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Upregulation of Phospholipase D Expression and Activation in Ventricular Pressure-Overload Hypertrophy

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Abstract. Evidence for a role of phospholipase D (PLD) in cellular proliferation and differentiation is accumulating. We studied PLD activity and expression in normal and hypertrophic rat and human hearts. In rat heart, abdominal aortic banding (constriction to 50% of original lumen) caused hypertrophy in the left ventricle (as shown by weight index and ANP expression) by about 15% after 30 days without histological evidence of fibrosis or signs of decompensation and in the right ventricle after 100 days. The hypertrophy was accompanied by small increases of basal PLD activity and strong potentiation of stimulated PLD activity caused by 4β-phorbol-12β,13α-dibutyrate (PDB) and by phenylephrine. The mRNA expressions of both PLD1 and PLD2 determined by semiquantitative competitive RT-PCR were markedly enhanced after aortic banding. In the caveolar fraction of the rat heart, PLD2 protein determined by Western blot analysis was upregulated in parallel with the expression of caveolin-3. A similar induction of PLD mRNA and protein expression was observed in hypertrophied human hearts of individuals (39 – 45-year-old) who had died from non-cardiac causes. In conclusion, PLD1 and PLD2 expressions were strongly enhanced both in rat and human heart hypertrophy, which may be responsible for the coincident potentiation of the PLD activation by α-adrenoceptor and protein kinase C stimulation. These results are compatible with a significant role of PLD activation in cell signaling of ventricular pressure-overload hypertrophy.

Keywords: cardiac hypertrophy, signal transduction, phospholipase D, protein kinase C, α-adrenoceptor

Introduction

The present study on rat and human hearts was aimed at the question whether phospholipase D (PLD) is involved in the development of cardiac hypertrophy. PLD hydrolyzes phosphatidylcholine (PC) to generate choline and phosphatidic acid (PA), which may be further metabolized to lyso-PA (1) and to 1,2-diacylglycerol (DAG). PA exhibits multiple activities on regulatory proteins such as small G-proteins, protein and phospholipid kinases, phosphatases (2), and Ca^{2+}-regulatory transport proteins (3, 4). PA aids in recruitment of Raf-1 to membranes, finally leading to activation of the mitogen-activated protein kinase (MAPK) pathway (5, 6). All MAPK subfamilies have been implicated in hypertrophy (7).

The PLD-mediated generation of DAG has been studied in the rat heart upon G-protein and protein kinase C (PKC) activation (8). The continuous formation of DAG appears to be derived from PC hydrolysis rather than from phosphatidylinositol 4,5-bisphosphate (PIP_2) (2). DAG originating either from PC or PIP_2 activates members of the PKC subfamilies cPKC and nPKC.

Recent review articles have pointed out the discrepancy between the rich body of facts with regard to PLD enzymology and our limited knowledge of the cellular and organs functions of PLD (2, 9, 10). Nevertheless, the hypothesis that PLD is important for the development of cardiac hypertrophy is plausible (11, 12)

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considering the broad spectrum of regulatory functions of PLD on protein targets that are linked into the signaling cascade of cardiac hypertrophy. However, to date, there is no immediate experimental evidence for a role of PLD in cardiac hypertrophy.

In the present study, cardiac hypertrophy of rat heart was established by constriction of the infrarenal abdominal aorta, which causes a chronic pressure overload of the left ventricle. Consequently a "concentric" hypertrophy with increased wall thickness is induced (13). Concentric hypertrophy secondary to aortic stenosis and hypertension is caused also by activation of G-protein-coupled receptors (GPCRs). Norepinephrine (NE) (via α-adrenoceptors), endothelin-I, and angiotensin II activate a common class of G-proteins, the G_q class (14). Although PKC is linked into the complex GPCR-signaling pathway of cardiac hypertrophy (1, 15, 16), it is still unclear whether phospho-inositide-specific phospholipase C (PLC) (17) or PLD or both are responsible for the activation of PKC. Experimental evidence for any of these concepts is still lacking.

Materials and Methods

Animals and humans

Ventricular pressure-overload hypertrophy was elicited by constriction of the infrarenal abdominal aorta of male Sprague-Dawley rats (300 – 350 g) (18). Briefly, under Equithesin (a mixture of pentobarbital, propylene glycol, $MgSO_4$, and chloral hydrate; i.p. administration) anesthesia, the aorta was banded to a residual lumen of 50% through an abdominal incision using a titanium clip (Horizon™ ligating clip). Sham-operated rats underwent the same surgical procedure but without insertion of the clip. The animals were killed between 30 and 180 days after surgery and were grouped as follows: 30 – 50 days (group 1), 50 – 100 days (group 2), and 100 – 180 days (group 3) after surgery. Left ventricle (LV) and right ventricle (RV) were isolated, and a weight index (ventricular weight × 1000 per body weight, BW) was calculated for quantification of LV and RV hypertrophy (19). Tissue samples were either incubated in Tyrode's solution (for PLD activity assay) or stored at −80°C for later analysis by RT-PCR of PLD and ANF mRNAs or Western blot analysis of PLD proteins. The extent of hypertrophy of the human hearts was quantified using Photoshop-based image analysis as previously described in detail (20). Briefly, three 20× fields of each histological hematoxylin and eosin (H&E) section of the isolated tissue sample was imported into a MacIntosh G4 computer using a standard diagnostic microscope (BX45; Olympus, Tokyo) and a digital camera (Olympus Cameda 550). The area of mean cardiomyocyte size was calculated in the two-dimensional plane. For detection of fibrosis in rat heart, three 10× sections (basal, middle, apical region of rat heart) stained with picric acid/Sirius red were imported and the number of pixels of dark red stain (=collagen) calculated as a fraction of the light eosinophilic myocardial cytoplasm.

