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Sub-family selective actions in the ability of Erk2 MAP kinase to phosphorylate and regulate the activity of PDE4 cyclic AMP-specific phosphodiesterases

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1 Expressed in intact cells and *in vitro*, PDE4B and PDE4C isoenzymes of cyclic nucleotide phosphodiesterase (PDE), in common with PDE4D isoenzymes, are shown to provide substrates for C-terminal catalytic unit phosphorylation by the extracellular signal-regulated kinase Erk2 ($p42^{MAPK}$).

2 In contrast, PDE4A isoenzymes do not provide substrates for C-terminal catalytic unit phosphorylation by Erk2.

3 Mutant PDE4 enzymes were generated to show that Erk2 phosphorylation occurs at a single, cognate serine residue located within the C-terminal portion of the PDE4 catalytic unit.

4 PDE4 long-form isoenzymes were markedly inhibited by Erk2 phosphorylation.

5 The short-form PDE4B2 isoenzyme was activated by Erk2 phosphorylation.

6 These functional changes in PDE activity were mimicked by mutation of the target serine for Erk2 phosphorylation to the negatively charged amino acid, aspartic acid.

7 Epidermal growth factor (EGF) challenge caused diametrically opposed changes in cyclic AMP levels in COS1 cells transfected to express the long PDE4B1 isoenzyme compared to cells expressing the short PDE4B2 isoenzyme.

8 We suggest that PDE4 enzymes may provide a pivotal point for integrating cyclic AMP and Erk signal transduction in cells with 4 genes encoding enzymes that are either insensitive to Erk2 action or may either be activated or inhibited. This indicates that PDE4 isoenzymes have distinct functional roles, giving credence to the notion that distinct therapeutic benefits may accrue using either PDE4 subfamily or isoenzyme-selective inhibitors.

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Abbreviations: cyclic AMP, cyclic 3′:5′ adenosine monophosphate; EGF, epidermal growth factor; Erk2, extracellular signal-regulated kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PDE, cyclic nucleotide phosphodiesterase; PDE4, cyclic AMP-specific PDE isoenzyme family 4; PKA, cyclic AMP-dependent protein kinase

Introduction

Individual signalling systems consist of a range of discrete components. In a number of instances individual members of a signalling cascade have been shown to be subject to regulation through the action of other signalling systems (Houslay & Milligan, 1997). This, presumably, provides a route whereby signalling responses in cells can be integrated. Flexibility in such cross-talk reactions can be generated by the cell-specific expression of isoenzymes of particular components that are subject to different modes of regulation.

Cyclic AMP (adenosine 3′:5′-cyclic monophosphate) has served as a paradigm for an intracellular second messenger (Houslay & Milligan, 1997). It is involved in mediating the action of a host of processes in specialized cells, ranging from control of various metabolic events, e.g. muscle contraction, secretion and memory. There is now good evidence indicating that the functioning of this pathway is inextricably linked with that of the extracellular signal-regulated kinase (Erk)/mitogen-activated protein kinase (MAPK) pathway. The first evidence for this came from studies showing that the elevation of cyclic AMP in fibroblasts and vascular smooth muscle cells induced a profound inhibition of Erk activation by growth factors (see Burgering *et al*., 1993; Cook & McCormick, 1993; Graves *et al*., 1993; Sevetson *et al*., 1993; Wu *et al*., 1993; Hafner *et al*., 1994). This inhibition was shown to result from the phosphorylation of Raf. However, the effect was cell-type specific as only the Raf-1 isoform was subject to inhibitory regulation. This is not the only point of cross-talk, however, as it has been shown recently that cyclic AMP-dependent protein kinase (PKA) phosphorylation within the KIM region of the PTP and PTP-SL tyrosyl phosphatases prevents their binding to Erk and thus their ability to de-phosphorylate and de-activate this kinase (Blanco-Aparicio *et al*., 1999; Saxena *et al*., 1999). Thus, in cells where these or similar phosphatases act as important regulators of Erk, then PKA activation can be expected to synergize and even sustain Erk activation by inhibiting its dephosphorylation.

The converse direction of regulation has been demonstrated by our observation that Erk2 can phosphorylate and inhibit the PDE4D3 cyclic AMP-specific phosphodiesterase,

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allowing Erk activation to increase cyclic AMP levels in cells where this isoenzyme is expressed (Hoffmann *et al*., 1999). PDE4D3 is one of 16 or more isoenzymes that are encoded by four PDE4 genes (4A, 4B, 4C and 4D) (Houslay *et al*., 1998). These enzymes appear to be vitally important since PDE4-selective inhibitors serve as potent anti-inflammatory agents and can also exhibit anti-depressant properties (Nicholson & Shahid, 1994; Cavalla & Frith, 1995; Dent & Giembycz, 1995; Schudt *et al*., 1995; Giembycz, 1996; Souness & Rao, 1997; Teixeira *et al*., 1997; Houslay *et al*., 1998; Rogers & Giembycz, 1998; Spina *et al*., 1998; Torphy, 1998; Schmidt *et al*., 1999; Torphy *et al*., 1999). The 'signature' feature of PDE4 enzymes is the presence of two highly conserved regions of sequence, called upstream coding region 1 (UCR1) and UCR2, which are found towards the N-terminus of these enzymes and which are distinct from the catalytic unit (Bolger *et al*., 1993; Bolger, 1994; Houslay *et al*., 1998). The so-called 'long' PDE4 isoenzymes exhibit both UCR1 and UCR2 whereas the 'short' PDE4 isoenzymes lack UCR1. Individual PDE4 isoenzymes are then each characterized by the presence of unique N-terminal regions that are believed to be involved in defining intracellular targeting through interaction with other proteins (Houslay *et al*., 1998).

