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RESEARCH PAPER

Short-term or long-term treatments with a phosphodiesterase-4 (PDE4) inhibitor result in opposing agonist-induced Ca^{2+} responses in endothelial cells

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Background and purpose: We previously reported that agonist-induced rises in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in human umbilical vein endothelial cells (HUVEC) were inhibited after a short-term (2 min) pre-treatment with cAMP-elevating agents. The aim of this work was to study the effects of longer term (8 h) pre-treatment with dibutyryl-cAMP (db-cAMP) or rolipram, a specific inhibitor of phosphodiesterase-4 (PDE4), on $[Ca^{2+}]_i$, cAMP levels and PDE activity and expression in HUVEC.
Experimental approach: $[Ca^{2+}]_i$ changes were measured in isolated HUVEC by Fura-2 imaging. Intracellular cAMP levels and PDE4 activity were assessed by enzyme-immunoassay and radio-enzymatic assay, respectively. PDE expression was measured by northern and western blot analysis.
Key results: Long-term pre-treatment of HUVEC with rolipram or db-cAMP significantly increased ATP-, histamine- and thrombin-induced $[Ca^{2+}]_i$ rises. Short-term pre-treatment with rolipram was associated with an increase in cAMP, whereas long-term pre-treatment was associated with a decrease in cAMP. Long-term pre-treatment with rolipram or db-cAMP induced a significant increase in PDE4 activity and the expression of 74 kDa-PDE4A and 73 kDa-PDE4B was specifically enhanced. All these effects were suppressed by cycloheximide.
Conclusions and implications: Our data suggest that sustained inhibition of PDE4 by rolipram induced an increase in PDE4 activity, possibly as a compensatory mechanism to accelerate cAMP degradation and that PDE4A and PDE4B were implicated in the regulation of $[Ca^{2+}]_i$. Thus, isozyme-specific PDE4 inhibitors might be useful as therapeutic agents in diseases where $[Ca^{2+}]_i$ handling is altered, such as atherosclerosis, hypertension and tolerance to β-adrenoceptor agonists.
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Keywords: calcium; cAMP; rolipram; PDE4; endothelial; human primary cell

Abbreviations: $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; db-cAMP, dibutyryl-cAMP; HUVEC, human umbilical vein endothelial cells

Introduction

Changes in cytoplasmic Ca^{2+} concentration in endothelial cells are generally accepted as key signalling events for the regulation of many endothelium-dependent processes, including the synthesis and release of vasoactive factors controlling vascular tone and endothelial permeability. Ca^{2+} handling in endothelial cells is modulated by intracellular cAMP levels, although the reported effects are contradictory and the underlying mechanisms are still unclear (Bolz and Pohl, 1997; Vischer and Wollheim, 1998; Hippenstiel *et al.*, 2002). We previously reported that agonist-induced $[Ca^{2+}]_i$ rises in human umbilical vein endothelial cells (HUVEC) were significantly reduced after 2 min pre-incubation with rolipram, a specific phosphodiesterase-4 (PDE4) inhibitor, mainly via inhibition of Ca^{2+} mobilization from intracellular stores (Campos-Toimil *et al.*, 2000).

Intracellular cAMP levels are regulated by eight families of cyclic nucleotide phosphodiesterases (Bender and Beavo, 2006; Lugnier, 2006), which are differently distributed in various endothelial cells (Keravis *et al.*, 2007). HUVEC express PDE2, PDE3 and PDE4 isozymes, with half of the cAMP-PDE activity being due to PDE4 (Favot *et al.*, 2003). In vascular smooth muscle cells, forskolin or 8-Br-cAMP

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treatment for long periods induces compensatory increases in cAMP-PDE activity (Rose *et al*., 1997; Maurice, 1998). This increase in cAMP-PDE activity could be related to PKA-dependent phosphorylation which increases PDE4 activity (Sette *et al*., 1994; Liu and Maurice, 1999) and/or to enhanced expression of two PDE4D variants (Liu *et al*., 2000). Similarly, upregulation of PDE4 activity occurs in human myometrial cells after long-term treatment with cAMP-elevating agents (Mehats *et al*., 1999). To our knowledge, no data dealing with long-term cAMP-dependent regulation of $[Ca^{2+}]_i$ and PDE in endothelial cells have been reported.

Therefore, we sought to characterize the long-term effects of cAMP-elevating agents on $[Ca^{2+}]_i$ handling in HUVEC and to determine if they would differ from those we previously described for a short-term (2 min) treatment (Campos-Toimil *et al*., 2000). HUVEC were thus pre-treated for 8 h with rolipram, a selective PDE4 inhibitor (Schwabe *et al*., 1976; Lugnier *et al*., 1983, 1986) or dibutyryl-cAMP (db-cAMP), a lipophilic non-hydrolyzable cAMP analogue, which directly activates PKA. Resting and agonist-induced rises in $[Ca^{2+}]_i$ were assessed in single HUVEC loaded with Fura-2. Total cAMP levels, cAMP-PDE and PDE4 activities from cells extracts were also determined, and PDE4 isozyme expression was analysed by northern and western blots.

Methods

Cell culture

Freshly delivered umbilical cords were obtained from a nearby hospital. HUVEC were isolated using 0.1% collagenase and grown in medium 199/RPMI 1640 (1:1, v:v) containing HEPES (10 mM), L-glutamine (2 mM), antibiotics/antimycotic (100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 0.25 μg ml^{-1} amphotericin B) and 20% (v:v) human serum (Klein-Soyer *et al*., 1986). Cells were stored in liquid N_2 at the first passage and used thereafter at third to fifth passages, because cellular responses were stable for these passages.

Measurement of $[Ca^{2+}]_i$

HUVEC were subcultured in 75 cm^2 flasks and for experiments in 35 mm Petri dishes in which a 20 mm diameter hole had been cut in the base and replaced by a thin (0.07 mm) glass coverslip. Plates were treated with polylysine (0.5 mg ml^{-1}), sterilized by UV light and then incubated with type I collagen (0.06 mg ml^{-1}) from rat tail at 4 °C overnight. Cells were seeded at low density (2×10^3 cells cm^{-2}), and kept in culture (37 °C, 5% CO_2 in air) for 2–4 days. Rolipram (50 μM), db-cAMP (100 μM) and cycloheximide (100 μM) were added for 8 h and then washed out. Ca^{2+}-imaging experiments were carried out on single cells as described previously (Lynch *et al*., 1994; Campos-Toimil *et al*., 2000). Agonists (10 μM histamine, 10 μM ATP, 5000 IU l^{-1} thrombin) were locally applied by pressure ejection from a glass pipette (2 μm i.d.) placed 200–300 μm from target cells.

cAMP measurements

For cAMP determination, HUVEC were seeded on 96-well plates (50 000 cells per well) for 24 h (37 °C, 5% CO_2 in air). Cells were incubated with rolipram (50 μM), db-cAMP (100 μM), vehicle (controls) and/or cycloheximide (100 μM) for 2 min or 8 h and then washed out. Forskolin (100 μM; 2 min) was used as a positive control (data not shown). Cells were lysed using dodecyltrimethylammonium bromide (0.5% w:v) with continuous shaking for 10 min. Total cAMP of 100 μl aliquots from each non-acetylated sample was determined by enzyme-immunoassay (cAMP Biotrak kit) following the manufacturer's instructions and evaluated using a multi-well plate reader (Titertek Multiskon® PLUS MKII, Titertek, Huntsville, AL, USA).

