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Phorbol 12-myristate 13-acetate Triggers the Protein Kinase A-Mediated Phosphorylation and Activation of the PDE4D5 cAMP Phosphodiesterase in Human Aortic Smooth Muscle Cells through a Route Involving Extracellular Signal Regulated Kinase (ERK)

GEORGE BAILLIE, SIMON J. MACKENZIE, and MILES D. HOUSLAY

Molecular Pharmacology Group, Division of Biochemistry & Molecular Biology, Institute of Biomedical & Life Sciences, University of Glasgow, Glasgow, Scotland, United Kingdom

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ABSTRACT

Phosphodiesterase 4D5 is the sole PDE4D cAMP phosphodiesterase isoform expressed in human aortic smooth muscle cells (HASMC). Phorbol 12-myristate 13-acetate (PMA) challenge of HASMC rapidly activated PDE4D5 through a process ablated by the mitogen-activated protein kinase kinase inhibitor PD98059. PMA elicited an inhibitory effect on PDE4D5 activity in HASMC treated with the cyclooxygenase (COX) inhibitor indomethacin, the COX-2 selective inhibitor NS-398, the phospholipase A_2 inhibitor quinacrine, and the cAMP-dependent protein kinase A (PKA) inhibitor H89. PMA challenge of COS-1 cells elicited the rapid inhibition and phosphorylation of both recombinant and endogenous PDE4D5 in a manner ablated by PD98059 and not seen in S651A mutant PDE4D5. PMA promoted the generation of PGE_2 in the medium of HASMC and caused activation of both extracellular signal-regulated kinase (ERK) and PKA through a process ablated by indomethacin, NS-398, quinacrine, and PD98059. Exogenous prostaglandin (PG) E_2 increased cAMP levels and activated PKA in HASMC. COX-2 was expressed in HASMC but not in COS-1 cells. Forskolin challenge of COS-1 cells activated PDE4D5 by causing the PKA-mediated phosphorylation of Ser126 as detected using a novel phosphospecific antiserum. PMA challenge of HASMC elicited phosphorylation of the stimulatory PKA-specific phosphorylation site, Ser126 in PDE4D5 in a manner ablated by PD98059, indomethacin, and H89. We propose that, in HASMC, PMA activates PDE4D5 through an ERK-controlled autocrine mechanism. This involves PGE_2 generation, which causes activation of adenylyl cyclase, allowing PKA to elicit net activation of PDE4D5 by phosphorylation at Ser126.

The classical extracellular-signal regulated kinase (ERK) pathway governs fundamental processes such as cell proliferation, transformation, differentiation and survival (Lewis et al., 1998; English et al., 1999; Schaeffer and Weber, 1999). Equally ubiquitous is the cAMP signaling pathway. This serves to regulate processes similar to ERK as well as changes in contraction and metabolic events (Rubin, 1994; Houslay and Milligan, 1997; Colledge and Scott, 1999). Intriguingly, it seems that the cAMP and ERK signaling pathways are closely integrated at a number of levels (Houslay and Kolch, 2000).

cAMP phosphodiesterases (PDEs) provide a diverse range of proteins that differ markedly in their regulatory and kinetic properties, as well as in their intracellular localization (Thompson, 1991; Bolger, 1994; Beavo, 1995; Manganiello et al., 1995; Souness and Rao, 1997; Houslay et al., 1998; Torphy, 1998; Conti and Jin, 1999). They provide the sole means of degrading cAMP in cells and are thus poised to serve as potential key regulators of intracellular signaling events. There is much interest in the PDE4 family because selective inhibitors are being developed as potential therapeutic agents for treating a wide range of inflammatory diseases, certain cancers, and depression (Souness and Rao, 1997;

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G.B. and S.J.M. contributed equally to this study and should be considered joint first authors.

ABBREVIATIONS: ERK, extracellular regulated kinase; PDE, phosphodiesterase; PDE4, cAMP-specific phosphodiesterase family 4; UCR, upstream conserved region; PKA, protein kinase A; EGF, epidermal growth factor; RASM, rat aortic vascular smooth muscle; PMA, phorbol 12-myristate 13-acetate; MEK, mitogen-activated protein kinase kinase; HASM, human aortic smooth muscle; PGE_2, prostaglandin type E_2; VSV, vesicular stomatis virus; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); PKC, protein kinase C; P-ERK, phosphorylated extracellular regulated kinase; PLA_2, phospholipase A type 2; COX, cyclooxygenase.

Houslay et al., 1998; Rogers and Giembycz, 1998; Torphy, 1998). Four genes (*PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D*) each encode a series of isoforms that are identifiable by their unique N-terminal regions. Between these regions and the catalytic unit are upstream conserved regions (UCR) 1 and 2, which are unique to PDE4 enzymes (Bolger et al., 1993). Long isoforms posses both UCR1 and UCR2, whereas short isoforms lack UCR1. Isoforms from all PDE4 subfamilies, except PDE4A, can be phosphorylated by ERK at a cognate serine residue within their catalytic unit (Baillie et al., 2000). The functional output of ERK phosphorylation is orchestrated (Baillie et al., 2000; MacKenzie et al., 2000) by the N-terminal UCR1 and UCR2 regulatory regions that interact with each other to form a regulatory module (Lim et al., 1999; Beard et al., 2000). In the case of PDE4D long isoforms, such as PDE4D3 and PDE4D5, these regions direct ERK phosphorylation to cause inhibition. However, in short forms lacking UCR1, the lone UCR2 reprograms ERK phosphorylation to cause activation (MacKenzie et al., 2000). However, studies on PDE4D3 have shown that PKA can phosphorylate a single serine in UCR1, causing activation of this isoform (Alvarez et al., 1995; Sette and Conti, 1996; Hoffmann et al., 1998; MacKenzie et al., 2000). This action of PKA also negates the inhibitory effect of ERK phosphorylation on PDE4D3 (Hoffmann et al., 1999; MacKenzie et al., 2000). Thus the UCR1-UCR2 regulatory module serves to integrate the functional consequences of both PKA and ERK2 phosphorylation on PDE4 isoforms.

Activation of ERK by EGF in intact COS cells causes the marked inhibition of PDE4D long isoforms (Hoffmann et al., 1999; Baillie et al., 2000; MacKenzie et al., 2000). Activated recombinant ERK also causes the phosphorylation and inhibition of isolated recombinant PDE4D3 and PDE4D5 in vitro. Additionally, mutation of the serine target for ERK phosphorylation in these enzymes to the negatively charged aspartate residue also mimics this inhibitory effect. However, Liu and Maurice (1999) have indicated that, in rat aortic vascular smooth muscle (RASM) cells, ERK activation leads to a small increase in the activity of either or both the PDE4D3 and PDE4D5 long forms expressed in these cells. This was proposed on the basis that challenge of RASM cells with the protein kinase C activator PMA led to an increase in PDE4D activity that was ablated by the MEK inhibitor PD98059. Such an observation leads to the tantalizing possibility that ERK activation may elicit very different effects on the activity of PDE4D long forms, depending on the cell type in which they are expressed. Nevertheless, the basis for observing activation, rather than inhibition, in smooth muscle cells is unclear.

Here we have analyzed human aortic smooth muscle (HASM) cells and show that PMA treatment serves to activate PDE4D5, the sole long PDE4D isoform expressed in these cells. Intriguingly, activation is seemingly achieved through an ERK-driven autocrine loop that generates PGE_2 in the medium of HASM cells, thereby leading to an increase in PKA activity and the net stimulatory phosphorylation of the PDE4D5 long form. This study provides a new perspective on the complex, cell-type–specific cross talk that links the ERK and cAMP signaling pathways (Houslay and Kolch, 2000).

Experimental Procedures

Protease inhibitor tablets were obtained from Roche Molecular Biochemicals (Mannheim, Germany). [^{3}H]cAMP, protein-G Sepharose 4B fast flow and enhanced chemiluminescence reagent were from Amersham Pharmacia Biotech (Cardiff, Wales, UK). Dithiothreitol and Triton X-100 were obtained from Roche Molecular Biochemicals (Herts, UK). Bradford reagent was from Bio-Rad (Herts, UK). The Correlate-EIA Prostaglandin E_2 Enzyme Immunoassay kit was from Assay Design Inc. (Ann Arbor, MI). NS-398, PK(6-22) Amide and quinacrine dihydrochloride were from Calbiochem (Nottingham, UK). All other materials were from Sigma (Poole, UK).

