Induction of Phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in Human Peripheral Blood T-lymphocytes by 8-Bromo-cAMP and G₄-coupled Receptor Agonists

POTENTIAL ROLE IN β₂-ADRE诺ORECEPTOR DESENSITIZATION

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In this study, a potential mechanism of β₂-adrenoceptor desensitization has been explored that is based upon the enhanced degradation of cAMP by phosphodiesterase (PDE). Pretreatment of Jurkat T-cells with 8-bromo cAMP (8-Br-cAMP) or prostaglandin E₂ increased PDE3 and PDE4 activity in an actinomycin D- and cycloheximide-sensitive manner. This effect was associated with increased expression of HSPDE3B, HSPDE4A4, HSPDE4D1, HSPDE4D2, and HSPDE4D3 mRNAs. Western analysis reproducibly labeled a band of immunoreactivity in vehicle-treated cells that corresponded to HSPDE4A4 (125 kDa). Although the intensity of this band was unchanged in cells treated with 8-Br-cAMP, additional 68–72-kDa proteins (HSPDE4D2, HSPDE4D1) were labeled that were not detected after vehicle. Similar results were obtained with T-lymphocytes exposed to 8-Br-cAMP and fenoterol. However, in those experiments HSPDE4A4 and HSPDE4D1 appeared to be equally expressed in vehicle- and treated cells, whereas HSPDE4D2 (72 kDa) was detected only after 8-Br-cAMP. The up-regulation of PDE activity in Jurkat T-cells abolished the ability of isoproterenol to elevate cAMP, which was partially reversed by the non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine, and by the PDE3 and PDE4 inhibitors, Org 9935 and rolipram, respectively. Collectively, these data suggest that chronic treatment of T-cells with cAMP-elevating agents compromises β₂-adrenoceptor-mediated cAMP accumulation by increasing the expression of HSPDE3B and HSPDE4D1 gene products.

Cyclic nucleotide phosphodiesterases (PDEs) are a heterogeneous group of immunologically distinct enzymes whose sole function is to metabolize the second messenger purine nucleotides, cAMP and cGMP, to their biologically inactive nucleotide 5'-monophosphates. Currently, PDEs are categorized into seven broad families (see Ref. 1 for nomenclature) that are distinguished by a number of criteria including substrate specificity, kinetic properties, sensitivity to allosteric modulators and synthetic inhibitors, and primary amino acid sequence (2, 3). In many cases, these families comprise multiple subtypes, which suggests that the degradation of cAMP and cGMP is a highly complex and tightly regulated process.

Over the last decade, the possible applications of PDE inhibitors to the treatment of an array of inflammatory diseases including asthma and atopic dermatitis has been realized. In particular, the cAMP-specific PDE, or PDE4, is viewed by the pharmaceutical industry as a viable molecular locus amenable to therapeutic intervention with selective inhibitors. The selection of this enzyme family as a possible drug target comes from the knowledge that PDE4 is the predominant, if not exclusive, regulator of cAMP homeostasis in essentially all pro-inflammatory and immune cells. Moreover, PDE4 inhibitors suppress many functional indices of cell activation that are considered pro-inflammatory (4–7).

Currently, four genes (for PDE4A, PDE4B, PDE4C, and PDE4D) have been identified in rat (8–11), in mouse (12), and in humans (13–19) that can encode multiple, immunologically distinct PDE4 isoenzymes. The most extensively investigated gene is PDE4D, which can produce at least five structurally distinct proteins that are subject to different regulatory constraints (20, 21). PDE4D1 and PDE4D2 are so-called "short" PDE4D isoenzymes that arise from alternative mRNA splicing and are produced following activation of an intronic promoter (22). In addition, activation of other upstream promoters results in the ultimate translation of “long” forms of the enzyme that have been designated PDE4D3, PDE4D4, and PDE4D5 (14, 20, 23). Intriguingly, activation of the cAMP/PKA cascade can augment the activity of one or more PDE4 family subtypes by phosphorylation (22–26) and/or gene induction (23, 25, 27, 28), which provide highly coordinated processes for short and long term control of cAMP homeostasis. Preliminary data suggest that the structural organization of the PDE4A and PDE4B genes is similar to PDE4D in that both short and long forms of the enzyme can, theoretically, be transcribed (21). Recently, it has been suggested that induction of PDE4D could occur in certain diseases such as asthma when β₂-adrenorecep-

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The abbreviations used are: PDE, phosphodiesterase; PGE₂, prostaglandin E₂; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-6-phosphate dehydrogenase; FCS, fetal calf serum; 8-Br-cAMP, 8-bromo cyclic AMP; PKA, cyclic AMP-dependent protein kinase.
tor agonist therapy is taken chronically (27, 29). Although the functional consequences of this phenomenon are uncertain, a prediction based on in vitro experiments is a heterologous desensitization of G_{c}-coupled receptors (27, 29). Thus, in asthma, induction of PDE4 could prove detrimental by compromising the therapeutic effects of β_{2}-adrenoceptor agonists and the anti-inflammatory actions of endogenous activators of adenylyl cyclase (27, 29).

Of those cells that have been implicated in the pathogenesis of asthma, T-lymphocytes play a pivotal role and are believed to orchestrate the chronic eosinophilic inflammation that is a hallmark of this disease (30). Given that T-lymphocytes represent a therapeutic target for PDE4 inhibitors, the current study was performed to (i) identify the PDE4 subtypes in Jurkat T-cells and human peripheral blood T-lymphocytes, (ii) determine if those isoenzyme detected are up-regulated by cAMP, and (iii) assess if an increase in PDE4 affects cell signaling through G_{s}-coupled receptors. Since T-cells also express PDE3 (31, 32) and the recently discovered, rolipram-insensitive lipram and Org 9935, and PGE_{2} were made up as stock solutions of 100 mM in Me_{2}SO and ethanol, respectively, filtered sterile and diluted to the desired working concentration as indicated.

Dilutions for T-cell cultures were made in Iscove's modified Dulbecco's medium supplemented with the proteinase inhibitors benzamidine (2 mM), leupeptin (50 μM), phenylmethylsulfonyl fluoride (100 μM), and soybean trypsin inhibitor (20 μg/ml) and used immediately as the enzyme source. Assays were performed in duplicate at 37 °C and initiated by the addition of 30 μl of isoproterenol, at the concentrations indicated in the text and legends to relevant figures, in the absence and presence of IBMX (500 μM), rolipram (50 μM), Org 9935 (30 μM), or a combination of Org 9935 and rolipram, and subsequently terminated after 1 min by the addition of 300 μl of ice-cold trichloroacetic acid (1 M). After vortex mixing and centrifugation (3000 × g), 125 μl of EDTA (10 μM) was added to 500 μl of the supernatant followed by 500 μl of freshly prepared tri-n-acetyl-1,2,3-tri-ribofuranose-1,2,3-tri-ribofuranose (1.1:1 v/v). The resulting mixture was recentrifuged (10,000 × g), and aliquots of the supernatant were neutralized with NaHCO_{3} (10 mM) and acetylated by the consecutive addition of triethylamine and acetic anhydride. Cyclic AMP mass was measured immediately by radioimmunooassay. Briefly, to 200 μl of acetylated sample, were added 50 μl of adenine 3,5’-monophospho-2’-O-succinyl-5’-[14C]iodotyrosine methyl ester (approximately 2000–3000 dpm) in 0.2% bovine serum albumin and 100 μl of anti-cAMP antibody in 0.2% bovine serum albumin. After incubation overnight at 4 °C and charcoal precipitation, samples were quantified by γ-counting. The detection limit and sensitivity (IC_{50}) of this assay are 10 and 145 fmol of cAMP, respectively.

