Phosphorylation of cAMP-specific PDE4A5 (phosphodiesterase-4A5) by MK2 (MAPKAPK2) attenuates its activation through protein kinase A phosphorylation

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INTRODUCTION

MAPKs (mitogen-activated protein kinases) provide phosphorylation cascades that control a variety of key cellular functions [1]. The p38 MAPK signalling cascade is a key signal transduction pathway involved in the control of cellular immune, inflammatory and stress responses. Cellular analyses invariably employ anisomycin to achieve a robust activation of p38 MAPK [1]. However, p38 MAPK can also be activated by a variety of extracellular stimuli such as growth factors, UV light, ionizing radiation, oxidative stress, osmotic shock and cytokines, with the cytokine-suppressive anti-inflammatory drug SB203580 able to inhibit it selectively [1].

Activated p38 MAPK exerts its cellular actions by phosphorylating, and thus activating, the predominant downstream kinase MK2 (MAPK-activated protein kinase 2, also called MAPKAPK2) and its compensatory kinase, MK3 (MAPKAPK3) [2]. Surprisingly, however, given the importance of this pathway in inflammatory and cell-cycle pathways, relatively few substrates for MK2/3 have been identified to date.

cAMP is a universal second messenger in mammalian cells that regulates a wide variety of cellular processes, including exerting anti-inflammatory actions [3]. The only route to degrade this key messenger is by hydrolysis achieved through members of the cyclic-nucleotide-hydrolysing PDE (phosphodiesterase) superfamily [4]. Of these, selective inhibitors of the cAMP-specific PDE4 family have been shown to have profound anti-inflammatory actions, offering therapeutic potential that is the subject of evaluation in diseases such as COPD (chronic obstructive pulmonary disease) and asthma [5]. Four gene families (PDE4A/B/C/D) encode more than 20 different isoforms that are expressed on a cell-type-specific basis and appear to have non-redundant functional roles on the basis of siRNA (small interfering RNA)-mediated knockdown and dominant-negative and gene-targeting approaches [6]. Pivotal in this is the ability of particular isoforms to be directed to associate with specific signalling scaffolds, such as β-arrestin, DISC1 (disrupted in schizophrenia 1), RACK1 (receptor for activated C-kinase) and AKAPs (A-kinase-anchoring proteins) as well as receptors such as p75NTR (p75 neurotrophin receptor) and AIP (aryl hydrocarbon receptor-interacting protein), also called MAPKAPK2), protein kinase A (PKA), tumour necrosis factor α (TNFα).

cAMP-specific PDE (phosphodiesterase) 4 isoforms underpin compartmentalized cAMP signalling in mammalian cells through targeting to specific signalling complexes. Their importance is apparent as PDE4 selective inhibitors exert profound anti-inflammatory effects and act as cognitive enhancers. The p38 MAPK (mitogen-activated protein kinase) signalling cascade is a key signal transduction pathway involved in the control of cellular immune, inflammatory and stress responses. In the present study, we show that PDE4A5 is phosphorylated at Ser147, within the regulatory UCR1 (ultraconserved region 1) domain conserved among PDE4 long isoforms, by MK2 (MAPK-activated protein kinase 2, also called MAPKAPK2). Phosphorylation by MK2, although not altering PDE4A5 activity, markedly attenuates PDE4A5 activation through phosphorylation by protein kinase A. This modification confers the amplification of intracellular cAMP accumulation in response to adenylyl cyclase activation by attenuating a major desensitization system to cAMP. Such reprogramming of cAMP accumulation is recapitulated in wild-type primary macrophages, but not MK2/3-null macrophages. Phosphorylation by MK2 also triggers a conformational change in PDE4A5 that attenuates PDE4A5 interaction with proteins whose binding involves UCR2, such as DISC1 (disrupted in schizophrenia 1) and AIP (aryl hydrocarbon receptor-interacting protein), but not the UCR2-independent interacting scaffold protein β-arrestin. Long PDE4 isoforms thus provide a novel node for cross-talk between the cAMP and p38 MAPK signalling systems at the level of MK2.

