Phosphorylation-mediated Activation and Translocation of the Cyclic AMP-specific Phosphodiesterase PDE4D3 by Cyclic AMP-dependent Protein Kinase and Mitogen-activated Protein Kinases

A POTENTIAL MECHANISM ALLOWING FOR THE COORDINATED REGULATION OF PDE4D ACTIVITY AND TARGETING*

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In this study, we describe a novel mechanism by which a protein kinase C (PKC)-mediated activation of the Raf-extracellular signal-regulated kinase (ERK) cascade regulates the activity and membrane targeting of members of the cyclic AMP-specific phosphodiesterase D family (PDE4D). Using a combination of pharmacological and biochemical approaches, we show that increases in intracellular cAMP cause a protein kinase A-mediated phosphorylation and activation of the two PDE4D variants expressed in vascular smooth muscle cells, namely PDE4D3 and PDE4D5. In addition, we show that stimulation of PKC via the associated activation of the Raf-MEK-ERK cascade results in the phosphorylation and activation of PDE4D3 in these cells. Furthermore, our studies demonstrate that simultaneous activation of both the protein kinase A and PKC-Raf-MEK-ERK pathways allows for a coordinated activation of PDE4D3 and for the translocation of the particulate PDE4D3 to the cytosolic fraction of these cells. These data are presented and discussed in the context of the activation of the Raf-MEK-ERK cascade acting to modulate the activation and subcellular targeting of PDE4D gene products mediated by cAMP.

PDEs form a multigene family, with individual members classified using several criteria including substrate selectivity, inhibitor sensitivity, and molecular sequence (1–5). Individual members of each individual PDE type are encoded by as many as four different genes, each of which can in turn give rise to variants by alternate splicing of mRNA or the use of alternate promoters (2–5).

Recently, significant progress has been made in elucidating some of the mechanisms regulating the activity and expression of some of the PDE. In this regard, one family that has received a significant amount of attention has been the cAMP-specific, Rolipram-inhibited PDE4 (2, 4, 5). In human, rat and mouse, four distinct genes encode PDE4 (PDE4A, PDE4B, PDE4C, and PDE4D), with each, as a result of alternate splicing or the use of alternate promoters, potentially giving rise to multiple enzyme variants. As a result of early work by several laboratories, it has been established that several PDE4 variants can be expressed in individual tissues and that these genes are regulated by transcriptional and/or posttranslational mechanisms (2, 4). Thus, prolonged increases in cAMP in cells can cause marked increases in the expression of certain PDE4 variants and a PDE-mediated desensitization to the effects of activators of adenylyl cyclase (6–16). Although the cAMP-mediated expression of individual PDE4 genes can be cell type-specific, the generality of this observation and the cellular mechanisms that allow for the selectivity are not known. PDE4 variants are also regulated by protein phosphorylation (reviewed in Ref. 5). Thus, a protein kinase A (PKA)-mediated phosphorylation and short-term activation of a specific PDE4 variant, PDE4D3, has been reported (6–8). Although PKA-mediated phosphorylation was shown to selectively activate a subset of PDE4D3 with high affinity for the inhibitor Rolipram, the molecular basis of this selectivity is unknown at the present time. Phosphorylation of PDE4B2 by mitogen-activated protein kinase (MAPK) has also been reported, although this phosphorylation did not alter the enzymes activity or sensitivity to inhibitors (17). More recently, expression of PDE4A and PDE4D gene products in heterologous expression systems has shown that certain variants of PDE4A and PDE4D are targeted to selected membrane fractions (Ref. 18 and reviewed in Ref. 5). The relevance of these findings to the subcellular distribution of PDE4 variants expressed endogenously in nontransformed cells and the impact of selected subcellular expression of these enzymes on their activity and sensitivity to inhibitors have not yet been systematically or rigorously investigated. However, given that targeting of PKA to membrane compartments via specific anchoring proteins (19) has been shown to play a central role on the
function of this protein in cells, a similar impact of selective expression of PDE4 variants may perhaps be anticipated.

Presumably due to the lack of vasorelaxant effects of PDE4 inhibitor (20), very few studies have investigated the PDE4 gene products expressed in vascular tissues and the mechanisms regulating their activity and expression. In a previous study, we demonstrated that prolonged activation of adenylyl cyclase in aortic vascular smooth muscle cells (VSMCs) caused up-regulation of total cAMP PDE activity, with at least 60% of this effect due to increases in PDE4 (15, 16). In these earlier studies, we also demonstrated that the cAMP-mediated increase in PDE4 activity was partially responsible for a cAMP-mediated desensitization to activators of adenylyl cyclase in these cells (15, 16). More recently, we have reported that PDE4 inhibitors were potent regulators of VSMC migration and could potentiate the effects of inhibitors of other cAMP PDEs on VSMCs (21), a result similar to that previously reported for relaxation of blood vessel (22).

In this study, we have investigated the role that protein kinase C (PKC)-mediated activation of the Raf-MEK-ERK cascade plays in regulating the activity and subcellular targeting of the PDE4D variants expressed in VSMCs. Using a combination of pharmacological and biochemical approaches, we have delineated the signaling pathways that allow for the coordinated activation of these PDE4D variants by activators of PKC and PKA. Our data are consistent with a direct role for PKA in activating PDE4D3 and PDE4D5 and a role for PKC, via its effects on the Raf-MEK-ERK cascade, in the activation and translocation of the membrane associated fractions of PDE4D3.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents (Dulbecco's modified Eagle's medium, calf serum, HEPES, penicillin/streptomycin, Hanks' balanced salt solution (HBSS), and trypsin-EDTA) were from Life Technologies, Inc. Radioactive products (5'-[32P]cAMP, [3H]cAMP, and [3H]phosphoric acid) were from NEN Life Science Products. Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol-12,13-didecanoate (4α-PDD), angiotensin II (AngII), rapamycin, LY 294002, SB202190, Ro 20–1724, Ro-31-8220, bisindolylmaleimide I, and PDB0959 were from Calbiochem-Novabiochem Corporation, Ontario, Canada. Insulin was purchased from Amersham Pharmacia Biotech. 

