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Activation and Selective Inhibition of a Cyclic AMP-Specific Phosphodiesterase, PDE-4D3

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SUMMARY

Prostaglandin E_2 produces a transient increase in the intracellular concentration of cAMP in a human promonocytic cell line (U937). The temporal pattern consists of a rapid increase followed by a gradual decline to a new steady state. The decline phase coincides with an increase in the activity of a high affinity form of cAMP phosphodiesterase (PDE). Immunoprecipitation with specific antibodies revealed that the activated enzyme is a variant of PDE-4D. To confirm this observation, three isoforms of human PDE-4 (A, B, and D) were cloned and expressed in Sf9 cells with recombinant baculovirus infection. The activity of only one of the isoforms (PDE-4D3) increased after incubation with the catalytic subunit of protein kinase A and Mg-ATP. Hydrolytic activity of human PDE-4D3 was dependent on Mg^{2+}. Before phosphorylation, the concentration-response curve for Mg^{2+} was biphasic and ranged from 0.1 to 100 mM. Phosphorylation of PDE-4D3 by protein kinase A produced a monophasic Mg^{2+} response curve (0.5 V_{max} = 0.2 mM). Phosphorylation of PDE-4D3 increased the sensitivity of the enzyme to inhibition by RS-25344 (~100-fold) and RS-33793 (~330-fold). Thus, phosphorylation of PDE-4D3 induces an apparent conformation change that increases maximum velocity and sensitivity to inhibition by some analogues of nitraquazone. These observations provide the basis for a novel pharmacological strategy that targets an activated form of PDE in human leukocytes. Selective PDE-4D3 inhibitors may have useful anti-inflammatory properties with fewer adverse side effects than other PDE-4 inhibitors.

The primary functions of human leukocytes are inhibited by agents that produce an increase in the intracellular concentration of cAMP (1–3). These functions include the synthesis and release of inflammatory mediators, chemotaxis, and the proliferation of lymphocytes in response to antigen challenge. An increase in cAMP can be accomplished by agents that either stimulate adenylyl cyclase [ATP-pyrophosphate-lyase (cyclizing) EC 4.6.1.1] or inhibit cAMP PDE [3′:5′-nucleotidohydrolase, EC 3.1.4.17].

In vivo, PDE inhibitors may interact with endogenous stimulators of adenylyl cyclase to produce useful therapeutic effects. For example, Kuehl *et al.* (4) proposed that the anti-inflammatory effect of theophylline in the treatment of asthma is due to its ability to inhibit PDE activity in leukocytes. In this concept, theophylline synergizes with endogenous prostaglandins to increase cAMP and suppress the release of eicosanoids and other inflammatory mediators (4–6). Thus, PGE_2 functions as a negative feedback inhibitor, and this role is enhanced with theophylline.

At least seven different gene families of cyclic nucleotide PDE have been identified. The isoenzymes have different substrate affinities, and they are differentially regulated. The isoenzymes that preferentially hydrolyze cAMP are sensitive to selective inhibitors, and they are not uniformly distributed in different cell types (7, 8). These observations have renewed interest in the development of PDE inhibitors as pharmacological agents with the expectation that isoenzyme-selective compounds would exhibit greater specificity and produce fewer adverse side effects. In a search for novel anti-inflammatory agents, pharmacologists and medicinal chemists have focused on selective inhibitors of PDE-4 because it is the predominant form in human leukocytes (1–3, 8, 9).

PDE-4, a cAMP-specific enzyme with a high affinity for substrate, is selectively inhibited by rolipram and Ro 20–1724. The recent discovery of different cDNA isoforms and splice variants of PDE-4 suggests that the regulation of cAMP metabolism in leukocytes may be more complex than originally thought. Four distinct isoforms, provisionally designated PDE-4A, -4B, -4C, and -4D (8), have been cloned and expressed (10–14). mRNA for the C isoform was not detected by polymerase chain reaction analysis in human leukocytes

ABBREVIATIONS: PDE, phosphodiesterase; PG, prostaglandin; RS-25344, 8-aza-1-(3-nitrophenyl)-3-(4-pyridylmethyl)-2,4-[1-^{3}H]quinazoline dione; RS-33793, 8-(3-nitrophenyl)-6-(3-methyl-2-butenyl)pyrido [2,3a]pyridazin-5-one; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine albumin serum.

(9). An increase in intracellular cAMP induces the synthesis of the A, B, and D isoforms (9, 16), suggesting a role for these enzymes in the long term regulation of cAMP metabolism.

In contrast, a variant of rat PDE-4D (PDE-4D3) is subject to rapid activation after phosphorylation by protein kinase A (16–18). A 132-amino-acid domain, present in rat PDE-4D3, is phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (17). This domain contains two consensus sequences for protein kinase A phosphorylation.

Rapid, substrate-induced activation of PDE-4D may serve to modulate the elevation in cAMP after the receptor-mediated stimulation of adenylyl cyclase by hormones and neurotransmitters. For example, activation of PDE-4D3 would be expected to diminish the ability of PGE_2 to increase intracellular cAMP and to function as a feedback inhibitor of inflammatory responses.

The results of the present study demonstrate that endogenous PDE-4 activity is increased in a promonocytic cell line in response to PGE_2. Immunoprecipitation studies reveal that the activated enzyme is a variant of PDE-4D. Also, phosphorylation of cloned human PDE-4D3 results in activation and alters the sensitivity of the enzyme for Mg^{2+}. The phosphorylated enzyme, which may represent a good pharmacological target, exhibits a marked sensitivity to inhibition by two novel nitraquazone analogues, RS-25344 and RS-33793.

