

Selective Activation of Rolipram-Sensitive, cAMP-Specific Phosphodiesterase Isoforms by Phosphatidic Acid

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SUMMARY

In rat thymic lymphocytes, accumulation of phosphatidic acid (PA) occurs at the same time as decrease in cAMP levels and activation of a cAMP-specific phosphodiesterase (PDE) [type 4, EC 3.1.4.17 (PDE4)]. We investigated the nature of the PDE activated by PA and the mechanism of activation by using recombinant cAMP-specific PDE4 isoforms derived from three different genes (*PDE4A*, *PDE4B*, and *PDE4D*). The "long" variants expressed from each gene (*PDE4A5*, *PDE4B1*, and *PDE4D3*) were activated by PA, whereas the "short" variants (*PDE4A1*, *PDE4B2*, *PDE4D1*, and *PDE4D2*) were not. Phosphatidylserine was an activator that was as effective as PA, whereas phosphatidylcholine was ineffective, indicating that activation was restricted to anionic phospholipids. PA caused

an increase in the V_{\max} value of *PDE4D3* without affecting the K_m value of the enzyme for the cAMP substrate. PA also caused a change in the Mg^{2+} requirement for hydrolysis. Half-maximal stimulation of the PDE was obtained with $\sim 10 \mu\text{g/ml}$ PA. Although protein kinase A-mediated phosphorylation of *PDE4D3* produces effects similar to those elicited by PA, the mechanism of PA-induced activation was not found to involve a phosphorylation. Instead, several observations suggest that PA may directly interact with the enzyme. The stimulation of cAMP PDEs by PA and other acidic phospholipids may be a mechanism by which growth factors and hormones modulate the cAMP-dependent signal transduction pathway during cell stimulation.

PA is a phospholipid that is produced in several cell types on stimulation by hormones and growth factors (1). PA and LPA play a role as growth factors for various cells, probably acting through a G protein-coupled receptor (1, 2). Furthermore, PA has been proposed as an intracellular second messenger that is involved in many physiological responses (1). It is established that PA plays a crucial role in the proliferation of fibroblasts (3–5). Furthermore, a role of PA in intracellular signaling has been suggested for the endotoxin-stimulated cytokine secretion in monocytes (6), the activation and proliferation of T lymphocytes induced by anti-CD3 antibodies (7), and the activation of the respiratory burst in neutrophils (8–10). PA can be formed through hydrolysis of phospholipids by phospholipase D or through phosphorylation of DAG by DAG kinase (1). Intracellular accumulation of PA very rapidly follows the onset of hormonal stimulation, suggesting a role of this phospholipid in the early cell signaling. Although the physiological intracellular targets are not yet

known, the activity of several enzymes involved in signal transduction is modulated by PA in a cell-free system. The protein-tyrosine-phosphatase PTP1C is activated by PA, and the phospholipid may play a role in dephosphorylation/inactivation of the epidermal growth factor receptor (11, 12). Some isoenzymes of the PKC family (13), phospholipase C- γ 1 (14), PI4P-5-kinase (15), and the rolipram-sensitive cAMP-specific PDE4 (16, 17) are other examples of intracellular enzymes with catalytic activity that is enhanced in the presence of PA. The activation of a partially purified PDE4 from rat thymocytes by PA *in vitro* is particularly interesting in view of the finding that mitogenic stimulation of these cells by concanavalin A induces PA accumulation (17), activation of a PDE4 (16), and a simultaneous decrease in cAMP levels.¹ Because cAMP is a negative signal for T cell proliferation (18), it has been hypothesized that intracellular accumulation of PA may bring about a decrease in cAMP levels by activation of a PDE4, thus promoting the development of cell response (17). A negative effect of PA on the cAMP pathway

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¹ G. Némóz, unpublished observations.

ABBREVIATIONS: PA, phosphatidic acid; PDE, phosphodiesterase; LPA, lysophosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; DAG, diacylglycerol; PKA, cAMP-dependent protein kinase; PKI, cAMP-dependent protein kinase synthetic inhibitor peptide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

is known in other cell types as well. In preantral granulosa cells, gonadotropin-releasing hormone exerts an antidifferentiating effect by inhibiting the follicle-stimulating hormone-induced cAMP-dependent progesterone production and cell rounding (19). This effect is attributed to phospholipase D activation and PA accumulation in these cells on stimulation with gonadotropin-releasing hormone (19). Direct PA activation of PDE4 might be the link between these pathways.

Rolipram-sensitive cAMP-specific PDEs (PDE4) are a family of enzymes that hydrolyze cAMP with a high affinity and are specifically inhibited by the antidepressant compound rolipram (20). Four different genes (*PDE4A–4D*) are present in the rat, mouse, and human genomes (21–26), and different mRNA and protein variants are derived from each gene through alternate splicing and the use of different promoters (26–28). Different variants arising from the rat *PDE4D* gene are differentially regulated by cAMP in endocrine cells. Two variants (PDE4D1 and PDE4D2) are regulated at the level of transcription (28, 29); a third variant (PDE4D3) is activated by a PKA-mediated phosphorylation (28, 30). This dual regulation has been proposed to play a role in the short and long term desensitization of target cells under continuous hormonal stimulation (31). It was therefore of interest to determine the isoforms regulated by PA, which might be involved in a modulation of cAMP levels by phospholipid messengers. Here, we describe the effect of PA on different PDE4 isoforms and investigate the possible mechanism of this activation. Because conventional fractionation techniques cannot be used to separate these highly homologous isoforms, we studied the effect of PA on homogeneous recombinant enzymes overexpressed in heterologous systems.