Incubation medium and reagents

Tyrode's solution had the following composition: 149.3 mM Na^+, 2.7 mM K^+, 1.8 mM Ca^{2+}, 1.05 mM Mg^{2+}, 145.5 mM Cl^-, 11.9 mM HCO_3, 0.4 mM H_2PO_4, and 5.6 mM (+)-glucose. The medium was gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). PLD was activated by 4β-phorbol-12β,13α-dibutyrate (PDB) and phenylephrine (Sigma-Aldrich, Deisenhofen, Germany).

PLD activity assay

PLD activity was determined by a modified transphosphatidylation assay (21). Briefly, phosphatidylcholine (PC) as substrate of PLD activity was labelled with 30 μCi of ^{3}H-myristic acid (NEN, Dreieich, Germany) added to the incubation medium for 1 h. ^{3}H-Myristic acid almost selectively labels PC (22) with high reproducibility (23). ^{3}H-Phosphatidylethanol (^{3}H-PEth) was formed at the expense of ^{3}H-phosphatidic acid after exposure of the tissues to ethanol (0.5%) for 30 min with or without PDB or phenylephrine. PLD activity was expressed as formation of ^{3}H-PEth in % of ^{3}H-PC. Phospholipids including PC, phosphatidic acid, and PEth were extracted as described by Folch et al. (24) and separated by two-dimensional TLC using chloroform / methanol / 25% ammonia (65:35:5) in the first dimension and ethylacetate / isooctane / acetic acid / water (13:2:3:10) in the second dimension. The spots were visualized with iodine vapor, and spots corresponding to PC, phosphatidic acid, and PEth standards were scraped off and quantified by liquid scintillation counting.

RT-PCR analysis

In rat LV, the expression of PLD1 and PLD2 mRNA was studied using a modified semiquantitative competitive RT-PCR (25, 26). The following specific primers were designed: rat PLD1 (Gene-Bank accession number U69550) sense, 5'-GGG TGA ACA CTT CAC TGC-3' and antisense, 5'-GTG TAC AGG TTG ACA CTT CGC-3'; rat PLD2 (accession number D88672) sense, 5'-CTA TGG GGA CCT GAA CTC-3' and antisense, 5'-GAC TTT GTG TCT CTG GAG GTC-3'; rat GAPDH (accession number X02231/X00972) sense, 5'-GTG GAG TCT ACT GGC GTG TTC-3' and antisense, 5'-TGA CCT TGC CCA CAG CCT TGG-3'. Then we constructed a multicompetitor by adding these primer sequences to a spacer using chimeric primers in succes-

sive PCRs. The wild-type cDNA of PLD1, PLD2, and GAPDH would be amplified as fragments of 289-, 328-, and 376-bp length during PCR, while the amplicon sizes of competitors are 643, 685, and 727 bp, respectively. Total RNA was isolated from ventricular tissue using TRIzol (Gibco BRL, Grand Island, NY, USA). Reverse transcription of 5 μg RNA was initiated using random primers. Aliquots of serially diluted cDNA were then coamplified with a constant amount of competitor using the specific primers. PCRs were run with denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s for 35 cycles. The amplified fragments were identified by electrophoresis on a 1.5% agarose containing ethidium bromide. The intensity of the bands was measured by densitometry (Gel Print 2000i; MWG-Biotech, Ebersberg, Germany) using the Zero-DScan software. The semiquantification was performed by comparing the dilution of the templates, in which the wildtype cDNA and competitor give equal strong signals.

For the semiquantification of PLD in *human* tissues, a cycle dynamical procedure was performed as follows: equal amounts of total RNA isolated from ventricular tissue were reverse transcribed using random primers. The cDNAs were then amplified using the following specific primers: human PLD1 sense, 5'-ATA TGT GTT TTA CTC AAC AGA-3' and antisense, 5'-GTT TGG ACT TAA GAG ATA TTC A-3'; human PLD2 sense, 5'-AGC TCA TCT ACA TCC ACA GCA AGG-3' and antisense, 5'-GCA GCG GAA GAT CTG CTC ATA GAT-3'; human GAPDH sense, 5'-CCA TGG AGA AGG CTG GGG-3' and antisense, 5'-CAA AGT TGT CAT GGA TGA CC-3'; human ANP sense, 5'-CTG TCC ATG GTG CTG AAG TTT-3' and antisense, 5'-GCC TAG GGA CAG ACT GCA AG-3'. The amplicon sizes of PLD1, PLD2, GADPH, and ANP are 277, 338, 200, and 249 bp, respectively. PCRs were run in a total volume of 100 μl with denaturation at 94°C for 35 s, annealing at 54°C for 35 s, and extension at 72°C for 50 s. A 15-μl aliquot of the solution was removed after 26, 29, 32, and 35 cycles, respectively, and resolved on a 1.5% agarose gel containing ethidium bromide.

Preparation of rat heart tissue components

PLD1 and PLD2 and caveolin-3 proteins were studied using Western blot analysis. Ventricular tissue was processed for preparation of total cell lysate, sarcolemmal membrane fraction, and caveolae fraction. Tissue samples (350 – 400 mg) were homogenized at 700 U/min with 15 strokes in a buffer containing 25 mM MES (pH 6.5), 150 mM NaCl, 1% Triton X-100, and a mix of proteinase inhibitors (Sigma-Aldrich). A second homogenization was then followed using a dounce homogenisator by hand (40 strokes). The homogenate was adjusted to a volume of 2 ml containing 40% sucrose. A discontinuous gradient was formed by overlaying 4 ml of 30% and 5% sucrose each. After centrifugation at 190,000 × *g* for 18 h at 4°C in a SW41 rotor, fractions of 0.9 ml were collected beginning at the top of the gradient, and the pellet at the bottom was resuspended in MES-NaCl buffer to a total volume of 0.9 ml. The protein content of the samples had been determined by the Lowry method.