Ionomycin was from Sigma. Leupeptin and pepstatin were from Bio-Rad. ECL Western blot detection kit was purchased from Amersham Pharmacia Biotech. The ECL reagents were from Boehringer Mannheim. Affi-Gel 601 and the column supports were from Bio-Rad. Ionomycin was from Sigma. Leupeptin was from Boehringer Mannheim. Affi-Gel 601 and the column supports were from Bio-Rad.

Cell Culture—Primary cultures of rat aortic VSMCs were established following isolation from rat aortae as described previously and the identity of the VSMCs was confirmed using a-actin staining (15, 16). VSMCs were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 8 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 95% air:5% CO2 humidified atmosphere. Cells were passaged by washing once with HBSS (without CaCl2 or MgCl2) and then incubating with 1% trypsin-EDTA for 5 min to detach cells and resuspended in growth medium. To maintain cell stocks, 75-cm2 flasks were seeded with 10^5 cells and 20 ml of medium per flask. For all experiments, cells were used between passages 5 and 10.

Treatment of Cultured Rat Aortic VSMCs with Pharmacological Agents—In these experiments, 3 x 10^5 cells were seeded in 25-cm2 flasks, and the experiments were initiated when the cells reached confluence (3-4 days). Culture medium was removed and replaced with 5.0 ml of fresh culture medium supplemented with either (i) forskolin (0.1–100 μM), (ii) PMA (0.1–1 μM), (iii) angiotensin II (1 μM), (iv) ionomycin (1–100 μM), or (v) vehicle (0.1% dimethyl sulfoxide (Me2SO)) and incubated for various times. After treatment, cells were washed once with 6 ml of HBSS (with CaCl2 and MgCl2) and harvested in 1 ml of lysis buffer containing 50 mM Tris-Cl (pH 7.4), 5 mM MgCl2, 5 mM benzamidine, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 μM leupeptin, and 1 μM Triton X-100. Cells were removed from the flask by scraping. Cellular debris and unlysed cells were removed by centrifugation at 1000 x g for 5 min at 4 °C. The 1000 x g supernatant was transferred to microtubes and stored at 4 °C until assayed for cAMP PDE activity (see below). For some experiments, cytosolic and particulate fractions were obtained as indicated. In these experiments, VSMCs were lysed as described above, except that Triton X-100 was excluded from the lysis buffer, and the 1000 x g supernatant was subjected to a further centrifugation at 100,000 x g for 1 h at 4 °C.

Assay of cAMP Phosphodiesterase Activity—Cyclic nucleotide phosphodiesterase activity was assayed by a modification of the method of Davis and Daly (23). Reactions were carried out in a total volume of 100 μl containing 5 μM of Tris-Cl (pH 7.4), 0.5 mM of MgCl2, 10 μM of EGTA, and 0.1 nmol of [3H]cAMP (5500–60000 dpm). Following a preincubation period of 2 min at 30 °C, a sample of VSMC homogenate (5 μg of protein) was added, and the reaction was allowed to proceed at 30 °C for 30 min. The reaction was terminated by addition of 50 μl of 0.5 M ice-cold EDTA (pH 7.4). Recovery marker (0.1 μl of 5'-[3H]cAMP, 1800 cpm were added to the reaction mixture. 0.1 μl of NaCl and 0.1 μl of Tris-HCl (pH 8.5) was added to each sample prior to purification of the product of the reaction, 5'-[3H]cAMP. 5'-[3H]cAMP and 5'-[14C]cAMP were recovered by chromatography using a polyacrylamide-borate gel column (Affi-Gel 601, Bio-Rad; bed volume, 1 ml). Samples were applied following prewashing of the columns with 8 ml of HEPES-NaCl buffer. After four additional washes of the columns with 2 ml of HEPES-NaCl and equilibration of the columns with 1 ml of 0.05 mM sodium acetate (pH 4.8), the 5'-[3H]cAMP was eluted with 4 ml of 0.05 mM sodium acetate. The recovered 5'-[3H]cAMP was quantified by liquid scintillation counting, corrected for recovery of 5'-[14C]cAMP, and normalized to the total protein used in the assay, and the total activity was expressed as pmol min⁻¹ mg⁻¹ of protein.

Immunoblotting—Rat aortic VSMC cultures incubated with compounds of interest were homogenized in a buffer consisting of 20 mM Tris-Cl (pH 7.5), 1 mM MgCl2, 0.1 mM EGTA, 5 mM benzamidine, 1 μM/ml apronitin, and 1 μM/ml leupeptin (Buffer A). These samples (5–20 μg of total homogenous protein of or of protein from isolated subcellular fractions) were subjected to SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad), and the specific immunoreactive proteins, the partially purified samples were blocked with 5% nonfat dry milk in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20, and 0.1% SDS. The membranes were incubated for various times. After treatment, cells were washed once with 2 ml of HEPES-NaCl, 0.1% Tween-20 containing 0.5% powdered nonfat milk for 1 h. Blots were incubated with an appropriate dilution of primary antibodies for 1–2 h and rinsed three times with TBST. Rinsed blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for 1 h, then rinsed with TBST, and immunoreactivity was detected by chemiluminescence as per the manufacturer's recommendations (Amersham Pharmacia Biotech).

Metabolic Labeling with [32P]Orthophosphate—Rat aortic VSMCs were seeded in T75 boxes (Corning) and cultured as described above. At confluence, the growth medium was replaced with phosphate-free minimal essential medium containing 20 mM HEPES (pH 7.4) and carrier-free [32P]orthophosphate (0.2 μCi/ml), and cells were incubated for 2 h. During the last 0.5 h of the metabolic labeling, PMA (100 nm), FSK (100 μM), or Me2SO was added. At the end of this treatment, cells were washed twice with HBSS; harvested in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 100 μg of phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate; and homogenized. After centrifugation at 1000 x g for 5 min, supernatants were incubated batch-wise for 30 min with DEAE-Sepharose buffered with 200 mM sodium acetate, pH 6.5. The resin was washed twice with the same buffer, and the adsorbed proteins were eluted with 600 mM sodium acetate, pH 6.5. In order to remove non-specific immunoreactive proteins, the partially purified samples were incubated with 2.5 μg/ml of an irrelevant monoclonal antibody (anti-estase, mouse IgG2b) for 2 h at 4 °C and then with protein G beads (Amersham Pharmacia Biotech) for 30 min and centrifuged (1000 x g for 5 min). The supernatants were further incubated with 2.5 μg/ml of the PDE4D-specific monoclonal antibody (61D10E) for 16 h at 4 °C. Again, precipitation was achieved using protein G beads. The immunoprecipitates were washed five times and resuspended in 1% SDS in phosphate-buffered saline, diluted in SDS-PAGE sample buffer, and separated by 7.5% SDS-PAGE. Proteins were blotted onto an Immobilon membrane, and the radioactive bands were...
TSMs were incubated with forskolin (100 µM), PMA (100 nM), or AngII (100 nM) for 30 min. Following the incubation, cells were rinsed with HBSS, homogenized in lysis buffer containing 1% Triton X-100, and centrifuged at 1000 x g for 10 min. PDE activity was determined as described under “Experimental Procedures,” and cilostamide (1 µM) or Ro, 20–1724 (10 µM) was used to determine PDE3 and PDE4 activities, respectively. Values are mean ± S.E. from four determinations.