Key words: aryl hydrocarbon receptor-interacting protein (AIP, also called XAP2), cAMP-specific phosphodiesterase-4 (PDE4), disrupted in schizophrenia 1 (DISC1), mitogen-activated protein kinase-activated protein kinase 2 (MK2, also called MAPKAPK2), protein kinase A (PKA), tumour necrosis factor α (TNFα).
basis of PDE4 selective inhibitors [6]. Additionally, elevated cAMP levels cause PKA (protein kinase A) to phosphorylate, and thus activate, long PDE4 isoforms that have UCR (ultraconserved region) 1 and UCR2 regulatory regions, thereby providing a key part of the cellular desensitization system to cAMP [7,8].

In the present study, we identify cAMP-specific PDE4A5 [9] as a novel MK2 substrate and show that MK2 elicits the phosphorylation of PDE4A5 at Ser147 within its regulatory UCR1. This attenuates PDE4A5 activation by PKA and selectively disrupts its ability to be sequestered by those partner proteins whose interaction involves UCR2.

EXPERIMENTAL

Cell culture

COS1 and HeLa cell lines were maintained at 37 °C in an atmosphere of 5% CO2/95% air in complete growth medium [DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 0.1% penicillin/streptomycin, glutamine and 10% (v/v) FBS (fetal bovine serum)]. Cell lines were maintained at 37 °C in 5% CO2/95% air. The cells were passaged when approximately 90% confluence was reached. Where indicated, anisomycin was added at 10 μg/ml, SB203580 at 10 μM, forskolin at 100 μM and IBMX (isobutylmethylxanthine) at 100 μM.

DNA manipulation

Site-directed mutagenesis of plasmid DNA was carried out using the Stratagene QuickChange® Site-Directed Mutagenesis kit, using the method in the manufacturer’s instructions. The concentration of DNA samples was determined spectrophotometrically (A260). Purified plasmid DNA was produced using Qiagen QIAprep® kits and stored either at 4 °C when eluted in 10 mM Tris/HCl, pH 8.5, or at −20 °C when eluted in sterile water.

Transfection

COS1 cells were transfected using either Polyfect® reagent (Qiagen) or DEAE-dextran (Sigma–Aldrich) as described previously [10,11].

siRNAs

The PolyFect® method of mammalian cell transfection (Qiagen) was used for the transfection of COS1 cells with an MK2 siRNA nucleotide from Santa Cruz Biotechnology, as reported previously [12].

Immunoprecipitation and immunoblotting

Cell lysates from COS1 cells were prepared by washing cells in PBS before being scraped into 3T3 lysis buffer [25 mM Heps, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% (v/v) glycerol and 1% Triton X-100, with added Complete ULTRA® protease inhibitors (Roche; one tablet/10 ml of lysis buffer)] and mixed at 4 °C for 20 min. This was cleared by centrifugation before further procedures. Alternatively cells were washed in KHEM buffer [50 mM KCl, 10 mM EGTA, 1.92 mM MgCl2, 1 mM DTT (dithiothreitol) and 50 mM Heps, pH 7.2] before being scraped into KHEM buffer with added Complete ULTRA® protease inhibitors (Roche; one tablet/10 ml of lysis buffer), lysis by freeze–thaw and needle homogenization, and removal of insoluble material by centrifugation (16000 g, 4 min). Immunoblotting was performed using various antisera as indicated. PDE4A-specific antisera were as described previously [13–15], being either specific to PDE4A5, being raised to its unique N-terminal region, or specific to the PDE4A subfamily, being raised to the C-terminal region that is common to all active PDE4A isoforms but is distinct from the C-terminal region of PDE4B/C/D subfamilies, i.e. a specific ‘pan’-PDE4A antiserum.

Generation of GST (glutathione transferase) and MBP (maltose-binding protein) fusion proteins

The Escherichia coli strain JM109 was transformed with pGEX2T (Amersham Biosciences) for the production of GST or transformed with pMALpC2 (New England Biolabs) for the production of MBP. Clones encoding full-length rat PDE4A5 as an MBP fusion and AIP (XAP2) as a GST fusion were generated and purified as described previously [16].