Immunological Reagents. We used an antiserum described previously (Bolger et al., 1997) that was specific for isoforms of the PDE4D subfamily. This antibody was generated against the extreme C-terminal 65 amino acids of the PDE4D3 protein that are found in common in all five known PDE4D isoforms. The C-terminal regions are unique to each of the four PDE4 subfamilies. Thus the antisera used were specific for PDE4D isoforms and showed no cross-reactivity with any of the three other PDE4 subfamilies (Hoffmann et al., 1999; MacKenzie et al., 2000). In addition, we used an antiserum raised against a region within the unique N-terminal region of PDE4D5 that specifically identified this isoform by both Western blotting and immunoprecipitation (Hoffmann et al., 1999). We also used a peptide antibody able to detect specifically the N-terminal portion of the PDE4D5 isoform (Hoffmann et al., 1999; MacKenzie and Houslay, 2000). We also employed a novel polyclonal antiserum (PS54-UCR1-A1) able to detect specifically the (protein kinase A) phospho-serine* form of the Arg-Arg-Glu-Ser*-Phe motif found in the conserved UCR1 region of all long isoforms (a kind gift from Dr G van Heeke, Novartis, Horsham, UK; Mackenzie SJ, Baillie GS, McPhee J, et al., manuscript in preparation). This was generated using a phosphopeptide whose sequence was SQRRES*FLYRSDSDYDLSP. The phosphorylated serine residue is indicated (S*) as well as the PKA consensus sequence, which is underlined. This sequence reflects amino acids 49 to 67, inclusively, in the long PDE4D3 isoform. This region is completely conserved in PDE4D5 (Bolger et al., 1997). A monoclonal antibody was used to detect the active, phosphorylated forms (Thr202/Tyr204) of both ERK1 and ERK2 (p44/42 mitogen-activated protein kinase) as well as an antibody able to identify both of these ERK forms irrespective of their phosphorylation status (New England Biolabs, Hitchin UK) as we described previously (Hoffmann et al., 1999; MacKenzie et al., 2000). Immunoblotting was done as we described previously (Hoffmann et al., 1999; MacKenzie et al., 2000) using ~20-μg protein samples.

Transient Expression of PDE4D3 and PDE4D5 in COS-1 Cells. The generation of expression plasmids encoding the VSV epitope tagged forms of PDE4D3, PDE4D5 and their indicated mutants have been described previously in detail (Hoffmann et al., 1999; Baillie et al., 2000; MacKenzie et al., 2000). Transfection was done using the COS-1 simian virus 40-transformed monkey kidney cell line maintained at 37°C in an atmosphere of 5% CO_2/95% air in complete growth medium containing Dulbecco's modified Eagle's medium supplemented with 0.1% penicillin/streptomycin (10,000 units/ml), glutamine (2 mM), and 10% fetal calf serum. We have described details of these procedures previously (Hoffmann et al., 1999). Briefly, however, COS-1 cells were transfected using DEAE Dextran. The DNA to be transfected (5 μg) was mixed, and incubated for 15 min with 250 μl of 10 mg/ml DEAE Dextran (Sigma) in PBS to give a DNA-dextran mix. When cells reached 70% confluence in 100-mm dishes, medium was removed and the cells were given 10 ml of fresh Dulbecco's modified Eagle's medium containing 0.1 mM chloroquine and the DNA-dextran mix (250 μl). The cells were then incubated for 4 h at 37°C. After this period, the medium was removed and the cells shocked with 10% dimethyl sulfoxide in PBS. After PBS washing, the cells were returned to normal growth medium and left for a further 2 days before use. For determination of PDE activity, the cells were homogenized in PDE assay buffer. As described pre-

viously (Baillie et al., 2000; MacKenzie et al., 2000), in such transfected cells, >98% of the total PDE activity was due to the recombinant PDE4 isoenzyme. Transfected COS-1 cells were plated onto six-well plates for use in experiments. They were serum-starved over night before being treated with the indicated ligands for the stated lengths of time.

Culture of Vascular Smooth muscle cells. Primary human aortic smooth muscle cells (HASMCc; product code 2HC-3121) were obtained from TCS Biologicals Ltd (Buckingham, UK) as a frozen stock. These were grown according to the detailed method provided with the cells. Briefly, the cells were cultured in smooth muscle cell basal medium (TCS Biologicals) with the addition of smooth muscle cell growth supplement and antibiotic supplement (both TCS Biologicals). Cells were passaged when they reached 70% confluence in the culture flask into six-well plates, where they were then fed every second day until confluence was reached. Upon attaining confluence they were serum-starved overnight before being treated with inhibitors or PMA for the stated lengths of time. All experiments upon HASMC cells were performed within three passages from the cells having been broken out. HASM cells were plated onto six-well plates for use in experiments.

RT-PCR analyses. RNA from HASM cells was generated and used as we described previously (Kostic et al., 1997; Rena et al., 2001) to detect transcripts for specific PDE isoforms by RT-PCR. In this instance we used primer pairs that specifically detected PDE4D3 (Genbank accession number L20970) and PDE4D5 (Genbank accession number AF012074) long isoforms. For PDE4D3, these were GCGAACATGATGCACGTGAA (forward) and TGGCCAAGACCTGAGCAAAT (reverse) to amplify a 292-bp fragment. For PDE4D5, they were TGCCAGCTGTACAAAGTTGACC (forward) and TTCTCGGAGAGATCACTGGAGA (reverse) to amplify a 212-bp fragment. The PCR reaction components were 12.5 μl of 2× QIAGEN HotStarTaq PCR Mix (contains enzyme), 18.75 pmol of Forward Primer (at 10 pmol/μl), 18.75 pmol of Reverse Primer (at 10 pmol/μl) DNase-treated cDNA equivalent to 2.5 ng of input RNA made up to 25 μl with nuclease free water. Samples were heated to 95°C for 5 min (step 1), held at 94°C for 30 s (step 2), held at 60°C for 30 s (step 3), held at 72°C for 1 min (step 4), cycled to step 2 for 39 times (step 5), held at 94°C for 30 s (step 6), and, finally, held at 60°C for 30 s (step 7).

Immunoprecipitation. This was done as described previously for various cell lines (MacKenzie et al., 1998). For harvesting, HASM cells were first washed in PBS before being scraped into lysis buffer (25 mM HEPES, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM napyrophosphate, 10% glycerol, and 1% Triton X-100, pH 7.5, with added protease inhibitors) and mixed at 4°C for 20 min. This allowed for the solubilization of all PDE4D immunoreactivity. The lysates (150 μg of protein) were precleared by incubation with 20 μl of protein-G Sepharose 4B fast flow for 30 min at 4°C and the beads were removed by centrifugation; no loss of PDE4D immunoreactivity occurred. PDE4D was then specifically immunoprecipitated by incubation with the PDE4D-specific antibody for 2 h at 4°C. The immune complexes were then coupled to 50 μl of protein-G Sepharose with incubation for 1 h at 4°C, followed by centrifugation. The pellet fraction was washed with lysis buffer and finally with PDE assay buffer (20 mM Tris, pH 7.6, with protease inhibitors) before final analysis. An identical procedure was used to selectively immunoprecipitate PDE4D5 using the previously reported specific antiserum for this isoform (Hoffmann et al., 1999).

Metabolic Labeling with [^{32}P]Orthophosphate. This was done essentially as we described previously (Hoffmann et al., 1999). Confluent dishes (10-cm diameter) of COS-1 cells were incubated in phosphate-free medium for 2 h before the addition of 0.5 mCi of [^{32}P]orthophosphate to each dish. Four hours thereafter, some dishes were treated with UO126 (10 μM) or chelerythrine (10 μM) for 15 min. The PMA (100 nM) was added to all dishes, except the control, and incubation was continued for 20 min. Medium was then removed and each plate washed with ice-cold PBS (3×). Cells were then lysed, as above, and PDE4D5 specifically immunoprecipitated using an antibody raised against its unique N-terminal region (Hoffmann et al., 1999). Immunoprecipitated protein was separated by SDS-polyacrylamide gel electrophoresis and phosphorylated species visualized using a Molecular Imager FX (Bio-Rad, Hercules, CA).

Assay of cAMP PDE activity. PDE activity was determined by a modification of the two-step procedure of Thompson and Appleman (1971) as we described previously (Marchmont and Houslay, 1980). All assays were conducted at 30°C with initial rates taken from linear time-courses. Activity was linear with added protein concentration. Untransfected and mock (vector only) transfected COS-1 cells exhibited a PDE activity of 10 ± 0.5 pmol/min/mg of protein.

PGE_2 assay. The measurement of PGE_2 in the cell culture medium was carried out as described in the supplied protocol book using the Correlate-EIA Prostaglandin E2 Enzyme Immunoassay kit from Assay Design Inc. (Ann Arbor, MI).

PKA assay. The level of activated protein kinase A (PKA) within HASM cells was measured (Corbin, 1983) using Kemptide as a substrate (Kemp et al., 1977). Cells were lysed, after treatment, in homogenization buffer (50 mM Tris, 5 mM EDTA, pH 7.5) and passed 12 times through a 26-gauge needle. The homogenate was centrifuged for 2 min at 10,000 rpm to remove debris. The assay was started by adding homogenate to the assay mixture (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, 100 μM ATP, 50 μM Kemptide, with 20 μCi/ml of [γ-^{32}P]ATP), mixed and incubated at 30°C. The reaction was stopped after 10 min by spotting onto phosphocellulose paper squares. These were washed twice in 1% phosphoric acid and once in H_2O, then dried and counted in a scintillation counter. PKA activity present in the homogenate was compared with the maximal potential PKA activity attainable. This was elicited by treating the homogenate with 40 μM cAMP so as to activate PKA maximally. This allowed the calculation of PKA activity within the cells as a percentage of the total possible PKA activity. To control for other kinases, reactions using the cell homogenate were carried out as above, in the presence of 10 μM PKA inhibitor peptide, PK(6-22)-Amide.

Intracellular cAMP Determination. This was carried out on cells in six-well plates, prepared as for PKA assays, and stimulated with the same concentrations of PMA and PGE_2. The assay was performed as described previously (Heyworth and Houslay, 1983; Tang and Houslay, 1992).

Protein Analysis. Protein concentration was determined using bovine serum albumin as standard (Bradford, 1976). SDS-polyacrylamide gel electrophoresis was done as described by Laemmli (1970).