| Additions            | cAMP PDE | PDE3 | PDE4 | PDE4Δ |
|----------------------|----------|------|------|-------|
| None                 | 70.6 ± 1.2 | 10.6 ± 1.9 | 45.9 ± 0.5 | 6 |
| Forskolin (1.0 µM)   | 72.9 ± 0.8* | 11.3 ± 1.3 | 47.8 ± 0.9 | 4 |
| Forskolin (10 µM)    | 85.3 ± 2.0* | 14.3 ± 1.1 | 56.9 ± 1.8* | 24 |
| Forskolin (100 µM)   | 90.1 ± 2.9* | 12.9 ± 2.4 | 67.3 ± 2.0* | 47 |
| PMA (1.0 mM)         | 71.3 ± 1.0 | 10.9 ± 1.4 | 46.3 ± 0.9 | 1 |
| PMA (10 mM)          | 74.3 ± 1.5 | 11.3 ± 2.7 | 47.9 ± 1.2 | 4 |
| PMA (100 mM)         | 80.9 ± 1.7* | 9.8 ± 2.0 | 51.5 ± 1.7* | 12 |
| AngII (10 mM)        | 77.2 ± 0.9* | 11.7 ± 1.3 | 49.9 ± 0.8* | 9 |
| AngII (100 mM)       | 83.5 ± 1.3* | 14.9 ± 2.2 | 53.6 ± 1.1* | 17 |
| Forskolin (100 µM) + PMA (100 mM) | 115.0 ± 2.3* | 14.8 ± 2.0 | 74.9 ± 2.1* | 63 |
| Forskolin (100 µM) + AngII (100 mM) | 113.9 ± 2.9* | 13.7 ± 2.8 | 84.9 ± 8.8* | 85 |

* P < 0.05 in comparison to no addition value.

ERK and PKA Regulate PDE4D3 Activity and Targeting

**Table I**

**Incubation of VSMC with forskolin, PMA, and AngII increases PDE4 activity**

Visualized and quantitated with an Instant Imager (Packard Instrument Co.).

**Protein Assays**—Protein was determined using the BCA protein assay system from Pierce, according to the manufacturer’s protocol, with bovine serum albumin as the standard.

**Statistical Analysis**—Numerical data (PDE activities) and densitometric determinations of PDE4 phosphorylation status are presented as mean ± S.E. of at least three independent experiments. Immunoblots that are shown are representative of results obtained in at least three individual experiments. Statistical differences between cAMP PDE activities were determined using the Student’s t test for either paired or unpaired samples, with p < 0.05 considered significant.

**RESULTS**

**Incubation of VSMCs with forskolin, PMA, or Angiotensin II Increases PDE4 Activity**—Incubation of cultured VSMCs with the activator of adenylyl cyclase, forskolin, caused a time- and concentration-dependent increase in cAMP PDE activity (Table I). Similarly, incubation of these cells with PMA or with the vasoactive agent AngII caused time and concentration-dependent increases in cAMP PDE activity in these cells, although the absolute magnitude of the increase brought about with these agents was less marked than was obtained with forskolin (Table I). Coincubation of PMA or AngII with forskolin resulted in an additive increase in cAMP PDE activity when compared with the effects of the individual agents used alone (Table I). The inactive analogues of forskolin, 1,9-dideoxyforskolin, and forskolin (1.0 µM) or Ro, 20–1724 (10 µM) was used to determine PDE3 and PDE4 activities, respectively. Values are mean ± S.E. from four determinations.

Additions | cAMP PDE | PDE3 | PDE4 | PDE4Δ |
----------|----------|------|------|-------|
None      | 70.6 ± 1.2 | 10.6 ± 1.9 | 45.9 ± 0.5 | 6 |
Forskolin (1.0 µM) | 72.9 ± 0.8* | 11.3 ± 1.3 | 47.8 ± 0.9 | 4 |
Forskolin (10 µM) | 85.3 ± 2.0* | 14.3 ± 1.1 | 56.9 ± 1.8* | 24 |
Forskolin (100 µM) | 90.1 ± 2.9* | 12.9 ± 2.4 | 67.3 ± 2.0* | 47 |
PMA (1.0 mM) | 71.3 ± 1.0 | 10.9 ± 1.4 | 46.3 ± 0.9 | 1 |
PMA (10 mM) | 74.3 ± 1.5 | 11.3 ± 2.7 | 47.9 ± 1.2 | 4 |
PMA (100 mM) | 80.9 ± 1.7* | 9.8 ± 2.0 | 51.5 ± 1.7* | 12 |
AngII (10 mM) | 77.2 ± 0.9* | 11.7 ± 1.3 | 49.9 ± 0.8* | 9 |
AngII (100 mM) | 83.5 ± 1.3* | 14.9 ± 2.2 | 53.6 ± 1.1* | 17 |
Forskolin (100 µM) + PMA (100 mM) | 115.0 ± 2.3* | 14.8 ± 2.0 | 74.9 ± 2.1* | 63 |
Forskolin (100 µM) + AngII (100 mM) | 113.9 ± 2.9* | 13.7 ± 2.8 | 84.9 ± 8.8* | 85 |

* P < 0.05 in comparison to no addition value.