Preparation of human heart tissue components

The preparation followed in principle the method described by Park et al. (27). Human tissue was minced and homogenized in 0.6 M sucrose, 10 mM imidazole HCl, pH 7.0, and the homogenate was centrifuged at 12,000 × *g* for 30 min at 4°C. The resulting pellet (P1) was discarded, while the supernatant (S1) was diluted with 160 mM KCl, 20 mM MOPS (morpholinopropanesulfonic acid), pH 7.2 (KCl/MOPS), and centrifuged at 96,000 × *g* for 30 min. The supernatant (S2) was removed, and the pellet (P2) was resuspended in KCl/MOPS, layered on top of a 30% sucrose solution containing 0.3 M KCl, 0.1 M Tris-HCl, pH 8.3, and centrifuged at 95,000 × *g* (Beckman SW 41 rotor) for 90 min. The band at the sample-sucrose interface (sarcolemma, SL) was recovered, diluted with 5 vol KCl/MOPS, and centrifuged at 100,000 × *g* for 30 min. The SL pellet was resuspended in buffer A (50 mM HEPES, pH 7.3, 3 mM EGTA, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 80 mM KCl) and stored at −70°C until use. To generate the detergent-insoluble proteins, sarcolemmal membranes were incubated with buffer A containing 1% Triton X-100 for 30 min at 4°C. The detergent-insoluble pellets (CAV fraction) were separated by centrifugation at 100,000 × *g* for 30 min at 4°C and resuspended in buffer A without detergent.

Western blot analysis

Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked by 5% skim milk in T-TBS (Tween-Tris buffered saline: 45 mM Tris-HCl, 0.3 M NaCl, 0.1% TWEEN® 20) for 1 h. PLD was detected with a polyclonal anti-PLD primary antibody (supplied by Sung Ho Ryu and Jong Bae Park, Pohang University, South Korea; for details, see refs. 27 and 28) in a dilution of 1:1000 (milk/T-TBS), followed by incubation with horseradish peroxidase-linked goat anti-mouse IgG secondary antibody.

The bands were visualized by enhanced chemoluminescence using a commercial kit (Amersham

Pharmacia Biotech, Freiburg, Germany). To detect the caveolae marker protein (caveolin-3) in the recovered fractions of rat heart, an aliquot of 600 μl was concentrated to 50 μl using Vivaspin concentrators and transferred to a separate blot after SDS-PAGE. Caveolin-3 was detected with a specific monoclonal antibody (clone 26; BD Transduction Laboratories, Lexington, KY, USA) in a dilution of 1:5000 (milk/T-TBS).

Data analysis and ethical considerations

Results were expressed as means ± S.E.M. Differences among means, which were tested by the unpaired *t*-test, were considered statistically significant at $P<0.05$. Studies on human cardiac tissue was approved by the state ethics committee.

Results

The activity and expression of PLD was investigated in pressure-overload hypertrophy of rat LV caused by abdominal aortic banding of Sprague Dawley rats. Ventricular hypertrophy was verified by LV and RV weight (LVW and RVW) index and ANP mRNA expression. Development of fibrosis was excluded histologically by staining with picric acid/Sirius red for collagen (results not shown).

Rats were grouped depending on the time after aortic banding or sham operation as follows: after 30 – 50 days (group 1), 50 – 100 days (group 2), and 100 – 180 days (group 3). Body weight (BW) and heart weight increased with aging. Mean BW increased from 283 g (at operation) to 586 g (group 3) in sham-operated rats and from 266 g (at operation) to 600 g (group 3) in banding rats. The mean heart weight increased from 1.54 g (group 1) to 1.65 g (group 3) in sham-operated rats and from 1.59 g (group 1) to 1.74 g (group 3) in banded rats. In sham-operated as well as in banded rats, the ratios LVW/BW and RVW/BW decreased significantly ($P<0.05$) with age because BW increased faster than heart weight (Table 1). Aortic banding had no significant effect on age-dependent growth of BW and overall heart weight.

Ventricular hypertrophy

Table 1 shows that aortic banding caused significant mean increases of the LVW index (LVW/BW) in all of the three groups by 13%, 18%, and 13%, respectively, of that of the sham-operated rats (100%). The RVW index was also increased, but only after a delay of >100 days (+19%). The expression of ANP mRNA in rat LV 30 – 50 days after aortic banding was analyzed by semi-quantitative competitive RT-PCR. The level of ANP expression was rather low in sham-operated rats and was increased 26-fold (26 ± 5, $n = 11$) after aortic banding above that of sham-operated rats.

PLD activity of rat LV and RV

PLD activity was determined using the specific transphosphatidylation reaction (see Material and Methods), which, in the presence of ethanol, leads to the formation of PEth. We have reported recently that in the heart, PEth is degraded at a fast rate (29). Therefore special care was taken to make sure that changes in the cumulative formation of PEth were not due to inhibition of degradation.

