDNAs of either PLD1 or PLD2 genes was performed using specific primers and the Superscript Preamplification System (Life Technology). Temperatures used for the polymerase chain reaction (PCR) were as follows: denaturation at 94°C for 30 s, annealing at 62°C for 60 s, and extension at 68°C for 120 s with a final extension for 7 min; 25 amplification cycles for each individual primer sets were carried out. For the purpose of normalization of the data, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward, 76-101, 5'-TGAAG-

Table 1 General characteristics of rats at moderate stage congestive heart failure.

Parameter	Control	CHF
Body weight, g	509 ± 14	483 ± 19
Viable LV weight, g	0.89 ± 0.04	1.13 ± 0.06*
Viable LV/body weight (mg/g)	1.75 ± 0.05	2.33 ± 0.12*
Lung wet weight/dry weight ratio	4.07 ± 0.36	5.47 ± 0.51*
Scar weight, g	-	0.33 ± 0.05

Values are means ± S.E. of 5 experiments with 5 different control or CHF animals. The non-infarcted LV weight of the experimental animals (8 weeks post MI) refers to the weight of LV free wall (plus septum) after removal of scar tissue. The ratio of wet to dry weight for lungs was obtained as indicated elsewhere [22]. LV, left ventricle; CHF, congestive heart failure. *P<0.05 *vs.* sham control.

GTCGGTGTCAACGGATTTGGC-3' and reverse, 806-783, 5'-GCATGTCAGATCCACAACGGATAC-3') 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) as a ratio of a target gene over GAPDH.

Statistical analysis

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

Results

Cardiac SL PLD isozyme protein abundance and activities

The activity of PLD1 and PLD2 was determined in purified SL membranes from sham, viable LV and scar tissues from failing hearts. GTP-γS as well as PMA stimulated PLD1 activity was significantly decreased in failing hearts as compared to sham controls (Figure 1A). In agreement with our earlier study [20], a significant increase in the oleate-dependent SL PLD2 activity was observed in CHF (Figure 1B). Western blotting and subsequent densitometric analysis of band intensities revealed that both SL PLD1 (120 KDa) and PLD2 (90 KDa) protein contents were elevated in the failing heart SL preparations (Figure 2). With respect to scar tissue only PLD2 protein was detectable, albeit a very weak band (data not shown) and consequently only PLD2 activity was detected in crude scar tissue membrane, which was higher than the sham control value, but lower than the activity measured in the viable tissue in post-infarction failing hearts (Figure 1B).