PDE activity, northern and western blots

Confluent HUVEC at passage 3 in 75 cm^2 flasks were incubated for 8 h with rolipram (50 μM), db-cAMP (100 μM), rolipram (50 μM) + cycloheximide (100 μM), or vehicle (controls) in culture medium. Cells were washed twice with phosphate-buffered saline (in mM: 0.9 $CaCl_2$, 0.5 $MgCl_2$, 137 NaCl, 2.6 KCl, 10 Na_2HPO_4, 1.7 KH_2PO_4, 5.0 glucose, pH 7.3) and then harvested by scraping. Cell suspensions were centrifuged (170 *g*, 5 min) and pellets were stored at −80 °C.

To assay PDE activity, pellets were homogenized in buffer (in mM: 250 saccharose, 5 EGTA, 2 Mg acetate, 20 Tris, 1 dithiothreitol, 0.33 Pefabloc, 2.10^6 IU l^{-1} aprotinin, 10 mg l^{-1} soybean trypsin inhibitor, 10 mg l^{-1} leupeptin, 0.2 g l^{-1} BSA; pH 7.5) with an Ultra-Turrax homogenizer (3×10 s at 4 °C) and stored at −80 °C. Protein concentration was evaluated (Lowry *et al*., 1951). PDE activity was determined by radioenzymatic assay using [^{3}H]-cAMP (Keravis *et al*., 1980, 2005). Total cAMP-PDE activity was assessed at 1 μM cAMP in presence of 1 mM EGTA; PDE4 activity (cAMP-PDE sensitive to rolipram) was assessed by including 50 μM rolipram in the assay.

For western blots, pellets were homogenized at 4 °C with a glass–glass Potter homogenizer (2×15 strokes) in buffer (in mM: 250 NaCl, 25 Tris, 5 EDTA, 1 Pefabloc, 2 mg l^{-1} aprotinin, 2 mg l^{-1} leupeptin, 2 mg l^{-1} pepstatin A, 1% SDS; pH 7.5). Homogenates were centrifuged (14 000 *g*, 2×10 min, 4 °C), and supernatants were aliquoted and stored at −80 °C. Protein samples (10 μg) were denatured and solubilized for 5 min at 95 °C in Laemmli buffer, electrophoresed on SDS-8% polyacrylamide gel and electrotransferred onto polyvinylidene fluoride membranes (Favot *et al*., 2004). Membranes were immunoblotted with anti-PDE4A (1:2000), anti-PDE4B (1:2000), anti-PDE4C (1:1000) and anti-PDE4D (1:2000) antibodies. Immobilized antigens were detected by chemiluminescence using horse radish peroxidase-conjugates as secondary antibodies (1:60 000), an ECL kit and autoradiography films. The amounts of protein in each lane were checked by re-probing the membranes with an antibody directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping protein (anti-GAPDH; 1/60 000). Autoradiography signals were captured on a GeneGenius Bio Imaging System (Syngene) using the GeneSnap soft wear and analysed using the GeneTool software. Relative values for PDE signals in treated HUVEC

were calculated as a percentage of untreated HUVEC, corrected for the density of GAPDH bands. GAPDH was shown not to be affected by the treatments.

For northern blots, total RNA was extracted from pellets using phenol-chloroform-guanidium thiocyanate (Chomczynski and Sacchi, 1987), quantified at 260 nm and stored at −80 °C with two volumes of formamide as 20 μg aliquots. RNA was size-fractionated by agarose-formaldehyde gel electrophoresis (Lehrach *et al*., 1977), transferred to nylon membranes by upward capillary transfer and immobilized by UV crosslinking. Membranes were hybridized at 68 °C in Denhardt's reagent using cDNA probes for PDE4A, PDE4B, PDE4C and PDE4D radiolabeled with [α-^{32}P]-dCTP by random priming (Prime-It II kit) and then washed twice in 20 × saline Na citrate (SSC) in presence of 0.1% SDS at 20 °C for 2 × 10 min (20 × SSC contained 3 M NaCl, 0.3 M Na citrate, pH 7.0). Membranes were subjected to increasing stringencies for 2 × 15 min (0.2 × SSC at 20 °C and 42 °C, 0.1 × SSC at 42 °C) followed by autoradiography (Hyperfilms MP). RNA in each lane was quantified by monitoring 18S and 28S ribosomal RNA on ethidium bromide-stained agarose gels.

Data presentation and statistical analysis

Ca^{2+} data are expressed as mean ± s.e.mean of at least 18 individual cells. The area under the Ca^{2+} curve obtained from individual cells was determined by the trapezoid rule (Prism 2 software; Graphpad): the curve is divided into series of trapezoids, and then the area of each trapezoid is calculated individually (Burden and Faires, 2005). cAMP data are expressed as % of basal cAMP value (39.4 ± 7.2 pmol 10^{-6} cells) and are mean ± s.e.mean of three independent experiments. PDE activities are expressed in pmol min^{-1} mg^{-1} as mean ± s.d. of two independent experiments. Northern and western blots were evaluated using a GeneGenius Bio Imaging System (Syngene) and GeneSnap and GeneTools software. Relative values for western blot PDE signals in treated HUVEC were calculated as % of untreated HUVEC, corrected for GAPDH density. Results are expressed as mean ± s.e.mean of three independent experiments.

Student's 2-tailed *t*-test for unpaired data or one-way ANOVA (followed by a Bonferroni's *post hoc* test when appropriate) were used for statistical analysis, with $P<0.05$ being considered significant.

Drugs and chemicals

[^{3}H]-cAMP (34 Ci $mmol^{-1}$), [α-^{32}P]-dCTP (3000 Ci $mmol^{-1}$), dodecyltrimethylammonium bromide, ECL kits, Hybond-P polyvinylidene fluoride membrane, Hyperfilms MP and Kodak Biomax films were from Amersham, Little Chalfont, England. Aprotinin, leupeptin, pepstatin and 4-(2-aminoethyl)-benzenesulphonyl fluoride HCl (Pefabloc) were from Interchim. Agarose, L-glutamine, medium 199 and RPMI 1640 were from Gibco, Cergy Pontoise, France. Endotoxin-free human serum was from 'Etablissement de Transfusion Sanguine' (Strasbourg, France; the serum was obtained from a pool of 13–15 healthy donors negative for hepatitis B virus and HIV and it was complement-inactivated at 56 °C for 30 min). Collagenase B was from Roche Diagnostics, Meylan, France. Horse radish peroxidase-conjugated secondary antibodies were from Promega, Charbonnières-les-Bains, France. Anti-GAPDH and anti-PDE4C antibodies were respectively from FabGennix, Frisco, Texas, USA. Acrylamide/bis-acrylamide (29:1 mix ratio; 30% solution), amphotericin B, ATP, BSA, chloroform, cycloheximide, db-cAMP, forskolin, histamine, penicillin, poly-L-lysine hydrobromide and streptomycin were from Sigma, l'Isle d'Abeau Chenes, Saint Quentin Fallavier, France. Prime-It II kit was from Stratagene, Amsterdam, The Netherlands. Denhardt's reagent, formaldehyde, guanidium thiocyanate, Tris, NaCl, nylon membrane, phenol and SDS were from Q.BIOgene. All other reagents were analytical grade (Merck, Darmstadt, Germany).

