The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579

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The extracellular receptor stimulated kinase ERK2 (p42^{MAPK})-phosphorylated human cAMP-specific phosphodiesterase PDE4D3 at Ser579 and profoundly reduced (~75%) its activity. These effects could be reversed by the action of protein phosphatase PP1. The inhibitory state of PDE4D3, engendered by ERK2 phosphorylation, was mimicked by the Ser579→Asp mutant form of PDE4D3. In COS1 cells transfected to express PDE4D3, challenge with epidermal growth factor (EGF) caused the phosphorylation and inhibition of PDE4D3. This effect was blocked by the MEK inhibitor PD98059 and was not apparent using the Ser579→Ala mutant form of PDE4D3. Challenge of HEK293 and F442A cells with EGF led to the PD98059-ablatable inhibition of endogenous PDE4D3 and PDE4D5 activities. EGF challenge of COS1 cells transfected to express PDE4D3 increased cAMP levels through a process ablated by PD98059. The activity of the Ser579→Asp mutant form of PDE4D3 was increased by PKA phosphorylation. The transient form of the EGF-induced inhibition of PDE4D3 is thus suggested to be due to feedback regulation by PKA causing the ablation of the ERK2-induced inhibition of PDE4D3. We identify a novel means of cross-talk between the cAMP and ERK signalling pathways whereby cell stimuli that lead to ERK2 activation may modulate cAMP signalling.

Keywords: cyclic AMP phosphodiesterase/ERK2/MAP kinase/phosphorylation/protein kinase A

Introduction

Cyclic AMP (cAMP) exerts tissue-specific effects on a variety of cellular functions including growth and differentiation. The levels of this second messenger are determined through the concerted action of synthesis achieved through adenylyl cyclase activity and degradation through cAMP phosphodiesterase (PDE) activity (Beavo, 1995; Manganiello *et al.*, 1995; Houslay and Milligan, 1997; Houslay *et al.*, 1997). In each case, the proteins which exhibit these various activities are encoded by large

multi-gene families, producing isoenzymes with distinct regulatory properties and intracellular localizations (Houslay and Milligan, 1997). This diversity is matched by a large family of distinct anchor proteins (AKAPs) for cyclic AMP-dependent protein kinase (protein kinase A, PKA), which serve to locate PKA activity to distinct intracellular sites (Rubin, 1994; Scott and McCartney, 1994; Faux and Scott, 1996). Thus, the regulation and organization of cAMP signalling in cells is provided by a complex network of proteins.

cAMP phosphodiesterases provide the sole means of degrading cAMP in cells and thus are placed in a key position for regulating cellular processes by altering cAMP levels (Thompson, 1991; Torphy *et al.*, 1993; Beavo *et al.*, 1994; Bolger, 1994; Conti *et al.*, 1995; Manganiello *et al.*, 1995; Houslay *et al.*, 1997). Indeed, in many cell types PDE inhibitors have been shown to be capable of increasing cellular cAMP levels and modulating a variety of physiological responses. The members of the PDE4 enzyme family show widespread expression in a variety of cell types and tissues (Torphy *et al.*, 1993; Bolger, 1994; Conti *et al.*, 1995; Houslay *et al.*, 1997; Hughes *et al.*, 1997). They specifically hydrolyse cAMP, for which they exhibit low K_m values, indicating a pivotal role for these enzymes in modulating cellular cAMP levels and hence cellular function and, indeed, PDE4 selective inhibitors can serve as potent anti-inflammatory agents (Dent and Giembycz, 1995; Souness and Rao, 1997; Torphy, 1998). PDE4 enzymes are encoded by four genes, each of which generates a number of distinct isoenzymes by alternative mRNA splicing (Bolger, 1994; Conti *et al.*, 1995; Houslay *et al.*, 1997). Products of any single PDE4 gene all have a common unit, consisting of a catalytic region together with a C-terminal region. Each isoenzyme is then distinguished by a unique N-terminal region. The so-called 'long' PDE4 isoenzymes are characterized (Bolger, 1994) by two PDE4-specific homology regions termed upstream conserved regions 1 and 2 (UCR1 and UCR2, respectively). These are located between the unique N-terminal region and the catalytic region. The 'short' PDE4 isoenzymes lack UCR1. The N-terminal and UCR regions appear to have regulatory properties associated with intracellular targeting to distinct sites (Shakur *et al.*, 1993; Scotland and Houslay, 1995; Houslay *et al.*, 1997) and also for conferring susceptibility to regulation by phosphorylation (Conti *et al.*, 1995; Sette and Conti, 1996).

It has recently been shown (Alvarez *et al.*, 1995; Sette and Conti, 1996; Hoffmann *et al.*, 1998) that the commonly expressed long PDE4D3 form can be phosphorylated by PKA. This occurs at two distinct sites: Ser13 within the unique N-terminal region of the enzyme and Ser54, which is found within the UCR1 region. It is the phosphorylation of Ser54 by PKA which leads to a marked activation of PDE4D3 (Sette and Conti, 1996; Hoffmann *et al.*, 1998),

a state which can be mimicked by the Ser54→Asp mutant of PDE4D3. Such PKA-mediated phosphorylation of PDE4D3 has been suggested (Sette *et al.*, 1994; Conti *et al.*, 1995) to provide part of a cellular desensitization mechanism which is related to the action of hormones that stimulate adenylate cyclase.

The receptor tyrosyl kinase EGF has been shown to be able to activate adenyl cyclase. This involves the physical interaction (Sun *et al.*, 1997) of the EGF receptor with the stimulatory G-protein G_s and the subsequent tyrosyl phosphorylation of this G-protein (Poppleton *et al.*, 1996). It is also dependent upon the expression of the adenyl cyclase type-V isoform (Chen *et al.*, 1995). A major signalling role of the EGF receptor is to stimulate the p42/44^{MAP} kinase (extracellular signal-related kinase, ERK)-related pathway which leads, amongst other actions, to effects on cellular transcription processes. In cells that express c-Raf, it has been shown that under conditions of adenylate cyclase stimulation the increased cAMP levels and ensuing activation of PKA led to the phosphorylation and inhibition of c-Raf (Cook and McCormick, 1993; Wu *et al.*, 1993; Hordijk *et al.*, 1994; Kolch *et al.*, 1996; Mischak *et al.*, 1996). Thus, an EGF-mediated increase in cAMP levels offers the possibility of modulating the activity of this pathway in cells where the c-Raf isoenzyme is expressed.

In this study we identify a new route whereby EGF and, presumably, other ligands able to activate ERK2 may regulate intracellular cAMP levels. This is based upon our observations that the cAMP-specific phosphodiesterase, PDE4D3, has an ERK consensus phosphorylation site at Ser579; a residue located towards the C-terminal end of the catalytic domain of PDE4D3. We show here that PDE4D3 can be phosphorylated at Ser579 by the action of ERK2 (p42^{MAPK}) both in transfected COS1 cells challenged with EGF and also *in vitro* using recombinant ERK2. Such phosphorylation by ERK2 led to a profound inhibition in the activity of PDE4D3 with accompanying elevation of intracellular cAMP levels. The inhibited state of ERK2-phosphorylated PDE4D3 could be mimicked by the Ser579→Asp mutant. We suggest that the ERK2 phosphorylation and inhibition of PDE4D3 may provide a novel route for effecting changes in cAMP signalling, highlighting a new point of cross-talk between key signalling pathways.

