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Activation and induction of cyclic AMP phosphodiesterase (PDE4) in rat pulmonary microvascular endothelial cells

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Abstract

Regulation of the rolipram-sensitive cAMP-specific phosphodiesterase 4 (PDE4) gene family was studied in rat pulmonary microvascular endothelial cells (RPMVECs). Total PDE4 hydrolysis was increased within 10 min after addition of forskolin (10 μM), reached a maximum at 20–40 min, and then gradually declined in the cells. A similar activation of PDE4 activity was observed using a protein kinase A (PKA) activator, N^6-monobutyryl cAMP. Both the forskolin and the N^6-monobutyryl cAMP activated PDE4 activities were blocked by the PKA-specific inhibitor, H89. This forskolin-stimulated and PKA-mediated short-term activation of PDE4 activity was further confirmed by in vitro phosphorylation of 87 kDa PDE4A6 and 83 kDa PDE4B3 polypeptides using exogenous PKA Cα. Increased immunoreactivity of phosphorylated PDE4A6 in situ was detected in Western blots by a PDE4A-phospho antibody specific to the putative PKA phosphorylation sites. Following long-term treatment of RPMVECs with rolipram and forskolin medium (RFM) for more than 60 days, PDE4 activity reached ten-fold higher values than control RPMVECS with twenty-fold increases detected in intracellular cAMP content. The RFM cells showed increased immunoreactivities of the constitutive 4A6 and 4B3 isoforms plus two novel splice variants at 101 kDa (4B1) and 71 kDa (4B2). Treatment with H89 did not inhibit the PDE4 elevation in RFM cells. In addition to the increased levels of PDE4 in RFM cells, immunofluorescence showed a translocation of PDE4A and 4B to a nuclear region, which was normally not observed in RPMVECs. The PDE4 activity in RFM cells decayed rapidly with an even faster decline of intracellular cAMP content when forskolin/rolipram were removed from the medium. These results suggest that both the activation (short-term) and induction (long-term) of PDE4A/4B isoforms in RPMVECs are closely modulated by the intracellular cAMP content via both post-translational and synthetic mechanisms.

Keywords: Cyclic AMP phosphodiesterase; Activation; Induction; Forskolin; Rolipram; Pulmonary microvascular endothelial cell

1. Introduction

Cyclic nucleotide phosphodiesterases (CN PDEs) consist of 11 gene families that hydrolyze cyclic AMP and cyclic GMP [1–4]. PDEs control a wide variety of biological, physiological and pathological events by regulating basal levels and the intensity and duration of agonist-induced changes of intracellular cAMP and cGMP content. One of the gene families, PDE4, is well characterized by its cAMP-specificity and rolipram-inhibitory sensitivity. PDE4 isozymes consist of four subfamilies (4A, 4B, 4C and 4D) encoded by distinct genes [5–9]. PDE4 isotype expression and activity can be regulated differentially by various agents, including β-adrenergic receptor-linked adenylyl cyclase agonists [10], cAMP analogs and PDE4 selective inhibitors [11], hormones [12], and lipopolysaccharide [11,13]. The regulation of PDE4 is dependent upon the type of agonist and the expression of PDE4 subfamily types in specific cells or tissues. For example, PDE4 activity in RFTL-5 thyroid cells is activated transiently by thyroid-stimulating hormone (TSH) [14]; U937 promonocytic cell PDE4 is increased by PKA-mediated phosphorylation of a specific splice variant PDE4D3 [15]. The in vitro activation of recombinant PDE4D by PKA

Abbreviations: PDEs, phosphodiesterases; 6-MB-cAMP, N^6-monobutyryl cAMP; RPMVECs, rat pulmonary microvascular endothelial cells; RFM, rolipram and forskolin medium; PKA, cyclic AMP dependent protein kinase; TM-PI, Tris–HCl–$MgCl_2$ buffer with protease inhibitors; DMEM, Dulbecco's modified Eagle's medium; PE, phycoerythrin; FBS, fetal bovine serum

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phosphorylation has also been demonstrated [15–18]. Moreover, sustained increases of intracellular cAMP enhance gene expressions of PDE 4A/4B in U937 cells [19] and 4B/4D in human myometrial cells [20] by different mechanisms.

The expression of different PDEs in cultured endothelial cells shows a pattern of variation among different species and culture conditions [21]. However, a prototype expression of a specific PDE4 gene family has been found in rat pulmonary microvascular endothelial cells (RPMVECs) that remains relatively stable in cell culture [22]. PDE4 is the predominant PDE isoform in this endothelial cell type and, as such, plays a key role in regulating the intracellular cAMP concentration [22]. Furthermore, the intracellular cAMP concentration is one of the most important factors involved in modulating the barrier permeability that is formed by the pulmonary microvascular endothelium [23–27]. Therefore, possible regulations of PDE4 in response to cAMP elevation in RPMVECs was tested in this study. The results of a portion of these studies have been published in abstract form [28].

2. Materials and methods

2.1. Materials

[2,8-^{3}H] cyclic AMP (specific activity 21.3 Ci/mmol) and [8-^{3}H] cyclic GMP (specific activity 5.8 Ci/mmol) and Dowex-1X8-400 resin were prepared as described previously [29]. Benzamidine, tosyl-lysl-chloro-ketone (TLCK), aprotinin, and snake venom were from Sigma, pepstain A and leupeptin from Peninsula Laboratories, and rolipram from Biomol. Forskolin, 6-MB-cAMP, and cycloheximide were from Calbiochem. Forskolin was dissolved in 100% ethanol and diluted into medium with a final concentration of ethanol at 0.1%. Other drugs were dissolved in DMSO and diluted to less than 0.5% in the PDE assays or in cell cultures. Recombinant murine PKA catalytic domain (PKA Cα), expressed and purified from *Escherichia coli* strain BL21(DE3)/PlysS carrying the plasmid pET9d.CαG1A [30], was kindly provided by Dr. E. Reiman. The protein kinase inhibitors, PKI and H89 (PKA), chelerythrine (PKC), and H7 (non-selective to PKA, PKG and PKC) were purchased from Calbiochem. Antibody to PDE4, subfamily-selective antibodies to 4A, 4B, and 4D, and antibodies to other types of PDEs used in this study were as described previously [8,15,22,31–33]. An affinity purified PDE4-phospho antibody specific to the putative PKA-mediated phosphorylation sites was purchased from FabGennix Inc. A PE (phycoerythrin)-labeled goat anti-rabbit IgG suitable for immunocytochemistry was obtained from Southern Biotechnology Associates and peroxidase-coupled goat anti-rabbit or rabbit anti-sheep IgG used for Western blots was from Zymed.

2.2. Culture and treatment of microvascular endothelial cells

RPMVECs were isolated in our laboratory from perfused rat lungs and grown in DMEM medium containing 10% FBS and antibiotics [22,24]. For long-term treatment, RPMVECs were cultured for an additional 10–15 passages starting at passage 5 in a medium supplemented with 10 μM rolipram and 10 μM forskolin, RFM. The cells in RFM were continuously cultured for a minimum of 60 days with the medium changed twice weekly and cell passage once a week until the measurable PDE4 activity in the cells plateaued. The RFM cells were kept in the same medium with continued passages of cells in the range of 15–30. Both RPMVECs and RFM cells were characterized to endothelial cell features by morphology, specific antibodies for surface receptors, and cell function as previously described [24,34,35].