Rolipram and human α-thrombin were gifts from Schering AG (Berlin, Germany) and Dr JM Freyssinet (INSERM U143, Strasbourg), respectively. Anti-PDE4A (AC55), anti-PDE4B (K118) and anti-PDE4D (M3S1) (Jin *et al*., 1998) antibodies were gifts of Dr Marco Conti (Stanford University). Rat PDE4-cDNAs were also obtained from Dr Conti: testis 1.0 kb PDE4A-cDNA, Sertoli cell 2.4 kb PDE4B-cDNA, testis 2.3 kb PDE4C-cDNA and Sertoli cell 1.9 kb PDE4D-cDNA (Swinnen *et al*., 1989).

All drugs used were dissolved in water, except for rolipram, forskolin and cycloheximide, which were dissolved in DMSO (final concentration ⩽0.2%). Rolipram was used also to assess PDE4 activity and DMSO was present in the assay at a final concentration of 1%. Controls were treated with solvent alone.

Results

Effects of rolipram and db-cAMP on agonist-induced $[Ca^{2+}]_i$ rises

In normal external solution (containing 2 mM Ca^{2+}), the basal $[Ca^{2+}]_i$ in control HUVEC was 86. 6 ± 0.5 nM ($n=84$) and was unchanged over the experimental time course. After 8 h pre-treatment with rolipram (50 μM) or db-cAMP (100 μM), basal $[Ca^{2+}]_i$ was unchanged.

In control cells, local applications of agonist (histamine, ATP, thrombin) for 60 s, caused rapid increases in $[Ca^{2+}]_i$ accompanied by sustained plateau-like elevations of varying duration (Figure 1). Return to near basal levels occurred within 120–140 s for histamine (Figure 1a) and ATP (Figure 1b), and within 180–200 s for thrombin (Figure 1c). The percentage of responsive cells in a given culture did not vary significantly and was 65–70% for ATP and 90–95% for histamine and thrombin. After 8 h pre-treatment with 50 μM rolipram, $[Ca^{2+}]_i$ increases induced by the same agonists were significantly potentiated (Figure 1, left panels). In a similar fashion, agonist-induced increases in $[Ca^{2+}]_i$ were significantly potentiated after 8 h pre-treatment with 100 μM db-cAMP (Figure 1, right panels). Note that the amplitudes of both the initial peak and the plateau phase were increased. The potentiation of histamine-induced $[Ca^{2+}]_i$ responses caused by 8 h pre-treatment with rolipram and db-cAMP was abolished when 100 μM cycloheximide was included during the pre-treatment (Table 1).