Results

ERK2-mediated phosphorylation of HSPDE4D3 *in vitro*

PDE4D3 is a commonly expressed isoenzyme which serves as a target for phosphorylation by PKA, leading to a marked enzyme activation (Sette *et al.*, 1994; Alvarez *et al.*, 1995; Hoffmann *et al.*, 1998). The consensus sequence for phosphorylation of target proteins by ERKs (p42/44^{MAPK}) has been demonstrated to be of the form $PX_n(S/T)P$, where X_n is normally one, but can be two residues which are either neutral or basic, but not acidic in nature (Alvarez *et al.*, 1991; Gonzalez *et al.*, 1991). We were intrigued to note that Ser579, a residue located towards the C-terminal end of the catalytic region of PDE4D3, lies within such a consensus sequence, being provided by PQSP. We thus set out to determine whether

Fig. 1. Schematic of PDE4D3 showing PKA and ERK phosphorylation sites. The human PDE4D3 isoform (DDBJ/EMBL/GenBank accession No. L20970) consists of 673 amino acids (Bolger *et al.*, 1997). It contains a putative catalytic unit which is thought to extend from Met225 to Asp611 (Houslay *et al.*, 1997). It exhibits the two characteristic features of long PDE4 isoforms (Bolger *et al.*, 1993), namely the UCR1, extending from residues Arg51 to Asn109, and UCR2, extending from Glu134 to Pro213. LR1 and LR2 are the two linker regions that connect UCR1 to UCR2, and UCR2 to the catalytic unit, respectively (Houslay *et al.*, 1997). At its extreme N-terminus is a 16-residue region which is unique to this particular PDE4 isoform and which arises through alternative mRNA splicing (Bolger *et al.*, 1996). Shown are the two sites for phosphorylation by PKA, of which modification at Ser54 causes enzyme activation (Alvarez *et al.*, 1995; Sette and Conti, 1996; Hoffmann *et al.*, 1998). Also shown is the ERK2 phosphorylation site analysed in this study. The proposed consensus sequences for PKA and ERK action are indicated, where 'x' is any amino acid, Ø indicates a hydrophobic residue and others are single letter amino acid code. The predicted size of PDE4D3 is 77 kDa but, as with all other long PDE4 isoforms, it migrates on SDS-PAGE with an aberrantly high molecular size of 95 kDa (Bolger *et al.*, 1997; Houslay *et al.*, 1997). There is a rat homologue to human PDE4D3 (Swinnen *et al.*, 1989) (DDBJ/EMBL/GenBank accession No. U50159).

PDE4D3 could be phosphorylated by ERKs and if this had any potential functional significance. In these studies we wished to be able to analyse phosphorylated and mutant forms of PDE4D3, free of any possible contamination of endogenously expressed PDE4D species that might be found in COS-1 cells. To obviate this we chose to use, as we have described before (Hoffmann *et al.*, 1998), an epitope-tagged version of human PDE4D3, which allowed us to immunoprecipitate the recombinant enzyme selectively for analysis. We placed this small epitope tag at the C-terminus of PDE4D3, as current evidence indicates that this would be unlikely to alter enzyme properties (Lobban *et al.*, 1994; Houslay *et al.*, 1997), which indeed proved to be the case (Hoffmann *et al.*, 1998). As we (Hoffmann *et al.*, 1998) and others (Sette *et al.*, 1994; Sette and Conti, 1996) have shown before, PDE4D3 could be phosphorylated by protein kinase A *in vitro* (Figure 2A). However, we show here (Figure 2A) that PDE4D3 could also be phosphorylated *in vitro* by ERK2 but not by various other kinases such as raf1, MEK, p90rsk and MAPKAP kinase 2 (MAPKAPK2). No phosphorylation ensued in the absence of added kinases. These data suggest that PDE4D3 may provide a substrate for ERK2 as well as for PKA.

In order to assess whether indeed Ser579 did provide the site for phosphorylation *in vitro* by ERK2, we generated the Ser579→Ala mutant so as to disrupt the putative target serine for ERK action (Figure 1). Consistent with Ser579 providing the target for phosphorylation, we were able to demonstrate (Figure 2B) that the Ser579→Ala mutant of PDE4D3 could not be phosphorylated by ERK2. In contrast, mutation of a nearby residue, namely Ser581→Ala, which lay just outside the putative ERK consensus motif (Figure 1), did not prevent ERK2 from phosphorylat-

Fig. 2. PDE4D3 provides a substrate for *in vitro* phosphorylation by ERK2. (A) This shows an autoradiograph of an SDS-PAGE gel for recombinant human PDE4D3 (95 ± 3 kDa) treated with the indicated protein kinases in a buffer containing [³²P]ATP as described in Materials and methods. This led to the labelling of a single 95 kDa species when incubations were performed with PKA and ERK2, but not with raf1, MEK, p90rsk and MAPKAPK2. (B) This shows autoradiographs of SDS-PAGE gels for recombinant mutant forms of human PDE4D3 treated either with (+) or without (–) the protein kinase ERK2 in a buffer containing [³²P]ATP as described in Materials and methods. Data is shown for the Ser579→Ala PDE4D3 mutant (Ala579) and the Ser581→Ala PDE4D3 mutant (Ala581). The position expected on the gel for migration of PDE4D3 is indicated. (C) ³²P-labelled PDE4D3 was generated by the action of ERK2. This was treated either with (+) or without (–) the protein phosphatase PP1 before analysis by SDS-PAGE and subsequent autoradiography. These are typical data from experiments performed at least three times.

ing this mutant enzyme (Figure 2B). Such data support the notion that ERK2 phosphorylates PDE4D3 and that this occurs at a single site, namely Ser579. As such a site is within a potential consensus motif for ERK action, then we consider it probable that ERK2 serves to phosphorylate PDE4D3 directly.

We then determined the activity of PDE4D3 under conditions where no further increase in labelling of PDE4D3 by ERK2 had occurred (30 min) and, presumably, phosphorylation had been completed. This demonstrated that the $V_{\max}$ of the enzyme had decreased to $25 \pm 12\%$ of that of the original activity (mean ± SD; $n = 5$ separate experiments). In control experiments performed with PDE4D3 incubated in the absence of added ERK2, no change in PDE4D3 activity occurred, with activity being $96 \pm 7\%$ of that of the starting value ($n = 3$). The ERK2-mediated inhibition of PDE4D3 could be reversed by treatment of ERK2-phosphorylated PDE4D3 with protein phosphatase 1 (PP1), whereupon PDE activity returned to $91 \pm 8\%$ of that of the original ($n = 3$). This was concomitant with the loss of radiolabel (³²P) from the enzyme (Figure 2C). In contrast, treatment with PP2A failed to cause the dephosphorylation of PDE4D3 (data not shown). The inhibitory phosphorylation of PDE4D3, caused by ERK2, was restricted to an effect on the $V_{\max}$ of PDE4D3 as the K_m for cAMP hydrolysis by this phosphodiesterase was unchanged, being $0.5 \pm 0.11 \mu\text{M}$ for the native enzyme and $0.6 \pm 0.2 \mu\text{M}$ for ERK2 phosphorylated PDE4D3 ($n = 3$).