2.3. Measurement of cAMP-PDE activity in permeabilized intact RPMVECs

RPMVECs were grown to 95% confluence in 24-well tissue culture plates. Before the experiment, the growth medium was changed to control medium or medium containing the drugs. The pre-incubation time for protein kinase inhibitors and cycloheximide was at 30 min and 2 h, respectively. After incubation with forskolin or 6-MB-cAMP, the cells were washed with DMEM, followed by cold PBS (2×) and placed on ice in 700 μl TM-PI buffer: Tris–HCl (20 mM; pH 7.4), $MgCl_2$ (5 mM) buffer supplemented with protease inhibitors (PI: 10 μM TLCK, 2 μM leupeptin, 2 μM pepstatin A, 10 μM benzamidine, 2000 U aprotinin/ml). A substrate mixture (100 μl) containing 7% Triton X-100, snake venom (0.5 mg/ml), 2 μM ^{3}H-cAMP was added to start the reaction with incubation for 10–15 min at 30 °C. Reactions were terminated by the addition of 900 μl methanol. Samples were applied to Dowex 1-X8 columns with a second 900 μl methanol washing and contents of ^{3}H in total Dowex column elutants were counted.

2.4. Total cellular cyclic nucleotide content

Cells were grown to pre-confluency on 100 mm dishes. Cells were washed with cold PBS twice and lysed in 2 ml 0.2 N HCl/50% methanol. The cell lysates were scraped on ice and collected by low speed centrifugation. The supernatants were diluted in 50 mM Tris–HCl (5 ml) to neutrality and applied to Dowex 1-X8 columns. Cyclic nucleotides were continuously eluted from columns with 0.02N HCl/50% methanol for cAMP and 0.2N HCl/50% methanol for cGMP. Samples were lyophilized and acetylated with triethylamine and acetic anhydride. Radioimmunoassay (RIA) was performed immediately using a modification of the methods previously described [36] with I^{125} labeled cAMP/cGMP-S-TME and specific antibodies.

2.5. Western blot analysis

Crude 100,000 × *g* supernatants obtained from RPMVECs, forskolin activated cells, and RFM cells were used for immunoblot analysis as described previously [21,22] with the TM-PI buffer supplemented with EDTA (0.5 mM) and NaF (10 mM). Briefly, near confluent cells were collected as low-speed pellets and homogenized in TM-PI buffer as described above. Fifty microgram of total protein was precipitated from 100,000 × *g* supernatant using methanol–chloroform (4:1), re-dissolved in loading buffer, and subjected to 7.5% polyacrylamide gel electrophoresis followed by the electrotransfer to a nitrocellulose membrane (Schleicher & Schuell, Inc.). After 1 h pre-incubation with 1% blocking buffer (Boehringer Mannheim) at room temperature, immunoblotting was performed with different antibodies as described previously [22]. The POD conjugated secondary IgG antibodies were used to visualize the immunoreactivity using detection reagents of BM Teton POD substrate (Boehringer Mannheim) and 1.5% hydrogen peroxide.

2.6. RT-PCR

RT-PCR for PDE4 isoforms mRNA expression in RPMVECs and RFM cells were performed as described previously [22]. Briefly, total RNA from preconfluent RPMVECs or RFM cells grown in 100 mm dishes were isolated by RNA isolation kit (Roche) and cDNA prepared by First Strand RT-PCR kit (Stratagene) with random primers. PCR were performed with identical amounts of total RNA(cDNA) extracted from RPMVECs and RFM cells. The amplification protocol with a PerkinElmer 9600 thermocycler was optimized to compare the differences in PDE4 mRNA between RPMVECs and RFM cells based on the numbers of cycles and the annealing temperatures required for each PCR reaction. Forward and reverse primers were used for PDE4 to resolve the subfamilies of 4A and 4B at their expected sizes: PDE4 (all families), 5′-CTGCGACATCTTCCAGAACCTCAG-3′ and 5′-CCGGTTGTCTTCCAGCGTGTCCAGGAT-3′ (476 bp); PDE4A (all splice variants), 5′-TCAGCT-GGAGGCCGCTCACTC-3′ and 5′-GCTGAGGTTCTGGAAGATGTCGCAG-3′ (426 bp); PDE4B (all splice variants), 5′-ATGACCCAGATAAGTGGAGTGAAG-3′ and 5′-TCAGGCTGAACCAGGTCT-GCCCAG-3′ (998 bp). Each positive band obtained with 1.2% Agarose gels was cloned to pCR2.1 vector (Invitrogen), and then sequenced at the USA Biopolymer Core laboratory using an ABI 377 automated sequencer.

2.7. CN PDE fractionation with DEAE-Trisacryl M chromatography

Approximately four hundred million cells from preconfluent RPMVECs or RFM cells were washed and collected in cold PBS. The cell pellets were re-suspended in 20 ml TM-PI buffer as described above and homogenized on ice with a 25 ml Duall type glass homogenizer. The homogenate was centrifuged at 100,000 × *g* for 60 min at 4 °C. The supernatant fraction was diluted with 80 ml of the same buffer and applied to a DEAE-Trisacryl M column (10 cm × 1.5 cm), pre-equilibrated with TM buffer without PI. After washing the column with 60 ml of TM buffer, PDE activities were eluted with 120 ml of two successive linear gradients of NaCl (0–200, 200–500 mM in TM buffer) at a flow speed of 1.0 ml/min. Seventy-five fractions were collected and assayed for cAMP hydrolytic activities in every third fraction.

2.8. Immunoprecipitation and phosphorylation

The soluble extracts from RPMVECs or RFM cells were prepared as described for Western blot analysis. Cell extraction and affinity purified rabbit PDE4A or 4B antisera were co-adsorbed to protein A/Agarose beads (Calbiochem) by incubating the suspensions in TB-PI buffer (40 mM Tris–HCl, pH 8.0, containing 0.5 mg/ml BSA and protease inhibitors) for 2 h at 4 °C under continuous rotation. At the end of incubation, the complex immunoadsorbed to the beads was washed twice with cold TB-PI buffer and kept on ice. Phosphorylation reactions were performed by the addition of beads to a reaction mixture containing 10 mM KPO_4 buffer (K_2HPO_4/KH_2PO_4, pH = 7.0), 5 mM $MgAC_2$, 50 μM ATP (10 μCi of γ-^{32}P ATP), and 100 U of murine recombinant PKA Cα. For PKA inhibition, the selective peptide inhibitor, PKI, was added at a final concentration of 1 μM. The mixture was incubated at 30 °C for 30 min and beads were washed twice in cold PBS. The bead pellets were mixed with 30 μl SDS loading buffer and subjected to 7.5% polyacrylamide gel electrophoresis. The gels were stained with Coomassie-blue and dried before exposure to film overnight for autoradiography.

2.9. Fluorescent immunocytochemistry

RPMVECs and RFM cells were grown to pre-confluence on sterile coverslips pre-coated with gelatin (100 μg/ml; 75 °C) for 1 h and rat collagen type IV (1 μg/ml; 37 °C) for 20 min. For drug withdrawal, the RFM was changed to fresh medium without rolipram and/or forskolin for 12, 24 and 48 h before the immunofluorescent staining. Cells were washed with PBS three times, fixed with Histochoice MB (Amresco) for 20 min, and permeabilized with acetone on ice for 5 min. The fixed cells were rinsed with cold PBS and incubated with PDE4A or PDE4B antisera for 60 min at room temperature, then incubated with PE-labeled goat anti-rabbit IgG for 60 min in the dark. Antisera blocked by PDE4A or 4B specific peptides were used as negative controls. Cell fluorescence was analyzed by confocal microscopy using a ACAS 570 (Meridian Instruments) with a laser light source. Fluorescent images

were collected with 100× and 200× oil objective in 3–6 cells using a series section mode at 0.3 micron section from the bottom to the top (approximately 10 microns). The fluorescent images were transferred and calibrated to digital images using different colors for the relative amounts of fluorescent density stained by PDE4A and 4B antibodies.