Basal activity of PLD (expressed as ^{3}H-PEth in % of ^{3}H-PC) was enhanced in the hypertrophied LV at various times after banding (Fig. 1). In the RV, there was a slight, though statistically non-significant, increase after >100 days. The phorbol ester PDB was much more effective in the hypertrophied ventricle than after sham operation (Fig. 2). Banding potentiated PDB-evoked PLD activity of the LV throughout the entire period of observation (30 – 180 days). Interestingly, in the RV, a similar potentiation developed after a latency of one month, reached statistical significance after 50 – 100 days, and preceded the manifestation of the RV hypertrophy (compare Fig. 2B and Table 1).

In analogy to PDB, phenylephrine (PE, 20 μM), an α_1-adrenoceptor agonist, enhanced PLD activity of the LV

Table 1. Weight index of LV and RV after abdominal aortic banding

Days after surgery	LVW / BW (mg/g)		RVW / BW (mg/g)		n
	Sham	Banding	Sham	Banding	
30 – 50 days	2.30 ± 0.08	2.59 ± 0.09*	0.75 ± 0.03	0.72 ± 0.03	9/9
50 – 100 days	2.31 ± 0.07	2.73 ± 0.19*	0.71 ± 0.07	0.73 ± 0.09	7/4
100 – 180 days	1.89 ± 0.02	2.14 ± 0.06**	0.52 ± 0.02	0.62 ± 0.02*	16/16

Weight index: LVW (left ventricular weight) and RVW (right ventricular weight) were expressed per body weight (BW). Weight index was determined after banding or sham operation as indicated. *$P<0.05$, **$P<0.001$ *vs* sham-operated rats. Data are expressed as the mean ± S.E.M. of n experiments (sham/banding); n indicates the number of animals used.

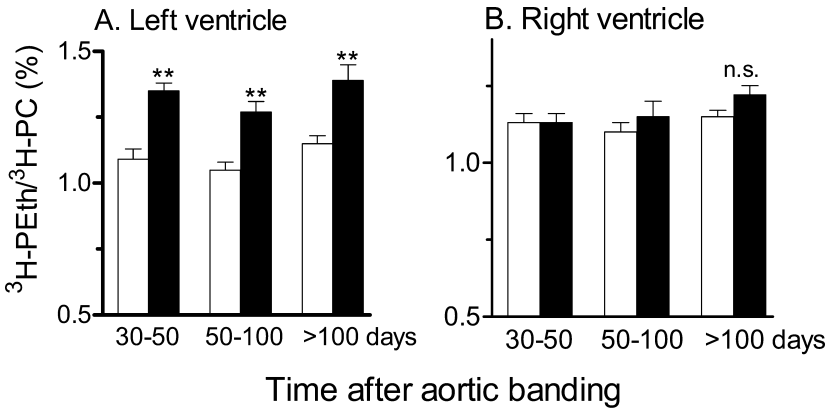

Fig. 1. The stimulatory effect of aortic banding on basal PLD activity in LV (A) and RV (B). PLD activity expressed as formation of ^{3}H-PEth in % of ^{3}H-PC was measured at various times (as indicated) after abdominal aortic banding (black columns) or sham operation (open columns). $^{**}P<0.01$; n.s., not significant ($P = 0.16$). Data are expressed as the mean ± S.E.M. of 4 – 7 or 22 – 25 (>100 days) experiments (for further details, see Table 1); for each experiment, another animal was used.

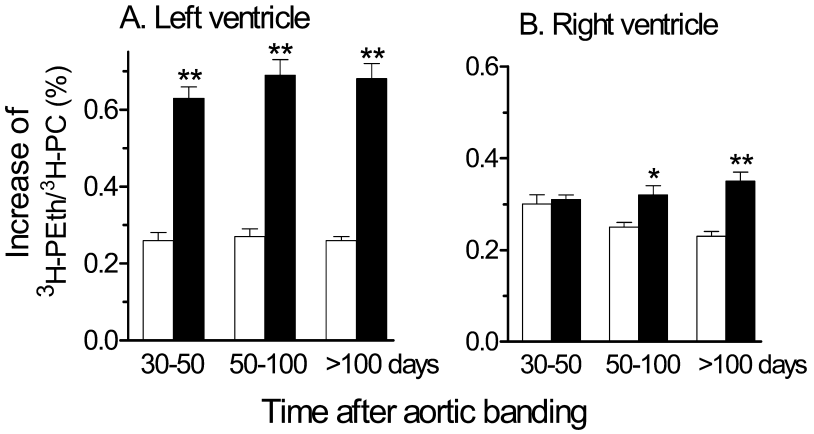

Fig. 2. The stimulatory effect of PDB on PLD activity in LV (A) and RV (B) from banded and sham-operated rats. Columns indicate absolute increases of ^{3}H-PEth in % of ^{3}H-PC. Basal values are shown in Fig. 1. The PDB-evoked increases of PLD activity were measured at various times (as indicated) after abdominal aortic banding (black columns) or sham operation (open columns). PDB (0.6 μM) was added to the incubation medium together with 0.5% ethanol for 30 min. $^{*}P<0.05$, $^{**}P<0.01$. Data are expressed as the mean ± S.E.M. of 4 – 7 or 13 – 16 (>100 days) experiments (for further details, see Fig. 1).