Inhibitory Erk2 phosphorylation of the long isoenzyme, PDE4D3, has been shown to occur at a single site (Ser^{579}) (Hoffmann *et al*., 1999) that lies at the C-terminal end of the PDE4D3 catalytic unit. However, we have recently shown (MacKenzie *et al*., 2000) that this inhibitory effect of Erk2 phosphorylation is directed by the UCR1 and UCR2 regions that have been shown to interact with each other and are believed to form a module whose function is to regulate the activity of the catalytic unit (Beard *et al*., 2000). In contrast to this, the PDE4D1 short form has been shown to be activated by Erk2 phosphorylation. This functional change appears to be directed by the presence of a complete UCR2 in the absence of UCR1 (MacKenzie *et al*., 2000).

PDE4D3 can also be phosphorylated at two sites by PKA. Of these, it is the modification of Ser^{54} in UCR1 that leads to enzyme activation (Sette *et al*., 1994; Alvarez *et al*., 1995; Sette & Conti, 1996; Hoffmann *et al*., 1998). Intriguingly, when PDE4D3 is modified by both Erk2 and PKA, its activity is similar to that of the unmodified enzyme, offering a potential feedback mechanism whereby Erk-mediated inhibition of PDE4D3 can trigger an increase in cyclic AMP levels, allowing PKA to elicit the phosphorylation and re-activation of PDE4D3 (Hoffmann *et al*., 1999).

On the basis of studies done on PDE4D isoenzymes it would appear that long and short isoenzymes behave very differently to the action of phosphorylation by Erk2. However, it is not known whether members of the three other PDE4 families can also be phosphorylated by Erk2 and what, if any, the functional consequences are. PDE4 subfamilies differ in sequence, both within their catalytic units and around the serine residue cognate to that phosphorylated in PDE4 enzymes (Houslay *et al*., 1998). Thus there is no *a priori* reason to expect that members of the different PDE4 families will either provide substrates for Erk2 phosphorylation or, if phosphorylated, show similar changes in activity. Here we show that PDE4B and PDE4C isoenzymes, but not the PDE4A cyclic AMP phosphodiesterase isoenzymes, can be phosphorylated by Erk2. In addition, we demonstrate that Erk2 phosphorylation leads to inhibition of long PDE4B and PDE4C isoenzymes but activation of the commonly expressed PDE4B2 short form.

Methods

Protein concentration was determined using BSA as standard (Bradford, 1976). Sequencing was done on an automated sequencing machine (ABI model 373 or 377; Perkin-Elmer, Beaconsfield, Buckinghamshire, U.K.) with reactions 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 done 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 unit of Taq-DNA-polymerase. SDS – PAGE was performed according to Laemmli (1970). The monoclonal antibodies (mAb) used were for the vsv epitope (Sigma Chemical Co., Poole, Dorset, U.K.) and for the specific C-terminal regions of PDE4A, PDE4B and PDE4C (a kind gift from Dr K. Ferguson and Dr S. Wolda, ICOS Corp., Seattle, U.S.A.) (Bolger *et al*., 1997) and for Erk2 (a kind gift from Dr M. Harnett, University of Glasgow, U.K.). We also employed polyclonal antisera specific for the various PDE4 forms (Shakur *et al*., 1995; Huston *et al*., 1996; 1997; Bolger *et al*., 1997).

Site-directed mutagenesis of PDE4 species

The expression plasmids encoding the various PDE species used in this study have all been described previously. These are PDE4A1 (Sullivan *et al*., 1998), PDE4A8 (Bolger *et al*., 1996), PDE4B1 and PDE4B2 (Huston *et al*., 1997) and PDE4C2 (Owens *et al*., 1997). The various other mutations described in this study were done using the QuickChange® site-directed mutagenesis kit from Stratagene Cloning Systems (La Jolla, CA, U.S.A.) according to the manufacturer's instructions. All mutant constructs were verified by sequencing before use. 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.

Cell culture and transfection

This was done using the COS1 SV40-transformed monkey kidney cell line maintained at 37°C in an atmosphere of 5% CO_2 in complete growth medium containing Dulbecco's modified Eagle medium (DMEM) supplemented with L-glutamine (2 mM), penicillin (10 units ml^{-1}), streptomycin (10 $\mu g\ ml^{-1}$) and 10% foetal calf serum. Details have been described before by us (Hoffmann *et al*., 1999).

Stimulation of COS-1 cells, harvesting and preparation of cell extracts for analysis

This was done as described in detail before by us (Hoffmann *et al*., 1999).

Western blot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS – PAGE) and subsequent immunoblotting for these PDE4 species was done as described before by us (Huston *et al*., 1996; 1997; Bolger *et al*., 1997; Hoffmann *et al*., 1998; 1999; MacKenzie *et al*., 1998; McPhee *et al*., 1999; MacKenzie & Houslay, 2000) using approximately 20 μg protein samples and detection using either mAb or polyclonal antibodies specific for the indicated PDE4 family.