Materials and Methods

cAMP accumulation in U937 cells. U937 cells (American Type Culture Collection) were cultured at 37° in 7% CO_2 in RPMI 1640 containing HEPES buffer supplemented with L-glutamine, penicillin/streptomycin, and 10% heat-inactivated fetal calf serum. The cells were grown to a density of 1–2 × 10^6/ml. The cell suspension (~3 × 10^8 cells) was centrifuged at 120 × *g* for 10 min at room temperature. Pellets were resuspended in 10 ml RPMI 1640 containing 15% Nu-Serum and 0.1 μM [2,8-^{3}H]adenine (50 μCi, DuPont-NEN). The cells were preincubated for 45 min at 37° to permit incorporation of [^{3}H]adenine into the intracellular ATP pool. The suspension was then centrifuged at 120 × *g* for 5 min and resuspended in fresh RPMI 1640 containing 10% Nu-Serum. Washing was repeated twice to remove extracellular [^{3}H]adenine. The final pellet was resuspended in 10 ml RPMI 1640 containing 15% Nu-Serum. The final reaction volume was 200 μl, consisting of 170 μl of cell suspension, 20 μl of PGE_2, and 10 μl of rolipram or solvent. The incubation period was initiated by the addition of the cell suspension. The reaction was terminated after 0–10 min by the addition of 20 μl 2.2 N HCl containing 0.005 μCi [^{32}P]cAMP (DuPont-NEN) followed by rapid mixing. The isolation of cAMP was performed according to a previously described method (19).

PDE assay. The PDE incubation medium contained 10 mM Tris·HCl buffer, pH 7.7, at 22°, 1 mM $MgSO_4$, and 1.0 μM [^{3}H]cAMP (0.2 μCi, DuPont-NEN) in a total volume of 0.2 ml. After the addition of the enzyme, the contents were mixed and incubated for 10 min at 30°. The reaction was terminated by immersing the tubes in a boiling water bath for 60 sec. After the tubes were cooled in an ice-water bath, 20 μl (20 μg) of 5′-nucleotidase from snake venom (*Crotalus atrox*, V-7000, Sigma Chemical Co.) was added to each tube. The contents were mixed and incubated for 30 min at 30°. The nucleotidase reaction was terminated by immersion of the tubes in a boiling water bath for 60 sec. Labeled adenosine was isolated from alumina columns according to a previously described method (19). Assays were performed in triplicate. Hydrolysis of cAMP ranged from 10% to 20%. IC_{50} and EC_{50} values were visually determined from linear-log plots of the data generated by Kaleidagraph, a graphing program for Macintosh computers.

Effect of PGE_2 on PDE activity in U937 cells. Before the assay, the U937 cell suspension was centrifuged at 250 × *g* for 10 min in a table-top clinical centrifuge. The pellet was resuspended in Dulbecco's phosphate-buffered saline. The cell suspension (90 μl) was preincubated with 10 μl PGE_2 (10 μM) or solvent control for 0–10 min. The cell suspension was frozen in a dry ice/ethanol bath and thawed at 30° for 1 min to lyse the cells. The PDE reaction was initiated by the addition of 100 μl of incubation medium to yield a final concentration of 1 μM [^{3}H]cAMP, 1 mM $MgSO_4$, and 50 μg/ml saponin. The PDE reaction was performed at 30° for 5 min.

Immunoprecipitation of PDE activity in U937 cells. The soluble extracts from control or PGE_2-treated U937 cells were immunoprecipitated with anti-PDE-4 antiserum selective for either PDE-4A (AC55) or PDE-4B (K118) or with a monoclonal anti-PDE-4D antibody (M3S1) using protein G- sepharose 4B as previously described (17). PDE activity was measured in the resuspended pellets and the supernatants after immunoprecipitation. Adsorbed proteins were eluted with 1% SDS in phosphate buffered saline and separated by SDS-PAGE as previously described (18). Proteins were blotted on Immobilon membranes and analyzed by Western blot with an anti-PDE-4 antibody (K116) (18).

