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The cAMP-specific Phosphodiesterase PDE4D3 Is Regulated by Phosphatidic Acid Binding

CONSEQUENCES FOR cAMP SIGNALING PATHWAY AND CHARACTERIZATION OF A PHOSPHATIDIC ACID BINDING SITE*

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Hormones and growth factors induce in many cell types the production of phosphatidic acid (PA), which has been proposed to play a role as a second messenger. We have previously shown in an acellular system that PA selectively stimulates certain isoforms of type 4 cAMP-phosphodiesterases (PDE4). Here we studied the effect of endogenous PA on PDE activity of transiently transfected MA10 cells overexpressing the PA-sensitive isoform PDE4D3. Cell treatment with inhibitors of PA degradation, including propranolol, induced an accumulation of endogenous PA accompanied by a stimulation of PDE activity and a significant decrease in both cAMP levels and protein kinase A activity. Furthermore, in FRTL5 cells, which natively express PDE4D3, pretreatment with compounds inducing PA accumulation prevented both cAMP increase and cAMP-responsive element-binding protein phosphorylation triggered by thyroid-stimulating hormone. To determine the mechanism of PDE stimulation by PA, endogenous phospholipids were labeled by preincubating MA10 cells overexpressing PDE4D3 with [^{32}P]orthophosphate. Immunoprecipitation experiments showed that PA was specifically bound to PDE4D3, supporting the hypothesis that PDE4D3 activation occurs through direct binding of PA to the protein. PA binding site on PDE4D3 was characterized by engineering deletions of selected regions in the N-terminal regulatory domain of the enzyme. Deletion of amino acid residues 31–59 suppressed both PA-activating effect and PA binding, suggesting that this region rich in basic and hydrophobic residues contains the PA binding site. These observations strongly suggest that endogenous PA can modulate cAMP levels in intact cells, through a direct activation of PDE4D3.

Phosphatidic acid (PA)[1] is a quantitatively minor phospholipid produced in the cell via different pathways and in particular as a result of phospholipase D or diacylglycerol kinase activation. Although it has long been considered a mere intermediate in the biosynthetic pathways of the major phospholipids or a precursor of lipid mediators such as diacylglycerol and lyso-PA, it is now suspected to play a role as a second messenger involved in several signal transduction pathways (1, 2). In response to hormones or growth factors, PA accumulates very rapidly in a large number of cell types (3). A number of studies have implicated PA in a variety of cellular functions. PA appears to be involved in cell proliferation induced by growth factors (4–6) or cytokines (7). Phospholipase D overexpression promotes cytoskeletal reorganization (8), and phospholipase D-mediated PA generation is an essential step in the stimulation of actin stress fiber assembly (9). A role of PA in membrane trafficking is supported by several observations (10–12). On the other hand, many reports have pointed to the ability of PA to modify, in cell-free conditions, the activity of proteins and enzymes playing key roles in signal transduction. Examples include phospholipase Cγ1 (13), the Ras-GTPase activating protein (14, 15), phosphatidylinositol-4-phosphate 5-kinase (16, 17), phosphatase PTP1C (18), and, directly relevant to the present work, a subset of cAMP-phosphodiesterases (19, 20). This nonexhaustive list of PA-sensitive proteins underscores the potential role of PA as an important regulator of cell functions. However, by comparison, rather little information is available on the functional significance of the regulations observed with broken cell preparations. Raf-1 kinase function constitutes one of the few cases in which the essential role of PA has been demonstrated. Indeed, a specific PA association to the Raf-1 protein has been characterized (21), and pharmacological or genetic blockade of PA synthesis has been shown to prevent the Raf-1 kinase translocation triggered by phorbol esters in Madin-Darby canine kidney cells (21) or by insulin in Rat-1 fibroblasts (22). It thus appears that PA takes part in the Ras-mitogen-activated protein kinase signaling pathway by controlling recruitment to the membrane and activation of Raf-1.

We have previously observed that PA selectively activates some isoforms of the type 4 PDE family *in vitro* (19). The type 4 PDEs are expressed from four genes, which give rise to numerous isoforms deriving from alternative splicing and usage of different promoters (23, 24). They have in common the specificity for cAMP hydrolysis and the sensitivity to inhibition by the antidepressant compound rolipram. The activity of type

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[1] The abbreviations used are: PA, phosphatidic acid; PCR, polymerase chain reaction; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; UCR, upstream conserved region; TSH, thyroid-stimulating hormone; CREB, cAMP-responsive element-binding protein; PBS, phosphate-buffered saline.

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4 PDEs is closely involved in the control of cAMP levels in a number of cell models, including lymphocytes (25, 26). The "long isoforms" expressed from the four genes coding for PDE4s are characterized by the presence of a conserved sequence termed UCR1 in their N-terminal regulatory domain (24). Only long PDE4 isoforms were found to be sensitive to PA activation (19). PA rapidly accumulates in rat thymic lymphocytes and human peripheral blood lymphocytes, following a mitogenic stimulation. This response precedes an increase in the activity of cAMP-phosphodiesterase and a lowering of cAMP levels. Furthermore, we observed that a pharmacological blockade of PA synthesis induced both an increase in cAMP levels and suppression of the lymphoproliferative response. We thus hypothesized that the regulation of PDE4 by PA has a functional relevance in the lymphoid cells, through its influence on levels of cAMP, a major negative effector of cell response (27–29).

The present study aims to investigate the hypothesis of the physiological relevance of PDE4 regulation by PA and to shed some light on the biochemical mechanism of this regulation. We investigated the effects of pharmacological treatments able to induce an accumulation of endogenous PA in intact cells and observed that they can alter the activity of type 4 PDEs and have marked consequences on the cAMP signaling pathway. This effect was observed with transfected MA10 cells overexpressing the PA-sensitive PDE4D3 isoform as well as with FRTL5 cells, which natively express PDE4D3, suggesting that modulation of the cAMP pathway by the action of PA on phosphodiesterase may have functional implications in various cell types. We have detected direct binding of PA to PDE4D3 and investigated the mechanism of PA-induced PDE4D3 activation by creating deletion mutations in the N-terminal regulatory domain of the enzyme. We have thus identified a region rich in basic and hydrophobic residues, overlapping into the UCR1 domain, that is required for PA association and PA-induced activation of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—*Pwo* DNA polymerase and dNTP were from Roche Molecular Biochemicals. Restriction enzymes were from Roche Molecular Biochemicals or Amersham Pharmacia Biotech. T4 DNA ligase, calf intestinal alkaline phosphatase, Shephaglas TM Band Prep Kit, protein G-Sepharose, ECL Western blot detection kit, and [^{3}H]cAMP were obtained from Amersham Pharmacia Biotech. Plasmid Maxi kit or Plasmid Mini kit were supplied by Qiagen. Fetal calf serum was from Life Technologies, Inc. Immobilon membrane was from Millipore Corp. AG1-X8 resin was from Bio-Rad. [^{3}H]Arachidonic acid (7.4 TBq/mmol) was from Isotopchim (Ganagobie-Peyruis, France). cAMP ^{125}I radioimmunoassay kit and [^{32}P]orthophosphoric acid were from PerkinElmer Life Sciences. Silicagel G60 plates were from Merck. All solvents were of analytical grade and were purchased from SDS (Peypin, France). Waymouth MB 752/1 medium, Coon's modified Ham's F-12 medium (Coon's F-12), Minimum essential Eagle's medium without sodium phosphate, horse serum, bovine insulin, bovine TSH, human transferrin, glutamine, bovine serum albumin (fraction V), PA from egg yolk lecithin, gelatin, penicillin, streptomycin, gentamicin, pepstatin, leupeptin, aprotinin, staurosporine, desipramine, racemic propranolol, *R*-propranolol, *S*-propranolol, and *Crotalus atrox* snake venom were from Sigma/Aldrich. Anti-phospho-CREB rabbit polyclonal antibody was purchased from New England Biolabs (Beverly, MA).