immunoreactive species with electrophoretic mobilities by SDS-PAGE characteristic of previously described PDE4D variants (Fig. 1). The smaller more abundant PDE4D4 species migrated with an observed molecular mass of 95 ± 2 kDa by SDS-PAGE, a size similar to that of the human recombinant PDE4D3 (Fig. 1). Based on these characteristics, we identified this protein as PDE4D3. The larger, less abundant PDE4D species migrated with an observed molecular mass of 105 ± 3 kDa (Fig. 1), a mobility consistent with PDE4D5 (18). Because this immunoreactive protein was significantly smaller than the other known PDE4D species, PDE4D4, which has been shown to migrate with an observed molecular mass of approximately 119 kDa (18), we identified the larger VSMC PDE4D variant as PDE4D5 (5, 18). Consistent with this identification, polymerase chain reactions carried out using oligo(dT)18-primed VSMC mRNA as a template and PDE4D3- and PDE4D5-specific oligonucleotide primers allows the amplification of both PDE4D3 and PDE4D5 (not shown). When cells were lysed in the absence of detergent, PDE4D3 was found primarily in the supernatant fraction, with approximately 17% present in the 100,000 x g pellets (Fig. 1). In contrast, PDE4D5 was present in both the supernatant and particulate fractions in roughly equal amounts (Fig. 1). Although the amount of PDE4D5 detected in our experiments was variable, it was never present at more than 15% of the level of PDE4D3. The variability in the levels of PDE4D5 detected in our studies may be related to our observation that the fraction of PDE4D5 expressed in the particulate fraction was not efficiently solubilized with Triton X-100, the detergent used in most of our studies (not shown).

In most previous reports, phosphorylation of PDE4D3 has been studied by quantitating the fraction of PDE4D3 that exhibited a retarded migration by SDS-PAGE, an event that is detected as an upward shift in the electrophoretic mobility of PDE4D3. Using this approach, all of our data were consistent with a PKA-mediated phosphorylation of PDE4D3 in response to incubation of VSMCs with forskolin. Thus, incubation of VSMCs with forskolin or with a membrane-permeant analogue of cAMP, 8-Br-cAMP, caused a time- and concentration-dependent shift in the electrophoretic mobility of PDE4D3 in these cells (Figs. 2 and 3), an effect that was maximal following treatment of cells with 100 µM forskolin for 20 min. Incubation of VSMCs with forskolin also caused a similar shift in the electrophoretic mobility of the PDE4D5 expressed in these cells; however, owing to the low abundance of this protein in VSMCs, and its limited extraction from the particulate fraction with Triton X-100, this effect was more subtle and only detected in some experiments (for example, see Fig. 4).
In onset and saturated after only 20 min, the PMA-induced course of the PMA-induced shift in PDE4D3 migration was qualitatively similar to that caused by forskolin, the time Although the shift of PDE4D3 caused by PMA or AngII was dependent shift in the electrophoretic mobility of PDE4D3. 

4)orAngII(notshown)alsoresultedinatime-andconcentration-

imental Procedures”). VSMC lysates were centrifuged at 1000 

10, 20, and 30 min. At the end of the incubation period, VSMCs were washed with HBSS and lysed in ice-cold lysis buffer (see under “Experimental Procedures.”) VSMC lysates were centrifuged at 1000 

3. Incubation of VSMCs with PMA (Fig. 

FIG.2 . Subcellular localization of VSMC PDE4D3 and PDE4D5. Confluent VSMCs were homogenized and centrifuged at 100,000 × g for 1 h at 4 °C. After centrifugation, equivalent amounts (20 μg) of each the pellet and supernatant fractions were electrophoretically resolved by SDS-PAGE and blotted to nitrocellulose membranes as described under “Experimental Procedures.” Immunoblots were probed with a PDE4D-selective monoclonal antibody (61D10E), and PDE4D3 and PDE4D5 were visualized by ECL as per the supplier’s recommendation using a horseradish peroxidase-conjugated goat anti-mouse IgG, as described under “Experimental Procedures.”

FIG.1 . Time-dependent, forskolin-induced phosphorylation of VSMC PDE4D3 and PDE4D5. Confluent VSMCs were incubated with fresh culture medium supplemented with forskolin (100 μM) for 0, 2.5, 5, 10, 20, and 30 min. At the end of the incubation period, VSMCs were washed with HBSS and lysed in ice-cold lysis buffer (see under “Experimental Procedures”). VSMC lysates were centrifuged at 1000 × g for 5 min, the supernatant fractions were separated by SDS-PAGE and analyzed by immunoblot with a PDE4D-specific monoclonal antibody (61D10E), and the percentage of PDE4D3 phosphorylated at different times was determined by scanning densitometry of the shifted band relative to the intensity of all of the PDE4D3 present in that lane using Corel Photo-Paint 7.0 software as per the manufacturer’s recommendations.

the 30 experiments in which the effects of forskolin or 8-Br-cAMP were determined was either agent capable of causing a shift of all of the PDE4D3 present in the cell lysates to the slower migrating species. In fact, as determined by densitometry, the maximum amount of PDE4D3 shifted following incubation of VSMCs with 100 μM forskolin for 30 min (maximal conditions) was 82 ± 3%. Incubation of VSMCs with PMA (Fig. 4) or AngII (not shown) also resulted in a time- and concentration-dependent shift in the electrophoretic mobility of PDE4D3. Although the shift of PDE4D3 caused by PMA or AngII was qualitatively similar to that caused by forskolin, the time course of the PMA-induced shift in PDE4D3 migration was very different. Thus, whereas the effects of forskolin were rapid in onset and saturated after only 20 min, the PMA-induced shift in mobility of PDE4D3 occurred more slowly and only reached a maximum after 1–2 h (Fig. 4). Also, incubation of VSMCs with PMA or AngII caused a much smaller fraction of the total PDE4D3 to shift. In the 17 experiments in which this was measured, incubation of VSMCs with a maximal concentration of PMA (100 nM) caused 17 ± 4% of the PDE4D3 to shift to the slower migrating species. Interestingly, co-incubation of VSMCs with forskolin (100 μM) and PMA (100 nM) resulted in a significant increase in the amount of PDE4D3 that was shifted to the more slowly migrating species, relative to the effect of either agent alone (Fig. 4). Indeed, in the 10 experiments in which this was measured, incubation of these cells with both 100 μM forskolin and 100 nM PMA for 30 min caused a shift of almost all of the PDE4D3 (95 ± 4%) to the slower migrating species. In our experiments, incubation of VSMCs with PMA was not seen to cause a change in the electrophoretic mobility of PDE4D5, although a shift in a small percentage of the PDE4D5 present in these cells would most likely have gone undetected in our experiments.