In vitro phosphorylation

A 1 μg portion of the indicated MBP or GST fusion protein and a defined amount of recombinant protein kinase as per the manufacturer’s instructions (Upstate Technology) was diluted in 20 mM Mops, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM DTT to a final volume of 40 μl. A 10 μl volume of 1 μCi/μl [32P]ATP diluted in 75 mM MgCl2 and 500 μM ATP was added to the target recombinant fusion protein and specific kinase mixture. The reaction mixture was incubated at 30 °C for 10 min. The reaction was stopped by the addition of 12.5 μl of 5× SDS sample buffer (10% SDS, 300 mM Tris/HCl, pH 6.8, 0.05% Bromophenol Blue, 50% glycerol and 10% 2-mercaptoethanol). The reaction tubes were mixed and heated for 10 min at 70 °C on a heating block. The tubes were then centrifuged at 10000 g for 1 min, and 30 μl of the sample was subjected to SDS/PAGE (4–12% gel) and transferred on to a nitrocellulose membrane. The nitrocellulose membranes were then air-dried and scanned using a Personal Molecular Imager FX® instrument (Bio-Rad Laboratories) to resolve the radioactive regions utilizing phosphorimage-screen technology. The Discovery Series™ Quantity One® 1-D Analysis Software (version 4.4.0; Bio-Rad Laboratories) was used to perform densitometry and analysis of the radioactive regions. In all cases, the extent of the phosphorylation was corrected for the immuno-reactive amount of the target protein.

Metabolic labelling of PDE4A5 with [32P]orthophosphate

COS1 cells were transfected with VSV (vesicular stomatitis virus)-tagged PDE4A5. At 48 h post-transfection, the culture medium was aspirated and the monolayer was washed with phosphate-free DMEM. The monolayer was incubated with phosphate-free DMEM containing 2% (v/v) FBS, 20 mM Heps, pH 7.4, and 100 mCi of [32P]orthophosphate overnight leading to isotopic equilibration of the ATP pool [17]. After treatments with anisomycin or other reagents, the labelling medium was discarded and cells were washed with ice-cold PBS before disruption in ice-cold lysis buffer [100 mM sucrose, 80 mM 2-glycerophosphate, 20 mM EGTA, 15 mM MgCl2, 1 mM DTT, 2 mM ATP and 0.5% NP-40 (Nonidet P40), containing 1 μg protease inhibitor cocktail (Roche)]. VSV–PDE4A5 was immunoprecipitated as described above, subjected to SDS/PAGE (4–12% gel) and visualized using a phosphorimager.
PDE and cAMP assays

For determination of PDE activity, COS1 cells were transfected as described above and then homogenized in KHEM buffer. As in our previous study [16], in transfected cells ≥98% of the total PDE activity was due to the recombinant PDE4 construct. PDE activity was assayed using 1 μM cAMP as substrate, as described previously [18]. Intracellular cAMP was determined using a Cyclic AMP Competitive ELISA kit (Thermo Fisher).

Pull-down assays and SDS/PAGE

These were carried out as described in detail previously [11,16,19].

Protein assay

The protein concentration of purified recombinant proteins or cell lysates (whole or subcellular fractions) was determined using BSA as a standard in a spectrophotometric assay with Bradford reagent (Bio-Rad Laboratories).

Macrophage analyses

MK2/3-null C57/BL6 mice [20] were generously provided by Matthias Gaestel (Department of Biochemistry, Medical School Hanover, Hanover, Germany). Bone-marrow-derived macrophages were isolated from the femurs of adult C57/BL6 mice (wild-type and MK2/3-null) as previously described [21]. Bone marrow cells were differentiated for 7 days on bacteria-grade plates in DMEM containing 10% (v/v) FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), L-glutamine (5 mg/ml) and mouse recombinant colony-stimulating factor (5 ng/ml; R&D Systems). After 7 days, adherent cells were removed using Versene solution (Gibco) and replated on tissue-culture-grade plates. At 24 h after replating, cells were stimulated.