PCR Amplification—The oligonucleotides used as primers for the PCR amplifications were as follows. A (5′-GC**GAATTC**GATATGGACAATGGCACATCA-3′) corresponded to the rat PDE4D3 sequence from base 49 to base 72. The G^{55} was replaced by an A in order to create an ATG start codon (underlined). At the 5′-end, the additional 5 bases created an *Eco*RI site (in boldface type). B (5′-GTTTCTGGT**AGGCCT**CCTCTGTG-3′) corresponded to the antisense sequence of rat PDE4D3 from base 396 to base 418 and included a *Stu*I site (in boldface type). C (5′-CTC**CCTTGG**ACAGCGATTATGACCTCTCTC-3′) corresponded to the sequence of rat PDE4D3 from base 178 to base 197. The additional 8 bases at the 5′-end created a *Sty*I site (in boldface type). D (5′-CTAGTT**CCTTGG**CCAGGACATC-3′) corresponded to the antisense sequence of rat PDE4D3 from base 766 to base 787 and included a *Sty*I site (in boldface type). E (5′-CGAC**TTCGAA**CCATCACAGAGGAGGCCTACCAG-3′) corresponded to the sequence of rat PDE4D3 from base 391 to base 414. At the 5′-end the additional 9 bases created a *Sfu*I site (in boldface type). F (5′-GGTTAG**TTCGAA**CTGGAATTTTTCAGT-3′) corresponded to the antisense sequence of rat PDE4D3 from base 1783 to base 1809 and included a *Sfu*I site (in boldface type).

PCRs were performed with 2.5 units of *Pwo* DNA polymerase and rat PDE4D3 cDNA as template in a buffer containing a 0.5 μM concentration of each primer, a 0.2 mM concentration of each dNTP, 20 mM Tris-HCl, pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. The reaction mixture was subjected to 20 cycles of denaturation (1 min, 94 °C), annealing (30 s, 55 °C), and elongation (45 s, 72 °C). The PCR products were separated by electrophoresis on a 1% agarose gel with ethidium bromide and visualized under UV light. DNA bands of the correct size were excised and purified from the gel with the Amersham Pharmacia Biotech Band Prep Kit following the instructions of the manufacturer.

Preparation of the Deletion Mutant Constructs—The purified PCR products were digested by the appropriate restriction enzymes and were inserted with the T4 DNA ligase into the pCMV5-PDE4D3 vector (30) previously restricted with the corresponding restriction enzymes and dephosphorylated by alkaline phosphatase (with the exception of mutant construct Δ(31–59), which was first inserted in pBS-skt plasmid). All of the mutated rat PDE4D3 cDNAs were finally inserted into the *Eco*RI site of the pCMV5 polylinker. *Escherichia coli* strain DH5α or NM522 was transformed with the rat PDE4D3-pCMV5 mutant constructs using the conventional procedure described in Ref. 31. Plasmid DNAs were prepared from the ampicillin-resistant transformed clones using the Plasmid Mini kit from Qiagen, and were examined by restriction enzyme analysis for the presence of the insert and for its correct orientation.

For mutant Δ(2–19), primers A and B were used to amplify a fragment of 374 bases, starting at the 3′-end of the sequence to delete, and ending at the *Stu*I site of PDE4D3 cDNA. The PCR fragment was digested by *Eco*RI and *Stu*I. Rat PDE4D3 cDNA was also digested by *Eco*RI and *Stu*I. The resulting largest fragment, of 1671 base pairs (starting with the *Stu*I site and ending at the 3′-extremity of the PDE4D3 cDNA by an *Eco*RI site) was reinserted together with the digested PCR product in the *Eco*RI site of the pCMV5 polylinker. The resulting mutant construct is deleted of the DNA sequence coding for amino acids 2–19 of the PDE4D3 protein, MHVNNFPFRRHSWICFDV. For mutant Δ(31–59), the sequence of 87 bases to delete is at the 3′-position of the first of two *Sty*I sites on rat PDE4D3 cDNA. Since there are several *Sty*I sites in pCMV5, PDE4D3 cDNA was subcloned in another vector, pBS-skt in the *Eco*RI site of the polylinker. pBS-PDE4D3 was restricted by *Sty*I. A fragment of 690 base pairs between the two *Sty*I sites of PDE4D3 cDNA was eliminated. Primers C and D were used to amplify by PCR a fragment of 617 base pairs, starting at the 3′-end of the sequence to delete and ending at the second *Sty*I site on PDE4D3 cDNA. This PCR product was restricted by *Sty*I and ligated into the *Sty*I-digested and dephosphorylated pBS-PDE4D3. The deleted PDE4D3 cDNA was cloned back into pCMV5 at the *Eco*RI site of the polylinker. This mutant construct is deleted of the DNA sequence coding for amino acids 31–59 of the PDE4D3 protein, DPMTSPGSGLILQANFVHSQRRESFLYRS. For mutant Δ99–130, the 96-base sequence to delete is at the 3′-position of the first of two *Sfu*I sites on the PDE4D3 cDNA. The vector pCMV5-PDE4D3 was restricted by *Sfu*I, and the sequence of 1508 base pairs between the two *Sfu*I sites was eliminated. Primers E and F were used to amplify by PCR a fragment of 1428 base pairs, starting at the 3′-end of the sequence to delete and ending at the second *Sfu*I site on the PDE4D3 cDNA. This PCR product was restricted by *Sty*I and ligated into the *Sfu*I-digested and dephosphorylated pCMV5-PDE4D3. This mutant construct is deleted of the DNA sequence coding for the amino acids 99–130 of the PDE4D3 protein, TVRNNFAALTNLQDRAPSKRSPMCNQPSINKATIT.

Cell Cultures—Rat thyroid follicular FRTL-5 cells were cultured in Coon's F-12 medium supplemented as described previously (32). Mouse Leydig tumor cells (MA10) were grown in Waymouth MB752/1 medium modified as previously reported (33).

Transient Transfection of MA10 Cells with Wild Type or Mutant PDE4D—Untransfected or mock-transfected MA10 cells express no immunodetectable amounts of type 4 PDE, and PDE activity of cell homogenates is insensitive to activation by PA (19). Therefore, the selective overexpression of individual PDE4D isoforms, which increased cell PDE activity by 20–100-fold above basal level, allowed us to study the effects of endogenous PA accumulation on a well defined PDE4 isoform. MA10 cells were transiently transfected using the calcium phosphate procedure as described previously (34), with 10–15 μg of

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vector pCMV5 containing the corresponding cDNA. Mock-transfected cells were treated in the same way but did not receive plasmidic DNA.

Metabolic Labeling of MA10 Cells with [^{32}P]Orthophosphate—24 h after the transfection, MA10 cells were incubated in Waymouth medium in which the serum was replaced by 0.1% bovine serum albumin for 2 h. The cells were then washed twice with phosphate-free minimal essential medium containing 0.1% bovine serum albumin and incubated with 0.25–0.5 mCi/ml [^{32}P]orthophosphate for additional 2 h. During the last 30 min of the labeling incubation, 250 μM propranolol was added to the cells. At the end of the treatment, cells were washed three times with cold PBS, harvested, and homogenized, and PDE was immunoprecipitated as described below.

