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Regulation of Distinct Cyclic AMP-Specific Phosphodiesterase (Phosphodiesterase Type 4) Isozymes in Human Monocytic Cells

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SUMMARY

Many functions of the immune and inflammatory responses are inhibited by agents that increase intracellular levels of cAMP. Recent investigations have revealed that cAMP levels in inflammatory cells are regulated by cyclic nucleotide phosphodiesterases (PDEs) belonging to the PDE4 family (cAMP-specific PDEs). At least four different genes are known to encode PDE4 isozymes, which are characterized by their selectivity for cAMP over cGMP and their sensitivity to the antidepressant drug rolipram. The aim of our studies was to investigate whether monocytic cells could regulate PDE4 activity and whether certain PDE4 isozymes were expressed preferentially over others. Our results showed that treatment of peripheral blood monocytes or closely related Mono Mac 6 cells with dibutyryl-cAMP or other cAMP-elevating agents transiently increased rolipram-sensitive PDE4 activity 2–3-fold, without concomitant increases in cGMP-inhibited PDE (PDE3) activity. PDE4 activity was predominantly cytosolic, whereas PDE3 activity was localized to the particulate fraction. Our Northern and Western blot studies with reagents recognizing three distinct PDE4 gene products (PDE4A, PDE4B, and PDE4D) revealed that their expression is transcriptionally regulated in monocytic cells. Although none of the three isozymes was detectable under normal culture conditions, all of these were up-regulated when Mono Mac 6 cells were exposed to dibutyryl-cAMP. Distinct differences were observed in their temporal patterns of expression. Endotoxin lipopolysaccharide, a potent monocyte stimulus, also enhanced PDE4 activity in monocytic cells. These data indicate that monocytic cells may express different PDE4 isozymes, depending on their state of activation or differentiation. These isozymes could thus regulate intracellular cAMP levels at various stages of monocyte activation and could thereby be important in limiting the inflammatory response.

Numerous studies show that agents elevating intracellular cAMP levels inhibit some but not all functions of cells involved in the inflammatory response (see Ref. 1 for review). For example, when cAMP levels are increased the production of tumor necrosis factor-α in monocytes is drastically reduced; however, synthesis of certain other cytokines, such as interleukin-1β and -6, is not diminished (2). Proliferation of lymphocytes (3) and production of superoxide by neutrophils (4) are also sensitive to inhibition by cAMP. The discovery of therapeutic agents that selectively regulate cAMP levels in inflammatory cells has thus attracted much interest, because such agents are expected to dampen both chronic and acute phases of inflammatory responses.

The intracellular level of cAMP is regulated by the rates of synthesis and degradation of cAMP and is readily manipulated pharmacologically. *In vitro*, cell-permeable analogues of cAMP are commonly added to cells to investigate whether cellular functions are sensitive to cAMP. Hormonal agonists, such as PGEs, or β-adrenergic agonists, which activate adenylate cyclase through G protein-coupled receptors, also are used to stimulate synthesis of cAMP in leukocytes (5). Degradation of cAMP is accomplished by PDEs, which are present in the cytosol or membranes of most cells (6). Addition of PDE inhibitors, such as 3-isobutyl-1-methylxanthine, greatly increases cAMP levels in conjunction with prostaglandins or β-adrenergic agonists and thereby promotes the down-regulation of many leukocyte functions (1, 7).

Multiple PDE isozymes have been characterized in mammalian cells. On the basis of their substrate selectivity, kinetic properties, intracellular modulators, regulatory control, and inhibition by selective inhibitors, these enzymes are classified into at least seven families (reviewed in Refs. 8–10; see Ref. 11 for PDE nomenclature). The comparison of available primary sequences has confirmed that a single family of

ABBREVIATIONS: PGE, prostaglandin E; PDE, cyclic nucleotide phosphodiesterase; LPS, lipopolysaccharide; HSPDE4, human phosphodiesterase type 4; RNPDE4, rat phosphodiesterase type 4; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

PDEs may contain multiple genes encoding distinct isozymes. For instance, at least four genes that code for the PDE4 family are present in human and rat, designated HSPDE4 A, B, C, and D for human and RNPDE4 A, B, C, and for rat, respectively. Three of the seven families of PDEs, namely PDE3, PDE4, and PDE7 (HCP1-PDE), hydrolyze cAMP with low K_m (0.1–3 μM) and can be pharmacologically distinguished from each other. PDE3 isozymes are inhibited by cGMP and are sensitive to cardiotonic and platelet antiaggregant drugs, such as milrinone and cilostamide. Both PDE4 and PDE7 isozymes are insensitive to cGMP, and only PDE4 isozymes are inhibited by antidepressant agents, such as rolipram and Ro 20–1724. HCP1-PDE is not sensitive to any known PDE inhibitors.

The predominant form of PDE in leukocytes appears to belong to the rolipram-sensitive PDE4 isozyme group. Indeed, rolipram has recently been shown to inhibit multiple leukocyte functions and to have anti-inflammatory effects *in vivo* (12). However, PDE4 isozymes are also predominant in other tissues, such as brain and testis, and thus PDE4-selective inhibitors may produce undesired side effects during the treatment of inflammatory diseases. It is therefore important to understand the subcellular localization and expression profiles of PDE4 isozymes in inflammatory cells and to determine whether development of inhibitors that are selective for one isozyme over the others within the PDE4 family is warranted.