Results

Smooth muscle cells are highly differentiated; as such, they have very specific properties. Using PMA to challenge primary rat aortic smooth muscle cells, Liu and Maurice (1999) suggested that ERK activation caused a small increase in the activity of the long PDE4D3 isoform. This contrasted with the inhibition of the activity of the long PDE4D3 and PDE4D5 isoforms that we have observed (Hoffmann et al., 1999; Baillie et al., 2000; MacKenzie et al., 2000) (1) upon EGF-stimulation of ERK activity in COS cells; (2) using recombinant purified ERK to phosphorylate single serine target residues within the catalytic site of both PDE4D3, at ser579, and PDE4D5, at ser651 and (3) using mimicking mutant forms where the target serine residue for ERK phosphorylation was mutated to the negatively charged aspartate. Here, we have investigated the action of PMA on the PDE4D activity found in HASM cells and on the recombinant PDE4D3 and PDE4D5 long isoforms expressed in COS-1 cells.

Human Aortic Smooth Muscle Cells Express a Single PDE4D Isoform, PDE4D5. Immunoblotting HASM cells using either a monoclonal antibody or a polyclonal antiserum (Fig. 1a), both shown (Bolger et al., 1997) to be specific for PDE4D isoforms identified a single immunoreactive species. This species (105-kDa) comigrated (Fig. 1a) with recombinant PDE4D5 and not any of the other four known PDE4D isoforms, including the more slowly migrating (95-kDa) PDE4D3 isoform [note that the PDE4D2 short isoform migrates similarly to the short PDE4D1 isoform (data not shown; see Bolger et al., 1997)]. Consistent with the single PDE4D immunoreactive species being PDE4D5, we were able to detect a similarly migrating species using a PDE4D5-specific antiserum (Fig. 1b). This was raised to a peptide representing a portion of the unique N terminus of PDE4D5 and has been shown to detect PDE4D5 specifically (Hoffmann et al., 1999; MacKenzie and Houslay, 2000). This antiserum successfully recognized a single 105-kDa immunoreactive species found in HASM cells (Fig. 1b) that comigrated with recombinant PDE4D5 expressed in COS-1 cells. It did not, however, recognize recombinant PDE4D3 (Fig. 1b). To address further the notion that HASM cells expressed PDE4D5, but not PDE4D3, we performed a transcript analysis using RT-PCR. Primers designed to be specific for PDE4D5 generated a product of the predicted size (292 bp) from HASM cells and also when we used a PDE4D5 encoding plasmid, but not when we used a plasmid encoding PDE4D3 as a template (Fig. 1c). Conversely, primers designed to be specific for PDE4D3 failed to generate a product using mRNA from HASM cells and also when using a PDE4D5-encoding plasmid, but they did generate an appropriately sized species (212 bp) when a plasmid encoding PDE4D3 was used as a template (Fig. 1d). These data show that HASM cells express a single PDE4D isoform, namely the long PDE4D5 isoform.

Homogenates of HASM cells had a specific activity of 33 to 39 pmol/min/mg of protein assayed at 1 μM cAMP substrate concentration (range, $n = 5$ separate studies). Of this, some 35 $\pm$ 4% (mean $\pm$ S.D., $n = 5$ separate studies) was attributable to PDE4 activity; namely, that fraction of PDE activity that was inhibited by the PDE4 selective inhibitor, rolipram (10 μM). Selective immunoprecipitation of the PDE4D subclass using a specific polyclonal antibody (MacKenzie and Houslay, 2000) showed that PDE4D activity accounted for some 28 $\pm$ 4% of the total PDE4 activity in HASM cells (mean $\pm$ S.D., $n = 5$ separate studies). This would allow a notional specific activity of 3.2 to 3.8 pmol/min/mg of protein for PDE4D5 in HASM cells.

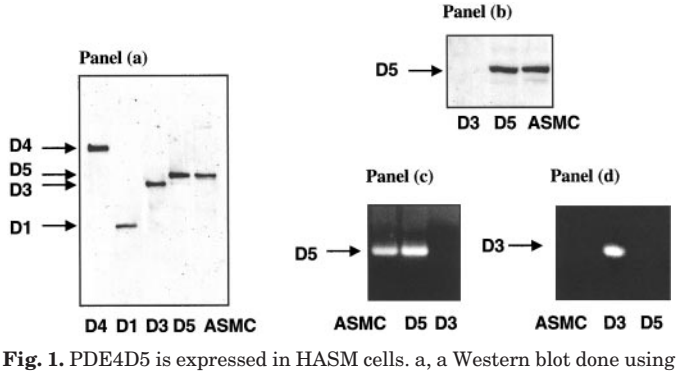

Fig. 1. PDE4D5 is expressed in HASM cells. a, a Western blot done using a PDE4D specific antiserum. Recombinant standards are shown for the 119-kDa PDE4D4 (D4), the 68-kDa PDE4D1 (D1), the 95-kDa PDE4D3 (D3), and 105-kDa PDE4D5 (D5) together with a homogenate of HASM cells (ASMC). b, a Western blot done using a PDE4D5 isoform-specific antiserum. Recombinant standards are shown for PDE4D3 (D3) and PDE4D5 (D5) together with a homogenate of HASM cells (ASMC). c and d, shows the results of RT-PCR analyses done using, as a source, RNA from HASM cells (ASMC) and plasmid preparations for PDE4D3 (D3) and PDE4D5 (D5) with primers specific for either PDE4D5 (c) or PDE4D3 (d). The size of fragments amplified were 292 bp for PDE4D3 and 212 bp for PDE4D5. These are typical of experiments done at least three times.

PMA Stimulates PDE4D5 Activity in Human Aortic Smooth Muscle Cells. As we have shown here (Fig. 1), of the five known PDE4D isoenzymes (Bolger et al., 1997), only the PDE4D5 isoform is expressed. To determine changes in PDE4D5 activity consequent upon challenge of HASM cells with PMA and other ligands, we set out to immunopurify PDE4D5 selectively using procedures that we have developed previously (Bolger et al., 1997; MacKenzie and Houslay, 2000). We did this using a PDE4D-specific antiserum because we have shown previously that PDE4D isoforms can be immunoprecipitated from solubilized cell lysates using such C-terminal directed antisera without incurring any change in PDE activity (Hoffmann et al., 1998, 1999; MacKenzie and Houslay, 2000). In performing these studies, we ensured that, as we described previously (Hoffmann et al., 1998, 1999; MacKenzie and Houslay, 2000), sufficient PDE4D-specific antiserum was added to immunoprecipitate all of the PDE4D5 under the various experimental conditions used. In addition, we ascertained that no other PDE4 family isoenzymes were pulled down (data not shown) by probing the immunoprecipitates with antisera specific for the three other PDE4 enzyme families (MacKenzie and Houslay, 2000).

Using such a procedure, we were able to demonstrate (Fig. 2) that PMA challenge of human HASM cells caused a clear increase in PDE4D5 activity. This activity increase was, seemingly, due to altered PDE4D5 activity rather than the induction of PDE4D5 or any other PDE4D isoform. Thus PMA treatment did not cause any change in the expression levels of PDE4D5 in HASM cells over a 60-min challenge, as detected using either a PDE4D-selective antiserum (Fig. 3a) or a PDE4D5-selective antiserum (Fig. 3b). Neither did PMA challenge induce any other known PDE4D isoenzyme over this period (Fig. 3a).

The PMA-mediated increase in PDE4D5 activity was attenuated by the PKC inhibitor chelerythrine chloride and was ablated by the MEK inhibitor PD98059 (Fig. 2). This clearly indicates the involvement of both PKC and ERK in eliciting such a stimulatory effect. Indeed, HASM cells express both ERK1 and ERK2, identified as 42- and 44-kDa immunoreactive species (data not shown), and their challenge with PMA led to the presumed activation of these isoforms, as detected immunologically by the appearance of their phosphorylated (P-ERK) forms (Fig. 3c). The generation of P-ERK species by PMA challenge was also inhibited by both the PKC-selective inhibitor chelerythrine chloride and the MEK inhibitor PD98059 (Fig. 3c), consistent with the action of such selective inhibitors in attenuating PMA stimulation of PDE4D3 in HASM cells (Fig. 2).

Action of PMA on PDE4D3 and PDE4D5 Expressed in COS-1 Cells. We have shown previously that ERK activation achieved by EGF challenge of transfected COS cells led to the inhibition of both PDE4D3 and PDE4D5 activity (Hoffmann et al., 1999; MacKenzie et al., 2000). Although both EGF and PMA are known to activate ERK, they exert a rather different range of effects on cells. It was important

then to distinguish whether the ability of PMA to activate PDE4D5 in HASM cells was either caused by to a cell-type–specific effect or was elicited by some particular action uniquely elicited by PMA compared with that exerted by EGF. Thus COS-1 cells were transiently transfected to express either PDE4D3 or PDE4D5. As described previously (Hoffmann et al., 1999; Yarwood et al., 1999; MacKenzie et al., 2000), under such conditions >98% of the total PDE activity was caused by the recombinant enzyme. In such transfected COS-1 cells, challenge with PMA elicited the inhibition of the activity of both PDE4D5 (Fig. 4a) and PDE4D3 (Fig. 4b) in a fashion similar to that shown previously employing EGF (Hoffmann et al., 1999; MacKenzie et al., 2000). PMA challenge also led to the rapid generation of the phosphorylated and thus presumably activated forms of both ERK-1 and ERK-2 isoenzymes in COS-1 cells (Fig. 4c). The MEK inhibitor PD98059 (Fig. 4d) ablated the PMA-mediated activation of ERK1/2 as well as the PMA-mediated inhibition of both PDE4D5 and PDE4D3 (Fig. 4, a and b). The inhibitory effect that PMA exerted upon these two long PDE4D isoforms also involved PKC activation; it was attenuated by the presence of the PKC inhibitor chelerythrine chloride (Fig. 4, a and b).