We have previously shown (Hoffmann *et al.*, 1998) that it was possible to mimic the PKA activated state of PDE4D3 using the Ser54→Asp mutant. We thus generated

Fig. 3. Phosphorylation of PDE4D3 mutants *in vitro* by ERK2 and PKA. The indicated recombinant human PDE4D3 were treated with the indicated protein kinases in a buffer containing [³²P]ATP as described in Materials and methods prior to SDS-PAGE. Subsequent analysis of phosphorylation was done by phosphorimager (upper panels) and the amount of PDE4D3 detected by immunoblotting with a mAb specific for the vsv epitope, and visualization using ECL (lower panels). (A) PKA was used to phosphorylate wild-type PDE4D3 (wt) as well as the Ser579→Asp (Asp579) and the Ser579→Ala (Ala579) mutant forms. Labelling was identical in all instances (<5% difference for three separate experiments of which a typical one is shown here). (B) ERK2 was used to phosphorylate wild-type PDE4D3 (wt), as well as the Ser554→Asp (Asp54) single and the Ser13→Asp:Ser54→Asp (Asp13, 54) double mutant forms. Labelling was identical in all instances (<5% difference for three separate experiments, of which a typical one is shown here).

the Ser579→Asp mutant of PDE4D3 in order to try to analyse a form of PDE4D3 which might mimic an enzyme preparation that had been stoichiometrically phosphorylated by ERK2. The $V_{\max}$ of the Ser579→Asp mutant was $21 \pm 8\%$ of that of the native enzyme, with a K_m of $0.45 \pm 0.12 \mu\text{M}$ cAMP ($n = 3$). In contrast, the $V_{\max}$ of the Ser579→Ala 'null' mutant was unchanged at $95 \pm 8\%$ of that of the native PDE4D3 with a K_m of $0.47 \pm 0.17 \mu\text{M}$ cAMP ($n = 3$). As described above, treatment of ERK2 phosphorylated PDE4D3 with protein phosphatase 1 (PP1) could both cause dephosphorylation (Figure 2C) and restore PDE activity to a level comparable to that seen using native PDE4D3 which had not been phosphorylated by ERK2. In contrast, we found that treatment of the Ser579→Asp mutant PDE4D3 form with PP1 did not affect phosphodiesterase activity of this mutant PDE. Rather, it remained at a level which was $25 \pm 3\%$ that of the wild-type enzyme which had not been phosphorylated by ERK2 ($n = 3$). Such data indicated that the Ser579→Asp mutant did indeed mimic the inhibited ERK2 phosphorylated state of PDE4D3. We suggest that the ERK2-mediated phosphorylation of PDE4D3, at Ser579, elicits the inhibition of PDE4D3 and that this effect can be reversed by PP1 phosphatase action.

If PDE4D3 was to become inhibited by ERK2 phosphorylation in intact cells, then any subsequent increase in cAMP that might ensue could be expected to lead to PKA activation. We therefore needed to know whether ERK2-phosphorylated PDE4D3 could provide a substrate PKA and vice versa. In order to assess this unequivocally, it is crucial to have preparations of PDE4D3 that have been stoichiometrically phosphorylated by the first kinase to present as substrates for the second kinase. Such a situation is difficult to achieve unless high amounts of recombinant enzyme are available for analysis, which was not the case here. However, as specific aspartate mutants of PDE4D3 appear to mimic the PKA and ERK2 phosphorylated

states, they potentially provide useful models of stoichiometrically phosphorylated species. In Figure 3 we show that the Ser579→Asp mutant of PDE4D3, which mimics the ERK2 phosphorylated state, can provide a substrate for PKA. Conversely, the Ser54→Asp and the Ser13→Asp:Ser54→Asp PDE4D3 mutants can both provide a substrate for ERK2 (Figure 3). Thus, it appears that phosphorylation of PDE4D3 by either ERK2 or PKA is unlikely to prevent the other kinase from acting upon PDE4D3; i.e. the modifications in phosphorylation status are not mutually exclusive.

As the activities of the Ser54→Asp and Ser579→Asp mutants, respectively, mimic the activity of the PKA and ERK2 fully phosphorylated forms of PDE4D3, we determined the activity of the Ser54→Asp:Ser579→Asp double mutant. We surmised that such a mutant might reasonably be expected to reflect the combined effect of stoichiometric stimulatory phosphorylation by PKA at Ser54 and stoichiometric inhibitory phosphorylation by ERK2 at Ser579. Intriguingly, this double mutant enzyme had a $V_{\max}$ some $82 \pm 9\%$ of that of the wild-type enzyme, whilst exhibiting a K_m value for cAMP which was similar to that of the wild-type enzyme at $0.52 \pm 0.07 \mu\text{M}$ ($n = 3$). Such a value contrasts with a $V_{\max}$ of $272 \pm 21\%$ expressed by the Ser54→Asp mutant, relative to that of the native enzyme. Such an activity status of the Ser54→Asp:Ser579→Asp double mutant suggests that if PDE4D3 was to be phosphorylated by the combined actions of both PKA and ERK2, then the activity of such a doubly modified enzyme might be expected to be similar to that of the unmodified native enzyme. One might then surmise that if phosphorylation and inhibition of PDE4D3 by ERK2 led to an increase in intracellular cAMP levels, then any subsequent activation of PKA might cause the phosphorylation of PDE4D3 and ablation of its ERK2-inhibited state.

As a control for these studies we also generated the double mutant Ser54→Ala:Ser579→Ala. This mutant PDE4D3 form exhibited a $V_{\max}$ value some $93 \pm 7\%$ of that of the wild-type enzyme, with a K_m value of $0.46 \pm 0.12 \mu\text{M}$ cAMP ($n = 3$), indicating that little or no change in activity resulted from mutation of these two serine residues to alanine.

