Phosphorylation and Activation of a cAMP-specific Phosphodiesterase by the cAMP-dependent Protein Kinase

INVolVEMENT OF SERINE 54 IN THE ENZYME ACTivation*

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A cAMP-specific phosphodiesterase (PDE4D3) is activated in rat thyroid cells by TSH through a cAMP-dependent phosphorylation (Sette, C., Iona, S., and Conti, M. (1994) J. Biol. Chem. 269, 9245–9252). This short term activation may be involved in the termination of the hormonal stimulation and/or in the induction of desensitization. Here, we have further characterized the protein kinase A (PKA)-dependent phosphorylation of this PDE4D3 variant and identified the phosphorylation site involved in the PDE activation. The PKA-dependent incorporation of phosphate in the partially purified, recombinant rat PDE4D3 followed a time course similar to that of activation. Half-maximal activation of the enzyme was obtained with 0.6 μM ATP and 30 nM of the catalytic subunit of PKA. Phosphorylation altered the Vmax of the PDE without affecting the Km for cAMP. Phosphorylation also modified the Mg²⁺ requirements and the pattern of inhibition by rolipram. Cyanogen bromide cleavage of the 32P-labeled rat PDE4D3 yielded two or three major phosphopeptide bands, providing a first indication that the enzyme may be phosphorylated at multiple sites in a cell-free system. Site-directed mutagenesis was performed on the serine residues present at the amino terminus of this PDE in the context of preferred motifs for PKA phosphorylation. The PKA-dependent incorporation of 32P was reduced to the largest extent in mutants with both Ser13 → Ala and Ser54 → Ala substitutions, confirming the presence of more than one phosphorylation site in rat PDE4D3. While substitution of serine 13 with alanine did not affect the activation by PKA, substitution of Ser54 completely suppressed the kinase activation. Similar conclusions were reached with wild type and mutated PDE4D3 proteins expressed in MA-10 cells, where the endogenous PKA was activated by dibutyryl cAMP. Again, the PDE with the Ser54 → Ala substitution could not be activated by the endogenous PKA in the intact cell. These findings support the hypothesis that the PDE4D3 variant contains a regulatory domain target for phosphorylation at the amino terminus of the protein and that Ser54 in this domain plays a crucial role in activation.

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The intracellular concentrations of the cyclic nucleotides cAMP and cGMP are determined by their rates of synthesis and degradation. The hormonal regulation of cyclic nucleotide synthesis by adenylyl and guanylyl cyclases was the first to be recognized, and most of the steps involved have been elucidated (reviewed in Refs. 2 and 3). The exact role of cyclic nucleotide degradation by phosphodiesterases (PDEs) during hormonal stimulation is less well understood (reviewed in Ref. 4). A large number of PDE forms are present in a cell and may be activated by hormones via different mechanisms. These isoenzymes may be divided into seven classes (PDE1 to PDE7) according to Beavo

1 The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; cAMP-PDE, cAMP-specific PDE; TSH, thyroid-stimulating hormone; PKA, protein kinase A (cAMP-dependent protein kinase); PKG, protein kinase G (cGMP-dependent protein kinase); PKC, protein kinase C (cAMP-dependent protein kinase); B12cAMP, N6,N4-dibutyryl cAMP; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; PCR, polymerase chain reaction. The nomenclature and classification of the cyclic nucleotide phosphodiesterase is according to Beavo et al. (1).
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PKA, with the former kinase having higher affinity for the PDE (20). This PDE is also phosphorylated in primary culture of rat vascular muscle cells in response to stimulation with atrial natriuretic factor (21). The function of this phosphorylation is not clear, even though a recent report showed that phosphorylation of a PDE5 isoform by PKA produces activation of the enzyme (22). Finally, it has been shown that the α, β, and γ subunits of the photoreceptor PDE (PDE6) are also phosphoproteins (23–26). Phosphorylation of the γ inhibitory subunits by PKC increases their affinity for the α and β catalytic subunits and could play a role in the adaptation to light (26).

In rat thyroid cells, TSH induces activation of a cAMP-PDE (PDE4D) (27). The activation is mediated by a cAMP-dependent phosphatase and can be reproduced in a cell-free system by incubation of the native or recombinant PDE4D3 with the catalytic subunit of PKA (27, 28). The PDE4D gene encodes three or more mRNA variants that differ in the 5′ region (28). PDE4D1 (encoded protein of 72 kDa) and PDE4D2 (67–68 kDa) differ in the presence (PDE4D1) or removal (PDE4D2) of a short intron sequence (29) in the mRNA, and are regulated by cAMP at the level of transcription or mRNA stability (28, 30). Hormones that raise intracellular cAMP levels in Sertoli and thyroid cells induce the expression of these two PDE4D variants (28, 30, 31). PDE4D3 mRNA is instead constitutively expressed in thyroid cells (28, 32). This mRNA codes for a protein with an additional 132-amino acid domain at the amino terminus, which, according to our hypothesis, renders the protein sensitive to PKA activation (28). In this study we characterized the phosphorylation and activation of the PDE4D3 protein and investigated the role of this regulatory domain by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials—Coons modified Ham's F-12 medium (Coon's F-12), bovine insulin, human transferrin, and Crotalus atrox snake venom were purchased from Sigma; bovine TSH (1.23 units/ml) for culture from Armour; Pansorbin cells from Calbiochem; Immobilon membrane from Millipore Corp.; the C subunit of protein kinase A from Promega, the synthetic inhibitor of protein kinase A from Sigma (2.8–3.4 nM) (20–30 Ci/mmol) and [12]-Phosphothioic acid (800–1000 Ci/mmol) from Du Pont NEN; [3H]JATP (10 mCi/ml) from Amersham Corp.; AG 1-X8 resin from Bio-Rad; ECL Western blot detection kit from Amersham. Rolipram (4-(3-butoxy-4-methoxybenzyl)imidazo[2,1-α]-pyridin) was obtained from Syntex. Exonecto, where otherwise designated, are all chemicals were the purest grade available from Sigma.