Thus, as found with EGF challenge of COS-1 cells (Hoffmann et al., 1999; MacKenzie et al., 2000), the inhibitory effect that PMA exerted upon the activity of recombinant forms of both PDE4D3 and PDE4D5 seemed to be mediated by ERK because it was ablated by the MEK inhibitor PD98059 (Fig. 4, a and b). Consistent with this, we show here (Fig. 4, a and b) that PMA-mediated inhibition was not evident using either the ser651ala-PDE4D5 mutant or the ser579ala-PDE4D3 mutant, where the target serine for direct phosphorylation by ERK2 (Hoffmann et al., 1999) was replaced with alanine. These data indicate that PMA closely mimics the action of EGF in causing inhibition of both PDE4D3 and PDE4D5 in transfected COS-1 cells.

As with EGF challenge (Hoffmann et al., 1999; MacKenzie et al., 2000), the PMA-mediated inhibition of both PDE4D3 and PDE4D5 was transient (Fig. 4, a and b) despite the fact that the ERK isoenzymes remained in their activated, phosphorylated state over the time of the experiment (Fig. 4c). We have shown previously (Hoffmann et al., 1999) that similar levels of PDE4D3 inhibition in COS-1 cells sufficed to cause an increase in cAMP levels, allowing PKA to phosphorylate PDE4D3 at ser54 in UCR1. This served to ablate the inhibitory effect of ERK phosphorylation of PDE4D3, thus accounting for the transient form of ERK inhibition in these cells. Consistent with this, the addition of the PKA inhibitor H89 attenuated the transience of the inhibition of both PDE4D3 and PDE4D5 activity (Fig. 4, a and b) caused by PMA challenge. This action was similar to that shown previously (Hoffmann et al., 1999) using challenge with EGF together with H89.

COS-1 cells, however, express both PDE4D3 and PDE4D5 endogenously (Fig. 4e). A specific antiserum for PDE4D5 has been shown to be able to immunoprecipitate this isoform selectively without altering its activity (Hoffmann et al., 1999). Using this, we were able to selectively immunoprecipitate PDE4D5 from COS-1 cells (Fig. 4d) and determine that it constituted some 14 ± 3% (mean ± S.D.; $n = 3$) of the total PDE activity in these cells, representing a notional specific activity of 1.3 ± 0.1 pmol/min/mg of protein. Note that the percentage contribution of endogenous PDE4D5 to total PDE activity on both COS-1 and HASM cells is very similar (10–14%), with the specific activity seen in HASM cells about three times higher than in COS-1 cells. They thus seem to provide a comparable system in terms of PDE4D5 expressed activity. We were able to ascertain that PMA challenge of COS-1 cells led to a transient inhibition of endogenous PDE4D5 activity (Fig. 4e) in a fashion similar to that seen for the recombinant enzyme expressed in these cells (Fig. 4a). In addition, we showed that PMA challenge caused the phosphorylation of endogenous PDE4D5 in COS-1 cells (Fig. 4 g). This action was ablated not only by the PKC inhibitor chelerythrine, but also by PD98059, the inhibitor of ERK activation (Fig. 4g). Such PMA-mediated phosphorylation was also seen for wild-type recombinant PDE4D5 in transfected COS cells (Fig. 4 h). However, PMA-induced labeling of PDE4D5 was not seen in the ser651ala-PDE4D5 mutant whose ERK phosphorylation site was ablated (Fig. 4h). Furthermore, inhibition of ERK activation using PD98059 (Fig. 4c) prevented PMA causing the phosphorylation of both endogenous (Fig. 4g) and recombinant (Fig. 4h) PDE4D5 in COS-1 cells. These data suggest that, in COS-1 cells, PMA solely causes ERK phosphorylation of PDE4D5 rather than, for example, any direct or other action of PKC.

Thus PMA challenge of COS-1 cells transfected to express either PDE4D3 or PDE4D5 caused the ERK-mediated inhi-

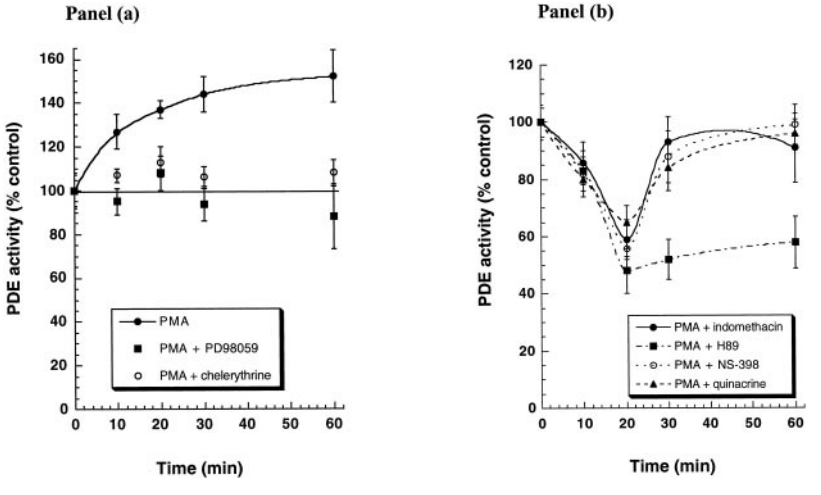

Fig. 2. Changes in PDE4D5 activity mediated by PMA treatment of HASM cells. In a, HASM cells were challenged for the indicated times with PMA (100 nM), either alone or after 20 min of preincubation with either PD98059 (50 μM) or chelerythrine (10 μM). In b, HASM cells were challenged for the indicated times with PMA (100 nM) after 20 min preincubation with either H89 (10 μM), indomethacin (10 μM), quinacrine (10 μM), or NS-398 (10 μM). They were then harvested, lysed, and PDE4D5 immunopurified before being subjected to PDE assay as described under Experimental Procedures. Activity is expressed as a percentage of that observed in preparations from control (untreated) cells. Data are given as means ± S.D. for $n = 3$ separate experiments.

bition of these isoforms in a fashion identical to that elicited by EGF (Hoffmann et al., 1999; MacKenzie et al., 2000). Also, a similar inhibitory effect was seen for endogenous PDE4D5 in PMA-treated COS-1 cells. Thus we infer that the PMA-mediated differences in regulation of PDE4D5 activity seen in COS-1 cells (Hoffmann et al., 1999; MacKenzie et al., 2000; this study) compared with that in HASM cells is extremely likely to be a cell-type–specific effect. Similarly, we would also expect this to be the case for the PMA activation of the PDE4D3 isoform expressed in rat aortic smooth muscle cells (Liu and Maurice, 1999).

Indomethacin Switches PMA to Cause Inhibition of PDE4D5 Activity in Human Aortic Smooth Muscle Cells. It has been demonstrated (Lin et al., 1993) that ERK can cause the phosphorylation and activation of PLA_2. This has also been shown to occur in smooth muscle cells, where the arachidonic acid generated can subsequently be metabolized by COX-2 to generate PGE_2 (Graves et al., 1996; Karim et al., 1997; Pyne et al., 1997). PGE_2 can then be expected to cause the autocrine-stimulation of adenylyl cyclase upon binding to cell surface receptors (Pyne et al., 1997; Stillman et al., 1999). Challenge with PMA may thus be able to cause the ERK-mediated activation of PKA through such an autocrine loop. Assuming that this occurs, then one might predict that PKA phosphorylation of PDE4D5 would ensue and that this would negate any inhibitory effect of ERK phosphorylation, as shown in the COS-1 cell model system (Hoffmann et al., 1999; MacKenzie et al., 2000). Such a mechanism may provide an explanation as to how PMA-mediated ERK activation in smooth muscle cells could lead to a net increase in the activity of long form PDE4D isoforms, such as we have noted here for PDE4D5 and has been suggested to occur for PDE4D3 (Liu and Maurice, 1999). We thus set out to evaluate such a possibility in HASM cells.