In order to more directly test whether the forskolin- or PMA-mediated shifts in the electrophoretic mobility of PDE4D3 and PDE4D5 were the result of phosphorylation of these proteins and to determine the relationship between this event and the increase in PDE4 activity that results from these treatments, we carried out two separate series of experiments. First, we...
treated lysates isolated from control or drug-treated cells with calf intestinal alkaline phosphatase (CIAP). These experiments were carried out because we reasoned that if the observed shifts in mobility were related to phosphorylation of these proteins, removal of the phosphate should return the more slowly migrating proteins to their original mobility and normalize the activity to unstimulated levels. Our data are entirely consistent with this (Figs. 5 and 6). Thus, CIAP treatment of an aliquot of lysates generated from cells incubated with forskolin, PMA, or Ang II alone or these agents in combination caused a downward movement of all shifted bands present in the isolates. Consistent with a role of the phosphorylation in the increased PDE4 activity, CIAP also reversed the forskolin- or PMA-induced increases in PDE4 activity in the isolates. Thus, treatment of VSMC lysates generated from VSMCs incubated with 100 μM forskolin or with 100 nM PMA with CIAP reduced the drug-mediated increases in PDE4 activities from 139 ± 4% and 118 ± 4% of control values to 104 ± 5% and 97 ± 5%, respectively, of levels obtained when lysates obtained from control untreated cells were incubated with CIAP. Second, we metabolically labeled VSMCs with [32P]orthophosphate and immunoprecipitated PDE4D immunoreactive proteins following incubation of the metabolically labeled cells with forskolin, PMA, or MeSO4, the drug vehicle. Fig. 6 shows results of such an experiment. Thus, although no 32P-containing PDE4D-immunoprecipitated proteins were detected in immunoprecipitates of VSMCs incubated with MeSO4, an approximately 94-kDa radiolabeled anti-PDE4D-immunoprecipitated protein was detected in pellets obtained from lysates of VSMCs incubated with either forskolin or PMA. The electrophoretic mobility of the labeled anti-PDE4D-immunoprecipitated protein was consistent with it being PDE4D3, the major PDE4 present in these cells, and the major protein with a mobility shift following incubation of VSMCs with forskolin, PMA, or Ang II. Similar amounts of PDE4D immunoreactive protein were immunoprecipitated under these conditions (Fig. 6B). The amount of PDE4D3 expressed endogenously in VSMCs (approximately 3–5 ng/mg of protein) made any further analysis of the phosphorylated immunoprecipitated protein impossible.

Activation of PKA and of the Raf-MEK-ERK Cascade in VSMCs Causes PDE4D3 Phosphorylation—Consistent with a role for PKA in the effect of forskolin, incubation of VSMCs with 8-Br-cAMP caused a similar shift in the mobility of PDE4D3 (Fig. 3), whereas addition of the inactive forskolin analogue 1,9-dideoxyforskolin did not result in a retarded electrophoretic mobility for PDE4D3 (not shown). In addition, preincubation of VSMCs with the highly selective PKA inhibitor, H89 (24), completely abolished the forskolin-mediated shift in the mobility of this protein (Fig. 7). In contrast to its antagonism of the effects of forskolin, H89 did not reduce the phosphorylation of PDE4D3 caused by either PMA or AngII (not shown), a result consistent with the involvement of another kinase catalyzing the phosphorylation of PDE4D3 brought about by incubation of cells with these agents. Consistent with a role for PKC in mediating the phosphorylation of PDE4D3 by PMA or AngII, down-regulation of PKC following a 16-h preincubation period with PMA (25) inhibited phosphorylation of PDE4D3 by either PMA (Fig. 4) or AngII (not shown), and incubation of the VSMCs with the inactive phorbol ester, 4aPDD, did not affect the electrophoretic mobility of PDE4D3 (not shown). Also consistent with a role for PKC in these effects, inclusion of either Ro-318220 (not shown) or bisindolylmaleimide I (Fig. 8), two highly selective PKC inhibitors (26, 27), completely blocked the PMA- or AngII-induced phosphorylation of PDE4D3. Consistent with PKC not being involved in the effects of forskolin in these cells, bisindolylmaleimide I had...
Confluent rat aortic VSMCs were incubated with fresh culture medium supplemented with either vehicle (MeSO) or H89 (1–10 μM) for 20 min. After this preincubation period, forskolin (100 μM) was added, and VSMCs were incubated for a further 30 min. Treated VSMCs were washed with HBSS and lysed in ice-cold lysis buffer (see under “Experimental Procedures”). VSMC lysates were centrifuged at 1000 × g for 5 min, and PDE4D in the supernatant fractions was detected by immunoblotting as described in the legend to Fig. 2.

Following this preincubation period, PMA (100 nM), forskolin (100 μM), or both agents were added, and the incubation was allowed to proceed for a further 0.5 or 1 h. At the end of the incubation period, VSMCs were washed with HBSS and lysed in ice-cold lysis buffer, and lysates were centrifuged at 1000 × g for 5 min. Supernatant fractions were separated by SDS-PAGE and analyzed by immunoblot with an antibody that specifically recognizes the activated forms of ERK1 and ERK2 (Anti-ACTIVETM MAPK pAb, Promega), and the activated proteins were visualized as described under “Experimental Procedures.”