Immunoprecipitation

Cell lysates from COS-1 cells were prepared as described previously (MacKenzie *et al*., 1998). For harvesting, the 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 sodium pyrophosphate, 10% glycerol, 1% Triton X-100, pH 7.5, with added protease inhibitors). This was cleared by centrifugation before further procedures were carried out. For immunoprecipitation, the lysates (150 μg protein) were pre-cleared by incubation with 20 μl of protein-G sepharose 4B fast flow (Amersham) for 30 min at 4°C. This was removed by centrifugation and the PDE immunoprecipitated by incubation with a 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 pellets were washed with lysis buffer and finally with PDE assay buffer (20 mM Tris pH 7.6 with protease inhibitors). Where indicated, immunoprecipitation was also done using either a polyclonal antiserum or a mAb specific for the indicated PDE4 species as described before by us (Huston *et al*., 1996; 1997; Bolger *et al*., 1997; Hoffmann *et al*., 1998; 1999; MacKenzie *et al*., 1998; McPhee *et al*., 1999; MacKenzie & Houslay, 2000).

Assay of cyclic AMP PDE activity

PDE activity was determined by a modification of the two-step procedure of Thompson & Appleman (1971) as described previously by us (Marchmont & Houslay, 1980). All assays were conducted at 30°C with initial rates taken from linear time-courses. Activity was linear with added protein concentration. Transfection of COS1 cells led to the newly expressed PDE4 activity comprising >97% of the total cell activity. Mock transfection (vector only) did not alter the endogenous COS cell PDE activity. As a routine we subtracted the residual endogenous COS-1 cell PDE activities done in parallel experiments from those activities found in the PDE4 transfected cells. Untransfected and mock (vector only) transfected COS1 cells exhibited a PDE activity of 10 ± 5 pmol min^{-1} mg protein^{-1}.

Phosphorylation in vitro *of PDE4 isoenzymes*

This was done as described previously (Hoffmann *et al*., 1998; 1999). PDE4 from 5×10^5 transfected COS1-cells was immunoprecipitated as a complex with protein G-sepharose. This was incubated for 30 min at 4°C with 1 volume of phosphorylation buffer (100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 30 mM beta-mercaptoethanol, 10% glycerol) containing 0.1 mM [γ-^{32}P]-ATP (100 MBq mmol^{-1}). The reaction was started by introduction of each of the various indicated protein kinases and allowed to continue for up to 30 min at 30°C, unless stated otherwise. The sepharose was washed four times with 1 ml PDE buffer and resuspended in PDE buffer for analysis.

Intracellular cyclic AMP determination

This was done as described before by us (Heyworth & Houslay, 1983).

Results

Erk2 causes the inhibition and phosphorylation of PDE4B and PDE4C long forms but not PDE4A long forms

Here we have analysed whether Erk2 can exert actions on recombinant long forms of the PDE4A, PDE4B and PDE4C enzyme families. Doing this we found that epidermal growth factor (EGF) stimulation of Erk2 was able to inhibit both the PDE4B1 and PDE4C2 long isoenzymes when transiently expressed in COS1 cells (Figure 1a,b). Such inhibition was rapid, occurring within around 5 min. Consistent with the action being mediated by Erk action, it was ablated by the MAPK/Erk kinase (Mek) inhibitor, PD98059 (Figure 1a,b).