3. Results

3.1. Short-term activation of PDE4 in RPMVECs by forskolin and 6-MB-cAMP

A rapid increase of PDE4 activity in intact RPMVECs occurred as early as 10 min after the addition of 10 μM forskolin to the cells (Fig. 1A). The increased PDE4 activity reached the maximum level at about 20 min of incubation, plateaued at 40 min, and then declined gradually over 2 h. A secondary delayed increase in PDE4 activity was also observed at the 12 h period. The EC_{50} value for forskolin activation, determined at the 20 min period of stimulation, was 0.44 μM (Fig. 1B).

The activation of PDE4 by 10 μM of forskolin at 20 min was further studied in the presence of cycloheximide or selective inhibitors of protein kinase. Pre-incubation of 20 μM cycloheximide for two hours at a concentration that inhibited de novo synthesis of proteins (data not shown), did not affect forskolin-stimulated PDE4 activity (Table 1). Conversely, the cell-permeable PKA selective inhibitor H89 did inhibit PDE4 activation by 10 μM forskolin in a concentration-dependent manner with an IC_{50} value of 0.8 μM (Fig. 1C). However, other kinase inhibitors, e.g. the PKC inhibitor chelerythine, the non-selective inhibitor H7,

Table 1
Effect of cycloheximide on forskolin simulated short-term activation of PDE4 activity in RPMVECs

Treatment	PDE4 activity (pmol/(min mg protein))
Control	1.79 ± 0.09
Control + cycloheximide	1.75 ± 0.07 (NS)
Forskolin	3.25 ± 0.07*
Forskolin + cycloheximide	3.06 ± 0.14 (NS)

RPMVECs were preincubated for 2 h in the absence or presence of 20 μM of cycloheximide. Cells were treated with 10 μM of forskolin or 0.1% ethanol as vehicle control for an additional 20 min. PDE4 activity was determined by the permeabilized cell activity assay. Values represent mean ± S.E.M. from three experiments. *$P < 0.05$ for Forskolin alone as compared with the control group; NS: not significant, versus control or forskolin alone.

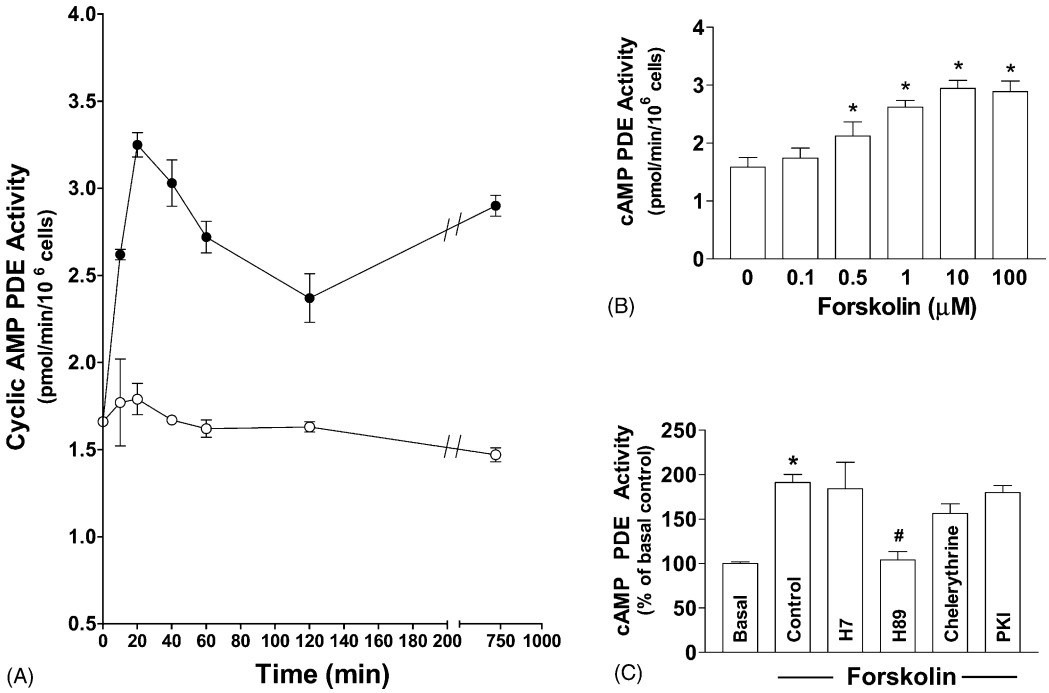

Fig. 1. Activation of PDE4 in RPMVECs by forskolin. (A) RPMVECs were incubated in the absence (open circle) or presence (filled circle) of 10 μM forskolin for the time as indicated. At the end of incubation, cells were rinsed and the PDE activity determined in permeabilized intact cells as described in Section 2. (B) RPMVECs were incubated with increasing concentrations of forskolin for 20 min. The EC_{50} value for forskolin to stimulate PDE4 activity is 0.44 μM. (C) Cells were treated with or without protein kinase inhibitors: H7 (10 μM), H89 (10 μM), chelerythrine (10 μM), or PKI (4.5 μM) for 30 min and incubated with 10 μM forskolin for another 20 min. The concentration dependence of H89 inhibition against 10 μM forskolin induced PDE4 activation was also performed under the same condition (data not shown) and the IC_{50} value for H89 inhibition was calculated at 0.8 μM. The values represent the mean ± S.E.M. of five independent experiments performed in triplicate. *$P < 0.05$ versus RPMVECs basal activity; #$P < 0.05$ versus forskolin activation control in C.

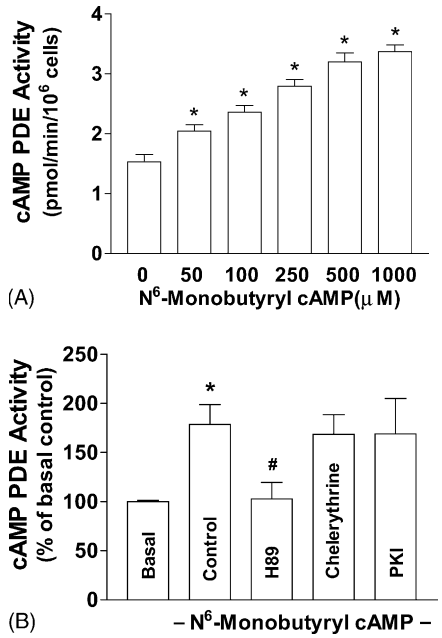

Fig. 2. Activation of PDE4 activity in RPMVECs by N^6-monobutyryl cAMP (6-MB-cAMP). (A) RPMVECs were incubated with increasing concentrations of 6-MB-cAMP for 20 min and the EC_{50} value for 6-MB-cAMP to stimulate PDE4 activity was calculated at 177 μM. (B) Cell treatments with 500 μM 6-MB-cAMP and protein kinase inhibitors. PDE4 activity determination was described as in the legend of Fig. 1. The values represent the mean ± S.E.M. of four independent experiments performed in triplicate. $^{*}P < 0.05$ versus RPMVECs basal control; $^{\#}P < 0.05$ versus 6-MB-cAMP activation control in B.