Experimental Procedures

Materials. Sf9 cells were cultivated in Sf900 II medium (GIBCO BRL, Baltimore, MD). MA-10 cells were cultivated in Waymouth's medium (GIBCO BRL). PKA catalytic subunit was supplied by Promega (Madison, WI). Protein G/Sepharose was from Pharmacia Biotech (Piscataway, NJ). AG1-X8 resin was from BioRad (Hercules, CA). [γ - 32 P]ATP (3000 Ci/mmol) was supplied by DuPont-New England Nuclear (Boston, MA). PA (from egg yolk lecithin), PS (from bovine brain), PC (from egg yolk), DAG, LPA, PKI (synthetic protein kinase inhibitor peptide P0300), and staurosporine were obtained from Sigma Chemical (St. Louis, MO). Immobilon P membranes were supplied by Millipore (Bedford, MA).

Preparation of recombinant PDE4 isoforms. Human PDE4A5 and PDE4B1 and rat PDE4A1, PDE4B2, and PDE4D3 were expressed by recombinant baculovirus infection of Sf9 insect cells as previously described (32). Briefly, Sf9 cells were grown at 27° with orbital shaking in Sf900 medium containing 50 μ g/ml gentamicin until they reached 1.2×10^6 cells/ml. At this phase, cells were infected with the corresponding recombinant baculovirus, and growth was continued for 3 days. During the infection, growth medium was supplemented with 1% fetal calf serum and 4% feed stock (prepared by mixing 20 ml of Yeastolate Ultrafiltrate 50X, 10 ml of lipid concentrate, 10 ml of 20% glucose, and 40 ml of 2.5% glutamine in Sf900 medium). At the end of the infection, cells were collected through centrifugation (1000 rpm for 10 min) and resuspended in 40 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM EGTA, 50 mM benzimidazole, 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, 4 μ g/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, and 2 mM phenylmethylsulfonyl fluoride. Cells were homogenized and centrifuged for 10 min at $14,000 \times g$ at 4°. PDE-specific activities in the crude extracts from infected cells were consistently higher by 1000-fold than the activity of uninfected cell extracts. Thus, the endogenous PDE activity ac-

counted for <0.1% of the recombinant PDE activity. The soluble extracts were diluted to 33% ethylene glycol and stored at –20° for further studies. Rat PDE4D1, PDE4D2, and PDE4D3 were expressed in MA-10 Leydig tumor cells by calcium phosphate transfection with 20 μ g of pCMV5 vectors containing the corresponding cDNAs/10-cm dish, as previously described (33). At 24 hr after transfection, the cells were harvested in the Tris-HCl buffer described above and homogenized. After a 10-min centrifugation at $14,000 \times g$, soluble extracts were diluted to 33% ethylene glycol and stored at –20° for further studies. Untransfected and mock-transfected MA-10 cells were devoid of detectable type 4D PDEs, and their basal PDE activity (10 pmol of cAMP hydrolyzed/min \times mg of protein) was not sensitive to PA stimulation. Transfection with pCMV5-PDE plasmids routinely increased PDE specific activity in cell extracts to 200–1000 pmol of cAMP hydrolyzed/min \times mg of protein.

PDE assay. PDE activity was measured using 1 μ M cAMP as substrate according to the method of Thompson and Appleman (34). Samples were assayed in a total volume of 200 μ l of reaction mixture including 40 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1.25 mM 2-mercaptoethanol, 1 μ M cAMP (0.1 μ Ci of [3 H]cAMP), and 0.5 mg/ml gelatin. For Mg^{2+} dose-response curves, increasing concentrations of Mg^{2+} (0–100 mM) were added separately to the reaction mixture in different tubes. After incubation at 34° for 5–15 min, the reaction was terminated by the addition of 200 μ l of 40 mM Tris-Cl, pH 7.5, containing 10 mM EDTA, followed by heat denaturation for exactly 1 min at 100°. To each reaction tube, 50 μ g of *Crotalus atrox* snake venom was added, and the incubation was continued for an additional 15 min at 34°. The reaction products were separated by anion exchange chromatography on AG1-X8 resin, and the amount of radiolabeled adenosine collected was quantified by scintillation counting.

Activation of PDE4 isoforms by PA and PKA. For determination of the effect of lipids, PA, PC, and DAG stock solutions in chloroform were evaporated under a nitrogen flux. The phospholipids were then resuspended at the concentration of 800 μ g/ml in 40 mM Tris-HCl, pH 8.0, and the suspensions were obtained by sonicating the lipid films with a probe sonicator (three cycles of 15 sec). Lipid suspensions were diluted directly at the final concentration in the reaction mixture for the PDE assay.

For PKA-mediated activation, recombinant rat PDE4D3 expressed in MA-10 cells was diluted in 40 mM Tris-HCl, pH 7.4, containing 10 mM magnesium acetate, and incubated for 10 min at 30° with or without 0.1 mM ATP and either in the presence or absence of 0.1 μ M of the catalytic subunit of PKA. At the end of the incubation, samples were assayed for PDE activity in the absence or presence of 200 μ g/ml PA in the reaction mixture. In some experiments, either the PKA inhibitor PKI (2 μ g/ml) or staurosporine (1 μ M) was added during the 10-min incubation and the PDE assay.

Immunoprecipitation of recombinant rat PDE4D3. The monoclonal anti-PDE4D antibody M3S1 (30) was preadsorbed onto protein G/Sepharose beads by incubating a 1:3 suspension of beads in phosphate-buffered saline containing 0.01% bovine serum albumin with the antibody (1:50) for 90 min at 4°. The complex was washed once with 20 mM Tris-HCl, 0.5 M NaCl, pH 7.8, and then twice with 20 mM Tris-HCl, pH 7.8. The immobilized antibody was incubated under continuous shaking with the extract from MA-10 cells transfected with the pCMV5-PDE4D3 expression vector at 4° for 2 hr with or without the addition of 100 μ g/ml PA. At the end of the incubation, complexes immunoadsorbed to the beads were rinsed four times with 40 mM Tris-HCl, pH 8.0, containing 1 mg/ml gelatin and resuspended in the same buffer. PDE activity of the resuspended samples was assayed as described above.