Expression of Recombinant PDE4D3 Enzymes—For the sake of simplicity, the oligonucleotides used for PCR amplification are identified by letters as follows; the mutated base is underlined: A, 5'-GGTCTGAGAAAGGATCCTGCTAGATG-3' (corresponding to the rat PDE4D3 sequence from base 23 to 47); B, 5'-CCGGGAGGCTCTCCGACATG-3' (corresponding to the rat PDE4D3 sequence from base 153 to 173); C, 5'-CTAGACAGGAAGGCTCTCCGGG-3' (corresponding to the antisense sequence of rat PDE4D3 from base 153 to 173); D, 5'-TCCAGAACAGGGTCCGTTAGGC-3' (corresponding to the sequence of rat PDE4D3 from base 211 to 231); E, 5'-GGTCTGAGAAAGGATCCTGCTAGATG-3' (corresponding to the rat PDE4D3 sequence from base 215 to 300; F, 5'-ACCAATGAGGACACCTGTCG-3' (corresponding to the antisense sequence of rat PDE4D3 from base 211 to 231; G, 5'-ACCAATGAGGACACCTGTCG-3' (corresponding to the rat PDE4D3 sequence from base 211 to 231); H, 5'-CCGGGAGGCTCTCCGACATG-3' (corresponding to the rat PDE4D3 sequence from base 215 to 300; I, 5'-CCGGGAGGCTCTCCGACATG-3' (corresponding to the rat PDE4D3 sequence from base 153 to 173; J, 5'-TGTTGAGAAAGGATCCTGCTAGATG-3' (corresponding to the sequence of rat PDE4D3 from base 23 to 47); K, 5'-CCGGGAGGCTCTCCGACATG-3' (corresponding to the rat PDE4D3 sequence from base 153 to 173)

Expression of Recombinant PDE4D3 Enzymes—To express the recombinant PDE4D3 enzymes in Sf9 insect cells, the wild type and mutated PDE4D3 cDNAs were excised from the pCMV5 constructs by EcoRI digestion and ligated to the pCMV5 vector that had been digested with EcoRI followed by dephosphorylation. The correctness of the constructs was verified by sequencing.

To express the recombinant PDEs in Sf9 insect cells, the wild-type and mutated PDE4D3 cDNAs were excised from the pCMV5 constructs by EcoRI digestion and ligated to the pCMV5 vector pSYN XIV VI/X3. An ATG internal to the pCMV XIV VI/X3 vector was used in order to increase the efficiency of expression of recombinant proteins. Therefore, recombinant proteins were expressed as fusion proteins containing six additional amino acids (Met-Gly-Ser-Ser-His-Gly) at the amino terminus.

Cell Culture—FRTL-5 cells (ATCC number CRL8305), a line of rat thyroid follicular cells developed by Dr. F. S. Ambesi-Impiombato et al. (35), were generously provided by Dr. Leonard Kahn (Section of Cell Reproduction, NIDDK, National Institutes of Health and the Intramural Research Foundation (Baltimore, MD). Cells were routinely cultured in Coon's F-12 medium supplemented with 5% calf serum and a mixture of three hormones (3H) including TSH (1 milliunit/ml), insulin (10 μg/ml), and transferrin (5 μg/ml). Mouse Leydig tumor cells (MA-10) (36) were grown in the Waymouth MB752/1 modified to contain 1.1 g/liter NaHCO3, 20 mM Hepes, 50 mM L glutamate (pH 7.4 (growth medium)). FRTL-5 cells and MA-10 cells were cultured in 75-cm flasks (Corning, Corning, NY) at 37 °C in an atmosphere of 95% air, 5% CO2 in a humidified incubator. Sf9 cells were kindly provided by Dr. Earl Shelton (Syngent) and grown in SF900 medium (Life Technologies, Inc.) supplemented with 50 μg/ml Gentamicin in a shaking incubator at 27 °C.