Measurement of cAMP PDE Activity—Cells were lysed osmotically in ice-cold buffer B (20 mM triethylamine, pH 8, 1 mM EDTA) supplemented with the proteinase inhibitors benzamidine (2 mM), leupeptin (50 μM), phenylmethylsulfonyl fluoride (100 μM), bactinacin (100 μg/ml), and soybean trypsin inhibitor (20 μg/ml) and used immediately as the enzyme source. Assays were performed in duplicate at 37 °C and initiated by the addition of 30 μl of cell lysate to 270 μl of a reaction mixture containing (final concentration) 20 mM triethylamine, pH 8.0, 5 mM dithiothreitol, 500 μM adenosine 5’-monophosphate (500 μM), 500 μM magnesium acetate, 0.25 μl of alkaline phosphatase, 1 μl of EDTA, 1 μl cAMP (supplemented with ~250,000 dpm of [8-3H]cAMP and ~5000 dpm of [8-14C]adenosine to estimate recovery) and any drug(s) under evaluation or its vehicle. The reaction was terminated by the addition of 1 ml of a mixture of Dowex AG 1×8 (methanol:water (1:2.1)), vortex-mixed, and placed in an ice bath until the end of the assay. Samples were then diluted with buffer B for 10 min before being centrifuged (3000 × g) for 5 min at 4 °C. The radioactivity in 700–900 μl of the supernatants was determined by liquid scintillation counting in 2 ml of ACS II scintillant (Amersham Pharmacia Biotech) at a counting efficiency of approximately 60%. PDE activity is expressed as the formation of nucleotide 5′-monophosphate from cAMP/min/10^6 cell equivalents (i.e. the lysate derived from 10^6 cells) at 37 °C after correction for the recovery ( routinely 65–85%) of [8-14C]adenosine. In this study, PDE3 and PDE4 are defined as cAMP hydrolytic activity inhibited by 500 μM magnesium acetate, 50% and 60–80%, respectively, by 50 μM rolipram.

Classification of Cyclic Nucleotide PDEs—Cyclic nucleotide PDEs are classified according to the nomenclature outlined by Beavo et al. (1). Thus, HSPDEs to 7 refer to the Ca^{2+}/calmodulin-dependent, cGMP-stimulated, cGMP-inhibited, cAMP-specific, cGMP-specific, photoreceptor- and rolipram-insensitive, cAMP-specific PDE families, respectively, where the prefix HS refers to the species Homo sapiens. Semi-quantitative RT-PCR—Total RNA was extracted from 10^6 T-cells using the guanidinium isothiocyanate method detailed by Chomczynski and Sacchi (34). One microgram of RNA was reverse transcribed in a total volume of 20 μl in the presence of 8 μl of Avian myeloblastosis virus reverse transcriptase, 0.5 μl of dNTPs (200 μM), 0.2 μg of random hexamers (Pharmacia, Uppsala, Sweden), and 1 μl deoxyribonucleotides. RT-generated cDNAs encoding the PDE3, PDE4, and PDE7 genes were amplified by PCR using specific primers designed from the reported primary sequences (14, 15, 33, 35–37) deposited with the GenBank data base (Table 1). To confirm the integrity of RNA and equal loading of sample, RT-PCR analysis of the GAPDH gene was performed with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM t-glutamate, and 2.5 μg/ml amphotericin B, grown to a density of 5 × 10^{6} ml and passed after three days. Cells (10^6 in 2 ml of Iscove’s medium) were subsequently cultured for another 0.5–24 h in the presence of 0.1 μM rolipram (1 μM), rolipram (50 μM), PGE1 plus rolipram (50 μM) (as rolipram was depleted as needed). Cells were harvested by centrifugation, washed three times in ice-cold Heps buffer (without Ca^{2+} and Mg^{2+}) and resuspended as necessary. In some experiments, actinomycin D (10 μg/ml) and cycloheximide (10 μg/ml) were also added to the cultures.

Measurement of cAMP—Cells were treated for 24 h with vehicle or a combination of rolipram (50 μM) and PGE1, PGE2 (1 μM), rolipram (50 μM), and subsequently terminated after 1 min by the addition of 300 μl of ice-cold trichloroacetic acid (1 M). After vortex mixing and centrifugation (3000 × g), 125 μl of EDTA (10 mM) was added to 500 μl of the supernatant followed by 500 μl of freshly prepared tri-n-acetyl-1,2,3-tri-ribofuranose-1,2,3-tri-ribofuranose (1.1:1 v/v). The resulting mixture was recentrifuged (10,000 × g), and aliquots of the supernatant were neutralized with NaHCO_{3} (10 mM) and acetylated by the consecutive addition of triethylamine and acetic anhydride. Cyclic AMP mass was measured immediately by radioimmunoassay. Briefly, to 200 μl of acetylated sample, were added 50 μl of adenine 3,5’-monophosphate (500 μM), 500 μM magnesium acetate, 0.25 μl of alkaline phosphatase, 1 μl of EDTA, 1 μl cAMP (supplemented with ~250,000 dpm of [8-3H]cAMP and ~5000 dpm of [8-14C]adenosine to estimate recovery) and any drug(s) under evaluation or its vehicle. The reaction was terminated by the addition of 1 ml of a mixture of Dowex AG 1×8 (methanol:water (1:2.1)), vortex-mixed, and placed in an ice bath until the end of the assay. Samples were then diluted with buffer B for 10 min before being centrifuged (3000 × g) for 5 min at 4 °C. The radioactivity in 700–900 μl of the supernatants was determined by liquid scintillation counting in 2 ml of ACS II scintillant (Amersham Pharmacia Biotech) at a counting efficiency of approximately 60%. PDE activity is expressed as the formation of nucleotide 5′-monophosphate from cAMP/min/10^6 cell equivalents (i.e. the lysate derived from 10^6 cells) at 37 °C after correction for the recovery ( routinely 65–85%) of [8-14C]adenosine. In this study, PDE3 and PDE4 are defined as cAMP hydrolytic activity inhibited by 500 μM magnesium acetate, 50% and 60–80%, respectively, by 50 μM rolipram.

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reaction volume of 25 μl, 8-Br-cAMP (1 mM), or their respective vehicle. At the appropriate time, cells were lysed and PDE activity determined at a substrate concentration of 1 μM cAMP. Data represent the mean ± S.E. of three determinations performed with different cell preparations. See “Experimental Procedures” for further details. *, P < 0.05 significant increase in cAMP PDE activity with respect to time-matched control values.

Fig. 1. Up-regulation of cAMP PDE activity. Jurkat T-cells (panel a) were cultured for 1–24 h in medium containing 8-Br-cAMP (1 μM), PGE2 (1 μM), or their respective vehicle. Similarly, T-lymphocytes (panel b) were cultured for 6 or 24 h in medium containing fenoterol (10 μM), 8-Br-cAMP (1 μM), or their respective vehicle. At the appropriate time, cells were lysed and PDE activity determined at a substrate concentration of 1 μM cAMP. Data represent the mean ± S.E. of three determinations performed with different cell preparations. See “Experimental Procedures” for further details. * P < 0.05 significant increase in cAMP PDE activity with respect to time-matched control values.

