Salbutamol Up-regulates PDE4 Activity and Induces a Heterologous Desensitization of U937 Cells to Prostaglandin E₂

IMPLICATIONS FOR THE THERAPEUTIC USE OF β-ADRENOCEPTOR AGONISTS*

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Theodore J. Torphy, Han-Liang Zhou, James J. Foley, Henry M. Sarau, Carol D. Manning, and Mary S. Barnette

From the Division of Pharmacological Sciences, UW2532, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406-0939

Previous studies with U937 cells, a human monocyte cell line, have shown that the activity of cyclic nucleotide phosphodiesterase 4 (PDE4) is increased by agents that elevate cyclic AMP content. The present experiments were conducted to determine 1) whether an increase in PDE4 steady-state message and/or protein accompanies the up-regulation of PDE4 activity and 2) whether the up-regulation changes the functional responses of U937 cells to activators of adenylyl cyclase. To up-regulate PDE4 activity, U937 cells were treated for 4 h with a combination of 1 μM salbutamol, a β-adrenoceptor agonist, and 30 μM rolipram, a PDE4 inhibitor. Cells were washed extensively to remove drugs and used immediately in various experimental protocols.

Cyclic nucleotide phosphodiesterases (PDEs)¹ are a family of isozymes that catalyze the hydrolysis of the 3'-phosphoester bond on adenosine cyclic 3',5'-monophosphate (cAMP) and guanosine cyclic 3',5'-monophosphate to form the inactive 5'-monophosphate products. Consequently, PDEs have a major role in regulating cellular cyclic nucleotide content. It is now recognized that PDEs represent a diverse family of isozymes, each with different kinetic and physical characteristics, tissue distribution, and sensitivity to endogenous regulators (Beavo,

¹ The abbreviations used are: PDE, phosphodiesterase; LTD₄, leukotriene D₄; hPDE, human recombinant phosphodiesterase; PDE4, cAMP-specific PDE; PGE₂, prostaglandin E₂; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; bp, base pairs.

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†To whom correspondence should be addressed: SmithKline Beecham Pharmaceuticals, Div. of Pharmacological Sciences, 709 Swedeland Rd., UW2532, King of Prussia, PA 19406-0939. Tel.: 610-270-6821; Fax: 610-270-5381.
Up-regulation of PDE4 Desensitizes U937 Cells

these cells (Torphy et al., 1992). This increase in activity develops after 2–4 h of agonist exposure and is: 1) preceded by an increase in V_{\text{max}} with no change in the CAMP K_{i}^{*}; 3) mimicked by 8-bromo-cAMP; 4) marked by an activity; 2) potentiated by cotreatment with rolipram, a PDE4 inhibitor; 3) preceded by an increase in V_{\text{max}} with no change in the CAMP K_{i}^{*}; 5) abolished by inhibitors of mRNA or protein synthesis; and 6) reversible within 3 h of agonist removal. In light of these results, the present experiments were conducted to determine whether treatment of U937 cells with b-adrenoceptor agonists increases the steady-state levels of PDE4 protein or transcript and, if so, whether this phenomenon changes the functional responsiveness of these cells to activators of adenyl cyclase.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Pretreatments—U937 cells from the American Type Culture Collection were grown in plastic flasks (80 cm²) in Roswell Park Memorial Institute (RPMI-1640) supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. Culture medium was changed every 2–3 days and always 24 h before harvest. Cells were seeded at densities of 0.5–10 × 10⁶ cells/ml in 118 mM NaCl; 4.6 mM KCl, 24.9 mM NaHCO₃, 1 mM KH₂PO₄, 11.1 mM glucose, 1 mM CaCl₂, 1.1 mM MgCl₂, and 5 mM HEPES, pH 7.4. Cells were then resuspended at a final concentration of 10⁶ cells/ml in Krebs-Ringer-Henseleit buffer containing 0.1% bovine serum albumin (radioimmunossay grade) and treated for the indicated times with different concentrations of PGE₂. Incubation buffer, pH 7.4, treated for the indicated times with different concentrations of PGE₂, in the absence of reverse transcriptase as a control for each RNA sample. Reactions were 1 min at 72 °C for a subsaturating cycle number (35 or 40 cycles). Products were electrophoresed on SDS-8% polyacrylamide gels and electroblotted 1:100 into a lysate of either the nontransfected PDE-deficient Saccharomyces cerevisiae strain GL62 (control) or the same strain engineered to express a 668-residue fragment of human recombinant PDE4A (hrPDE4A) containing the conserved catalytic domain of PDE4. The mixtures were incubated overnight at 4 °C with gentle agitation. The antibody was used at a final dilution of 1:2000 in PBS/Tween-20, 1% nonfat dry milk.