Consistent with a central role for this MAPK pathway in mediating the effects of this agent, in all of our experiments, ERK1 and ERK2 activation by PMA or AngII was reduced in the presence of forskolin (Fig. 9), an effect presumably related to PKA regulation of an upstream event, perhaps activation of Raf (25, 28). Inhibitors of other kinase cascades that could potentially be affected by activation of PKC in our cells had no effect on forskolin-mediated phosphorylation of PDE4D3 (Fig. 8). Because several recent reports have shown that activation of PKC by PMA can lead to a PKC-dependent activation of ERK1 and ERK2 (25, 28) and that some of the effects of PMA have been shown to be dependent on activation of these kinases, their involvement in the PMA- and AngII-mediated phosphorylation of PDE4D3 was investigated. Because no specific ERK1/ERK2 inhibitors exist, for these studies, we used a selective inhibitor of MEK (PD98059) (29), the upstream kinase responsible for the activation of ERK1 and ERK2 in cells. Consistent with a central role for this MAPK pathway in mediating the PMA- or AngII-induced phosphorylation of PDE4D3, PD98059 inhibited both the PMA-induced phosphorylation of both ERK1 and ERK2 (Fig. 9) and the phosphorylation of PDE4D3 caused by these agents (Fig. 10). In our studies, PD98059 had no effect on the shift in PDE4D3 induced by forskolin, a result excluding a role for this pathway in mediating the effects of this agent. In all of our experiments, ERK1 and ERK2 activation by PMA or AngII was reduced in the presence of forskolin (Fig. 9), an effect presumably related to PKA regulation of an upstream event, perhaps activation of Raf (25, 28). Inhibitors of other kinase cascades that could have been shown to be dependent on activation of these kinases, their involvement in the PMA- and AngII-mediated phosphorylation of PDE4D3 was investigated.
maximum of 20% of total PDE4D3, whereas treatment with forskolin caused a maximum of 80% of the PDE4D3 to be phosphorylated. Because these percentages of PDE4D3 were roughly equal to the fractions of PDE4D3 detected in the 100,000 × g particulate and supernatant fractions of these cells, respectively, we investigated the possibility that PMA and forskolin could have subcellular selective effects. Our data from these experiments are consistent with this. Thus, whereas immunoblots of unfractionated VSMC lysates incubated with forskolin (100 μM) or PMA (100 nM) alone or in combination, identified the previously described pattern of PDE4D3 phosphorylation (Fig. 11), immunoblot analysis of resolved particulate and supernatant fractions showed that the PDE4D3 in these fractions was differentially affected. Thus, whereas treatment of VSMCs with forskolin for 30 min resulted in the phosphorylation of more than 80% of the supernatant PDE4D3, less than 40% of the particulate PDE4D3 was phosphorylated under these conditions (Fig. 11). Similar results were seen when cells were incubated with forskolin for periods between 5 min and 1 h (not shown). Incubation of VSMCs with PMA also resulted in a lower percentage of particulate PDE4D3 phospho-
rylation, an effect that was most marked when VSMCs were incubated simultaneously with both PMA and forskolin (Fig. 11). Indeed, when both agents were added together, a very marked reduction in total PDE4D3 was observed in the particulate fraction of treated cells (Fig. 11). Based on densitometric analysis, approximately 70% less PDE4D3 was present in the particulate fraction of VSMCs treated with both forskolin and PMA than when samples derived from forskolin-treated cells were analyzed (Fig. 11). Because pharmacological agents that discriminate between the different PDE4 variants do not exist, a strategy of selective pharmacological inhibition of the different PDE4 variants present in VSMC particulate fractions was not possible. Therefore, to more directly assess the effects of our treatments on PDE4D3 activity in the particulate fraction of VSMCs, we used a strategy of selective PDE4D3 immunoprecipitation. Our data from these experiments are consistent with a forskolin- or PMA-mediated activation of particulate PDE4D3 and with a translocation of PDE4D3 from this fraction upon co-incubation with both these agents (Table II). Thus, incubation of VSMCs with either forskolin or PMA for 30 min resulted in an 8- or 4-fold increase in the PDE4 activity present in PDE4D immunoprecipitates obtained from the particulate fractions, respectively; when cells had been incubated with both forskolin and PMA for 30 min (Table II), there was a marked reduction in the immunoprecipitatable PDE4D3 activity in this fraction. In fact, only slightly more (approximately 40%) PDE4D3 activity was present in the particulate fraction of cells incubated with both these agents than was obtained in immunoprecipitates of control untreated cells. Interestingly, not all of the PDE4 activity present in the particulate fraction was immunoprecipitated with the anti-PDE4D antibody in our studies. Because there was no PDE4D3 detectable by immunoblotting in the particulate fraction following PDE4D immunoprecipitation (not shown), these data are consistent with the presence of another PDE4 variant in this fraction. However, because the particulate PDE4 activity not immunoprecipitated by the PDE4D antibody was equivalent between fractions prepared from forskolin and forskolin plus PMA-treated VSMCs, we can conclude that the loss of PDE4 activity in this fraction is entirely attributable to the loss of PDE4D4 (Table II and Fig. 11). Although preliminary data obtained in our laboratory are consistent with the presence of both PDE4A and PDE4B variants in the VSMCs particulate fractions isolated from untreated cells (not shown), the identity of the PDE4 variant activated by forskolin but not translocated in response to PMA (Table II) will require further work.

DISCUSSION

The PDE4D gene encodes two distinct short forms (PDE4D1 and PDE4D2) and at least three long forms, PDE4D3, PDE4D4, and PDE4D5 (18). Although all five PDE4D variants contain identical catalytic domains, the three long forms also contain two amino-terminal regions that have been shown to be highly conserved in all PDE4 genes, but not in other PDE families. These regions have been termed upstream conserved regions 1 and 2 (UCR1 and UCR2) and have been postulated to play important roles in both catalysis by these enzymes and their susceptibility to inhibition with PDE4-selective agents, such as Rolipram (reviewed in Ref. 5). Although PDE4D1 and PDE4D2 have been shown to be soluble proteins (14, 16), when expressed in heterologous expression systems, PDE4D3, PDE4D4 and PDE4D5 were recovered in both the supernatant and particulate fractions (18). The data presented in this study identify the PDE4D variants expressed in VSMCs as PDE4D3 and PDE4D5, characterize the subcellular expression pattern of these enzymes, describe a cAMP/PKA mediated regulation of both PDE4D4 variants, and delineate a previously unreported role for the Raf-MEK-ERK cascade in regulating the activity and the subcellular localization of PDE4D3.

Using a PDE4D-selective antibody, we identified PDE4D3 and PDE4D5 as the PDE4D variants expressed in aortic VSMCs and showed that PDE4D5 was expressed at approximately 10% the level of PDE4D3. Whereas PDE4D3 was predominantly a soluble enzyme, with less than 10% expressed in the particulate fraction, PDE4D5 was found in equal amounts in both the soluble and particulate fractions of lysed cells. Because in our studies virtually all of the PDE4D3 and less than 10% of the PDE4D5 expressed in the particulate fraction were solubilized with Triton X-100, our data are consistent with the idea that PDE4D3 and PDE4D5 are expressed in different compartments or that they localize to a similar compartment as a consequence of different molecular interactions. Further studies will be required to fully address this issue.