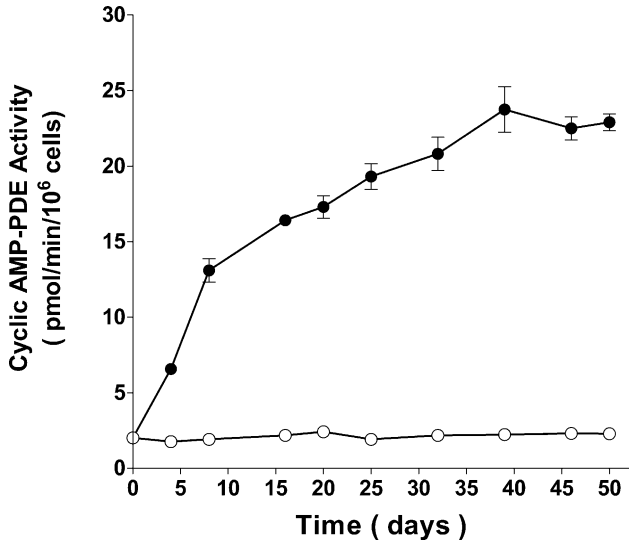

Fig. 3. PDE 4 activity accumulation in RPMVECs following long-term culture in rolipram and forskolin medium (RFM). At passage five, RPMVECs were cultured in DEME medium supplied with 10 μM rolipram and 10 μM forskolin. The RFM medium for cell culture were changed twice weekly and cell passage once per week. The 100,000 × g cell supernatant obtained by ultracentrifugation was prepared as described in Section 2. The cAMP hydrolytic activity of PDE 4 in the supernatant of the passage control RPMVECs (open circle) or RFM treated cells (filled circle) was measured during the medium change or at different cell passages until the activity reached a plateau (at nearly 45 days). All data shown are mean ± S.E.M. of triplicate flasks of cells.

and the non-permeable PKA inhibitory peptide (PKI), did not inhibit forskolin-induced PDE4 activation at concentrations above their IC_{50} values reported to inhibit respective protein kinases (Fig. 1C).

Treatment of RPMVECs with 500 μM 6-MB-cAMP, a cell permeable and site-A preference activator for type I PKA, increased PDE4 activity two-fold at 20 min with an EC_{50} value of 177 μM (Fig. 2A). H89 also blocked the activation of PDE4 by 6-MB-cAMP, whereas other kinase inhibitors were ineffective (Fig. 2B). The role of PKA in phosphorylation of PDE4A and 4B isoforms in this short activation was further evaluated (see Sections 3.3 and 3.4).

3.2. Long-term treatment of RPMVECs by rolipram and forskolin

Significant increases of PDE4 activity have been previously noted following continuous treatment of cells with cAMP elevating agents over hours to days [19,20]. However, in most cases, these long-term treatment paradigms showed variable expressions of PDE4 activity with time of exposure. To demonstrate a sustained increase of PDE4 activity in pulmonary endothelial cells, we treated the RPMVECs continuously with 10 μM of rolipram and forskolin until the PDE4 activity reached a maximum level of activity (Fig. 3). During the first 10 days of treatment the cAMP hydrolytic activity in RPMVECs increased markedly, which was followed by a much smaller increase over the next three to four weeks of treatment. The total cAMP hydrolytic activity in final RFM cells was 10-fold higher than that of the normal RPMVEC cells with the $T_{1/2}$ of maximum increase seen at about 18 days. Despite the large increases in the activity, supernatant extracts of the RFM cells were still sensitive to in vitro inhibition by the PDE4-selective inhibitor rolipram (data not shown). However, an additional short-term incubation of the RFM cells with "fresh" forskolin and rolipram at 10 μM did not increase the activity further (Fig. 4). Treatment of the RFM cells with the PKA inhibitor H89 for 30 min at 10 μM for 30 min had no effect on the elevated PDE4 activity. (These values were 21.5 ± 0.3 pmol/min per 10^6 cells for RFM cell control versus 20.6 ± 0.4 pmol/min per 10^6 cells for RFM cell plus 10 μM of H89 for 30 min, $P > 0.05$, $n = 3$).

Twenty-fold increases in cAMP content were measured by radioimmunoassay in RFM cells with only a modest 50% increase in cGMP content (Fig. 5). However, a rapid decline in the cAMP content, but not cGMP, occurred within 2 h following removal of forskolin and rolipram (M-RF) from the medium (Fig. 5). PDE4 activity also decreased rapidly after removal of these drugs (Fig. 6). The time course of the PDE4 decay showed a $T_{1/2}$ at 3.6 h, which was much shorter (about 120 times) than the time noted to increase PDE4 activity in RFM-treated cells (see Fig. 3).

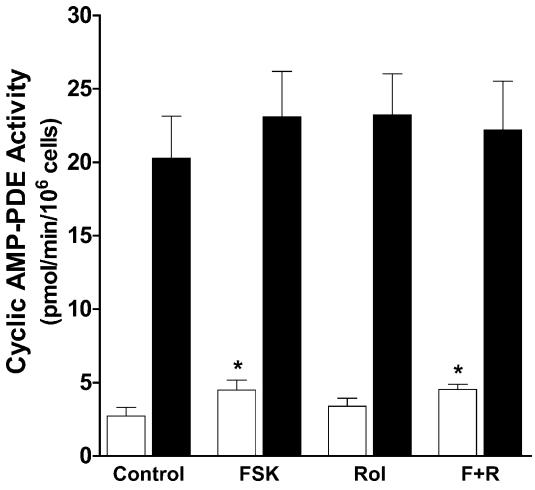

Fig. 4. Comparison of PDE 4 activities in un-treated RPMVECs and long-term RFM cells in response to forskolin and rolipram. RPMVECs (open bars) and RFM cells (solid bars) were cultured in 24-well plates for two days and treated with fresh medium minus drugs (control), or in the presence of 10 μM forskolin (FSK), 10 μM rolipram (Rol), or both drugs (F + R) for 20 min before PDE assay. The cAMP hydrolysis in RPMVECs or RFM was measured in permeabilized intact cells as described in Section 2. Values represent the mean ± S.E.M. of four (RPMVECs) or three (RFM) independent experiments performed in triplicate. $^{*}P < 0.05$ versus corresponding control.

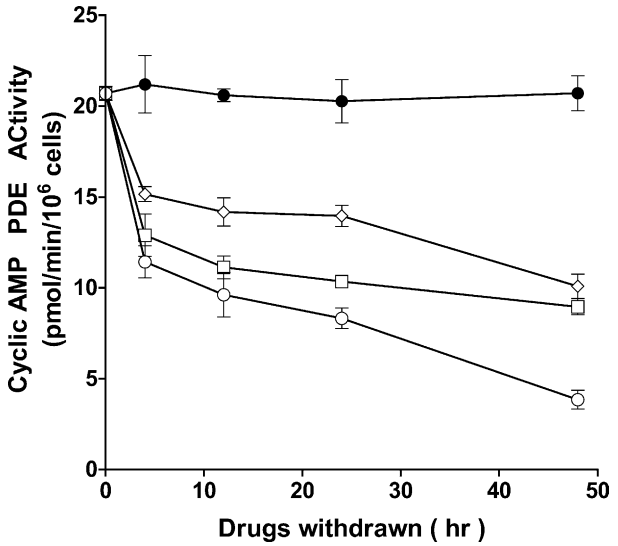

Fig. 6. Decay of cAMP PDE activities in rolipram and/or forskolin withdrawn RFM cells. The RFM cells were cultured in flasks for two days and medium was changed with fresh medium containing two drugs (filled circle), only forskolin (open diamond), only rolipram (open square), or drug-free medium (open circle) at indicated times before the PDE assay. The preparation of cell supernatant and measurement of cAMP-hydrolytic activity were performed as described in the legend for Fig. 3. All data shown are mean ± S.E.M. of triplicate samples from one experiment repeated three times.

3.3. Changes of mRNA and immunoreactivities of PDE4 isoforms in activation

PDE4A and PDE4B subtypes were identified in RFMVECs using PDE4 subfamily-selective antibodies for immunoblotting of cytosolic extracts that contain more than 90% of total cAMP hydrolytic activity in the cells [22]. Two major splice variants were detected at 87 and 83 kDa with a PDE4A-specific and a PDE4B-specific antibody, respectively. These were designated PDE4A6 (87 kDa) and PDE4B3 (83 kDa) [22]. RT-PCR also confirmed the existence of a transcript message of PDE4A6 without expression of mRNAs of other known splice variants of PDE4A, e.g. PDE4A1 and A5A (data not shown). The 83 kDa of PDE4B in rat lung endothelial cells was similar in size to PDE4B3 of rat brain [37,38] but smaller than the human PDE4B3 with a reported molecular weight of 103 kDa [39,40]. No PDE4D isoform could be detected in cytosolic extractions from either control or treated RPMVECs (data not shown) using the PDE4D specific antibody (M3S1), which does recognize two PDE4D isoforms (68 and 105 kDa) in rat kidney [22]. Other PDE isoforms, e.g. PDE2 and PDE5, were also undetectable following either short- or long-term treatment of RPMVECs when the family selective antibodies were used to probe these molecules (data not shown). We found that the mRNA for PDE4, PDE4A, and PDE4B were increased in long-term treated RFM cells (Fig. 7A) by using the RT-PCR methods.