PDE4A5 foci analyses

HEK-293 cells (human embryonic kidney cells) were grown in six-well plates at approximately 70% confluency overnight in DMEM containing 10% (v/v) FBS, 1% penicillin/streptomycin and 1% L-glutamine. Cells were transfected with plasmids encoding either wild-type or the MK2-null S147A-PDE4A5–MBP (green fluorescent protein) mutant using the Polyfect® transfection protocol in the manufacturer’s instructions. After 48 h, cells were treated with or without 5 μM rolipram overnight followed by treatment with or without 10 μg/ml anisomycin for 60 min. Confocal analyses and foci quantification were as described previously [22,23].

RESULTS

PDE4A5 (GenBank® accession number L27057) is uniquely defined by its isoform-specific N-terminal region of 103 amino acids and is classified as a long PDE4 isoform due to the presence of the paired UCR1 and UCR2 regulatory domains (Figure 1a) [16]. Its catalytic domain is highly conserved within the PDE4 family, and its C-terminal domain is unique to the PDE4A subfamily and is found in all members from that subfamily [16]. We chose PDE4A5 as an exemplar in our studies because of its importance, as indicated by its up-regulation in sleep deprivation underpinning cognitive deficits [24], whereas its human orthologue (PDE4A4) is up-regulated in macrophages of patients with COPD [25]. Furthermore, PDE4A5 becomes inhibited upon binding AIP [16], and chronic exposure to the PDE4 selective inhibitor rolipram causes it to form cytosolic aggregates (foci) that are dynamically linked to autophagy [22].

In vitro phosphorylation of PDE4A5 by MK2

Sequence inspection of PDE4A5 reveals one serine residue that lies within a perfect motif for phosphorylation by MK2 on the basis of a suggested consensus phosphorylation motif [26] of Ψ-Xaa-Arg-Xaa-Xaa-Ser, where Ψ is a hydrophobic amino acid and Xaa is any amino acid. This MK2 phosphorylation target residue is Ser147, which is located within the UCR1 regulatory domain and, specifically, the sequence Leu-Tyr-Arg-Ser-Asp-Ser [47] (Figure 1a; Table 1). However, we also noted that another residue, namely Ser161, lies within a suboptimal MK2 phosphorylation motif of Met-Ser-Arg-Asn-Ser-Ser [48], which starts with a hydrophilic methionine residue rather than a hydrophobic amino acid, as required for MK2 action (Table 1).

Setting out to determine whether PDE4A5 could be phosphorylated by MK2, we first evaluated this in vitro using PDE4A5 purified as an N-terminal MBP fusion protein treated with purified activated recombinant MK2 in the presence of [γ-32P]ATP (Figure 1b). We showed that PDE4A5 is phosphorylated by MK2, but not by p38 MAPK (Figure 1b).

The two potential MK2 phosphorylation sites of PDE4A5 were separately mutated to alanine with recombinant proteins purified as N-terminal MBP fusion proteins (S147A and S161A) and treated with purified activated recombinant MK2 in the presence of [γ-32P]ATP. Although the S147A-PDE4A5–MBP mutant was not phosphorylated by MK2, the S161A-PDE4A5–MBP mutant was (Figure 1c). Indeed, wild-type PDE4A5–MBP could be phosphorylated stoichiometrically by MK2 (0.93 ± 0.11 mol of phosphate/mol; mean ± S.D., n = 3). Taken together, these results show that MK2 phosphorylates PDE4A5 on a single residue, namely Ser147.

In vivo phosphorylation of PDE4A5 by MK2

To evaluate whether PDE4A5 could be phosphorylated by MK2 in intact cells, we used COS1 cells transfected to transiently express recombinant PDE4A5. Treatment of these cells with anisomycin, a well-established activator of the p38 MAPK pathway [1], caused the time-dependent stimulatory phosphorylation of both p38 MAPK and MK2 (Figures 2a and 2b) through a process ablated by the p38 MAPK specific inhibitor SB203580 [1].