Effect of Fenoterol, PGE2, and 8-Br-cAMP on PDE Activity—To determine if PDE could be up-regulated by cAMP, Jurkat T-cells were cultured for 1–24 h with PGE2 (1 μM) and 8-Br-cAMP (1 μM). In resting Jurkats, the basal rate of cAMP hydrolysis was 2–3 pmol/min/10^6 cells. After a lag period of approximately 2 h, PDE activity increased steadily in response to PGE2 and 8-Br-cAMP such that at 24 h the rate of cAMP hydrolysis was elevated 1.46-fold (to 5.57 ± 0.17 pmol/min/10^6 cells) and 2.95-fold (to 8.96 ± 0.18 pmol/min/10^6 cells), respectively (Fig. 1a). In contrast, PDE activity was not increased at 24 h in Jurkat T-cells cultured with fenoterol (10 μM) under identical experimental conditions (data not shown).

Protein synthesis was blocked by cycloheximide or actinomycin-D. Following five 5-min washes in buffer G (0.05% Tween 20, 0.05% milk in PBS), the membranes were incubated for 90 min with a goat, anti-rabbit peroxidase-conjugated IgG antibody (Amersham) diluted 1:10,000 in buffer G, and then washed again (five 5-min washes). Antibody-labeled proteins were subsequently visualized by enhanced chemiluminescence.

To check for specificity, the PDE4 antisera was preabsorbed with the peptide used as immunogen (40), incubated overnight at 4 °C with gentle agitation, and diluted to the desired working dilution. The membranes were stripped and then reprobed with the “blocked” antibody as described above.

Analysis of Data and Statistics—Data in the text and figures represent the mean ± S.E. of n independent determinations. Where appropriate, statistics were performed on non-transformed data using Student’s unpaired t-test or, in the case of multiple groups, analysis of variance followed by a Dunnett’s multiple comparison test. The null hypothesis was rejected when P < 0.05.

RESULTS

Effect of Fenoterol, PGE2, and 8-Br-cAMP on PDE Activity—To determine if PDE could be up-regulated by cAMP, Jurkat T-cells were cultured for 1–24 h with PGE2 (1 μM) and 8-Br-cAMP (1 μM). In resting Jurkats, the basal rate of cAMP hydrolysis was 2–3 pmol/min/10^6 cells. After a lag period of approximately 2 h, PDE activity increased steadily in response to PGE2 and 8-Br-cAMP such that at 24 h the rate of cAMP hydrolysis was elevated 1.46-fold (to 5.57 ± 0.17 pmol/min/10^6 cells) and 2.95-fold (to 8.96 ± 0.18 pmol/min/10^6 cells), respectively (Fig. 1a). In contrast, PDE activity was not increased at 24 h in Jurkat T-cells cultured with fenoterol (10 μM) under identical experimental conditions (data not shown).

Similar data were obtained with freshly isolated human peripheral blood T-lymphocytes. Thus, 8-Br-cAMP up-regulated PDE activity from a resting rate of 0.22 ± 0.01 pmol/min/10^6 cells to 0.28 ± 0.01 and 0.36 ± 0.01 pmol/min/10^6 cells at 6 and 24 h, respectively (Fig. 1b). PDE activity was also significantly augmented (from 0.20 ± 0.01 to 0.29 ± 0.02 pmol/min/10^6 cells) in T-lymphocytes treated with fenoterol (10 μM) for 6 h; this effect seemed to be transient since activity returned to baseline levels (0.21 ± 0.1) at 24 h. A striking finding was that on a per cell basis the basal rate of cAMP hydrolysis was approximately 10-fold lower in T-lymphocytes than in Jurkat T-cells.

Effect of Cycloheximide and Actinomycin-D on the Up-regulation of PDE Activity—The increase in PDE activity evoked by 8-Br-cAMP in Jurkat T-cells and human T-lymphocytes was abolished by the inclusion of actinomycin D (10 μg/ml) and cycloheximide (10 μg/ml) in the culture medium indicating a requirement for de novo protein synthesis (Fig. 2). In fact,
actinomycin D and cycloheximide reduced cAMP hydrolysis to a rate significantly lower than basal PDE activity in both vehicle- and 8-Br-cAMP-treated cells implying that the turnover of PDE isoenzymes is rapid (<24 h) in cells in which PDE is not induced (Fig. 2). Identical results were obtained when fenoterol and PGE2 were used as stimuli (data not shown). Preliminary studies established that neither cycloheximide nor actinomycin D affected T-cell viability as assessed by measuring the mitochondria-dependent reduction of 3-(4,5-dimethy-lthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (an index of cell respiration).

Effect of IBMX, Rolipram, and Org 9935 on the Up-regulation of PDE Activity—Further experiments were performed to determine what isoenzyme families were responsible for the increase in PDE activity described above. T-Cells exposed to 8-Br-cAMP (1 mM) were selected for this analysis since they showed the greatest induction. The non-selective PDE inhibitor, IBMX (500 μM), markedly attenuated cAMP hydrolysis in vehicle- and 8-Br-cAMP-treated Jurkat T-cells by 77.7% and 76.3% respectively (A and B in Fig. 3a). However, the residual activity in cells in which PDE was up-regulated (0.92 ± 0.12 pmol/min/million cells) was consistently greater than in vehicle-treated cells (0.28 ± 0.06 pmol/min/million cells) (C + D versus C in Fig. 3a) indicating an increase (3.2-fold) in the activity of an IBMX-insensitive isoenzyme (C + D/A in Fig. 3a). The identity of the PDE isoenzymes that were induced by 8-Br-cAMP was investigated by the use of isoenzyme-selective inhibitors. The PDE4 inhibitor, rolipram (50 μM), and the PDE3 inhibitor, Org 9935 (30 μM) (41), significantly attenuated cAMP hydrolysis in vehicle- and 8-Br-cAMP-treated cells by 49.2% and 39.1%, and 47.3% and 27.6%, respectively (Fig. 3, b and c). Quantification of this effect (B/A in Fig. 3, b and c) indicated that PDE3 and PDE4 were up-regulated 2.3- and 3.1-fold, respectively. When used in combination, rolipram and Org 9935 suppressed PDE activity in control and treated Jurkat T-cells (Fig. 3d) to the same extent as IBMX, suggesting that the IBMX-inhibitable activity was due solely to PDE3 and PDE4.

Using the same method, T-lymphocytes treated with 8-Br-cAMP for 24 h resulted in a 1.7-fold increase in the rate of cAMP hydrolysis of which PDE3 and PDE4 were up-regulated 1.39- and 1.87-fold respectively (Fig. 4, a and b). When used in combination, rolipram and Org 9935 abolished the increase in cAMP hydrolysis effected by 8-Br-cAMP, indicating that this effect was attributable solely to PDE3 and PDE4 (Fig. 4c). Consistent with data obtained in Jurkat T-cells, approximately 20% of the total PDE activity in untreated T-lymphocytes was insensitive to rolipram and Org 9935 (Fig. 4c).