Phosphorylation of rat PDE4D3. The soluble extracts from Sf9 cells infected by recombinant rat PDE4D3 baculovirus were diluted in 40 mM Tris-HCl, pH 7.4, containing 10 mM magnesium acetate and 0.1 mM ATP (10 μ Ci of [γ - 32 P]ATP) and incubated for 10 min at 30° with a 0.1 μ M concentration of the catalytic subunit of PKA, 200 μ g/ml PA, or 200 μ g/ml PC. The control sample, without additions,

was incubated under the same conditions. At the end of the incubation, reactions were stopped by dilution in SDS-PAGE sample buffer, and samples were used for Western blot analysis and autoradiography as previously described (30).

Results

Selective activation of recombinant PDE4 isoforms by PA. Each of the *PDE4A*, *PDE4B*, and *PDE4D* gene encodes at least two protein variants (for a review, see Ref. 20). These variants can be classified as “long” (90–130 kDa; PDE4A5, PDE4B1, and PDE4D3) or “short” (66–74 kDa; PDE4A1, PDE4B2, PDE4D1, and PDE4D2) (Fig. 1). Recombinant PDE4 isoforms were obtained through infection of Sf9 insect cells with recombinant baculoviruses or through calcium phosphate transfection of MA-10 Leydig tumor cells as described in Experimental Procedures. The activity of the recombinant PDE4 isoforms was assayed in the absence or presence of 200 $\mu\text{g}/\text{ml}$ PA. The results in Fig. 2 indicate that although all the long isoforms (i.e., PDE4A5, PDE4B1, and PDE4D3) were activated by the phospholipid, the short variants (i.e., PDE4A1, PDE4B2, PDE4D1, and PDE4D2) were not significantly affected. To test whether the activation by PA was specific, the activity of recombinant PDE4D3 was measured in the presence of different lipid molecules (Fig. 3). PC had no significant effect on the PDE activity, whereas DAG and LPA had a weak effect. PS produced an activation similar to that of PA (Fig. 3), suggesting that the long PDE4 isoforms are selectively activated by anionic phospholipids. None of the lipids tested affected the activity of the PDE4D1 isoform (Fig. 3). A dose-response study showed that near-maximal activation of PDE4D3 was obtained with 50 $\mu\text{g}/\text{ml}$

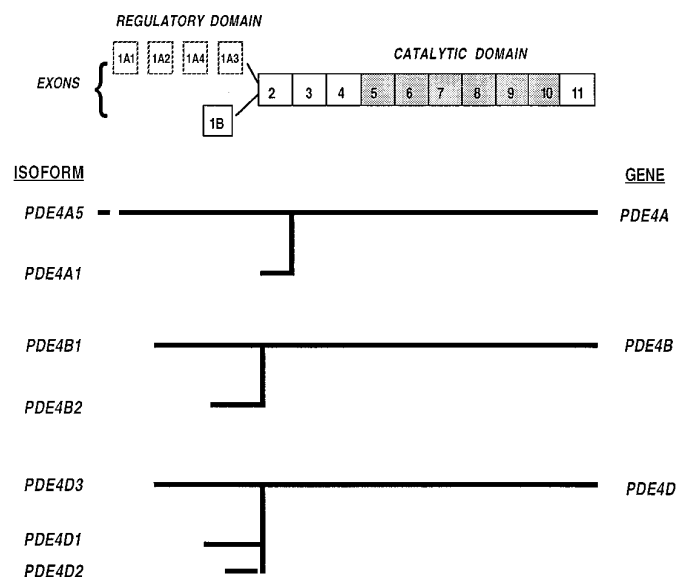


Fig. 1. cDNAs encoding the different recombinant PDE4 isoforms used in the current study. The seven cDNAs used are derived from three genes: *PDE4A*, *PDE4B*, and *PDE4D*. A complete description of the different variants expressed in human and rat from these genes is given in Ref. 20. Briefly, exons 1A1, 1A2, 1A3, and 1A4 are inferred from the homology with the *Drosophila* dunce PDE. Exon 1B is expressed in variants PDE4D1, PDE4D2, and PDE4B2. Exons 2–11 are present in all transcripts, with the exception of PDE4A. PDE4D and PDE4B variants start in an identical position at the 5' boundary of exon 2. These splicing variants are derived from the activity of two promoters that direct the transcription of exons 1A1–4 or 1B, respectively.