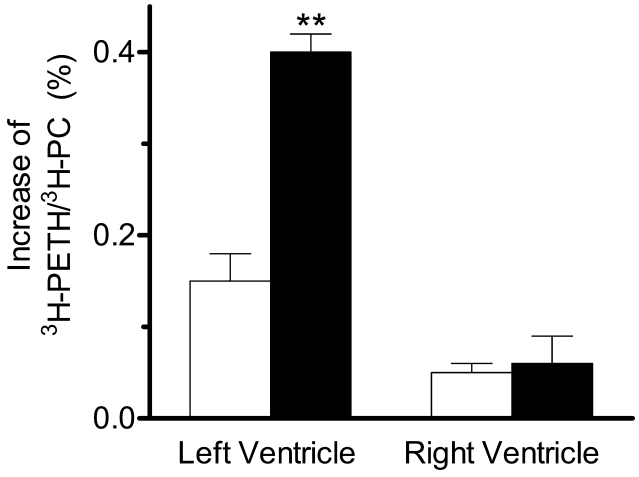

Fig. 3. The stimulatory effect of PE on PLD activity in LV and RV from banded and sham-operated rats. Columns indicate absolute increases of ^{3}H-PEth in % of ^{3}H-PC. The PE-evoked increases of PLD activity were determined in rats 30 – 50 days after aortic banding (black columns) or sham operation (open columns). PE (20 μM) was added to the incubation medium together with 0.5% ethanol for 30 min. $^{**}P<0.001$. Data are expressed as the mean ± S.E.M. of 4 – 8 experiments (for further details, see Fig. 1 and Table 1).

to a greater extent after banding than after sham operation. The very small phenylephrine-evoked activation of PLD in the RV was not changed by aortic banding 30 – 50 days after surgery (Fig. 3). This may be due to the previous observation (30) that the density of α-adrenoceptors is lower in the RV than in the LV of the rat heart.

RT-PCR analysis

The expression of PLD1 and PLD2 mRNA in the hypertrophied LV was studied using specific primers for semi-quantitative competitive RT-PCR. Figure 4 shows the predominance of PLD1 mRNA over PLD2 and also the marked upregulation of both PLD1 and PLD2 mRNA 30 – 50 days after aortic banding. The expression of the household enzyme GAPDH mRNA was not significantly altered.

Western blot analysis

The protein expression of PLD1 and PLD2 was analyzed in various fractions collected from the sucrose gradient. Due to the high protein content of cardiomyocytes, Western blot analysis of cardiac tissue was

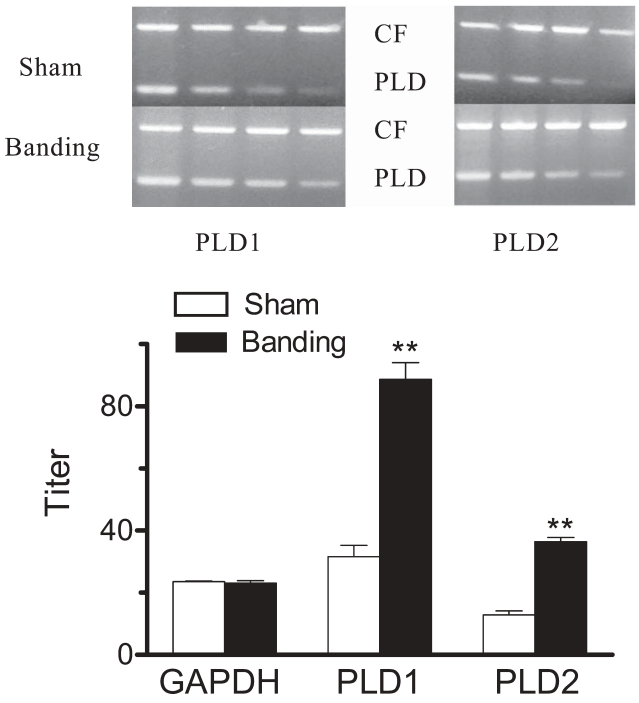

Fig. 4. PLD1 und PLD2 mRNAs in normal and hypertrophied LV of banded and sham-operated rats. Isolated total RNAs from ventricular tissue were analyzed by semiquantitative competitive RT-PCR. Upper panel, resolution of PCR products with four amplification cycles on agarose gel (CF, competitive fragment). Lower panel, densitometric evaluation. Data are expressed as the mean ± S.E.M. of 3 experiments each. ***P*<0.01.

more difficult than the analysis of the astrocytes (31), which were used as reference cells (Fig. 5). As the present study was not designed to comparatively analyze subcellular PLD fractionation, we focussed on fraction 5, the caveolin-enriched membrane microdomain (CAV) as a region with particular importance for GPCR-triggered signal transduction. Figure 5 shows both PLD isozymes in the CAV fraction of the rat heart. Aortic banding induced the protein expression of PLD1 and PLD2 in parallel with that of caveolin-3. Yet, the effect on PLD1 was statistically not significant ($P = 0.11$).

Human LV: PLD1 and PLD2 expression

mRNA and protein expressions of PLD1 and PLD2 were studied in 12 human LVs. The material was obtained from humans who died from non-cardiac causes (pulmonary embolism, head trauma) and were designated as donors for transplantation of their hearts, which could not be used, however, for this purpose because of various reasons. In addition, material was obtained from heart surgery of "hypertrophic transaortic myoectomy" and from surgical replacement of heart valves. In summary, we only used material that would have been discarded. Phenotyping "hypertrophy" (19) was based on gross observation (wall thickness, ventricular lumen), ANP mRNA expression, and morphometric verification (cardiomyocyte size). One heart was excluded because of histological evidence of marked