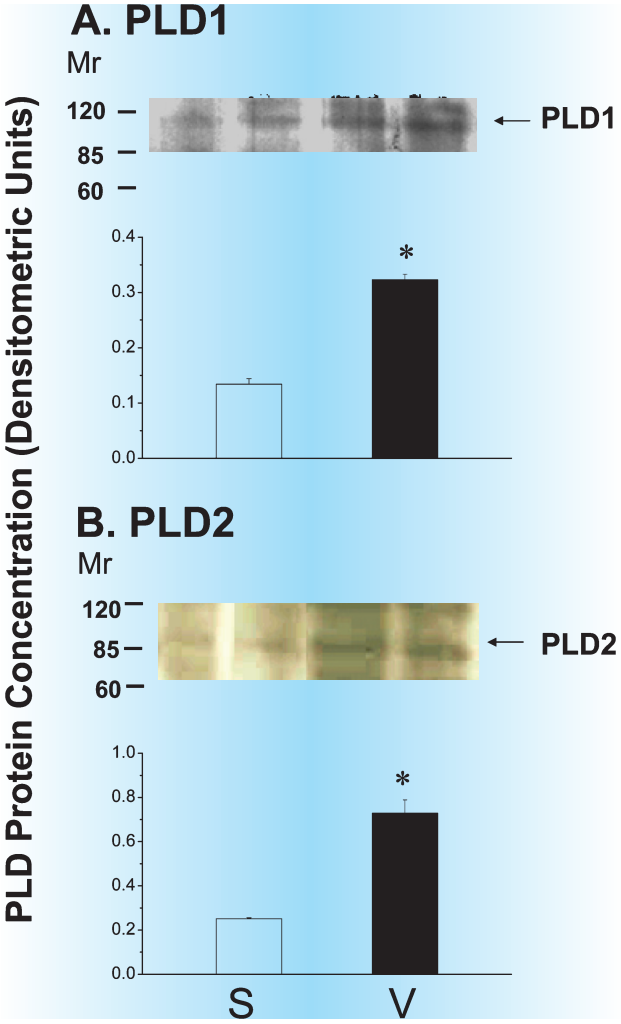

Fig. 2 Sarcolemmal PLD isozyme protein contents in left ventricle of sham control and failing hearts. A. Representative Western blots showing 120 KDa PLD1 and quantified data on PLD1 isozyme protein content. Molecular weight markers are shown on the left [relative molecular weight (Mr) expressed as X 10^3]. Data are means ± S.E. of 3 experiments. B. Representative Western blots showing 90 KDa PLD2 and quantified data on PLD2 isozyme protein content. Molecular weight markers are shown on the left [relative molecular weight (Mr) expressed as X 10^3]. Data are means ± S.E. of 3 experiments. S, sham control; V, viable left ventricular tissue. *$P<0.05$ *vs.* sham control.

Myocardial PLD1 and PLD2 mRNA expression levels

PLD1 and PLD2 mRNA expression was detected in post-MI viable LV tissue and in the infarct scar using RT-PCR. Resultant bands were quantified by