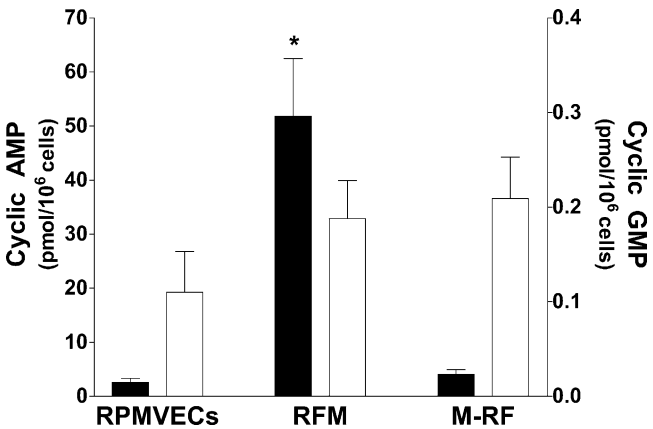

Fig. 5. Total intracellular cyclic nucleotide content in RPMVECs, RFM and the cells in which the drugs were withdrawn (M-RF). Cells were grown to pre-confluency and washed with cold PBS before cell collection. The medium in drug withdrawn cells was changed two hours before cell collection in the rolipram/forskolin free medium. The cAMP (solid bars) and cGMP (open bars) levels in whole cell extractions were measured as described in Section 2. Values represent the mean ± S.E.M. of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus RPMVECs.

The selective 4A and 4B antibodies were then used to study PDE4 expression in the treated cells following short-term and long-term treatment with forskolin and rolipram. No significant changes of PDE4A6 immunoreactivities at 87 kDa were found following treatment of forskolin over a 2 h period using the PDE4A specific antibody which detect the total amounts of the polypeptides (Fig. 7B, left). Under the same conditions, the immnuoreactivity of PDE4B was also unaffected (data not shown). A PDE4A-phospho antibody specific to the putative PKA-mediated phosphor-

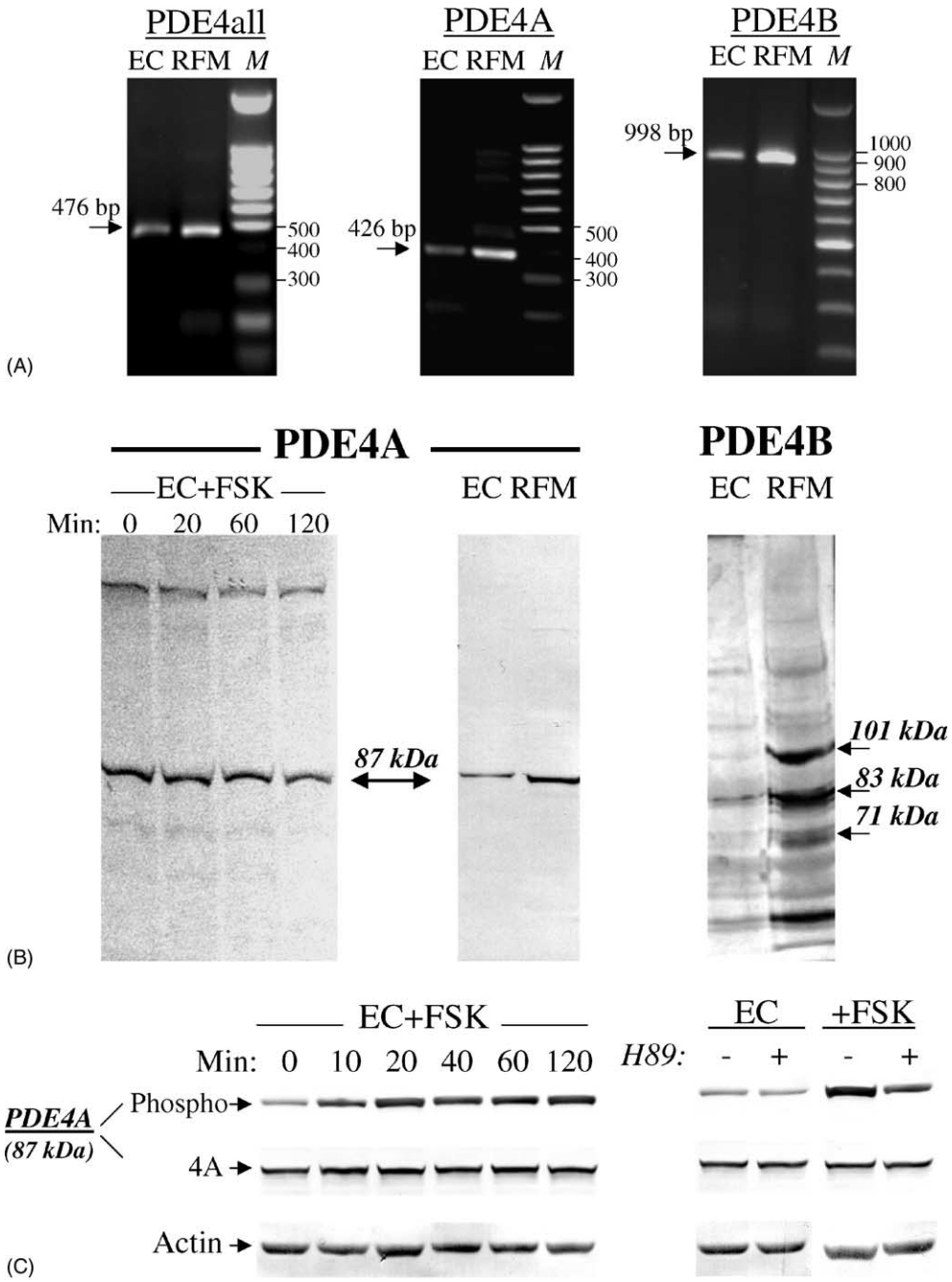

Fig. 7. RT-PCR, immunoreactivities, and phosphorylation status for PDE4A and 4B isoforms in activated RPMVECs. Panel A, increased mRNA levels for PDE4 (all subfamilies), PDE4A, and PDE4B were found increased in long-term treated RFM cells than PMVECs. The RT-PCR were performed with specific primers using identical amounts of total RNA/cDNA obtained from RPMVECs and RFM cells. Panel B, left, unchanged immunoreactivities of 87 kDa PDE4A6 in short-term forskolin stimulation. RPMVECs were treated with fresh medium containing 10 μM forskolin (FSK) for 0–120 min before cell collection. The immunoreactivities of 83 kDa PDE4B3 were also not changed under the same conditions (data not shown). Middle and right, increased immunoreactivities of 87 kDa PDE4A6 and 83 kDa 4B3, and novel isotypes of PDE4B at 71 and 101 kDa, were identified in long-term RFM cells as compared with the passage control of RPMVECs (EC). Panel C, left, increased phosphorylation of PDE4A6 in short-term FSK stimulation was detected by a PDE4A-phospho antibody specific to the putative PKA-mediated phosphorylation sites as compared to the unchanged amount of PDE4A6 at 87 kDa by the PDE4A-specifc antibody. The ECs were treated with FSK for 0–120 min as indicated in panel B. Right, to study effects of H89, 10 min of pre-incubation with 10 μM H89 (+) or the same volume of vehicle (−) was followed by a treatment of 10 μM FSK in 20 min. For all blots, the 100, 000 × *g* supernatant extracts were prepared and subjected to 7.5% SDS–PAGE at 50 μg/lane and then transferred to a 0.2 μm nitrocellulose membrane. Affinity purified 4A and 4B subfamily selective antisera and PDE4A-phospho antibody were used in Western blots as described in Section 2. The POD-linked secondary antibodies were used for detection using BM Teton and 0.02% hydrogen peroxide as the substrates. Actin was used to control the amount of protein applied to the gel.