Assembled with three washes with PBS/Tween-20 blots were then incubated for 1 h with horseradish peroxidase-linked anti-rabbit Ig whole antibody from donkey (Amersham) and washed five times with PBS/Tween-20. Immunoactive proteins were detected by chemiluminescence (Amersham ECL reagents).

Reverse Transmission and Amplification by Polymerase Chain Reaction (RT-PCR)—RNA was purified using the single-step total RNA isolation procedure (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987). Washed and pelleted monocytes (28.6 × 10⁶ cells/ml) were resuspended in denaturing solution (4 μM guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-mercaptoethanol, 0.5% N-lauroylsarcosine, 0.5% sodium deoxycholate, and 100 mM sodium dodecyl sulfate). After four washes with PBS, the mRNA preparations were DNase-treated for 15 min at 37 °C and repurified by phenol/chloroform extraction and ethanol precipitation. RT-PCR was carried out using a commercial RNA PCR kit (Saiki et al., 1988). First strand cDNA was generated from total RNA using random hexamers to prime the reverse transcription and was then amplified by PCR following the addition of specific primer pairs (0.36 μg/tube) and Ampli-taq DNA polymerase. Oligonucleotide primers were: PDE4A₅, 5′-AACAGGCGCTGAAAGCC-3′ and 5′-TCTACGTTACGACCGATAGTTCC-3′. Cells were transfected with either the nontransfected PDE-deficient Saccharomyces cerevisiae strain GL62 (control) or the same strain engineered to express a 668-residue fragment of human recombinant PDE4A (hrPDE4A) containing the conserved catalytic domain of PDE4. The mixtures were incubated overnight at 4 °C with gentle agitation. The antibody was used at a final dilution of 1:2000 in PBS/Tween-20, 1% nonfat dry milk.

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exposed to vehicle or rolipram (10 μM) for 1 min before the addition of various concentrations of PGE2. One min after the addition of PGE2, cells were challenged with 3.3 mM leukotriene D4 (LTD4), and Ca2+ mobilization was monitored and quantified via fura-2 fluorescence (Winkler et al., 1988).

Results

PDE4 Subtype Gene Expression—PDE4 subtype-specific RT-PCR products in control and PDE4-up-regulated cells are shown in Fig. 1. Transcripts for PDE4B and PDE4D were detected in untreated cells, whereas only a faint band corresponding to PDE4C was observed. The PCR products in control and PDE4-up-regulated cells are shown in Fig. 1. Transcripts for PDE4B and PDE4D were determined by an unpaired or paired Student’s t test. Differences among means were accepted as significant at \( p < 0.05 \). Additionally, the effect of up-regulating PDE4 activity on PGE2-stimulated phosphodiesterase activity was confirmed by conducting an analysis of variance and a Newman-Keuls multiple comparison test.

Western blot analysis of PDE4 immunoreactivity in U937 cells and human monocytes using antibody raised against GalK-hrPDE4A. Panel A, U937 cells were untreated or exposed to 1 μM salbutamol and 30 μM rolipram for 4 h. After the treatment period, RNA was prepared, reverse transcribed into cDNA, and amplified using primer sets specific for the four subtypes of PDE4. The PCR products were electrophoresed on 3% agarose gels and visualized with ethidium bromide. RNA normalization for control and treated samples was confirmed by conducting RT-PCR reactions using primers for β-actin RNA (–). Lack of DNA contamination was confirmed by conducting the β-actin reaction in the absence of RT (–). DNA molecular mass standards appear in the lane on the far left. The results are representative of three experiments using material from three different cell preparations.

Cells were treated with salbutamol and rolipram a band of immunoreactivity derived from PDE4B and PDE4D, but no hrPDE4A (Fig. 3 A). Although a faint band of immunoreactivity in U937 cells was observed that corresponded to a large molecular mass protein (~60–120 kDa) in untreated U937 cells (Fig. 2A). However, after 4 h of treatment with salbutamol and rolipram a band of immunoreactivity was consistently observed. The band corresponded to a molecular mass of approximately 125-kDa, identical in size to a band of immunoreactivity detected in human blood monocytes. As expected, the antibody also bound to a truncated form of hrPDE4A that lacks 264 N-terminal amino acids. To demonstrate that the 125-kDa protein was indeed PDE4, studies were conducted in which the antibody was saturated with the truncated hrPDE4A before it was exposed to the immunoblot (Fig. 2B). Preabsorbing the antibody in this manner virtually abolished its ability to detect either hrPDE4A or U937 cell PDE4. Note that the preabsorption procedure also reduced or eliminated the immunoreactivity directed against several other minor bands in U937 cells, suggesting that these proteins represented fragments of PDE4.

