Biochemical Pharmacology, Vol. 56, pp. 799–805, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00636-9

Phospholipase D in Rat Myocardium: Formation of Lipid Messengers and Synergistic Activation by G-Protein and Protein Kinase C

*Ruth Lindmar and Konrad Löffelholz**
DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF MAINZ, MAINZ, GERMANY

ABSTRACT. Activation of phospholipase D (PLD) and phosphoinositide-specific phospholipase C (PI-PLC) by fluoride, to stimulate heterotrimeric G-proteins, and by phorbol esters, to stimulate protein kinase C (PKC), was studied in rat atria. Fluoride and 4β-phorbol-12β,13α-dibutyrate (PDB), in contrast to 4β-phorbol-13α-acetate (PAc), activated PLD, catalyzing the formation of [^{3}H]-phosphatidylethanol ([^{3}H]-PETH), [^{3}H]-phosphatidic acid ([^{3}H]-PA), choline and *sn*-1,2-diacylglycerol (DAG). Basal PLD activity was resistant to drastic changes in Ca^{2+} and to Ro 31-8220, a PKC inhibitor, but was decreased by genistein, an inhibitor of tyrosine kinase, and increased by vanadate, a tyrosine phosphatase inhibitor; both effects were, however, very small. Fluoride-evoked PLD activity was resistant to Ro 31-8220 and to genistein, but was Ca^{2+}-dependent. The rate of fluoride-induced PLD activation was maintained for at least 60 min. In contrast, PDB-mediated PLD activity was blocked by Ro 31-8220 and was resistant to extracellular Ca^{2+}-depletion and desensitized within *ca.* 15 min. PDB markedly potentiated the fluoride-evoked generation of [^{3}H]-phosphatidylethanol and of choline, but inhibited the formation of [^{3}H]-inositol phosphates ([^{3}H]-IP_{1-3}). Ethanol (2%) blocked the PDB-evoked generation of both [^{3}H]-phosphatidic acid and of *sn*-1,2-diacylglycerol, whereas fluoride-evoked responses were reduced only to approximately 50%. In conclusion, the trimeric G-protein–PLD pathway in heart tissue did not enclose PKC activation and was long-lasting and Ca^{2+}-dependent; there was no evidence for an involvement of tyrosine phosphorylation. However, PKC activation modulated G-protein-coupled PLD and PI-PLC activities in opposite directions. PLD activity significantly contributed to the mass production of *sn*-1,2-diacylglycerol in the heart. The evidence for a pathophysiological role of PLD activation in cardiac hypertrophy and in ischemic preconditioning is discussed. BIOCHEM PHARMACOL **56**;7:799–805, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. heart; phospholipase D; phospholipase C; protein kinase C; diacylglycerol

The breakdown of phosphatidylcholine through phospholipases D, C and A_2 has the potential to transduce cellular signals to yield a physiological response [1]. In recent years, increasing research activities have been focused on PLD† and its lipid second messengers PA and DAG; PA is dephosphorylated to yield DAG by the widespread enzyme PA phosphohydrolase. Although the formation of PA contributes to the mass of DAG produced upon receptor stimulation in many tissues and cells [1–3], this may not always be the case [4,5].

Signal-dependent activation of PLD has been observed in a variety of cells, such as blood cells, muscle cells, neuronal cells, glial cells, fibroblasts and hepatocytes. However, the signal transduction leading to PLD activation seems to be rather complex, and a single widely employed signal cascade does not seem to exist. Thus, PLD may be stimulated by various receptor types coupled to heterotrimeric G-proteins, tyrosine kinase and small G-proteins such as ARF, Rac and RhoA [3]. These observations have helped to tentatively connect PLD activation with physiological roles in, for example, growth, vesicular trafficking and secretion of lysosomal enzymes in granulocytes, actin polymerization, meiosis and mitosis [6]. The existence of PLD isozymes could explain the divergent properties observed in certain cells and tissues, such as different dependencies on cations, on PKC isozymes and on small G-proteins [3].

What is known about receptor-controled activity of PLD in the heart? Ten years ago, a receptor-mediated hydrolysis of PC via PLD was hypothesized when we studied the effect of muscarinic receptor agonists in isolated heart preparations [7–9]. Recently, receptor coupling of PLD activity has also been shown for angiotensin-II [10] and endothelin-1 [11,12]. The physiological role of receptor-mediated PLD activation in the heart is a matter of speculation which is focused on three aspects: 1) Na^+-Ca^{2+} exchange and positive inotropism; 2) hypertrophy of myocardial cells; and 3) ischemic preconditioning (see Discussion).

* Corresponding author: K. Löffelholz, Department of Pharmacology, Obere Zahlbacher Str. 67, University of Mainz, 55101 Mainz, Germany; E-mail: LOEFFELH@mzdmza.zdv.uni-mainz.de

† *Abbreviations:* ARF, ADP-ribosylating factor; DAG, *sn*-1,2-diacylglycerol; G-Protein, GTP-binding protein; IP, inositol phosphate; PA, phosphatidic acid; PAc, 4β-phorbol-13α-acetate; PDB, 4β-phorbol-12β,13α-dibutyrate; PETH, phosphatidylethanol; PI-PLC, phosphoinositide-specific phospholipase D; PKC, protein kinase C; and PLD, phospholipase D.