Expression of Recombinant PDE4D3 Enzymes—To express the recombinant PDE4D3 wild-type and mutants in MA-10 cells, the pCMV5 vectors containing the respective cDNAs were transfected by the CaPO4 method (37). Cells were harvested 24 h after transfection in a homogenization buffer containing 20 mM Tris-Cl, pH 8.0, 1.0 mM EDTA, 0.2 mM EGTA, 0.4 M NaF, 10 mM sodium pyrophosphate, 50 mM benzamidine, 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. Cells were then homogenized and centrifuged for 10 min at 14,000 × g. The PDE activity was measured in the homogenates or in the soluble extracts. Protein concentration was measured according to Bradford et al. (38). To express recombinant PDE4D3 wild-type and mutants in Sf9 insect cells, the rat PDE4D3 cDNA was subcloned in the expression vector pSYN XIV VI/X3. The plasmid was cotransfected with BaculoGold virus DNA (PharMingen) by lipofection. The recombinant virus stock was made from a single plaque isolated after 6 days. The virus was amplified by infection of 10 ml of growing Sf9 cells in a shaking incubator at 27 °C for 5 days. After the incubation, the infected cells were separated by centrifugation at 1,500 × g for 10 min, and the medium containing the virus was collected and stored at 4 °C in the dark. To prepare recombinant PDE4D3, 30–50 ml of Sf9 cells at a density of 0.8–1.0 × 106
cells/ml were infected with recombinant baculovirus and grown for 3 days in SF900 medium (Life Technologies, Inc.) containing 1% heat-inactivated fetal calf serum, 50 μg/ml Gentamicin, and 4% fed stock. After 3 days, cells were collected by centrifugation and resuspended in lysis buffer containing 40 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.2 mM EGTA, 50 mM benzamidine, 0.5 μM leupeptin, 0.7 μg/ml peptatin, 4 μg/ml aprotinin, 1 mM 3-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride. Cells were homogenized and centrifuged for 10 min at 14,000 g at 4°C. Soluble extracts were centrifuged for 1 h at 100,000 g at 4°C. The cytosolic fraction was then diluted in 200 mM sodium acetate, pH 6.5, and loaded onto a DEAE ion exchange HPLC column equilibrated with 200 mM sodium acetate, pH 6.5, at a flow rate of 3 ml/min. Washing the column with 2–3 volumes of SDS-PAGE sample buffer. Bound proteins were eluted with a 200–750 mM sodium acetate pH 6.5 linear gradient. PDE activity was assayed in each fraction, and fractions containing PDE activity were diluted to 33% ethylene glycol and stored at −20°C for further studies. To determine the specific activity of the wild type and the double mutant PDE, the fractions of the DEAE containing the peak of activity were pooled and injected on a TSK-Phe nyl 5PW-HPLC column equilibrated at 70 mM sodium acetate, pH 6.5. Proteins were then eluted with a gradient of ethylene glycol (0–35%) and assayed for PDE activity. Details of the purification have already been reported elsewhere (39). The activities of these preparations were measured as described below. Protein content was measured either by the Lowry procedure or by quantitation of the Coomassie Blue bound to the protein, using bovine serum albumin as the standard.

PDE Assay—PDE activity was measured using 1 μM cAMP as substrate, according to the method of Thompson and Appleman (40) and as detailed previously (41). Samples were assayed in a total volume of 200 μl of reaction mixture containing 40 mM Tris-HCl (pH 8.0), 1 mM MgCl2, 1.25 mM 2-mercaptoethanol, 1 μM CAMP, 0.14 mg of bovine serum albumin, and [3H]AMP (0.1 μCi/tube). In some experiments serial dilutions (1 nm to 10 μM) of rolipram were added to the reaction mixture. After incubation at 34°C for 5–15 min, the reaction was terminated by adding an equal volume of 40 mM Tris-Cl, pH 7.5, containing 10 mM EDTA, followed by heat denaturation for exactly 1 min at 100°C. To each reaction tube 50 μg of C. atrax venom snake venom phosphodiesterase was added and incubated at 34°C for 30 min. The reaction products were separated by anion exchange chromatography on AG1-X8 resin, and the amount of radiolabeled adenosine collected was quantitated by scintillation counting.

Metabolic Labeling of PDE4D3 with [32P]Orthophosphate—FRTL-5 cells were seeded in 96-mm dishes (Corning) and cultured as described above. Cells were made quiescent by replacing the serum and the hormon in 10% in the medium with 10% in serum albumin with 0.1% in sodium acetate as substrate. After induction of quiescence, the medium was replaced with phosphate-free minimal essential medium containing 20 mM Hepes, pH 7.4, and carrier-free [32P]Orthophosphate (0.2–0.3 μCi/ml), and cells were incubated for an additional 2 h. During the last 15 min of incubation, part of the cells were treated with 10 mM TSH. At the end of the treatment, the cells were washed 3 times with Hanks' balanced salt solution, harvested in homogenization buffer, and homogenized. Supernatants from a 10-min centrifugation at maximum speed in a microcentrifuge at 4°C. Pellets containing immunoadsorbed proteins were first washed with buffer containing 80 mM Tris-Cl, pH 8.0, 0.7 M NaCl, 0.1% SDS, and 0.4% Triton X-100 and then eluted with 1% SDS in PBS. The eluted protein was diluted with concentrated SDS-PAGE sample buffer (4 X) and separated by SDS-PAGE.