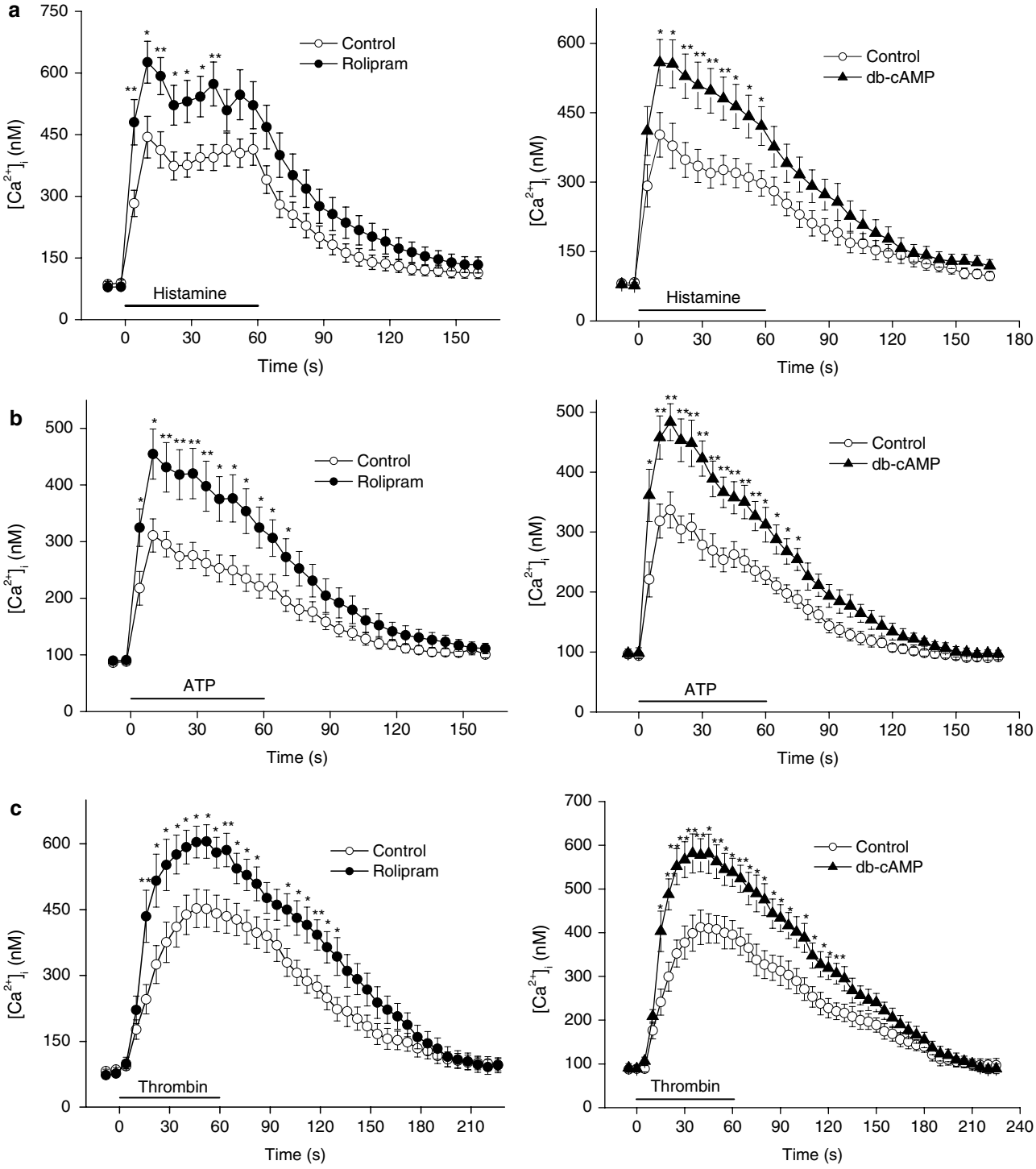

Figure 1 Effects of 8 h pre-treatment of HUVEC with 50 μM rolipram (left panels) or 100 μM db-cAMP (right panels) on $[Ca^{2+}]_i$ increases induced by local applications of 10 μM histamine (**a**), 10 μM ATP (**b**) and 5000 IU l^{-1} thrombin (**c**) in a 2 mM Ca^{2+}-containing external solution. Data are mean ± s.e.mean from at least 19–21 individual cells. The areas under the Ca^{2+} response curves are: (**a**) 26.8 ± 2.8 for control and 44.2 ± 4.5* for rolipram, 25.8 ± 3.4 for control and 38.8 ± 5.0* for db-cAMP (**b**) 15.6 ± 2.4 for control and 26.7 ± 3.6* for rolipram, 16.6 ± 3.0 for control and 29.6 ± 3.3* for db-cAMP; (**c**) 44.1 ± 4.7 for control and 66.9 ± 5.3* for rolipram, 39.5 ± 4.0 for control and 57.7 ± 4.1* for db-cAMP. *$P<0.05$, **$P<0.01$ with respect to control values.

Effects of rolipram and db-cAMP on histamine-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-free solution

In absence of external Ca^{2+}, histamine application evoked a rapid elevation in $[Ca^{2+}]_i$ that returned to basal values within 80–90 s. The initial peak of the histamine-induced response was similar to that observed in the presence of 2 mM Ca^{2+}, whereas the plateau phase was completely suppressed (Figure 2a). Thus, as commonly held, initial $[Ca^{2+}]_i$ responses are largely due to release of Ca^{2+} from intracellular stores while the sustained plateau phase

Table 1 Cycloheximide reverses effects of pre-treatment with rolipram or db-cAMP on histamine-induced $[Ca^{2+}]_i$ increases in HUVEC, bathed in normal Ca^{2+} solution

Control	29.1 ± 3.2
50 μM rolipram	41.5 ± 4.1*
50 μM rolipram + 100 μM cycloheximide	27.8 ± 4.2
Control	28.1 ± 2.3
100 μM db-cAMP	39.5 ± 4.0*
100 μM db-cAMP + 100 μM cycloheximide	29.5 ± 3.3

Data represent the area under Ca^{2+} response curves from individual cells (mean ± s.e.mean from ⩾16 cells). *$P<0.05$ with respect to control.
Pre-treatment with rolipram or db-cAMP, with or without cycloheximide, was for 8 h. The cells were then washed and stimulated with histamine (10 μM).

requires Ca^{2+} entry (Bregestovski *et al*., 1988; Duchêne and Takeda, 1997). The potentiation of the histamine-induced Ca^{2+} response after 8 h pre-treatment with rolipram (Figure 2b) or db-cAMP (Figure 2c) was preserved in the absence of external Ca^{2+}.

Effects of rolipram and db-cAMP on intracellular cAMP levels

The basal cAMP level measured immediately in HUVEC was 39.4 ± 7.2 pmol 10^{-6} cells ($n=3$) and was unchanged after 8 h. As shown in Figure 3, a 2 min or 8 h pre-treatment with 100 μM cycloheximide caused no significant changes in basal cAMP levels. A 2 min pre-incubation of HUVEC with 50 μM rolipram led to a 84% increase in cAMP, that was not affected by cycloheximide (Figure 3a). In contrast, an 8 h pre-treatment with rolipram provoked a significant decrease (62%) of cAMP levels and the presence of cycloheximide was able to restore the basal level (Figure 3a). On the other hand, both 2 min and 8 h pre-treatments with 100 μM db-cAMP increased cAMP levels (209 and 188%, respectively) and cycloheximide was without any significant effect (Figure 3b).

Effects of rolipram and db-cAMP on cAMP-PDE activity

PDE activity was mainly directed towards cAMP in control HUVEC, with cAMP-PDE activity being seven-fold higher than that of cGMP-PDE (respectively, 66.8 ± 1.5 vs 10.0 ± 0.5 pmol min^{-1} mg^{-1}). An 8 h pre-treatment of HUVEC with 50 μM rolipram or 100 μM db-cAMP increased total cAMP-PDE activity in cell extracts and the effect of rolipram was abolished by cycloheximide (Figure 4a). The PDE4 activity of control extracts (27.6 ± 2.9 pmol min^{-1} mg^{-1}) represents 41% of total cAMP-PDE activity (66.8 ± 1.4 pmol min^{-1} mg^{-1}). An 8 h pre-treatment of HUVEC with 50 μM rolipram or 100 μM db-cAMP increased the PDE4 activity of extracts and cycloheximide blocked the effect of rolipram (Figure 4b).

After rolipram pre-treatment, the increase in PDE4 activity (+134%; Figure 4b) was two-fold higher than the increase in total cAMP-PDE activity (+62%; Figure 4a), indicating that an increase in PDE4 activity was mainly involved. After db-cAMP pre-treatment, the total cAMP-PDE and PDE4 activities were increased similarly (+54%; Figures 4a and b). Cycloheximide suppressed the increase in PDE4 activity due to rolipram (Figure 4b), suggesting that this increase could be due to increased synthesis of PDE4. Thus, we investigated the effects of these pre-treatments on PDE4 expression.