EGF-stimulated phosphorylation of HSPDE4D3 in intact cells

As a test system to evaluate whether PDE4D3 might be phosphorylated and inhibited through ERK action in intact cells, we employed COS1 cells that had been transfected to express PDE4D3. We were able to demonstrate (data not shown) that in COS1 cells, EGF served to cause the tyrosyl phosphorylation of a 170 ± 3 kDa species (Figure 4A) which, presumably, reflected the autophosphorylation of the EGF receptor itself. Such a receptor tyrosyl kinase is known to activate the $p42^{\text{MAPK}}/p44^{\text{MAPK}}$ (ERK) pathway in COS (Lange-Carter *et al.*, 1993; Fanger *et al.*, 1997) and other cells (Cook *et al.*, 1993). Here we show that EGF treatment caused a diminution in the mobility of the 42 kDa ERK2 species found in COS1 cells (Figure 3C). This is consistent with EGF causing the phosphorylation of ERK2 to form the more slowly migrating phosphorylated form of ERK2 (Blumer and Johnson, 1994). Such an effect was observable within 5 min of exposure of COS1

Fig. 4. EGF challenge of COS1 cells elicits the activation of ERK2 and the phosphorylation of PDE4D3. **(A)** COS1 cells were challenged with EGF (50 ng/ml) in either the presence (+) or absence (–) of 20 μM PD98059. This was performed for the indicated times prior to harvesting and subjecting extracts to SDS–PAGE prior to immunoblotting with an ERK-specific antibody. In untreated cells, a single 42 kDa species was evident which indicates the presence of ERK2 ($p42^{\text{MAPK}}$). After 5 and 10 min exposure to EGF, a novel, more slowly migrating species was evident which suggest that EGF causes ERK2 activation and phosphorylation, yielding the more slowly migrating phospho-ERK2. The ability of EGF to elicit the formation of the more slowly migrating phospho-ERK2 was attenuated by the MEK inhibitor PD98059. **(B)** COS1 cells were transfected with vsv-epitope tagged PDE4D3, labelled with $[^{32}\text{P}]\text{P}_i$ and then challenged for 5 min either with or without 50 ng/ml EGF. After cell harvesting and lysis, PDE4D3 was immunoprecipitated using a mAb to the vsv-epitope, subjected to SDS–PAGE and autoradiography. A single labelled 96 kDa species was evident in the EGF-treated cells. Label was lost if the immunoprecipitated PDE4D3 was treated with the alkali KOH. **(C)** Experiments were done as described above using either wild-type (wt) PDE4D3 or the Ser579→Ala PDE4D3 mutant (Ala579), as indicated. EGF (50 ng/ml) and PD98059 (20 μM) were added as indicated. A single labelled species of 96 kDa was evident in extracts from COS1 cells transfected with wild-type PDE4D3 and treated with EGF. These are typical data from experiments performed at least three times.

cells to EGF (Figure 3C). This mobility shift was ablated (Figure 3C) if the cells were treated with the MEK inhibitor, PD98059 (Pang *et al.*, 1995). These data are consistent with the presence of a functional EGF receptor in COS1 cells that is able to activate ERK2 through a pathway involving MEK.

In order to evaluate whether PDE4D3 could become phosphorylated as a result of EGF challenge of COS1 cells, we labelled them with $[^{32}\text{P}]\text{P}_i$ and then used an anti-VSV monoclonal antibody (mAb) to immunoprecipitate PDE4D3 selectively. This clearly showed (Figure 4A) that, after 5 min challenge of cells with EGF, there was a profound increase in the labelling of PDE4D3. Furthermore, such an increase in labelling was ablated when cells were treated with the MEK inhibitor, PD98059 (Figure 4A). The EGF elicited increase in labelling of PDE4D3 was also alkali labile, indicating that it was due to either serine or threonine phosphorylation of the enzyme and not tyrosyl phosphorylation (Figure 4B). Indeed, probing immunoprecipitates of PDE4D3 from EGF-treated COS1

Fig. 5. Inhibition of PDE4D3 and PDE4D5 in COS1 cells challenged with EGF. COS1 cells were transfected with the indicated vsv-epitope tagged forms of either PDE4D3 or PDE4D5. They were then incubated for the indicated times with 50 ng/ml EGF prior to harvesting, disruption and immunoprecipitation of PDE4D3 with an anti-VSV mAb as described previously (Hoffmann *et al.*, 1998). The cAMP-PDE activity in the immunoprecipitates was determined and expressed as a percentage of the value noted at the start of the experiment for each of the PDE4D3 forms. Analyses were performed (A) on wild-type PDE4D3 (wt 4D3); the Ser579→Ala PDE4D3 mutant (Ala579) and wild-type PDE4D3 analysed in the presence of cells which had also been treated with the MEK inhibitor PD98059 (20 μ M) together with EGF (wt 4D3 + PD98059); (B) on COS1 cells transfected to express wild-type PDE4D5 (wt 4D5), and also in COS1 cells which had been treated with the MEK inhibitor PD98059 (20 μ M) together with EGF (wt 4D5 + PD98059); (C) on wild-type PDE4D3 analysed in the presence of cells which had also been treated with 0.5 μ M H89, the selective PKA inhibitor (wt 4D3 + H89); the Ser13→Ala:Ser54→Ala double mutant which does not form a substrate for PKA action (Hoffmann *et al.*, 1998); and the Ser54→Ala mutant which is not activated by PKA (Hoffmann *et al.*, 1998). Data shown are from three separate experiments showing means $\pm$ SD.

cells with a mAb specific for phosphotyrosine indicated that PDE4D3 was not tyrosyl phosphorylated (data not shown).

No such increase in the labelling of PDE4D3 was seen in cells that had been transfected with the Ser579→Ala mutant of PDE4D3. These data indicated that EGF stimulation of ERK2 in intact COS1 cells led to the ERK2-mediated phosphorylation of PDE4D3, confirming the *in vitro* studies which suggest that PDE4D3 provides a substrate for ERK2 action.

Challenge of COS1 cells with EGF causes the inhibition of PDE4D3

In order to evaluate whether changes in PDE4D3 activity occurred during EGF challenge of COS1 cells, we determined the cAMP PDE activity of recombinant PDE4D3 that had been immunoprecipitated using the VSV-mAb (Figure 5A). Such analyses demonstrated clearly that challenge of COS1 cells with EGF caused a marked reduction in the activity of PDE4D3. This occurred without achieving any change in the levels of expression of PDE4D3 as determined by immunoblotting with the anti-VSV mAb (data not shown). Indeed, in all of the assays described, equivalent amounts of PDE4D3 immunoreactive protein were taken for analysis on the basis of quantification, using immunoblotting of the immunoprecipitated enzyme with the anti-VSV mAb.

In contrast to this, no such reduction in activity occurred if PDE4D3-transfected COS1 cells were treated with EGF in the presence of the MEK inhibitor PD98059 (Figure 6), indicating that inhibition of PDE4D3 was dependent upon ERK2 activation. In addition to this, we did not observe (Figure 5A) any change in PDE4D3 activity when analysing COS1 cells that had been transfected with the Ser579→Ala PDE4D3 mutant which, unlike the wild-type enzyme, does not provide a substrate for ERK2 (Figure 2). Such studies suggest that PDE4D3 can be inhibited by ERK2-mediated phosphorylation in intact cells and are consistent with the *in vitro* analyses of PDE4D3 described above. These, together, indicated that the ERK2-mediated phosphorylation of PDE4D3 at Ser579 led to a diminution in PDE activity and that this can be observed both *in vitro* and in intact cells.