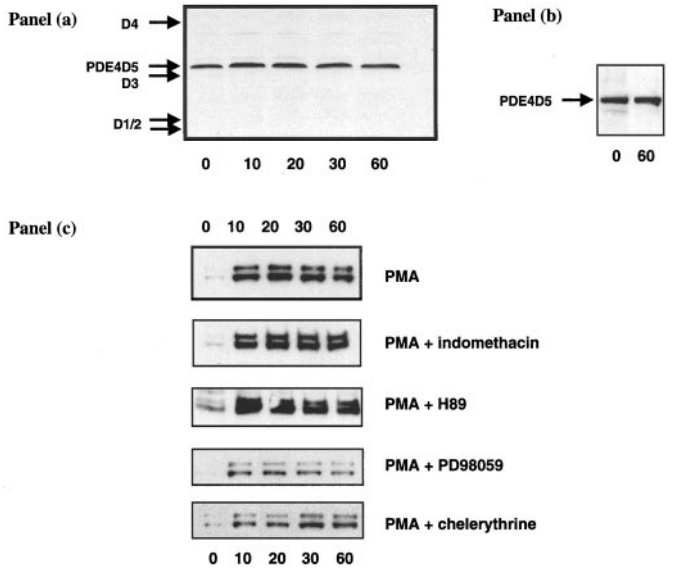

Fig. 3. ERK phosphorylation and PDE4D5 expression in PMA-treated HASM cells. a, Western blotting analysis of HASM cells using an antiserum able specifically to detect all known PDE4D isoenzymes (Bolger et al., 1997). Cells were challenged for the indicated times with PMA (100 nM). They were then harvested and 40-μg protein samples were taken for immunoblotting. Under all conditions, a single 105-kDa immunoreactive species was identified that comigrated with recombinant PDE4D5. The positions in which recombinant PDE4D3, PDE4D4, and PDE4D1/2 would be expected to migrate is shown. In b, immunoblotting was done with a PDE4D5 specific antiserum for untreated HSM cells and those challenged for 60 min with PMA (100 nM). In c, HASM cells were challenged for the indicated times with PMA (100 nM) either alone or after 20 min of preincubation with PD98059 (50 μM) or H89 (10 μM) or chelerythrine (10 μM) or indomethacin (10 μM). They were then harvested and 40-μg protein samples taken for Western blotting analyses. Shown here are Western blots using an antibody specific for identifying the phosphorylated, activated forms of ERK-1 and ERK-2 that migrate at 42 and 44 kDa (arrows). Not shown are blots using an antibody able to detect ERK1 and ERK2 in both phosphorylated and nonphosphorylated states, which yielded identical responses in all tracks, showing that no change in expression/loading of ERK occurred. The data are typical of experiments done at least three times.

Intriguingly, treating HASM cells with the COX inhibitor indomethacin served to change dramatically the action of PMA on PDE4D5 activity in HASM cells (Fig. 2). In the presence of indomethacin, PMA challenge now exerted a transient inhibitory effect on PDE4D5 activity (Fig. 2) that was akin to the inhibitory effect the PMA exerted on both PDE4D5 and PDE4D3 activity in transfected COS-1 cells (Fig. 4, a and b). A similar effect was apparent (Fig. 2) using the COX-2–selective inhibitor NS-398 (Ouellet and Percival, 1995). Thus, the PMA-mediated activation of PDE4D5 in HASM cells would indeed appear to require COX-2 activity. In addition to this, the action of PMA on PDE4D5 activity was also converted into a transient inhibitory effect (Fig. 2) when HASM cells were treated with the PLA_2 inhibitor, quinacrine (Sakuta and Yoneda, 1994).

PMA Causes the Generation of PGE_2 in the Medium of HASM Cells. The data described above suggest that the ERK-mediated stimulation of PDE4D5 in HASM cells may indeed be mediated via an autocrine effect, allowing for the PGE_2-stimulated activation of PKA. To explore this, we set out to assess whether PGE_2 could be generated in the medium of HASM cells. Indeed, we were able to demonstrate that challenge of HASM cells with PMA led to a rapid increase in extracellular PGE_2 (Fig. 5a). This effect, however, was ablated by treatment of HASM cells with indomethacin, NS-398, and quinacrine as well as PD98059 (Fig. 5a). Thus PMA-mediated PGE_2 generation was, seemingly, dependent upon the action of ERK, PLA_2 and COX2.

These data suggest that COX is present in HASM cells. Indeed, we were able to detect, through immunoblotting with a specific antiserum, the presence of the 72-kDa form of COX-2 (Fig. 5b). In contrast to this, we were unable to identify COX-2 in COS-1 cells even after 60 min of challenge with PMA (Fig. 5b). In HASM cells, however, COX-2 was seemingly fully induced because no changes occurred in the levels of its expression over a 60-min challenge of cells with PMA (Fig. 5b). In neither cell type were we able to detect, immunologically, the presence of COX-1 (data not shown). This is consistent with our observations that the COX-2 selective inhibitor NS-398 was as effective as the nonselective COX inhibitor indomethacin in exerting actions on HASM cells.

We also noted that under the conditions in which we normally began our experiments (16-h serum-starved; see *Experimental Procedures*) we were able to detect PGE_2 in the medium of these resting cells and this was at a concentration of around 1.0 ± 0.2 μM (mean $\pm$ S.D.; $n = 3$). However, after 60 min of challenge with PMA, the PGE_2 level in the medium rose (Fig. 5a) to some 4.8 ± 0.3 μM (mean $\pm$ S.D.; $n = 3$). We wished to determine whether the PMA-elicited changes in medium concentrations of PGE_2 that we observed here were sufficient both to elevate cAMP levels and to activate PKA. Certainly, we were able to demonstrate that PMA challenge

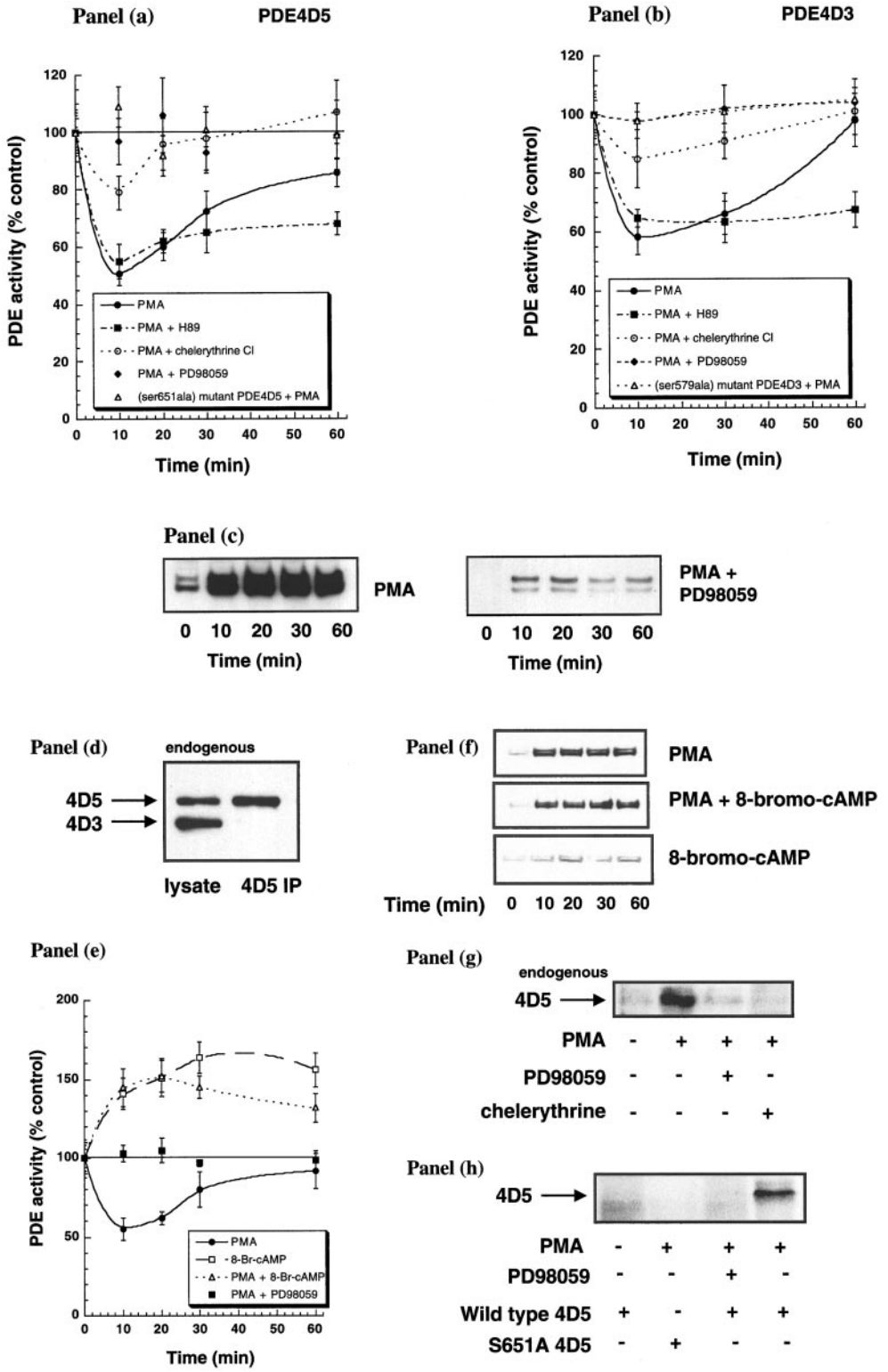

Fig. 4. Challenge of COS-1 cells with PMA. In a, COS-1 cells were transfected to overexpress PDE4D5 and then challenged for the indicated times with PMA (100 nM) either alone or after 20 min of preincubation with PD98059 (50 μM), H89 (10 μM), or chelerythrine (10 μM). They were then harvested, lysed, and subjected to PDE assay as described under *Experimental Procedures*. The wild-type form of PDE4D5 was employed unless specified otherwise when either a mutant form of PDE4D5 with obliterated ERK phosphorylation site was used (ser651ala-PDE4D5). In b, cells were transfected with PDE4D3 rather than PDE4D5 when either wild-type PDE4D3 was used or a mutant form with obliterated ERK phosphorylation site (ser579ala-PDE4D3) (Hoffmann et al., 1999; MacKenzie et al., 2000). Activity is expressed as a percentage of that observed in preparations from control (untreated) cells. Data are given as means ± S.D. for $n = 3$ separate experiments using different transfections. In all of these studies, >98% of the lysate PDE activity was due to the transfected PDE4 species. The specific activity values were in the range of 532 to 585 pmol of cAMP hydrolyzed/min/mg of protein for transfections done with these various PDE4D isoforms. In c, COS-1 cells were challenged for the indicated times with PMA (100 nM), either alone (left part of c) or after 20-min preincubation with PD98059 (50 μM) (right part of c). They were then harvested and 20 μg of protein samples taken for Western blotting analyses. Shown here are Western blots using an antibody specific for identifying the phosphorylated,