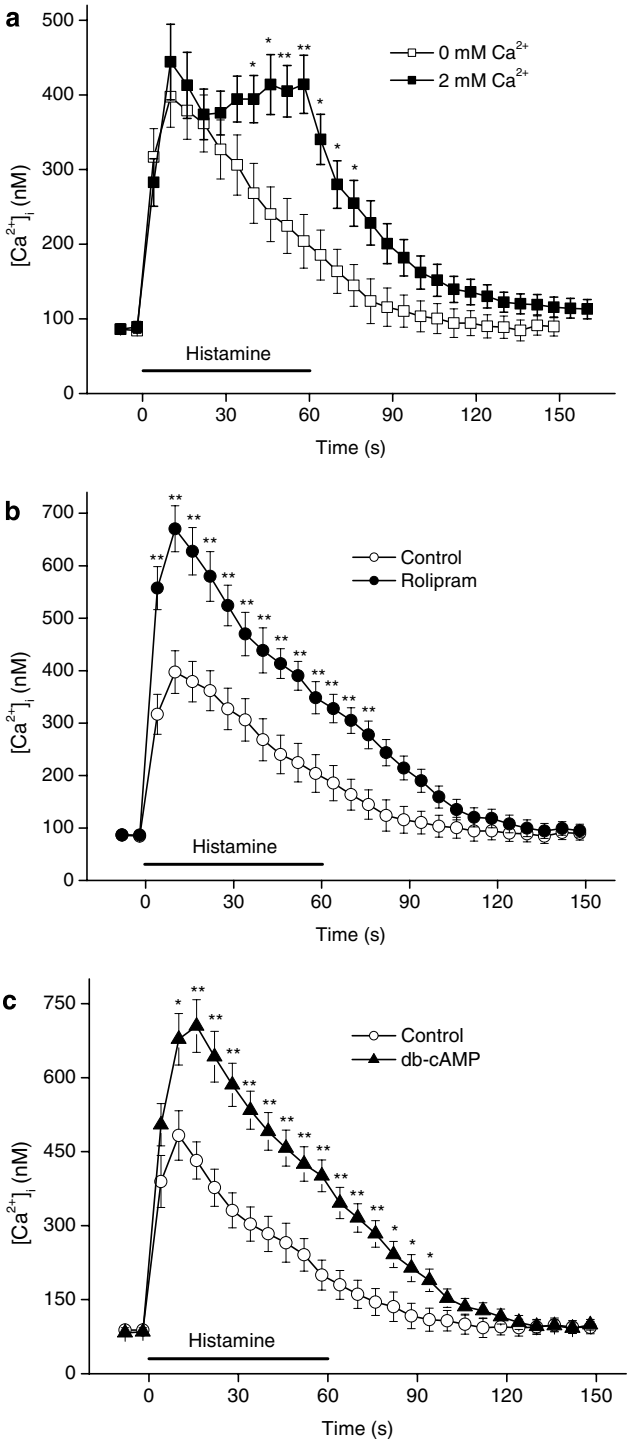

Figure 2 (**a**) $[Ca^{2+}]_i$ increases produced by local application of 10 μM histamine in Ca^{2+}-free external solution compared with 2 mM Ca^{2+}-containing external solution. Data are mean ± s.e.mean from at least 18 individual cells. *$P<0.05$, **$P<0.01$ with respect to values in absence of external Ca^{2+}. An 8 h pre-treatment with 50 μM rolipram (**b**) and 100 μM db-cAMP (**c**) potentiates histamine-stimulated $[Ca^{2+}]_i$ increases in Ca^{2+}-free external solution. Data are mean ± s.e.mean from at least 18 individual cells. The areas under the Ca^{2+} response curves are: (**b**) 21.4 ± 3.6 for control and 40.3 ± 3.4** for rolipram; (**c**) 25.8 ± 3.6 for control and 40.0 ± 3.9** for db-cAMP. *$P<0.05$, **$P<0.01$ with respect to control values.

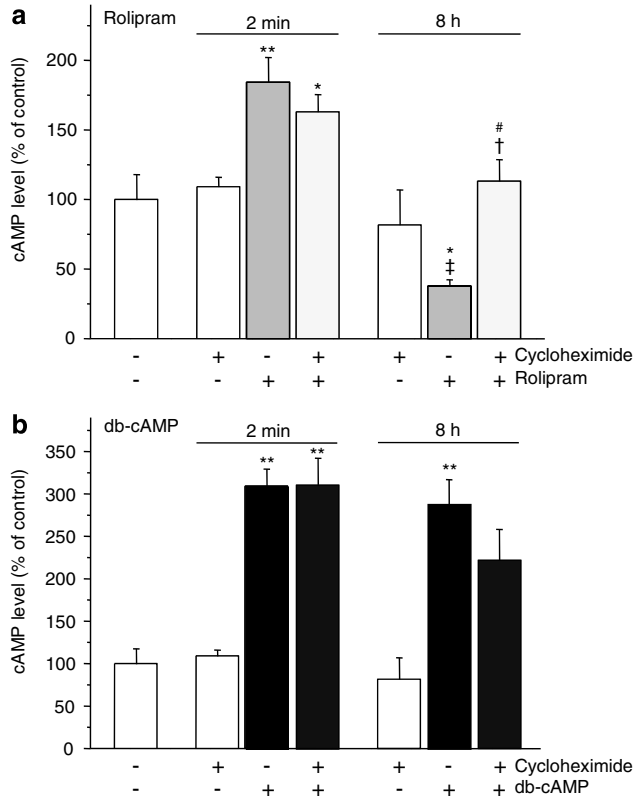

Figure 3 Intracellular cAMP levels. HUVEC were pre-treated for 2 min or 8 h with 50 μM rolipram (**a**) or 100 μM db-cAMP (**b**), in presence or absence of 100 μM cycloheximide. Data are mean ± s.e.mean of three independent experiments. Results are expressed as % of basal cAMP (39.4 ± 7.2 pmol 10^{-6} cells). $^{*}P<0.05$, $^{**}P<0.01$ with respect to basal value. $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$ with respect to the value after 2 min of incubation with the same agent. $^{\#}P<0.05$ with respect to the value after 8 h of incubation with rolipram in the absence of cycloheximide.

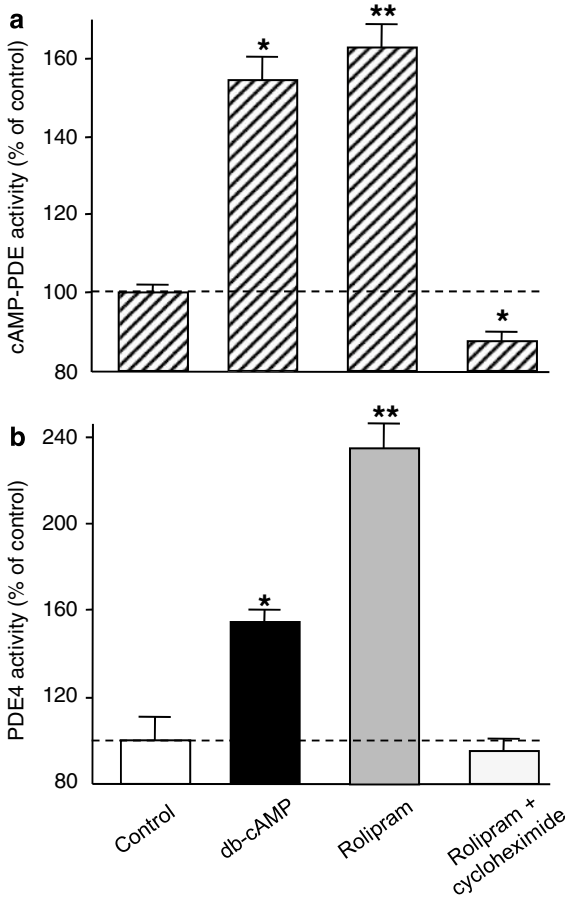

Figure 4 Effects of rolipram and db-cAMP pre-treatment (8 h) on cAMP-PDE activity, in presence or absence of 100 μM cycloheximide. Total cAMP-PDE activity (**a**) and PDE4 activity (**b**) were assessed as described in Methods. Data are mean ± s.d. of two independent experiments and are expressed as % of control values of total cAMP-PDE activity (66.8 ± 1.4 pmol min^{-1} mg^{-1}) and PDE4 activity (27.6 ± 2.9 pmol min^{-1} mg^{-1}), respectively.