Received 30 July 1997; accepted 20 October 1997.

TABLE 1. Ca^{2+} dependency of PLD activity

Incubation medium (mM)	Basal PETH formation	PDB (0.6 μM)	Fluoride (10 mM)
0 mM Ca^{2+} + 0.5 mM EGTA	0.31 ± 0.02% (4)*	1.90 ± 0.31% (4)*	0.76 ± 0.03% (4)*
1.8 mM Ca^{2+}	0.45 ± 0.03% (13)	3.22 ± 0.29% (4)	2.21 ± 0.14% (6)
10.0 mM Ca^{2+}	0.43 ± 0.05% (5)	0.60 ± 0.04% (3)*	0.63 ± 0.07% (4)*

[^{3}H]-PETH expressed as percent of lipid label was determined in rat atria after 60 min of incubation. The standard medium contained 1.8 mM Ca^{2+} and 1.0 mM Mg^{2+}. Data are expressed as means ± SEM (N).
*Significantly different from control (standard medium).

In this study, we analyzed the PLD activation by stimulating PKC and G-proteins in incubated rat atria using phorbol esters (PDB, PAc) and fluoride as tools. In particular, we studied: a) time-course and cation dependency of the PKC- and G-protein-mediated PLD activation; b) the role of PKC and tyrosine phosphorylation in G-protein–PLD pathway; c) the role of PKC in G-protein coupling to PI-PLC activity; and d) whether and to what extent activation of PLD, besides that of PI-PLC, contributes to the mass production of DAG.

MATERIALS AND METHODS

Isolated atria from male Wistar rats were incubated in 4 mL of Tyrode solution at 35°. Tyrode solution was gassed with 95% O_2/5% CO_2 and had the following composition (in mM): Na^+ 149.3, K^+ 2.7, Ca^{2+} 1.8, Mg^{2+} 1.05, Cl^- 145.5, HCO_3 11.9, and H_2PO_4 0.4; (+) glucose 5.6. Choline was determined in the incubation medium by a chemoluminescence assay. Phospholipids were radioactively labeled by incubation of the atria with 40 μCi of [^{3}H]-glycerol (DuPont/NEN, Dreieich, Germany) for 3 hr. The labeling period was followed by a 15-min wash in oxygenated HEPES buffer salt solution at pH 7.2. Thereafter, the test substances (PDB, PAc, sodium fluoride) were added together with ethanol (2%) or in the absence of ethanol; only Ro 31-8220 was introduced 10 min before ethanol. The phospholipids were extracted and separated by two-dimensional TLC using chloroform/methanol/25% ammonia (65:35:5) in the first dimension. In the second dimension, chloroform/acetone/methanol/acetic acid/water (60:24:12:12:3) was used for the separation of [^{3}H]-PA, and the upper phase of ethylacetate/isooctane/acetic acid/water (13:2:3:10) for the separation of [^{3}H]-PETH. The spots were visualized with iodine vapor, and spots corresponding to [^{3}H]-PA and [^{3}H]-PETH were scraped off and quantified by liquid scintillation counting. The amounts of radioactive lipid were expressed as percent of total labeled phospholipids (for further details, see Lindmar and Löffelholz [13]). Total DAG in extracted lipids (see above) was determined with a radioactive assay kit (Amersham, Little Chalfont, U.K.). In the presence of [^{32}P]-ATP and DAG kinase, DAG was converted to [^{32}P]-PA, which was separated by TLC and counted.

Inositol phosphates were measured according to Berridge et al. [14; see also Ref. 8]. Rat atria were incubated for 4 hr in 10 mL of Tyrode solution containing [*myo*-^{3}H]-inositol (12.8 Ci/mmol). After a 60-min wash in Tyrode solution containing 10 mM LiCl, the atria were exposed to the test drugs (PDB, fluoride) and to LiCl. Thereafter, the tissue was homogenized in 12 mL of chloroform:methanol (1:2). For the estimation of *myo*-inositol phosphates, chloroform and water were added to 9 mL of the homogenate to obtain a two-phase system. After centrifugation at 3000 *g* and 4° for 20 min. the aqueous phase was transferred to a column containing 1 g of anion exchange resin (Bio Rad AG 1-X8, 100–200 mesh, formate form). The phosphate esters were then eluted by the stepwise addition of solutions containing increased concentrations of formate. The 1.0-mL fractions eluted from the columns were counted for radioactivity using a liquid scintillation counter.

Drugs used were PDB, PAc and sodium orthovanadate (Sigma, Deisenhofen, Germany), genistein (RBI, Biotrend Chemikalien, Cologne, Germany), sodium fluoride (Merck, Darmstadt, Germany) and Ro 31-8220 (Roche, Welwyn Garden City, U.K.). Data are presented as means ± SEM of N experiments. Statistical significance was evaluated by *t*-test.