The antibody raised against the GalK-hrPDE4A also detected hrPDE4D, but not hrPDE4B (Fig. 2B). A Western blot analysis of PDE4 immunoreactivity in U937 cells and human monocytes using antibody raised against GalK-hrPDE4A. Panel A, U937 cells were untreated or exposed to 1 μM salbutamol and 30 μM rolipram for 4 h. After the treatment period, RNA was prepared, reverse transcribed into cDNA, and amplified using primer sets specific for the four subtypes of PDE4. The PCR products were electrophoresed on 3% agarose gels and visualized with ethidium bromide. RNA normalization for control and treated samples was confirmed by conducting RT-PCR reactions using primers for β-actin RNA (–). Lack of DNA contamination was confirmed by conducting the β-actin reaction in the absence of RT (–). DNA molecular mass standards appear in the lane on the far left. The results are representative of three experiments using material from three different cell preparations.
were untreated or exposed to 1 μM salbutamol and 30 μM rolipram for 4 h. After the treatment period cells were washed extensively and lysed. Supernatant fractions were then prepared and identical amounts of protein (~100 μg) were subjected to SDS-polyacrylamide gel electrophoresis. Immunoreactivity was determined using antibody raised against GaIK-hrPDE4A. Also run in these studies were lysates from yeast engineered to express hrPDE4A (full-length), PDE4B, or PDE4D. The data are representative of results from four (U937 cell lysates) or two (hrPDE4 subtypes) experiments. Panel B, additional experiments were conducted to detect PDE4B immunoreactivity in control U937 cells and cells treated for 4 h with salbutamol (1 μM) and rolipram (30 μM). For reference purposes, hrPDE4B was also run. The antibody used in these studies was raised against a unique carboxyl-terminal PDE4B peptide. The data are representative of two experiments.

PGE2-stimulated cAMP Accumulation—To determine whether up-regulating PDE4 activity had an impact on agonist-induced cAMP accumulation, U937 cells were treated for 4 h with vehicle or a combination of salbutamol (1 μM) and rolipram (30 μM). At the end of the 4-h pretreatment period, cells were washed extensively to remove drugs before being resuspended in drug-free Krebs-Ringer-Henseleit buffer. The cells were then exposed to 1 μM of PGE2 and cAMP was measured at various times over a 15-min period (Fig. 4). In vehicle-pretreated cells, basal cAMP content (1.7 ± 0.2 pmol/10^6 cells) was increased by 25-fold (41.5 ± 7.8 pmol/10^6 cells) within 2 min of being exposed to PGE2. In cells in which PDE4 activity had been up-regulated, basal cAMP content (2.4 ± 0.3 pmol/10^6 cells) was not significantly different from control cells. However, PGE2-stimulated cAMP accumulation was substantially less, reaching a maximum of only 23.3 ± 7.3 pmol/10^6 cells within 2 min. A pattern virtually identical to this was observed in separate studies using a larger concentration (10 μM) of PGE2 (data not shown).

PGE2-induced cAMP accumulation in control and PDE4 up-regulated cells was also assessed in the presence of 30 μM rolipram (Fig. 4). We reasoned that if the decrease in PGE2-stimulated cAMP accumulation observed in salbutamol-pretreated cells was due, at least in part, to an up-regulation of PDE4 activity, then inhibiting PDE4 in these cells with rolipram would tend to normalize their responsiveness to PGE2. Indeed, in the presence of rolipram, PGE2-stimulated cAMP accumulation over the 15-min time course was virtually identical in control versus PDE4-induced cells (Fig. 4). Only after 15 min of exposure to PGE2 was cAMP content slightly less in PDE4 up-regulated cells (68.2 ± 11.2 pmol/10^6 cells) than in control cells (89.4 ± 9.6 pmol/10^6 cells).

The ability of a range of PGE2 concentrations (1 nm-10 μM) to elevate cAMP content in control and PDE4-up-regulated U937 cells is shown in Fig. 5. The conditions of these experiments were identical to those of the time course studies, except that cells were treated with various concentrations of PGE2 for a single fixed time (15 min) before cAMP content was determined. In control cells PGE2 produced a large, concentration-related increase in cAMP accumulation. For example, basal cAMP content (0.99 ± 0.14 pmol/10^6 cells) was increased to 52.9 ± 2.9 pmol/10^6 cells by 10 μM PGE2, greater than 50-fold over the basal level. In contrast, PGE2 had much less effect on cAMP content in cells in which PDE4 activity had been up-regulated. In fact, cAMP accumulation stimulated by 10 μM PGE2 was only 5.4 ± 0.5 pmol/10^6 cells, nearly 10-fold less than in control cells and only 5-fold above basal cAMP content (0.97 ± 0.12 pmol/10^6 cells).