In the present study, we utilized a human monocytic cell line, Mono Mac 6, as a model of human monocytes to determine the localization and expression of different PDE4 isozymes at the transcriptional and translational levels. To gain insights into the regulation of these enzymes, we also treated these cells with cAMP-elevating agents or with bacterial LPS, a classical activator of monocytes and macrophages (13). Our studies demonstrated that PDE4 activity in normal human monocytes and Mono Mac 6 cells was predominantly cytosolic and was increased 2–3-fold under these conditions. At least three different PDE4 genes are expressed in these cells, and their expression appears to be regulated at the transcriptional and/or translational level.

Materials and Methods

Reagents. RPMI 1640 medium, fetal bovine serum, pyrogen-free PBS, penicillin-streptomycin, and pooled, type AB, human serum were obtained from GIBCO (Grand Island, NY). Lymphocyte separation medium was from Organon Technica (Durham, NC). Monoclonal antibody 234/0/10 was produced by the Glaxo Monoclonal Antibody Facilities and specifically recognizes HSPDE4B isozymes. Monoclonal antibodies 66C12H and 61D10E were generous gifts from Dr. K. Ferguson (ICOS, Bothell, WA) and recognize HSPDE4A and HSPDE4D isozymes, respectively. All antibody specificities for the various recombinant PDE4 isozymes were confirmed using human recombinant proteins.[1] These antibodies recognize the carboxyl termini of the three gene products.

Cell culture. Mono Mac 6 cells (14) were obtained from Dr. Ziegler Heitbrock (Institut für Immunologie, Universität München, Munich, Germany) and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin.

Isolation and culture of human monocytes. Blood from healthy volunteers was collected into heparin (10 units/ml) and diluted 1/2 with PBS containing 5 mM EDTA. The mononuclear cell fraction was collected after sedimentation over lymphocyte separation medium, and monocytes were further purified by elutriation (15). Monocytes were cultured in 24-well plates in RPMI 1640 medium supplemented with penicillin/streptomycin and 2.5% heat-inactivated, pooled, human AB serum.

Preparation of membrane and cytosolic fractions. The permeabilization procedure described here was optimized for freshly isolated human monocytes and Mono Mac 6 cells, at a concentration of 1×10^7 cells/ml in PBS. Permeabilization was performed by incubating the cells for 20 min on ice with different concentrations of digitonin in PBS, pH 7.2, with 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, and 100 μg/ml soybean trypsin inhibitor. The permeabilized cells were centrifuged at 1000 × *g* for 5 min and the supernatant (cytosol) and pellet (membranes) were saved as separate fractions. The two fractions were adjusted to equal volumes with 0.1% Triton X-100 in PBS, pH 7.2, and stored at −80°.

Enzyme assays. cAMP-specific PDE activity was assayed using 96-well microtiter plates. The reaction mixture contained 50 μl of the following reagents: 20 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 0.3 mg/ml bovine serum albumin, 0.1 mM EDTA, 10 μg/ml 5′-nucleotidase, 3 μM [2,8,-^{3}H]cAMP (28.9 Ci/mmol), and 0.3 μM [8-^{14}C]adenosine (56.5 mCi/mmol). Serial dilutions of the cell extracts were added to the reaction mixture to identify conditions that produce a linear time course and use <10% of the substrate. The reaction was incubated for 30 min at 30° and was terminated by transferring 40 μl of the reaction mixture into a 96-well filter plate containing 200 μl of a 50% QAE-Sephadex (40–120 μm) mixture in 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 10. The product was collected by ultrafiltration into a 96-well micro-β-scintillation plate containing 120 μl of scintillation fluid. PDE activity was measured in the presence of vehicle (dimethylsulfoxide), 10 μM cilostamide (a PDE3 inhibitor), 10 μM rolipram (a PDE4 inhibitor), or 10 μM zaprinast (a PDE5 inhibitor). Lactate dehydrogenase activity (a cytosolic marker) was measured using a cytotoxicity assay, as indicated by the manufacturer (Promega Corp., Madison, WI). β-Glucuronidase activity (a lysosomal marker) was measured as reported (16).

Immunodetection of PDE4 isozymes in Mono Mac 6 cells. Digitonin-soluble extracts equivalent to 1×10^6 cells were mixed with an equal volume of 2× Laemmli buffer (17), boiled for 5 min, and separated on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) as described (18). The blots were blocked for 1 hr at 50° with 5% (w/v) bovine serum albumin in 10 mM Tris-buffered saline, pH 7.3, containing 0.2% (w/v) Tween-20. After blocking, the blots were incubated with monoclonal antibodies specific for HSPDE4A (66C12H), HSPDE4B (234/0/10), or HSPDE4D (61D10E). Horseradish peroxidase-linked anti-mouse antibodies were used with Amersham enhanced chemiluminescence reagents to detect immunoreactive bands, according to the manufacturer's protocol. Amersham Rainbow prestained markers were used to estimate molecular weights.

Hybridization probes. Four synthetic, single-stranded oligodeoxyribonucleotides were used for Northern blot hybridizations. These oligomers are complementary in sequence to unique 5′ portions of HSPDE4A5, HSPDE4B2, and HSPDE4D1 mRNA. Based on the report of Bolger *et al.* (19), the sequences and positions of the four oligomers are as follows: 5′-CCGCTGCCGGTGCCAGTGCCATGGAAGGACGAGGGCCAGGACATGC-3′ and 5′-GCGGCCAGGAGATGGTGTCGGCCCATTCTCTGCCTCGAAGCGCCTGCT-3′, which correspond to the antisense sequence of HSPDE4A5 cDNA (clone PDE46) from base 408 to base 363 and from base 475 to base 428, respectively, 5′-CCTTCATTATTTGCACGCTGGCTCCTTCCTTCCAGCT-3′, which corresponds to the antisense sequence of HSPDE4B2 cDNA (clone PDE32) from base 772 to base 736, and 5′-GGACTCAGTTCTCAAGCGCTTCACGGGTCCGCTAGCGAGTTCAA-3′, which corresponds to the antisense sequence of

[1] K. Ferguson, personal communication.