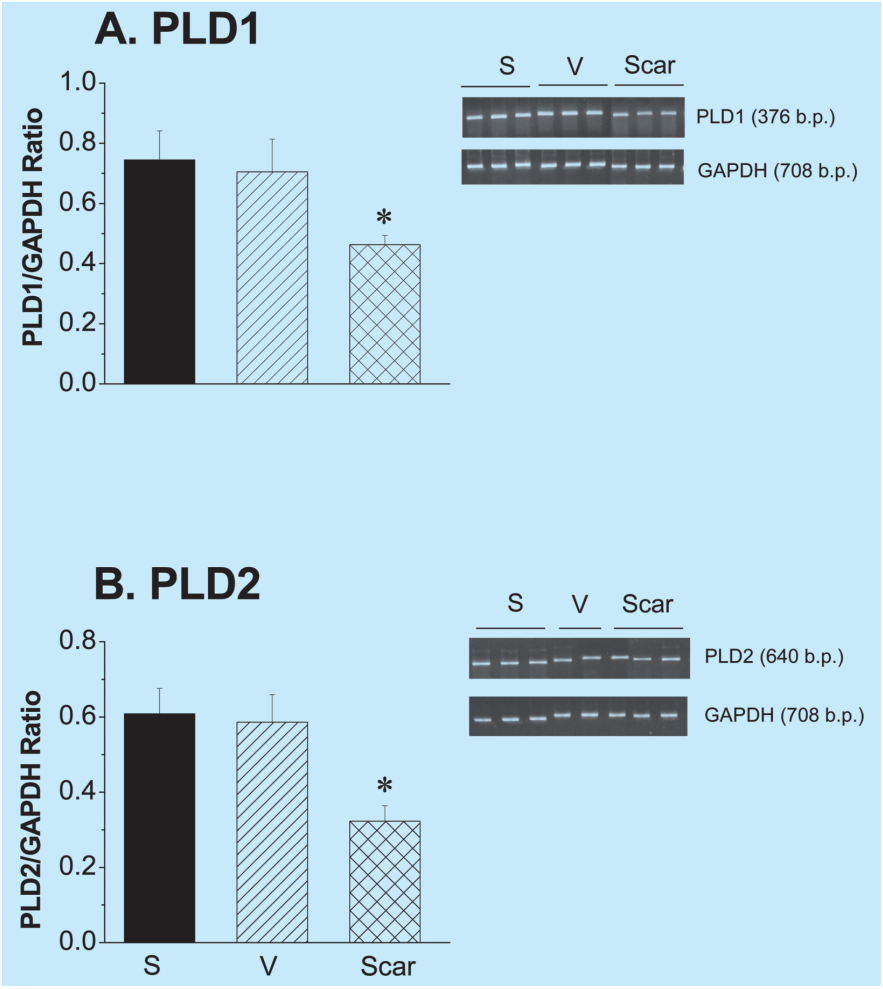

Fig. 3 PLD1 and PLD2 mRNA levels in cardiac left ventricular and scar tissues of rats at moderate stage of congestive heart failure. Representative blots showing PLD1 (A) and PLD2 (B) mRNA levels as well as the PLD isozyme /GAPDH mRNA signal ratio. The data are means ± S.E. of 3 different experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; S, sham control; V, viable left ventricular tissue. *P<0.05 *vs.* sham control.

densitometric analysis, and these data were normalized for loading differences by comparison to cardiac GAPDH. The mRNA levels of both PLD1 and PLD2 were unchanged in viable LV as compared to sham control values (Figure 3), whereas PLD1 and PLD2 mRNA levels in the scar were less than the amounts detected in sham control and viable LV tissue.

General characteristics of animals and LV function

Surgical coronary occlusion resulted in the presence of reproducible transmural infarcts in the LV free wall of experimental rats. In agreement with our earlier studies [21-24], the remnant heart muscle of the experimental animals underwent hypertrophy during the 8 weeks post-MI, indicated by an increase of the viable LV (septum and non-infarcted LV free wall remote from infarct) weight and by the augmented ratio of viable LV weight to body weight *vs.* sham control values (Table 1). An increase in the wet to dry weight ratio of the lungs revealed the presence of pulmonary edema in 8 wk post-MI animals. Increased LVEDP with concomitant loss of LV function (+ dP/dt_{max} and - dP/dt_{max}) was detected in the MI group (Figure 4). These results are consistent with earlier observations in this model indicating that at 8 weeks after the induction of MI these animals were in a moderate stage of CHF [21-24].

Discussion

Although we have previously reported an increase in the SL PLD2 activity in CHF due to MI [20,21], the present study was undertaken to examine the