Expression of HSPDE3, HSPDE4, and HSPDE7 mRNAs—The presence of mRNAs for the HSPDE3 and HSPDE4 subtypes expressed in Jurkat T-cells and human T-lymphocytes was evaluated by RT-PCR using primers (Table I) that recognize unique sequences in the relevant human genes (14, 15, 33, 35–37). In view of the rolipram- and Org 9935-resistant cAMP hydrolytic activity, an identical approach was employed to assess whether the recently discovered HSPDE7 gene (33) is also transcribed by these cells. In those studies, generic primers were designed to detect both HSPDE7A1 and HSPDE7A2 (42). Fig. 5 shows ethidium bromide-stained agarose gels of representative experiments. In four independent determinations using different cell preparations, RT-PCR revealed products corresponding to the predicted sizes of HSPDE3B, (676 bp),
Gene Expression—To determine the identity of the PDE isoenzymes at the mRNA level as Jurkat T-cells were treated with 8-Br-cAMP, a combination of Org 9935 and rolipram (panel A) was used to suppress cyclic AMP hydrolysis. The ability of Org 9935 (30 μM; panel A) to induce PDE activity (Fig. 1), whereas message for HSPDE4D3 lagged significantly behind. Noteworthy is the increase in HSPDE4A4, HSPDE4D1, and HSPDE7A mRNAs also were increased (1.7-fold) under identical experimental conditions (Fig. 8). Again, this effect was time-dependent but the expression of the three HSPDE4D splice variants followed markedly different kinetics. Thus, message for HSPDE4D3 prior to the up-regulation of HSPDE4A4 that occurred quite rapidly (t1/2 ~ 80 min), peaked between 2 to 6 h and then returned to basal levels within 24 h (Fig. 7). Although the mean increase was modest, significant variability was noted between experiments. For example, the HSPDE4A4/GAPDH ratio calculated from the dot-blot shown in Fig. 7b, indicated a 5-fold increase in mRNA at 6 h. The steady-state level of HSPDE4D1, HSPDE4D2, and HSPDE4D3 mRNAs also were increased (~3.5-fold) under identical experimental conditions (Fig. 8). Again, this effect was time-dependent but the expression of the three HSPDE4D splice variants followed markedly different kinetics. Thus, message for HSPDE4D1 and HSPDE4D2 increased in parallel (t1/2 values ~ 75 min), peaked at 4 h, and remained elevated for up to 24 h (Fig. 8). In contrast, message for HSPDE4D3 increased much more slowly (t1/2 > 6 h), and was generally detected only at the 6- and 24-h time points (Fig. 8). It is noteworthy that the increase in HSPDE4A4, HSPDE4D1, and HSPDE4D2 mRNA was detected prior to the up-regulation of PDE activity (Fig. 1), whereas message for HSPDE4D3 lagged significantly behind.

Jurkat T-cells exposed to fenoterol and 8-Br-cAMP showed a 1.56- and 2.65-fold increase in HSPDE3B mRNA transcripts at 24 h (Fig. 9a). Fenoterol similarly increased transcripts for HSPDE4A4 (570 bp), HSPDE4D1 (530 bp), HSPDE4D2 (456 bp), HSPDE4D3 (516 bp), and HSPDE7A (286 bp). The identity of the PCR products was confirmed by cloning into a pGEM5z vector followed by double-stranded sequencing. HSPDE3A, HSPDE4B1, and HSPDE4C1 mRNAs were not detected in any preparation of Jurkat T-cells after 40 cycles of amplification. Identical experiments were performed to identify the PDE mRNAs in human T-lymphocytes. PCR products corresponding to HSPDE4A4, HSPDE4B1, HSPDE4D3, and HSPDE7A were unambiguously detected (data not shown) confirming the presence of the PCR products in human T-lymphocytes. In none of the experiments was GAPDH significantly induced or repressed by cAMP (Figs. 7–9). Culture of Jurkat T-cells with 8-Br-cAMP (1 mM) resulted in a time-dependent, 1.7-fold increase in mRNA for HSPDE4A4 that occurred quite rapidly (t1/2 ~ 80 min), peaked between 2 to 6 h and then returned to basal levels within 24 h (Fig. 7). Although the mean increase was modest, significant variability was noted between experiments. For example, the HSPDE4A4/GAPDH ratio calculated from the dot-blot shown in Fig. 7b, indicated a 5-fold increase in mRNA at 6 h. The steady-state level of HSPDE4D1, HSPDE4D2, and HSPDE4D3 mRNAs also were increased (~3.5-fold) under identical experimental conditions (Fig. 8). Again, this effect was time-dependent but the expression of the three HSPDE4D splice variants followed markedly different kinetics. Thus, message for HSPDE4D1 and HSPDE4D2 increased in parallel (t1/2 values ~ 75 min), peaked at 4 h, and remained elevated for up to 24 h (Fig. 8). In contrast, message for HSPDE4D3 increased much more slowly (t1/2 > 6 h), and was generally detected only at the 6- and 24-h time points (Fig. 8). It is noteworthy that the increase in HSPDE4A4, HSPDE4D1, and HSPDE4D2 mRNA was detected prior to the up-regulation of PDE activity (Fig. 1), whereas message for HSPDE4D3 lagged significantly behind.

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### Table I

| Gene product | GenBank accession | Deoxyligand sequence | Coordinates of PCR product in human gDNA sequence | Product size (base pairs) | Annealing temperature |
|--------------|-------------------|----------------------|-----------------------------------------------|--------------------------|-----------------------|
| HSPDE3A      | M91667            | Forward: 5'-TCA CTC CCA GAG GAC CCT TGC-3' | 1609-2312                                      | 709                      | 59 °C for 60 s         |
| HSPDE3B      | X95520            | Reverse: 5'-TCT TCC AAC CAT GGA CCA CC-3' | 1549-2224                                      | 676                      | 65 °C for 30 s         |
| HSPDE4A4B    | L20965            | Forward: 5'-ACC AAT GGG CTC TGC AGT-3'  | 710-1279                                       | 570                      | 66 °C for 30 s         |
| HSPDE4B1A    | L20966            | Reverse: 5'-GCC TCG ACC TGA ATC CGA CA-3' | 56-419                                         | 364                      | 62 °C for 30 s         |
| HSPDE4C1B    | Z46662            | Forward: 5'-GGG TGT CAA GAT CCA GTT ACT-3' | 2169-2504                                      | 336                      | 71 °C for 30 s         |
| HSPDE4D1A    | U50137            | Reverse: 5'-AAG AGA CAA CCA GGA CAT CGT-3' | 55-584                                         | 530                      | 65 °C for 30 s         |
| HSPDE4D2A    | U50158            | Forward: 5'-AGG GCA CCA GAC GAC TCC TGC-3' | 43-498                                         | 456                      | 68 °C for 30 s         |
| HSPDE4D3A    | L20970            | Reverse: 5'-CGG TCA CCA GAC TCT GTC-3'  | 461-976                                        | 516                      | 68 °C for 30 s         |
| HSPDE4D3C    | L20152            | Forward: 5'-AGG GCA CAG TCA ACC-3'     | 1153-1438                                      | 286                      | 59 °C for 30 s         |
| GAPDH        | J04038            | Reverse: 5'-CCA CCC ATG GCA TAA ACC-3'  | 146-733                                        | 598                      | 58 °C for 30 s         |

*a PDE nomenclature of Beavo et al. (1) is used.*

FIG. 4. Effect of rolipram and Org 9935 on cAMP PDE activity in human T-lymphocytes. The ability of Org 9935 (30 μM; panel A) and a combination of Org 9935 and rolipram (panel C) to suppress cyclic AMP hydrolysis was assessed in T-lymphocytes that had been cultured for 24 h with 8-Br-cAMP (1 mM) or vehicle. See legend to Fig. 3 for details. Data represent the mean ± S.E. of eight determinations performed with different cell preparations. See "Experimental Procedures" for further details. *, p < 0.05, significant inhibition of PDE activity.
HSPDE4D1 (5.1-fold; Fig. 9c), HSPDE4D2 (5.24-fold; Fig. 9d), and HSPDE4D3 (4.7-fold; Fig. 9e), which was of comparable magnitude to the elevation produced by 8-Br-cAMP (4.7-, 5.5-, and 5.4-fold, respectively; Fig. 9, c–e). In contrast, transcripts for HSPDE4A4 were not elevated in Jurkat T-cells at 24 h in response to fenoterol and 8-Br-cAMP (Fig. 9b).