COS1 cells transfected to express VSV-epitope-tagged PDE4A5 were labelled with 32P and then challenged with either anisomycin or anisomycin + SB203580 prior to immunoprecipitation for analysis. Recombinant VSV–PDE4A5 was selectively immunoprecipitated from the cells using VSV antiserum coupled to Protein G–agarose (Figure 2c, lane IP) but not using Protein G–agarose beads alone [Figure 2c, lane b (‘beads only’ control)]. Anisomycin challenge alone caused the time-dependent phosphorylation of PDE4A5 in these cells and this phosphorylation was ablated by SB203580 (Figures 2c–2e). Therefore activation of the p38 MAPK pathway elicits the phosphorylation of PDE4A5 in COS1 cells. Comparison of the time course in which phosphorylation of MK2 occurs with the time course in which PDE4A5 phosphorylation occurs under anisomycin stimulation implies a role for MK2 in phosphorylation of PDE4A5 (Figure 2e).

A transiently transfected N-terminally truncated mutant of PDE4A5 lacking the first 103 amino acids, which are unique to this isoform, was phosphorylated in response to anisomycin...
Table 1 Consensus motif for phosphorylation by MK2

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Figure 1 MK2 phosphorylates PDE4A5 in vitro

(a) Schematic representation of the long PDE4A5 isoform with its isoform-defining N-terminal region, common long form region (CLF), regulatory UCR1 and UCR2 modules, linker regions (LR1 and LR2), catalytic domain and subfamily-specific C-terminal domain. The sites for phosphorylation by PKA (Ser140) and MK2 (Ser147) are shown. Also shown are the binding sites for AIP (also called XAP2), which binds at both UCR2 and the N-terminal region, DISC1, which binds at both UCR2 and the catalytic unit, and β-arrestin, which binds at the catalytic unit only. (b) Autoradiograph (upper panel) and loading immunoblot for PDE4A5 (lower panel) from in vitro phosphorylation experiments with [32P]ATP and no added kinase (Ctr), purified MK2 or purified p38 MAPK together with purified MBP–PDE4A5. Results shown are typical of three experiments. (c) Quantification of the experiments in (b) using the indicated protein kinases and wild-type MBP–PDE4A5 (WT) or the S147A or S161A mutants of MBP–PDE4A5. Results are means ± S.D. (n = 3) with phosphorylation levels given as intensities from phosphorimager analyses relative to the maximum level of phosphorylation observed in experiments with wild-type MBP–PDE4A5 and added MK2.
Phosphorylation of PDE4A5 by MK2

Figure 2 MK2 phosphorylates PDE4A5 in vivo

(a) Immunoblots of cell lysates performed to assess activation of MK2 and p38 MAPK in resting cells (Ctrl), those treated with anisomycin (10 μg/ml) (A) and those treated with both anisomycin (10 μg/ml) and SB203580 (10 μM) (A + S). (b) Time course of phosphorylation of both p38 MAPK and MK2 in cells treated with anisomycin (10 μg/ml) for the indicated times. (c) Phosphorylation of VSV-tagged recombinant PDE4A5 expressed ectopically in COS1 cells labelled with [32P]Pi by phosphorimager analysis. Cells were subjected to challenge (60 min) with either anisomycin (A) alone or together with SB203580 (A + S) or with no additions (Ctrl), as indicated. PDE4A5 was immunopurified and then subjected to SDS/PAGE with subsequent phosphorimager analysis (upper panel) and immunoblot analysis (VSV epitope) for a VSV–PDE4A5 loading control (lower panel). (d) As in (c), but showing a time course for the phosphorylation of VSV–PDE4A5 with the indicated times of anisomycin treatment. (e) COS1 cells were treated with anisomycin (10 μg/ml) for the indicated times and samples were taken for determination of the MK2 phosphorylation status (pSer147) of VSV–PDE4A5 (phosphorimager analysis of pooled data as in (d)) with the p38 MAPK-mediated phosphorylation status of MK2 (densitometry analysis of pooled data as in (a)); results are means ± S.D. (n = 3).