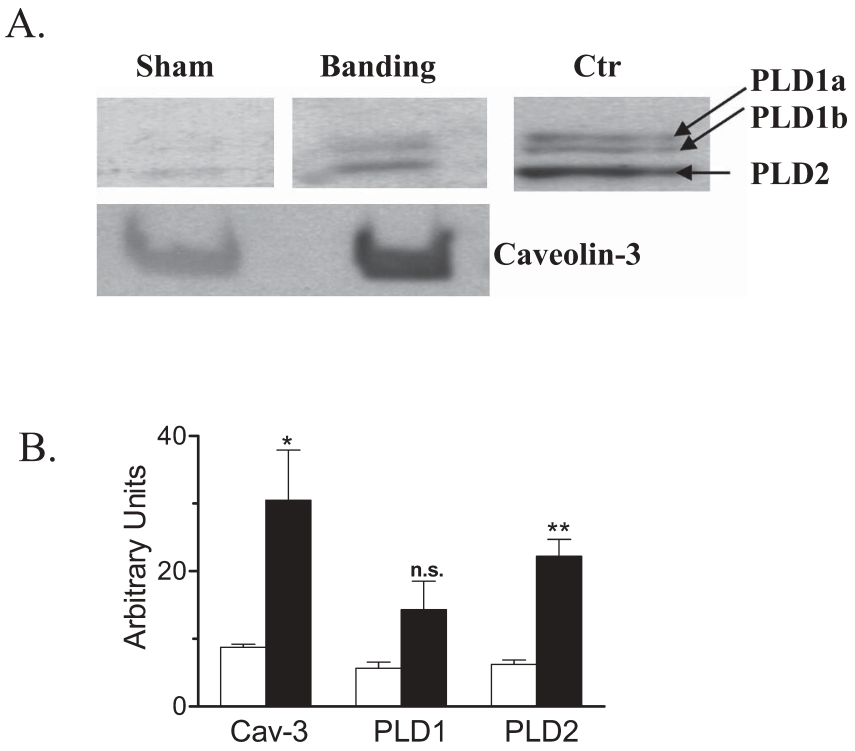

Fig. 5. Western blotting analysis of PLD1, PLD2, and caveolin-3 in fraction 5 ('detergent-insoluble, caveolin-rich membrane microdomain') of rat LV. Ventricular tissue from rats 30 – 50 days after aortic banding (black columns) or sham operation (open columns) was homogenized and subjected to SDS-PAGE. After being transferred to nitrocellulose membrane, PLD1 (120 kDa) and PLD2 (108 kDa) were detected using specific antibodies. A: Ctr, cultured astrocytes; note that the splice variants PLD1a and PLD1b were clearly identified only in astrocytes used as reference cells (31). B: Densitometric quantification of the relative band intensity presented in arbitrary units. Data are expressed as the mean ± S.E.M. of 4 experiments each. **P*<0.05, ***P*<0.01; n.s., not significantly different from sham-operated rats.

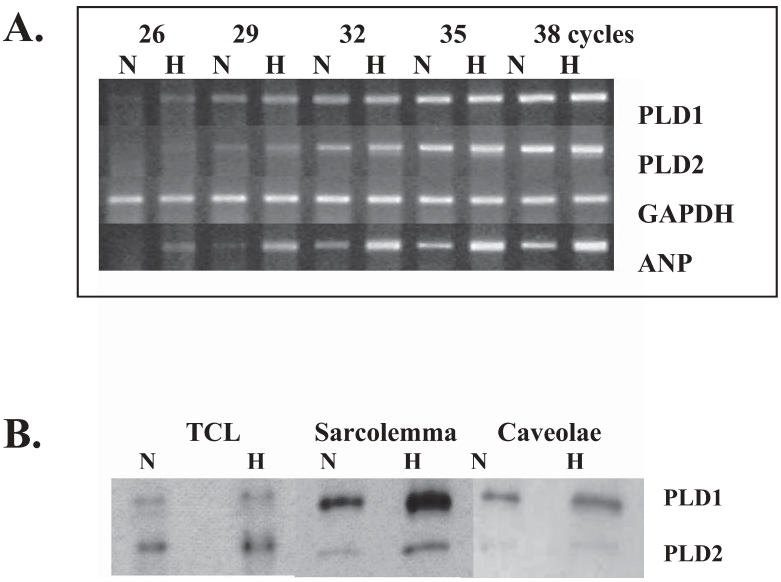

Fig. 6. Expression of PLD1 and PLD2 in a normal (N) and a hypertrophied human LV (H). A: PLD RNA analyzed by RT-PCR. PCR was performed with different amplification cycle numbers for a semi-quantative estimation. B: PLD protein analyzed by Western blotting. Equal amounts of protein from total cell lysate (TCL), sarcolemmal, and caveolar fractions were seperated on SDS-PAGE and transferred to nitrocellulose membrane. PLD1 (120 kDa) and PLD2 (108 kDa) were detected using specific antibodies. For further details and clinical data, see the text. Identical results have been obtained in a second pair (N/H) of hearts.

fibrosis. Two pairs of a normal (N) and a hypertrophied (H) heart each fulfilled the above criteria. The four selected human individuals were males, about the same age (39 – 45-year-old), and died unexpectedly from non-cardiac causes. The small number and heterogeneity of the four individuals allowed only a casuistic study of PLD expression. The results obtained from these two pairs by RT-PCR and Western blot analysis were almost identical. Figure 6 shows the results of one of these two pairs. The heart of the patient with the heart hypertrophy was initially considered for transplantation, but was rejected for transplantation because of a so far undetected LV hypertrophy and aortic stenosis. The cardiomyocyte areas were 417 μm^2 (N) and 558 μm^2 (H) (corresponding to an increase of cellular mass by about 60%), and the ANP mRNA expression was markedly elevated in the hypertrophic LV (Fig. 6A). The PLD1 and PLD2 expressions of mRNA (Fig. 6A: cycles 26 to 32) and protein (Fig. 6B) were upregulated in the hypertrophied LV.