In T-lymphocytes, 8-Br-cAMP produced effects on PDE3 and PDE4 mRNA expression that were qualitatively similar to those observed in Jurkat T-cells albeit smaller in magnitude at 24 h (Fig. 10). Thus, 8-Br-cAMP (1 mM for 24 h) elevated message for HSPDE3B (1.6-fold; Fig. 10a), HSPDE4D1 (1.7-fold; Fig. 10c), HSPDE4D2 (2.7-fold; Fig. 10d), and HSPDE4D3 (2.1-fold; Fig. 10e). HSPDE4A4 transcripts were also elevated (1.7-fold; Fig. 10b) in cells exposed to 8-Br-cAMP at 24 h in contrast to data obtained in Jurkat T-cells (Fig. 9b). Modest increases in HSPDE3B (1.3-fold; Fig. 10a), HSPDE4A4 (1.5-fold, Fig. 10b), HSPDE4D1 (1.5-fold; Fig. 10d), and HSPDE4D3 (1.2-fold; Fig. 10e) also were effected by fenoterol (10 μM; 24 h) but were consistently less that the changes seen in Jurkat T-cells at the same time-point (cf. Fig. 9).

In none of the experiments was mRNA for HSPDE3A and HSPDE4C1 detected in fenoterol- and 8-Br-cAMP-treated Jurkat T-cells or human T-lymphocytes at any time point examined after 40 cycles of amplification (data not shown). Similarly, neither fenoterol nor 8-Br-cAMP increased basal HSPDE7 mRNA transcripts under identical experimental conditions (Figs. 9f and 10f). The abundance of mRNA for HSPDE4B1, which we detected only in T-lymphocytes, varied markedly between donors and was not consistently altered by 8-Br-cAMP (data not shown).

Fig. 5. Qualitative RT-PCR analysis of PDE3, PDE4 and PDE7 mRNA isoforms in Jurkat T-cells. RT-PCR was performed using RNA from untreated Jurkat T-cells and the products subjected to electrophoresis on 2% agarose gels. The figure shows representative ethidium bromide-stained gels where lanes 1–4 represent sample cDNA, sample processed in the absence of reverse transcriptase (to control for genomic contamination), total lung cDNA (positive control), and sterile water (negative control), respectively. RT-PCR product sizes for HSPDE4A4, HSPDE4B1, HSPDE4C1, HSPDE4D3, HSPDE7 (panel a), HSPDE4D1, HSPDE4D2 (panel b), HSPDE3A and HSPDE3B (panel c) were 570 bp (32 cycles), 364 bp (40 cycles), 516 bp (38 cycles), 286 bp (30 cycles), 530 bp (34 cycles), 456 bp (38 cycles), 709 bp (40 cycles), and 676 bp (29 cycles), respectively. The left-hand lane (denoted M in each gel) shows molecular weight markers (0.5 μg of 1-kb ladder, Life Technologies, Inc.). Data are representative of four experiments conducted with different cell preparations. See “Experimental Procedures” for further details.

Fig. 6. Qualitative RT-PCR analysis of HSPDE4D1, HSPDE4D2 (panel a), and HSPDE3A and HSPDE3B (panel b) mRNA isoforms in human T-lymphocytes. RT-PCR was performed using RNA from untreated human T-lymphocytes as described under “Experimental Procedures” and the legend to Fig. 5. Data are representative of four experiments conducted with different cell preparations.

Effect of Rolipram and PGE₂ on HSPDE4D1 and HSPDE4D2 Gene Expression—Fig. 11 shows the results of experiments designed determine if HSPDE4D1 and HSPDE4D2 mRNA was elevated in Jurkat T-cells exposed to PGE₂ (1 μM) and rolipram (50 μM) given alone and in combination. After 4 h, mRNA for both gene products was increased by 491 ± 95 and 446 ± 161%, respectively, in response to PGE₂, and 220 ± 34 and 242 ± 82%, respectively, in response to...
rolipram. When cells were incubated with a combination of PGE2 and rolipram, the mean increase in HSPDE4D1 (561 ± 10%) and HSPDE4D2 (459 ± 120%) message did not exceed that produced by PGE2 alone (Fig. 11, a and b), although in two of the four experiments synergy was clearly observed (Fig. 11, c and d).

**Effect of 8-Br-cAMP on HSPDE4 Isoenzyme Expression**—Western analyses were performed to determine the identity of the PDE4 isogene products expressed in vehicle- and 8-Br-cAMP-treated Jurkat T-cells and human T-lymphocytes to establish that the cycloheximide-sensitive increase in cAMP hydrolysis was associated with an increase in the expression of one or more PDE4 isoenzymes. The specificity of the antibody α-galK-PDE1 against HSPDE4 has been validated previously by the ability of the peptide used as immunogen to block selectively the recognition of both human recombinant and native PDE4 isoforms (40). We have also confirmed the specificity of α-galK-hPDE1 in Jurkat T-cells and human T-lymphocytes (data not shown).

In Jurkat T-cells treated with vehicle for 24 h, α-galK-hPDE1 labeled a band that migrated as a 125-kDa protein on SDS-polyacrylamide gels (Fig. 12). This protein, which was identical in size to a band of immunoreactivity detected in human monocytes (40), U-937 cells (27), Mono Mac 6 cells (28), and human peripheral blood mononuclear cells (37), is similar to the predicted size of HSPDE4A4 (14), a finding consistent with the unequivocal detection of HSPDE4A4 mRNA in these cells (see above). A much fainter band of immunoreactivity also was observed in Jurkat T-cells that corresponded to a molecular mass of 93 kDa, which, in agreement with the PCR data, suggested the presence of HSPDE4D3 (Fig. 12). After exposure of Jurkat T-cells to 8-Br-cAMP (1 mM) for 24 h, the intensities of the 93- and 125-kDa bands were unchanged (Fig. 12). Identical results were obtained at 6 h, a time when HSPDE4A4 was increased at the mRNA level (data not shown). However, additional bands of immunoreactivity were detected by α-galK-hPDE1 that were not observed in vehicle-treated cells (Fig. 12). The predominant polypeptide migrated with a mass of 72 kDa, suggesting the expression of HSPDE4D1 together with a less abundant 68-kDa protein corresponding to the expected molecular weight of HSPDE4D2 (25, 37). In some experiments a faint, relatively small (−49 kDa) protein was detected (Fig. 12) that presumably reflected a proteolytic fragment of one or more bona fide PDE4 isoenzymes since it was not seen in “antibody-blocked” experiments (data not shown).