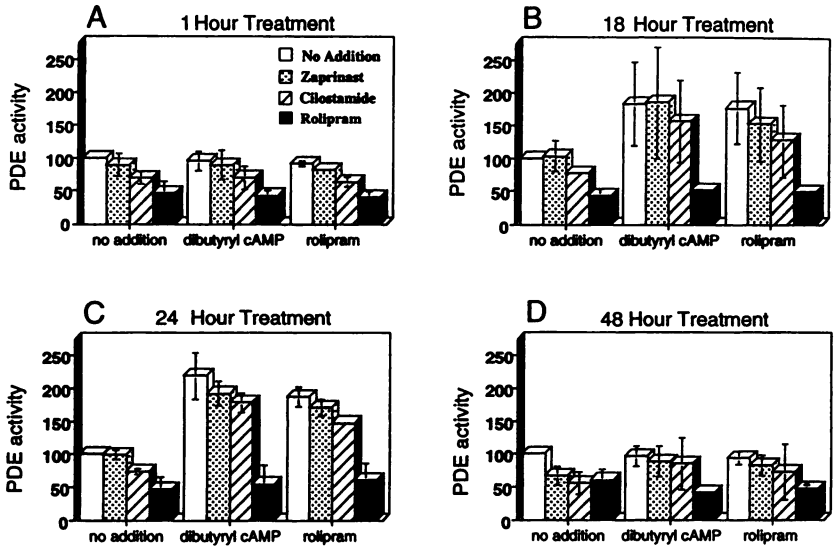

Fig. 1. Effects of dibutyryl-cAMP or rolipram treatment of human monocytes on PDE4 activity. Adherent human monocytes were cultured in 24-well plates in the presence of buffer, dibutyryl-cAMP (100 μM), or rolipram (10 μM) for 1, 18, 24, or 48 hr. Total cellular cAMP-specific PDE activity was assayed in permeabilized cells (three freeze/thaw cycles). Rolipram (10 μM), cilostamide (10 μM), and zaprinast (10 μM) were included as inhibitors of PDE4, PDE3, and PDE5 isozymes, respectively. Data were normalized to buffer-treated cells at each time point, where cAMP hydrolysis was 12.4 ± 0.7, 4.1 ± 0.9, 7.3 ± 2.1, and 5.8 ± 2.8 nmol/hr/10^5 cells at 1, 18, 24, and 48 hr, respectively (three experiments).

HSPDE4D1 cDNA from base 560 to base 517 (clone PDE11).[2] This HSPDE4D1 cDNA represents an alternate splicing variant that corresponds to a rat homologue, RNPDE4D1 cDNA (rat PDE3.1 in Ref. 20). The four oligomers were labeled at the 3′-hydroxyl termini using [α-^{32}P]dATP or [α-^{32}P]dCTP and terminal deoxynucleotidyltransferase (GIBCO-BRL), as described (21). To enhance the hybridization signal, the two oligomers with sequences specific to HSPDE4A5 cDNA were combined to probe our Northern blots. A control cDNA probe encoding human β-actin was labeled using a random-primed labeling kit, following the manufacturer's instructions (GIBCO-BRL). The probe was used at a concentration of 1–2 × 10^6 cpm/ml of hybridization buffer.

RNA extraction and Northern blot hybridization. Total RNA was prepared from Mono Mac 6 cells by the guanidine thiocyanate/cesium chloride method, as described previously (22). Twenty-five micrograms of total RNA were denatured in glyoxal and dimethylsulfoxide and then size-fractionated on 1% agarose gels (23). After electrophoresis, samples were transferred from the gels to Biotrans nylon membranes and baked at 80° for 2 hr. Blots were prehybridized overnight at 37°, followed by hybridization with ^{32}P-labeled probes for 16–24 hr at the same temperature. Prehybridization and hybridization were performed in 50% formamide, 50 mM sodium phosphate, pH 6.5, 5× SSC (20× SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 1× Denhardt's solution (100× Denhardt's solution contains 2% bovine serum albumin, 2% Ficoll, and 2% polyvinylpyrrolidone), 0.5% SDS, 75 μg/ml sonicated denatured salmon sperm DNA. After hybridization, blots were washed in 2× SSC/0.1% SDS at room temperature for 10 min and twice in 0.2× SSC/0.1% SDS at 46–50° for 20 min, followed by exposure to XAR-5 X-ray film, using intensifing screens. Blots that had been hybridized with the oligomer probes were stripped with 0.1× SSC/0.1% SDS at 90–100° and then rehybridized with a 2-kilobase human β-actin cDNA probe, which served as a control that similar amounts of RNA were loaded into each lane of a Northern blot. Hybridization and washing conditions for the actin probe were more stringent than those for the oligomer probes and were detailed in Ref. 22.