In the presence of 10 μM rolipram, PGE2-induced cAMP accumulation was greater in both control cells and, even more impressively, in cells in which PDE4 activity had been up-regulated. Overall, inhibiting PDE4 activity with rolipram increased PGE2-stimulated cAMP accumulation by 2-fold in control cells and 12-fold in PDE4 up-regulated cells. Thus, in the presence of rolipram, maximal PGE2-stimulated cAMP accu-
mulation in cells in which PDE4 activity had been up-regulated was only 2-fold less than in control cells. This contrasts with the 10-fold difference detected in the absence of rolipram.

Effect of PDE4 Up-regulation on Agonist-stimulated Ca²⁺ Mobilization—Consistent with the results of other studies (Saussy et al., 1989; Winkler et al., 1990), LTD₄ produced a concentration-dependent Ca²⁺ mobilization with an EC₅₀ = 3–5 nM. Note that up-regulating PDE4 activity had no effect on the LTD₄ concentration-response curve (Fig. 6).

Although up-regulation of PDE4 activity had no direct effect on LTD₄-stimulated Ca²⁺ mobilization, it had a substantial effect on the ability of PGE₂ to inhibit this response (Fig. 7). In control cells PGE₂ suppressed maximal Ca²⁺ mobilization induced by 3.3 nM LTD₄ with an IC₅₀ = 30 nM and a maximal inhibitory effect of 70 ± 1% (Fig. 7A). Prior exposure of U937 cells to salbutamol (1 µM) and rolipram (30 µM) for 4 h to induce PDE4 resulted in a substantial reduction in the inhibitory effect of PGE₂. In these cells, PGE₂ suppressed Ca²⁺ mobilization with an IC₅₀ = 150 nM, 5-fold greater than in untreated cells, and had a maximal inhibitory effect of only 21 ± 2%.

The heterologous desensitization to the inhibitory effect of PGE₂ in PDE4 up-regulated cells was largely reversed in the presence of 10 µM rolipram (Fig. 7B). For example, although the maximal inhibitory effect of PGE₂ on Ca²⁺ mobilization was statistically less in PDE4 up-regulated cells even in the presence of 10 µM rolipram, the difference from control cells was extremely small (70 ± 3%, control versus 65 ± 1%, up-regulated). Qualitatively, similar results were obtained in studies in which PGE₂-stimulated cAMP accumulation was assessed in the presence of 100 µM rolipram, although a significant diminution in the ability of PGE₂ to inhibit Ca²⁺ mobilization in PDE4 up-regulated cells under these conditions was observed only at one concentration of PGE₂ (10 nM PGE₂ data not shown). No statistically significant difference was observed with any of the other concentrations of PGE₂. Thus, the ability of PGE₂ to inhibit Ca²⁺ mobilization in cells that have increased PDE4 activity is largely recovered in the presence of a PDE4 inhibitor.

**DISCUSSION**

PDE4 is the predominant cAMP-metabolizing enzyme family in inflammatory cells and has been identified as an important new molecular target for novel antiasthmatic and anti-inflammatory drugs (Torphy and Undem, 1991; Giembycz and Dent, 1992). Based upon the results of recent studies in which Sertoli cells were used as a model system (Sette et al., 1994a, 1994b), considerable attention has been focused on two general mechanisms by which the activity of PDE4 is regulated by hormones, particularly those that stimulate adenyllyl cyclase activity. One regulatory mechanism, designated “short term activation,” involves a protein kinase A-mediated phosphorylation of a specific splice variant PDE4D. This phosphorylation results in an increase in catalytic activity, perhaps by allosteric modification of the catalytic domain (Sette et al., 1994b). A second regulatory mechanism, designated “long term activation,” occurs with two other splice variants of PDE4D (Swinnen et al., 1989, 1991). Activation of protein kinase A in intact cells increases the expression of these latter forms by enhancing mRNA synthesis or increasing mRNA stability.