Consistent with earlier reports using several different cell types, incubation of VSMCs with agents that elevated cAMP or with cAMP analogues caused a time- and concentration-de-
ERK and PKA Regulate PDE4D3 Activity and Targeting

**Table II**

Effects of incubation of VSMCs with forskolin and/or PMA on particulate PDE4D3

| Additions | Particulate cAMP PDE activity (pmol/min/μl) | PDE4D immunoprecipitates |
|-----------|---------------------------------------------|--------------------------|
|           | | Pellet | Supernatant | Re | Ro | Re | Ro |
| None      | 31 ± 3 | 20 ± 2 | 3 ± 1 | 15 ± 3 | 0 ± 1 |
| Forskolin (100 μM) | 254 ± 13* | 110 ± 7 | 4 ± 2 | 121 ± 8 | 18 ± 6 |
| PMA (100 nM) | 69 ± 4* | 10 ± 3 | 1 ± 2 | 12 ± 5 | 4 ± 3 |
| Forskolin (100 μM) + PMA (100 nM) | 138 ± 8* | 117 ± 11 | 16 ± 4 | 21 ± 3 | 1 ± 2 |

*P < 0.05 in comparison to no addition value.

We have published previously that long-term treatments with agents that elevate cAMP caused a transcriptionally regulated increase in PDE4 activity (6, 7, 10–16). Although we have published previously that long-term treatments with agents that elevate cAMP caused a transcriptionally regulated increase in PDE4 activity (6, 7, 10–16), the effects described in this report, using short (<30 min) treatment regimens, were not sensitive to cycloheximide or to actinomycin D, demonstrating that de novo protein synthesis was not required. In fact, several pieces of evidence were consistent with a PKA-mediated phosphorylation of VSMC PDE4D3 and PDE4D5 being responsible for the forskolin-induced increase in PDE4 activity in these cells. First, incubation of VSMCs with forskolin resulted in the phosphorylation of both proteins as determined by in vivo labeling, shifts in their electrophoretic mobilities as well as the effects of incubation of treated VSMC cell lysates with the phosphatase (CIAP). In addition, direct evidence of a central role for PKA in these effects was obtained using a PKA-selective inhibitor, H89 (24). Although PDE4D5 is present at much lower abundance than PDE4D3, our data identify VSMC PDE4D5 as a substrate for PKA. Although PDE4D5 encodes the serine residue previously shown to be required for PKA-mediated phosphorylation and activation of PDE4D3 (Ser-54 in PDE4D3 and Ser-126 in PDE4D5; see Ref. 18), further work will be required to identify the site(s) phosphorylated by PKA on this protein.

A novel finding of our work was that incubation of VSMCs with PMA or with AngII increased PDE4 activity. Although PMA had been shown to increase PDE4 activity in rat renal collecting tubule (30), the enzyme involved and the mechanism that accounted for this activation were not described. In these studies, we show that this activation is a consequence of protein phosphorylation and that a PKC-dependent activation of the Raf-MEK-ERK cascade catalyzes this phosphorylation-mediated activation of PDE4 activity. Thus, down-regulation of PKC activity (24, 27), addition of selective PKC inhibitors (Ro-318220 or bisindolylmaleimide I), and addition of the MEK inhibitor (PD98059) all abolished the PMA- and AngII-induced phosphorylation of PDE4D3. Because neither the PMA pretreatment nor the PKC or MEK inhibitors had any effect on the phosphorylation of PDE4D3 in response to addition of forskolin and because forskolin did not activate either ERK1 or ERK2, our results confirm that PKC and the Raf-MEK-ERK pathways were not involved in the phosphorylation of PDE4D3 and PDE4D5 caused by cAMP elevating agents. In a previous report, phosphorylation of PDE4B2 within a MAPK consensus sequence was reported, although this modification had no effect on enzyme function (17). Increases in intracellular Ca\(^{2+}\) concentrations have been shown to occur upon activation of the Raf-MEK-ERK cascade in cardiac myocytes (31), and several Ca\(^{2+}\)-activated kinases have been described (32). In our experiments, addition of the calcium ionophore ionomycin to VSMCs had no effect on PDE4 activity or on the phosphorylation status of PDE4D3. Similarly, selective inhibitors of either p70 S6-kinase, phosphatidylinositol 3-kinase, p38 MAPK, or tyrosine kinases were without effect on either the forskolin- or PMA-induced phosphorylation and activation of PDE4D3 in our cells. The residue phosphorylated in PDE4D3 as a consequence of the PKC-mediated activation of the Raf-MEK-ERK cascade described in this report is at present unknown, as is the mechanism that allows for activation of PDE4D3 in response to this phosphorylation event. However, because our experiments have shown that the site(s) phosphorylated as a consequence of the PKC-mediated activation of the Raf-MEK-MAPK pathway in these cells caused a shift-coupled activation of the enzyme, very similar to that caused by PKA-mediated phosphorylation of Ser-54 (in PDE4D3), it is perhaps reasonable to postulate that the site(s) will be in relatively close proximity to the site phosphorylated by PKA. Based on these considerations, we would propose that Ser-119, which is in an ERK consensus sequence, could perhaps be an excellent candidate site for this phosphorylation. Clearly, further experimentation will be required to validate this supposition.