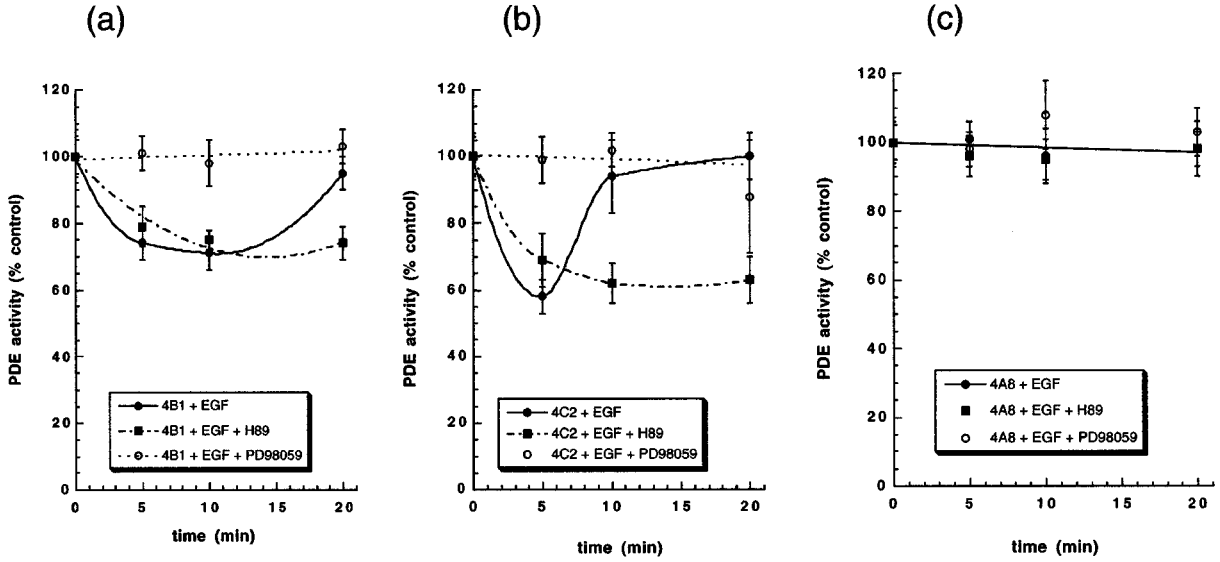

Figure 1 Action of EGF on COS cells expressing long PDE4 isoenzymes. COS1 cells were transfected with the indicated long PDE4 isoenzyme and challenged with EGF (50 ng ml^{-1}) in the absence or presence of the Mek inhibitor, PD98059 (20 μM), or the PKA inhibitor, H89 (0.5 μM), for the indicated time prior to harvesting for the determination of PDE4 activity. Data are mean $\pm$ s.d. for three separate experiments. Various long isoenzymes, namely (a) PDE4B1, (b) PDE4C2 and (c) PDE4A8, were used in these studies, with activities expressed relative to that in the untreated control cells (100%) which were 350–410, 510–620 and 800–870 pmol min^{-1} mg^{-1} protein (range; $n=3$) for PDE4B1, PDE4C2 and PDE4A8, respectively.

In contrast, the activity of the long PDE4A8 isoenzyme was unaffected by challenge of transfected COS1 cells with EGF either in the presence or absence of PD98059 (Figure 1c). This lack of effect on PDE4A activity could be due either to an inability of Erk2 to phosphorylate PDE4A8 or to differences in the structure of this isoenzyme preventing any phosphorylation event resulting in inhibition of PDE activity. To address these alternatives we set out to determine whether Erk2 could phosphorylate these various enzymes. Consistent with PDE4B1 and PDE4C2 being inhibited as a result of Erk2 activation, we were able to show that recombinant Erk2 caused the phosphorylation of both of these isoenzymes (Figure 2a,b). The single target residue for Erk phosphorylation in PDE4D3 is Ser^{579}, which lies within the catalytic unit of this enzyme (Hoffmann *et al*., 1999). PDE4B1 and PDE4C2 both have cognate serine residues. For PDE4B1 this is Ser^{659} and for PDE4C2 it is Ser^{535} (Houslay *et al*., 1998). In order to evaluate whether these serine residues did indeed provide the site for Erk2 phosphorylation we mutated the presumed target serine residue to alanine in each of these two PDE4 isoenzymes. Doing this we found that neither the Ser^{659}Ala-PDE4B1 mutant nor the Ser^{535}Ala-PDE4C2 mutant could be phosphorylated by Erk2 (Figure 2a,b). These data demonstrate that Erk2 phosphorylates these two long PDE4 isoenzymes at a single serine residue that is cognate to the Ser^{579} site that Erk2 phosphorylates in the PDE4D3 long form (Hoffmann *et al*., 1999). In marked contrast to this, however, Erk2 failed to elicit the phosphorylation of the long PDE4A8 isoenzyme (Figure 2c).

We have shown previously that the attenuated activity of Erk2 phosphorylation of PDE4D3 can be mimicked by replacing the serine target for phosphorylation with the negatively charged amino acid, aspartate (Hoffmann *et al*., 1999; MacKenzie *et al*., 2000). Here, we show that the cognate Ser^{659}Asp-PDE4B1 and Ser^{535}Asp-PDE4C2 mutants exhibited activities that were some 67 ± 9 and $68 \pm 4\%$ (mean $\pm$ s.d.; $n = 3$ separate experiments), respectively, of the activity of the wild-type enzymes (100%). In contrast, replacing the serine with the neutral alanine residue, as in the Ser^{659}Ala-PDE4B1 and Ser^{535}Ala-PDE4C2 mutants, had no effect on activity, being unaltered at 94 ± 6 and $94 \pm 5\%$ ($n = 3$), respectively, compared to the activity of the wild-type enzymes (100%). The reduced activities of the aspartate mutants are consistent with Erk2 phosphorylation, eliciting the inhibition of these long PDE4B and PDE4C isoenzymes.

We also noted in these studies that EGF was able to effect a more sustained inhibition of the PDE4B1 and PDE4C2 long forms when cells were also treated with the PKA-selective inhibitor, H89 (Figure 1a,b).