Results

Effect of dibutyryl-cAMP or rolipram on cAMP-specific PDE activity in human monocytes. To determine whether PDE4 activity could be regulated, human monocytes were treated with dibutyryl-cAMP or 10 μM rolipram, which is known to elevate intracellular cAMP in these cells (24). As demonstrated in Fig. 1, total cellular PDE4 activity was increased at least 2-fold 18–24 hr after treatment with either of these agents. After 48 hr of treatment, PDE activity returned to normal. Untreated cells contained predominantly PDE4 isozymes but also expressed some PDE3 activity, as demonstrated by the 50% and 25% inhibition of PDE activity by rolipram and cilostamide, respectively. Zaprinast, an inhibitor of cGMP-specific PDE, served as a control in these assays. The enhanced PDE activity present at 18–24 hr was completely inhibited by rolipram, suggesting that these increases resulted from alterations in PDE4 isozymes.

Distribution of cAMP-specific PDE in human monocytes. cAMP-specific PDE was localized within human monocytes by following the digitonin permeabilization procedure described in Materials and Methods. As a result of the high affinity of digitonin for cholesterol-rich membranes, the plasma membrane is disrupted at lower concentrations of digitonin than are intracellular vesicles (25). Fig. 2 shows that lower concentrations of digitonin were needed for the

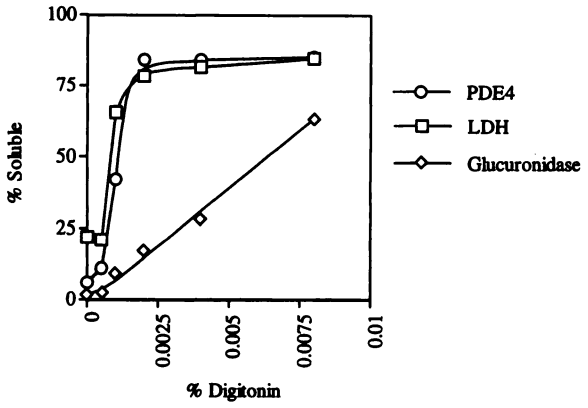

Fig. 2. Localization of PDE4 to cytosol in monocytes. Cytosolic localization of PDE4 was tested by incubating human monocytes with different concentrations of digitonin, as described in Materials and Methods. The permeabilized cells were pelleted by centrifugation and the soluble and particulate fractions were assayed for lactate dehydrogenase (*LDH*), β-glucuronidase, and rolipram-sensitive PDE4 activity. Values are reported as a percentage of the total enzyme (100% = 10,029 cpm) released by digitonin treatment. Representative data are given from one of three experiments. Each *point* represents the average of duplicate PDE assays.

[2] K. Ferguson, unpublished observations.

release of cytosolic lactate dehydrogenase than the release of lysosomal β-glucuronidase from permeabilized cells. It is important to note that cytosolic lactate dehydrogenase and PDE4 isozymes were released from the cells at similar concentrations of digitonin. Exposure to different concentrations of digitonin did not change the total PDE activity. These results suggest that PDE4 isozymes are predominately localized to the cytosol within monocytes.

Effect of dibutyryl-cAMP or LPS on particulate and cytosolic PDE4 activity in human monocytic cells. Because cAMP-elevating agents increased PDE4 activity, we wanted to investigate whether monocyte activation with LPS has similar effects. The most striking increases in PDE4 activity were observed in the cytosolic fraction rather than the particulate fraction obtained from cells treated with either dibutyryl-cAMP or LPS (Table 1). Like dibutyryl-cAMP, LPS also appeared to regulate only PDE4 isozymes, as demonstrated by the increased inhibition of PDE activity by rolipram but not by cilostamide. The LPS treatment resulted in significant loss of enzyme activity in the particulate fraction, but the reason for this is unclear. It is possible that LPS could promote redestribution of enzyme activity to the soluble fraction. This possibility was not pursued further, due to the low level of PDE activity present in the particulate fraction.

To eliminate any possible influences of contaminating lymphocytes, we repeated these experiments with Mono Mac 6 cells, a human monocytic cell line that expresses a more mature monocyte phenotype than the commonly used THP-1 and U-937 monocytic cell lines (Table 2). Although Mono Mac 6 cells expressed less PDE activity on a protein basis, the profile of alterations in PDE4 versus PDE3 resembled that in peripheral blood monocytes. As expected, PGE_1 enhanced PDE4 activity in these cells. Because LPS may increase production of prostaglandins in some monocytic cells, we added indomethacin to block cyclooxygenases, which are required for prostaglandin synthesis. Clearly, the effects of LPS on PDE4 activity were not significanly inhibited by indomethacin.

Time course of dibutyryl-cAMP-induced PDE4 activity in particulate and cytosolic fractions from Mono Mac 6 cells. Mono Mac 6 cells were used to confirm whether this human monocytic cell line retained the temporal and spatial distributions of PDE4 activity that were observed in fresh human monocytes. As found in human monocytes (Fig. 2), the soluble fraction accounted for the majority of the PDE4 activity in this cell line (Fig. 3). The particulate fraction contained the majority of the PDE3 activity. Also as observed for human monocytes (Fig. 2), treatment of Mono Mac 6 cells with dibutyryl-cAMP increased PDE4 but not PDE3 activity (Fig. 3). Taken together, these observations suggest that human monocytes and Mono Mac 6 cells regulate PDE4 activity by similar mechanisms.