PDE4D5 is a recently cloned long PDE4D isoenzyme (Bolger *et al.*, 1997) which differs from PDE4D3 solely by virtue of its unique N-terminal region. It thus shares with PDE4D3 a consensus site for phosphorylation by ERK2, in this case Ser651 in the motif PQSP. We see here that, as with PDE4D3, when PDE4D5 was transiently expressed in COS1 cells then EGF challenge caused a marked inhibition of the activity of this PDE4D isoenzyme (Figure 5B). Inhibition of PDE4D5 was ablated if cells were challenged with EGF in the presence of PD98059 (Figure 5B), consistent with the inhibitory effect being due to the action of ERK2.

EGF challenge of HEK293 and 3T3-F442A cells causes the inhibition of endogenously expressed PDE4D3 and PDE4D5

In order to address the possibility that overexpression of PDE4D3 in transiently transfected COS1 cells might have led it to provide an inappropriate substrate for ERK2 action, we set out to determine whether natively expressed

Fig. 6. Inhibition of endogenously expressed PDE4D3 and PDE4D5 in HEK293 cells challenged with EGF. (A) Extracts of HEK293 cells were immunoblotted with a PDE4D-specific mAb, identifying the 95 kDa PDE4D3 and 105 kDa PDE4D5 isoenzymes (lane 1), which co-migrated with recombinant forms (not shown). An antiserum specific for the PDE4D5 isoenzyme allowed for the selective immunoprecipitation of this isoenzyme (lane 2) and subsequent selective immunoprecipitation of the PDE4D3 isoenzyme (lane 3). (B) HEK293 cells were challenged with either EGF (50 ng/ml) alone (●,○) or together with PD98059 (◇,▲) for the indicated times prior to harvesting, disruption and the selective immunoprecipitation and activity assay of PDE4D3 (●,◇) and PDE4D5 (○,▲). The specific activity of PDE4D3 and PDE4D5 was 5.2 ± 0.3 and 5.2 ± 0.1 pmol/min/mg protein in extracts from untreated cells. Data are given as means $\pm$ SD for $n = 3$ independent experiments.

PDE4D3 could be inhibited. To do this we analysed the human kidney epithelial (HEK293) cells which express both the 95 kDa PDE4D3 isoenzyme, and another long PDE4D species, the 105 kDa PDE4D5 isoenzyme (Figure 6A). These isoenzymes differ solely by virtue of having distinct N-terminal regions, arising through alternative mRNA splicing (Bolger *et al.*, 1997). EGF treatment of these cells caused the activation of ERK2 (data not shown) in a similar fashion to that seen using COS1 cells (Figure 3C). PDE4D3 and PDE4D5 were selectively immunoprecipitated (Figure 6A) from HEK293

cells so as to determine their activity. This allowed us to show that challenge of HEK293 cells with EGF caused a similar rapid inhibition of the activity of both PDE4D3 and PDE4D5 (Figure 6B). In contrast, EGF failed to cause any reduction in the activity of either PDE4D3 or PDE4D5 if cells were also treated with the MEK inhibitor, PD98059 (Figure 6B). Challenge of cells with PD98059 alone did not alter (<5% change) the activity of either enzyme over the timecourse of the experiment.

In a similar fashion we analysed the mouse fibroblast 3T3-F442A cell line. This expresses murine versions of PDE4D3 and PDE4D5, which have been shown to be detected by the same antisera used to analyse the human enzymes due to sequence conservation (MacKenzie *et al.*, 1998). In these cells, PDE4 activity constitutes some $46 \pm 4\%$ of the total PDE activity (mean $\pm$ SD), with PDE4D3 and PDE4D5 providing some $16 \pm 2\%$ and $18 \pm 3\%$ of the total PDE4 activity, respectively. The specific activities of PDE4D3 and PDE4D5 were 1.10 ± 0.05 and 1.31 ± 0.06 pmol/min/mg protein, respectively. After challenge of F442A cells for 10 min with EGF (50 ng/ml), PDE4D3 and PDE4D5 activities dropped to $68 \pm 3\%$ and $71 \pm 5\%$, respectively, of that observed in extracts from untreated cells. In contrast to this, if cells were challenged with EGF in the presence of PD98059 (20 μ M), then PDE4D3 and PDE4D5 activities were $105 \pm 8\%$ and $99 \pm 3\%$, respectively, that observed in extracts from untreated cells ($n = 3$; 1 μ M cAMP substrate). Treatment of cells with PD98059 alone had no effect (<5%) on the activities of either PDE4D3 or PDE4D5.

Such data indicate that natively expressed PDE4D3 and PDE4D5 can be inhibited by challenge of both HEK293 and F442A cells with EGF and that this process requires MEK activation as it is inhibited by PD98059. These data are consistent with the notion that PDE4D3 and PDE4D5 can be inhibited in intact cells through ERK action.

EGF elicits changes in the intracellular cAMP levels of COS1 cells transfected with HSPDE4D3

We set out to discover whether the ERK2-mediated inhibition of PDE4D3 could have a role in the modulation of intracellular cAMP levels in COS1 cells challenged with EGF. Such experiments showed (Figure 7) that only when COS1 cells were transfected with PDE4D3 did cAMP levels rise upon challenge with EGF. This increase in [cAMP] paralleled the decrease in PDE4D3 activity (Figure 5A), signifying an inverse relationship. We have shown previously (Bolger *et al.*, 1997; Hoffmann *et al.*, 1998) that PDE4D3 activity accounts for >96% of the total PDE activity in homogenates of PDE4D3-transfected COS1 cells. Thus, the activity of this recombinant enzyme will dominate cAMP catabolism in such transfected cells, which account for ~50% of the total. In non-transfected or vector-only transfected cells then EGF did not increase cAMP levels (Figure 7), providing a null background for this cell population. Such an observation indicates that EGF was unable to activate adenylate cyclase in COS1 cells. Thus, the change in cAMP levels elicited by EGF in COS1 cells which had been transfected with PDE4D3 appears to be due solely to an action at the PDE level; namely inhibition of recombinant PDE4D3. Although EGF has been suggested to be able to activate adenylate cyclase, this process does not appear to occur in all cell types and

Fig. 7. Intracellular cAMP levels in COS1 cells challenged with EGF. Cells were transfected with the indicated vsv-epitope tagged forms of PDE4D3. They were then incubated for the indicated times with 50 ng/ml EGF prior to harvesting for the analysis of intracellular cAMP levels. Changes in cAMP levels are shown as percentages of the starting values. Analyses were performed on wild-type PDE4D3 (wt 4D3); the Ser579→Ala PDE4D3 mutant (Ala579); wild-type PDE4D3 analysed in the presence of cells which had also been treated with the MEK inhibitor PD98059 (20 μ M) (wt 4D3 + PD98059); the Ser579→Ala PDE4D3 mutant analysed in the presence of cells which had also been treated with the MEK inhibitor PD98059 (20 μ M) (Ser579→Ala + PD98059); and COS1 cells which had not been transfected with PDE4D3 (control). This reflects data from three separate experiments showing means $\pm$ SD. Intracellular cAMP concentrations in untreated cells were in the range of 0.35–0.45 pmol/10⁶ cells.