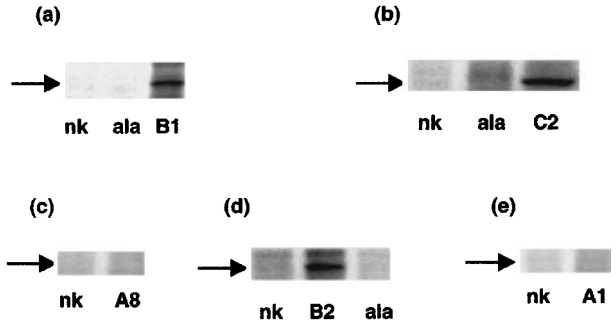

Figure 2 Erk2 phosphorylation of PDE4 isoenzymes. As detailed in Methods, the indicated recombinant PDE4 isoenzymes were immunoprecipitated and then incubated with Erk2 under phosphorylating conditions *in vitro* and then subjected to SDS–PAGE with visualization by phosphorimager. The arrows indicate the expected position for migration of these species. This was confirmed by immunoblotting (not shown). Data are typical of experiments done at least three times. In all cases 150 μg of transfected COS1 cell lysate was taken for the phosphorylation analyses. The various PDE4 species were immunoprecipitated using class-specific antibodies directed at epitopes within their unique C-terminal regions. Tracks are of samples with Erk2 addition to the phosphorylation experiment unless indicated as 'nk'–no kinase (Erk2) addition. Wild type enzymes were used except that in some instances mutant species were used where the putative serine target for Erk2 action was replaced with alanine. The various isoenzymes and constructs used were: (a) 104-kDa long PDE4B1 (B1) also showing the Ser^{659}Ala mutation (ala); (b) 80-kDa long PDE4C2 (C2) with Ser^{535}Ala (ala) mutant; (c) 98-kDa long PDE4A8 (A8); (d) 80-kDa short PDE4B2 (B2) plus the Ser^{487}Ala mutant (ala) and (e) 79-kDa short PDE4A1 (A1). These data are typical of experiments done at least three times.

Erk2 leads to the phosphorylation and activation of the short PDE4B2 isoenzyme

In profound contrast to the inhibitory action of Erk2 on the long PDE4B1 isoenzyme, we observed that EGF challenge of COS1 cells transfected to express the PDE4B2 short form led to an increase in its activity (Figure 3a). This EGF-mediated effect was ablated by PD98059, consistent with such activation being an effect exerted by Erk2.

In the unlikely event that such an effect was related in some way to the recombinant enzyme, we also sought to determine if the endogenously expressed PDE4B2 short form could similarly be activated through Erk2. To do this we chose to investigate the human U937 monocytic cell line, as the only PDE4B species that these cells express is the short isoenzyme, PDE4B2 (Wang *et al*., 1999; MacKenzie & Houslay, 2000). We have shown (MacKenzie & Houslay, 2000) that it is possible to immuno-purify PDE4B2 selectively for analysis using a C-terminal antiserum that is specific for PDE4B enzymes and does not affect enzyme activity (Huston *et al*., 1997). Challenge of U937 cells with EGF in order to activate Erk led to a rapid increase in the activity of endogenous PDE4B2 in a manner that was ablated by PD98059 (Figure 3b). Thus, the PDE4B2 short form, expressed natively in U937 cells, appears also to be activated by ERK action.

Consistent with an action of Erk2 on PDE4B2 we then demonstrated that treatment of recombinant PDE4B2 with recombinant Erk2 (Figure 2d) caused the phosphorylation of this short PDE4 isoenzyme. In contrast, alanine mutation of the putative target site, Ser^{487}, generated a mutant PDE4B2 species that was neither phosphorylated by Erk2 *in vitro* (Figure 2d) nor activated in transfected COS1 cells treated with EGF (Figure 3a). This indicates that PDE4B2 is phosphorylated at a single site within its catalytic unit by Erk2.

Using such phosphorylating conditions *in vitro* (see Methods), we were also able to show that treatment of recombinant PDE4B2 with Erk2 led to an increase in its activity to $132 \pm 5\%$ of the untreated enzyme ($n = 3$). In contrast to this, incubation without Erk2 had no effect ($< 5\%$) on activity. Additionally, we determined the relative activity of a PDE4B2 short-form mutant where the target serine for Erk phosphorylation was replaced by the negatively charged, aspartate residue. This was generated so as to mimic the effect of a form of PDE4B2 that had been stoichiometrically phosphorylated by Erk2. This Ser^{487}Asp-PDE4B2 mutant had an activity that was increased relative to that of the wild-type enzyme, being some $127 \pm 5\%$ compared to wild-type PDE4B2 ($n = 3$). In contrast, the activity of the

Ser^{487}Ala-PDE4B2 (control) mutant was unchanged at $99 \pm 3\%$ ($n=3$) of that of the activity of wild-type PDE4B2.

We also noted that EGF challenge of COS cells transfected to express the short PDE4A1 isoenzyme did not elicit any change in PDE activity (Figure 3b). This is consistent with the lack of effect seen when analysing the long PDE4A8 isoenzyme (Figure 1a). Additionally, treatment of PDE4A1 with recombinant Erk2, under conditions shown to phosphorylate the PDE4B2 short form, caused neither the phosphorylation of PDE4A1 (Figure 2e) nor any change in its activity ($98 \pm 7\%$ of control; $n=3$). That two PDE4A long forms were unable to be phosphorylated by Erk2 suggests that this is likely to be a property of PDE4A family isoenzymes in general rather than being restricted to a particular isoenzyme.

No PDE4C short forms were examined as none have been identified to date (Houslay *et al*., 1998). Indeed, analysis of the structure of the *PDE4C* gene has led to the suggestion that it is highly unlikely that this gene will encode any short isoenzymes (Sullivan *et al*., 1999).

EGF promotes diametrically opposed changes in intracellular cyclic AMP levels in COS cells transfected with either a long or a short PDE4B isoenzyme

When COS1 cells are transfected with plasmids encoding PDE4 isoenzymes, the activity of the recombinant enzymes accounts for over 98% of the total cellular PDE activity and this dominates cyclic AMP degradation (Hoffmann *et al*., 1999). We have shown previously (Hoffmann *et al*., 1999) that EGF challenge of native COS1 cells or those transfected with empty vector failed to cause any change in their cyclic AMP levels. Here we show that challenge with EGF increased intracellular cyclic AMP levels in COS1 cells that had been transfected to express the long PDE4B1 form (Figure 4). However, in marked contrast to this, EGF challenge led to a clear decrease in cyclic AMP levels of COS cells that had been transfected to express the short PDE4B2 isoenzyme (Figure 4). These effects are consistent with Erk2 causing activation of the short PDE4B2 form and inhibition of the long PDE4B1 form. Such effects were not seen in cells that had been treated with PD98059, as expected for an action that involved Erk2 (Figure 4).