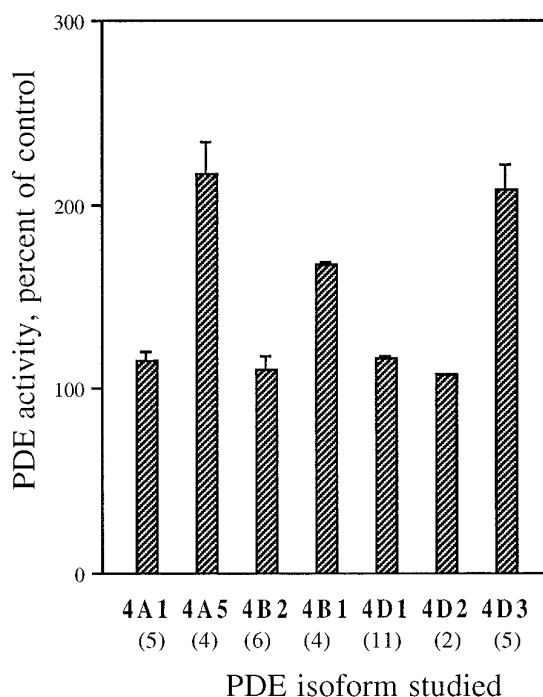


Fig. 2. Activation of the different PDE4 isoforms by PA. The activity measured in the presence of 200 $\mu\text{g}/\text{ml}$ PA is expressed as a percentage of the control value (activity of the considered isoform without any addition). Recombinant PDE4D1 and PDE4D2 isoforms were expressed in MA-10 cells; all of the other recombinant isoforms were expressed in Sf9 cells. The mean \pm standard error of n independent determinations are shown; n is given in parentheses. The average basal specific activities of the preparations used (in nmol of cAMP hydrolyzed/min \times mg of protein) were 0.85 and 0.62 for the isoforms expressed in MA-10 cells, PDE4D1 and PDE4D2, respectively (the specific activity of mock-transfected MA-10 cells was 0.01), and 57, 90, 45, 120, and 85 for the isoforms expressed in Sf9 cells, PDE4A1, PDE4A5, PDE4B1, PDE4B2, and PDE4D3, respectively (the specific activity of mock-transfected Sf9 cells was 0.03).

PA, whereas half-maximal activation occurred at a concentration of ~ 10 $\mu\text{g}/\text{ml}$ (Fig. 4). These concentrations of PA correspond to the physiological ranges reached on hormonal stimulation of target cells (35). A similar response was observed with a purified recombinant PDE4D3, which was $>90\%$ pure as assessed by SDS-PAGE analysis (not shown). In contrast, the short variant PDE4D1 was not significantly affected by PA, even at the highest concentrations tested (Fig. 4). Similar results were obtained when long and short variants from the *PDE4A* and *PDE4B* genes were used (data not shown). For this reason, PDE4D variants were used as representative of the long and short variants for the remainder of the study.

Effect of PA on the sensitivity of PDE activity toward Mg^{2+} concentration and enzyme kinetics. PDE4 hydrolyzing activity requires Mg^{2+} as a cofactor. In the absence of PA, PDE4D3 showed a shallow Mg^{2+} response curve. Indeed, the catalytic activity of the enzyme at the physiological range of the cation (1–10 mM) was lower than half-maximal. An increase in Mg^{2+} from 10 to 100 mM produced a 2.4-fold increase in PDE4D3 activity. The addition of PA profoundly modified the PDE4D3 requirement for Mg^{2+} . In the presence of the phospholipid, a near-maximal activity was reached at 3 mM Mg^{2+} , and the dose-response curve showed a plateau at higher concentrations (Fig. 5). As a

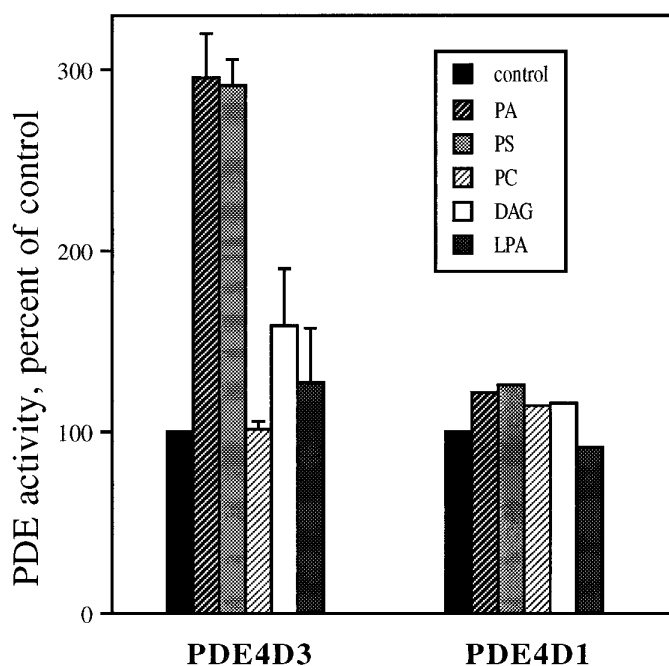


Fig. 3. Effect of different lipids on the activity of recombinant PDE4D1 and PDE4D3 expressed in MA-10 cells. All of the tested compounds were added at a concentration of 200 $\mu\text{g/ml}$ in PDE assay. PDE activity is expressed as a percentage of control values measured without any addition. The values shown for PDE4D3 are the mean \pm standard error of three to five independent determinations. For PDE4D1, the values are the mean of two independent determinations with similar results.

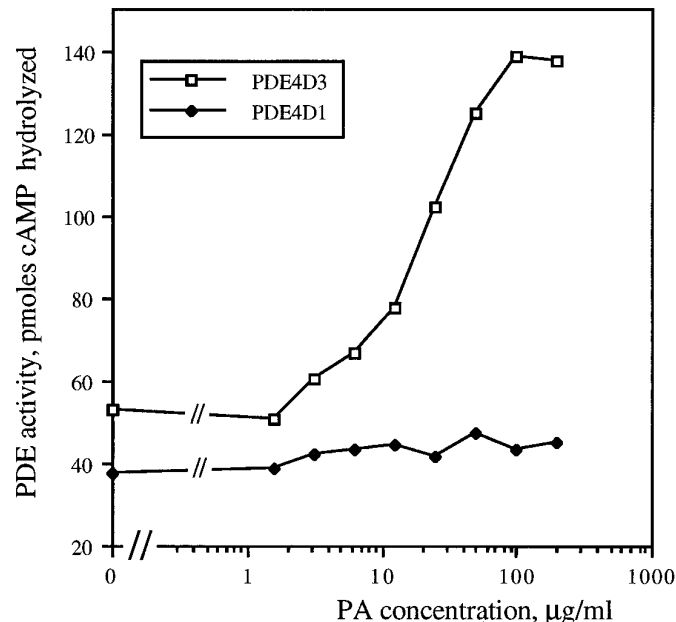


Fig. 4. Concentration dependence of the PA stimulation of recombinant PDE4D1 and PDE4D3 expressed in MA-10 cells. Results are from one typical experiment. The PA concentration producing half-maximal activation of PDE4D3 was $10.8 \pm 3.4 \mu\text{g/ml}$ (mean \pm standard error), as measured in five independent experiments.