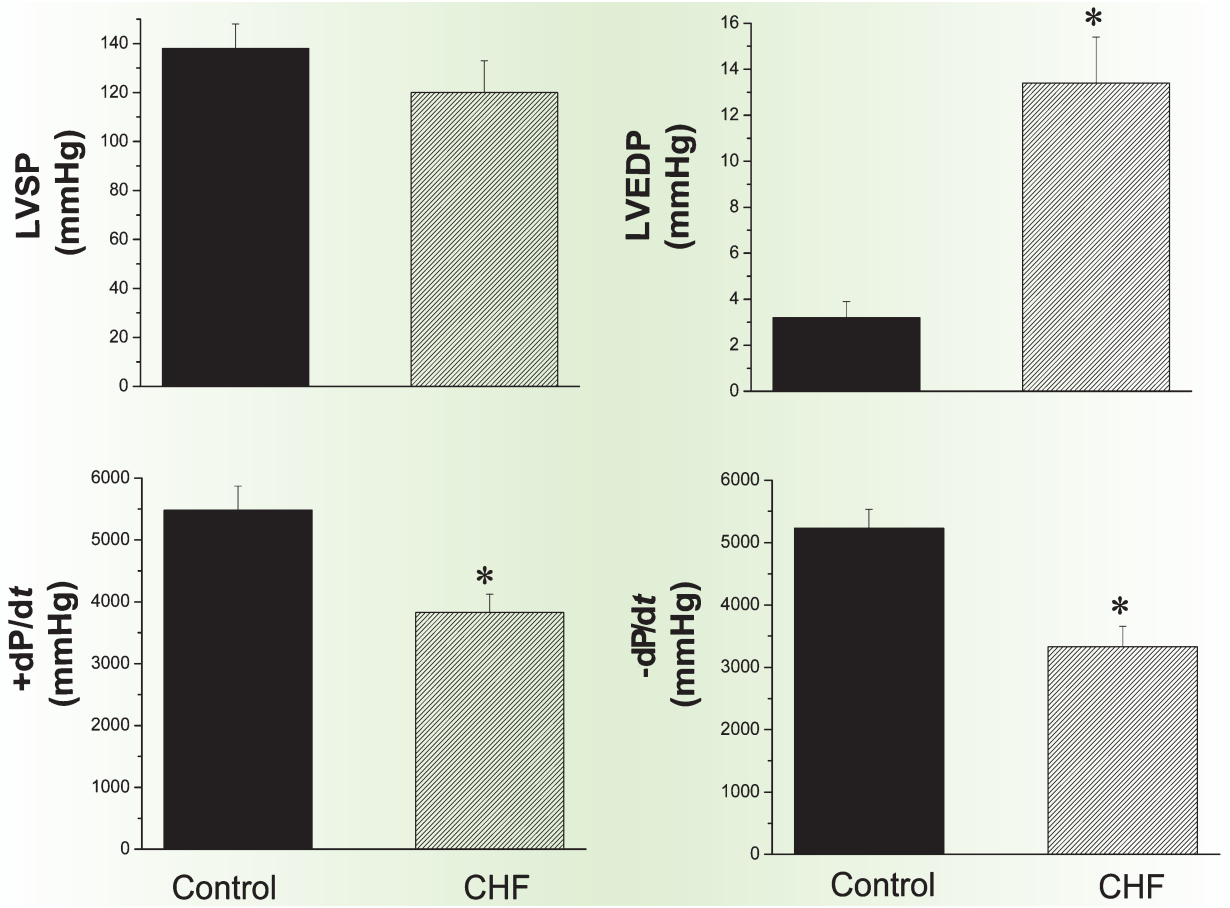

Fig. 4 Left ventricular function of rats at moderate stage of congestive heart failure. Values are means ± S.E. of 12-16 animals in each group. CHF, congestive heart failure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; $+dP/dt_{max}$, maximum rate of pressure development; $-dP/dt_{max}$, maximum rate of pressure decay. *P<0.05 *vs.* sham control.

changes in the activity of another cardiac SL PLD isozyme, PLD1, as well as addressing whether the changes in the activities of PLD1 and PLD2 isozymes in CHF are due to their altered gene and protein expression. In addition, PLD isozyme activities, protein contents and mRNA levels were also determined in the scar tissue of the failing heart. Our findings have demonstrated that while PLD1 and PLD2 mRNA levels were unchanged, their protein contents at the SL membrane were elevated. Although these changes indicate that the mechanisms involved in the transcription of the PLD1 and PLD2 genes are unaltered in CHF, the mechanisms involved in the translation process of PLD isozyme mRNA to protein were upregulated. However, a decrease in PLD1 activity was detected, whereas PLD2 activity was increased in the SL membrane of the failing heart. The occurrence of the differential changes in the PLD isozyme activities is suggestive that different activation mechanisms could play a role in modulating PLD1 and PLD2 isozyme activities [26]. In this regard, we have reported that a Ca^{2+}-independent phospholipase A_2 (PLA_2) and subsequent mobilization of the unsaturated fatty acid has been shown to modulate the activity of PLD2 in heart SL [16], it is therefore possible that the observed increase in PLD2 activity in the failing heart may be due to this mechanism. However, it has been shown that the activity of this PLA_2 isoform is downregulated in CHF [24]; and therefore the increased PLD2 activity observed in this study as well as our earlier study [21] is most likely due to its increased SL abundance. The assay system employed for the

detection of PLD1 activity included GTP and PMA (phorbol myristate acetate, a potent PKC activator). Since PLD1 is activated by GTP and PKC, the reduced SL PLD1 activity in the failing heart, despite its increased SL content, may be due to a defect in its responsiveness to these activating factors.