RESULTS

Activation of PLD by Phorbol Ester

The activity of PLD was determined in rat atria by measuring the formation of [^{3}H]-PETH (expressed as percent of total phospholipid label) which occurred in the presence of 2% ethanol. The enzyme activity was measured 5, 15 and 60 min after exposure to ethanol. Under basal conditions, [^{3}H]-PETH formation was 0.12 ± 0.01% (N = 4) after 5 min and 0.45 ± 0.03% (N = 13) after 60 min (Table 1).

After 60 min, the phorbol ester PDB (0.6 μM) caused a six-fold enhancement of the basal formation of [^{3}H]-PETH by 2.8 ± 0.3% of phospholipid label (N = 4) (Fig. 1). The EC_{50} value for PDB was ca. 0.1 μM (not illustrated). PDB also accelerated choline efflux (Fig. 2) and increased the formation of [^{3}H]-PA (Fig. 1) and of unlabeled *s,n*-1,2-DAG (Fig. 3). The finding that the inactive phorbol ester PAc (0.6 μM) was ineffective (not documented) and that the PKC inhibitor Ro 31-8220 (10 μM) blocked the response to PDB (Fig. 4) strongly indicates that PDB enhanced PLD activity by stimulating PKC. Ethanol almost blocked the effects of PDB on PA and DAG (Fig. 3), which

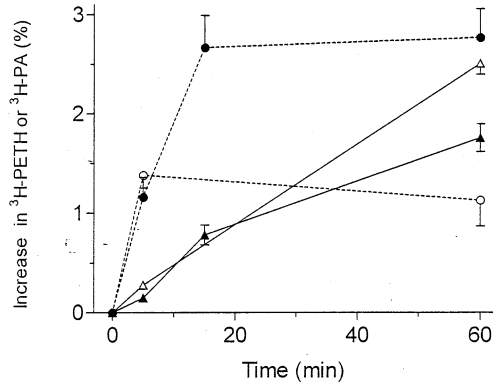

FIG. 1. Time course of effects evoked by PDB and by fluoride on the generation of [^{3}H]-PETH and [^{3}H]-PA. Incubated rat atria were exposed to 0.6 μM PDB (circles, broken line) and to 10 mM fluoride (triangles, solid line) for 5, 15 or 60 min. The tissue was prelabeled with [^{3}H]-glycerol and exposed to PDB along with 2% ethanol; after extraction, the radioactivity associated with PETH (closed symbols) and PA (open symbols) was separated by TLC and counted. Shown are increases expressed as % of lipid label. The basal values after 60 min were 0.45 ± 0.03% of lipid label for [^{3}H]-PETH and 0.73 ± 0.04% for [^{3}H]-PA (N = 13). Data are expressed as means ± SEM.

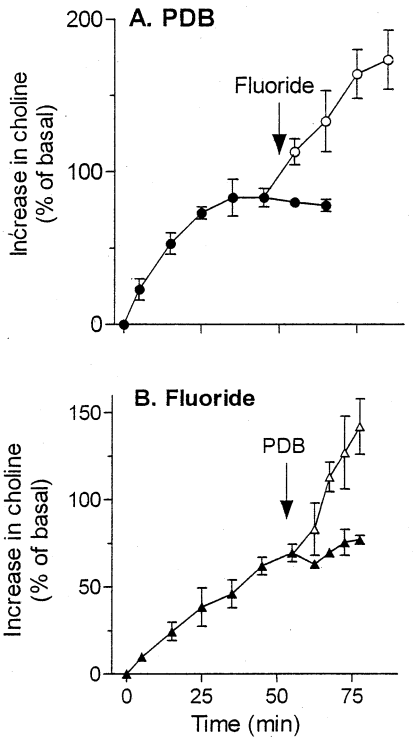

FIG. 2. Changes in choline efflux from incubated rat atria: interaction of PDB and fluoride. The rate of choline efflux (nmol/g/min) was determined in 10-min samples under basal conditions and after addition of 0.6 μM PDB (A) or of 10 mM fluoride (B). The rate was expressed in percent of basal rate, which was constant at *ca.* 1 nmol/g/min throughout the experiment. After *ca.* 50 min (arrow), the tissue was exposed to both fluoride and PDB (A and B: open symbols). Data are expressed as means ± SEM (N = 3–6).

suggests that activation of PLD was responsible for the formation of PA and DAG.

After only 5 min of incubation, [^{3}H]-PETH, [^{3}H]-PA and choline were clearly increased, whereas DAG was still unchanged (control value: 160 ± 5 pmol/mg, N = 6; Fig. 3). Fig. 1 shows that the PDB-induced formation of [^{3}H]-PETH and of [^{3}H]-PA increased rapidly to reach a plateau after 15 min. In other words, PLD activation by PDB unexpectedly desensitized within a short time.

Activation of PLD by Fluoride

After 60 min of exposure to fluoride (0.1–10 mM sodium fluoride), the formation of [^{3}H]-PETH was found to be enhanced by maximally 1.76 ± 0.14% of lipid label (N = 6). The EC_{50} of fluoride was 3 mM (not illustrated). Fluoride (10 mM), like PDB, accelerated choline efflux (Fig. 2) and increased the formation of [^{3}H]-PA and DAG (Figs. 1 and 3). However, in contrast to the effects of PDB, the fluoride-evoked increases in [^{3}H]-PETH, [^{3}H]-PA and choline were strictly linear up to 60 min, i.e., PLD activation did not show desensitization.