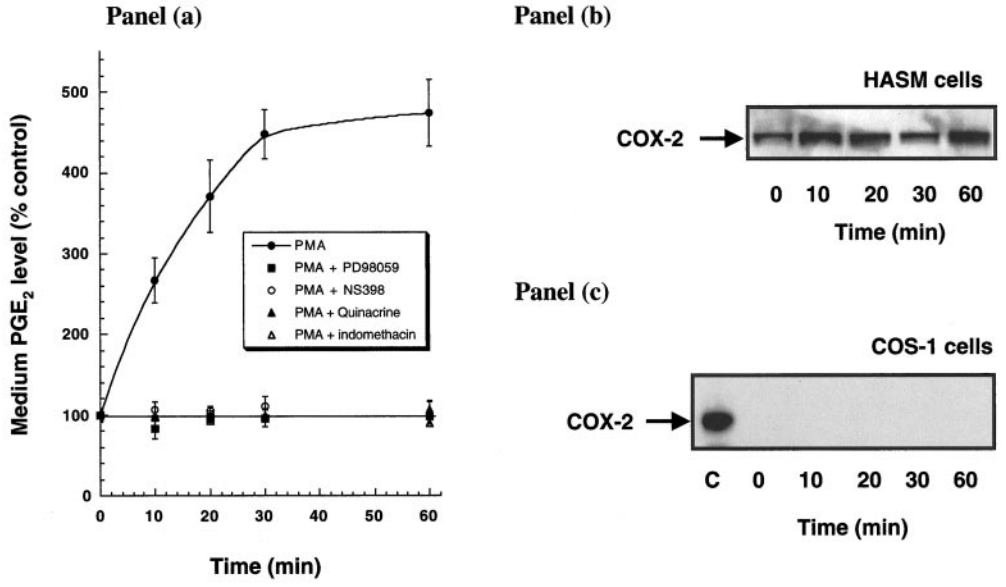

Fig. 5. PMA stimulates PGE_2 generation in HASM cells but does not alter COX-2 expression. a, the change in generation of PGE_2 in the medium of HASM cells. HASM cells were first serum starved for 16 h and then challenged with PMA (100 nM), either alone or together with one of indomethacin (10 μM), PD98059 (50 μM), quinacrine (10 μM), or NS-398 (10 μM). At the indicated times, samples of medium were taken for determination of PGE_2 content as shown under *Experimental Procedures*. The percentage change in the level of PGE_2 in the medium is shown. Data are give as means ± S.D. for $n = 3$ separate experiments. b, HASM cells that were challenged for the indicated times with PMA (100 nM) before being harvested for Western blotting analysis using an antiserum able specifically to detect COX-2. Protein samples (40 μg) were taken in each case. Under all conditions a single immunoreactive species of the expected size, 72 kDa, was identified. c, shows COS-1 cells that were challenged for the indicated times with PMA (100 nM). They were then harvested and 40-μg protein samples were taken for Western blotting analysis using an antiserum able specifically to detect COX-2. No observable immunoreactive species was evident. A positive control (C) for the 72-kDa COX-2 was used. Using an antibody specific for COX-1, we failed to observe an immunoreactive species in homogenates from either HASM cells or from COS-1 cells (data not shown). The data are typical of experiments done at least three times.

of HASM cells caused PKA activation to occur with a time course (Fig. 6a) closely following that for both the stimulation of PGE_2 production (Fig. 5a) and the activation of PDE4D5 (Fig. 2) by PMA in these cells. We believe that PMA-mediated PKA activation in HASM cells occurred primarily through the proposed autocrine loop rather than, for example, any direct stimulatory effect of PKC on adenylyl cyclase. This is because PKA activation was ablated (Fig. 6a) by treatment of HASM cells by not only blocking ERK activation with PD98059 but also by inhibiting COX-2 activity with either indomethacin or NS-398 and PLA_2 activity with quinacrine. Consistent with this notion, the ability of PMA to increase cAMP levels in ASM cells was also inhibited by various of these agents (Fig. 6d). Furthermore, we observed (Fig. 6b) that by directly adding exogenous PGE_2 to HASM cells, over a concentration range that encompassed changes elicited by PMA, PKA activation ensued. The magnitude of this change (Fig. 6b) was similar to that elicited by PMA addition (Fig. 5a). Indeed, the addition of exogenous PGE_2 to HASM cells, over such a range, also caused an increase in the intracellular concentration of cAMP (Fig. 6c). These various data are consistent with PMA serving to activate PKA in HASM cells via a pathway that involves the action of ERK, PLA_2, and COX.

PMA Elicits the Phosphorylation of PDE4D5 at the PKA Target Site, ser126. The highly conserved UCR1 region found in all long form PDE4 enzymes contains a consensus site for phosphorylation by PKA, namely Arg-Arg-Glu-Ser-Phe (Houslay et al., 1998). Detailed studies done on PDE4D3 (Alvarez et al., 1995; Sette and Conti, 1996; Hoffmann et al., 1998) have shown that this provides a target for PKA phosphorylation both in vitro and in intact cells. Such phosphorylation of PDE4 long forms leads to their activation (Alvarez et al., 1995; Sette and Conti, 1996; Hoffmann et al., 1998) and also to the ablation of the inhibitory effect of phosphorylation by ERK (Hoffmann et al., 1999; Baillie et al., 2000; MacKenzie et al., 2000). Here, we show (Fig. 7a) that challenge of transfected COS-1 cells with the adenylyl cyclase stimulator forskolin leads to a time-dependent increase in PDE4D5 activity that is similar to that shown previously for PDE4D3 (Hoffmann et al., 1998). Such an increase in activity was not seen using the Ser126Ala mutant form of

activated forms of ERK-1 and ERK-2 that migrate at 42 and 44 kDa (arrows). Not shown are blots using an antibody able to detect ERK1 and ERK2 in both phosphorylated and nonphosphorylated states, which yielded identical response in all tracks, showing that no change in either expression of ERK or loading of gels occurred. d, a Western blot, using a PDE4-specific antisera, identifying recombinant PDE4D3 and PDE4D5 standards; endogenous PDE4D3 (95 kDa) and PDE4D5 (105 kDa) in COS-1 cell extracts and endogenous PDE4D5 that had been selectively immunoprecipitated from COS-1 cells using a PDE4D5-specific antiserum. e, the change in selectively immunoprecipitated, endogenous PDE4D5 activity, relative to control (100%), from COS-1 cells treated with for the indicated times with PMA (100 nM) alone or with 8-bromo-cAMP (100 μM) alone. Also, cells were treated with PMA together with either PD98059 (10 μM) or 8-bromo-cAMP (100 μM). f, shows imaging analysis of endogenous PDE4D5 selectively immunoprecipitated from COS-1 cells labeled with $[^{32}P]P_i$. Shown are control, untreated cells and those treated for 10 min with either PMA (100 nM) alone or with one of PD98059 (10 μM) or chelerythrine (10 μM). g, shows imaging analysis of recombinant VSV-epitope-tagged PDE4D5 selectively immunoprecipitated, using a VSV-specific mAb, from COS-1 cells labeled with $[^{32}P]P_i$. Shown are control, untreated cells and those treated for 10 min with either PMA (100 nM) alone or with one of PD98059 (10 μM) or chelerythrine (10 μM). In one experiment, as indicated, the S651A mutant form of PDE4D5 was used whose ERK phosphorylation site is disrupted. The data are typical of experiments done at least three times.

PDE4D5, where the target site for PKA phosphorylation in UCR1 was destroyed (Fig. 7a). Neither was it observed if cells were incubated with the PKA-selective inhibitor, H89 (Fig. 7a).

Using a novel polyclonal antiserum (PS54-UCR1-A1) raised against a phospho-peptide encompassing the PKA target serine located in UCR1 of PDE4D5 and PDE4D3, we were able to detect a single immunoreactive species in PDE4D5 transfected COS-1 cells that had been treated with forskolin to activate adenylyl cyclase (Fig. 7b). This species migrated with an identical molecular size (106 kDa) to that of PDE4D5 visualized using a PDE4D-specific antibody. The immunoreactive species identified by the phospho-RRESF antiserum was not seen using forskolin-treated COS-1 cells transfected with the Ser126Ala mutant form of PDE4D5 (Fig. 7b). Neither was it seen in forskolin-treated cells to which the PKA inhibitor H89 had been added (Fig. 7b). We also noted that it did not detect recombinant PDE4D5 that had been phosphorylated in vitro with recombinant ERK2 (data not shown). These data suggest that the phospho-RRESF antiserum can specifically detect the PKA-activated form of PDE4D5 when it is phosphorylated at Ser126 within UCR1. We used this antiserum to probe PDE4D5 that had been immunoprecipitated using a PDE4D specific antiserum from HASM cells (Fig. 7c). This showed that PMA treatment did indeed cause the PKA-mediated phosphorylation of PDE4D5 in these cells. Such an action was ablated by PD98059, indomethacin, and also by H89 (Fig. 7c).