Preparation of recombinant human PDE-4 isoforms. An insect cell line (Sf9), derived from pupal ovarian tissue of the Fall army worm, *Spodoptera frugiperda*, was infected on day 0 with a recombinant baculovirus expressing PDE-4D3. Actively growing Sf9 cells at mid log stage and a density of 1.2 × 10^6 cells/ml were infected (virus/cell ratio = 0.5) and grown at 27° with orbital shaking at 160 rpm in Ex-Cell 400 media (JRH Biosciences). At the time of infection, the cell cultures were supplemented with 1% (f.c.) heat-inactivated fetal bovine serum (16140–014, GIBCO-BRL), 50 μg/ml gentamicin, and 4% feed stock [80 ml of feed stock is prepared by mixing 20 ml Yeastolate Ultrafiltrate, 50× (18200–014, GIBCO-BRL) plus 10 ml lipid concentrate, 100× (21900–014, GIBCO-BRL) plus 40 ml 2.5% glutamine in Ex-Cell 400 plus 10 ml 20% glucose]. The recombinant virus stock was made from an isolated plaque from the cotransfection of Sf9 cells with the plasmid expression vector pSYN XIV VI$^+$X/3-PDE IV (pHPD-43T) and BaculoGold virus DNA [a derivative of the *Autographa californica* nuclear polyhedrosis virus (21100D, PharMingen)]. On day 3 after infection, a cellular lysate was prepared with all steps performed at 0–4°, and the cells were harvested by centrifugation (10 min at 1000 × *g*) and suspended in lysate buffer equal to 20% of the original culture volume. The lysis buffer contained 5% glycerol, 45 mM Tris·HCl, pH 7.7, 0.5 mg/l leupeptin, 0.7 mg/l pepstatin, 0.2 mM phenylmethylsulfonylfluoride, and 0.1 mM sodium vanadate. The cells were homogenized with 5 strokes of a Dounce homogenizer using pestle B (Kontes). The lysate was clarified by centrifugation (10 min at 10,000 × *g*) and stored frozen at −80°. Aliquots (0.3 ml) were stored in ethylene glycol (30% f.c.). Before use in the PDE assay, the enzyme was diluted 1:400 into 10 mM Tris·HCl buffer, pH 7.4, containing 100 μg/ml BSA to stabilize the enzyme preparation.

For inhibitor screening assays, the remaining human PDE-4 isoforms were similarly prepared. Baculovirus expression of PDE-4A5 from plasmid pHPA-3T (PDE sequence of 837 amino acids starting AEDE) (13) and PDE-4C from plasmid pHPC-150 (20), which includes UCR-2 (13) from intron B (15) to the carboxyl terminus, were prepared as described for PDE-4D3 (above). PDE-4B1 was expressed in mouse MA-10 cells with the use of plasmid pCMV5-B72S, and the enzyme was provided by Dr. Al Baecker (Syntex Discovery Research, Palo Alto, CA).

Phosphorylation of human PDE-4D3. PDE-4D3 was preincubated in the presence or absence of the catalytic subunit of protein kinase A (1500 units/ml) for 8 min at 30°. The tubes contained 40 mM Tris·HCl buffer, pH 7.4, 0.2 mM ATP, 20 mM $MgSO_4$, and 0.05% BSA in a total volume of 0.1 ml. The kinase preincubation step was terminated by a 1:100 dilution with 10 ml ice-cold 10 mM Tris·HCl

buffer, pH 7.4, containing 0.2 mM EDTA. The diluted enzyme was added to test tubes containing 1 μM [^{3}H]cAMP, 100 mM NaCl, and $MgSO_4$, as indicated, to initiate the PDE assay. The reaction was performed for 10 min at 30°.

Chemicals. Rolipram, trequinsin, RS-25344, and RS-33793 were synthesized in the Institute of Organic Chemistry (Syntex Discovery Research, Palo Alto, CA). Stock solutions for these inhibitors were prepared in 100% dimethylsulfoxide. The final concentration of dimethylsulfoxide in the enzyme assay was 1%.

Results

Stimulation of PDE activity in U937 cells. PGE_2 increases intracellular cAMP in a promonocytic cell line (U937). Examination of the time course reveals a rapid increase in cAMP followed by a gradual decline to a new steady state level (Fig. 1A). Rolipram, a selective inhibitor of PDE-4, prevents the decrease in cAMP, permits a time-dependent accumulation of the cyclic nucleotide (Fig. 1A), and promotes a synergistic increase in the efficacy and potency of PGE_2 (Fig. 1B). These data resemble those obtained with prostaglandins and isobutyl-methyl xanthine on cAMP accumulation in human platelets (21). By analogy with these and subsequent studies (22), they suggest that protein kinase A stimulation of endogenous PDE activity produces the gradual decline in cAMP.

To test this possibility, we examined PDE activity in U937 cells. Hydrolytic activity increased after a brief incubation (2–10 min) with PGE_2 (Fig. 2). The PDE activity was completely inhibited by 10 μM rolipram and not blocked by 10 μM cycloheximide (data not shown). Results similar to those shown in Figs. 1 and 2 were obtained when histamine (0.1 mM) was used instead of PGE_2 (data not shown). Thus, agents capable of stimulating adenylyl cyclase and of increasing intracellular cAMP in U937 cells stimulate PDE-4 activity. The sequential activation of adenylyl cyclase and PDE-4 by these agents accounts for the observed rapid increase in cAMP accumulation followed by a gradual decline.

Identification of the activated PDE in U937 cells. Previous studies have shown that three of the four isoforms of PDE-4 (A, B, and D), may be present in U937 cells (9). A variant of the rat homologue of one of these forms, PDE-4D, is stimulated by the catalytic subunit of cAMP-dependent protein kinase (16–18). Subsequent studies revealed that rat and human PDE-4A and -4B were not significantly stimulated by the kinase (data not shown). These observations suggest that the isoform activated in U937 cells, after an increase in intracellular cAMP, is PDE-4D. To examine this hypothesis, we used isoform-selective PDE-4 antibodies to immunoprecipitate PDE activity from lysates of control and PGE_2-stimulated cell suspensions. The results indicate that the predominant isoform in U937 cells is PDE-4D, followed by PDE-4B, with PDE-4A as a minor component of total