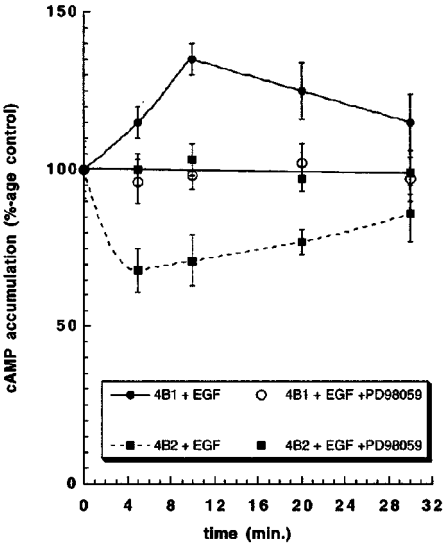

Figure 4 EGF-mediated changes in COS cell cyclic AMP levels. COS1 cells were transfected to express either the long PDE4B1 isoenzyme or the short PDE4B2 isoenzyme. They were then challenged with EGF (50 ng ml^{-1}) and harvested at the indicated time for determination of intracellular cyclic AMP concentration. Data are shown for cells that had been incubated in the absence or presence of PD98059 (20 μM). In untransfected or empty vector-transfected cells EGF did not elicit any change in COS cell cyclic AMP levels. Data represent mean $\pm$ s.d. of $n=3$ separate experiments. In the absence of EGF the intracellular cyclic AMP concentrations were 0.91 pmol 10^{-6} cells in PDE4B1-expressing cells and 1.2 pmol 10^{-6} cells in PDE4B2-expressing cells. These data are typical of experiments done at least three times.

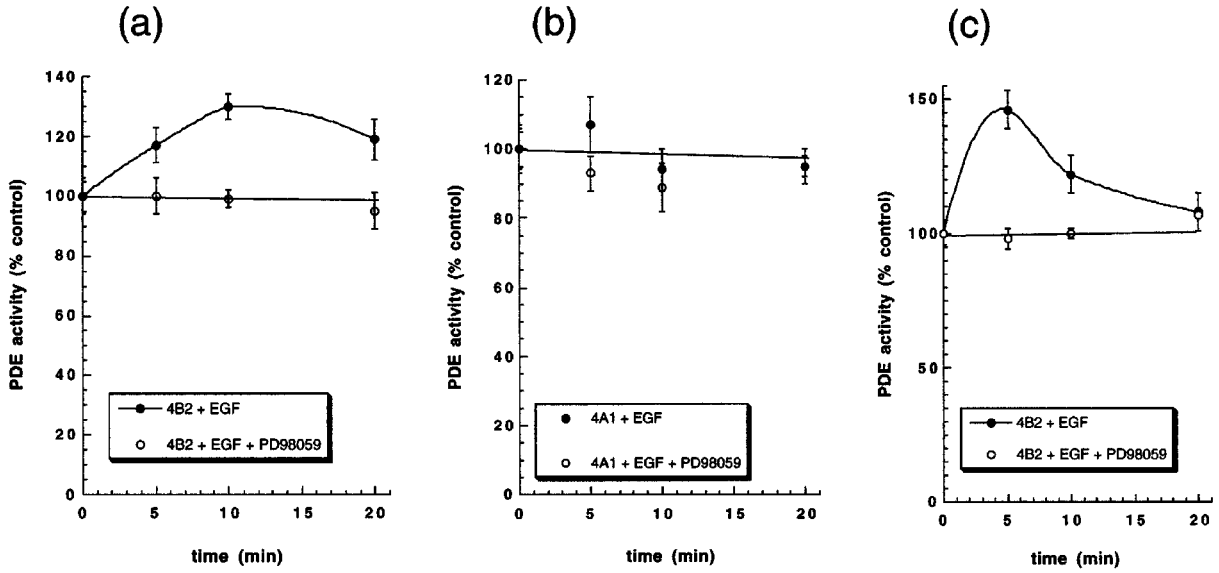

Figure 3 Action of EGF on the activity of short PDE4B2 and PDE4A1 isoenzymes. Experiments were done as per the legend for Figure 2. Shown here are the (a) PDE4B2, (b) PDE4A1 short isoenzymes. Activities are shown relative to those of the untreated controls (100%) with mean $\pm$ s.d. ($n=3$ separate experiments shown). The specific activities of the lysates for COS cells expressing these species were 840–950 and 1150–1220 pmol min^{-1} mg^{-1} protein, respectively, for the species listed above. In addition, we show (c) the effect of EGF treatment on the endogenously expressed short PDE4B2 form found in U937 monocytic cells in the absence and presence of PD98059 (20 μM), the inhibitor of Mek activation. These data are typical of experiments done at least three times.