consequence, in the 1–3 mM Mg^{2+} concentration range, PA produced a 3-fold stimulation of the enzyme. These data suggest that PA induced a considerably higher affinity of PDE4D3 for the cofactor Mg^{2+} . The short variants PDE4D1 and PDE4D2 displayed a different requirement for Mg^{2+} . In

the absence of PA, the Mg^{2+} response curve was shallow, but an increase in the cation from 10 to 100 mM produced only a 1.3-fold increase in the catalytic activity of these two PDE4D variants. Furthermore, the addition of PA did not significantly change the requirement for Mg^{2+} of PDE4D1 and PDE4D2, and it produced <30% activation of these isoforms (Fig. 5). To further investigate the activation of PDE4D3 by PA, the kinetic parameters of the enzyme were measured at low (1 mM) and high (30 mM) concentrations of Mg^{2+} in the absence or presence of 200 $\mu\text{g/ml}$ PA. The Lineweaver and Burk plots in Fig. 6 indicate that PA activated PDE4D3 by increasing the V_{max} value (2.3-fold at 1 mM Mg^{2+} and 1.5-fold at 30 mM Mg^{2+}) of the enzyme without significantly affecting the K_m value at both concentrations tested.

Mechanism of activation of PDE4D3 by PA. Previous studies from our laboratory have shown that PDE4D3 is activated by a PKA-dependent phosphorylation (28, 30). Activation also produced similar increases in the affinity for Mg^{2+} and the V_{max} of the enzyme, without affecting the K_m (32).

To investigate whether activation by PA was also mediated by phosphorylation, and possibly by kinases present in the cytosolic extracts used, the effects of PA and PKA on the activity of PDE4D3 were tested in the presence of different protein kinase inhibitors. Recombinant PDE4D3 was pretreated or not pretreated with the catalytic subunit of PKA, and the catalytic activity of the enzyme was measured in the presence of PA. Both PKA and PA alone induced a 2.5–3.5-fold increase of the PDE4D3 activity, but no additivity of the activation was obtained when PA was added to PDE4D3 previously incubated with PKA (Fig. 7). Activation by PKA was completely suppressed by the addition of a synthetic PKA inhibitor (PKI), whereas activation by PA was unaffected (Fig. 7). The protein kinase inhibitor staurosporine partially suppressed PKA activation. It slightly inhibited both the basal and PA-stimulated PDE activities without modifying the extent of activation (Fig. 7). This indicates that at the rather high concentration used (1 μM), staurosporine slightly inhibited the catalytic activity of PDE without altering the activating effect of PA. We previously showed that phosphorylation and activation of PDE4D3 by PKA display similar time courses, and they reach a maximum level within 10 min. In contrast, activation by PA did not require preincubation, and it was constant for ≥ 15 min (Fig. 8). Finally, incubation of PDE4D3 with either PKA or PA in the presence of [γ - ^{32}P]ATP showed that although PKA induced phosphorylation of the enzyme, no phosphate incorporation was obtained in the presence of PA or in the presence of the nonactivating phospholipid PC (Fig. 9). The addition of 0.1 mM CaCl_2 or 0.1 mM Ca^{2+} -chelator EGTA to the incubation medium did not modify the basal or PA-stimulated enzyme activities (data not shown). These data rule out the possibility that the PDE4D3 activation by PA is mediated by its Ca^{2+} -chelating action (36).

Several reports have shown that PA and PS exert their effect by binding to specific domains on the target enzymes (37, 38). In a preliminary attempt to define the mechanism of the activation of PDE4D3 by PA, we investigated the effect of dilution or immunoprecipitation of the enzyme on PA activation. PDE4D3 was preincubated with or without PA (100 $\mu\text{g/ml}$). The enzyme was then diluted to decrease the concentration of PA to 1 $\mu\text{g/ml}$, and the PDE activity was measured.