Western Blot Analysis—Samples were prepared in 1 × sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (w/v) bromphenol blue), subjected to electrophoresis on an 8% SDS-polyacrylamide gel (SDS-PAGE), and then blotted onto Immobilon membrane. Western blot analysis was performed using a monoclonal antibody (M3S1) raised against the carboxyl-terminal region of rat PDE4D3 (39). Immunoreactive bands were detected by using peroxidase-conjugated goat anti-mouse IgG (Amer sham) at a dilution of 1:5000 and the ECL detection reagents (Amer sham).

Phosphopeptide Analysis—Partially purified rat PDE4D3 was incubated for 15 min with the catalytic subunit of PKA (0.1 μM) in the presence of 0.1 mM [γ-32P]ATP (0.5 μCi/μl) at 30°C. At the end of the incubation, the reaction was stopped by the addition of SDS-PAGE sample buffer, and the sample was boiled for 5 min at 100°C. Proteins were separated by 8% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was exposed to autoradiography, and the phosphorylated rat PDE4D3 band was excised from the membrane. The membrane was rehydrated in water and then incubated for 90 min with 100 mg/mi cyanogen bromide in 70% formic acid at room temperature. At the end of the incubation, the samples were centrifuged for 5 min at 14,000 × g, and the supernatant was collected and lyophilized. The pellet was resuspended in SDS-PAGE sample buffer and boiled for 5 min at 100°C. Peptides were separated by SDS-Tricine-Tris 16% PAGE and blotted onto Immobilon membrane. The membrane was dried and exposed to autoradiography. In some experiments the SDS-Tricine gel was dried and directly used for autoradiography. Omission of the Immobilon transfer step yielded the same phosphopeptides but higher background.

Time Course of the Activation of PDE4D3 by PKA—Recombinant PDE4D3 proteins were diluted in a reaction buffer containing 40 mM Tris-Cl, pH 7.4, 2 mM magnesium acetate, 0.1 mM ATP, 1.25 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin and incubated at 30°C for increasing intervals of time in the absence or presence of the catalytic subunit of protein kinase A (0.1 μM) (Fig. 2). When samples were assayed for PDE activity, 1 μM [3H]AMP (0.3 μCi/ml) was used as substrate. When 32P incorporation was measured, 0.1 mM [γ-32P]ATP (0.5 μCi/μl) was added to the reaction. The final volume in each tube was 2 ml, 200-μl aliquots of the reaction mixtures were withdrawn at different times, and the reaction was terminated as described for the PDE assay. For the ATP dose-dependent activation of PDE4D3 by PKA (0.1 μM), the reaction buffer was modified to contain 20 mM magnesium acetate and serial dilutions of ATP (0–0.4 mM). For the PKA dose-dependent activation, the reaction buffer was modified to contain 20 mM magnesium acetate, 0.1 mM ATP, and serial dilutions of the catalytic subunit of PKA (0.1–1 μM). In these two last experiments, after a 15-min incubation at 30°C, samples were diluted in 40 mM Tris-Cl, pH 7.4, 5% nonfat powdered milk, and 0.1% bovine serum albumin, and the incorporation was measured as described above. 32P incorporation was measured by densitometry of the autoradiogram using the NIH Image software.

RESULTS

PKA-mediated Phosphorylation of Rat PDE4D3—The activity of a cAMP-PDE is stimulated in the intact thyroid cell by TSH (32) and in a cell-free system by incubation with the catalytic subunit of PKA (28). Together with PDE activation, short-term treatment of quiescent FRTL-5 thyroid cells with TSH induced an increase in 32P incorporation in a polypeptide of 93–97 kDa (32) (Fig. 1A). When recombinant PDE4D3 was phosphorylated by cAMP-PDE...
incubated in a cell-free system with the catalytic subunit of PKA (Fig. 1B), a polypeptide with electrophoretic properties identical to the phosphoprotein derived from intact cells was phosphorylated. Similar results were obtained whether the recombinant PDE was derived from expression in mammalian cells or in insect cells as a fusion protein (see below). To investigate the phosphorylation/activation of the enzyme by PKA in more detail, the recombinant PDE4D3 was partially purified by a DEAE ion exchange HPLC column as described under “Experimental Procedures.” Incubation of PDE4D3 with the catalytic subunit of PKA produced a 2–3-fold increase of phosphodiesterase activity (Fig. 2A). In Fig. 2, A and C, the time courses of activation and the incorporation of $^{32}$P in the enzyme are reported. In the absence of the kinase, the activity of PDE4D3 was constant over 15 min, and a trace amount of $^{32}$P incorporation was detected (Fig. 2C). The activation of PDE4D3 by PKA was completely suppressed both by the addition of a PKA inhibitor and by the omission of ATP from the assay (data not shown). Incubation of rat PDE4D3 with similar concentrations of cGMP-dependent protein kinase or protein kinase C, under appropriate conditions (see “Experimental Procedures”), neither produced changes in the phosphodiesterase activity nor caused any detectable $^{32}$P incorporation in the PDE4D3 protein (data not shown). PKA induced activation of rat PDE4D3 by increasing the maximal velocity of cAMP hydrolysis (8.13 pmol/min versus 4.18 pmol/min) without affecting the $K_m$ for cAMP (1.26 $\mu$M versus 1.51 $\mu$M). The stimulation of PDE activity by PKA was dependent on ATP concentration, with half-maximal activation at 0.6 $\mu$M nucleotide (data not shown). In the presence of 100 $\mu$M ATP and 20 $\mu$M Mg$^{2+}$, activation of rat PDE4D3 was dependent on the concentration of the catalytic subunit of PKA. Half-maximal activation of rat PDE4D3 was achieved with 30 nM catalytic subunit of PKA (data not shown), suggest-