Ethanol reduced the fluoride-induced formation of [^{3}H]-PA by *ca.* 50% of control (Fig. 3). A similar observation was made for DAG, which was reduced to 36% of control. These data indicate that only approximately half of the two lipid messengers formed by fluoride were due to activation of PLD; the other half most likely originated from the activation of PI-PLC (see below).

The PKC inhibitor Ro 31-8220 (10 μM) failed to inhibit the PLD response to fluoride (Fig. 4). There was even a slight increase by Ro 31-8220 alone as well as in combination with fluoride. Genistein (50 μM), an inhibitor of protein tyrosine kinases, caused a small attenuation of the basal PLD activity determined after 60 min (0.31 ± 0.02% of lipid label, N = 4), but failed to significantly reduce the response to fluoride (1.70 ± 0.38%; N = 4) (for control values see Table 1). Vanadate (100 μM) significantly increased the basal PLD activity from 0.45% by 0.15 ± 0.02% of lipid label (N = 4), which is neglegible (1.3-fold) when compared with the 5-fold increase induced by fluoride.

Potentiation by PDB of Fluoride-induced Activation of PLD

Fluoride in the highest concentration applied (10 mM) caused a steady increase in the rate of choline efflux for 80 min (Fig. 2). When PDB (0.6 μM) was added after 50 min, there was a further marked acceleration. A similar synergistic effect was observed when the sequence was reversed: PDB was introduced first and fluoride was added when the PDB-evoked choline efflux had reached a plateau (Fig. 2). Clear evidence for a potentiating effect of PDB on the fluoride-evoked activation of PLD was obtained by the formation of [^{3}H]-PETH as shown in Fig. 5. PDB (0.6 μM) drastically shifted the concentration-response curve for fluoride (the contribution of +2.8% of lipid label caused by

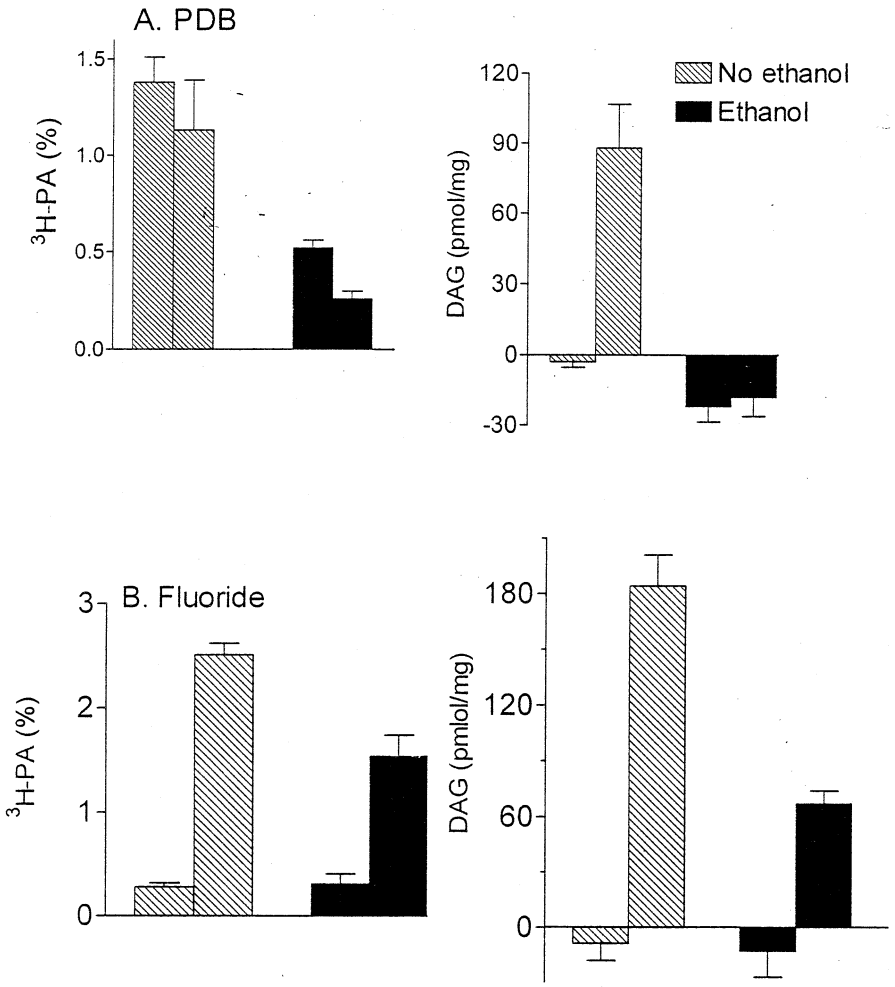

FIG. 3. Effect of ethanol on the formation of [^{3}H]-PA and DAG evoked by PDB (A) or fluoride (B). Unlabeled *sn*-1,2-DAG (pmol/mg ww) was measured in the tissue by a radiometric assay kit. Shown are changes expressed as % of lipid label after 5 and 60 min as indicated by each pair of columns. Basal values after 60 min were 0.73% [^{3}H]-PA (N = 13) and 160 ± 5 pmol/mg ww for DAG (N = 6). Data are expressed as means ± SEM. For further details see Fig. 1.