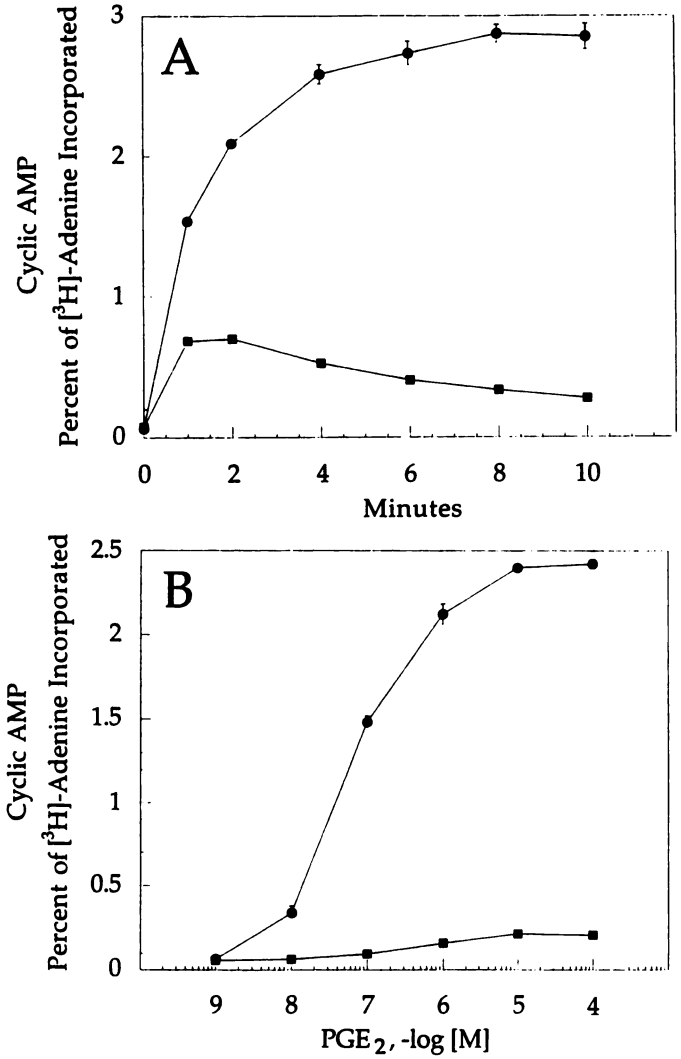

Fig. 1. Accumulation of cAMP in U937 cells. A, cAMP was measured as a function of time after incubation with 10 μM PGE_2 in the presence (●) or absence (■) of 10 μM rolipram. B, Concentration-response curves for PGE_2 incubated for 10 min in the presence (●) or absence (■) of 10 μM rolipram. The EC_{50} value for PGE_2 alone was 0.45 μM; it decreased to 0.06 μM when combined with rolipram. Values represent the mean ± standard deviation of triplicate determinations. Data presented are from a representative experiment that was repeated three times with similar results. Accumulation of cAMP was measured as described in Materials and Methods.

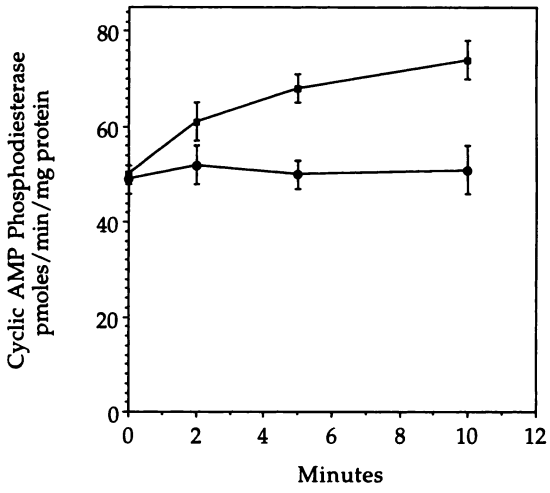

Fig. 2. Effect of PGE_2 on PDE activity in U937 cells. A suspension of U937 cells in Dulbecco's phosphate-buffered saline was preincubated with 10 μM PGE_2 (■) or solvent control (●) for the times indicated. The cell suspension was frozen in a dry ice/ethanol bath and thawed at 30° for 1 min to lyse the cells. The PDE reaction was initiated by the addition of substrate, and the reaction was performed at 30° for 5 min. Values represent the mean ± standard deviation of triplicate determinations. Data presented are from a representative experiment that was repeated three times with similar results.

enzyme activity (Fig. 3). PGE_2-stimulated activity is present only in the pellet produced by the antibody to PDE-4D (Fig. 3). Western blot analysis of the PDE activity precipitated by M3S1 or with a nonselective anti-PDE-4 antibody (K116) showed the presence of two immunoreactive polypeptides of 93 and 100 kDa, respectively. The 93-kDa band comigrates with a recombinant human PDE-4D3 protein on SDS-PAGE gels. The 100-kDa protein does not correspond to any of the recombinant PDE-4 enzymes reported to date. It is possible, however, that a larger PDE-4D isoform is expressed in U937 cells because a fourth mRNA variant has been cloned that is larger than the mRNA for PDE-4D3 (13).

We have previously shown that rat PDE-4D3 is activated by hormones through phosphorylation mediated by protein kinase A (17, 18). These data prompted additional studies on human PDE-4D3 and the activation process.