![FIG. 1. Phosphorylation of native PDE4 in intact FRTL-5 cells and of recombinant rat PDE4D3 in a cell-free system.](image)

![FIG. 2. Time course of the activation/phosphorylation of rat PDE4D3.](image)
PKA phosphorylation for some substrates, at least similar to the RX motif with predicted sizes of 3.5 and 4.1 contain Ser13 and Ser54. In Fig. 5 the predicted cyanogen bromide cleavage in vitro where the concentration of the kinase ranges between 0.2 and 0.7 μM (42). It was also observed that phosphorylation causes a decrease in the Mg^{2+} concentration required for cAMP hydrolysis (Fig. 3).

Rolipram inhibition of phosphorylated rat PDE4D3—PDE4 isoenzymes are selectively inhibited by the antidepressant rolipram (1, 43). To investigate the effect of phosphorylation on rolipram inhibition, recombinant rat PDE4D3 was incubated for 15 min in the absence or presence of PKA (0.1 μM), and the PDE activity was measured in the presence of increasing concentrations of rolipram (Fig. 4A). In the absence of PKA, the inhibition profile of the PDE4D3 activity showed a biphasic curve, with a high affinity inhibition at 10^{-9} M rolipram and a low affinity inhibition at 10^{-6} M rolipram. The addition of the kinase produced a stimulation of the PDE activity that is inhibited by rolipram with a high affinity (Fig. 4A). To determine whether the presence of two rolipram affinity states was an artifact induced by the partial purification of the enzyme, a similar experiment was performed using FRTL-5 cells. These cells were chosen because the predominant PDE activity expressed can be activated by short term incubation of quiescent cells with TSH (32). A similar inhibition profile was observed with soluble extracts from quiescent and TSH-stimulated FRTL-5 cells (Fig. 4B). TSH treatment induced activation only of the PDE that displayed a high affinity for rolipram, reproducing the effect obtained by PKA in a cell-free system (Fig. 4, A and B).

Site-directed mutagenesis of PDE4D3—The amino-terminal region of PDE4D3 contains 19 Ser and 6 Thr residues (Fig. 5). The sequence surrounding Ser^{13} (RRHSW) and Ser^{54} (RRESF) is identical to the PKA preferred motif (RRX(S/T*)X) found in many physiological substrates of the kinase (44) (Fig. 5). Two additional Ser in the amino terminus of PDE4D3 (Ser^{74} and Ser^{119}) are preceded by basic residues. These sequences are similar to the RX(S/T*) motif, which has been shown to be the site of PKA phosphorylation for some substrates, at least in vitro (44). In Fig. 5 the predicted cyanogen bromide cleavage sites in this region of PDE4D3 are also indicated. Two peptides with predicted sizes of 3.5 and 4.1 contain Ser^{13} and Ser^{54}. An additional peptide of 5.5 kDa contains serines 74 and 119. A phosphopeptide analysis of rat PDE4D3 cleaved with cyanogen bromide yielded two major phosphopeptide bands of 5.3 and 4.5 kDa (Fig. 6). In several experiments, the 4.5-kDa band could be resolved into two components (data not shown). This experiment provided a first indication that PDE4D3 is phosphorylated at multiple sites under these cell-free conditions.

The possibility that Ser^{13} and Ser^{54} are indeed phosphorylated and involved in the PDE4D3 activation by PKA was further tested by site-directed mutagenesis. Serine-to-alanine substitutions were inserted by PCR amplification with oligomers containing a single base change (as described under “Experimental Procedures”). The insertion of the mutations in PDE4D3 was confirmed by sequencing of the cDNAs. The wild type and mutant PDE4D3 enzymes were then expressed in MA-10 cells by transient transfection or by infection in insect cells. Comparable results were obtained with proteins derived from the two expression systems. To test the degree of phosphorylation of the PDE4D3 wild type and mutant proteins, phosphorylation was carried out in the presence of [γ-32P]ATP (Fig. 7). Substitution of Ser^{13} or Ser^{54} caused a reduction in 32p incorporation in PDE4D3 when compared with the wild type enzyme (Ser^{13} → Ala, 48.6 ± 3.9% of wild type, n = 5; Ser^{54} → Ala, 58.2 ± 10.0 of wild type, n = 4). The protein with double substitutions at Ser^{13} and Ser^{54} incorporated much less 32p than the wild type PDE (20.1 ± 8.7 of wild type, n = 3). Under these experimental conditions of maximal phosphorylation, it was estimated that approximately 2 mol of phosphate/mol of PDE were incorporated in the wild type PDE, while the double mutant incorporated less than 0.4 mol/mol of PDE. This experiment indicated that Ser^{13} and Ser^{54} were the preferential sites of phosphorylation but that additional sites were also used in this cell-free model.