where it does occur the process does not involve ERK action (Nair *et al.*, 1990; Chen *et al.*, 1995; Sun *et al.*, 1997). The EGF-mediated increase in intracellular cAMP levels of PDE4D3-transfected cells was abolished if cells were treated with the MEK inhibitor, PD98059 (Figure 7), being consistent with EGF increasing cAMP levels through the ERK2-catalysed phosphorylation of PDE4D3 rather than any action on adenylate cyclase. In addition, we were able to show (Figure 7) that if COS1 cells were transfected with the Ser579→Ala PDE4D3 mutant, which is unable to be phosphorylated by ERK2 action, then challenge with EGF failed to elicit any change in intracellular cAMP levels in such transfected cells. These data clearly demonstrate that the ERK2-mediated phosphorylation and inhibition of PDE4D3 was paramount in eliciting changes in intracellular cAMP concentrations in COS1 cells transfected to express recombinant PDE4D3.

We did observe, however, that the EGF-mediated inhibition of PDE4D3 expressed in COS1 cells appeared to be transient (Figure 5A). Indeed, reversal of this inhibitory state appeared to begin at a point where cAMP levels had achieved a maximum value. Our *in vitro* studies described above indicated that the PKA-mediated phosphorylation of PDE4D3 served to reverse the inhibitory effect of ERK2 action on PDE4D3 activity. It thus seemed possible that the transience of the EGF-mediated inhibition in intact cells might have been due to subsequent action of PKA

on PDE4D3 as a result of the EGF-mediated increase in intracellular cAMP levels. To address this possibility, we analysed the activity of the Ser54→Ala mutant of PDE4D3 (Figure 5C), which cannot be activated by PKA action (Sette and Conti, 1996; Hoffmann *et al.*, 1998). Such a species was rapidly inhibited, some 5 min after EGF challenge of COS1 cells, in a similar fashion to wild-type PDE4D3. However, in contrast to wild-type PDE4D3, this mutant form remained in a stably inhibited state over the time course of the experiment (Figure 5C). Similarly, if analyses of the wild-type PDE4D3 were done using cells that had been incubated with the PKA selective inhibitor H89 {*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide} (Chijiwa *et al.*, 1990), then as with the Ser54→Ala mutant of PDE4D3, EGF engendered a stably inhibited state (Figure 5C). These independent approaches suggest that the transience of the inhibitory response seen using wild-type PDE4D3 over the period analysed was primarily due to the negation of ERK2-mediated inhibition by the subsequent phosphorylation of PDE4D3 at Ser54 by PKA. Such an effect may also characterize the PDE4D5 isoenzyme. Whilst treatment of PDE4D5-transfected COS1 cells with the PKA inhibitor H89 (0.5 μ M) had no effect (<5%) on PDE4D5 activity, it served to enhance the inhibitory effect of EGF in a fashion similar to that seen in cells transfected to express PDE4D3. Thus, after 20 min challenge with EGF (50 ng/ml) alone, PDE4D5 activity in COS1 cells was $74 \pm 5\%$ of that seen in control cells, whereas PDE4D5 activity fell to $38 \pm 7\%$ in cells that had been treated with both EGF and H89 ($n = 3$). By analogy with PDE4D3, the PDE4D5 isoenzyme may be subject to stimulatory regulation by PKA where this action serves to attenuate the inhibitory effect of ERK2.

Discussion

We demonstrate here that EGF can cause the rapid inhibition of the human PDE4D3 cAMP-specific phosphodiesterase expressed in intact COS1 cells. This effect is mediated by the action of ERK2 which causes the phosphorylation of PDE4D3 on Ser579. Such a phosphorylation process, and accompanying inhibition of PDE4D3 activity, could also be demonstrated *in vitro* using purified ERK2. In addition, this inhibitory state of PDE4D3 could be mimicked by the Ser579→Asp mutant form of PDE4D3. This effect is not limited to only *in vitro* and transfected systems, as we were able to demonstrate that EGF could inhibit endogenously expressed PDE4D3 through a process ablated by the MEK inhibitor PD98059 in both HEK293 and F442A cells.

EGF has been shown to be able to elicit an increase in the intracellular levels of cAMP in certain cells by an ERK-independent action on adenylyl cyclase (Nair *et al.*, 1990; Chen *et al.*, 1995; Sun *et al.*, 1997). The data presented here indicate an alternative route through which EGF may serve to effect an increase in intracellular cAMP levels, namely by an inhibitory action on the cAMP-specific phosphodiesterase, PDE4D3. This route, however, would be expected to be exploited by any cellular stimulus that led to ERK2 activation. Seemingly, such a response is subject to feedback modulation, as any subsequent phosphorylation of PDE4D3 by PKA serves to ablate the

inhibitory effect of ERK2 phosphorylation on this enzyme. The kinetics of such feedback inhibition can be expected to be dependent upon a number of factors which serve to influence the activation of PKA. One factor in this regard might be the intracellular compartmentalization of PKA isoforms (Scott and McCartney, 1994), serving to exert selective actions on PDE4 isoenzymes. However, a key factor (Houslay and Milligan, 1997) influencing responses is likely to be the magnitude of basal adenylyl cyclase activity in particular cell types. If basal adenylyl cyclase activity is of a sufficient magnitude then PDE inhibition will serve to increase cAMP levels to an extent where PKA activation can ensue. This, seemingly, was the case in COS1 cells. However, in certain cells, e.g. hepatocytes (Houslay, 1990), basal adenylyl cyclase activity is so low that PDE inhibition does not suffice to increase cAMP concentrations to a level able to activate PKA. In such instances, however, any inhibition of PDE activity will serve to accelerate the rate of cAMP accumulation when cells are challenged with an agonist able to activate adenylyl cyclase through a G_s -mediated process (Houslay and Milligan, 1997). Thus, one might expect that the prior ERK2-mediated inhibition of PDE4D3 would serve to sensitize cells with low basal adenylyl cyclase activity to the action of G_s -coupled receptor agonists.

