Full Paper

Effects of Norepinephrine and Cardiotrophin-1 on Phospholipase D Activity and Incorporation of Myristic Acid Into Phosphatidycholine in Rat Heart

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Abstract. The present study is part of a project on phospholipase D (PLD) in cardiac hypertrophy and analyzed effects on PLD activity of two growth stimuli, norepinephrine (NE) and cardiotrophin-1 (CT-1), in incubated rat heart. Phosphatidycholine (PC) was labeled by \( ^3\)H-myristic acid. PLD produced \( ^3\)H-phosphatidylethanol (\( ^3\)H-PEth) from \( ^3\)H-PC in the presence of ethanol and maintained a basal formation of \( ^3\)H-PEth. Short-term and long-term exposure to NE for 2 or 13 h, respectively, enhanced the formation of \( ^3\)H-PEth, which was blocked by prazosin. Long-term pretreatment with NE or CT-1 increased the incorporation of \( ^3\)H-myristic acid into PC, which was blocked by atenolol. When the \( ^3\)H-PEth formation was expressed as a fraction of \( ^3\)H-PC, PLD activity seemingly was unchanged (NE) or markedly reduced (CT-1); the true effects, namely, stimulation by NE and nonresponsiveness towards CT-1, were unraveled by atenolol (NE) or when PLD activity was expressed as \( ^3\)H-PEth per ng protein. In conclusion, \( \alpha\)-adrenoceptor activation increased PLD activity. Long-term treatment with NE (via \( \alpha\)-receptors) or CT-1 enhanced the \( ^3\)H-myristic acid incorporation into a PC compartment, that was not available for the \( \alpha\)-receptor-mediated PLD activation. These results were discussed in regard to cellular mechanisms of cardiac hypertrophy and to the transphosphatidylation assay of PLD.

Keywords: phosphatidycholine, phospholipase D, transphosphatidylation assay, adrenoceptor, heart

Introduction

Phospholipase D (PLD) hydrolyzes phosphatidycholine (PC) to choline and the second messengers phosphatidic acid and subsequently diacylglycerol (1–3). Experimental evidence for almost two decades indicates that PLD is an important “receptor-activated” signalling enzyme that controls cell growth and proliferation, cytoskeletal organization, membrane traffic, and exocytosis (1, 3). Park et al. (4) provided evidence for a major role of cardiac PLD in the reorganization of the actin cytoskeleton.

The present study is part of a current research project on the role of PLD in heart hypertrophy and analyzes effects of norepinephrine (NE) and cardiotrophin (CT)-1, which produce cardiac hypertrophy under long-term conditions (reviewed by 5 and 6). NE is an effective growth promotor via the \( \alpha\)-adrenoceptor-G\(_4\) pathway (7, 8) and via \( \beta\)-adrenoceptors (9, 10). The receptor of CT-1 belongs to the cytokine class-specific gp130/LIF (leukemia inhibitory factor) receptor, which activates the ras mitogen-activated protein (MAP) kinase pathway (11). Direct effects on PC metabolism have been described for NE, but not for CT-1. \( \alpha\)-Adrenoceptor stimulation activates PLD and phosphoinositide-specific phospholipase C in heart and other organs (see Discussion) and \( \beta\)-adrenoceptor stimulation mediates activation of phospholipases A\(_1\) and A\(_2\) (PLA\(_1\) and PLA\(_2\)) in the perfused heart (12, 13) and in cardiomyocytes (14).
PLA activation is part of the deacylation-reacylation cycle (15) and thereby is involved in the remodeling of membrane phospholipids, for example, during hypertrophic cell growth.

Materials and Methods

Experiments were carried out on small pieces of ventricular tissue of adult and 1-week-old Sprague Dawley rats. The tissue pieces were incubated at 37°C in a 5-ml bath of Tyrode solution that had the following composition: 149.3 mM Na+, 2.7 mM K+, 1.8 mM Ca2+, 1.05 mM Mg2+, 145.5 mM Cl−, 11.9 mM HCO3−, 0.4 mM H2PO4−, 11.2 mM (+)-glucose, 0.1 mM ascorbic acid. The medium was gassed with a mixture of 5% CO2 and 95% O2 (pH 7.4).

PC as substrate of PLD activity was labelled with 30 μCi of 3H-myristic acid (NEN, Dreieich, Germany) added to the incubation medium for 1 h (short-term experiments) or 2 h (long-term experiments). 3H-Myristic acid almost selectively labels PC (16, 17). 3H-Phosphatidylethanol (3H-PEth) was formed at the expense of 3H-phosphatidic acid after exposure to ethanol (0.5%). In the long-term experiments, ethanol was added for 30 min after exposure to NE or CT-1 for 13 h and 1H-labeling for 2 h. In the short-term experiments, ethanol was added together with NE or CT-1 for 2 h after exposure to 3H-myristic acid for 1 h. The PLD-mediated formation of PEth by the transphosphatidylation reaction (18) was expressed as 3H-PEth per ng protein or as fraction of 3H-PC. PLD activity was determined under basal conditions and after exposure to NE bitatrate (Sigma Chemie, Deisenhofen, Germany) or CT-1 (Chemicon Int., Inc., Temecula, CA, USA). The phospholipids (PC, phosphatidic acid, PEth) were extracted as described by Folch et al. (19) 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. Protein was determined according to Lowry. Differences of 3H-PEth and 3H-PC between two experimental groups were analyzed by the paired or unpaired Student’s t-test.