The effect of the different mutations on the PKA-dependent
activation of the PDE was next tested in a cell-free system. The Ser\textsuperscript{13} → Ala substitution did not affect the activation by PKA (Fig. 8). By contrast, mutation Ser\textsuperscript{54} → Ala completely suppressed the effect of the kinase (Fig. 8). The double mutation of Ser\textsuperscript{13}/Ser\textsuperscript{54} → Ala in the conserved consensus sites behaved in a manner identical to the Ser\textsuperscript{54} → Ala mutant (Fig. 8). Since the PDE4D3 sequence contains two additional serines (Ser\textsuperscript{74} and Ser\textsuperscript{119}), which reside in the less conserved consensus for PKA (Fig. 5), phosphorylation of these residues may occur under the conditions used, as suggested by the phosphorylation studies and the phosphopeptide map analysis. To determine whether phosphorylation of these two additional sites is important for activation of PDE4D3 by PKA, site-directed mutagenesis of the two residues was performed. Neither substitution Ser\textsuperscript{74} → Ala nor substitution Ser\textsuperscript{119} → Ala affected the activation of PDE4D3 by the kinase (data not shown). This indicated that, even if phosphorylated, these two residues do not play a crucial role in PDE activation.

The lack of stimulation of the PDE bearing the Ser\textsuperscript{54} → Ala substitution may be the consequence of a change in conformation of the protein rendering it constitutively activated. This constitutive activation unrelated to phosphorylation would prevent or mask a further activation by PKA. If this were the case, the specific activity of PDE4D3 Ser\textsuperscript{13}/Ser\textsuperscript{54} → Ala would be 2–3-fold higher than that of wild type PDE4D3. To investigate this possibility, the recombinant proteins expressed as fusion proteins in the baculovirus-Sf9 cell system were purified by two chromatographic steps to 90–95% homogeneity as determined by SDS-PAGE and Coomassie Blue staining (data not shown). In the two experiments performed, the specific activities of wild type and mutated proteins were comparable (PDE4D3 wild type 11.4 ± 2.6 mol/min/mg; the double mutant PDE4D3 6.64 ± 0.17 mol/min/mg), indicating that the double mutation does not yield a constitutively activated enzyme.

The whole of these data then indicate that, under the cell-free conditions used, PKA phosphorylates PDE4D3 on several residues. Ser\textsuperscript{54} was the only residue involved in the kinase activation of the enzyme.

Activation of PDE4D3 Mutants in Intact MA-10 Cells—To determine whether Ser\textsuperscript{54} is also important for activation of the rat PDE4D3 in the intact cell, MA-10 cells were transfected with rat PDE4D3 wild type or with rat PDE4D3 mutant cDNAs. After transfection, cells were incubated in the presence or absence of Bt\textsubscript{2}cAMP to activate the endogenous PKA, and the PDE activity was measured in the cell homogenates. Bt\textsubscript{2}cAMP treatment induced a 50% stimulation of PDE activity in cells transfected with PDE4D3 wild type cDNA (Table I). Similar activation was observed in cells transfected with mutant Ser\textsuperscript{13} → Ala (46%), whereas in cells transfected with mutant Ser\textsuperscript{54} → Ala or Ser\textsuperscript{13}/Ser\textsuperscript{54} → Ala, the activation was negligible (2–9%). (Table I). These data indicate that Ser\textsuperscript{54} of PDE4D3 is a residue necessary for PKA-dependent activation also in the intact cell.

**DISCUSSION**

Serine 54 of the cAMP-specific phosphodiesterase variant PDE4D3 was identified as the phosphorylation site crucial for the activation of the recombinant enzyme both in a cell-free...
system and in the intact cell. This finding strongly suggests that this PDE is a substrate for PKA in vivo and that TSH activates this PDE via a PKA-dependent phosphorylation. Furthermore, the identification of this residue at the amino terminus of this variant opens the possibility that this region of the protein encodes a regulatory domain. The presence of this domain distinguishes this protein from the previously described variants (PDE4D1 and PDE4D2) derived from the same gene, and explains why this variant is the only one activated by a PKA-dependent phosphorylation (28).