Effects of rolipram and db-cAMP on PDE4 isozyme expression

The PDE4 isozyme family comprises four subtypes: PDE4A, PDE4B, PDE4C and PDE4D. Western blot analysis was performed to investigate whether a specific isozyme was responsible for the increased PDE4 activity. The PDE4 isozyme expression was as follows: a single signal of 74 kDa for PDE4A; three signals of 102, 89 and 73 kDa respectively in a ratio of 1, 1.5 and 0.9 for PDE4B; a single signal of 74 kDa for PDE4C; a single signal of 64 kDa for PDE4D.

On western blots, PDE4A-protein was expressed as a single band at 74 kDa (Figure 5a). The amounts of PDE4A were significantly increased by pre-treatment with db-cAMP and rolipram, with cycloheximide reversing the rolipram-induced increase. Northern blot analysis (Figure 5b) shows that PDE4A-mRNA was expressed as a 3.7 kb signal which was increased by pre-treatments with db-cAMP (+133%) and rolipram (+74%). Cycloheximide abolished the rolipram increase of the PDE4A transcript.

PDE4B-protein was expressed as three bands of 102, 89 and 73 kDa (Figures 6a–c). Pre-treatments with db-cAMP and rolipram induced no change in the 102 and 89 kDa bands. However, the 73 kDa band was significantly increased by db-cAMP and rolipram with the latter increase being abolished by cycloheximide. Northern blot analysis shows that PDE4B-mRNA was expressed as a 3.7 kb signal which was increased by db-cAMP (+95%) and rolipram (+24%); cycloheximide abolished the rolipram-induced increase of the PDE4B transcript (Figure 6d).

PDE4C was expressed as a 74 kDa protein and a 3.7 kb transcript. The amounts of protein and mRNA were not significantly modified by db-cAMP or rolipram pre-treatments (Figure 7).

PDE4D-protein (Figure 8a) was expressed as a single band at 64 kDa, which was significantly increased by db-cAMP and rolipram pre-treatment. In this case, including cycloheximide together with rolipram during pre-treatment still resulted in increased PDE4D expression (+27%). Northern blot analysis (Figure 8b) shows that the 3.9 kb PDE4D-mRNA was increased by db-cAMP (+58%), rolipram (+32%) and rolipram + cycloheximide (+25%) pretreatment. Again, cycloheximide did not reverse the rolipram-induced increase of the 3.9 kb PDE4D-transcript. A 7.8 kb transcript for PDE4D was also detected and was not modified by either db-cAMP or rolipram. However, cycloheximide + rolipram increased the expression of this transcript by 139% (Figure 8b).

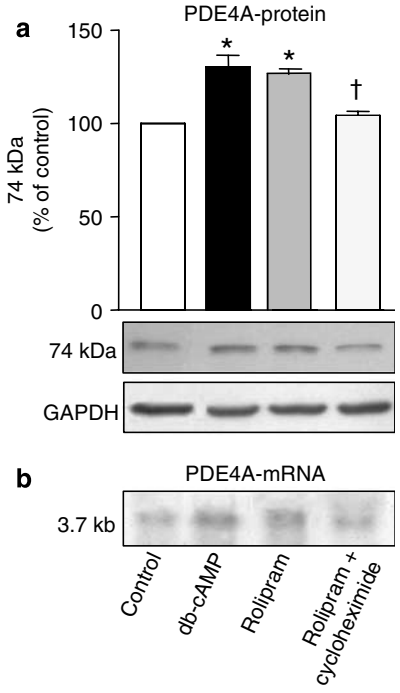

Figure 5 Effects of rolipram and db-cAMP pre-treatment (8 h) on expression of PDE4A. (**a**) Amounts of PDE4A protein were determined by western blot using the AC55 anti-PDE4A antibody, as illustrated by a representative gel, followed by densitometric analysis. Results are expressed as % of control values, with histograms representing mean ± s.e.mean of three independent experiments. *$P<0.05$, compared with control; $^{\dagger}P<0.05$, compared with rolipram. (**b**) PDE4A-mRNA expression was revealed by northern blot with a 1.0 kb PDE4A-cDNA.

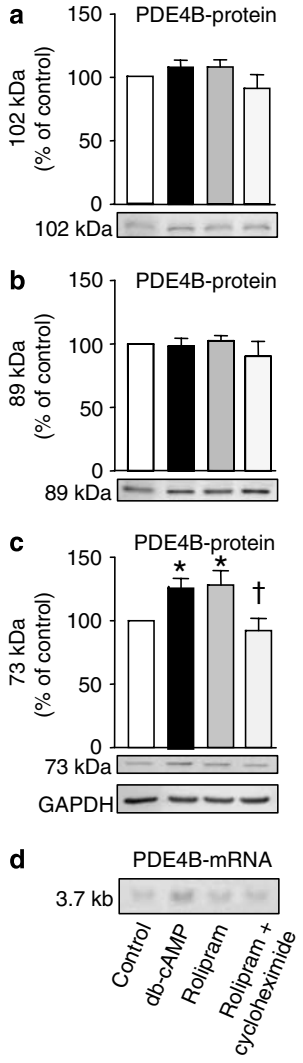

Figure 6 Effects of rolipram and db-cAMP pre-treatment (8 h) on expression of PDE4B. (**a–c**) Amounts of PDE4B-protein were determined by western blot using the K118 anti-PDE4B antibody, as illustrated by representative gels, followed by densitometric analysis. Results are expressed as % of control values, with histograms representing mean ± s.e.mean of three independent experiments. *$P<0.05$, compared with control; $^{\dagger}P<0.05$, compared with rolipram. (**d**) PDE4B-mRNA expression was revealed by northern blot with the 2.4 kb PDE4B-cDNA.

Discussion

In the present study, we demonstrate that 8 h pre-treatment of HUVEC with rolipram (a specific PDE4 inhibitor) or db-cAMP (a permeant non-hydrolyzable cAMP-analogue) significantly increased agonist-induced $[Ca^{2+}]_i$ rises. Including cycloheximide during pre-treatment blocked the potentiation of histamine-induced $[Ca^{2+}]_i$ increases, indicating that protein synthesis was necessarily involved. The results presented here clearly contrast with our previous report on the effects of short term (2 min) pre-treatment of HUVEC with the same agents, which resulted in a significant decrease of agonist-induced $[Ca^{2+}]_i$ rises (Campos-Toimil *et al*., 2000).