PDB alone was subtracted). Fluoride at the threshold concentration of 0.3 mM fluoride showed a significant effect in the presence of PDB, which was equal to the effect of fluoride at its highest concentration (10 mM) without PDB. The combination of 10 mM fluoride and PDB increased [^{3}H]-PETH by 8.27% of lipid label, whereas the increases by fluoride alone (+1.77%) and by PDB alone (+2.8%) were considerably smaller.

Effects of PDB and Fluoride on PI-PLC Activity

The activation of PI-PLC was studied by measuring IP_1, IP_2 and IP_3. As illustrated in Fig. 6, fluoride (10 mM) present for 60 min markedly enhanced the formation of the sum of IPs; the strongest effect was on IP_1 (7.4-fold), whereas IP_3 remained unchanged (not illustrated). PDB (0.6 μM) did not influence the basal generation of IPs, but reduced the fluoride-evoked enhancement of IPs by ca. 50% (Fig. 6).

Divalent Cations and PLD Activity

Basal PLD activity was easily detected in the absence of extracellular Ca^{2+} (0.5 mM EGTA in Ca^{2+}-free medium) (Table 1). Addition of 1.8 mM standard medium slightly increased basal PLD activity (+50%), but had no effect on the relative increase caused by PDB, which was ca. six-fold both in the absence and presence of extracellular Ca^{2+}. In contrast, the G-protein-mediated coupling to PLD was found to be Ca^{2+}-dependent: the response to fluoride was decreased from 6- to 2.5-fold upon chelating extracellular Ca^{2+}. High Ca^{2+} (10 mM) markedly reduced both the PDB- and fluoride-evoked PLD activities. Variation of Mg^{2+} between 0 and 10 mM did not modulate basal or evoked PLD activity (not documented).

DISCUSSION

PLD Activity in the Heart

Basal PLD activity in the heart is high as compared to other organs [15] and is both cytosolic and bound to membranes [16,17] and cell nuclei [18]. It has been known for 10 years that muscarinic receptor agonists and oleate enhance cardiac hydrolysis of phosphatidylcholine [7–9] and of phosphoinositides [19,20]. In mammalian heart, the only other receptor agonists so far shown to activate PLD are endothelin-1, atrial natriuretic factor and angiotensin II [12,20]. Endothelin stimulated both PLD and PI-PLC activities [12].

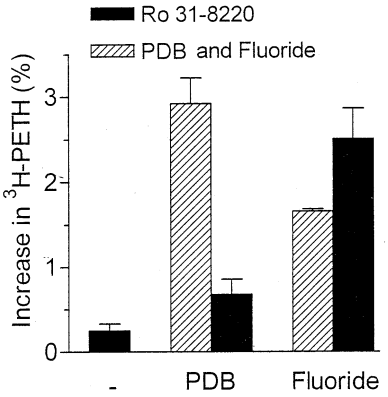

FIG. 4. Role of PKC in the PDB- and fluoride-evoked activation of PLD. Rat atria were exposed to 0.6 μM PDB and to 10 mM fluoride for 60 min. The PKC inhibitor Ro 31-8220 (10 μM) was added alone (first column) or along with PDB or fluoride. Ro 31-8220 was introduced 10 min before ethanol plus PDB/fluoride. Shown are changes expressed as % of lipid label. Data are expressed as means ± SEM (N = 4–6). For further details see Fig. 1.

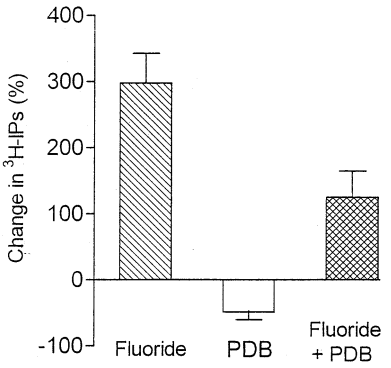

FIG. 6. Effect of PDB on the formation of [^{3}H]-IPs caused by fluoride in rat atria. Presented are increases in [^{3}H]-IPs (sum of IP_1, IP_2 and IP_3) expressed as percent of total lipid label. The tissue was exposed to 10 mM fluoride, 0.6 μM PDB or fluoride plus PDB for 60 min. Data are expressed as means ± SEM (N = 4).

Activation of PLD: Role of PKC and Tyrosine Phosphorylation

Cardiomyocytes express the Ca^{2+}-dependent α-PKC and the Ca^{2+}-independent ε- and δ-isoforms [21]. In addition, PKC activation was found to be the most powerful stimulus of PLD activity in the heart. The effect of phorbol ester was mediated by activation of PKC, as the response was blocked by Ro 31-8220 and was not imitated by the inactive phorbol ester PAc. Unexpectedly, we observed that the effect of PDB, in contrast to that caused by fluoride, was transient and disappeared within ca. 15 min. It is unlikely that this phenomenon is due to down-regulation of PKC, which normally takes several hours to develop, or to desensitization of PLD itself. The result is best explained by the assumption [3] that PKC–PLD coupling involves an intermediate small G-protein, such as Rho or ARF, which may be responsible for the observed desensitization caused by phorbol ester [22,23].