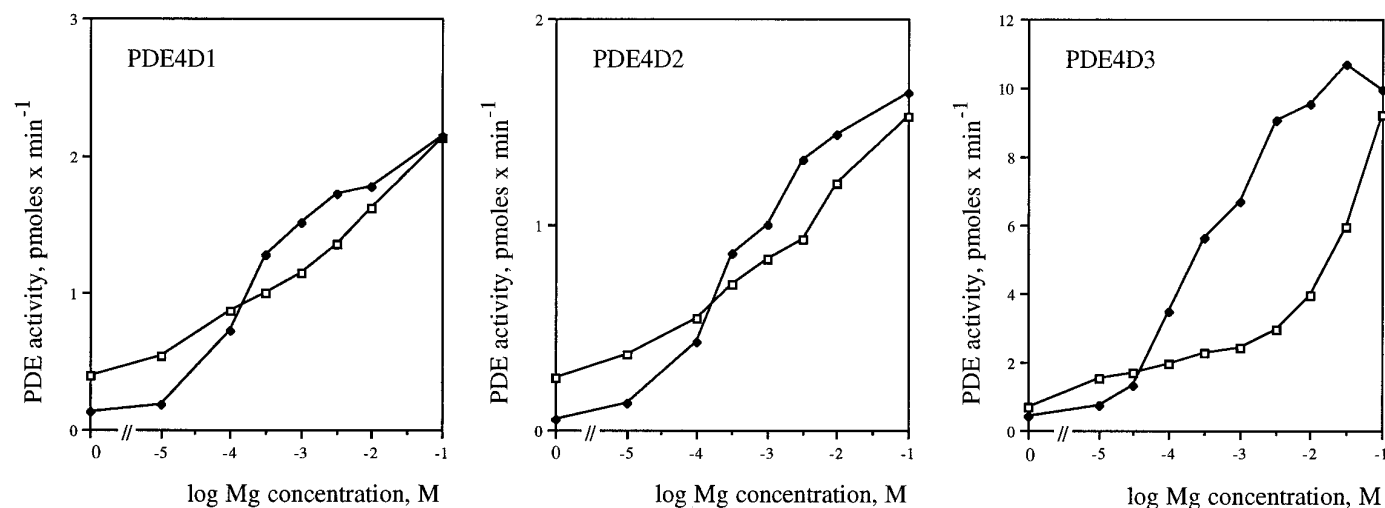


Fig. 5. Effect of the addition of PA on the requirement for Mg^{2+} of PDE4D1, PDE4D2, and PDE4D3 recombinant isoforms expressed in MA-10 cells. PDE was assayed in the (◆) presence or (□) absence of 200 $\mu\text{g}/\text{ml}$ PA. Results are from one experiment representative of three independent experiments.

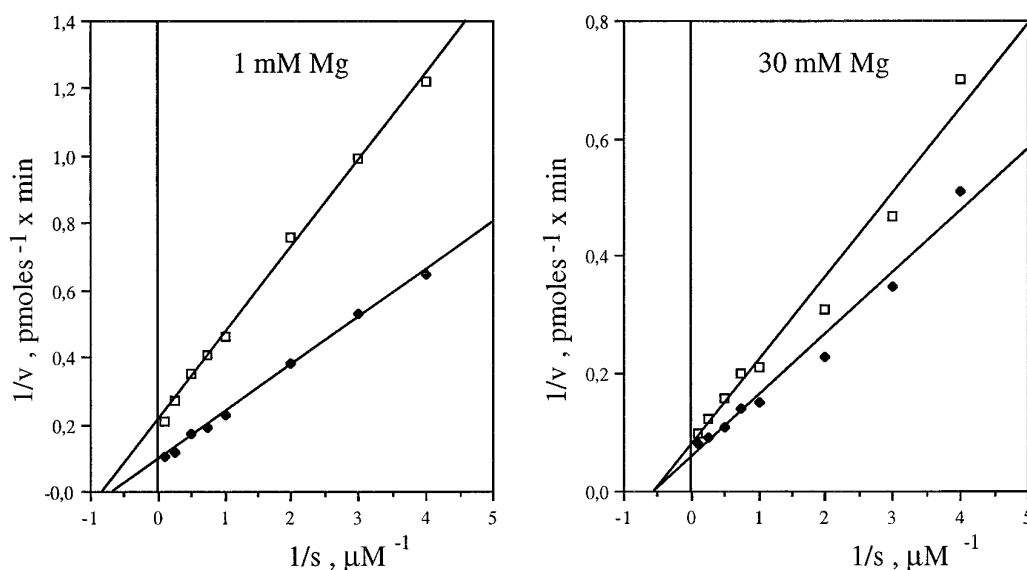


Fig. 6. Kinetics of cAMP hydrolysis by recombinant PDE4D3 expressed in MA-10 cells. PDE activity was assayed in the (◆) presence or (□) absence of 200 $\mu\text{g}/\text{ml}$ PA. *Left*, PDE activity was assayed in the presence of 1 mM Mg^{2+} . K_m and V_m values were 1.23 μM and 4.71 pmol/min without PA and 1.54 μM and 11.0 pmol/min with PA, respectively. *Right*, PDE activity was assayed in the presence of 30 mM Mg^{2+} . K_m and V_m values were 1.95 μM and 12.69 pmol/min without PA and 1.95 μM and 18.83 pmol/min with PA, respectively.

PA still induced a significant activation of PDE4D3 under these conditions ($+64 \pm 4\%$, mean \pm standard error of three independent experiments, $p < 0.01$; data not shown), although the activation was smaller than that obtained with 100 $\mu\text{g}/\text{ml}$ PA in the final assay ($+187 \pm 33\%$). It is worth noting that 1 $\mu\text{g}/\text{ml}$ PA did not exert an effect on PDE4D3 activity when no previous incubation of the enzyme with higher PA concentration was carried out (Fig. 4). This suggested that PA may remain tightly bound to the enzyme and maintain activation on dilution. To confirm this point, an immunoprecipitation experiment was performed. PDE4D3 was incubated for 2 hr with or without PA in the presence of a monoclonal anti-PDE4D antibody (M3S1) previously adsorbed to protein G/Sepharose. At the end of the incubation, the Sepharose beads were thoroughly washed before measurement of the bound PDE activity. Fig. 10 shows that the enzyme preincubated with PA was more active than control (by $\sim 70\%$), suggesting that PA activation was maintained on immunoprecipitation under conditions in which no free PA was present.

Discussion

It has been known for years that some naturally occurring lipids, particularly phospholipids, can activate calmodulin-dependent PDE *in vitro* (39, 40). Phospholipids have also been shown to activate low- K_m particulate PDEs in microsomes and plasma membranes of adipocytes (41). The regulation of PDEs by phospholipids has gained renewed interest in view of the finding that phospholipid metabolites, particularly PA, are messengers involved in many physiological functions. The effect of PA on PDE4 isoforms partially purified from rat thymocytes (17) or human promonocytic cells (42) has been reported, and it was hypothesized that this activation may play a signaling role in cells in which both the cAMP pathway and the phospholipid turnover exert a control on physiological responses.