Activation of recombinant human PDE-4D3 by phosphorylation. The cloned isoform of human cAMP PDE (PDE-4D3) used in these experiments was expressed in baculovirus-infected insect cells (Sf9). PDE activity was linear as a function of time for 30 min, proportional to enzyme concentration and specific for the hydrolysis of cAMP, with a K_m of 3.1 μM. To determine the effect of phosphorylation on human PDE-4D3, we incubated the enzyme in the presence or absence of the catalytic subunit of protein kinase A and Mg-ATP for 12 min. Incubation with the kinase increased the activity of the human PDE-4D3, and this effect was dependent on the presence of Mg-ATP in the reaction buffer (Fig. 4). Activation of the enzyme is mediated by phosphorylation, as demonstrated by the incorporation of ^{32}P into human PDE-4D3 after the addition of protein kinase A (Fig. 4). As previously shown for the rat homologue of PDE-4D3, phosphorylation of the enzyme is associated with a shift in protein migration on SDS-PAGE (16, 17).

Phosphorylation of PDE-4D3 and the requirement for Mg^{2+}. Catalytic activity of the soluble enzyme (674 amino acids) was dependent on Mg^{2+}, with a biphasic response curve (Fig. 5). In addition, we conclude that the Mg^{2+} activation site is associated with the catalytic domain because a truncated recombinant protein (391 amino acids) lacking the first 298 amino acids from the amino terminus displayed Mg^{2+}-dependent PDE activity (data not shown). To determine the effect of phosphorylation on the Mg^{2+} requirement of human PDE-4D3, the enzyme was incubated with the catalytic subunit of cAMP-dependent protein kinase before PDE activity was measured in the presence of different concentrations of Mg^{2+}. Incubation with protein kinase and Mg-ATP converted the biphasic Mg^{2+} response curve to a monophasic curve (0.5 V_{max} = 0.2 mM; Fig. 5). Under these conditions, there was no significant change in the affinity for cAMP as the substrate (K_m = 3 μM).

Selective inhibitors of PDE-4. RS-25344, an analogue of nitraquazone, is a potent and selective inhibitor of the endogenous PDE-4 in human lymphocytes with an IC_{50} value of 0.3 nM (23). IC_{50} values for the inhibition of PDE-1, -2, and -3 with this compound are >100 μM (23). Under basal conditions, shallow concentration-response curves (>4 log intervals) for the inhibition of PDE-4D3 were obtained with RS-25344 (data not shown). This observation suggested that the inhibitor may be interacting with two or more different forms of the cloned enzyme with dissimilar affinities for RS-25344. Such forms could arise by partial phosphorylation of newly synthesized enzyme.

Selective inhibition of recombinant human PDE-4D3. Subsequent experiments, designed to test this possibility, revealed that phosphorylation of PDE-4D3 produced an enzyme that was approximately 100 times more sensitive to inhibition by RS-25344 than control enzyme preparations (Fig. 6). In addition, the concentration-response curve obtained with the phosphorylated form of PDE-4D3 conformed more closely to typical saturation kinetics expected for a competitive inhibitor. In contrast, the IC_{50} value (1 μM) for trequinsin was not altered by phosphorylation (Fig. 7). Inhibition of the activated form of PDE-4D3 by RS-25344 was competitive (Fig. 8). Similarly, trequinsin exhibited competitive inhibition. Thus, phosphorylation produces an activated form of PDE-4D3 with increased sensitivity to some, but not all, inhibitors.

To obtain structure-activity relationships and search for a more selective PDE-4D inhibitor than RS-25344, a large

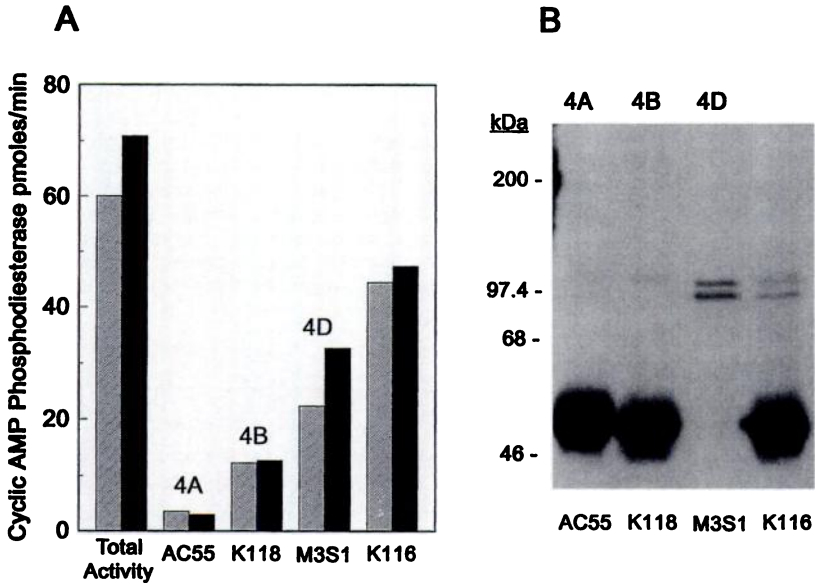

Fig. 3. Effect of PGE_2 on PDE activity and Western blot analysis of U937 extracts. U937 cells were incubated for 10 min in the absence or presence of PGE_2 as described in Materials and Methods. At the end of the treatment, cells were homogenized and centrifuged for 10 min at 14,000 × *g*. Soluble extracts were incubated at 4° for 90 min with protein G- preadsorbed to an anti-PDE-4A antiserum (*AC55*), an anti-PDE-4B antiserum (*K118*), or an anti-PDE-4D monoclonal antibody (*M3S1*). At the end of the incubation, samples were centrifuged to separate free and immunoadsorbed proteins. A, Immunoadsorbed proteins were resuspended in PBS containing 0.05% BSA and PDE activity was assayed. Data represent PDE activity in the presence (*solid bars*) or absence (*striped bars*) of 10 μM PGE_2. Values are from a single experiment that was repeated three times with similar results. B, Immunoabsorbed proteins in PBS containing 1% SDS were analyzed by SDS-PAGE and Western blot with an isoform-nonselective anti-PDE-4 antiserum (*K116*).