We have used PDE4D3 as a model to analyse ERK2 action for a variety of reasons. First, its regulation by PKA-mediated phosphorylation is well defined (Sette and Conti, 1996; Hoffmann *et al.*, 1998). Secondly, PDE4D3 is expressed in a large number of cell types (Conti *et al.*, 1995). Also, the importance of this enzyme has been inferred from observations (Barnette *et al.*, 1998) showing that an inhibitor, SB207499, which has selectivity for the PDE4D subfamily serves as a potent anti-inflammatory agent. SB207499 lacks the side effects of nausea seen with rolipram {4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone}, a compound which exhibits no selectivity for PDE4D enzymes over those of other PDE4 species (Houslay *et al.*, 1997). However, the related long PDE4D isoenzyme, PDE4D5 is also widely expressed (Bolger *et al.*, 1997; Houslay *et al.*, 1997) and we show here that this isoenzyme can also be inhibited by EGF treatment of COS1 cells. PDE4D5 differs from PDE4D3 in having a distinct N-terminal region (Bolger *et al.*, 1997), but shares the ERK2 phosphorylation site, in this instance Ser651. That the inhibitory effect of EGF on PDE4D5 showed a similar time course to that seen with PDE4D3 and was similarly ablated by PD98059 (Figure 5B) suggests that, as with PDE4D3, it occurred through an ERK2-mediated event. Indeed, that the near reversal of the inhibition of PDE4D5 after 20 min exposure to EGF could be blocked by the PKA inhibitor H89 indicates that PDE4D5 may be subject to a stimulatory phosphorylation by PKA in a similar fashion to PDE4D3; it certainly shares a cognate consensus phosphorylation site for PKA in Ser126. It should be noted in this context that the short form products of the PDE4D gene, PDE4D1 and PDE4D2, lack the UCR1 region which contains the site which allows PKA phosphorylation and activation. Thus, in contrast to the PDE4D long forms, the predicted ERK2-mediated inhibition of these short isoenzymes will not be subject to feedback ablation by PKA. This may provide a key reason

for the selective expression of PDE4D isoenzymes in different cell types.

It is quite possible that other PDE4 enzymes may be regulated by ERK action. PDE4B isoenzymes share an identical motif (PQSP) to that found in members of the PDE4D family and, indeed, bacterially expressed PDE4B2 can be phosphorylated *in vitro* by MAPK (Lenhard *et al.*, 1996). PDE4C enzymes exhibit, in a cognate location, a slightly different motif (PRSP) which is also likely to provide a substrate for ERK2 action (Alvarez *et al.*, 1991; Gonzalez *et al.*, 1991). In contrast to this, PDE4A enzymes have the motif RQSP which is not normally thought to provide a consensus motif for ERK action (Alvarez *et al.*, 1991; Gonzalez *et al.*, 1991). Indeed, we have shown (MacKenzie *et al.*, 1998) that PDE4A5, expressed natively in F442A cells, can in fact be activated by growth hormone and that this is achieved through a process which lies downstream of both PI-3 kinase and p70S6 kinase. The magnitude of this stimulation was not, however, affected by treating cells with PD98059, despite the fact that growth hormone activated ERK2 in these cells (MacKenzie *et al.*, 1997). This might imply that PDE4A species may not provide substrates for ERK2. There are, however, >16 known PDE4 isoenzymes, and the analysis of their complex regulation by phosphorylation will be both challenging and formidable and will undoubtedly have to be done on an isoform-by-isoform basis.

Here, we identify a novel means of cross-talk between the cAMP and ERK2 signalling pathways. This highlights a novel means whereby EGF and other agents able to activate ERKs might increase intracellular cAMP levels, shown here in a model system using a widely expressed PDE4 isoenzyme subject to multisite phosphorylation. The ability of different cell types to express specific complements of PDE4 isoenzymes may thus offer the possibility of tailoring cAMP responsiveness.

Materials and methods

Protein concentration was determined using bovine serum albumin (BSA) as standard (Bradford, 1976). Sequencing was done on an automated sequencing machine (ABI model 373 or 377, Perkin-Elmer) with assays containing 1.5 µg plasmid DNA and 15 pmol of appropriate sequencing oligonucleotide (DNA sequencing kit, Dye Terminator Cycle Sequencing Ready Reaction, Perkin-Elmer). All PCRs were performed in a volume of 25–50 µl containing 200 µM dNTP, 20 pmol of each oligonucleotide, 50–100 ng of DNA-template and 0.2–1 U of *Taq* DNA polymerase. SDS-PAGE was performed as described previously (Laemmli, 1970). mAbs used were for the vsv epitope (Sigma), the C-terminus of PDE4D (a kind gift from Drs K.Ferguson and S.Wolda, ICOS Corp., WA) and ERK2 (a kind gift from Dr M.Harnett, University of Glasgow, UK).

Site-directed mutagenesis of HSPDE4D3

A cDNA encoding the cAMP-specific human phosphodiesterase, PDE4D3, was originally isolated by us using RT-PCR from neuroblastoma cells (Wilkinson *et al.*, 1997). As we described earlier (Hoffmann *et al.*, 1998), this was subcloned into the expression vector pCIneo and a C-terminal VSV epitope tag (Kreis and Lodish, 1986) of amino acid sequence Tyr-Thr-Asp-Ile-Leu-Met-Asn-Arg-Leu-Gly-Lys-STOP was added. The construction of the various Ser13 and Ser54 mutants of HSPDE4D3 has been described in detail previously by us (Hoffmann *et al.*, 1998). The mutations of Ser579 and Ser581 to either alanine or aspartate in HSPDE4D3 was done using the 'QuickChange' Site-Directed Mutagenesis Kit from Stratagene Cloning Systems (La Jolla, CA) according to manufacturer's instructions. Plasmid DNA was prepared from bacterial colonies which showed undigested PCR products on agarose gels and was then sequenced in order to confirm the correct

mutation. The expression plasmid encoding human PDE4D5 has been described previously (Bolger *et al.*, 1997).

Cell culture and transfection

This was performed using the COS1 SV40-transformed monkey kidney cell line maintained at 37°C in an atmosphere of 5% CO₂, 95% air in complete growth medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% penicillin/streptomycin (10 000 U/ml) and 10% fetal calf serum (FCS). Details have been described previously (Bolger *et al.*, 1997; Hoffmann *et al.*, 1998).

EGF stimulation of COS-1 cells

For all stimulation experiments, cells were divided 24 h post DNS-transfection into equal subpools in 6-well culture plates, to guarantee the same amount of expressed protein in every cell extract. After another 24 h of cultivation, the medium was aspirated and cells overlaid with growth medium containing 50 ng/ml EGF. Incubation was 5–30 min. At different time points the medium was discarded, and cells were washed once with PBS before disrupting cells by adding ice-cold buffer C [25 mM Tris-phosphate pH 7.5, 10% glycerol, 2 mM dithiothreitol (DTT), 2 mM EDTA, 1% Triton X-100] supplemented with 10 µM okadaic acid, and 1 mM sodium orthovanadate and protease inhibitor mix (final concentration of 40 µg/ml PMSF, 156 µg/ml benzamide and 1 µg/ml each of aprotinin, leupeptin, pepstatin A and antipain).