challenge at a similar level to wild-type overexpressed PDE4A5 (Figure 3a, delta NT). However, further N-terminal truncation, which removes UCR1, generated a construct that failed to become phosphorylated upon anisomycin challenge (Figure 3a; delta NT/UCR1). These observations are consistent with the notion that all long PDE4 isoforms are potential substrates for MK2/3 as they each contain the conserved consensus MK2 phosphorylation motif in their UCR1 (Table 1). Consistent with the in vitro analyses above (Figures 1b and 1c), anisomycin challenge of COS1 cells failed to elicit phosphorylation of the S147A-PDE4A5 mutant, but allowed phosphorylation of the S161A-PDE4A5 mutant (Figure 3a). This is again consistent with Ser147 providing the sole site in PDE4A5 for phosphorylation by MK2.

Owing to the lack of MK2-specific inhibitors, in order to support the contention that MK2 can phosphorylate PDE4A5 in cells, we used an siRNA strategy to knock down MK2 expression that has been proven previously to be both effective and specific [12]. Treatment with MK2-specific siRNA, but not with a control siRNA, achieved a profound, time-dependent, knockdown of MK2 (Figures 3a and 3b). This treatment affected neither p38 MAPK expression levels nor its ability to become phosphorylated in anisomycin-challenged cells (Figure 3c). However, it did ablate the ability of anisomycin challenge to elicit the phosphorylation of PDE4A5 in COS1 cells (Figure 3a).

Although not exhaustively examined, the MK2-related kinase MK3 is considered to have an overlapping substrate specificity to MK2 and, in cells where it is expressed, plays a backup role to the dominant kinase, MK2 [27]. Although we readily detected MK2 in COS1 cells, we singularly failed to identify MK3 using specific antisera (results not shown). This, coupled with the fact that the siRNA reagents we employed were MK2-specific, indicates that it is MK2 that phosphorylates PDE4A5 in COS1 cells. However, such observations do not rule out the possibility that, in cell
MK2 phosphorylates PDE4A5 at Ser147 in vivo

(a) COS1 cells were transfected to express either the wild-type (wt) or indicated mutant forms of VSV–PDE4A5, loaded with [32P]Pi and challenged for 60 min with anisomycin (10 μg/ml) (A) or with both anisomycin (10 μg/ml) and SB203580 (10 μM) (A + S) prior to immunopurification of VSV–PDE4A5, SDS/PAGE and subsequent quantification by phosphorimager analysis. For the siRNA experiments, cells were subjected to knockdown of MK2 using targeted siRNA (MK2 siRNA) or treated with a control (Ctr) scrambled siRNA (scr siRNA) prior to transfection with wild-type VSV–PDE4A5, treatment with anisomycin (10 μg/ml) and SB203580 (10 μM) (A + S), and phosphorimager analysis performed as described in the Experimental section. Results are means ± S.D. (n = 3). (b) Immunoblot of cell lysates immunoblotted for MK2 at the indicated times for cells treated with either control scrambled siRNA or siRNA targeted to MK2. (c) Immunoblot of lysates from cells treated with either control scrambled siRNA or siRNA targeted to MK2 and then either challenged with anisomycin (A) or not (C), for detection of both total p38 MAPK and p-p38MAPK. (d) Lysates of cells challenged for the indicated times with TNFα (10 ng/ml) prior to immunoblotting for either total MK2 or p-MK2. (e) Phosphorylation of VSV-tagged recombinant PDE4A5 expressed ectopically in cells labelled with [32P]Pi by phosphorimager analysis. Cells were subjected to challenge (20 min) with TNFα (10 ng/ml) alone (T) or together with SB203580 (T + S) or with no additions (C), as indicated. PDE4A5 was immunopurified and then subjected to SDS/PAGE with subsequent phosphorimager analysis (upper panel) and immunoblot analysis (VSV epitope) for a VSV–PDE4A5 loading control (lower panel). Results shown are typical of at least three experiments.