A dose-response curve was obtained to determine the optimal concentration of dibutyryl-cAMP needed for induction of PDE4 activity in Mono Mac 6 cells. Fig. 4 shows that maximal stimulation of cytosolic PDE4 activity was observed after 4 hr of incubation in the presence of 0.5–1.0 mM dibutyryl-cAMP. As expected, a similar increase in PDE4 activity was not observed in the particulate fraction. The effects of dibutryl-cAMP were not mediated through butyric acid, because 0.5 mM dibutyryl-cGMP increased the activity of PDE4 by only 8, 16, and 5% at 2, 4, and 24 hr, respectively, compared with the effects of 0.5 mM dibutyryl-cAMP.

Regulation of steady state PDE4 mRNA levels in Mono Mac 6 cells. The results described above indicated that PDE4 isozymes were expressed in human monocytic cells. To examine the expression of individual isozymes, total RNA was isolated from Mono Mac 6 cells and hybridized with single-stranded oligomer probes that were specific in sequence for HSPDE4A5, HSPDE4B2, and HSPDE4D1 mRNA. Northern blot analyses indicated that the three PDE4 mRNAs expressed in these cells were at the limit of detection under normal culture conditions. However, dibutyryl-cAMP significantly increased the steady state mRNA levels (Figs. 5 and 6). As shown in Fig. 5, the hybridization signals of the three PDE4 mRNAs were apparent upon exposure of Mono Mac 6 cells to 0.1 mM dibutyryl-cAMP. The increase in steady state levels of the three PDE4 mRNAs by dibutyryl-cAMP appeared to be dose dependent. The time course of changes in the PDE4 mRNA levels in Mono Mac 6 cells treated with 0.5 mM dibutyryl-cAMP is presented in Fig. 6. After a time lag of about 1 hr, the levels of HSPDE4A5 and HSPDE4D1 mRNAs increased, reaching a maximum between 2 and 8 hr. The hybridization signal for HSPDE4B2 mRNA, however, was readily detected at 1 hr, and the mRNA level reached a maximum between 2 and 3 hr and gradually decreased after 4 hr of treatment. At 24 hr, all three PDE4 mRNAs decreased to nearly basal levels.

TABLE 1

Dibutyryl-cAMP or LPS increases PDE4 activity in human monocytes

Elutriated monocytes were cultured for 18 hr in 24-well plates, in the presence of buffer, 100 μM dibutyryl-cAMP, or 10 ng/ml LPS. Cells were washed and permeabilized with 0.05% digitonin to release cytosolic PDE, as described for Fig. 1. Cytosolic and particulate cAMP-specific PDE activity was assayed in the presence or absence of 10 μM rolipram or cilostamide, to inhibit PDE4 or PDE3, respectively. Data are averages ± standard errors from four separate experiments.

Treatment	cAMP production			Inhibition	
	Control	Rolipram	Cilostamide	Rolipram	Cilostamide
	pmol/min/mg			%	
Cytosolic:					
Buffer	70 ± 26	33 ± 10	56 ± 18	50 ± 5	13 ± 12
Dibutyryl-cAMP	392 ± 132	58 ± 11	331 ± 115	83 ± 3	17 ± 3
LPS	166 ± 39	54 ± 12	141 ± 35	68 ± 1	15 ± 1
Particulate					
Buffer	56 ± 44	35 ± 23	16 ± 8	26 ± 5	52 ± 16
Dibutyryl-cAMP	56 ± 38	19 ± 10	26 ± 14	57 ± 8	44 ± 8
LPS	9 ± 9	6 ± 6	4 ± 4		

TABLE 2

PGE_1 or LPS up-regulates PDE4 activity in human Mono Mac 6 cells

Mono Mac 6 cells were cultured for 3 hr in 24-well plates, in the presence of buffer, 100 μM dibutyryl-cAMP, 1 μM PGE_1, or 10 ng/ml LPS. Indomethacin 1 μM was included as indicated, to inhibit formation of PGE_1. Cells were washed and the cytosolic fraction of digitonin-permeabilized cells was collected for PDE assays, as described for Fig. 1. cAMP-specific PDE activity was assayed in the presence or absence of 10 μM rolipram or cilostamide, to inhibit PDE4 or PDE3, respectively. Data are averages ± standard errors from two separate experiments.

Treatment	cAMP production			Inhibition	
	Control	Rolipram	Cilostamide	Rolipram	Cilostamide
	pmol/min/mg			%	
Buffer	28 ± 4	14 ± 2	27 ± 4	49 ± 14	3 ± 2
Dibutyryl-cAMP	89 ± 7	9 ± 8	83 ± 4	91 ± 8	6 ± 5
PGE_1	89 ± 7	10 ± 8	72 ± 5	90 ± 9	18 ± 1
LPS	47 ± 9	12 ± 1	42 ± 12	75 ± 4	12 ± 7
LPS + indomethacin	41 ± 7	13 ± 1	42 ± 10	68 ± 9	−5 ± 5