Results

The present study is focussed on the short-term and long-term effects of NE and of CT-1 on PLD activity. PLD activity was measured in incubated ventricular pieces using the transphosphatidylation assay (18). This assay takes advantage of the fact that PLD specifically forms PEth from PC in the presence of ethanol (0.5%). 3H-Myristic acid labels the substrate PC with high selectivity (17).

Effects of NE

We observed that 5 μM NE doubled the incorporation of 3H-myristic acid into PC (3H-PC per ng protein) in heart tissue from adult rats and 1-week-old rats under long-term conditions (pretreatment with NE for 13 h, Fig. 1), but not under short-term conditions (exposure to NE for 1 h; 88.7 ± 11.2% of control, N = 8). The effect was blocked by the β-receptor antagonist atenolol (20 μM, Fig. 1), but not by the α-receptor antagonist prazosin (20 μM) (data not shown); atenolol alone had no effect. The increase caused by NE in atria and ventricles of adult rats was 68 ± 16% (N = 8) and 100 ± 23% (N = 21), respectively.

Figure 2 shows the α-receptor-mediated activation of PLD by 5 μM NE in short-term and long-term experiments. In short-term experiments, ventricular tissue was exposed to 3H-myristic acid for 1 h and thereafter to 5 μM NE plus 0.5% ethanol (see Materials and Methods). NE (Fig. 2) or phenylephrine (+12 ± 2% of control, N = 8) enhanced the basal formation of 3H-PEth expressed as fraction of 3H-PC even in the absence of atenolol.

In long-term experiments, heart tissue was exposed to 5 μM NE for 13 h and to 3H-myristic acid for 2 h.
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followed by washout and addition of 0.5% ethanol for 30 min. Long-term pretreatment with NE (5 μM) had the same effect on PLD activity as NE in the short-term experiments. As expected, the effect was blocked by 20 μM prazosin (Fig. 2). When the formation of 3H-PEth was expressed as fraction of 3H-PC, the α-adrenergic effect on PLD activity could be shown in the presence of the β-blocker atenolol (20 μM).

These experiments uncovered a methodological phenomenon in regard to the transphosphatidylation assay of PLD activity: In the absence of atenolol, NE seemingly had no effect on PLD activity (98.2 ± 6.9% of control, N = 27) because the β-receptor-mediated 3H-labeling of PC apparently neutralized the α-receptor-mediated 3H-PEth formation. It is concluded that the 3H-PC formed from 3H-myristic acid in response to β-receptor stimulation was not available for the α-adrenergic PLD-mediated hydrolysis of PC.

Effects of CT-1

These experiments were carried out like the above long-term experiments with NE. It was found that pretreatment with 3 nM CT-1, similar to NE, doubled the incorporation of 3H-myristic acid into PC (Fig. 3a). CT-1 did not affect the formation of 3H-PEth when expressed per ng protein, which indicates a lack of an effect on PLD activity (Fig. 3b). The results demonstrate again the above described methodological trap of the PLD activity assay. When the formation of 3H-PEth was expressed as a fraction of 3H-PC, PLD activity seemingly was reduced to half (Fig. 3c), because the 3H-PC compartment formed under the influence of CT-1 was not available for the basal PLD activity.

Discussion

Effects of NE and CT-1 on PC metabolism

The present study analyzes long-term effects on PLD activity and on PC metabolism caused by the growth-
promoting signals NE and CT-1. Prolonged activation of PLD has been linked to cardiac hypertrophy potentially through the formation of phosphatidic acid (20) and subsequently of diacylglycerol (4, 6).

The growth-promoting effects of NE are mediated by \( \alpha_{1r} \) as well as \( \beta_{1} \)-adrenoceptors (10). \( \alpha_{1r} \)-Adrenoceptors like other Gq-coupled receptors play a major role in cardiac hypertrophy (6, 9), which involves activation of protein kinase C and the ras MAP kinase pathway (7, 8, 21, 22). The \( \beta_{1} \)-adrenoceptor-mediated hypertrophy is due either to the enhanced higher basal cardiac work load (via activation of the cAMP pathway) or to activation of the MAP kinase pathway (9, 21). The receptor of CT-1 belongs to the cytokine class-specific gp130/LIF receptor (11). Following CT-1 receptor stimulation, intracellular signaling procedes through several cascades, most notably the pathway of Janus kinase (Jakt), signal transducers and activators of transcription (Stat) and finally the ras MAP kinase pathway. For a general overview of the cellular mechanisms of cardiac hypertrophy, see recent review articles (5, 6, 10).