Phosphorylation of PDE4A5 by MK2 attenuates its activation by PKA phosphorylation

Anisomycin-induced MK2 phosphorylation of VSV–PDE4A5 has little or no effect on either PDE catalytic activity (Figure 4a) or its sensitivity to inhibition by the archetypal PDE4-selective inhibitor rolipram. Assayed using 1 μM cAMP as substrate, rolipram IC50 values (means ± S.D., n = 3) of 1.4 ± 0.5 μM for untreated cells and 1.8 ± 0.4 μM for anisomycin-treated cells (60 min) were obtained for PDE4A5.

UCR1 is a regulatory domain that is unique to PDE4 long isoforms and is the site of their stimulatory phosphorylation by PKA, which occurs at Ser147 in PDE4A5 [7]. Upon discovering that MK2 phosphorylates PDE4A5 within UCR1, we set out to determine whether such MK2 phosphorylation exerted any functional effect on stimulatory PKA phosphorylation. COS1 cells, expressing VSV–PDE4A5, were thus pre-treated with anisomycin (60 min) prior to challenge with the adenylate cyclase activator forskolin (100 μM) and the reversible non-selective PDE inhibitor IBMX (100 μM) in order to increase intracellular cAMP and activate PKA, as shown previously [7]. Using a previously described phospho-specific antiserum to the PKA phosphorylation site in UCR1 that is common to all PDE4 long isoforms (phospho-UCR1; [7]), we observed that prior challenge with anisomycin, in order to phosphorylate PDE4A5 at Ser147, had no observable effect on the ability of PKA to phosphorylate PDE4A5 at Ser147 (Figures 4b and 4c). We also noted that S147A-PDE4A5 was phosphorylated at Ser147 by PKA to a similar extent to wild-type PDE4A5 in cells challenged in a similar way (Figure 4e). These results show that MK2 phosphorylation of PDE4A5 does not alter PKA phosphorylation of the enzyme.

However, in contrast with this, we observed that the ability of PKA phosphorylation to activate PDE4A5 was attenuated...
Phosphorylation of PDE4A5 by MK2

(a) Relative cAMP PDE activity of VSV–PDE4A5 immunopurified from COS1 cells treated for the indicated times with anisomycin to elicit the MK2 phosphorylation of VSV–PDE4A5; results are means ± S.D. (n = 3). (b) A phospho-UCR1 antiserum [7] was used to assess the PKA phosphorylation status of the serine residue in the conserved PKA motif located in the conserved UCR1 of all PDE4 long isoforms, which is Ser^{147} in PDE4A5. In the present study, VSV–PDE4A5 was first immunopurified from COS1 cells treated for the indicated times with forskolin (100 μM) (Fsk) plus IBMX (100 μM) that either had (anisomycin; open squares) or had not (control; closed circles) been pre-treated for 60 min with anisomycin (10 μg/ml), and was then immunoblotted using phospho-UCR1 antiserum; results are means ± S.D. (n = 3). (c) Typical set of phospho-UCR1 immunoblots for the experiments described in (b), with PDE4D5 loading detected by blotting for the VSV epitope tag. (d) Experiments were performed as in (b), except that the cAMP PDE activity of immunopurified PDE4A5 was determined subsequent to forskolin/IBMX treatment for the indicated times in cells that either had (anisomycin; open squares) or had not (control; closed circles) been pre-treated for 60 min with anisomycin (10 μg/ml); results are means ± S.D. (n = 3). (e) As in (b), but using the S147A mutant form of VSV–PDE4A5; results are means ± S.D. (n = 3). (f) As in (d) but using the Ser147A mutant form of VSV–PDE4A5; results are means ± S.D. (n = 3). (g) COS1 cells, transfected to express PDE4A5, were either untreated or were treated, as indicated, with either anisomycin (10 μg/ml; 60 min) or forskolin/IBMX (100 μM each for 20 min together) or were treated first with anisomycin followed by forskolin/IBMX. Lysates from cells were incubated at 55°C and samples removed at the indicated times and immediately flash-cooled on ice. These were then taken for assay of cAMP PDE activity and the results plotted in a semi-log fashion as Log % of PDE4 activity remaining (no heat treatment was taken as 100 %) against time. This shows a typical example of an experiment performed three times. All immunoblots shown are typical of blotting experiments performed at least three times.