Discussion

The Erk MAP kinase system provides a pivotal route whereby a variety of growth factors and hormones exert actions on key transcriptional and other cellular processes (Blumer & Johnson, 1994). This pathway has been shown to be subject to cross-talk through interaction with the cyclic AMP signalling pathway at the level of the protein kinase, Raf. Such regulation, however, is cell-type specific, being dependent upon which selection of the three Raf isoenzymes is present. It would appear that, in the PDE4 enzyme family, we have uncovered a complementary family of differentially regulated isoenzymes in the cyclic AMP signalling system. As with Raf isoenzymes, PDE4 isoenzymes are expressed in a cell type specific fashion (Houslay *et al*., 1998) and are differentially regulated through cross-talk with other signalling pathways.

Erk2 has been shown to phosphorylate the long PDE4D3 isoenzyme at a single site (Ser^{579}), leading to inhibition of PDE activity (Hoffmann *et al*., 1999). This was shown *in vitro* by treating recombinant enzyme with Erk2. It was also demonstrated in intact COS1 cells transfected to express PDE4D3 that were challenged with EGF so as to cause Erk2 activation. Consistent with this effect being mediated by Erk, these EGF-induced actions upon PDE4D3, expressed in COS1 cells, were ablated by PD98059, an inhibitor of Mek activation. Similar results were also obtained using the PDE4D5 long isoenzyme (Hoffmann *et al*., 1999).

We show here that PDE4B and PDE4C isoenzymes provide substrates for regulatory phosphorylation by Erk2. In each case, this occurs at a single serine residue located within the catalytic unit. For both the PDE4D isoenzymes, shown previously by us to be Erk substrates (Hoffmann *et al*., 1999; MacKenzie *et al*., 2000), and the PDE4B isoenzymes, this serine residue lies within the motif PQSP while for PDE4C isoenzymes this residue lies within the motif PRSP. Both of these motifs are of the form (P-x-S/T-P) that might reasonably be expected to provide a site for phosphorylation by Erk2 (Alvarez *et al*., 1991). In each instance, the functional consequences of Erk phosphorylation could be mimicked by mutating the serine target residue to aspartate. This suggests that it is the placement of a negative charge at this site in the catalytic unit that triggers the effect on phosphodiesterase activity.

In marked contrast to its actions on the PDE4B, PDE4C and PDE4D enzymes, Erk2 failed to elicit the phosphorylation of the long PDE4A8 isoenzyme (Figure 2c). The PDE4A catalytic unit does, however, contain a cognate serine residue, Ser^{621} in PDE4A8, to that phosphorylated by Erk2 in long isoenzymes from the three other PDE4 families. However, in the case of PDE4A family members this residue lies within the motif RQSP. The replacement of the first proline with arginine in the Erk2 consensus motif, P-x-S/T-P can be expected to severely affect the ability of Erk2 to recognize PDE4A enzymes as effective substrates (Alvarez *et al*., 1991). From this we conclude that activation of Erk2 in COS cells does not inhibit the PDE4A8 long form because it is unable to phosphorylate the cognate serine within its catalytic unit. Thus long PDE4B, PDE4C (this study) and PDE4D isoenzymes (Hoffmann *et al*., 1999; MacKenzie *et al*., 2000) provide substrates for C-terminal catalytic unit phosphorylation by Erk2. The functional consequence of such Erk2 phosphorylation is to cause inhibition of these long forms. In marked contrast to this, the PDE4A gene appears likely to provide a family of enzymes where Erk2 is unable to elicit regulatory phosphorylation through modification of their catalytic unit.

Intriguingly, the transient nature of the Erk-mediated inhibition of the long PDE4B and PDE4C isoenzymes was converted to a more sustained effect when cells were treated with the PKA selective inhibitor, H89 (Figure 1). We have made a similar observation before using COS1 cells expressing the PDE4D3 long form (Hoffmann *et al*., 1999). This was demonstrated to be due to an intriguing feedback mechanism (Hoffmann *et al*., 1999) where, as a consequence of the Erk-mediated inhibition of PDE4D3, cyclic AMP levels rose causing PKA to phosphorylate PDE4D3. PKA has been shown to phosphorylate PDE4D3 at two sites, of which Ser^{54} in UCR1 serves to effect enzyme activation (Sette *et al*., 1994; Alvarez *et al*., 1995; Sette & Conti, 1996; Hoffmann *et al*., 1998). However, we have shown for PDE4D3 that phosphorylation of Ser^{54} in UCR1 by PKA serves to negate the inhibitory effect of Erk phosphorylation and hence to re-activate PDE4D3 (Hoffmann *et al*., 1999). The long PDE4B and PDE4C isoenzymes have a cognate serine residue in UCR1 that lies in a consensus site for PKA phosphorylation. Thus our observation that inhibition of PKA activity using H89 serves to sustain the inhibitory effect of Erk action indicates that these two long PDE4 isoenzymes are likely to be regulated by a similar feedback regulatory mechanism involving PKA action (Hoffmann *et al*., 1999).