A completely novel finding that emerged from our studies relates to the observation that the combined actions of PMA and forskolin or AngII and forskolin caused a translocation of PDE4D3 from the particulate to the soluble fraction of these cells, with a resulting decrease in particulate PDE4 activity relative to that caused by forskolin alone. This conclusion was supported by the loss of significant amounts of phosphorylated PDE4D3 in the particulate fraction of PMA-treated VSMCs and by the marked loss of PDE4D3 in the particulate fraction of VSMCs incubated with both forskolin and PMA. Based on our PDE4D-specific immunoblotting and immunoprecipitation studies, changes in PDE4D activity and targeting entirely accounts for the PMA-induced increase in particulate PDE4 activity, as well as the reduction in PDE4 activity following the coordinated phosphorylation of particulate PDE4D3 in the presence of both forskolin and PMA. Although further studies will be required to fully assess the significance of the translocation event described in this work, some of its implications are clear. First, although the increase in soluble PDE4D3 activity caused by the PKC-Raf-MEK-ERK cascade-induced translocation in these cells would most likely be small, the loss of PDE4D3 in the particulate fraction could have very significant functional consequences. For example, if the location of PDE4D3 coincided with that of cAMP effector proteins, such as PKA, significant changes in cAMP-mediated signaling might be anticipated following treatment of cells with activators of PKC and PKA. In order to assess this, studies are under way in...
our laboratory to determine whether activation of the Raf-MEK-ERK cascade could alter the ability of PDE4 inhibitors to affect cell functions, such as proliferation and migration. In this context, we recently reported that PDE4 inhibitors were potent inhibitors of PDGF-BB-mediated VSMC migration (21). Clearly, if differences in the ability of PDE4 inhibitors to antagonize migration induced by chemotactic factors was shown to be dependent on the relative contribution of the Raf-MEK-ERK cascade to the effects of the chemotactic factor studied, the mechanism of PDE4D3 regulation described here might have significant functional relevance in many cell types. Because our data show that not all of the increase in particulate PDE4 caused by forskolin can be attributed to PDE4D, and variants of PDE4A and PDE4B are also known to also have amino-terminal MAPK consensus phosphorylation sites (5, 18), the generality of this mechanism will need to be further investigated.

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REFERENCES
1. Beavo, J. A., and Reifsnyder, D. H. (1990) Trends Pharmacol. Sci. 11, 150–155
2. Bolger, G., Michaeli, T., Martins, T., St. John, T., Steiner, B., Rodgers, L., Riggs, M., Wigler, M., and Ferguson, K. (1993) Mol. Cell. Biol. 13, 6558–6571
3. Manganiello, V. C., Taira, M., Degerman, E., and Belfrage, P. (1995) Cell Signal. 7, 445–455
4. Loughney, K., and Ferguson, K. (1996) in The Handbook of Immunopharmacology: Phosphodiesterase Inhibitors (Schudt, C., Dent, G., and Rabe, K. F., eds) pp. 1–14, Academic Press, London
5. Houslay, M. D., Sullivan, M., and Bolger, G. B. (1998) in Advances in Pharmacology, (August, T. J., Murad, F., Schudt, C., Dent, G., Rabe, K. F., and Rabe, K. F., eds) Vol. 44, pp. 225–342, Academic Press, London
6. Sette, C., Iona, S., and Conti, M. (1994) J. Biol. Chem. 269, 9245–9252
7. Alvarez, R., Sette, C., Yang, D., Eglen, R., Wilhelm, R., Shelton, E. R., and Conti, M. (1995) Mol. Pharmacol. 48, 616–622
8. Sette, C., and Conti, M. (1996) J. Biol. Chem. 271, 16526–16534
9. Swinnen, J. V., Joseph, D. R., and Conti, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8197–8201
10. Torphy, T. J., Zhou, H.-L., and Cieslinski, L. B. (1992) J. Pharmacol. Exp. Ther. 263, 1195–1205
11. Torphy, T. J., Zhou, H.-L., Foley, J. J., Sarau, H. M., Manning, C. D., and Barnette, M. S. (1995) J. Biol. Chem. 270, 23598–23604
12. Vértesy, M. W., McConnell, R. T., Lenhard, J. M., Hamacher, L., and Jin, S.-L. C. (1995) Mol. Pharmacol. 47, 1184–1171
13. Manning, C. D., McLaughlin, M. M., Livin, G. P., Cieslinski, L. B., Torphy, T. J., and Barnette, M. S. (1996) J. Pharmacol. Exp. Ther. 276, 810–818
14. Erdogan, S., and Houslay, M. D. (1997) Biochem. J. 321, 165–175
15. Rose, R. J., Liu, H., Palmer, D., and Maurice, D. H. (1997) Br. J. Pharmacol. 122, 233–240
16. Maurice, D. H. (1998) Cell Biochem. Biophys. 29, 35–47
17. Lenhard, J. K., Kassel, D. B., Rocque, W. J., Hamacher, L., Holmes, W. D., Patel, I., Hoffman, C., and Luther, M. (1996) Biochem. J. 316, 751–758
18. Bolger, G. B., Erdogan, S., Jones, R. E., Loughney, K., Scotland, G., Hoffman, R., Wilkinson, I., Farrell, C., and Houslay, M. D. (1997) Biochem. J. 232, 539–548
19. Scott, J. D. (1997) Soc. Gen. Physiol. Ser. 52, 227–239
20. Polson, J. B., and Strada, S. J. (1996) Ann. Rev. Pharmacol. Toxicol. 36, 403–427
21. Palmer, D., Tsoi, K., and Maurice, D. H. (1998) Circ. Res. 82, 852–861
22. Komas, N., Lugnier, C., and Stoclet, J.-C. (1991) Br. J. Pharmacol. 104, 495–503
23. Davis, C. W., and Daly, J. W. (1979) J. Cyclic Nucleotide Res. 5, 495–503
24. Findik, D., Song, Q., Hidaka, H., and Parker, P. J. (1998) Mol. Cell. Biol. 18, 790–798
25. Beltman, J., McCormick, F., and Cook, S. J. (1996) J. Biol. Chem. 271, 27018–27024
26. Gekeler, V., Boer, R., Uherzall, F., Ise, W., Schubert, C., Uts, I., Hofmann, J., Sanders, K. H., Schachtete, C., Klemm, K., and Grunwiche, H. (1996) Br. J. Cancer 74, 897–905
27. Schudt, C., Marais, R., Marshall, C. J., and Parker, P. J. (1998) Mol. Biol. Cell. 18, 790–798
28. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7689
29. Tetsuka, T., Usama, K., Takada, S., Hemma, S., Yoshida, I., Ando, Y., and Azano, Y. (1995) Am. J. Physiol. 268, F808–F814
30. Ho, P. D., Zechner, K., He, H., Dillmann, W. H., Glombotski, G. C., and McDonough, P. M. (1998) J. Biol. Chem. 273, 21730–21735
31. Schulman, H. (1993) Curr. Opin. Cell Biol. 5, 247–253