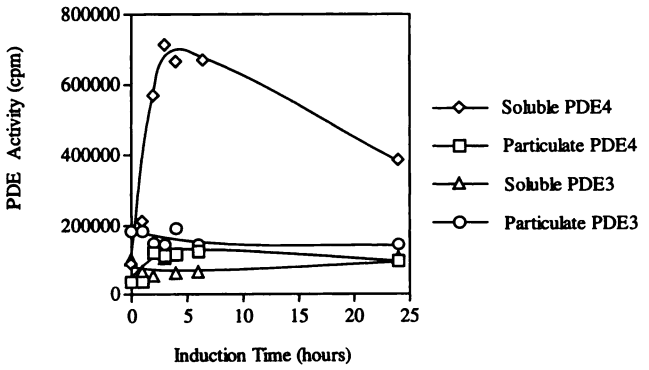

Fig. 3. Increase by dibutyryl-cAMP of PDE4 but not PDE3 activity in cytosol from Mono Mac 6 cells. Mono Mac 6 cells were incubated for different periods of time in the presence of 0.5 mM dibutyryl-cAMP. The cells were permeabilized with 0.004% digitonin as described in Materials and Methods. The permeabilized cells were collected by centrifugation and PDE activity in the soluble and particulate fractions was assayed in the absence or presence of 10 μM rolipram or 10 μM cilostamide. Dibutyryl-cAMP increased PDE4 activity (i.e., rolipram-sensitive PDE) in the cytosolic fraction but not the particulate fraction. In contrast, PDE3 activity (i.e., cilostamide-sensitive PDE) remained unchanged in soluble and particulate fractions. Each *point* represents the average of duplicate PDE assays from one of three experiments.

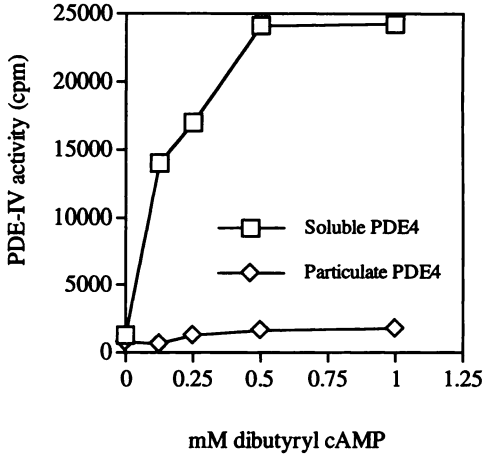

Fig. 4. Maximal cytosolic PDE4 activity obtained by incubating Mono Mac 6 cells with 0.5–1.0 mM dibutyryl-cAMP. Mono Mac 6 cells were incubated for 4 hr in the absence or presence of various concentrations of dibutyryl-cAMP. The cells were permeabilized with digitonin and collected by centrifugation as described in Materials and Methods. PDE activity in the soluble and particulate fractions was assayed in the absence or presence of 10 μM rolipram. Values are reported as rolipram-inhibitable PDE activity (i.e., PDE4 activity). Each *point* represents the average of triplicate PDE assays from one of two experiments.

Regulation of PDE4 protein levels in Mono Mac 6 cells. Western blots with specific monoclonal antibodies were performed to determine which PDE4 isozymes were present in digitonin extracts from Mono Mac 6 cells. No immunoreactive bands for PDE4 isozymes were detected unless the cells were treated with dibutyryl-cAMP (Fig. 7). Dibutyryl-cAMP increased the intensity of immunoreactive HSPDE4B (72 kDa), HSPDE4D (72 and 74 kDa), and HSPDE4A (130 kDa). Maximal increases in HSPDE4B and HSPDE4D protein were seen at 3 and 5 hr of dibutyryl-cAMP treatment, respectively. Similarly, maximal PDE4 activity was observed between 3 and 6 hr after treatment of Mono Mac 6 cells with dibutyryl-cAMP (Fig. 3). The signal for both of these proteins decreased by 24 hr. In contrast, the signal for HSPDE4A protein gradually increased through the 24-hr dibutyryl-cAMP treatment.

Discussion

The pharmacological profile of cAMP-specific PDE activity observed in our studies indicated that human monocytic cells expressed predominantly PDE4 isozymes. Cytosolic PDE4 activity was dramatically increased when fresh human monocytes were exposed to agents that elevate intracellular cAMP (e.g., dibutyryl-cAMP, rolipram, or PGE_1). Similarly, the cytosol of Mono Mac 6 cells also displayed increased PDE4, but not PDE3, activity when cells were treated with LPS, PGE_1, or increasing concentrations of dibutyryl-cAMP. These data are consistent with the observed increases in cytosolic PDE4 activity in U-937 cells treated with β-adrenergic agonists or prostaglandins in the presence of rolipram (26). Activation of monocytes or Mono Mac 6 cells with LPS also increased cytosolic PDE4 activity, but these effects did not appear to be attributable to changes in intracellular cAMP levels. Interestingly, PDE3 activity in monocytic cells was particulate and did not increase in response to elevated intracellular cAMP levels.

The differences in the subcellular localization of PDE3 and PDE4 enzymes may be important for the regulation of specific cell functions. For example, PDE3 is in the particulate fraction of rabbit or dog ventricles but is cytosolic in rat ventricles (27). These differences may account for the discrepant effects of PDE3 inhibitors on cardiac function of dogs and rabbits versus rats (28). Similarly, PDE4 activity is the major PDE in cytosol of rat epididymal fat cells (29) but is membrane bound in COS cells transfected with recombinant vector pSVL-RD1 (30). It has been suggested that the intra-