Preparation of cell extracts

Forty-eight hours post-transfection, cell monolayers were washed once with PBS and scraped from the culture dish in 1 ml PBS per 9 cm plate, collected by centrifugation and resuspended in 200 µl PDE extraction buffer (20 mM Tris-HCl pH 7.5, 20% glycerol, 15 mM β-mercaptoethanol, 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100) supplemented with 1 mM DTT and a mixture of protease inhibitors at a final concentration of 40 µg/ml PMSF, 156 µg/ml benzamide and 1 µg/ml each of aprotinin, leupeptin, pepstatin A and antipain. Cells were sonicated (Branson sonifier microtip) and then centrifuged for 5 min at 14 000 g. The supernatant was then analysed for the presence of VSV-tagged protein, by immunoblotting using a specific monoclonal antibody as described in detail (Kreis and Lodish, 1986; Soldati and Perriard, 1991), and for cAMP phosphodiesterase activity.

Western blot analysis

SDS-PAGE and subsequent immunoblotting for these PDE4D species was performed as described previously (Bolger *et al.*, 1997; Hoffmann *et al.*, 1998) using ~20 µg protein samples and detection using anti-VSV and anti-PDE4D mAbs and, in some instances, polyclonal antisera specific for either all PDE4D family members or specific for PDE4D5. Analysis of phosphotyrosine proteins was done with PY99 anti-phosphotyrosine antiserum. Analysis of ERK2 used a specific mAb.

Immunoprecipitation

As performed previously (MacKenzie *et al.*, 1998), cell lysate from 5 × 10⁵ transfected COS1-cells was generated using lysis buffer (25 mM HEPES, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100 pH 7.5) containing protease inhibitors. This was incubated with 3–5 µl of anti-VSV mAbs for 60 min on ice followed by 30 min at room temperature. Antigen-antibody complexes were bound to 30 µl of protein G-coupled fast-flow Sepharose for 60 min at room temperature. The immobilized material was collected by centrifugation, washed three times with 1 ml of PDE buffer (20 mM Tris-HCl pH 7.7, 1 mM MgSO₄, 15 mM β-mercaptoethanol) followed by three wash cycles with 1 ml PDE buffer containing 0.5% Triton X-100 and 50 mM NaCl. A further wash cycle was performed with 1 ml of PDE buffer and the Sepharose gel pellet was finally resuspended in PDE buffer. Where indicated, immunoprecipitation was also performed using a polyclonal antiserum and a mAb specific for PDE4D (Bolger *et al.*, 1997). Immunoprecipitations were performed similarly using both HEK293 and F442A cells, although in these cases a polyclonal antiserum specific for PDE4D5 was used to first immunoprecipitate PDE4D5 selectively prior to the use of a PDE4D mAb to immunoprecipitate the PDE4D3 isoenzyme present in these cells.

PDE assays

PDE activity was determined by a modification of the two-step procedure of Thompson and Appleman (1971) as we have described previously (Marchmont and Houslay, 1980). All assays were conducted at 30°C with initial rates taken from linear time courses. Activity was linear in relation to added protein concentration. Kinetic parameters were

determined from PDE assays conducted using 10–15 different cAMP concentrations (0.1–100 μ M). Transfection of COS1 cells led to the novel PDE4 activity comprising >97% of the total cell activity. Mock transfections (vector only) did not alter the endogenous COS cell PDE activity. As a routine, we subtracted the residual endogenous COS1 cell PDE activities done in parallel experiments from those activities found in the PDE4D-transfected cells. Representative studies were also performed on PDE4D species immunoprecipitated using the anti-VSV mAb, with identical results being obtained. Relative specific PDE activity values for the various mutant forms were determined immunologically as described in detail previously (McPhee *et al.*, 1995; Shakur *et al.*, 1995). Untransfected and mock (vector only)-transfected COS1 cells exhibited a PDE activity of 18 ± 5 pmol/min/mg protein, PDE4D3 transfected cells exhibited a PDE activity of 785 ± 22 pmol/min/mg protein and PDE4D5 transfected cells exhibited a PDE activity of 525 ± 12 pmol/min/mg protein ($n = 6$).

Phosphorylation in vitro of PDE4D3

This was performed as described previously (Hoffmann *et al.*, 1998). HSPDE4D3 from 5×10^5 transfected COS1 cells was immunoprecipitated as a complex with protein G–Sepharose. This was incubated for 30 min at room temperature with 1 vol. of phosphorylation buffer (100 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 30 mM β -mercaptoethanol, 10% glycerol) containing 0.1 mM [γ -³²P]ATP (100 Mbq/mmol). The reaction was started by introduction of each of the various indicated protein kinases and allowed to continue for up to 30 min at room temperature, unless stated otherwise. The Sepharose was washed four times with 1 ml PDE buffer and resuspended in PDE buffer for analysis. The concentrations of kinases used was 200 mU Raf-1, 60 mU-ERK2, 100 mU MAPKAPK2, 200 mU MEK1, 200 mU p90rsk and 1 mU PKA.

Dephosphorylation of PDE4D3

Dephosphorylation was analysed using the serine/threonine-specific protein phosphatases PP1 and PP2A. This was done by adding one 'bead-volume' of protein phosphatase buffer (100 mM Tris–HCl pH 7.5, 10% glycerol, 30 mM β -mercaptoethanol, 10 mM MnCl₂) to one 'bead volume' of immunoabsorbed, ERK2-phosphorylated, ³²P-labelled PDE4D3. The dephosphorylation reaction was then started by the introduction of 1 mU of the purified catalytic subunit of either protein phosphatase PP1 or protein phosphatase PP2A in the two reactions, respectively. After an incubation period of 30 min at room temperature, the assays were analysed by SDS–PAGE and then by autoradiography for changes in the radiolabelling of the various PDE4D3 species as well as by assessing cAMP PDE activity.

Metabolic labelling of human PDE4D3 with [³³P]orthophosphate

Transfected COS1-cells (5×10^5) were subdivided after 24 h of cultivation into six-well tissue-culture plates and incubated at 37°C in 5% CO₂, 95% air for another day. The culture medium was aspirated followed by a wash with phosphate-free DMEM and covering of the monolayer with 350 μ l/well of labelling medium containing phosphate-free DMEM, 2% dialysed FCS, 20 mM HEPES pH 7.4, the indicated enzyme inhibitors and 100 μ Ci [³³P]orthophosphate. Cells were incubated overnight under standard conditions which led to isotopic equilibrium of the ATP pool (Hoffmann *et al.*, 1998). They were then treated for 5 min with 50 ng/ml EGF before harvesting on ice. The labelling medium was discarded, cells washed twice with ice-cold PBS and subsequently disrupted in ice-cold cell culture lysis buffer (Clontech) supplemented with 10 μ M okadaic acid. Radiolabelled PDE4D3 was immunoprecipitated as described above and then subjected to SDS–PAGE and autoradiography. In some instances, immunoprecipitated PDE4D3 was treated with KOH as described before (Hoffmann *et al.*, 1998).

Intracellular cAMP determination

This was performed as described previously (Heyworth and Houslay, 1983; Tang and Houslay, 1992).

Acknowledgements

This work was supported by a grant from the Medical Research Council and also by a Wellcome Trust capital equipment grant to M.D.H.

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Received October 21, 1998; revised and accepted December 22, 1998