yaltion sites was used to test the possible change of in situ amount of PDE4A phosphorylation following forskolin treatment. Immunoreactivity to the PDE4A-phospho antibody at the position of 87 kDa PDE4A6 increased as early as 10 min after forskolin addition, reached a maximum at 20 min (five-fold above basal levels), and maintained to 120 min, whereas the amount of total PDE4A6 was unchanged (Fig. 7C, left). After pre-treatment with H89, basal phosphorylation of PDE4A6 was not changed whereas forskolin-induced phosphorylation measured at the 20 min period was blocked (Fig. 7C, right).

Western blots showed that the intensities of both 87 kDa PDE4A6 and 83 kDa 4B3 immunoreactivities were significantly increased in long-term RFM cells (Fig. 7B, middle and right). These results are consistent with the increased amount of mRNA. New polypeptides of 71 and 101 kDa for PDE4B immunoreactivities were detected in RFM cells that were not identified in the control cells. The 71 kDa PDE4B immunoreactivity in RFM cells is identical in molecular weight to the PDE4B2 isoform expressed in rat brain [37], or the recombinant polypeptide expression noted in MA-10 cells [38], and is similar to the 65–67 kDa proteins for T cell lines [41]. However, it is smaller than the PDE4B2 of human recombinant proteins and the isoform reported in rat heart with a molecular weight of 78 kDa [40]. The101 kDa 4B in RFM is possibly an endothelial specific rat PDE4B1 or a novel "long-form" of 4B (see Section 4).

Because the expression spectrum of PDE4A and 4B in long-term RFM cells differed from that of control cells, we studied the enzyme elution profiles of the cells following the isolation of specific PDE4 isozymes. Following DEAE chromatograpy, a much larger cAMP hydrolytic peak and a shift of peak position from low salt elution fractions (100–120 mM NaCl) to higher salt fractions (150–180 mM NaCl) was found in RFM cells as compared to the control RPMVECs (Fig. 8).

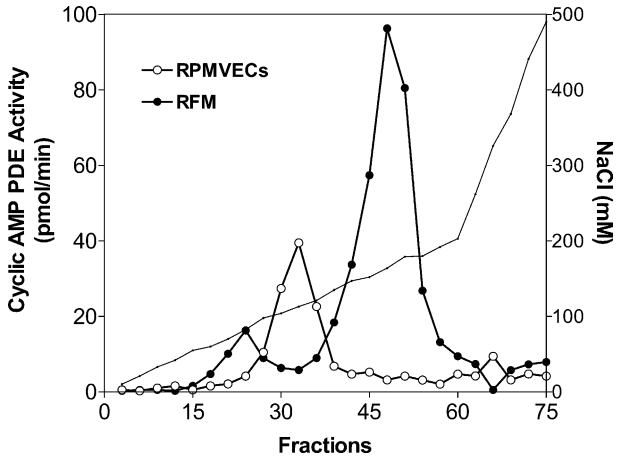

Fig. 8. Anion-exchange chromatography of RPMVECs and RFM cells cAMP phosphodiesterase. Cell supernatants of RPMVECs or RFM cells (400 million cells) were fractionated by Trisacryl-M DEAE-cellulose columns (10 cm × 1.5 cm) as described in Section 2. PDE activities were eluted with two successive linear gradients of 0–200 mM and 200–500 mM NaCl in homogenization buffer at flow rates of 1.0 ml/min. The elution profiles of cAMP activities in every third fraction were measured in RPMVECs (open circle) and RFM cells (filled circle) using 0.25 μM ^{3}H-cAMP as substrate. No significant cGMP hydrolysis was detected in the cell supernatants or column fractions obtained from RPMVECs or RFM cells.

3.4. In vitro phosphorylation of PDE4A and 4B by PKA

We could immunoprecipitate PDE4 activities from soluble extracts of RPMVECs and RFM cells by PDE4A or 4B antibodies on protein A/Agarose pre-coated beads (data not shown). Therefore, the immunoprecipitated polypeptides of PDE4A or 4B on the beads were co-incubated with PKA Cα and ^{32}P-ATP for in vitro phosphorylation studies. Using this protocol, a single ^{32}P incorporated band of PDE4A was noted at 87 kDa in the RPMVECs (Fig. 9A, left), which showed an identical molecular weight for PDE 4A6 in Western blots (see Fig. 7). A secondary 83 kDa band was phosphorylated by PKA under the same conditions with RPMVEC extracts immunoprecipitated by PDE4B antibody (Fig. 9A, right). These phosphorylations of both bands were blocked by a 1 μM concentration of the PKA specific inhibitory peptide, PKI. Protein kinase G showed no phosphorylation of PDE4A/4B immunoprecipitated proteins under similar experimental conditions (data not shown). Subsequent sequence analysis showed that the putative PKA phosphorylation sites exist in the regulatory domains of both PDE4A6 and 4B3 (see Section 4), which is the same specific motif that is recognized by the PDE4A-phospho antibody (Fig. 7). Increased intensities for 87 kDa PDE4A6 and 83 kDa PDE4B3, and two novel PDE4B isoforms at 101 and 71 kDa, were identified as polypeptides phosphorylated in RFM cells extracts using identical amounts of proteins in the immunoprecipitation and PKA reactions, and in gel loading (Fig. 9B).

3.5. Immunocytochemical localization of PDE4A and 4B following long-term treatment with forskolin and rolipram

We used confocal microscopy to study the intracellular localization of PDE4A and 4B in RPMVECs. Fixed and permeabilized RPMVECs showed immunoreactivities to PDE4A and 4B that were present throughout the cytoplasm but clustered primarily toward perinuclear regions of the cell. Minimal nuclear or peripheral cell membrane staining was observed previously [22].

Compared with un-treated RPMVECs, two major changes of PDE4A/B immunoreactivities were found in the long-term treated RFM cells: (1) the entire distribution pattern throughout the cell was significantly increased; (2) a new subcellular locale of the enzyme was observed in nuclear regions of the cell, which now showed the highest intensity of immunofluorescence (panel A in Fig. 10). In these images, the intensity of immunofluorescence in