Immunoprecipitation of Mutant or Wild Type PDE4D Isoforms—Cells were harvested by scraping into the homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM EGTA, 0.04% 2-mercaptoethanol, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and 2 mM phenylmethylsulfonyl fluoride. They were homogenized using a Dounce homogenizer, by 2 × 30 strokes and centrifuged for 10 min at 14,000 × g and 4 °C. The supernatant was incubated at 4 °C for 2 h under continuous shaking with the monoclonal anti-PDE4D antibody M3S1 (32) preadsorbed onto protein G-Sepharose beads (a 1:3 suspension of beads in phosphate-buffered saline containing 0.05% gelatin (PBS-gelatin) and the antibody (1:50) was shaken for 90 min at 4 °C; the complex was then washed once with 20 mM Tris-HCl, pH 7.8, 0.5 M NaCl and twice with 20 mM Tris-HCl, pH 7.8). At the end of the incubation, complexes immunoadsorbed to the beads were washed four times with PBS-gelatin and separated into two fractions. One part was subjected to a lipid extraction followed by phospholipid analysis and PA quantification. The second part of the immunoprecipitate was resuspended in PBS containing 1% SDS, shaken for 15 min at room temperature, and the supernatant was diluted in sample buffer to be analyzed for PDE4 expression by Western blotting as described below.

Western Blot Analyses—Immunoprecipitated proteins or cell extracts were diluted in Laemmli sample buffer, submitted to SDS-polyacrylamide gel electrophoresis, transferred onto an Immobilon-P membrane (Millipore), and analyzed by Western blotting using either the rabbit polyclonal K116 antiserum (1:500) for PDE4 detection (32) or rabbit polyclonal anti-phospho-CREB antibody (1:1000) for the detection of CREB phosphorylated on serine 133. Second antibody incubation was carried out with anti-rabbit-IgG conjugated to peroxidase, and immunoreactive bands were detected by the ECL method (Amersham Pharmacia Biotech).

Lipid Extraction, Phospholipid Separation, and PA Quantification—For the immunoprecipitated samples, the final immunoprecipitation pellet was resuspended in 0.5 ml of HCl-PBS, pH 3. For MA10 cell extracts, cells were first labeled for 2 h with 1 μCi of [^{3}H]arachidonic acid and then treated with 0.25 mM propranolol. At the end of the incubation, cells were washed twice with ice-cold PBS and scraped from the cell culture dishes with 0.5 ml of HCl-PBS, pH 3. Then, in both cases, lipids were extracted by the Bligh and Dyer (35) method in the presence of 50 μM butylhydroxylated toluene as an antioxidant. PA was separated from the other phospholipids by a bidimensional TLC as described by Meskini *et al.* (36). TLC plates were stained by Coomassie Brilliant Blue according to Nakamura and Handa (37). PA was quantified by videodensitometry (Bioprofil Vilber Lourmat/Fröbel) in comparison with standards of egg yolk PA chromatographed on the same plate. Phospholipid spots were scraped and mixed with Picofluor (Packard) in order to determine their radioactivity by liquid scintillation counting. The PA-associated ^{3}H radioactivity was expressed as a percentage of the total phospholipid-incorporated radioactivity.

For the analysis of lipid extracts obtained from the immunoprecipitation pellets, unlabeled egg yolk PA was added to the samples before the bidimensional TLC separation. The TLC plates were then stained and subjected to an autoradiography. PA-associated ^{32}P radioactivity was quantified by videodensitometry analysis of the autoradiography films.

cAMP Assay—Before harvesting, cells were washed twice with ice-cold PBS, and 0.5 ml of ice cold 10% trichloroacetic acid was added. 15 min × 10,000 × g supernatants were extracted five times with water-saturated diethylether to eliminate trichloroacetic acid, and cAMP was assayed by radioimmune assay according to the manufacturers' recommendations, using the acetylation procedure.

PKA Assay—MA10 cells were washed twice with ice-cold PBS, scraped into 200 μl of hypotonic buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2 mM EGTA, 30 mM NaF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin) and homogenized in a Dounce glass/glass homogenizer by 30 strokes. The homogenate was centrifuged for 10 min at 10,000 × g and 4 °C. cAMP-dependent protein kinase activity was quantified by measuring the incorporation of labeled phosphate from [γ-^{32}P]ATP into the synthetic peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), as described in Ref. 38. PKA activity was evaluated as the fraction of activity specifically inhibited by protein kinase inhibitor Sigma catolog number P 0300.

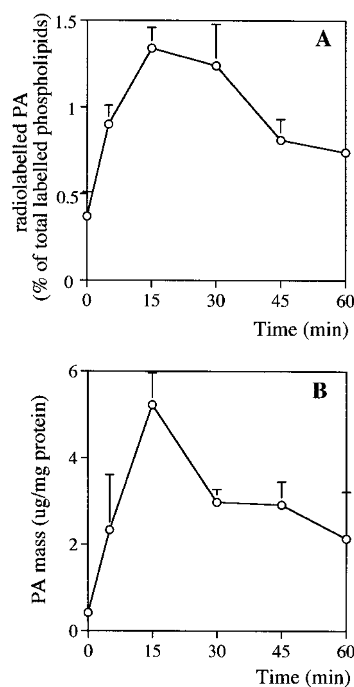

FIG. 1. **Propranolol increases the PA content of MA10 cells.** MA10 cells were labeled with 0.5 μCi/ml [^{3}H]arachidonic acid for 2 h, washed, and treated with 250 μM (R/S)-propranolol for the indicated times. Cells were harvested in PBS-HCl, pH 3, and lipids were extracted, separated by bidimensional TLC, and dyed with Coomassie Blue. *A*, phosphatidic acid was quantified by measuring the radioactivity of the PA spot. *B*, PA mass was evaluated by videodensitometry analysis of the PA spot by comparison with PA standards chromatographed on the same plate. Results are means ± S.E. of three independent experiments.

Adenylyl Cyclase Assay—FRTL5 cell membranes were prepared from quiescent cells (cultivated for 24 h without serum and hormones), according to the procedure described in Ref. 39. Adenylyl cyclase activity was assayed according to Ref. 40, with some modifications (1 mM isobutylmethylxanthine, 1 mM EDTA, 5 units/ml adenosine deaminase were added to the assay medium) in the presence or absence of 0.5 units/ml TSH.

Preparation of Phosphatidic Acid Suspension—To evaluate the sensitivity of PDE4D preparations to activation by PA, PA stock solution in chloroform was evaporated under nitrogen flux, and PA was resuspended at a concentration of 400 μg/ml in Tris-HCl, pH 8.0. Suspension was obtained by sonicating the lipid film with a probe sonicator (three cycles of 15 s). PA suspension was then appropriately diluted in the PDE assay buffer.

PDE Assay—PDE activity was assayed by a two-step radioisotopic procedure, using [^{3}H]cAMP as substrate, as described in Ref. 38.