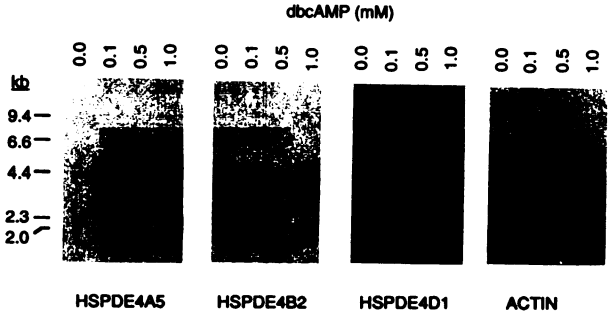

Fig. 5. Regulation of steady state PDE4 mRNA levels by dibutyryl-cAMP in Mono Mac 6 cells. Mono Mac 6 cells cultured at a density of approximately 7×10^5/ml were treated with different concentrations of dibutyryl-cAMP (*dbcAMP*) (0, 0.1, 0.5, and 1 mM). After 3 hr of treatment, cells were washed with PBS and lysed with 4 M guanidine isothiocyanate, followed by centrifugation through a 5.7 M CsCl cushion to pellet total RNA. Aliquots (25 μg each) of isolated total RNAs were size fractionated by electrophoresis on 1% agarose gels and were blotted to Biotrans nylon membranes. Blots were hybridized with ^{32}P-labeled oligonucleotide probes corresponding to human HSPDE4A5, HSPDE4B2, or HSPDE4D1 cDNA sequences. After hybridization, blots were washed and exposed to X-ray films. To determine whether similar amounts of RNAs were loaded into each lane, blots were stripped of hybridized probes, rehybridized with a human β-actin cDNA probe, and autoradiographed.

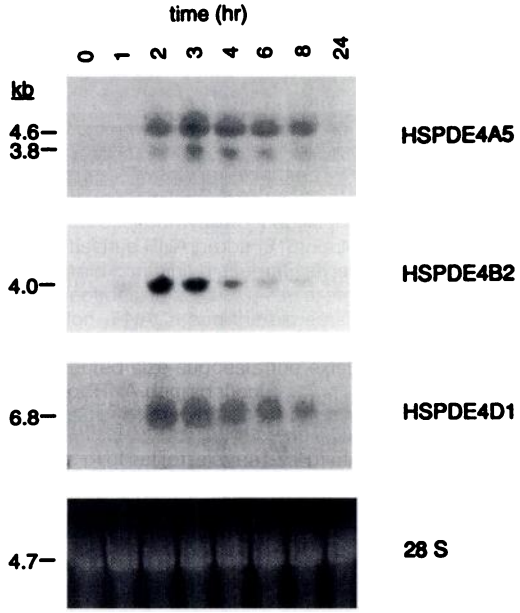

Fig. 6. Time course of the effect of dibutyryl-cAMP on the steady state mRNA levels of PDE4 in Mono Mac 6 cells. Mono Mac 6 cells were treated with 0.5 mM dibutyryl-cAMP for the indicated times. Northern blot hybridization was performed as described for Fig. 5. Because the hybridization signal intensity for β-actin RNA decreased at 24 hr, the RNA samples were analyzed by electrophoresis on ethidium bromide-containing agarose gels. Similar intensities of 28 S rRNA in each lane served as an alternate control for similar amounts of RNAs applied to the gels.

cellular distribution of PDEs may control 'local' concentrations of cAMP (30). Alternatively, the restricted localization of PDE isozymes may control the interaction of PDEs with other proteins and their function.

Evidence is accumulating that different PDE4 isozymes are expressed in a cell- and tissue-specific manner and can be regulated independently. In rat testis, for example, rat PDE1 (RNPDE4C1) and rat PDE2 (RNPDE4A?) are predominantly

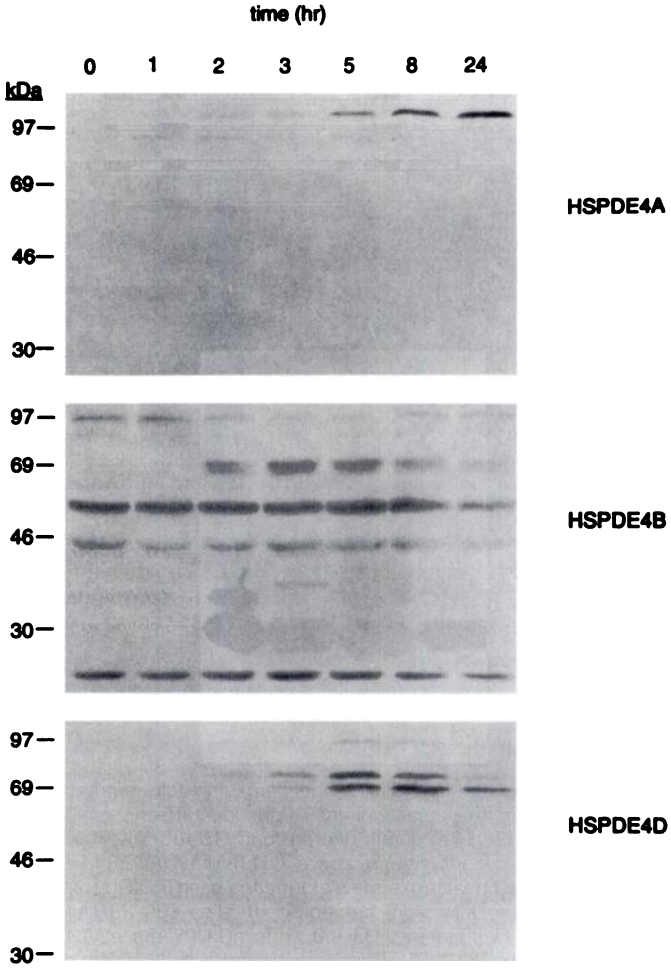

Fig. 7. Immunoblot analysis of PDE4 regulation by dibutyryl-cAMP in Mono Mac 6 cells. Cells were cultured in the presence or absence of 0.5 mM dibutyryl-cAMP for the indicated times. Cell extracts were prepared as described in Fig. 1. Aliquots of extract equivalent to 1×10^6 cells were subjected to SDS-PAGE and immunoblotting with PDE4-specific antibodies, as described in Materials and Methods.