**Incorporation of \( ^{3}H \)-myristic acid into PC**

The present study provides evidence for the first time that NE (via \( \beta_{1} \)-adrenoceptors) and CT-1 enhanced the incorporation of a fatty acid (\( ^{3}H \)-myristic acid) into PC under long-term conditions (pretreatment for 13 h), but not in the short-term experiments (see also Ref. 16). The mechanism of incorporation is unclear, but is probably mediated by the acyl-CoA: lysophosphatidylcholine acyltransferase, which is part of the deacylation-reacylation cycle of phospholipids (15, 23). There is no evidence for a receptor-mediated regulation of the acyltransferases. We assume that the enhanced incorporation of \( ^{3}H \)-myristic acid into PC is caused indirectly by activation of a PLA\(_{1}\)/PLA\(_{2}\) and enhanced formation of lyso-PC as substrate of the acyltransferases (see the schematic diagram in Fig. 4). It was, indeed, previously shown that the activities of PLA\(_{1}\)/PLA\(_{2}\) were markedly enhanced in the perfused heart by \( \beta \)-receptor stimulation (12) and in cardiomyocytes (14). Earlier findings by our group had indicated a \( \beta \)-receptor-mediated PLA\(_{1}\)/PLA\(_{2}\) activation in the perfused heart, as isoprenaline enhanced the efflux of choline in a mepacrine- and Ca\(^{2+}\)-sensitive manner (13). PLA may be stimulated directly or indirectly by activation of MAP kinases as described for the cytosolic PLA\(_{2}\) (24, 25). The MAP-kinase – PLA\(_{2}\) connexion may be responsible also for the observed effect of CT-1 on the incorporation of \( ^{3}H \)-myristic acid into PC (see above).

Whatever mechanism is responsible for the enhanced acylation of lyso-PC and biosynthesis of PC caused by the growth stimuli NE and CT-1, the effects may reinforce phospholipid membrane remodeling in cardiac hypertrophy.

**Activation of PLD**

The present study confirmed previous reports on an \( \alpha_{1r} \)-adrenoceptor-mediated hydrolysis of PC through activation of PLD. The first evidence for such an effect was obtained in brain cortical slices in 1992 (26). The observation was then confirmed for various cell types and organs: isolated astrocytes (27), rat fibroblasts (28), rat tail artery (16), rat aorta (29), ventricular myocytes (30, 31), and isolated perfused rat heart (32). Activation of PLD produces, besides choline, the second messengers phosphatidic acid and subsequently diacylglycerol (1 – 3), both known to activate certain protein kinase C-subtypes. Protein kinase C seems to play a central role in \( \alpha \)-receptor-mediated growth of cardiomyocytes (6, 8, 21). Additional evidence led to the conclusion that phosphatidic acid (formed by PLD) may be a signal transducer for cardiac hypertrophy (20).

A schematic diagram of the \( \alpha_{1r} \) and \( \beta_{1} \)-adrenoceptor-mediated effects on PC metabolism is shown in Fig. 4. Both effects may be significant in the cellular mechanisms of NE-evoked cardiac hypertrophy.

**Methodological aspects and PC compartmentation**

Morris et al. (33) speculated that PLD activities are localized to specific membrane compartments where PC compartments may be restricted. The effects of NE and CT-1 on PC metabolism and on PLD activity observed in the present study provide evidence in favor of such compartmentation. The two compartments were defined by the above \( \beta \)- and \( \alpha \)-receptor-mediated effects on PC
metabolism. Although it is likely that these compartments are localized both within the myocardial cell, their presence in different cell types cannot be excluded. In any case, the phenomenon of compartmentation may lead to false conclusions about changes of PLD activity, especially when the formation of H-PC, a commonly used parameter of PLD activity, remained unchanged.

The true effects of NE and CT-1 on PLD activity were unraveled in the presence of atenolol (NE experiments) or when PLD activity was expressed as fraction of H-PC, a commonly used parameter of PLD activity. Sarri et al. (34) were the first to draw attention to this important methodological aspect. This is exemplified by the present study: the β-receptor-mediated formation of H-PC from H-myristic acid apparently neutralized the α-receptor-mediated H-PEth formation because the H-PC formed from H-myristic acid in response to β-receptor stimulation was not available for the α-adrenergic PLD-mediated hydrolysis of PC.

A similar compartmentation was observed in the experiments with CT-1, which like NE-enhanced the labeling of PC, but in contrast to NE, had no effect on the H-PEth formation. Thus the quotient H-PEth per H-PC was markedly reduced, although PLD activity remained unchanged.

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