RESULTS

Effect of Inhibitors of PA Degradation on PA Levels in MA10 Cells—In an attempt to demonstrate the functional relevance of PDE4 regulation by PA in intact cells, transiently transfected MA10 Leydig tumor cells overexpressing different isoforms of PDE4 were studied. To increase PA levels in these cells, we used the conventional inhibitor of PA-phosphohydrolase, propranolol (41). Treatment of MA10 cells with 250 μM propranolol induced a marked accumulation of PA, reaching a maximum at 15 min (Fig. 1, *A* and *B*). At that time, PA mass was increased by 12-fold compared with the control levels. Such a PA accumulation probably reflects an efficient *de novo* phosphoglyceride synthesis in these cells. The smaller increase

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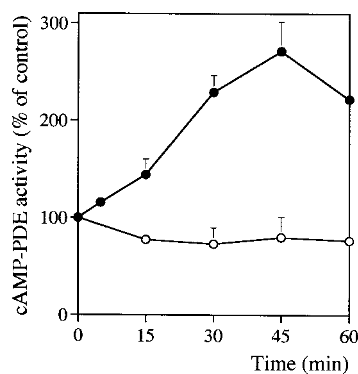

FIG. 2. **Propranolol selectively increases PDE activity in MA10 cells overexpressing PDE4D3.** MA10 cells grown in 10-cm dishes were transfected with 10 μg of plasmids pCMV5-PDE4D3 (*filled symbols*), or pCMV5-PDE4D1 (*open symbols*), using the calcium phosphate procedure. 24 h later, transfected cells were treated by 250 μM propranolol for the indicated times and homogenized, and cAMP-PDE activity was immediately assayed. Results are means ± S.E. of 3–6 independent experiments and are expressed as percentages of control values.

measured for radiolabeled PA (3-fold) may be attributed to a high rate of exchange of the labeled fatty acid moiety. However PA mass and labeled PA variations followed similar time courses, and both were still elevated after 1 h of propranolol treatment (5-fold for PA mass and 2-fold for [^{3}H]PA). Very similar changes in PA levels were induced by propranolol treatment in transfected MA10 cells overexpressing the PDE4D3 phosphodiesterase (not shown). We established that 250 μM propranolol was the most efficient concentration (not shown), as also observed by Lauritzen *et al.* (42) with rat Leydig cells. This concentration was shown to be nontoxic for Leydig cells for the duration of treatment (42).[2] Another amphiphilic and cationic PA-phosphohydrolase inhibitor, desipramine (43) (200 μM), produced a comparable PA accumulation in MA10 cells (not shown).

Effect of Inhibitors of PA Degradation on PDE Activity in MA10 Cells Overexpressing PDE4D Isoforms—We previously showed that PA selectively stimulates in cell-free preparations "long isoforms" of type 4 phosphodiesterases carrying a highly conserved region (UCR1) in their amino-terminal domain, whereas the "short isoforms," lacking this regulatory region, are insensitive to PA activation (19). We thus investigated whether propranolol-induced endogenous PA accumulation had an effect on PDE activity in MA10 cells overexpressing a "long" and a "short" PDE4D isoform, respectively. Fig. 2 shows that propranolol induced a strong stimulation of PDE activity in MA10 cells overexpressing the PDE4D3 long isoform. The activation was already marked at 15 min and peaked at 45 min of propranolol treatment (3-fold compared with the control value). Stimulation of PDE activity measured in the transfected cells is comparable with that observed *in vitro* with recombinant PDE4D3 and exogenous PA (19, 20). As a control, the absence of direct effect of propranolol on the activity of recombinant PDE4D3 in a broken cell assay was verified (not shown). Furthermore, propranolol treatment did not have any effect on the PDE activity of MA10 cells overexpressing the short PDE4D1 isoform, insensitive to PA activation in a cell-free assay (Fig. 2). We also observed PDE4D3 activation following a treatment of the transfected cells with two other PA phosphohydrolase inhibitors, desipramine (200 μM) and bromoenol lactone (44) (50 μM). Yet the levels of stimulation measured with both compounds were smaller as compared with

[2] M. Grange, C. Sette, M. Cuomo, M. Conti, M. Lagarde, A.-F. Prigent, and G. Némoz, unpublished observations.

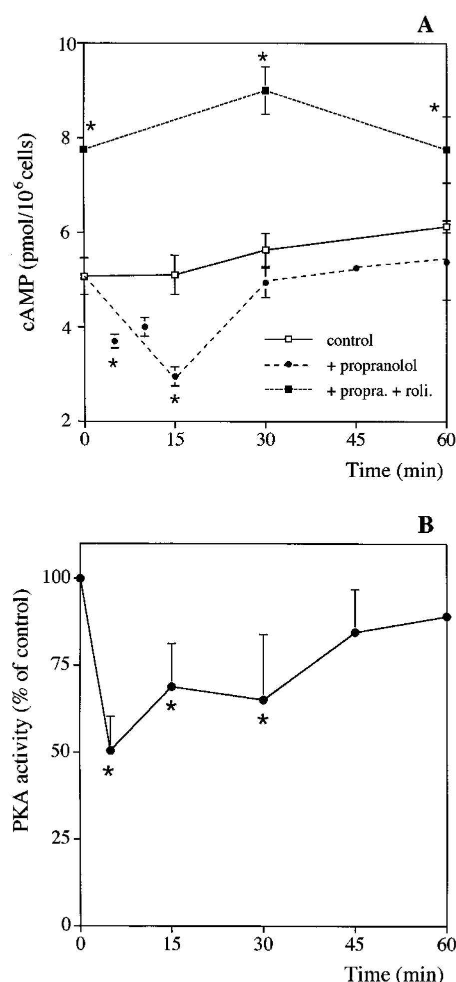

FIG. 3. **cAMP levels and PKA activity are decreased in MA10 cells overexpressing PDE4D3 under propranolol treatment.** MA10 cells transiently transfected with pCMV5-PDE4D3 plasmid were treated with 250 μM propranolol (●) or left untreated (□) for the indicated times. In some cases, cells were preincubated for 30 min with 10 μM rolipram, which remained present during the propranolol treatment (■). *A*, 10% trichloroacetic acid was added, and intracellular cAMP was then assayed by radioimmune assay. Results are means ± S.E. of 4–7 assays. *, significantly different from control values ($p < 0.05$). *B*, alternatively, cells were homogenized, and PKA activity was assayed by measuring the incorporation of [^{32}P]phosphate into Kemptide in the presence or absence of the PKA-specific protein kinase inhibitor. Results are means ± S.E. of 2–8 independent experiments. *, significantly different from the control ($p < 0.05$).

propranolol effect (69 and 75% above control value after a 45-min treatment for desipramine and bromoenol lactone, respectively; results not shown).

Propranolol Effect on cAMP Levels and PKA Activity in MA10 Cells Overexpressing PDE4D3—To evaluate the functional consequences of PDE activation caused by a treatment inducing PA accumulation, we assayed cAMP levels and cAMP-dependent protein kinase (PKA) activity of PDE4D3-overexpressing MA10 cells. The results presented in Fig. 3*A* show a significant decrease of cAMP levels (−25%) after 5 min of propranolol treatment. The cAMP decrease reached −40% after 15 min, and cAMP levels returned to near control values within 30 min. Preincubation of the PDE4D3-transfected cells with the PDE4-specific inhibitor rolipram (10 μM), followed by the propranolol treatment, increased cAMP levels to values higher than basal, showing that the cAMP decrease observed under propranolol treatment alone resulted from PDE4 activation (Fig. 3*A*).