A role for SL PLD and its hydrolytic product, PA, in cardiac function has been indicated in a number of studies [27-30]. For example, the cardiac SL Na^+ - Ca^{2+} exchanger activity has been shown to be increased with the treatment of the membranes with exogenous PLD [28]. Such a treatment has also been shown to increase Ca^{2+}-binding and force of contraction of the heart [29]. The observation that exogenous addition of PA or PLD generates Ca^{2+}-dependent slow action potentials in depolarized rat atrium supports the role of PA in mediating the increase of Ca^{2+} influx into the cardiac cells [30]. Furthermore, PA has been shown to stimulate the L-type Ca^{2+}-channels and thus represents an important component of the PA-induced increase in adult cardiomyocytes [6]. Therefore, increased PLD2 activity and subsequent increase in the SL PA formation may contribute to the occurrence of Ca^{2+} -overload in CHF due to MI [31-33]. On the other hand, the current treatment for acute MI focuses on re-establishing blood flow (reperfusion). Paradoxically, reperfusion itself may cause additional injury to the heart. In this regard, PLD is activated during myocardial ischemia and reperfusion and has been suggested to be beneficial for the heart [34-37].

From the aforementioned, it is clear that PLD has a significant role to play in cardiac physiology under normal and pathophysiological conditions; however, the specific role of PLD isozymes is not fully established. Although an oleate-dependent PLD activity is drastically increased during apoptosis of Jurkat T cells [38], an increased PLD2 activity has been shown to reduce hypoxia-induced death of PC12 cells [39], while a hypoxia-induced activation of PLD1 in smooth muscle cells has also been identified [40]. Furthermore, PA has been reported to be protective against staurosporine-induced apoptosis in cultured neuronal cells [41]; these studies suggest that PLD isozymes play a role in cellular apoptosis. It is interesting to note that in smooth muscle cells, activation of the phosphatidylinositol-specific phospholipase C (PLC) is essential for triggering contraction, while PLA_2 is involved in the sustained tonic contraction and PLD appears to play a role in triggering relaxation [42,43]. It is tempting to speculate that these phospholipases modulate contraction and relaxation of the heart in a similar fashion, particularly when we have previously reported abnormalities in PLC and PLA_2 in CHF due to MI [23,24].

MI is characterized by early infarct expansion and is followed by discrete scar formation via the wound healing process [44,45]. While PLD1 and PLD2 mRNA levels in the scar tissue were decreased, which could be due to either reduced gene transcription or reduced mRNA stability; only PLD2 protein was detectable and consequently only PLD2 activity was measurable. The significance of the detection of PLD2 protein and activity in the scar region is presently unclear, however, it can be suggested that PLD2 might be participating in ongoing scar remodeling. Scar formation is considered complete by 3 weeks post MI [46], however, our findings suggest that the scar is not quiescent even at 8 weeks post MI, as indicated by PLD2 activity in the scar tissue. This assumption is also borne out and supported by other recent findings of augmented smad protein-mediated signaling in fibroblasts and myofibroblasts [47,48] as well as increases in the mRNA levels of Giα2 and β-myosin heavy chain in infarct scar [49]. Therefore, it is conceivable that the mechanisms that are activated during the wound healing of the infarct may not be terminated within the defined period of infarct healing and therefore it is suggested that PLD2-mediated signaling may also be involved in the ongoing remodeling of scar morphology. In this regard, it is interesting to note that inhibition of PLD activity has been demonstrated to attenuate LV fibrosis and improve cardiac function [50].

In conclusion, the differential changes in PLD isozyme activities would seem to imply specific actions of the PLD isozymes. In fact, PA derived from PLD1 and PLD2 may be of different molecular species and thus may exhibit different intracellular messenger functions [51] with regard to cardiac hypertrophy, evolution of scar remodeling, fibrosis of the viable LV tissue, apoptosis as well as contractile function such possibilities warrant further investigation. Furthermore, the findings of

the present study as well as our earlier observation that inhibition of the angiotensin-converting enzyme with imidapril attenuates both the cardiac contractile impairment and the increased PLD2 activity [21], suggest that PLD isozymes may provide a novel therapeutic target for the treatment of CHF.

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