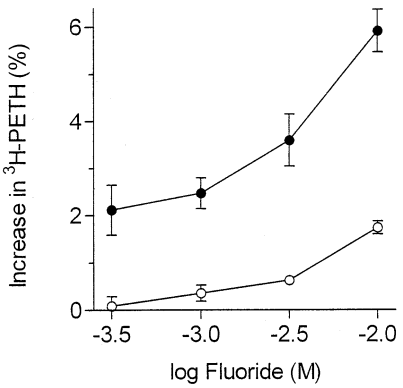

FIG. 5. Effect of PDB on the fluoride-induced increase of [^{3}H]-PETH. Rat atria were exposed to fluoride alone (open circles) or to fluoride plus 0.6 μM PDB (closed symbol) for 60 min. When the combination was tested, the PDB-evoked increase in [^{3}H]-PETH (+2.8% of lipid label; see Fig. 1) was subtracted from the results caused by the combination. Data are expressed as means ± SEM (N = 4–6). For further details see Fig. 1.

There is still an ongoing debate as to the role of PKC in the receptor- and G-protein-mediated activation of the enzyme. It is widely believed that the sequential activation "G-protein → PI-PLC → PKC → PLD" represents the normal pathway leading to PLD activation [3]. However, there is clear evidence that both the PKC-dependent and PKC-independent pathways of receptor-mediated PLD activation can be observed under certain conditions. We report here that Ro 31-8220 failed to reduce fluoride-stimulated PLD activity. Moreover, the effect of fluoride was maintained up to 60 min without any sign of a desensitization, which would be expected if PKC were involved. Thus, in the heart, PLD activity is stimulated via PKC and G-protein through independent pathways.

In several studies, tyrosine kinases have been found to be enclosed in PLD activation by G-protein-coupled receptors [3]. However, as shown by the present study, inhibition of protein tyrosine kinases by genistein did not decrease the five-fold increase in [^{3}H]-PETH formation induced by fluoride. Genistein caused a small decrease in basal PLD activity, which was slightly increased (1.3-fold) by vanadate (100 μM), a tyrosine phosphatase inhibitor. In conclusion, there is no evidence for an involvement of a protein kinase or tyrosine phosphatase in the "G-protein–PLD pathway". Only a small fraction of *basal* PLD activity seems to be regulated by tyrosine phosphorylation.

Synergistic Interaction between PKC Activation and G-Protein-mediated PLD Activity

In this study, we observed both the PKC-induced potentiation of PLD activation and the PKC-induced inhibition of PI-PLC activation [24]. At low concentrations, fluoride caused an activation of PLD only in the presence of PDB. This is remarkable because PKC is not integrated into the

G-protein–PLD pathway, which suggests that the site of synergistic interaction is at the enzyme itself (see above). Previously, a synergistic activation of PLD by GTPγS and phorbol ester was reported for preparations such as isolated platelet membranes, HL60 cells and neural-derived NG108-15 cells [25]. The mechanism of this interesting phenomenon is still unknown.

Cation Dependency of Basal and Stimulated PLD Activities

The basal and PDB-evoked PLD activities of the heart were easily detected even in the absence of extracellular Ca^{2+} and Mg^{2+}. They either did not respond or responded only slightly to the addition of 1.8 mM Ca^{2+} (standard medium). Only the fluoride-evoked PLD activity was reduced by the omission of Ca^{2+} (with or without EGTA). In general, the link responsible for the Ca^{2+}-dependency of PLD activation has not been identified yet; a Ca^{2+}-dependent PKC (and any other PKC subtype) could be excluded for the heart from the outset (see above). Moreover, the question whether the Ca^{2+}-dependency of the fluoride-activated PLD is due to an upstream link or to the enzyme itself cannot yet be answered. PLD isozymes with different Ca^{2+}-dependencies were found in HL60 and other cells [26]. In conclusion, the present data do not support a direct physiological control of cardiac PLD activity by changes in Ca^{2+} [3].

The Formation of DAG in Myocardial Tissue

A large part of the receptor-mediated formation of DAG may be due to parallel activation of PI-PLC and PLD [1,2,24]. However, PA formed by PLD activation is not necessarily transformed to yield DAG [4,5]. The fact that, in the presence of ethanol, [^{3}H]-PETH was formed at the expense of PA was utilized to determine the fraction of DAG that is derived from PA; ethanol served as an inhibitor of the production of PA [1]. It was found that PDB caused the generation of considerable amounts of [^{3}H]-PA and DAG (the latter after a delay of a few min), and that ethanol almost blocked this effect. Thus, DAG generated by phorbol ester was totally due to activation of PLD. In contrast, the fluoride-evoked formation of PA and DAG was only partially reduced by ethanol, indicating that both PLD and most likely PI-PLC were equally responsible for the DAG formed by G-protein activation. The data shown in Fig. 3 do not indicate a significant PA phosphatase inhibition by fluoride [18].