We found that the effects of rolipram on intracellular cAMP levels in HUVEC also differ depending on the time of pre-treatment. cAMP levels were significantly increased by 2 min pre-treatment with rolipram, in good agreement with results obtained using IBMX, a non-selective PDE inhibitor (Hopkins and Gorman, 1981) and RP73401, a selective PDE4 inhibitor (Keravis *et al*., 2007). This indicates that HUVEC have significant basal AC activity. In contrast, 8 h pre-treatment with rolipram surprisingly and significantly decreased cAMP levels. This last effect was abolished in the presence of cycloheximide, consistent with *de novo* PDE4 protein synthesis being involved in the rolipram-induced decrease in cAMP. As expected, a 2 min pre-treatment with db-cAMP significantly increased cAMP level. After 8 h pre-treatment of HUVEC with db-cAMP, cAMP levels were also markedly increased, indicating that the non-hydrolyzable db-cAMP is recognized as cAMP by enzyme-immunoassay, as reported previously (Hosokawa *et al*., 2001). Although db-cAMP is able to stimulate PDE4 expression, as it is not hydrolysed, its effect on intracellular cAMP level was not time-dependent. Therefore, the presence of cycloheximide did not modify the increased cAMP level.

Among the four PDE4 subtypes (PDE4A, PDE4B, PDE4C and PDE4D) present in HUVEC, the expression of 74 kDa-PDE4A and 73 kDa-PDE4B was clearly increased after sustained inhibition of PDE4 activity by 8 h pre-treatment with rolipram and these increases were reversed by cycloheximide. The effect of rolipram on PDE4 expression was

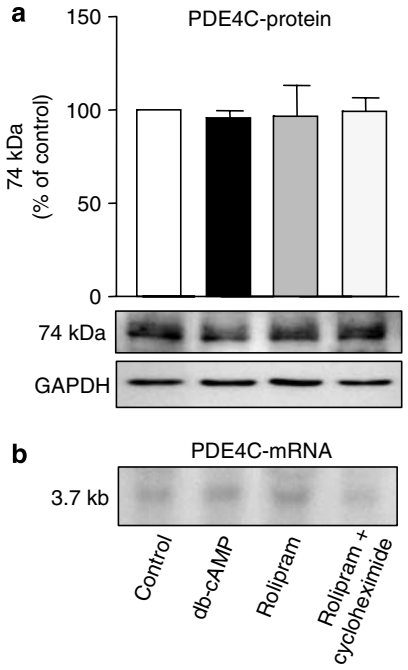

Figure 7 Effects of rolipram and db-cAMP pre-treatment (8 h) on expression of PDE4C. (**a**) Amounts of PDE4C-protein were determined by western blot using the FabGennix anti-PDE4C antibody, as illustrated by a representative gel, followed by densitometric analysis. Results are expressed as % of control, with histograms representing mean ± s.e.mean of three independent experiments. (**b**) PDE4C-mRNA expression was revealed by northern blot with the 2.3 kb PDE4C-cDNA.

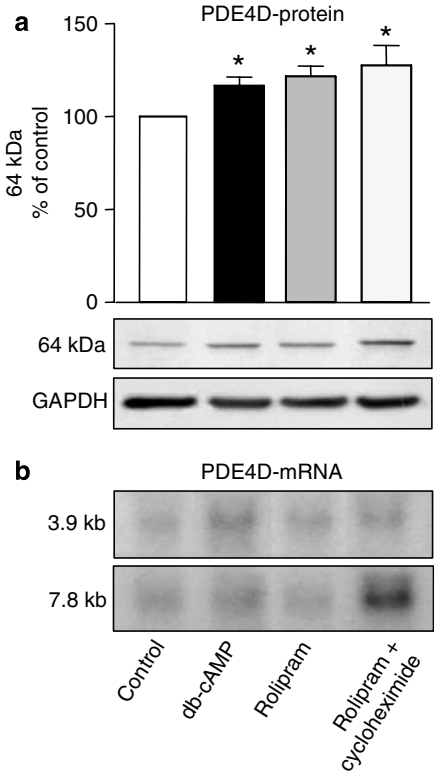

Figure 8 Effects of rolipram and db-cAMP pre-treatment (8 h) on expression of PDE4D. (**a**) Amounts of PDE4D-protein were revealed by western blot using the M3S1 anti-PDE4D antibody. Results are expressed as % of control values (mean ± s.e.mean of three independent experiments). *$P<0.05$, compared with control. (**b**) PDE4D-mRNA expression was revealed by northern blot with the 1.9 kb PDE4D-cDNA.

mimicked by db-cAMP pre-treatment. The increased PDE4A and PDE4B expression is likely to be mediated by the sustained elevation in cAMP, as previously described for PDE4D in smooth muscle cells (Rose *et al*., 1997; Maurice, 1998; Tilley and Maurice, 2002). Similarly, PDE4A and PDE4B were also induced in rat pulmonary microvascular endothelial cells, following long-term treatment with forskolin and rolipram, and this induction was modulated by intracellular cAMP content (Zhu *et al*., 2004).

Here, the increased expression of PDE4A and PDE4B after sustained pre-treatment was accompanied by an increase in total cAMP-PDE activity, due largely to an increase in PDE4 activity. However, rolipram pre-treatment induced a significantly greater contribution of PDE4 to the increase in total cAMP-PDE activity compared with db-cAMP. This may arise from a possible localized regulation of PDE4 as well as a non-specific effect of db-cAMP on PDE activity.

Interestingly, the expression of 64 kDa-PDE4D was regulated differently compared with the other PDE4 isozymes. As 64 kDa-PDE4D expression was increased by rolipram and db-cAMP pre-treatment, cycloheximide did not reverse the effect of rolipram. This suggests that PDE4D might be down-regulated by a repressor such as the inducible cAMP early repressor (Lamas and Sassone-Corsi, 1997) and consequently, differently involved in PDE activity and resulting cAMP levels. Indeed, it was shown that PDE4D is localized in the perinuclear region (Jin *et al*., 1998) and is associated with the nuclear envelope (Lugnier *et al*., 1999). This specific

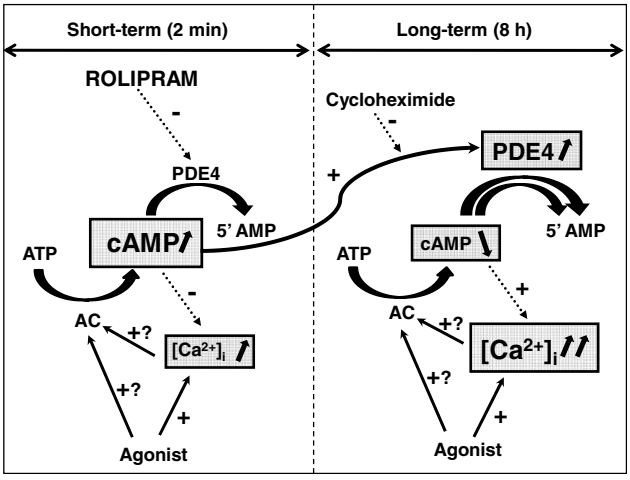

Figure 9 Proposed scheme accounting for the short- and long-term effects of rolipram pre-treatment in HUVEC. Agonists induce rises in $[Ca^{2+}]_i$ and may stimulate AC activity. After 2 min pre-treatment with cAMP-elevating agents (rolipram, db-cAMP), enhanced local increases of cAMP reduce agonist-stimulated $[Ca^{2+}]_i$ signals by inhibiting Ca^{2+} mobilization from internal stores. A sustained elevation of cAMP after 8 h pre-treatment with cAMP-elevating agents increases the expression and activity of PDE4 subtypes, which would speed up cAMP degradation. Induced synthesis of PDE4 is blocked by cycloheximide. In these conditions, cAMP levels are low and agonist-induced $[Ca^{2+}]_i$ rises are no longer inhibited, but rather Ca^{2+} responses are potentiated.

localization of the PDE4D subtype may regulate cAMP dependent transcription factors in the vicinity of the nuclear envelope.