In T-lymphocytes, α-galK-hPDE1 strongly labeled a 122-kDa polypeptide on SDS-polyacrylamide gels, which, based upon weight, the RT-PCR results, and selectivity of the antibody, was indicative of HSPDE4A4. Consistent with the Western
analyses of Jurkat T-cell lysates, this protein was not significantly induced in cells treated with 8-Br-cAMP for 6 or 24 h but was markedly reduced (>95%) by CHX (10 μg/ml; see Fig. 13 for 24-h data). The PDE4 antibody also routinely detected similar amounts of a constitutively expressed peptide in both vehicle and treated T-lymphocytes that had a molecular mass (67 kDa) predictive of HSPDE4D2. However, CHX did not have a major impact on the expression of this polypeptide indicating slow turnover relative to HSPDE4A4. In contrast, T-lymphocytes exposed to 8-Br-cAMP for 24 h expressed an additional 72-kDa band of immunoreactivity that was absent in vehicle-treated cells and that corresponded to the molecular mass of 67 kDa.
HSPDE4D1. Some unidentified bands (55, 83, 88, and 95 kDa; possibly HSPDE4D3) both smaller and larger than HSPDE4D1/2 were also labeled by α-galK-hPDE1 in vehicle- and 8-Br-cAMP-treated T-lymphocytes that were not expressed in cells exposed to CHX (Fig. 13).

Functional Consequences of PDE4 Up-regulation—Experiments were performed to determine the effect of up-regulating PDE on the ability of isoproterenol to elevate intracellular cAMP. Jurkat T-cells were chosen for these experiments since 8-Br-cAMP induced PDE to a greater extent than in T-lymphocytes. Cells were cultured for 24 h in medium supplemented with vehicle or a combination of rolipram (50 μM) and PGE₂ (1

**Fig. 10. Effect of 8-Br-cAMP and fenoterol on the steady state levels of PDE3, PDE4, and PDE7 mRNAs in human T-lymphocytes.** Cells were cultured for 24 h in medium containing vehicle, fenoterol (10 μM), or 8-Br-cAMP (1 mM) and processed as described in the legend to Fig. 10 and under "Experimental Procedures." Panels a-f show the mean results and a Southern blot for HSPDE3B, HSPDE4A4, HSPDE4D1, HSPDE4D2, HSPDE4D3, and HSPDE7A mRNA. Data are expressed relative to GAPDH (panel g), where 100% indicates control levels. Data represent the mean ± S.E. of four determinations performed with different cell preparations.
m), washed extensively to remove drugs, and resuspended in buffer. Each cell preparation was then exposed to isoproterenol (0.1 nM to 3 μM) in the absence and presence of IBMX (500 μM), and the cAMP accumulated after 60 s was extracted and subsequently measured by radioimmunoassay. In vehicle-treated cells, isoproterenol elicited a concentration-dependent increase in cAMP mass (from 0.54 to 2.77 pmol/10⁶ cells at 3 μM; 5-fold elevation) with an EC₅₀ of 17.1 nM (Fig. 14a). In contrast, the maximal increase in cAMP effected by isoproterenol in Jurkat T-cells in which PDE had been induced was very modest (from 0.39 to 0.48 pmol/10⁶ cells) and amounted to merely 2.9% of that elicited in vehicle-treated cells under identical experimental conditions (Fig. 14a). In the presence of IBMX, isoproterenol-induced cAMP accumulation was increased 3.4- and 31.9-fold in control and 8-Br-cAMP-treated T-cells, respectively; no change in EC₅₀ was detected between the two cell preparations (15.6 and 29.9 nM, respectively). Thus, the maximal increase in cAMP effected by isoproterenol in cells where PDE was induced was only 2.4-fold less than in vehicle-treated cells, compared with a 27.7-fold difference observed in the absence of IBMX.

The relative contribution of PDE3 and PDE4 to the desensitization is shown in Fig. 14b. Inhibitors of both isoenzyme families conferred sensitivity of Jurkat T-cells to isoproterenol although rolipram was more effective than Org 9935, suggesting that the induction of PDE4 played a greater role in the process of desensitization. Consistent with the results obtained with IBMX (Fig. 14a), rolipram and Org 9935 used in combination only partially restored the ability of isoproterenol to increase in the cAMP when compared with non-desensitized cells, providing additional support that multiple mechanisms account for the loss of sensitivity (Fig. 14b). However, it is noteworthy that isoproterenol-induced cAMP accumulation in desensitized cells was significantly greater in the presence of IBMX than when the Org 9935/rolipram combination was used (cf. Fig. 14, a and b).

FIG. 11. Effect of rolipram and PGE₂ on the steady state levels of HSPE4D1 and HSPE4D2 mRNA in Jurkat T-cells. Cells were cultured for 24 h in medium containing PGE₂ (1 μM), rolipram (50 μM), and a combination of both stimuli and processed as described in the legend to Fig. 10 and under “Experimental Procedures.” Panels a and b show the mean data for HSPE4D1 and HSPE4D2 mRNA respectively and represent the mean ± S.E. of four independent determinations performed with different cell preparations. Panels c and d show examples of Southern blots performed with the HSPE4D1 and HSPE4D2 cDNA probes, respectively. See “Experimental Procedures” for further details.

DISCUSSION

Although β₂-adrenoreceptor agonists are a mainstay in the treatment of asthma, they are not anti-inflammatory (43, 44) and are limited to providing symptomatic relief. One explanation for the failure of these drugs to resolve asthmatic inflammation is the development of tolerance by pro-inflammatory cells within the lung due to direct receptor desensitization.
mediated by one or more G-protein receptor-coupled kinases or PKA (45–48). In this study, we have tested an alternative and largely ignored hypothesis, which proposes that desensitization of Gs-coupled receptors is due to the up-regulation of PDE in relevant cells and a consequent increase in their ability to degrade cAMP (26, 28).

T-cells were cultured with 8-Br-cAMP, fenoterol, PGE₂, or a combination of PGE₂ and rolipram, for various times up to 24 h; cAMP hydrolysis, PDE isogene mRNA levels, protein expression, and the ability of isoproterenol to increase the cAMP content was subsequently determined. 8-Br-cAMP increased PDE activity in both Jurkat T-cells and T-lymphocytes by a mechanism that was prevented by actinomycin D and cycloheximide. Identical results were obtained with PGE₂ (Jurkat) and fenoterol (T-lymphocyte), suggesting that the increase in the rate of cAMP hydrolysis was due to enhanced transcription of one or more PDE genes (as reported in other cell types; Refs. 49 and 50) and/or the induction of other genes, which act to stabilize preexisting PDE mRNAs.