The propranolol-induced changes in cAMP levels were ac-

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companied by a reduction in PKA activity, which reached 50% after 5 min of treatment. The activity was maintained significantly lower than control until 30 min and later returned to control values (Fig. 3*B*). In broken-cell control experiments, propranolol exerted a moderate inhibitory effect on PKA activity, at the highest concentrations used (−20 to −24% at 100–250 μM; not shown). This direct effect probably does not account for PKA inhibition in intact cells, because lower intracellular propranolol concentrations are most likely attained. Moreover, if the PKA activity decrease observed after propranolol treatment of intact cells were due to a direct inhibitory effect, a constant time-independent decrease could be expected rather than the transient effect we observed.

cAMP Levels and CREB Phosphorylation in FRTL5 Cells Are Reduced by Inhibitors of PA Degradation—A potential limit of the model of PDE-overexpressing cells is that a high PDE activity is likely to prevent cAMP accumulation in the cells, thus minimizing cAMP variations (33, 45). To bypass this problem, we then investigated propranolol effects on the FRTL5 rat thyroid cell line, since PDE4D3 is the predominant PDE4 isoform expressed in these cells (32). cAMP production in FRTL5 cells can be stimulated by the addition of TSH. We first demonstrated that the PA-phosphohydrolase inhibitor propranolol causes substantial PA accumulation in FRTL5 cells, in a manner similar to that observed in MA10 cells (not shown). Propranolol treatment of FRTL5 cells induced a more modest increase in PDE activity, as compared with PDE4D3-overexpressing MA10 cells (45% above control), possibly because other PDE isoforms, insensitive to PA, are also expressed in FRTL5 cells (32).

TSH stimulation of FRTL5 cells caused a rapid increase in intracellular cAMP levels that was totally suppressed by propranolol treatment (Fig. 4*A*). As the *S*-stereoisomer of propranolol displays β−adrenergic receptor antagonist properties (46), we examined the effect of each of the *R*- and *S*-stereoisomers in comparison with racemic propranolol. Either of the enantiomers had the same efficiency, in decreasing basal cAMP levels and in abolishing TSH-induced cAMP elevation in FRTL5 cells, as racemic propranolol (Fig. 4*B*). This observation rules out any β−adrenergic receptor-related effect of propranolol on FRTL5 cAMP levels. Furthermore, by assaying adenylyl cyclase activity of FRTL5 cell membranes, we observed that the addition of up to 250 μM propranolol to assay medium inhibited neither basal nor TSH-stimulated activity (not shown), which excludes any direct effect of the compound at the level of cAMP synthesis.

To investigate the functional significance of variations in cAMP levels induced by propranolol in FRTL5 cells, we evaluated TSH-induced phosphorylation of the transcription factor CREB. PKA-dependent phosphorylation of CREB on Ser^{133} is a critical step involved in the transcriptional gene regulation by the cAMP pathway (47). Western blot analyses performed with an anti-phospho-Ser^{133} CREB antibody revealed that TSH induced a rapid increase in CREB phosphorylation, already maximal at 2 min of treatment (not shown). TSH-induced CREB phosphorylation was totally suppressed by pretreatment of the cells with propranolol (250 μM) (Fig. 5*A*). Racemic propranolol, *R*- and *S*-propranolol, and desipramine (100 μM), were equally efficient in abolishing CREB phosphorylation induced by TSH (Fig. 5*B*). Since propranolol has also been described as a possible protein kinase C inhibitor *in vitro* (48), we studied the effects of the protein kinase C inhibitor staurosporine (49) on TSH-induced CREB phosphorylation in FRTL5 cells. Staurosporine (0.1 μM) was without effect on CREB phosphorylation (Fig. 5*B*), which shows that inhibition of CREB phosphorylation by propranolol did not involve protein kinase C inhibition.

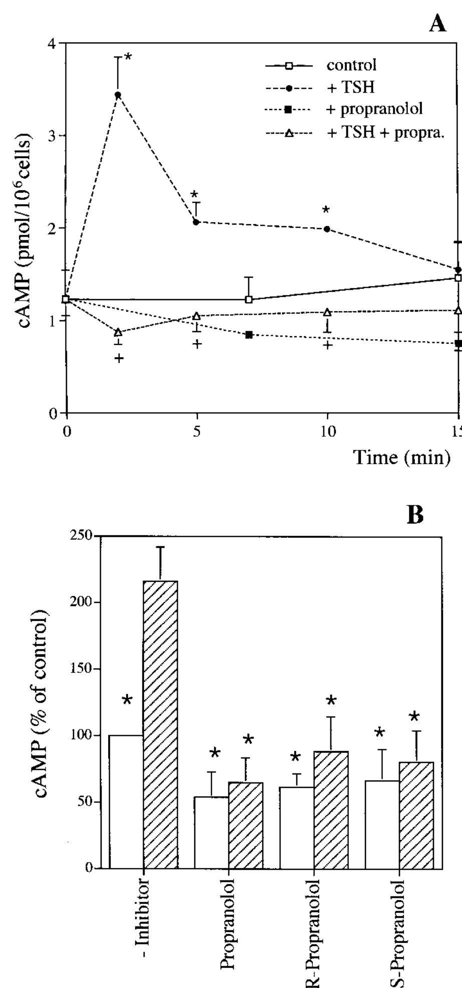

FIG. 4. **Propranolol prevents the cAMP-elevating effect of TSH on FRTL5 thyrocytes, in a nonstereospecific way.** *A*, 75% confluent FRTL5 cells were deprived of serum and hormones for 24 h. Cells were then treated with 250 μM R/S-propranolol for a total duration of 30 min; meanwhile, TSH (10 milliunits/ml) was added to part of the cells for the indicated period of time (△), while other cells did not receive any further treatment (■). Some cells were treated by TSH only (●), and control cells did not receive any treatment (□). 10% trichloroacetic acid was then added, and intracellular cAMP was assayed by radioimmune assay. Results are means ± S.E. of 4–6 assays. *, significantly different from the control at $t = 0$, $p < 0.05$; +, significantly different from the value with TSH without propranolol, measured at the same time, $p < 0.05$. *B*, FRTL5 cells were treated for 30 min by a 250 μM concentration of the tested compound, and 10 milliunits/ml TSH was added during the last 2 min (*hatched bars*) or not added (*empty bars*), and cAMP was assayed as above. Results are means ± S.E. of three or four independent experiments and are expressed as percentage of the control without inhibitor. *, significantly different from the value with TSH without propranolol, $p < 0.01$.

These results suggest that a pharmacologically induced accumulation of PA is able to prevent the PKA-mediated phosphorylation of CREB, in agreement with the observed suppression of the cAMP rise.

Expression and PA Sensitivity of PDE4D3 Deletion Mutants—We have previously proposed the hypothesis that PA activates the long isoforms of PDE through direct binding to the enzyme protein. Indeed, preliminary binding studies showed that exogenous radiolabeled PA binds to a recombinant PDE4D3 preparation in a saturable and displaceable manner, suggesting the presence of high affinity binding sites on the PDE4D3 isoform (20). Since PA does not activate short PDE4 isoforms lacking the N-terminal regulatory region containing the UCR1 domain (19), a localization of PA binding site within

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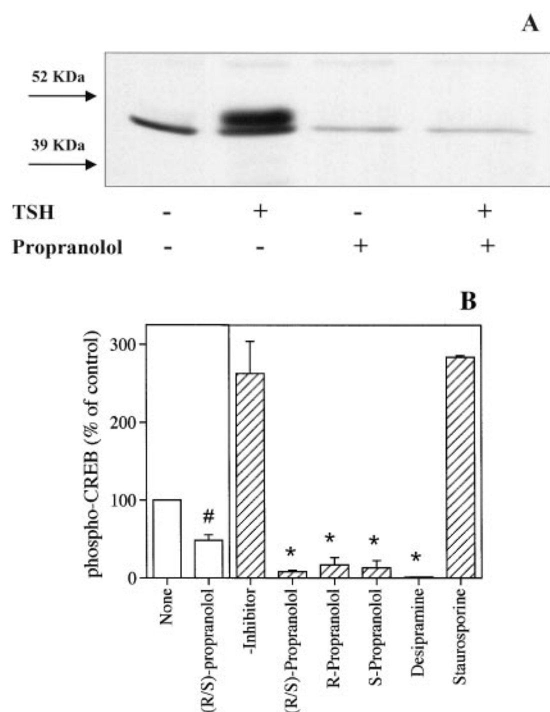