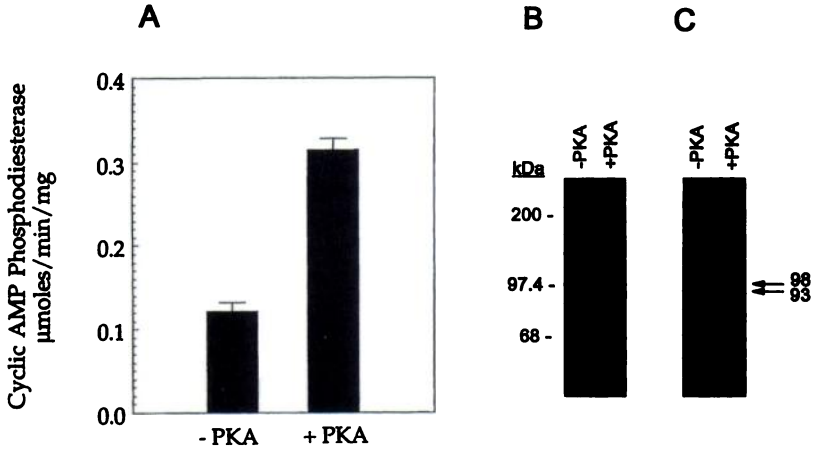

Fig. 4. Effect of protein kinase A on recombinant human PDE-4D3 activity and Western blot analysis. Recombinant human PDE-4D3 was incubated for 10 min in the presence (+*PKA*) or absence (−*PKA*) of the catalytic subunit of protein kinase A (0.2 µM) in a reaction buffer containing 40 mM Tris·HCl, pH 7.4, 10 mM MgOAc, and 0.2 mM ATP. A, At the end of the incubation, PDE activity was measured with 1 µM cAMP as the substrate. In some experiments, 10 µCi [γ-^{32}P]ATP was added to the reaction. At the end of the incubation, samples were diluted in SDS-PAGE sample buffer, and the proteins were separated by SDS-PAGE and transferred to Immobilon membranes. B, After the transfer, the membranes were subjected to autoradiography. C, Next, the membranes were analyzed by Western blot with an anti-PDE-4D monoclonal antibody (M3S1) (C).

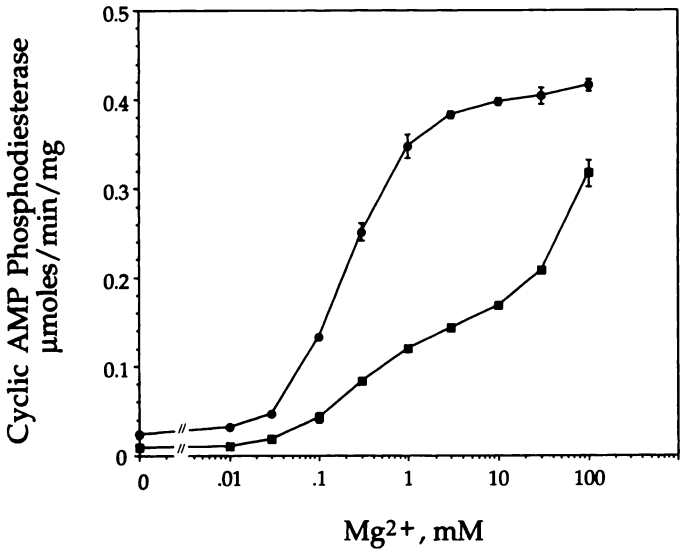

Fig. 5. Effect of phosphorylation of PDE-4D3 on the requirement for Mg^{2+}. PDE-4D3 was preincubated in the presence (●) or absence (■) of the catalytic subunit of protein kinase A for 8 min at 30°. The kinase preincubation step was terminated by dilution into ice-cold 10 mM Tris·HCl buffer containing 0.2 mM EDTA. The diluted enzyme was added to test tubes containing 1 µM [^{3}H]cAMP, 100 mM NaCl, and Mg^{2+}, as indicated. The PDE reaction was performed for 10 min at 30°. Values represent the mean ± standard deviation of triplicate determinations. Data presented are from a representative experiment that was repeated three times with similar results.