Putative Physiological Roles of PLD Activity in Myocardial Tissue

There is little evidence for a role of PLD activation in *rapid* neurotransmitter-evoked cell signaling. In the heart, the physiological role of agonist-evoked PLD activation is a matter of speculation [21], just as is that of PI-PLC [27]. Recently, it has been reported that PA in low concentrations is a potent activator of IP formation in adult ventricular myocytes [28].

Firstly, some years ago it was proposed that PLD might be crucial for stimulation of Na^+-Ca^{2+} exchange [29], possibly leading to positive inotropic responses of the heart [30,31]. This idea has not yet been substantiated.

Secondly, it deserves mention that PKC-δ seems to play a crucial role in the phenomenon of "ischemic preconditioning" in the heart [32], which describes the observation that a brief period of ischemia protects the heart against injury from a subsequent prolonged period of ischemia. Clear-cut evidence for a direct role of PLD activity in this phenomenon [21,33] has not yet been obtained.

Thirdly, there is increasing evidence that PLD and PI-PLC activation might be responsible for cardiac hypertrophy and gene expression. Endothelin-1-induced hypertrophy of rat neonatal cardiomyocytes was initiated by PI-PLC as well as by PLD activation; both enzymes seem to be activated by the atrial natriuretic factor and by angiotensin II [12,21]. Interestingly, mechanical load caused hypertrophy of myocardial cells and in parallel activated PLD, PI-PLC and also a plethora of other regulatory elements upstream or downstream of the phospholipases: PKC, tyrosine kinases, $p21^{ras}$, mitogen-activated protein kinases and finally c-*fos* gene expression [34,35]. The evidence for an involvement of a PKC isozyme in cardiac hypertrophy is strong. Ca^{2+}-independent PKC-ε is activated by phorbol esters and endothelin-1, which also cause hypertrophy [36] and activate PLD [12]. During development of left ventricular hypertrophy in rats induced by pressure overload, PKC-ε activity (besides that of PKC-$\beta_{1,2}$) was increased; PKC-ε was present mainly in the membrane and nuclear-cytoskeletal fractions [36]. Additional chronic effects on gene expression and cell growth may be mediated by the Ca^{2+}-independent δ-isoform [22]. Stimulation of Ca^{2+}-independent PKC isoforms, such as PKC-ε and PKC-δ, is also associated with PLD activated in the absence of Ca^{2+} increase rather than with PI-PLC activation [37].

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. We thank Dr. D. Bradshaw, Roche Research Centre, Welwyn Garden City, Hertfordshire, for providing Ro 31-8220.

References

1. Divecha N and Irvine RF, Phospholipid signaling. *Cell* **80:** 269–278, 1995.
2. Exton JH, Cell signalling through guanine-nucleotide-binding regulatory proteins (G proteins) and phospholipases. *Eur J Biochem* **243:** 10–20, 1997.
3. Exton JH, Phospholipase D: enzymology, mechanisms of regulation, and function. *Physiol Rev* **77:** 303–320, 1997.
4. Wright TM, Willenberger S and Raben DM, Activation of phospholipase D by α-thrombin or epidermal growth factor contributes to the formation of phosphatidic acid, but not to