Studies with selective inhibitors strongly indicated that, after 24 h, 8-Br-cAMP up-regulated PDEs and PDE4 in Jurkat T-cells and T-lymphocytes. Induction of PDE by cAMP has been reported in several cell types including macrophages (51), monocytes (40), U-937 cells (27, 52), Mono Mac 6 cells (28), and the human keratinocyte cell line, HeCaT (53). However, in contrast to the aforementioned cells, where the up-regulation of PDE is transient, PDE activity in Jurkat T-cells, which was monitored over a period of 24 h, increased more slowly. Although the reason for this discrepancy is unclear, several possibilities are worthy of consideration. A plausible explanation is a difference in the expression and/or regulation of PDE4 isogenes between cell types. Indeed, whereas mRNA for HSPDE4B1 was not detected in vehicle- and 8-Br-cAMP-treated Jurkat T-cells, other studies have unambiguously identified HSPDE4B in a number of cells (19, 27, 28, 40, 50, 54) including T-lymphocytes (Ref. 31 and this study) that is elevated in response to an increase in cAMP (27, 28, 40). This is significant since PDE4B appears to be induced far more rapidly than either PDE4A or PDE4D gene products (28). Unfortunately, considerable variability in the abundance of HSPDE4B1 mRNA transcripts in T-lymphocytes was noted between donors. Similarly, the effect of 8-Br-cAMP on PDE4B gene expression was routinely inconsistent which negated an analysis of the comparative kinetics of PDE4A, PDE4B, and PDE4D gene induction. An additional explanation is that PDE3 is either absent or not up-regulated in the other cell types examined, which could account for the difference in kinetics if these isoenzymes are induced over a time frame that differs from PDE4. The results of this study demonstrate that, of the two human PDE3 genes that have been identified (35, 36), Jurkat T-cells and human T-lymphocytes express the “adipocyte” or HSPDE3B isoenzyme. Although it is well established that cAMP can activate HSPDE3B by PKA-dependent phosphorylation (55, 56), the results of this study suggest that, in T-cells, gene induction is primarily responsible for the increase in Org 9935-sensitive activity as evinced from the enhanced expression of HSPDE3B mRNA transcripts and the ability of actinomycin D and cycloheximide to block the up-regulation of PDE3.

Although we failed to detect message for HSPDE4B1 (Jur-
kats only) and HSPDE4C1 after 40 cycles of amplification, transcripts for HSPDE4A4, which provides the only catalytically active product from this locus in humans (14) and is a major isoenzyme in peripheral blood monocytes (37), was reproducibly identified in both Jurkat T-cells and T-lymphocytes, together with HSPDE4D1, HSPDE4D2, and HSPDE4D3. The detection of spliced variants of the PDE4D gene is consistent with PDE4D multiplicity reported in the rat (22, 25) and humans (37), and supports the assertion that splicing of this gene is conserved during evolution (37).

Given that T-cells have the potential to express multiple cAMP PDE isoenzymes, experiments were performed to determine the extent to which of these could account for the increase in PDE activity. Detailed RT-PCR studies with 8-Br-cAMP-treated Jurkat T-cells identified HSPDE4A4, HSPDE4D1, and HSPDE4D2 as good candidates since kinetic studies demonstrated that message for these proteins was significantly elevated prior to a detectable increase in enzyme activity, which is what would be predicted if a causal relationship exists between these two events. In addition, a time-dependent increase in HSPDE4D3 mRNA was also detected but only several hours after PDE activity had started to rise. Although these data suggest that HSPDE4D3 is unlikely to contribute to the early increase in PDE activity, this isoenzyme was not immediately discounted since it could play a supportive role at late (24 h) time points. Transcripts for HSPDE3B and HSPDE4A4 and the D1, D2, and D3 splice variants of the PDE4 gene were also elevated in 8-Br-cAMP-treated T-lymphocytes at 24 h, indicating a similar mechanism of PDE gene expression to that observed in Jurkat T-cells.

To address which transcripts might account for the increase in the rate of cAMP hydrolysis, the PDE4 isoenzymes expressed by vehicle- and 8-Br-cAMP-treated Jurkat T-cells and T-lymphocytes were identified by immunoblotting using an antisera, α-galK-hPDE1, that recognizes all forms of PDE4 (27, 40). In both cell preparations, α-galK-hPDE1 labeled a strong band of immunoreactivity that was identical to the predicted size of HSPDE4A4 (14), an isoenzyme that has since been unambiguously identified in human monocytes and T-lymphocytes (27, 28, 37, 40). In Jurkat T-cells (and possibly T-lymphocytes), a weak 93-kDa band of immunoreactivity was also labeled by the antibody, which had a mass identical to that expected of HSPDE4D3 and was in keeping with the unequivocal identification of HSPDE4D3 mRNA in these cells. In addition, T-lymphocytes constitutively expressed a 67-kDa protein not detected in Jurkat T-cells of the predicted molecular weight of HSPDE4D2. Intriguingly, although 8-Br-cAMP increased message for HSPDE4A4, HSPDE4D2 and HSPDE4D3, it did not alter the intensity of the 125- and 93-kDa bands in Jurkat T-cells or the corresponding 122-, 95-, and 67-kDa bands in T-lymphocytes in Western blots after 6 and 24 h of culture. Thus, it would seem that these isoenzymes did not contribute significantly to the overall increase in PDE activity (see below) or that their turnover was rapid. In contrast, a strong and highly reproducible band of immunoreactivity was labeled by α-galK-hPDE1 in both cell preparation after 8-Br-cAMP that was not detected in cells exposed to vehicle. Although we cannot state with certainty the identity of this protein, it probably represents HSPDE4D1 since: (i) it had a molecular mass of 72 kDa, which is almost identical to the molecular mass of HSPDE4D1 extracted from mononuclear cells (28, 37); (ii) it routinely migrated with a fainter 68-kDa protein, which is consistent with the highly related splice variant, HSPDE4D2 (this protein is identical to HSPDE4D1 other than for an 86-base pair deletion in its 5’ alternatively spliced region, which results in the expression of a slightly smaller protein); and (iii) an increase in HSPDE4D1 and HDPDE4D2 message was reproducibly detected in Jurkat T-cell and T-lymphocytes exposed to 8-Br-cAMP. Taken together, these data indicate that as far as the PDE4 isoenzyme family is concerned, cAMP is hydrolyzed in unstimulated Jurkat T-cells principally by HSPDE4A4 and, to a lesser extent, HSPDE4D3, which act as “housekeeping” isoenzymes that are not markedly influenced by hormones at the protein level. In T-lymphocytes HSPDE4D2 might also regulate basal cAMP homeostasis. This contrasts with the increased rate of cAMP hydrolysis in cells exposed to 8-Br-cAMP, which is attributable to induction of the PDE4D gene via increased transcription and/or stabilization of preexisting mRNAs followed by an increase in catalytically active HSPDE4D1 and, in Jurkat T-cells, possibly HSPDE4D2.

Up-regulation of HSPDE4D1 and HSPDE4D2 mRNA was also seen in Jurkat T-cells exposed to PGE2 and, to a lesser extent, rolipram, indicating that this phenomenon can be also elicited by agents that activate Gs-coupled receptors and inhibit PDE4 respectively. Moreover, in two experiments PGE2 and rolipram clearly acted in a synergistic manner, which strongly supports a central role of cAMP in the up-regulation of HSPDE4D1 and HSPDE4D2 mRNAs.

A somewhat unexpected result was that 8-Br-cAMP failed to increase protein for HSPDE4A4 in Jurkat T-cells or T-lymphocytes. Although this is entirely consistent with the inability of cAMP-elevating drugs to up-regulate PDE4A4 in the murine MA-10 Leydig tumor cell line (50), it is nevertheless at variance with observations made with human monocytes (28, 40), Mono Mac 6 cells (28), and U-937 cells (27, 54) where HSPDE4A4 is induced in the mRNA and protein level and accounts, in part, for the increase in PDE4 activity. One broad interpretation of these results is that this isoenzyme can be differentially regulated in a cell-specific and species-independent manner, which suggests an additional level of complexity that is not necessarily predicted from the mere number of PDE4 isoenzymes.