To pursue the notion that PKA activation was indeed involved in the stimulatory effect of PMA on PDE4D5 activity in HASM cells, we challenged these cells with PMA in the presence of the PKA inhibitor, H89 (Fig. 2b). As with indomethacin addition (Fig. 2b), this served to switch the effect of PMA on PDE4D5 activity from one of activation to one of inhibition. Such observations underpin the notion that stimulation of PDE4D5 in HASM cells occurs through the action of PKA that has been activated by an ERK-controlled autocrine loop. Despite such profound changes in the effect of PMA on PDE4D5 activity in HASM cells caused by both indomethacin and H89, neither compound served to affect the ability of PMA to cause the generation of P-ERK forms (Fig. 3).

We then attempted to do the converse of the above experiment by seeing if we could ablate the ability of PMA to inhibit endogenous PDE4D5 activity in COS-1 cells by artificially elevating cAMP levels (Fig. 4e). To do this, we treated COS-1 cells with the cell-permeable cAMP analog 8-bromo-cAMP and simultaneously challenged them with PMA, which elicited the overall stimulation of PDE4D5 activity to a degree similar to that seen when cells were challenged with 8-bromo-cAMP alone (Fig. 4e). Under these conditions, the ability of PMA to activate ERK was unchanged (Fig. 4f).

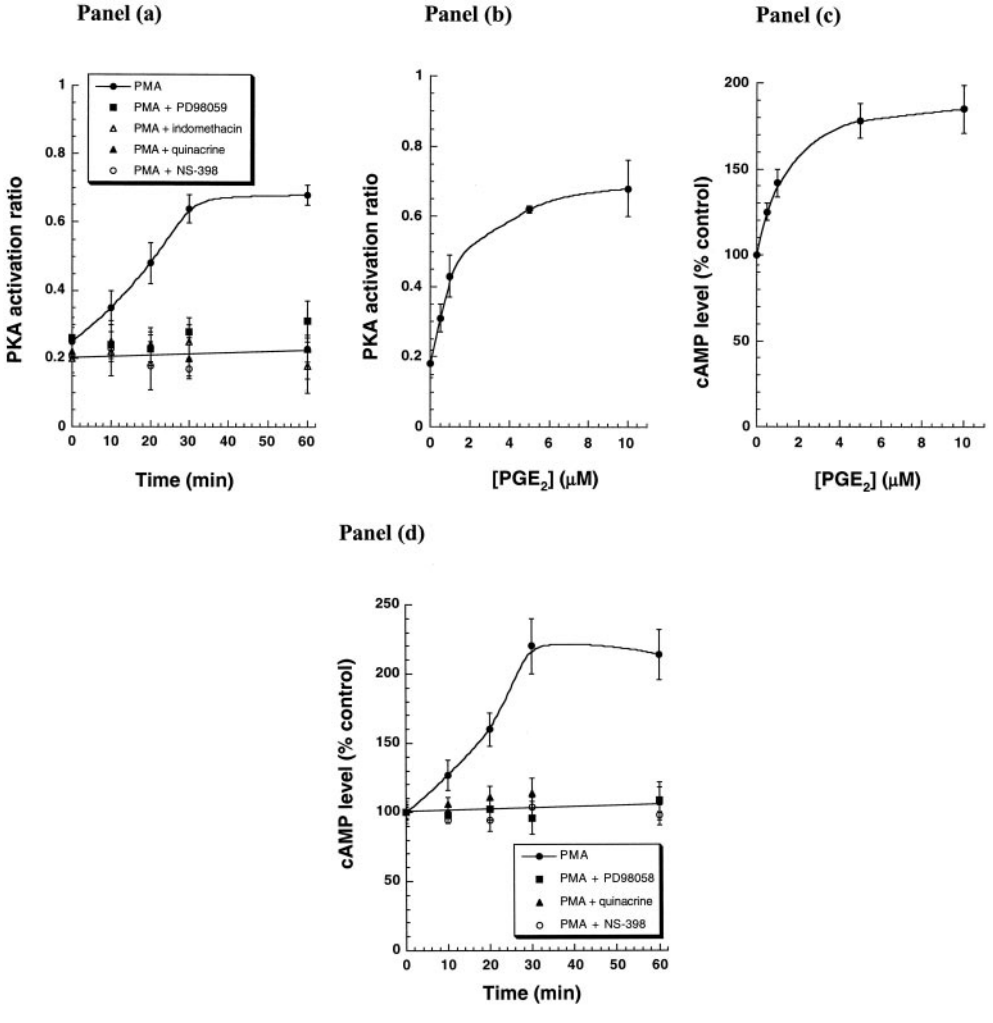

Fig. 6. Increases in PKA activity and cAMP accumulation in HASM cells. In a, HASM cells were challenged for the indicated times with either PMA (100 nM) alone or after 20 min of preincubation with either PD98059 (50 μM), indomethacin (10 μM), quinacrine (10 μM), or NS-398 (10 μM). Cells were then harvested for determination of PKA activity. This was expressed as an activation ratio by comparing these activities with the PKA activity under maximal cAMP stimulation as described under *Experimental Procedures*. In b, the activation ratio of PKA was similarly determined in HASM cells that had been treated for 5 min with the indicated range of PGE_2 concentrations. In c, the percentage change in the intracellular cAMP levels were determined in HASM cells that had been treated for 5 min with the indicated range of PGE_2 concentrations. In d, the percentage change in the intracellular cAMP levels were determined in HASM cells that had been treated with PMA (100 nM) for the indicated times in the absence or presence of either PD98059 (50 μM), quinacrine (10 μM), or NS-398 (10 μM). Data are given as means $\pm$ S.D. for $n = 3$ separate experiments.

These data are supportive of the notion that the inhibitory effect that ERK exerts on PDE4D5 can be negated if cAMP levels are elevated as seen in HASM cells.

Interestingly, PDE4D5 was transiently inhibited in HASM cells treated with PMA in the presence of indomethacin (Fig. 2b). This occurred in a fashion similar to that of PMA on both PDE4D3 and PDE4D5 activity in transfected COS-1 cells (Fig. 4, a and b) and for endogenous PDE4D5 in COS-1 cells (Fig. 4f). In studies done on transfected COS cells, we have shown (Hoffmann et al., 1999; MacKenzie et al., 2000) that the transient inhibitory effect is related to subsequent PKA activation. This occurs because the initial ERK-mediated inhibition of such long PDE4D isoforms causes an increase in cAMP levels, leading to the activation of PKA. The subsequent PKA-mediated phosphorylation of these PDE4D long isoforms serves to ablate ERK-mediated inhibition (Hoffmann et al., 1999).

Unlike the action of indomethacin, however, the PKA inhibitor H89 allowed PMA challenge of HASM cells to achieve a sustained rather than a transient inhibitory effect over the time course of the experiment (Fig. 2). Such a form of sustained inhibition of PDE4D5 was identical to that seen in COS-1 cells challenged with either EGF (Hoffmann et al., 1999; MacKenzie et al., 2000) or PMA (Fig. 4a) in the presence of H89. This is consistent with a role for PKA activation in defining the transient nature of the response of PDE4D5 to phosphorylation by ERK (Hoffmann et al., 1999).

Conclusion

We have shown previously that ERK-mediated phosphorylation within the catalytic unit of various PDE4 isoforms, including PDE4D5, exerts an inhibitory effect on their activity (Hoffmann et al., 1999; MacKenzie et al., 2000). In contrast to this, however, PKA can phosphorylate a target serine within the UCR1 region of long PDE4 isoforms, causing their activation (Sette and Conti, 1996; Hoffmann et al., 1998). Such stimulatory PKA phosphorylation also serves to ablate the inhibitory effect of ERK phosphorylation of PDE4 long isoforms (Hoffmann et al., 1999; Baillie et al., 2000; MacKenzie et al., 2000). We identify here an autocrine mechanism in HASM cells whereby ERK stimulation can lead to the activation rather than inhibition of a PDE4 long isoform (Fig. 8).

Studies by various investigators have shown that PMA challenge will lead to the PKC-mediated activation of ERK. This can be expected to cause the direct inhibitory phosphorylation of PDE4D5 (Fig. 8). However, in smooth muscle cells, activated ERK has been shown to cause the phosphorylation and activation of PLA_2 (Lin et al., 1993; Pyne et al., 1997). This provides arachidonic acid that can be metabolized by COX-2 (Graves et al., 1996; Pyne et al., 1997) to generate and release PGE_2. The released PGE_2 can then be expected to bind to stimulatory adenylyl cyclase-linked cell surface receptors (Graves et al., 1996; Stillman et al., 1999), thus leading to an increase in cAMP production and the activation