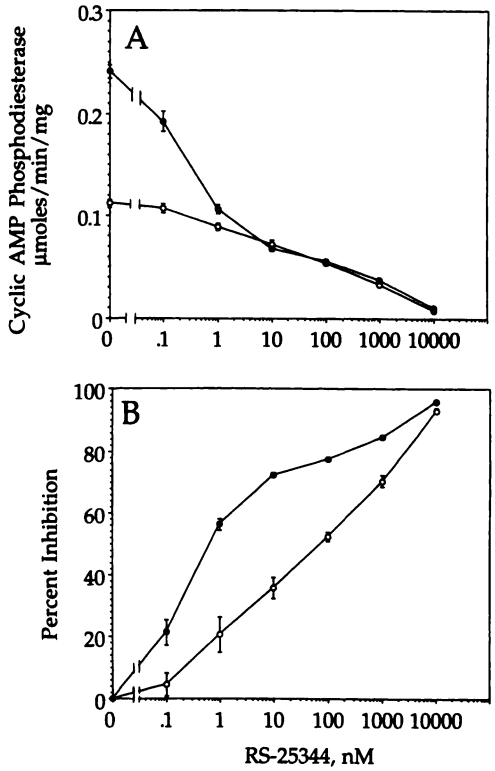

Fig. 6. Effect of phosphorylation on the sensitivity to inhibition by RS-25344. PDE-4D3 was preincubated in the presence (●) or absence (○) of the catalytic subunit of protein kinase A for 8 min at 30° as described in Materials and Methods. PDE-4D3 was diluted 1:500 with 10 mM Tris·HCl buffer containing 0.1 mg/ml BSA. The diluted enzyme was added to test tubes containing RS-25344, as indicated. The PDE reaction was performed for 10 min at 30° in the presence of 10 mM $MgSO_4$. A, The ordinate represents specific activity. B, The ordinate represents percent inhibition. Values represent the value ± standard deviation of triplicate determinations. Data presented are from a representative experiment that was repeated three times with similar results.

number of compounds (>300) were screened versus isoforms A, B, C, D, and phosphorylated D. From this screen, RS-33793 (Fig. 9) was identified as an inhibitor with marked selectivity (~330-fold) for the phosphorylated form of PDE-4D3. K_i values for RS-33793 were 49, 102, 49, 40, and 0.12 nM versus isoforms A, B, C, D, and phosphorylated D, respectively.

Discussion

The results of the present study indicate that U937 cells do not have the capacity to generate a sustained increase in cAMP in response to PGE_2 unless PDE activity is inhibited. The magnitude and duration of the increase in cAMP after exposure to PGE_2 appear to involve a cAMP-dependent stimulation of PDE activity, and this temporal pattern can be modulated by rolipram, a selective PDE-4 inhibitor.

Previous studies by Torphy *et al.* (24) demonstrated that the synthesis of PDE-4 was induced after prolonged (2–4 hr) exposure to PGE_2, salbutamol, and 8-bromo-cAMP. The increase in PDE-4 was abolished by the protein synthesis inhibitor cycloheximide. In contrast, the short (2–10 min) exposure to PGE_2 described in the present study produced a rapid increase in PDE-4 activity that was not blocked by 10 µM cycloheximide. From these data, we conclude that PGE_2 is

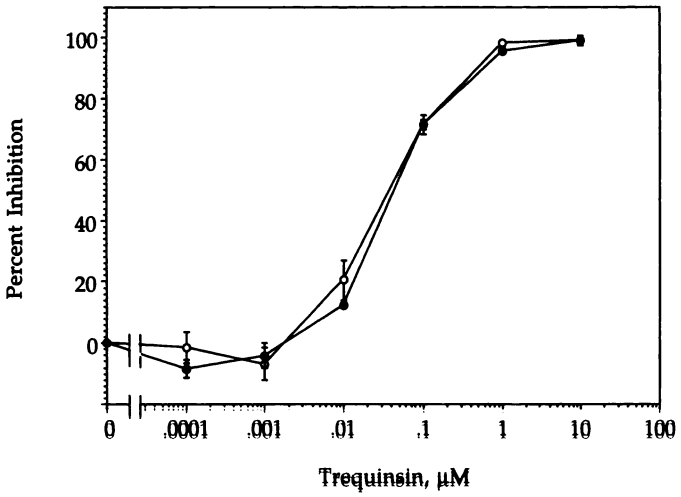

Fig. 7. Effect of phosphorylation on the sensitivity to inhibition by trequinsin. PDE-4D3 was preincubated in the presence (●) or absence (○) of the catalytic subunit of protein kinase A for 8 min at 30° as described in Materials and Methods. PDE-4D3 was diluted 1:500 with 10 mM Tris·HCl buffer containing 0.1 mg/ml BSA. The diluted enzyme was added to test tubes containing trequinsin, as indicated. The PDE reaction was performed for 10 min at 30° in the presence of 10 mM $MgSO_4$. Values represent the mean ± standard deviation of triplicate determinations. Data presented are from a representative experiment that was repeated three times with similar results. Protein kinase A stimulated PDE-4D3 activity 1.32-fold.

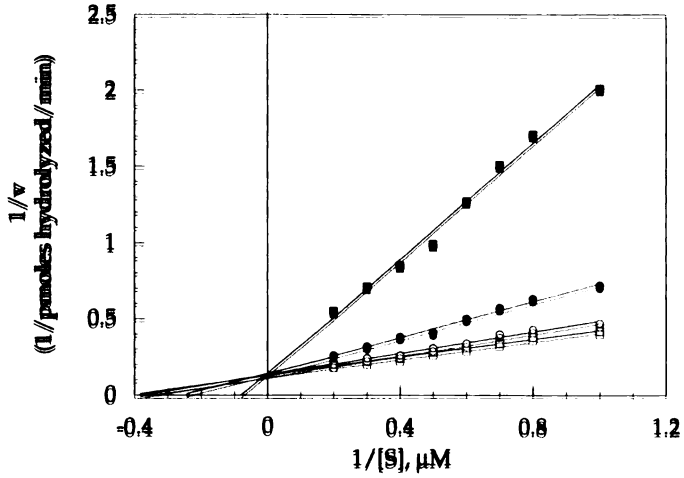

Fig. 8. Inhibition of phosphorylated PDE-4D3 by RS-25344. Data for velocity as a function of substrate concentration are presented as a Lineweaver-Burk plot. □, Control; RS-25344 concentrations: ○, 0.04 nM; ●, 0.2 nM; ■, 1 nM. PDE-4D3 was activated by phosphorylation as described in Materials and Methods. The PDE reaction was performed with 1 mM $MgSO_4$ for 10 min at 30°.

able to increase PDE-4 activity via two mechanisms: activation (rapid) and induction (delayed).