The protein expressions of the PLD isoenzymes were studied in 3 fractions, the total cell lysate (TCL), the sarcolemmal, and the CAV fractions (Fig. 6B). PLD1 expression dominated over that of PLD2 in all fractions and exhibited a pronounced upregulation in the sarcolemmal and CAV fractions.

Discussion

In this study, we examined the changes of activity and expressions of PLD1 and PLD2 in rat and human cardiac hypertrophy. In rats, ventricular pressure-overload hypertrophy was studied between 1 month and 6 months after abdominal aortic banding. Constriction to about 50% of the original lumen resulted after 30 days in about 15% increase in LVW index without fibrosis and signs of "decompensation". The RV hypertrophied much later, namely, >100 days after banding. A similar sequential LV and delayed RV hypertrophy following aortic banding had been described before (32). In the hypertrophic LV 30 – 50 days after banding, the PLD activation caused by the α1-adrenoceptor agonist PE and by the phorbol ester PDB were markedly potentiated, whereas the basal PLD activity was only slightly increased. Potentiation of the PDB-evoked PLD activation in the RV was observed as early as 50 days after banding, whereas the hypertrophic phenotype occurred more than 100 days after banding.

Theoretically, increases of the responsiveness of PLD activity could be due to an enhanced availability of the cofactor PIP_2 (33) (Fig. 7), possibly as part of a phospholipid membrane remodeling (23). The present study provided evidence, however, that the mRNA and protein expressions of PLD1 and PLD2 in the LV were markedly upregulated 30 days after banding. This was shown by semiquantitative competitive RT-PCR and Western blot analysis. A similar result was obtained in human LV hypertrophy. Of the 12 human hearts studied (6 normal and 6 hypertrophied hearts), only 2 pairs (N/H, see Fig. 6) fulfilled the strict criteria of normal and hypertrophied hearts and had no signs of heart failure and fibrosis. Thus, our investigations allowed only a casuistic evaluation. Recently Dent et al. (34) found that also in the viable LV of the failing rat heart, the sarcolemmal PLD1 and PLD2 protein expressions were elevated; however, in contrast to the present study on the

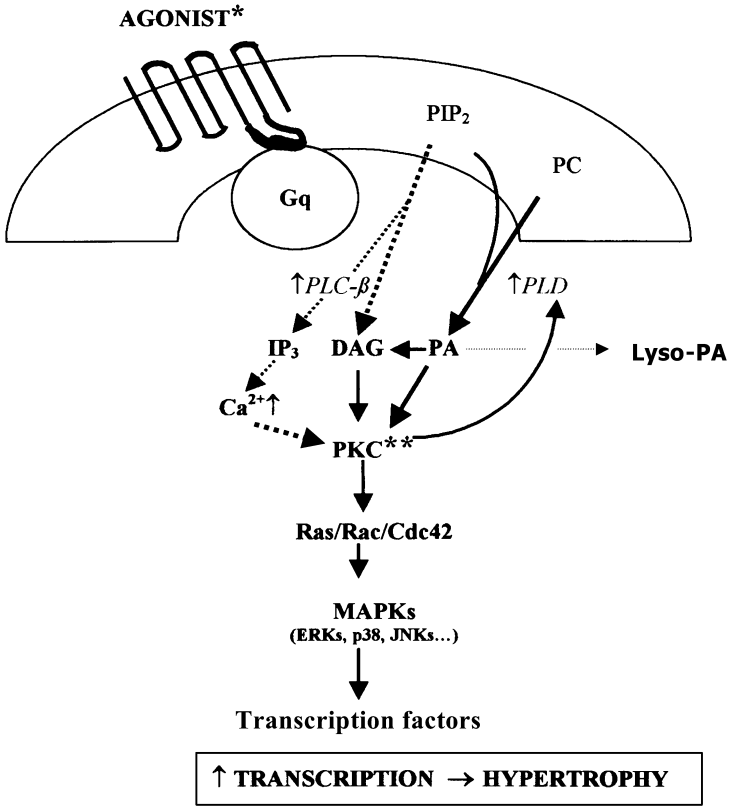

Fig. 7. Tentative scheme illustrating how PLD may be linked to the hypertrophic signaling pathway which is activated by GPCR-agonists and aortic banding. GPCR agonists such as NE and PE stimulate PLC and PLD (39) which produce lipid messengers (DAG, PA, and lyso-PA) and lead to activation of cPKC and nPKC, small G-proteins, and MAPK subfamilies (7): ERKs (extracellular responsive kinases), JNKs (cJun N-terminal kinases), and p38-MAPKs. PIP_2 is a substrate of PI-PLC and serves also as an allosteric regulator of the PLD activity (10, 33). Continuous PLD activation is regulated by a PKC-driven positive feedback loop. Finally, a specific hypertrophic program of MAPK-mediated transcription activation leads to the characteristic morphological pattern of concentric cellular hypertrophy. In the present study, PE and PDB were used to stimulate GPCR* and PKC**, respectively.

hypertrophied non-failing LV, PLD activation by a phorbol ester was decreased rather than potentiated.

Our observations on rat and human hearts corroborate the previously published hypothesis that PLD and its product PA might be linked to the hypertrophic signaling cascade (11, 12). This hypothesis was deduced from the broad spectrum of regulatory functions of PLD on protein targets in general and from the large variety of membrane receptor agonists stimulating PLD activity (2, 9, 10). Activation of PLD generates choline and PA from PC. PA exhibits multiple activities on regulatory proteins including small G-proteins, protein kinases (including PKCζ) and phospholipid kinases, phosphatases (2), and Ca^{2+}-regulatory transport proteins (3, 4). Xu et al. (35) observed that PA stimulated protein synthesis in cardiomyocytes.