Although indirect evidence suggests that activators of adenyllyl cyclase can regulate PDE4 activity in immune and inflammatory cells (Chan et al., 1982; Holden et al., 1987, Bourne et al., 1973; Torphy et al., 1992), definitive information on the precise nature of this phenomenon in these cells is not available. Moreover, despite the growing body of evidence suggesting that the activity of PDE4 can be up-regulated by hormonal stimulation, little is known about the biological importance of this regulation. We have begun to address these deficiencies by examining the nature and functional consequences of PDE4 up-regulation in U937 cells, a human monocytic cell line. As previously reported (Torphy et al., 1992), activation of the protein kinase A cascade in these cells by β-adrenoceptor agonists increases PDE4 catalytic activity. The magnitude of this up-regulation is enhanced if rolipram is included in the incubation medium, presumably because inclusion of a PDE inhibitor both heightens and prolongs the increase in cAMP content produced by β-adrenoceptor agonists. The increase is prevented by actinomycin D or cycloheximide, indicating that the up-regulation of PDE4 activity depends upon the synthesis of both mRNA and protein. The results of the present experiments are consistent with this conclusion. Treatment of U937 cells for 4 h with a combination of salbutamol and rolipram increased the amount of immunoreactive PDE4A detected in cell supernatants. The results were particularly striking in that PDE4A was virtually undetectable in untreated cells but...
clearly evident in the induced cells. An increase in PDE4B immunoreactivity also occurred in response to 4-h exposure to salbutamol and rolipram. In contrast, this treatment regimen had no effect on PDE4D levels. These studies do not, however, eliminate the possibility that PDE4D activity can be regulated directly by a protein kinase A-mediated phosphorylation pathway. Moreover, since our focus was on PDE4D, we cannot exclude the possibility that β-adrenoceptor agonists regulate the expression of other mRNA splice variants (i.e., PDE4D1 and PDE4D2).

mRNA transcripts encoding PDE4 subtypes were identified through RT-PCR methodology using subtype-specific oligonucleotide primers. In untreated U937 cells, the only PCR products detected were those corresponding to PDE4B and PDE4D. Although the technique utilized was not designed to be quantitative, distinct changes in the pattern of PCR products were detected after treatment with rolipram and salbutamol. Specifically, PCR product for PDE4A transcript was barely detectable in untreated cells, but a PCR product of the appropriate length was observed clearly and consistently in stimulated cells. In addition, the amount of PCR product corresponding to PDE4B appeared to increase, whereas that corresponding to PDE4D appeared to decrease. The functional consequences of these apparent changes in steady-state transcript levels as they relate to PDE4B and PDE4D protein expression are unknown. Consistent with the results of this study, Engels and colleagues (1994) also detected an increase in message for PDE4A and PDE4B in response to 18-h treatment of U937 cells with 0.5 mM dibutyryl cAMP.

Up-regulation of PDE4 activity in U937 cells had a substantial impact on their responsiveness to PGE2, an activator of adenylyl cyclase. As demonstrated in both time course and concentration-response studies, pretreatment of cells with a combination of salbutamol and rolipram substantially decreased the ability of PGE2 to elevate cAMP content. This loss of activity was mirrored by a decrease in the ability of PGE2 to inhibit LTD4-induced Ca2+ mobilization. We reasoned that if an increase in PDE4 activity had a role in producing the heterologous desensitization in pretreated U937 cells, then inhibiting PDE4 activity in these cells would tend to normalize their sensitivity to PGE2. This was indeed the case. Whereas there was a substantial difference in the ability of PGE2 to stimulate CAMP accumulation and suppress Ca2+ mobilization in desensitized versus control U937 cells, the difference was markedly reduced or, in some instances, virtually abolished when functional studies were carried in the presence of rolipram (10 μM).

It is not yet known whether regulation of PDE4 activity represents a general homeostatic mechanism by which all target cells, particularly inflammatory cells, modulate their responsiveness to hormones and autacoids that activate adenylyl cyclase. However, in support of the broad applicability of this phenomenon, we recently have demonstrated that PDE4 activity and steady-state protein levels in human monocytes is up-regulated by β-adrenoceptor agonists in a manner similar to that seen in U937 cells.2

The results of these studies have implications regarding the use of β-adrenoceptor agonists as bronchodilators in the treatment of asthma. Normally, endogenous activators of adenylyl cyclase such as epinephrine, PGE2, and prostacyclin act as natural anti-inflammatory and bronchodilator agents (Barnes, 1986; Kuehl et al., 1987), presumably by elevating CAMP content in the appropriate target tissues. In theory, the beneficial actions of these agents would be compromised if chronic

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2 C. D. Manning, T. J. Torphy, H.-L. Zhou, J. J. Foley, H. M. Sarau, and M. S. Barnette, unpublished observations.