In the current study, we have shown that PA induces activation of long PDE4A5, PDE4B1, and PDE4D3 isoforms without affecting the short PDE4 variants from the same genes. The activation is specific for anionic phospholipids

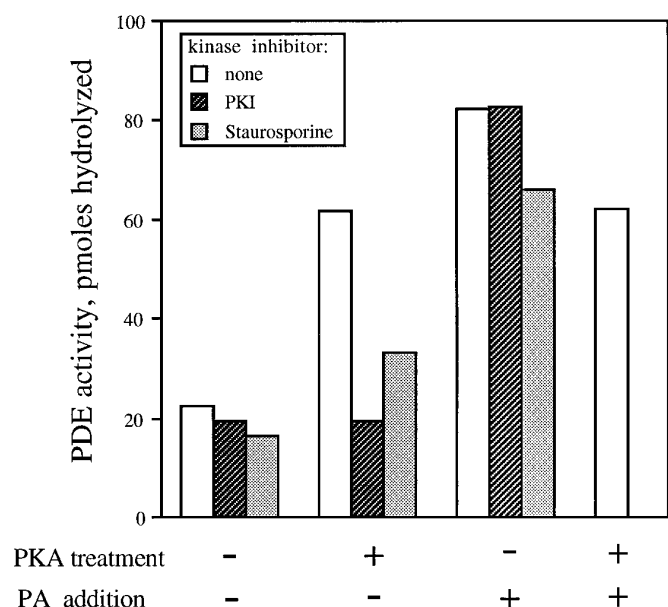


Fig. 7. Effect of kinase inhibitors or PKA pretreatment on PA stimulation of recombinant PDE4D3 expressed in MA-10 cells. Recombinant PDE4D3 was incubated in the presence or absence of exogenous ATP and PKA catalytic subunit, before PDE assay. PDE was assayed in the presence or absence of 200 $\mu\text{g/ml}$ PA. PKI (2 $\mu\text{g/ml}$), staurosporine (1 μM), or no inhibitor (*none*) was added to the phosphorylation medium and to the PDE assay medium. Results are from one experiment representative of three independent experiments.

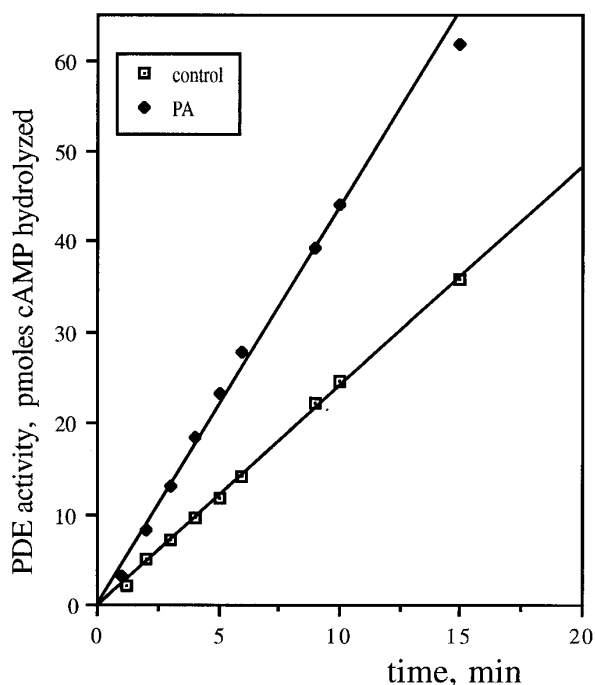


Fig. 8. Time course of the effect of PA on recombinant PDE4D3 expressed in MA-10 cells. PA was added at time 0 to the assay medium, and cAMP hydrolyzed at different times was assayed. A parallel control incubation was run without the addition of PA. Results are from one experiment representative of two independent experiments performed with similar results.

because PS produced a similar effect, whereas PC was unable to activate either long or short PDE4 variants.

It is noteworthy that the PDE4D3 isoform has been previously shown to be activated by a PKA-dependent phosphor-

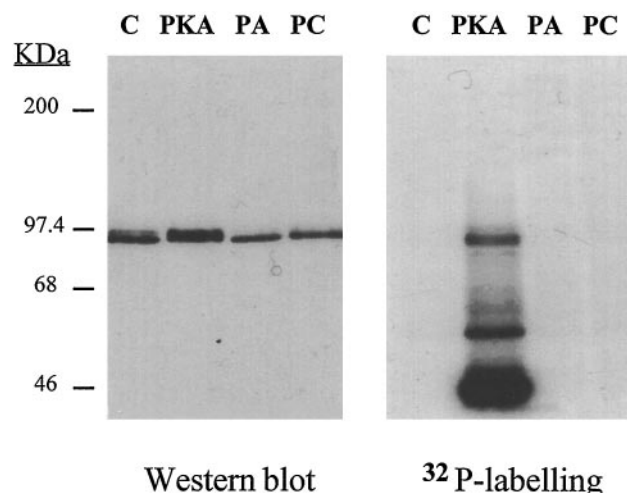


Fig. 9. Effect of PKA or phospholipid treatment on the incorporation of ^{32}P in PDE4D3 in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Recombinant PDE4D3 expressed in Sf9 cells was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of PKA, PA, or PC or without any addition (C). The samples were then submitted to SDS-PAGE, and the separated proteins were transferred onto an Immobilon P membrane. The membrane was autoradiographed (*right*) and then underwent Western blotting analysis by using a PDE4-specific antibody, K116 (30) (*left*).