FIG. 5. **Racemic propranolol and *R*- and *S*-propranolol similarly abolish CREB phosphorylation in TSH-stimulated FRTL5 cells.** 75% confluent FRTL5 cells were cultured for 48 h without serum and hormones. The cells were then treated for 30 min with either 250 μM (R/S)-propranolol, 250 μM *R*-propranolol, 250 μM *S*-propranolol, 100 μM desipramine, or 0.1 μM staurosporine. For the last 2 min of incubation, part of the cells were stimulated by 10 milliunits/ml TSH (*hatched bars*) or not (*empty bars*). *A*, at the end of incubation, cells were harvested directly in the sample buffer and submitted to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto an Immobilon P membrane, and an antibody recognizing CREB phosphorylated at serine 133 was used to blot the membrane. The experiment was repeated three times with similar results. *B*, the intensity of phospho-CREB bands was measured by videodensitometry and normalized by the amount of protein in each sample. Results are expressed as percentages of the control (cells without treatment) and represent the mean of 3–8 values. #, significantly different from the control (untreated cells), $p < 0.01\%$; *, significantly different from the value of cells only treated by TSH, $p < 0.01\%$.

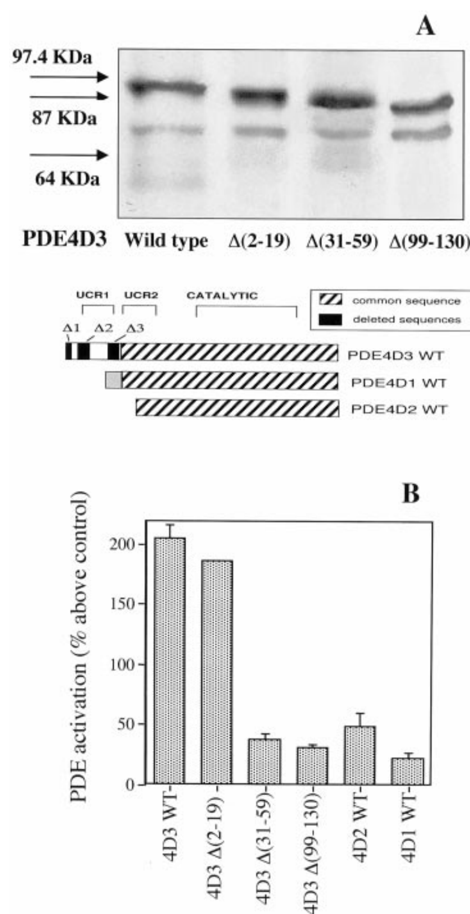

FIG. 6. **Expression and activation of PDE4D3 deletion mutants by PA.** Recombinant wild type PDE4D1, PDE4D2, PDE4D3, and deletion mutants PDE4D3Δ(Met[2]–Val[19]), PDE4D3Δ(Asp[31]–Ser[59]), and PDE4D3Δ(Thr[99]–Ala[130]) were expressed in MA10 cells by transient transfection. *A*, cell extracts were submitted to Western blot analysis, using K116, an antibody recognizing all of the type 4 PDE isoforms. *B*, PDE activity was assayed directly on the cell homogenates, in the presence or absence of 200 μg/ml PA. Results are expressed as percentage of activity above the control value obtained without PA addition. Results are means ± S.E. of 3–6 independent experiments.

the N-terminal domain could be expected. To further investigate the position of a PA binding site, deletions of selected regions were engineered in the N-terminal regulatory domain of PDE4D3. Three regions particularly rich in basic and hydrophobic amino acid residues, which are expected to give rise to interactions with an acidic lipid, were deleted using PCR-based strategies. The three constructs were expressed in MA10 cells by transient transfection. The deleted proteins had apparent molecular masses of 89.4 ± 0.2, 87.2 ± 1.7, and 84.0 ± 1.2 kDa for 4D3Δ(2–19), 4D3Δ(31–59), and 4D3Δ(99–130) respectively, compared with 93.0 ± 1.1 kDa for PDE4D3 wild type, as analyzed by Western blotting (Fig. 6*A*). PDE activity of the different transfected cell homogenates was measured, and specific activities were estimated by normalizing enzyme activities by the amounts of immunodetected proteins (as evaluated by videodensitometric analysis of the corresponding bands on ECL films). Relative specific activities were calculated by taking the specific activity of wild type enzyme, determined in the same experiment, as 1. The specific activity of mutant 4D3Δ(2–19) appeared slightly decreased with regard to wild type enzyme (0.8 ± 0.17, $n = 3$), whereas that of mutants 4D3Δ(31–59) and 4D3Δ(99–130) was increased (1.7 ± 0.15 ($n = 4$) and 1.7 ± 0.34 ($n = 4$), respectively). Sensitivity of the mutants to PA activation was then evaluated by the addition of exogenous PA to cell extracts. As shown in Fig. 6*B*, in the presence of 200 μg/ml PA, PDE4D3 wild type and deletion mutant 4D3Δ(2–19) were activated to similar extents, whereas deletion mutants 4D3Δ(31–59) and 4D3Δ(99–130) were almost insensitive to PA activation. Their level of activation was comparable with that of the PA-insensitive isoforms PDE4D1 and PDE4D2. These findings indicate an involvement of regions of the PDE4D3 protein comprised between amino acids 31 and 59 and between 99 and 130 in the PA activation process. Therefore, these regions might either include PA binding sites or be involved in intramolecular interactions responsible for the regulation of enzyme activity.

Endogenous PA Binding to Wild Type PDEs and PDE4D3 Deletion Mutants—To investigate the possibility that PA specifically binds to one of the above considered regions of the PDE4D3 N-terminal regulatory domain, we used MA10 cells overexpressing wild type PDE4D3 or deletion mutants and labeled the endogenous phospholipids by preincubating the cells with [^{32}P]orthophosphate. The cells were treated by propranolol to increase their radiolabeled PA content, and PDEs were then immunoprecipitated by the PDE4D-specific antibody M3S1. Lipids present in the immunopellets were extracted and analyzed by TLC. The autoradiography of the TLC plates showed that PA was the major phospholipid that co-precipi-

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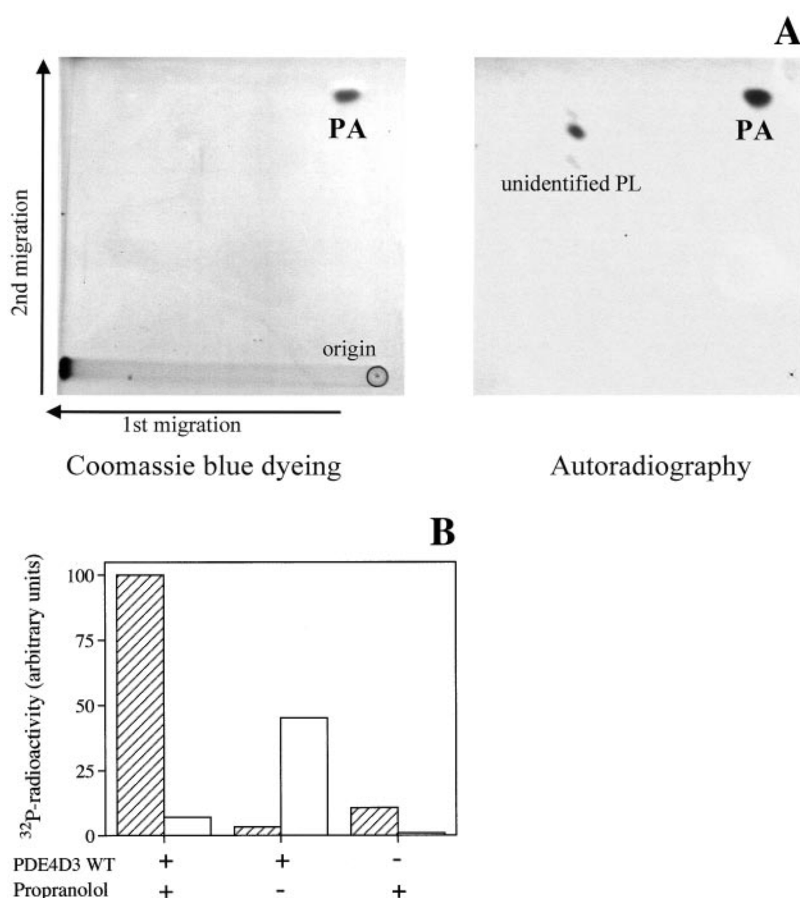