In general, some of the effects of PA could be attributed to lyso-PA (36) and DAG generated from PA by phospholipase A_2 and PA phosphohydrolase, respectively. DAG activates members of the PKC subfamilies cPKC and nPKC, which are important elements of the hypertrophic signaling cascade activated by the GPCR agonists NE, angiotensin II, and endothelin-1 (1, 16). Also, direct stimulation of PKC by phorbol esters mimicked the hypertrophic actions of NE (37). Aortic banding caused an upregulation of certain Ca^{2+}-dependent and Ca^{2+}-independent PKC isozymes in the LV and, after a delay, also in the RV (32). A similar delay of the RV response was observed in the present study with regard to the hypertrophic phenotype and also to the potentiation of the PDB-evoked PLD activation.

Adrenoceptors

We studied the α_1-adrenoceptor-activated PLD (present study) and, very recently, PLC (38) and found that both enzyme activations were upregulated in the hypertrophic LV. PE was selected as agonist because α_1-adrenoceptors play an important role in physiological and pathological cardiac hypertrophy (40, 41). We did not use NE because β-adrenoceptor activation may affect PC labeling with ^{3}H-myristic acid (23). Chronic exposure to NE induced hypertrophic growth and gene transcription via α_{1A}-adrenoceptor stimulation which could be a mechanism for sustained growth (42). α_1-Adrenoceptors activate three subfamilies of MAPKs (ERK, p38-MAPK, JNKs; Fig. 7) through the nPKC isoforms PKCδ and PKCε (43) and small G-proteins such as Ras (7, 44, 45).

PLD and the GPCR signaling pathway

There are several hypertrophic signaling pathways depending on the extracellular signal acting. Figure 7

shows how PLD may be linked to the GPCR-mediated hypertrophic signaling pathway. The pathway upstream of PLD was not analyzed in the present study. Previously we showed that immediate G-protein-mediated PLD activation in the rat heart was Ca^{2+}- and PKC-independent, but was drastically potentiated by simultaneous activation of PKC (8). This is easily explained by a positive feedback regulation of PLD as shown in Fig. 7. Permanent activation of PKC was found to induce the expression of PLD1b (28). In NIH 3T3 cells overexpressing PLD1, the GPCR-triggered activation of ERK was dependent on PKC and PLD1 generating PA (not DAG) (46). In general, the ERKs are strongly integrated in signal pathways triggered by those GPCR agonists that stimulate PKC and transcription of genes involved in cell differentiation, proliferation, growth, and hypertrophy (7, 16, 47).

PLD and hypertrophy

We could show that the α-adrenergic and PKC-evoked PLD activation and the expressions of PLD1 and PLD2 were upregulated in pressure-overload hypertrophy of rats caused by abdominal aortic banding. It has been shown previously that aortic banding lead to induction of the α_{1A}-adrenoceptor as did chronic NE exposure (42). This observation corroborated the recent finding that endogenous NE and epinephrine play an important role in the cardiac hypertrophy produced by aortic banding (41). In the present study, we found in the isolated rat heart tissue that aortic banding potentiated the PLD activation evoked by α1-adrenoceptor (by PE) and PKC (by PDB) stimulation. It seems likely that the effect occurred in cardiomyocytes because α_1-adrenoceptors are absent in cardiac fibroblasts (48).

PLD isozymes and location

Two mammalian PLD isozymes have been cloned, PLD1 and PLD2 (2, 9, 10). The expression of both PLD1 and PLD2 was enhanced in the hypertrophied rat LV after aortic banding (present study) and in the failing rat LV after occlusion of a coronary artery (34). In the hypertrophic LV, the upregulation of PLD1 may be, at least in part, responsible for the potentiated activation of PLD caused by α-adrenoceptor and by PKC stimulation because PLD2 is preferentially activated by receptor tyrosine kinase stimulation and is less responsive to PKC activation (9). However, selective upregulation of PLD2 in caveolar membranes of human cancer cells was accompanied by a potentiation of the phorbol ester-evoked PLD activity (49).

The subcellular location of the PLD isozymes may help to understand their functional roles. In general, PLD1 appears to be preferentially localized in intracellular membranes, whereas PLD2 is confined to the plasma membrane (10). Yet, this association is not exclusive. Thus, in the rat heart, PLD2 was identified in caveolae, that is, detergent-insoluble membrane microdomains (ref. 27 and the present study), but also PLD1 was present at these sites (refs. 50, 51 and the present study). These lipid rafts function as platforms for attachment of proteins and facilitate membrane trafficking and G-protein-coupled signaling; they concentrate many proteins that are linked to intracellular signal transduction including PLD (10, 51, 52). Caveolar PLD of non-cardiac cells was activated by stimulation of membrane receptors, G-proteins, and PKC (50, 51, 53, 54). In line with these previous data, we found in the CAV fraction of the hypertrophic rat hearts that caveolin-3 and both PLD1 and PLD2 proteins were upregulated.

In conclusion, ventricular pressure-overload hypertrophy is associated with activation of the GPCR-triggered signaling pathway. Elements of this pathway including α_{1A}-adrenoceptors and certain PKC isozymes have been shown to be upregulated. The present study adds evidence for an induction of PLD1 and PLD2 expression coincident with a potentiation of the PLD activation caused by α_1-adrenoceptor and by PKC stimulation. Direct evidence for a key-role of PLD is difficult to assess because specific PLD blockers suitable for the present experimental design are not available yet. Nevertheless, the present results corroborate the hypothesis of a role of PLD in the development or maintenance of cardiac hypertrophy (11, 12).

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