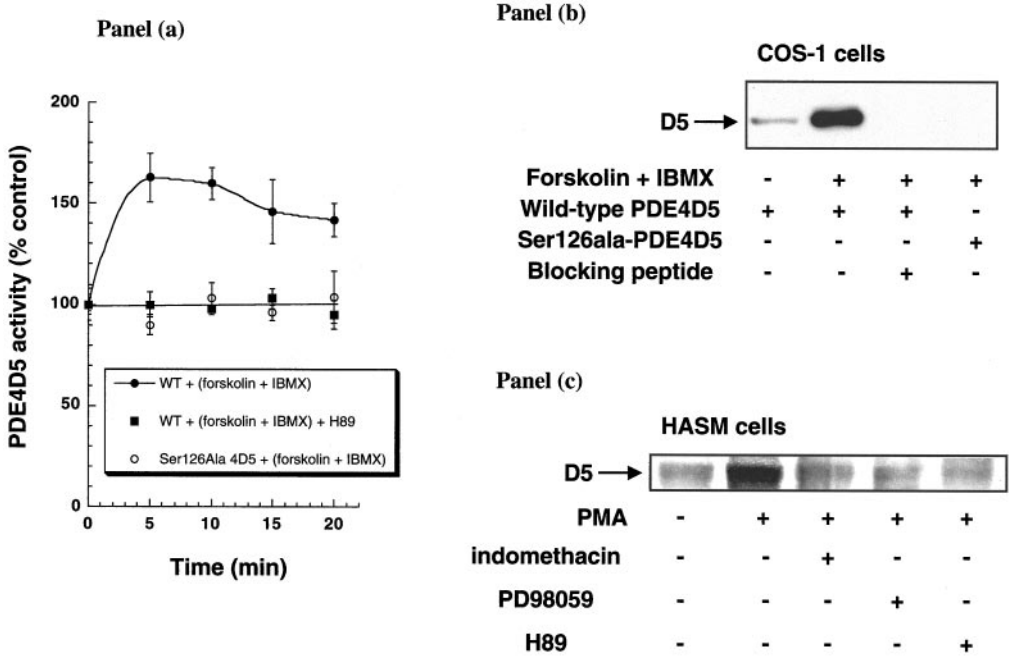

Fig. 7. Phosphorylation of ser126 in PDE4D5. a, data for the activity of recombinant wild type PDE4D5 or the Ser126Ala mutant form of PDE4D5, as indicated, in lysates from transfected COS-1 cells. Cells were treated with forskolin (100 μM) and IBMX (100 μM) together in either the absence or presence of H89 (10 μM) for the indicated time before harvesting, generation of lysates, and determination of PDE activity. Data are given as means $\pm$ S.D. for $n = 3$ separate transfections. The PDE activity in lysates of control (untreated) cells, transfected with either wild type PDE4D5 or the Ser126Ala mutant form of PDE4D5, did not change (<8%) over the 20-min period of the experiment. b, immunoblots done with the PS54-UCR1-A1 antiserum. This was designed to detect specifically the PKA phosphorylated, phospho-serine (S*) form of the Arg-Arg-Glu-Ser*-Phe PKA consensus motif found in the conserved UCR1 of all long PDE4 isoforms. This antiserum was generated using a phosphopeptide whose sequence was SQRRES*FLYRSDSDYDLSP. COS-1 cells were transfected so as to express either wild-type PDE4D5 or the Ser126Ala mutant form. As indicated, cells either were or were not treated as above with forskolin and IBMX to increase cAMP concentrations. This led to a 56 to 65% increase in the activity of PDE4D5 (range; $n = 3$). In one instance, the antiserum was preincubated with the peptide used to raise the antiserum (blocking peptide) before immunoblotting. In c, HASM cells were, as indicated, either treated or not with PMA (100 nM) for 15 min and in some instances after pretreatment for 20 min with either indomethacin (10 μM), PD98059 (50 μM), or H89 (10 μM). Cells were then harvested, lysed, and subject to immunoprecipitation with a PDE4D specific antiserum. The immunoprecipitates were then subject to immunoblotting with the phospho-RRESF antiserum. Data are representative of $n = 3$ separate experiments.

of PKA. Such activated PKA might then be expected to phosphorylate PDE4D5, causing the ablation of any inhibitory effect of direct phosphorylation by ERK and allowing for its net activation (Fig. 8). Consistent with this, we show that PMA exerted a net stimulatory effect on PDE4D5 activity that could readily be ablated by the inhibition of either PKC, PKA, PLA_2, or COX, as well as by preventing ERK activation. In addition to this, we have been able to show here that challenge of HASM cells with PMA led to the generation of PGE_2 in the medium as well as increased cAMP levels and the activation of PKA (Figs. 5a, 6, a and d). Furthermore, addition of exogenous PGE_2 to HASM cells, over a similar range of concentrations to that elicited in the medium by PMA challenge also led to PKA activation and increased accumulation of cAMP (Fig. 6, b and c). Indeed, the activation of PDE4D5 by PMA in HASM cells (Fig. 2) followed a time course similar to that seen for both the generation of extracellular PGE_2 (Fig. 5a) and the activation of PKA (Fig. 6a).

The inhibitory effect of PMA challenge on the activity of recombinant forms of either PDE4D3 or PDE4D5 expressed in COS-1 cells is thus very different from the stimulatory effect seen for PDE4D5 in HASM cells (this study) and PDE4D3/5 in RASM cells (Liu and Maurice, 1999). Perhaps this is true in part because COS-1 cells do not seem to express COX isoforms and hence lack a key component that would allow the activation of ERK to cause the activation of PKA as seen in ASM cells (Fig. 8). Nevertheless, an underlying inhibitory action of PMA on PDE4D5 activity in HASM cells, caused presumably through its direct phosphorylation by ERK, could be uncovered by ablating various stages of the pathway that led to PKA activation by ERK (Fig. 8), such as inhibition of COX-2 and PLA_2. This implies that at least a fraction of PDE4D5 in HASM cells is subject to direct phosphorylation by ERK. Unfortunately, we were unable to address this directly because we have been unable to generate phospho-specific antisera to the ERK site, unlike the PKA site in UCR1. Furthermore, the extremely low levels of PDE4D5 protein expression militate against any examination of labeled phospho-peptides to determine phosphorylation at multiple sites. The ability of PMA to cause the indirect activation of PKA allowed PKA to phosphorylate PDE4D5 at Ser126 in UCR1 and in so doing negate any inhibitory effect of direct phosphorylation of PDE4D5 by ERK (Hoffmann et al., 1999). That a net activation of PDE4D5 was seen here in HASM cells may result from differences in the relative efficiencies of both ERK and PKA to phosphorylate PDE4D5. There could be differences in the efficiency with which phosphatases dephosphorylate PDE4D5 at these two very different sites.

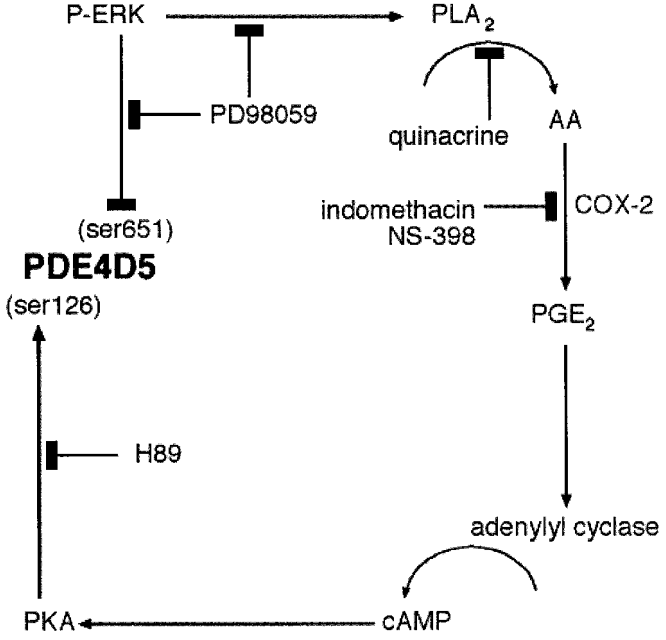

Fig. 8. Schematic of the network through which ERK may regulate PDE4D5 activity in HASM cells. Stimulatory processes are shown by arrows and inhibitory pathways by T-bars.

The *PDE4D* gene encodes a family of isoenzymes that are differentially regulated by ERK phosphorylation of their catalytic unit by virtue of the differences in their N-terminal UCR regulatory modules (Hoffmann et al., 1999; MacKenzie et al., 2000). The direct phosphorylation of the catalytic unit of long isoforms by ERK elicits inhibition that can be ablated by PKA phosphorylation of UCR1. In contrast to this, however, short forms are insensitive to intervention by PKA because they lack UCR1 and, in contrast to long isoforms, can either be activated (short) or slightly inhibited (super-short) by ERK phosphorylation (Baillie et al., 2000; MacKenzie et al., 2000). In this study, we have identified a further degree of sophistication in the panel of options that are available to ERK to alter the activity of PDE4D long isoenzymes such as PDE4D5. This is where the autocrine stimulation of PKA in smooth muscle cells leads to the indirect activation of PKA by ERK. This autocrine system serves to reprogram the cellular action of ERK on PDE4 long isoforms to elicit their net stimulation, rather than inhibition, as seen in this study for the long isoform PDE4D5 (Fig. 8). Thus, the cell-type–specific expression of PDE4D isoenzymes and their ability to be phosphorylated by PKA serves to direct very different responses of PDE4D isoenzyme activity to ERK action. Such differences may explain why ERK activation elicits a net increase in PDE4 activity in both vascular smooth muscle cells (Liu and Maurice, 1999) and in FDCP2 myeloid cells (Ahmad et al., 1999) but causes inhibition of long PDE4D isoforms expressed in COS-1 and 3T3-F442A fibroblasts and in human embryonic kidney 293 cells (Hoffmann et al., 1999; MacKenzie et al., 2000). Similar reprogramming may occur in cell types that express adenylyl cyclase isoforms able to be directly phosphorylated and activated by PKC, for this may also generate rapid PKA activation able to prevent the inhibitory effect of ERK phosphorylation of long PDE4 isoforms.

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Address correspondence to: Dr. Miles D. Houslay, Molecular Pharmacology Group, Division of Biochemistry & Molecular Biology, Davidson & Wolfson Buildings, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. E-mail: m.houslay@bio.gla.ac.uk