Taken together, our results indicate that a sustained elevation of intracellular cAMP after long term pre-treatment with rolipram or db-cAMP leads to increased expression and activity of PDE4, probably as a compensatory mechanism to accelerate cAMP degradation. We propose that this effect explains, at least in part, the differences in agonist-induced Ca^{2+} responses observed after short (Campos-Toimil *et al*., 2000) and long term pre-treatments with cAMP-elevating agents.

It is generally accepted that agonists like histamine, ATP and thrombin induce rises in $[Ca^{2+}]_i$ by activation of specific G-protein-coupled receptors, inositol 1,4,5-trisphosphate-(IP_3) mediated release of Ca^{2+} from internal stores and Ca^{2+} influx via several types of Ca^{2+}-permeable channels, including store-operated Ca^{2+} channels responsible for capacitative Ca^{2+} entry and agonist-activated nonselective cationic channels (Bregestovski *et al*., 1988; Carter *et al*., 1988; Hallam *et al*., 1988; von der Weid *et al*., 1993; Duchêne and Takeda, 1997; Nilius and Droogmans, 2001; Tiruppathi *et al*., 2006). In agreement, our results indicate that the initial transient histamine-induced elevation in $[Ca^{2+}]_i$ is due to mobilization of Ca^{2+} from intracellular stores, while the sustained plateau requires Ca^{2+} influx, as previously described in HUVEC (Oike *et al*., 1994; Lantoine *et al*., 1998; Campos-Toimil *et al*., 2000).

The interaction of cAMP with Ca^{2+} signals in HUVEC appears to occur during the mobilization of intracellular Ca^{2+} as the potentiation of histamine-induced rises in $[Ca^{2+}]_i$ after 8 h pre-treatment with rolipram or db-cAMP was also observed in the absence of extracellular Ca^{2+}. Moreover, in our previous short-term pre-treatment study (Campos-Toimil *et al*., 2000), reduction of agonist-induced rises in $[Ca^{2+}]_i$ by cAMP-elevating agents was mainly due to inhibition of Ca^{2+} mobilization from internal stores, suggesting that changes in cAMP levels may directly regulate release of intracellular Ca^{2+}. Finally, despite the existence of contradictory data, most studies indicate that elevated cAMP inhibits phosphoinositide hydrolysis and IP_3 generation in a variety of cell types (Socorro *et al*., 1990; Keularts *et al*., 2000; for review, see Bruce *et al*., 2003). Furthermore, inhibitors of cAMP-PDE decrease IP_3 generation in smooth muscle (Challiss *et al*., 1998) and this may involve specific PDE4 isozymes at the plasma membrane, as shown by cytochemistry (Okruhlicova *et al*., 1996). It is important to note that PDE4 isozymes may have different subcellular localizations (Jin *et al*., 1998; Lugnier, 2006; Houslay *et al*., 2007), thereby resulting in local regulation of discrete cAMP pools by specific PDE4 isozymes. The interaction between elevated cAMP and Ca^{2+} release could also occur by modulation of IP_3 receptors, as cAMP alters the characteristics of these receptors via PKA-dependent phosphorylation (Bugrim, 1999). This latter effect might involve a PDE4 isoform associated with the sarcoplasmic reticulum (Lugnier *et al*., 1993).

The reduced release of intracellular Ca^{2+} by elevated cAMP level may indirectly decrease the subsequent entry of Ca^{2+} via store-operated Ca^{2+} channels. This would represent another mechanism for cAMP regulation of endothelial function, as capacitative Ca^{2+} entry in HUVEC is crucial in controlling endothelial permeability and the release of endothelial factors, including NO (Lin *et al*., 2000; Nilius and Droogmans, 2001; Tiruppathi *et al*., 2006). It was also recently shown that an increase in Ca^{2+} caused relocalization of PDE4A1 from trans-Golgi to punctate structures, which would allow further possible crosstalk between cAMP and Ca^{2+} signalling pathways (Huston *et al*., 2006).

In view of the above considerations, our results and the derived mechanistic hypotheses are summarized in Figure 9. A brief elevation of cAMP due to short-term (2 min) inhibition of PDE4 with rolipram or directly due to addition of db-cAMP reduces agonist-induced Ca^{2+} rises, by decreasing Ca^{2+} release from internal stores, although Ca^{2+} influx may also be altered. This agrees with a decrease in histamine-induced Ca^{2+} signals by cAMP-elevating agents described in HUVEC (Bolz and Pohl, 1997), even if others found no significant effects of such agents on agonist-evoked rises in $[Ca^{2+}]_i$ (Carson *et al*., 1989; Vischer and Wollheim, 1998). A sustained elevation of cAMP induced by 8 h pre-treatment with rolipram leads to increased expression and activity of PDE4, resulting finally in a marked decrease of cAMP. This might result in an increase of 5′-AMP which would participate in activation of AMP-kinase (see McGee and Hargreaves, 2008). In these conditions, agonist-induced increases in $[Ca^{2+}]_i$ are potentiated as the inhibitory effect of cAMP, no longer exists. On the other hand, if a PDE4 inhibitor was permanently present, specific inhibition of the induced PDE4 isozymes might induce a decrease of $[Ca^{2+}]_i$.

In conclusion, we show for the first time that long-term pre-treatment with cAMP-elevating agents significantly increases agonist-induced $[Ca^{2+}]_i$ rises in HUVEC, probably via enhanced Ca^{2+} mobilization from intracellular stores. We suggest that this effect involves cAMP-dependent upregulation of PDE4 expression, in particular PDE4A and PDE4B. Consequently, PDE4 isozymes may represent useful targets for therapeutic intervention. The development of isozyme-specific PDE4 inhibitors could be helpful as selective therapeutic agents in vascular diseases where $[Ca^{2+}]_i$ handling is altered, such as atherosclerosis and hypertension. Finally, an increase in PDE4 activity may be involved in the development of tachyphylaxis following the administration of the β-adrenoceptor agonists (Mehats *et al*., 1999). Thus, cAMP-dependent upregulation of PDE4A and PDE4B may participate in tolerance to β-adrenoceptor agonists and consequently PDE4 isotype inhibitors could represent a new and promising therapy for tolerance induced by β-adrenoceptor stimulation.

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Conflict of interest

The authors state no conflict of interest.

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