expressed in germ cells, and rat PDE3 (RNPDE4D1) and rat PDE4 (RNPDE4B2) are found mainly in Sertoli cells (31). Rat Sertoli cells contain readily detectable mRNA for rat PDE4 under normal culture conditions, whereas rat PDE3 mRNA is detected only after the cells have been treated with follicle-stimulating hormone or other agents that elevate cAMP (32). In our earlier studies, we used a DNA fragment with a sequence common to both HSPDE4B1 and HSPDE4B2 cDNAs as a probe to screen a human monocyte cDNA library. We found that all 12 of our clones corresponded to the HSPDE4B2 sequence, suggesting that monocytes express HSPDE4B2 but not the HSPDE4B1 isozyme (data not shown). We also observed considerable donor variability in the abundance of HSPDE4B2 mRNA prepared from freshly isolated human monocytes, suggesting that the HSPDE4B2 isozyme can be transcriptionally regulated. Indeed, in Northern and Western blot experiments HSPDE4B2 was barely detectable unless blood monocytes were exposed to cAMP-elevating agents or to LPS (data not shown).

To avoid inherent variability associated with blood monocytes, we utilized Mono Mac 6 cells for further analyses of PDE4 isozyme expression and regulation. Our Northern and Western blot analyses of untreated Mono Mac 6 cells failed to

detect any signal for the three PDE4 isozymes. These results were not surprising, because cytosol obtained from unactivated Mono Mac 6 cells consistently expressed only about 50% as much rolipram-sensitive, cAMP-specific PDE activity as did cytosol from unactivated monocytes. We did not include particulate fractions in Western blot analysis, due to their low levels of PDE4 activity and the sensitivity of our isozyme-specific antibodies. When Mono Mac 6 cells were treated with increasing concentrations of dibutyryl-cAMP, mRNA levels for the three PDE4 isozymes examined began to increase, in a dose-dependent fashion, about 1–2 hr after treatment. Although mRNA hybridization signals for the three isozymes were no longer detectable after 24 hr of dibutyryl-cAMP treatment, the duration of the mRNA accumulation for the isozymes seemed to be significantly different. The signal for HSPDE4B2 mRNA was drastically reduced by 4 hr, whereas HSPDE4A5 and HSPDE4D1 mRNAs were still readily detectable at 8 hr.

Protein levels detected for the three isozymes in Mono Mac 6 cells in general correlated with their mRNA accumulation during dibutyryl-cAMP treatment, but some discrepancy was observed among the three isozymes. It should be kept in mind that Western blot data are useful for monitoring relative changes in protein levels for a given protein but are not quantitative when different antibodies must be used to detect different proteins. HSPDE4B protein appeared to be expressed first but decreased substantially by 24 hr, compared with HSPDE4A and -4D. This time course of HSPDE4B protein levels appeared to correlate with PDE4 activity in Mono Mac 6 cytosol, which peaked between 3 and 6 hr. HSPDE4A protein was detected later and maintained its high level until at least 24 hr after dibutyryl-cAMP treatment. This high protein level for the HSPDE4A isozyme at 24 hr was in contrast to the weak mRNA signal found by Northern blot analysis. At least two possibilities may explain this. 1) In addition to HSPDE4A5, alternate splicing variants from the HSPDE4A gene may be expressed in Mono Mac 6 cells. Our oligomer probes specific for the 5′ end of HSPDE4A5 cDNA would not detect these variants, although their protein product(s) could be recognized by our carboxyl terminus-specific antibody. 2) The protein stability of the HSPDE4A5 isozyme may contribute to this high protein level. These findings indicate that monocytes at different stages of activation or maturation display different sets of PDE4 isozymes.

PDE4 isozymes appear to be of major importance in regulating monocyte activation. This is demonstrated by the findings that rolipram and related compounds are consistently more effective than cilostamide and other PDE3 inhibitors at increasing cAMP levels and inhibiting tumor necrosis factor-α production in human monocytes (24). It is tempting to speculate on possible reasons why it might be useful for monocytic cells to express more than one PDE4 isozyme. Several studies have demonstrated that intracellular cAMP not only serves to inhibit certain functions of monocytes but also may act as a differentiating agent for myelocytic cells (33). For example, human promeylocytic HL-60 cells can be differentiated toward neutrophils with dibutyryl-cAMP or dimethylsulfoxide, but increases in PDE3 and PDE4 activity of whole-cell homogenates were detectable only after dibutyryl-cAMP treatment (data not shown). The orchestrated temporal expression of different PDE4 isozymes may therefore be important in shutting off monocyte activation or in enhancing monocyte differentiation under certain circumstances.

In summary, our results demostrate that human monocytic cells manipulate PDE4 activity through the regulated expression of at least three genes. It will be critical to determine which PDE4 isozymes are expressed by monocytes and other cells of the immune and inflammatory systems in different pathologies. Tools are now becoming available to determine the profile of PDE4 isozyme expression in tissues from appropriate disease states. It should therefore be possible to determine whether the pursuit of isozyme-selective PDE4 inhibitors would be expected to yield drugs with appropriate therapeutic effects.

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