Phosphorylation studies in a cell-free system indicate that PDE4D3 is a good substrate for PKA. Phosphorylation and activation occur at PKA concentrations well within the physiological range found in the cell. Several sites are phosphorylated in this cell-free system. Two of them have been identified as Ser13 and Ser54, since substitutions at the two sites cause a decrease in phosphate incorporation in the PDE protein. The finding that the double mutant is still phosphorylated by PKA, albeit at a much lower level, indicates that additional phosphorylation sites may be present in PDE4D3. Whether phosphorylation at these additional sites occurs also in vivo in the intact cell remains to be determined. Of the four potential phosphorylation sites studied, only mutation of Ser54 to Ala abolishes the PKA-dependent activation of the enzyme. That this residue plays an important role in the PDE activation is further indicated by studies in intact cells where the Ser54 → Ala mutant cannot be activated by treatment of the cells with

**TABLE 1**

| cDNA transfected | PDE activity |
|------------------|-------------|
| −Bt2cAMP | +Bt2cAMP |
| pmol/min · mg protein |

| Experiment 1 | |
|------------------|-------------|
| PDE4D3 wild type | 189 ± 10 | 266 ± 8 |
| PDE4D3 Ser13 → Ala | 92 ± 3 | 139 ± 7 |
| PDE4D3 Ser54 → Ala | 194 ± 1 | 216 ± 3 |
| PDE4D3 Ser13/Ser54 → Ala | 138 ± 2 | 130 ± 5 |
| Experiment 2 | |
| PDE4D3 wild type | 132 ± 7 | 204 ± 11 |
| PDE4D3 Ser13 → Ala | 152 ± 20 | 251 ± 21 |
| PDE4D3 Ser54 → Ala | 90 ± 2 | 102 ± 3 |
| PDE4D3 Ser13/Ser54 → Ala | 190 ± 17 | 210 ± 30 |
| Experiment 3 | |
| PDE4D3 wild type | 113 ± 6 | 198 ± 2 |
| PDE4D3 Ser54 → Ala | 123 ± 3 | 131 ± 6 |
dibutyryl cAMP. Thus, PKA activation in the intact cell phosphorylates PDE on Ser54, in turn producing its activation. An alternative explanation of the mutagenesis studies is that the Ser to Ala substitution produces a conformational change and a constitutively active enzyme that cannot be further activated by PKA. However, this hypothesis is in conflict with the finding that the specific activity of this mutant is comparable with or lower than that of the wild type enzyme. It is thus unlikely that the introduced mutations cause a constitutive activation of the PDE. Another possibility that cannot, at present, be excluded is that the introduced mutation causes a change in conformation of the protein that prevents phosphorylation at a site other than Ser54, thus disrupting the activation. Phosphopeptide mapping after metabolic labeling of the native enzyme in intact FRTL-5 cells and sequencing will be required to answer this question. In addition, it will be important to determine whether Ser54 is the only phosphorylation site involved in TSH activation in the intact cell.

In all experiments performed, phosphorylation of the cAMP-PDE produced a 2–3-fold increase in activity. It is worth noting that, although small, this activation is quantitatively similar to the activation of a cGMP-PDE by insulin or isoprenaline (45). Under basal conditions, i.e., without kinase treatment, substantial activity of the isoenzyme was detected. Measurements of the activity of this enzyme under conditions of complete dephosphorylation need to be performed to clarify this point. However, it should be noted that the PDE with substitution of the two major phosphorylation sites has substantial hydrolytic activity. Since it is assumed that the double mutants are recovered in the dephosphorylated state, the possibility needs to be entertained that PDE phosphorylation modifies the activity but does not produce a complete transition from an inactive to an active state. This possibility is at odds with the finding that rolipram, a specific inhibitor of PDE4, has no effect on cAMP levels in the intact FRTL5 cell under basal conditions (46), an indication that this enzyme is mostly inactive under basal conditions. A factor to be considered in the interpretation of the data is that the PDE assay used does not reflect the conditions in the intact cell. For instance, the Mg2+ concentration used in the PDE assay is about 10-fold higher than the concentration found in the cell. Indeed, if activation is measured at lower Mg2+ concentrations, basal activity is reduced, thus increasing the degree of activation brought about by the kinase treatment. It is also possible that the cell-free system used here is lacking additional regulatory components present in the cell. For instance, phosphorylation may affect the interaction of a putative inhibitor or activator with the PDE. These regulatory molecules may affect the conformation and the activation of the enzyme (see below).

It remains possible, however, that the cell requires only minor changes in PDE activity and that these small changes can greatly affect the response of a target cell to hormones. It has been reported that hCG-induced steroidogenesis in MA-10 Leydig tumor cells transfected with a cAMP-PDE was reduced by 80%, although only a 2–3-fold increase in PDE activity could be measured in extracts from these cells (46). Furthermore, FRTL-5 thyroid cells infected with a constitutively active Gsα protein showed a 10-fold increase in adenylyl cyclase activity but only a 40–50% increase in PDE activity compared with wild type FRTL-5 cells (46, 47). Since less than a 2-fold increase in intracellular cAMP levels was found in these cells, the small increase in PDE detected is sufficient to counteract the large increase in cyclase activity (46).