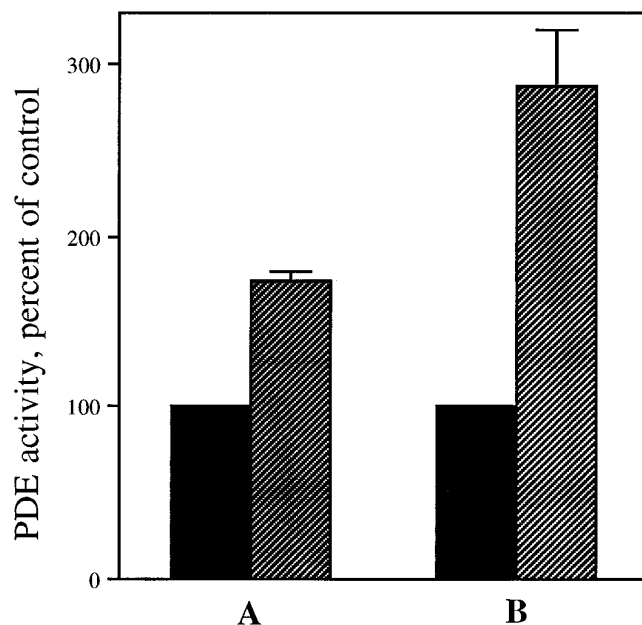


Fig. 10. PDE4D3 activation by PA is partially preserved on immunoprecipitation of the enzyme. A, Recombinant PDE4D3 obtained by expression in MA-10 cells was incubated with an anti-PDE4D antibody, M3S1 (30), complexed to protein G/Sepharose beads in the (*filled bars*) absence or (*hatched bars*) presence of 100 $\mu\text{g/ml}$ PA. PDE activity in the immunoprecipitation pellets was then evaluated in the absence of added PA. Preincubation with PA significantly increased PDE activity by $73 \pm 7\%$ (mean \pm standard error of three independent determinations, $p < 0.01$). B, For comparison, untreated PDE4D3 preparation was assayed for PDE activity in the (*filled bars*) presence or (*hatched bars*) absence of 100 $\mu\text{g/ml}$ PA.

ylation (28, 30, 32). PDE4D3 activation by PA very closely resembles the activation by phosphorylation. In both cases, activation correlated with a higher affinity of the enzyme for the cofactor Mg^{2+} and with similar increases in the V_{max} value of the enzyme (32). Furthermore, the two effects were

not additive, and PDE4D3 activated by PKA could not be further activated by PA, which might suggest that both effectors induced the same phosphorylation of the protein. The reported ability of PA to stimulate some PKC isoforms, and even specific kinases (13, 15, 35), supports this hypothesis. However, evidence that the effect of PA was not mediated by PKA or by other kinases present in the preparation of enzyme that was used was provided by experiments in which [γ - ^{32}P]ATP as substrate or protein kinase inhibitors were used. Although PKA induced ^{32}P incorporation in the PDE4D3 polypeptide, no incorporation was observed under conditions in which PA activated PDE4D3. Furthermore, the activation by PKA was completely suppressed by the addition of a PKA inhibitor (PKI), whereas the inhibitor had no effect on the activation of PDE4D3 by PA. Similarly, staurosporine, an inhibitor of a wide array of kinases, did not prevent the effect of PA on PDE4D3. We conclude that although the actions occur through different mechanisms, phosphorylation by PKA and activation by PA induce a similar conformational change of the enzyme, and PDE4D3 that is activated by either mechanism cannot be further activated. It is interesting to notice that although the long PDE4D3 variant is activated by PKA and by PA, the short variants encoded by the same gene are not. The difference between these variants resides in their amino-terminal region (28). The phosphorylation site important for *in vitro* PDE4D3 activation by PKA was recently mapped to Ser54 (43), in the region not present in the PDE4D1 variant. It is possible that PA induces PDE4D3 activation by interacting with the amino-terminal region of the enzyme and that both phosphorylation of Ser54 and interaction with PA produce a conformational change in the enzyme, removing an inhibitory constraint. Because chelation or the addition of free calcium ions to the medium did not affect the stimulating effect of PA, a calcium-dependent mechanism for PA/PDE interaction can be excluded.

PA has been shown to act as an allosteric activator of PLC- γ 1, and it has been hypothesized to bind to a phospholipid-binding site of the enzyme (14). Furthermore, a PS-binding site has been identified on PKC- γ and PS decarboxylase, and a consensus sequence has been implicated by using synthetic peptides (37). It is possible that PA activates PDE4D3 by binding to a specific sequence in the amino-terminal region of the enzyme. In fact, part of the activation by PA was maintained after washing the enzyme immunoadsorbed to protein G/Sepharose beads, which suggests a tight but reversible binding of the phospholipid on the enzyme. However, competition experiments with synthetic peptides and direct measurement of PA binding are needed to prove this hypothesis. An alternative explanation for PA activation could be that the lipid activator binds to a protein, which, in turn, interacts with PDE.

The amino-terminal region of PDE4D3 includes a sequence called UCR 1, which is highly conserved in the long variants of the other PDE4 genes (26). If the hypothesis of a specific binding site for PA on the enzyme holds true, it can be supposed that it is included in this UCR 1 region because the three variants that bear it are sensitive to PA activation. Because the phosphorylation site involved in the PKA-mediated activation of rat PDE4D3 also resides in this region, it is suggested that UCR1 is part of a regulatory domain of PDE4D3 and possibly of the other long PDE4 isoforms.

The demonstration of the activation of certain PDE iso-

forms by the binding of PA or PS would then open the possibility of a reversible interaction of the enzymes with cell membrane by translocation. Indeed, it has been shown that PKC preferentially interacts with PS-enriched domains formed in the membrane in the presence of bound proteins (44). Further studies will be required to shed light on this point.

The current results suggest that part of the signaling mediated by intracellular formation of PA in various cell types might rest on the stimulation of the catabolism of cAMP and a decrease in cAMP levels, with ensuing physiological responses. Such cross-talk between the cAMP pathway and the phospholipid metabolism could be of considerable importance in the mechanism of action of different hormones and growth factors. Because PDE4s constitute well-recognized targets for drug action, especially in the field of anti-inflammatory therapeutics (45), it will be important to characterize PDE regulation by the mediators stemming from phospholipid metabolism.

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