FIG. 7. **TLC analysis of ^{32}P-labeled phospholipids bound to immunoprecipitated PDE4D3.** Transfected MA10 cells overexpressing PDE4D3 or mock-transfected cells were labeled for 2 h with [^{32}P]orthophosphate and treated for 30 min with or without 250 μM propranolol. Cells were then homogenized, and PDE4D3 was immunoprecipitated with the PDE4D-specific antibody M3S1. The lipids of the immunopellets were extracted and analyzed by bidimensional TLC. The TLC plate was then autoradiographed for 20–72 h. *A*, an autoradiogram corresponding to the analysis of phospholipids present in the immunopellet from PDE4D3-overexpressing, propranolol-treated cells is shown, together with the dyed TLC plate. Similar results were obtained in all of the experiments performed. *B*, immunopellet-bound phospholipids from PDE4D3-overexpressing (+) or mock-transfected cells (−) were analyzed as above. The spots corresponding to the various phospholipids on the autoradiography films were analyzed by videodensitometry. The radioactivity of the spots was expressed in arbitrary units, relative to the intensity of the PA spot obtained with propranolol-treated PDE4D3-overexpressing cells taken as 100. *Hatched bars*, radioactivity of the PA spot; *open bars*, radioactivity of the other spots (unidentified phospholipids). Results of one representative experiment are shown.

tated with the wild type PDE4D3 isoform, following propranolol treatment (Fig. 7, *A* and *B*). Analysis of immunopellets from mock-transfected MA10 cells undergoing the same treatment showed that a negligible amount of phospholipids was precipitated in these conditions (Fig. 7*B*). This demonstrates that the presence of PDE4D3 in the immunocomplex was required for the precipitation of PA to occur and thus that PA actually bound to the PDE4D3 protein. With PDE4D3-overexpressing cells, in the absence of propranolol treatment, only little PA was co-precipitated, as compared with propranolol-treated cells (Fig. 7*B*). In untreated MA10 cells, about 20% of the radiolabel incorporated into phospholipids corresponded to PA, the remaining label being distributed among all of the other phospholipid classes. Under propranolol treatment, the distribution of labeling was modified, and two predominant pools were observed, phosphatidylinositol/phosphatidylserine and phosphatidate (which contained about 60 and 40% of the total label, respectively) (results not shown). This suggests that a substantial PA binding to PDE4D3 required an accumulation of the endogenous phospholipid. Moreover, it shows that PA is a preferential ligand of PDE4D3, since in the presence of propranolol PA contained only a minor part of the labeling, whereas the radiolabeled ligand bound to PDE4D3 was almost exclusively PA.

The amount of endogenous PA bound to each immunopre-

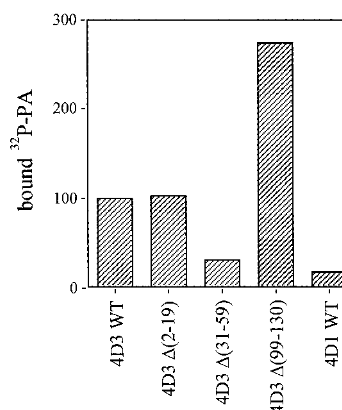

FIG. 8. **Binding of endogenous [^{32}P]PA to immunoprecipitated wild type PDE4D isoforms or PDE4D3 deletion mutants.** MA10 cells were transfected to overexpress wild type PDE4D1 or PDE4D3 or deletion mutants of PDE4D3. Endogenous phospholipids were labeled by cell incubation with [^{32}P]orthophosphate. The cells were then treated by 250 μM propranolol, and the amount of PA bound to PDE immunopellets was evaluated as described in the legend to Fig. 7. Results are expressed as percentages relative to the amount of radioactivity in the PA spot obtained with wild type PDE4D3-overexpressing cells in the same experiment. They are means of 2–4 experiments with similar results, and they are normalized by the amount of the considered protein in the immunopellet, as quantified by Western blotting.

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cipitated deletion mutant protein was also evaluated and compared with that bound to the wild type PDE4D3 and PDE4D1 proteins (Fig. 8). The PA-insensitive PDE4D1 isoform only weakly bound PA; the amount of PA associated to the PDE4D1 immunopellet was similar to that present in the immunopellet obtained from untransfected cells (Fig. 7*B*) and can be considered as nonspecific binding of the phospholipid to the immunoprecipitation complex. Deletion of amino acids 2–19 of the PDE4D3 protein, which did not alter the PA-activating effect, also did not modify PA binding as compared with the wild type protein. By contrast, deletion of amino acids 31–59, which suppressed PA activation, strongly decreased PA binding, confirming the involvement of this region in the association of PA to the protein. Surprisingly, deletion of amino acids 99–130, which also suppressed the PA-activating effect, markedly increased the capacity of the protein to bind PA.

DISCUSSION

In the present study, we provide evidence for the interaction between cAMP signaling and phospholipid metabolism in two different cell line models.

First, we have used MA10 cells overexpressing PDE4 isoforms because these cells express undetectable levels of type 4 PDEs, allowing the study of the effects of endogenous PA on a well defined PDE4 isoform. Accumulation of PA, induced by treatment of the intact cells with the PA-phosphohydrolase inhibitor propranolol, was accompanied by a marked increase in cAMP-PDE activity in cells overexpressing the PDE4D3 isoform. By contrast, cells overexpressing the PDE4D1 isoform displayed no increase in cAMP-PDE activity, showing that PDE4D3 activation was not related to aspecific effects of propranolol on PDE activity and suggesting that, as previously shown *in vitro* (19), PA can selectively stimulate the activity of certain PDE4 isoforms also in the intact cell. Maximal activation of PDE4D3 occurred after 45 min of propranolol treatment, while maximal PA accumulation was reached at 15 min, although PA levels remained elevated for more than 60 min. Such a delay suggests that an intermediate step takes place between PA synthesis and PDE activation (*e.g.* PA might be transported from its site of synthesis to another cell compartment, where it would get in contact with the enzyme). No evidence of a translocation of the enzyme could be obtained under our experimental conditions (not shown), although a translocation of PDE4D3 in smooth muscle cells simultaneously treated by forskolin and phorbol 12-myristate 13-acetate has been reported (50). PDE4D3 stimulation was also observed when intact cells were treated with other PA-phosphohydrolase inhibitors, which supports the involvement of endogenous PA in the increased enzyme activity. As expected, the propranolol-induced increase in PDE activity in PDE4D3-overexpressing cells was accompanied by a decrease in both cAMP levels and PKA activity. However, these latter changes were transient and of rather limited amplitudes, which may be related to the fact that in transient transfection experiments only part of the cells express the recombinant protein. The observed levels are thus likely to be averages between cells expressing high and low levels of PDE4, leading to underestimation of cAMP/PKA variations. In addition, overexpression of PDE4 activity may maintain cAMP levels in a low range of concentrations, which can hardly be further lowered. The observed time course discrepancy between the rise of PDE activity (maximum at 45 min) and the lowering of cAMP and PKA (minimum at 5–30 min) might be accounted for by adaptative changes in adenylyl cyclase function, as previously shown in LLC-PK1 renal epithelial cells (51) and FTC thyroid cells (24) overexpressing a PDE4 isoform. It is thus possible that a rapid and efficient cAMP synthesis is able to counteract the effect of increased PDE activity in transiently transfected MA10 cells.