The PDE4 isoenzymes are selectively inhibited by the antidepressant rolipram. The experiments reported in this study indicated that freshly isolated and partially purified rat PDE4D3 is inhibited by rolipram with low (EC50 = 1 μM) and high affinity (EC50 = 10 nM). Phosphorylation of the enzyme causes an increase exclusively in the activity inhibited by rolipram with a high affinity. These data are similar to what has been observed with the PKA activation of the human PDE4D3 and inhibition by analogs of nitraquazone (48). It has been previously shown that a rolipram high affinity (Kp = 1–10 nM) binding site copurifies with cAMP-PDEs from brain (49) and from recombinant yeast extracts expressing a cAMP-PDE (50). The affinity of this binding site is considerably higher than the affinity of rolipram for the catalytic site, as estimated from inhibition of the activity (Ki = 0.5–1 μM). To reconcile these differences, it can be hypothesized that PDE4D3 is recovered in two states. One conformation allows binding of rolipram with a high affinity and inhibition of the activity at low nanomolar concentrations. The PDE in this conformation can be activated by phosphorylation. The second state has low affinity binding for rolipram, and the catalytic activity is inhibited by micromolar concentrations of rolipram. This second state does not allow activation by phosphorylation. The physiological relevance of these two conformation states is uncertain, but the finding that some biological responses are inhibited by rolipram at very low concentrations suggests that a significant portion of these enzymes may be present in the cell in a high affinity conformation.

The PDE4D locus encoding the cAMP-PDE object of our study contains several transcriptional units under the control of different promoters (4). This feature and the occurrence of alternate splicing of the mRNAs indicate that distinct protein variants (28, 29) are derived from this gene. Several PDE proteins have indeed been identified immunologically as products of this gene. PDE4D1 is a short protein of 72 kDa with an amino terminus different from that of the long 93-kDa PDE4D3. The PDE4D2 (68 kDa) is derived from alternate splicing of the mRNA and encodes a truncated PDE (28). The presence of one or more additional variants has been inferred from cDNA cloning and from mRNA or Western blot analysis (48, 51). The observation that different regulatory mechanisms control these proteins provides a physiological explanation for the existence of these different forms. The expression of PDE4D1 and PDE4D2 is regulated by cAMP at the level of transcription and/or messenger stabilization in endocrine cells like the Sertoli cell (30, 31). Similar regulations have been demonstrated for FRTL-5 thyroid cells (32), inflammatory cells (52, 53, 54), glial cells (31), and skeletal muscle cells (55). The experiments described here strengthen the hypothesis that the variant PDE4D3 is activated by a different mechanism. Hormones that act through the cAMP-dependent pathway produce a rapid activation of this PDE form, and this activation is mediated by a PKA-dependent phosphorylation. Although this form may be phosphorylated at multiple sites at the amino terminus of the protein, our data indicate that the phosphorylation at Ser54 plays a crucial role in the PKA-dependent activation both in a cell-free system and in the intact cell. Since the other two variants studied, PDE4D1 and PDE4D2, are not good substrates for PKA and cannot be activated by phosphorylation (28), we propose that the longer PDE4D3 form contains a regulatory domain absent in the other variants. The identification of Ser54 as the most likely target for this phosphorylation confirms this view. Thus, the presence of different variants may be the result of the inclusion of different regulatory domains that modify the function of the protein. This points to the modularity of the PDE protein and suggests that all PDEs may have similar arrangements of regulatory and catalytic domains. Our observation made on these CAMP-PDEs is in complete agreement with early observations made on the CaM-
PDE and the cGS-PDE (56, 57), where regulatory domains are present at the amino terminus of these proteins.

Activation by phosphorylation is not unique to the rat PDE4D3 because human (48) and mouse recombinant PDE4D3 can also be activated by phosphorylation. The same consensus PDE and the cGS-PDE (56, 57), where regulatory domains are present in variants derived from the PDE4A, PDE4B, and PDE4C genes (51). On the basis of these structural similarities, one would predict that all variants containing that domain should be good substrates for PKA and that the phosphorylation should cause an activation of these additional forms. In preliminary experiments performed, PDE4D4 and PDE4B variants containing this domain could not be activated under conditions in which PDE4D3 activity is stimulated. The reason for this is unclear. It is possible that conditions for the PKA-dependent activation are not optimal to detect activation of these PDE4 variants derived from different genes. Alternatively, the large differences at the carboxyl terminus may influence the phosphorylation and activation of these two other variants. Finally, other kinases may be involved in the phosphorylation and activation of PDE4A, PDE4B, and PDE4C.

In summary, our results demonstrate that PKA phosphorylation activates rat PDE4D3 and the activation can be suppressed by substitution of Ser54 with Ala. It is proposed that the N terminus 132-amino acid domain of PDE4D3 is a regulatory domain that allows modulation of the activity of the protein by phosphorylation. The phosphorylated/activated PDE4D3 may be the preferred target of the antidepressant drug rolipram in the intact cell. These data, therefore, suggest that PDE4D3 plays a crucial role in the hormonal control of intracellular cAMP levels. The exact physiological significance of this regulation requires further investigation. Phosphorylation and activation of this PDE variant may occur at the same time as adenylyl cyclase activation to maintain cAMP in a narrow range of concentrations and to decrease the time required to attain a new steady state. In addition, this regulation may be a component of the mechanisms that terminate the hormone stimulation and produce cell adaptation or desensitization.

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