observed increases in 1,2-diacylglycerol. *Biochem J* **285** (Pt 2): 395–400, 1992.
5. Ward DT, Ohanian J, Heagerty AM and Ohanian V, Phospholipase D-induced phosphatidate production in intact small arteries during noradrenaline stimulation: involvement of both G-protein and tyrosine-phosphorylation-linked pathways. *Biochem J* **307** (Pt 2): 451–456, 1995.
6. Boarder MR, A role for phospholipase D in control of mitogenesis. *Trends Pharmacol Sci* **15:** 57–62, 1994.
7. Lindmar R, Löffelholz K and Sandmann J, Characterization of choline efflux from the perfused heart at rest and after muscarine receptor activation. *Naunyn Schmiedebergs Arch Pharmacol* **332:** 224–229, 1986.
8. Lindmar R, Löffelholz K and Sandmann J, On the mechanism of muscarinic hydrolysis of choline phospholipids in the heart. *Biochem Pharmacol* **37:** 4689–4695, 1988.
9. Klein J, Lindmar R and Löffelholz K, Muscarinic activation of phosphatidylcholine hydrolysis. *Prog Brain Res* **109:** 201–208, 1996.
10. Sadoshima J and Izumo S, Signal tranduction pathways of angiotensin II-induced c-*fos* gene expression in cardiac myocytes *in vitro*. Roles of phospholipid-derived second messengers. *Circ Res* **73:** 424–438, 1993.
11. Ye H, Wolf RA, Kurz T and Coss PB, Phosphatidic acid increases in response to noradrenaline and endothelin-1 in adult rabbit ventricular myocytes. *Cardiovasc Res* **28:** 1828–1834, 1994.
12. Lamers JM, Eskildsen-Helmond YE, Resink AM, de Jonge HW, Bezstarosti K, Sharma HS and van Heugten HA, Endothelin-1-induced phospholipase Cβ and D and protein kinase C isoenzyme signaling leading to hypertrophy in rat cardiomyocytes. *J Cardiovasc Pharmacol* **26 (Suppl 3):** S100–S103, 1995.
13. Lindmar R and Löffelholz K, Phospholipase D in heart: basal activity and stimulation by phorbol esters and aluminum fluoride *Naunyn Schmiedebergs Arch Pharmacol* **346:** 607–613, 1992.
14. Berridge MJ, Downes CP and Hanley MR, Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J* **206:** 587–595, 1982.
15. Kobayashi M and Kanfer JN, Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J Neurochem* **48:** 1597–1603, 1987.
16. Panagia V, Ou C, Taira Y, Dai J and Dhalla NS, Phospholipase D activity in subcellular membranes of rat ventricular myocardium. *Biochim Biophys Acta* **1064:** 242–250, 1991.
17. Wang P, Anthes JC, Siegel MI, Egan RW and Billah MM, Existence of cytosolic phospholipase D. Identification and comparison with membrane-bound enzyme. *J Biol Chem* **266:** 14877–14880, 1991.
18. Kanfer JN, McCartney D, Singh IN and Freysz L, Phospholipase D activity in rat brain neuronal nuclei. *J Neurochem* **67:** 760–766, 1996.
19. Löffelholz K, A novel source of diacylglycerol and phosphatidic acid. Receptor regulation of choline phospholipid hydrolysis. *Biochem Pharmacol* **38:** 1534–1549, 1989.
20. Woodcock EA, White LB, Smith AI and McLeod JK, Stimulation of phosphatidylinositol metabolism in the isolated, perfused rat heart. *Circ Res* **61:** 625–631, 1987.
21. Eskildsen-Helmond YE, van Heugten HA and Lamers JM, Regulation and functional significance of phospholipase D in myocardium. *Mol Cell Biochem* **157:** 39–48, 1996.
22. Pucéat M, Hilal-Dandan R, Strulovici B, Brunton LL and Brown JH, Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J Biol Chem* **269:** 16938–16944, 1994.
23. Kozma R, Ahmed S, Best A and Lim L, The GTPase-activating protein n-chimaerin cooperates with Rac1 and cdc42Hs to induce the formation of lamellipodia and filopodia. *Mol Cell Biol* **16:** 5069–5080, 1996.
24. Klein J, Chalifa V, Liscovitch M and Löffelholz K, Role of phospholipase D activation in nervous system physiology and pathophysiology. *J Neurochem* **65:** 1445–1455, 1995.
25. Liscovitch M and V Chalifa, Signal-activated phospholipase D. In: *Signal-activated phospholipases* (Ed. Liscovitch M), pp. 31–63; R.G. Landis, Austin, 1994.
26. Siddiqi AR, Smith JL, Ross AH, Qui RG, Symons M and Exton JH, Regulation of phospholipase D in HL60 cells. Evidence for a cytosolic phospholipase D. *J Biol Chem* **270:** 8466–8473, 1995.
27. Woodcock EA, Suss MB and Andersen KE, Inositol phosphate release and metabolism in rat left atria. *Circ Res* **76:** 252–260, 1995.
28. Kurz T, Wolf RA and Corr PB, Phosphatidic acid stimulates inositol 1,4,5-trisphosphate production in adult cardiac myocytes. *Circ Res* **72:** 701–706, 1993.
29. Philipson KD and Nishimoto AY, Stimulation of Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles by phospholipase D. *J Biol Chem* **259:** 16–19, 1984.
30. Burt JM, Rich TL and Langer GA, Phospholipase D increases cell surface Ca^{2+} binding and positive inotropy in rat heart. *Am J Physiol* **247** (5 Pt 2): H880–H885, 1984.
31. Pappano AJ, Matsumoto K, Tajima T, Agnarsson U and Webb W, Pertussis toxin-insensitive mechanism for carbachol-induced depolarization and positive inotropic effect in heart muscle. *Trends Pharmacol Sci* **9** (Suppl): 35–39, 1988.
32. Mitchell MB, Meng X, Ao L, Brown JM, Harken AH and Banerjee A, Preconditioning of isolated rat heart is mediated by protein kinase C. *Circ Res* **76:** 73–81, 1995.
33. Eskildsen-Helmond YE, Gho BC, Bezstarosti K, Dekkers DH, Soei LK, van Heugten HA, Verdouw PD and Lamers JM, Exploration of the possible roles of phospholipase D and protein kinase C in the mechanism of ischemic preconditioning in the myocardium. *Ann NY Acad Sci* **793:** 210–225, 1996.
34. Sadoshima J and Izumo S, Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J* **12:** 1681–1692, 1993.
35. Yazaki Y, Komuro I, Yamazaki T, Tobe K, Maemura K, Kadowaki T and Nagai R, Role of protein kinase system in the signal transduction of stretch-mediated protooncogene expression and hypertrophy of cardiac myocytes. *Mol Cell Biochem* **119:** 11–16, 1993.
36. Bogoyevitch MA, Parker PJ and Sugden PH, Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-ε is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin. *Circ Res* **72:** 757–767, 1993.
37. Nishizuka Y, Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* **9:** 484–496, 1995.