The regulation of PDE4D depends, in part, upon the spliced variants expressed by the cell of interest. Thus, PDE4D3 is phosphorylated at serine 54 by PKA, which promotes a rapid and reversible increase in activity (24–26, 57). This so-called short term regulation is distinguished from the up-regulation of PDE activity that follows an increase in synthesis and/or stability of PDE4D1 and PDE4D2 mRNAs, which has been designated long term regulation (10, 25, 50). The finding that long term (24 h) incubation of Jurkat T-cells and, to a more modest extent T-lymphocytes, with 8-Br-cAMP increased the steady-state level of HSPDE4D3 mRNA suggests that it is also regulated at the transcriptional level. This was an unexpected result in light of other studies, which failed to detect any increase in HSPDE4D3 mRNA in response to cAMP-elevating drugs (26, 40). However, in those reports, measurements were made on cells exposed to the inducing agent for relatively short periods of time (~4 h), which, based on our results, could be insufficient to increase HSPDE4D3 message.

Although mRNA for HSPDE4A4 and HSPDE4D3 was increased in both cell types exposed to 8-Br-cAMP, no increase in protein was detected using α-galK-hPDE1. Similarly, comparison of the PCR and Western analyses results demonstrated that strong signals for HSPDE4D1 and HSPDE4D2 mRNA were expressed in vehicle-treated Jurkat T-cells and T-lymphocytes despite the fact that their corresponding proteins (with the possible exception of HSPDE4D2 in T-lymphocytes) were not identified by α-galK-hPDE1. Identical results were observed in Jurkat T-cells exposed to fenoterol where transcripts for HSPDE3B, HSPDE4D1, HSPDE4D2, and HSPDE4D3 were significantly increased at a time (24 h) when no increase in enzyme activity was noted. The reasons for these marked dis-
crepancies are unclear, but instability of the active enzymes and/or a low translation rate of mRNA to protein are likely explanations. Indeed, Némox and colleagues (37) have recently reported similar results for the HSPE4D3 isoenzyme in human peripheral blood mononuclear cells.

The identification of a cAMP PDE activity in Jurkat T-cells and human T-lymphocytes that was insensitive to rolipram and Org 9935 is consistent with the report of Ichimura and Kase (58), who described a similar activity in several T-cell lines, and confirms the more recent finding of an atypical PDE in human T-lymphocytes (31, 32). Although the identity of this enzyme is uncertain, the unambiguous identification of PDE7 mRNA transcripts in human T-lymphocytes and the Jurkat and MOLT-4 T-cell lines (this study and Refs. 31 and 42) suggests that it might represent a HSPE7A gene product. Whether this enzyme accounts for the IBMX-insensitive activity detected in 8-Br-cAMP-treated Jurkats is uncertain given that (i) PDE7 mRNA levels were not increased, and (ii) PDE7 is inhibited by the concentrations of IBMX used in this study (58).

Concentration-response studies demonstrated that isoproterenol-induced cAMP accumulation in Jurkat T-cells was abolished when PDE was up-regulated. We reasoned that if this refractoriness is attributable to the induction of one or more PDEs, the responsiveness of cells to isoproterenol should be normalized, at least in part, by a PDE inhibitor. Since the activity of several isoenzymes/splice variants was increased, this possibility was initially assessed using the non-selective inhibitor, IBMX. The results from these experiments were equivocal. IBMX markedly restored the sensitivity of Jurkat T-cells to isoproterenol, implying that the functional desensitization was, indeed due to the increase in PDE activity. Identical experiments performed with Org 9935 and rolipram indicated that, although both families contributed to desensitization, PDE4 was the primary determinant. Taken together, these data are in broad agreement with another study where chronic exposure of U-937 cells to salbutamol and rolipram produced a state a desensitization that was the result of an increase in HSPE4D4 and HSPE4B gene products (27).

A highly reproducible finding was that IBMX was more effective at restoring the sensitivity of Jurkat T-cells to isoproterenol than the combination of Org 9935 and rolipram. Although we have not formally addressed possible mechanisms that could account for this disparity, Org 9935 and rolipram were equivalent to IBMX at suppressing cAMP hydrolysis in lysates prepared from desensitized cells, which suggests that the latter elevates cAMP mass in intact Jurkat T-cells by a mechanism(s) in addition to PDE inhibition. A number of explanations are worthy of consideration including the ability of IBMX to antagonize the actions of adenosine at receptors (A1/A2) that couple negatively to adenyl cyclase. Although this would have the effect of blocking the inhibitory effects of endogenously-released adenosine and augmenting cAMP accumulation, it is unlikely to explain the results of the present study as Jurkat T-cells do not express adenosine A1 or A2 receptors (59). Other possibilities include the ability of IBMX to prevent, directly, Gi from inhibiting adenyl cyclase (60) and/or to inhibit PDEs (e.g. PDE1 isoenzymes) that were not measured in a cell-free system.

Although this study has made a case for PDE induction in β2-adrenocceptor desensitization, it is important to emphasize that this molecular effect does not preclude the participation of G-protein receptor-coupled kinases, PKA, or other processes that can promote a state of tolerance and points to the fact that the molecular etiology of desensitization can be multi-factorial. Indeed, PDE inhibitors only partially restored the sensitivity of Jurkat T-cells to isoproterenol, a finding concordant with a previous study in U-937 cells (27), and might reflect the ability of rolipram and PGE2 also to reduce β2-adrenocceptor signaling by acting at the level of the receptor, Gβγ, or adenylyl cyclase.

In the context of therapeutics, tolerance to β2-adrenocceptor agonists has far reaching clinical implications and could go some way to explain why chronic or repeated exposure to these drugs has been associated with an increase risk from asthma deaths (61). It is tempting to speculate that a heterologous desensitization of Gβγ-coupled receptors on cells pertinent to the pathogenesis of airway inflammation could result from a progressive and generalized up-regulation of PDE (29). Theoretically, this would progressively compromise the ability of β2-adrenocceptor agonists to promote effective bronchodilation and, potentially, exacerbate airway inflammation by limiting the beneficial anti-inflammatory actions of endogenous activators of adenylyl cyclase such as circulating catecholamines, prostacyclin, and E-series prostaglandins (29, 62).

In conclusion, treatment of Jurkat T-cells and human T-lymphocytes with cAMP-elevating agents up-regulates catalytically active protein for HSPE4D1/4D2 and HSPE3B. The available evidence indicates that this occurs by stimulating transcription of the HSPE4D4 and HSPE3B genes and/or by stabilizing preexisting PDE mRNA. The significance of these findings is currently unclear. However, if up-regulation of PDE occurs in response to chronic treatment of asthmatic subjects with β2-adrenocceptor agonists, then the increased capacity of susceptible cells to degrade cAMP could reduce the effect of all agonists that act by increasing adenylyl cyclase activity. Since such ligands generally exert a beneficial effect in the airways of asthmatic subjects this biochemical change could be reflected by a deterioration in disease status.

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Addendum—Since submission of this manuscript, data similar to those reported herein have been reported for Jurkat T-cells exposed chronically to forskolin (Erdogan, S., and Houslay, M. D. (1997) Biochem. J. 312, 165–175).

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