Hydrolytic activity of PDE-4D3 is dependent on the presence of Mg^{2+} as an essential activator. The results presented demonstrate that phosphorylation of PDE-4D3 increases catalytic activity and alters the sensitivity of the enzyme to high concentrations (≥10 mM) of Mg^{2+}. In addition, phosphorylation increased the sensitivity of the enzyme to inhibition by RS-25344 (≈100-fold) and RS-33793 (≈330-fold). Thus, phosphorylation induces an apparent conformation change that increases the maximum velocity of the enzyme and sensitivity to inhibition by some analogues of nitraquazone but not trequinsin.

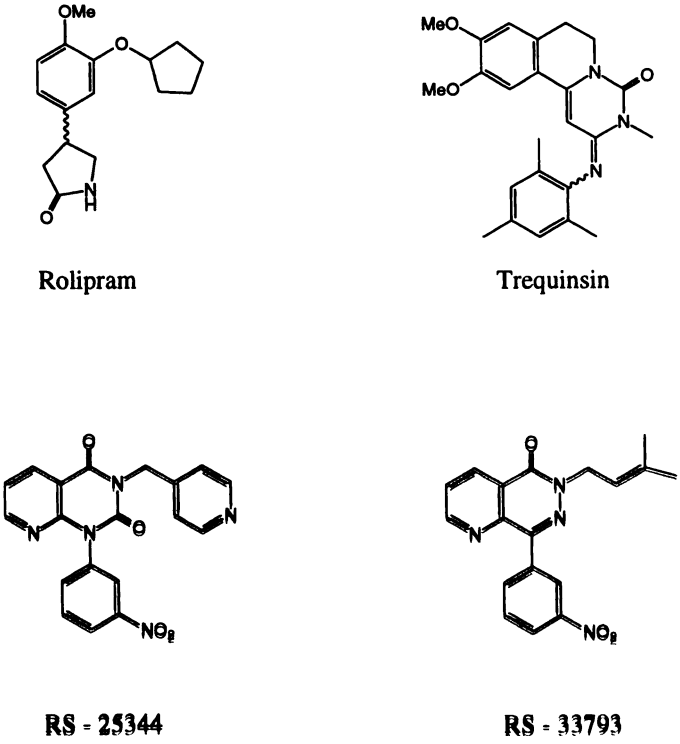

Fig. 9. Chemical structures of rolipram, trequinsin, RS-25344, and RS-33793.

Previous studies with thyroid cells demonstrated that PDE-4D3 is phosphorylated and activated by protein kinase A (18). Although some variants of PDE-4A and -4B are substrates for phosphorylation catalyzed by protein kinase A, we have been unable to detect a significant increase in enzyme activity with these variants. It is conceivable that phosphorylation of these variants induces a conformation change but the modification fails to increase catalytic activity.

RS-25344 produces a shallow inhibition curve over several log intervals, suggesting an interaction with two or more forms of the enzyme with different affinities for the inhibitor. After phosphorylation, the kinetic pattern by RS-25344 becomes typical of a competitive inhibitor, with the slope of the curve close to unity. In addition, PDE-4D3 becomes ≈100 times more sensitive to inhibition by RS-25344. This change in sensitivity is even more apparent with RS-33793 (data not shown). Our attempts to reverse the protein kinase-mediated activation of PDE-4D3 with alkaline phosphatase and other phosphatases have been unsuccessful.

The Mg^{2+} concentration-response curve for the recombinant PDE-4D3 not incubated with protein kinase A was biphasic. The metal ion appears to stimulate hydrolysis when included in the assay at high concentrations (>10 mM). This effect is unlikely to be due to an increase in ionic strength of the incubation medium because 100 mM NaCl was present in all tubes. After phosphorylation, the Mg^{2+} response becomes monophasic. One interpretation of these data is that phosphorylation converted low affinity binding sites for Mg^{2+} to high affinity binding sites. Additional experiments will be required to examine this phenomenon. Nevertheless, the available data suggest that a significant conformation change occurs after phosphorylation.

The present study demonstrates that PGE_2 stimulates the activity of a cAMP-specific PDE isoform identified as a vari-

ant of PDE-4D. The activation is mediated by cAMP and can be reproduced *in vitro* with recombinant enzyme and purified catalytic subunit of protein kinase A. The phosphorylation of PDE-4D3 changes the kinetic properties of the enzyme and alters the sensitivity to specific inhibitors. These observations provide the conceptual basis for a novel pharmacological strategy that targets an activated (phosphorylated) form of PDE in human leukocytes. Such compounds may have useful anti-inflammatory properties with fewer adverse side effects than other PDE-4 inhibitors.

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