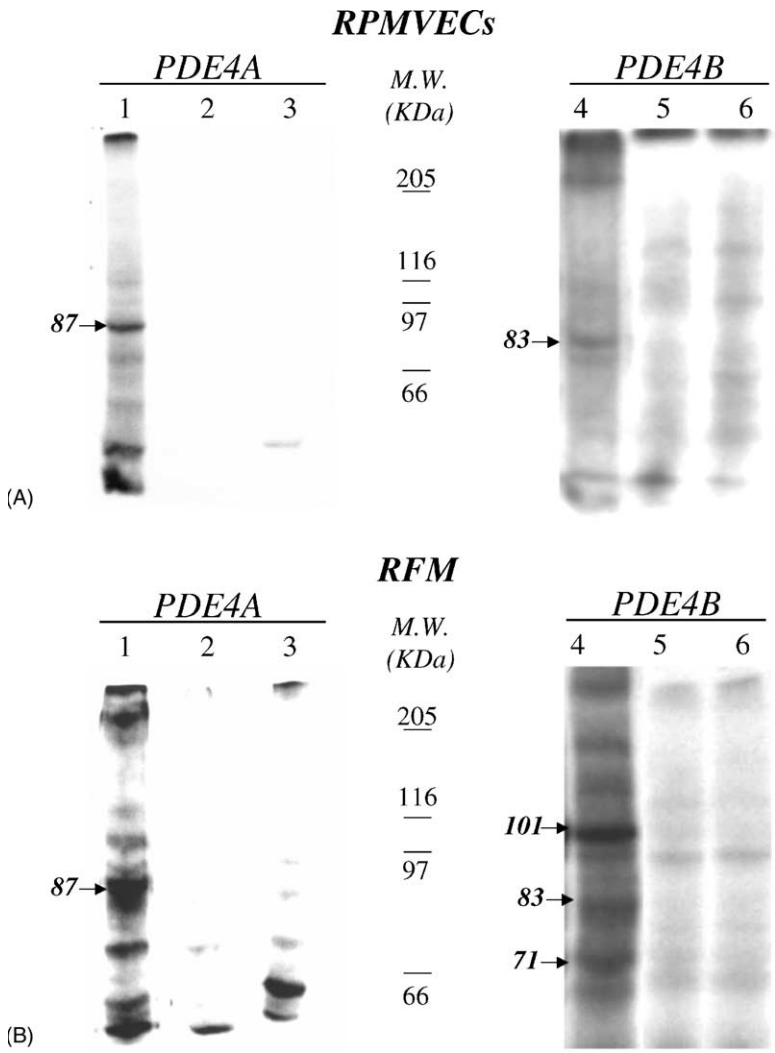

Fig. 9. In vitro phosphorylation of immunoprecipitated PDE4A and PDE4B by protein kinase A in RPMVECs (panel A) and RFM (panel B). The PDE4A (left, lanes 1–3) and PDE4B (right, lanes 4–6) from cell supernatants of RPMVECs and RFM cells were immunoadsorbed by PDE4A and PDE4B antisera pre-coated protein A/Agarose beads. The beads with PDE4A or 4B were incubated with 100 U of recombinant PKA Cα and 50 μM ^{32}P-ATP at 30 °C for 20 min. Lanes 1 and 4, PKA phosphorylation; lanes 2 and 5, PKA-free control; lanes 3 and 6, PKA with 1 μM PKI.

nuclear or perinuclear regions is about 2–3 times higher than the cytoplasmic area.

Removal of forskolin and rolipram from the culture medium showed a loss of the immunoreactivities for PDE4A and 4B within 48 h. The time-course of decay in the nuclear region occurred more rapidly than in peripheral areas. For example, nuclear immunoreactivties of PDE4A and 4B disappeared completely within 12 h after drug withdrawal (panel B in Fig. 10), whereas the immunofluorescence in the cytoplasm did not decline appreciably during the first 24 h (panel C) but instead required more than 48 h to return to the levels found in un-treated RPMVECs (panels D and E).

4. Discussion

This study demonstrates that the PDE4 activity expressed in cultured rat pulmonary microvascular endothelial cells can be activated (short-term) or induced (long-term) via increases of intracellular cAMP concentrations. It further confirms our previous finding regarding a characteristic cAMP turnover in this cell type [22] and extends the types of PDE4 regulation described for other cell types [14,15,19,20]. For example, the time-course and dose–response curves to forskolin agree with the previous studies on transient turnover of intracellular cAMP in RPMVECs following activation of adenylyl cyclase or PDE4 inhibition by selective inhibitors [22,42]. The EC_{50} values needed for forskolin to activate PDE4 (0.44 μM) in this study and increase cAMP conversion (1.1 μM) [22] were similar. Other experiments in the cells indicate that the short-term activation of PDE4 is mediated by a PKA in situ phosphorylation but is independent of the de novo synthesis of the enzyme since the activation is not blocked by cycloheximide. This interpretation is consistent with previous observations made in other studies with several cell types [14].

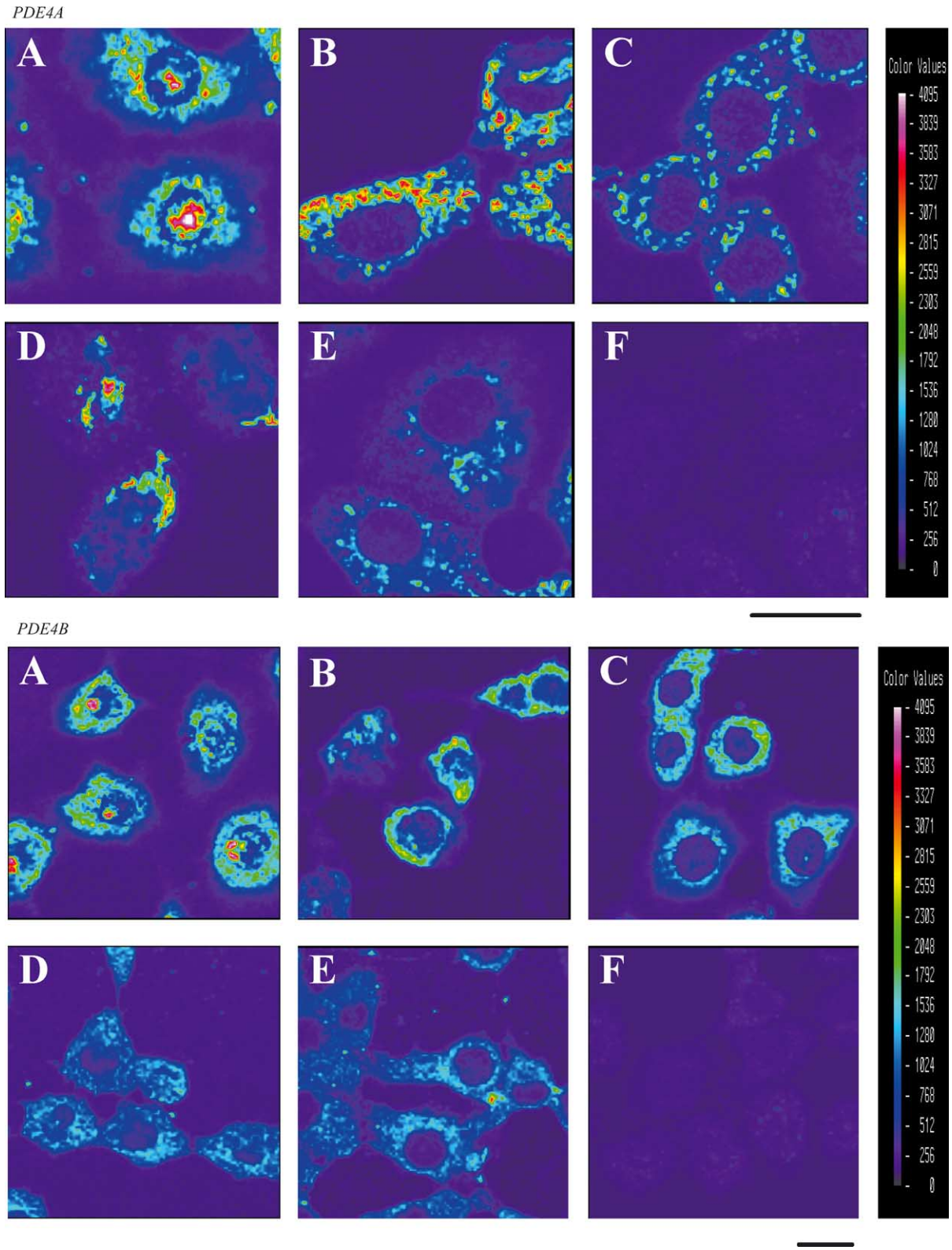

Fig. 10. Decay of relative amount of PDE4A and PDE4B immunofluorescence in RFM cells after withdrawal of forskolin and rolipram from the medium. The fluorescent staining shows PDE4A (top panels, 200×) and PDE4B (bottom panels, 100×) in RFM cells (A), drugs withdrawn at 12 h (B), 24 h (C), and 48 h (D), and control RPMVECs (E). The negative control for RFM staining was performed by antiserum pre-incubated with PDE4A or 4B specific peptides (F). RFM cells and RPMVECs were grown to pre-confluency on collagen/geltain coated glass coverslips. The cells were fixed and permeabilized before incubation with antisera as described in Section 2. A PE labeled second antibody was used for fluorescence. A series of sections at 0.3 μm were obtained under the confocal microscope. Different colors of digital image standard for the density of fluorescence and were calibrated as the value shown on the bar at far right. The ‘‘—’’ bar at the bottom of standards indicates the length of 20 μm.