In FRTL5 cells, which natively express the PA-sensitive PDE4D3 isoform (32), propranolol caused a moderate but significant increase in cAMP-PDE activity. In these cells, the hormone TSH induces a transient increase in cAMP level, which peaks within minutes and returns to basal after 15 min (32). Furthermore, we observed that TSH induced a rapid and marked increase in CREB phosphorylation, consistent with the kinetics of cAMP accumulation. The rapid decline in cAMP can be attributed to a progressive stimulation of PDE activity correlated to PKA-dependent phosphorylation of PDE4D3 (32, 52, 53). The existence of such a feedback loop shows that PDE4D3 activity tightly controls cAMP concentration in these cells. By pretreating FRTL5 cells with propranolol, we could decrease basal cAMP levels and, more importantly, completely suppress both the TSH-induced cAMP transient and phosphorylation of the transcription factor CREB. Interestingly, all of the different PA-phosphohydrolase inhibitors studied exhibited a similar effect on CREB phosphorylation. Considering that propranolol displayed no direct inhibitory effect on TSH-stimulated adenylyl cyclase, these results strongly support the view that accumulation of endogenous PA was able to preactivate PDE4D3 and, as a consequence, to prevent both cAMP accumulation and the functional consequences of the cAMP pathway stimulation in response to TSH.

We have also addressed the mechanism of PA-induced PDE4 activation by studying the co-immunoprecipitation of labeled endogenous PA with PDE4 isoforms overexpressed in MA10 cells. Several observations point to a specific binding of PA to the PDE4D3 isoform: (i) although PA is only a minor component of the labeled phospholipid pool, it was the most abundant phospholipid present in PDE4D3 immunoprecipitates; (ii) PA was almost absent in immunopellets resulting from mock-transfected cells, which shows that PA binding occurs on the PDE protein and not on other components of the immunocomplex; (iii) PA bound more efficiently to the PDE4D3 protein than to the PDE4D1 short variant. This last result strongly suggests that PA binding is responsible for PDE activation, because the PDE4D1 isoform is not sensitive to PA activation. Moreover, it suggests that PA binding to PDE4D3 must occur on the N-terminal regulatory domain of the protein, since PDE4D1 and PDE4D3 only differ in this extremity.

To define the region encompassing PA binding site, various deletions were engineered in the N-terminal domain of PDE4D3. The deleted regions were chosen for their particular richness in basic and hydrophobic residues. Indeed, our previous results suggested that both acidic groups and fatty acyl chains of PA are involved in the interactions giving rise to PDE activation (20, 28). Deletion of two different sequences within the N-terminal domain led to a complete loss of PDE4D3 activation by PA, suggesting that the deleted regions are involved in PA association. Alternatively, the activation loss could be due to a disruption of the normal mechanism of activity regulation. Indeed, the highly conserved UCR1 region present in the N-terminal domain is supposed to be involved in intramolecular interactions responsible for the regulation of activity in the long PDE4 isoforms (24). It has been shown that PKA-dependent phosphorylation of the serine 54 residue within UCR1 (52) as well as point mutations affecting PKA consensus site (52, 54) result in modifications of PDE4D3 activity, probably by modifying interactions of UCR1 with other downstream domains. Furthermore, studies using the yeast two-hybrid system have demonstrated a direct interaction of UCR1 domain with the downstream inhibitory domain UCR2 and confirmed that the association between these domains is modified by point mutations in the PKA site (55), or in the C-terminal

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extremity of UCR1 (56). Because the two deletions that suppress PA activation overlap into UCR1, they may well modify intramolecular interactions and thus prevent the normal transconformational regulatory processes. This contention is supported by the approximately 2-fold apparent increase in specific activity of the two mutants with respect to wild type enzyme, which suggests that the mutant proteins spontaneously adopt an activated state and as a consequence cannot further respond to PA activation. By studying endogenous PA binding to PDE4D3 deletion mutants, we could more clearly define the involvement of the considered regions in PA association. The only deletion that decreased PA binding was Δ(31–59), which suggests that the deleted sequence includes a PA binding site or at least part of it. Deletion of the N-terminal extremity (Δ(2–17)) had neither an effect on enzyme activation, nor on PA binding. Deletion Δ(99–130) markedly increased PA binding, which shows that this region does not overlap with the PA binding site, but it could be involved in intramolecular interactions regulating enzyme activity. The increase in PA binding to this mutant protein may be due to the removal of steric constraints imposed by the deleted sequence on the neighboring PA binding site or to an unveiling effect on the phospholipid binding site. The sequence delimited by deletion Δ(31–59) (see "Experimental Procedures" for the complete sequence) is characterized by the presence of a 13-amino acid fragment (residues 46–58) including 4 basic residues (arginines and histidine) and 5 hydrophobic residues (phenylalanines, tyrosine, leucine, valine). Interestingly, a basic structural motif of 12 amino acids comprising 4 basic and 3 hydrophobic residues mediates the specific association of various proteins with phosphatidylserine (57). Also, a specific PA-binding site, comprising a sequence of 12 amino acids (including 4 basic and 5 hydrophobic residues), has been identified in the C terminus of Raf-1 kinase (21). Thus, although no straightforward similarity of sequence is apparent, some common features emerge from the comparison of PDE4D3-(46–58) sequence and other reported acidic phospholipid binding sites.

An important point to be raised is the relationship between the regulation of PDE4D3 activity by PKA-dependent phosphorylation, and PA-induced regulation. The sequence of the presumed PA binding site includes serine 54, which is a target for PKA, and the phosphorylation of which correlates with PDE activation (32, 52). Interestingly, we have observed in previous studies that activation by PA and by PKA-dependent phosphorylation are mutually exclusive and that they induce similar changes in enzyme properties (*i.e.* similar increases in V_{max} and in affinity for Mg^{2+} ions) (19). It suggests that the presence in this region of UCR1 of either a bound acidic phospholipid or a covalently linked phosphate group has the same consequences on enzyme conformation, both resulting in activation.

On the whole, this study shows that regulation of a PDE4 isoform by phosphatidic acid has a significant functional relevance. The stimulation of PA production by hormones or growth factors is thus likely to interfere with the cAMP pathway of signaling in cells that express the long isoforms of PDE4, such as lymphocytes or thyrocytes. Such a cross-talk between the cAMP pathway and phospholipid signaling reinforces the concept that PA plays a role as a second messenger. PA-mediated activation of PDE4 can be expected to have physiological consequences on various cAMP-regulated cell functions. Particularly, it could remove the blockade of lymphocytes in a quiescent state maintained by high cAMP levels and thus contribute to promoting lymphoproliferative responses.

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