The most commonly expressed short PDE4 isoenzyme appears to be PDE4B2 (Houslay *et al*., 1998; Seybold *et al*., 1998; Wang *et al*., 1999). We show here that PDE4B2 is phosphorylated at a single site within its catalytic unit by Erk2 and that this is cognate to the site phosphorylated in the PDE4B1 long isoenzyme. This conclusion is also in agreement with mass spectrometry studies showing that Ser^{487} of *Escherichia coli*-expressed recombinant PDE4B2 was the *in vitro* site of phosphorylation elicited using a brain MAP kinase preparation (Lenhard *et al*., 1996). However, in marked contrast to the inhibitory action of Erk2 on the long PDE4B1 isoenzyme, the PDE4B2 short form was activated as a consequence of its phosphorylation by Erk. Previous studies by Lenhard *et al*. (1996) suggest that phosphorylation of PDE4B2 with a crude brain MAPK preparation did not generate any dramatic change in PDE activity. However, such analyses were not performed at a level detailed enough to have identified changes of the magnitude reported here. In addition, such studies might also have been affected by the use of both PDE4B2, expressed in *E. coli* rather than mammalian cells, and a crude brain MAP kinase preparation. Nevertheless, we show here, using consistent results from three independent types of analysis, that Erk2 phosphorylation of the PDE4B2 short form led to an augmentation of its catalytic activity, rather than the inhibition seen with the PDE4B1 long form. In this respect, such an observation is consistent with our demonstration that phosphorylation by Erk2 elicits the activation of the short isoenzyme, PDE4D1 (MacKenzie *et al*., 2000). Such observations are consistent with the notion that lack of UCR1 allows the intact UCR2 to direct Erk2 phosphorylation to cause activation of these short isoenzymes rather than the inhibition seen in long PDE4 enzymes (MacKenzie *et al*., 2000). These data indicate a functional role for UCR1 and UCR2. Indeed, these regions appear to interact with each other (Beard *et al*., 2000) and may thus form a regulatory module able to direct the functional outcome of phosphorylation of the catalytic site by Erk and, for long isoenzymes, to integrate the effect of PKA action through phosphorylation of UCR1. Certainly these regions are located in a cognate fashion, N-terminal to the catalytic unit, to paired regulatory regions found in both PDE1 and PDE2

isoenzymes, for example (Beavo, 1995; Manganiello *et al*., 1995; Conti & Lin, 1999).

The output of phosphorylation of PDE4 isoenzymes by Erk is, intriguingly, directed to elicit either inhibition or activation dependent upon whether the target is a long or short PDE4 isoenzyme. The PDE4 isoenzyme profile of a particular cell will thus be pivotal in appreciating the effect of processes that lead to Erk activation. In this regard, phorbol ester treatment of FDCP myeloid cells (Ahmad *et al*., 1999) and vascular smooth muscle cells (Liu & Maurice, 1999) appeared to cause a small activation of total PDE4 activity that was ablated by PD98059 action. It could be that a preponderance of Erk-activatable PDE4B and PDE4D short isoenzymes serves to explain such observations.

The discovery here that various PDE4 families and isoenzymes are differentially regulated by Erk phosphorylation may have consequences for the development of PDE4 subfamily-specific inhibitors as potential therapeutic agents. Pro-inflammatory mediators lead to the activation of both Erk and other MAPK signalling pathways (Tibbles & Woodgett, 1999) as well as the PI3-kinase pathway (Herrera-Velit, 1996; Herrera-Velit *et al*., 1997). As PDE4-selective inhibitors act as potent anti-inflammatory agents one might infer that activating PDE4 enzymes would facilitate the action of pro-inflammatory mediators. The identification of any such activated PDE4 isoenzymes may thus serve to highlight forms that it would be profitable to inhibit selectively in order to ablate inflammatory processes. In this regard, it has recently been suggested that selective inhibition of either or both PDE4A and PDE4B isoenzymes may be of particular importance in attenuating inflammatory cell function (Manning *et al*., 1999). While the commonly expressed PDE4A4 long isoenzyme is not affected by Erk action, pro-inflammatory mediators have been shown to lead to PDE4A4 activation in monocytic cells *via* a process that involves PI3-kinase activation (MacKenzie & Houslay, 2000). PDE4A4 is also expressed in T lymphocytes (Seybold *et al*., 1998) where PI3-kinase is activated by liganding of the CD28 receptor (Ward, 1999). This may give credence to the notion that inhibitors with selectivity for PDE4A are likely to be particularly effective. However, PDE4B2 is both the most commonly expressed short form and the most commonly expressed PDE4B form (Huston *et al*., 1997; Houslay *et al*., 1998) as well as being a major contributor to PDE4 activity in monocytic cells (Wang *et al*., 1999; MacKenzie & Houslay, 2000). It is, therefore, intriguing that this isoenzyme is stimulated by Erk2 activation, implying that it too may be an important enzyme to inhibit selectively. Strengthening such a notion is the observation that pro-inflammatory mediators also up-regulate this isoenzyme in monocytic cells (Wang *et al*., 1999). Thus the appreciation that members of the PDE4 enzyme family can be differentially regulated by Erk may provide a route for identifying key isoenzymes to target selective therapeutics in the future. Certainly the situation we have uncovered here offers the opportunity for Erk2 activation to lead to either an increase or a decrease in cyclic AMP levels dependent upon the PDE4 isoenzyme expression pattern. Indeed, as PDE4 isoenzymes appear to have distinct intracellular locations (Houslay *et al*., 1998), phosphorylation by Erk2 may in fact lead to localized (compartmentalized) changes in cyclic AMP levels that could alter the functioning of distinct PKA populations targeted to specific intracellular sites by binding to AKAP anchor proteins (Colledge & Scott, 1999).

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