The IC_{50} value of PKA–specific H89 to inhibit PDE4 activation by forskolin is comparable to its in vitro IC_{50} value for PKA inhibition. It is unclear, however, why the non-selective agent, H7, failed to exert any effect on forskolin response since it will inhibit PKA in vitro. Activation of PKA by the analogue 6-MB-cAMP, mimicked forskolin-induced PDE4 activation and was also inhibited by H89. These findings suggest that PKA-mediated phosphorylation in RPMVECs is the primary post-translational modification mechanism involved in the short-term activation of PDE4 following a transient cAMP elevation by forskolin. The in situ and in vitro phosphorylation of PDE4A6 and 4B3 by PKA strongly support this hypothesis. Not all PDE4 subfamilies or splice variants are substrates for PKA and only the so-called ''long form'' has the phosphorylation site. PDE4D families like 4D3 have been reported in the literature as one of the reported isoforms [14,15,43]. We have shown in RPMVECs that 4B3 is a PKA substrate and has been noted in the literature [39] that this splice variant is a ''long-form'' PDE4. The phosphorylation of PDE4A6 in RPMVECs is a novel finding and suggests that 4A6 is a ''long-form'' PDE4. The PKA putative phosphorylation sites (RRXSX) in these proteins were verified by gene sequence analysis. PDE4A6 contains this domain at residues 56–60 in N-terminus, and PDE4B3 contains the domain at residues 115–119. These results support the data on immunoprecipitated PDE 4A6 and 4B3 in PMVECs and RFM showing that they are in vitro substrates for PKA.

The relatively stronger, and apparently novel, 101 kDa PDE4B band seen in the RFM cells does not correspond to any known recombinants or native rat PDE4B isoforms that have been reported to date. Its molecular weight is near to that reported for the human recombinant 104 kDa PDE4B1 or 103 kDa PDE4B3, or 7-oxo-PGI_2 induced expression of PDE4B1 + 3 forms at rat heart [40], but higher than the 91–92 kDa PDE4B1 that has been found in rat brain [37,38]. A truncated cDNA sequence of rat PDE4B1 homologue to *Homo sapiens* encoding 563 amino acid of C-terminal (so called DPD) was also previously noted [44]. Therefore, this long-term activation, i.e. inducible 101 kDa 4B, may possibly be an endothelial specific rat PDE4B1 or a novel ''long-form'' of 4B. Sequence analysis of the corresponding peptides will be required to further substantiate this interpretation.

To study the effects of sustained, i.e. long-term elevation of cAMP in RPMVECs, rolipram and forskolin (RFM) were added to the cells for extended periods. This paradigm may be a relevant model for the response of airway cells to chronic use of hormones or β-adrenergic agonists for diseases such as asthma [19,45,46]. In addition to the constitutive PDE4A6 and 4B3 enzymes, 101 kDa PDE4B1 and 71 kDa PDE4B2 isoforms were found to accompany the sustained increase in intracellular cAMP concentrations in the RFM treated cells. The increased PDE4A/B immunocytochemical nuclear localization suggests a role for sustained increases in cAMP concentrations in regulating both PDE4 gene expression and the subcellular translocation of the enzyme, though the latter idea is more speculative.

The physiological significance of such a translocation is also unknown. However, one possibility is that compartmentation of cAMP in RFM cells may form a gradient from nuclear to cytoplasmic regions leading to the accumulation of newly synthesized PDE4 isoforms by chemotaxis. This interpretation is supported by data obtained in RFM cells in which the drugs are removed from the medium. In this situation, cAMP content decays much faster than PDE4 activity and PDE4 immunoreactivity in the nucleus decreases much more rapidly than it does in the cytoplasm. In this regard, several anchoring or recruitment factors for PDE4, e.g. A-kinase anchoring proteins (AKAP), may be the candidates involved in regulating complex formation of the PDE4A/B with binding proteins to aid the co-localization of PDE4 with perinuclear or cellular membranes [47–51]. Indeed, a 95 kDa DNA binding AKAP isoform has been detected in RPMVCEs and RFM (data not shown), suggesting a possible role for this molecule in regulating the translocation of PDE4 isoforms under the condition of long-term activation. Further studies are required to investigate the co-immunoprecipitation of PDE4A/4B with AKAP95 and their co-localization in the nucleus of RFM cells.

The upstream transcription elements regulating the PDE4 genes in any cell type are not well understood, whereas the downstream genes, including PDE4 and PKA, are known to be up-regulated when cAMP levels are increased [10]. A similar cAMP responsive element (CRE) is presumably involved in the up-regulation of PDE4 transcripts in RPMVECs. However, PKA catalyzed phosphorylation and activation of transcription factors such as CRE-binding protein (CERB) has been recently elucidated in certain but not all cells [13]. For example, up-regulation of PDE4 expression in human monocytes by LPS stimulation involves a different mechanism from PKA mediated transcriptional activation [13]. Similarly, we found that the up-regulation of PDE4 is less sensitive to the PKA inhibitor H89 in the RFM cells than is the short-term activation of the enzyme. For this reason, a cAMP-dependent but presumably PKA-independent mechanism of transcriptional activation of PDE4 expression may also exist in the forskolin/rolipram stimulated pulmonary microvascular endothelial cells. Studies are underway to investigate whether the same nuclear scaffolding proteins, e.g. AKAP95, will compartmentalize with cAMP in RFM.

In summary, the activity of PDE4 in RPMVECs can be up-regulated either by PKA via a direct phosphorylation of the enzyme following short-term activation or a cAMP-dependent induction of gene expression following long-term treatment with cAMP elevating agents. In long-term RFM cells, the elevation of total cellular PDE4 activity coincides with steady-state increases of PDE4A and 4B

proteins and a subcellular translocation of these isoforms in the nuclear region of the cells. From a functional standpoint, the up-regulation of PDE4 may mediate desensitization of the microvascular endothelial cells in response to extracellular signals, e.g. β-adrenoceptor agonists and hormones. Elevation of intracellular cAMP has been presumed as a factor that can account for the enhanced barrier formation of the pulmonary endothelium in biophysical and inflammatory conditions via the promotion of cell-cell and cell-matrix interactions, involving cAMP-dependent pathways that oppose the effects of increased intracellular Ca^{2+} [42,52,53]. In contrast to the effects observed in pulmonary conduit macrovascular endothelial cells, intracellular Ca^{2+} has minimal direct effects on the monolayer permeability of cultured RPMVECs [24] and the activation of store-operated Ca^{2+} (SOC) itself does not decrease intracellular cAMP content in RPMVECs [54]. Indeed, only following the inhibition of PDE4 activity by rolipram in RPMVECs does thapsigagin-induced SOC inhibit AC6-mediated conversion of ATP to cAMP that consequently influences the intracellular cAMP concentration [54]. These studies support the concept that the status of PDE4 activation/expression is of fundamental importance in governing signaling pathways that link the functional permeability processes in the pulmonary microvascular endothelium.

Acknowledgments

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