Figure 4 Phosphorylation by MK2 attenuates the action of stimulatory phosphorylation by PKA

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Figure 5  MK2 phosphorylation of PDE4A5 alters its ability to regulate intracellular cAMP concentrations

(a and b) COS1 cells were transfected with either wild-type PDE4A5–VSV (a) or S147A-PDE4A5–VSV (b), and then, as indicated, either wild-type or the S61A mutant form of PDE4D3 (c) such that the activity of these recombinant enzymes accounted for >98% of total cAMP PDE activity in these cells when lysates were assayed using 1 μM cAMP as substrate. Transfected cells were challenged with forskolin (Fsk; 100 μM) and samples were taken at the indicated times for determination of intracellular cAMP, which is expressed relative to basal levels in untreated cells (100%) with results shown as means ± S.D. (n = 3 separate experiments). Where indicated, cells were pre-treated with either anisomycin (Aniso; 10 μg/ml, 60 min pre-incubation) or the PKA inhibitor KT5720 (KT; 4 μM, 20 min pre-incubation). (c) Cells were challenged with similar concentrations of these ligands for the indicated times, but lysates were then used to assess changes in the activity of the recombinant PDE4D3 forms and expressed relative to control activity in untreated cells (100%). wt, wild-type. (d and e) Primary murine macrophages from either wild-type or double-knockout MK2−/−/MK3−/− (KO) animals were incubated with combinations of forskolin (100 μM), anisomycin (10 μg/ml, 60 min pre-incubation) and KT5720 (4 μM, 20 min pre-incubation) prior to harvesting at the indicated times for assessment of intracellular cAMP levels, which are expressed relative to the maximal levels achieved in each case (100%). Results are means ± S.D. for three separate experiments (n = 3), with individual cAMP determinations done in triplicate.

markedly upon pre-treatment of cells with anisomycin (Figure 5d). When cells were simply challenged with forskolin/IBMX, then PDE4A5 activity increased to 281 ± 12 % of the control (100%). However, when cells were pre-treated with anisomycin, subsequent forskolin/IBMX challenge increased PDE4A5 activity only by 174 ± 8 % of the control (values are means ± S.D., n = 3; Figure 4e). This action of anisomycin was not observed when the MK2 phospho-null S147A-PDE4A5 mutant was employed (Figures 4d and 4f). This MK2 phospho-null mutant could be phosphorylated at Ser140 by PKA to a similar extent to wild-type PDE4A5 in cells challenged with forskolin/IBMX alone (detected with anti-phospho-UCR1 antibody; results not shown), with its activity in untreated cells being identical with that of wild-type PDE4A5 (<5 % change). Furthermore, consistent with MK2 phosphorylation attenuating the activation of PDE4A5 by PKA, when cells expressing wild-type PDE4A5 were pre-treated with anisomycin in the presence of SB203580, challenge with forskolin/IBMX caused a similar increase in activation to that seen in cells subjected to forskolin/IBMX challenge only (276 ± 14%; mean ± S.D., n = 3).
These results imply that, through actions on UCR1, MK2 phosphorylation alters PDE4A5 conformation to attenuate the degree of activation caused by PKA phosphorylation. Although there is a wealth of structural data on the truncated PDE4 catalytic unit from all four PDE4 subfamilies (see, e.g., [28]), the structure of full-length PDE4 species remains to be resolved. Nevertheless, recent structural data [29] strongly support the notion that PDE4 enzymes can adopt different conformational states depending upon the folding of UCR2 across the catalytic unit and that such events are likely to be regulated by phosphorylation [30]. As the UCR1 and UCR2 regulatory domains can interact with each other to form a regulatory module [31,32], it is likely that phosphorylation of UCR1 will initiate distinct conformational changes in PDE4.

Alterations in protein conformation can lead to changes in thermal stability, which gives a simple means of gaining insight into possible conformation changes. This is because thermal inactivation of enzymes is a first-order process whose half-life ($t_{1/2}$) can be determined by following the log$_{10}$ PDE4 activity remaining when the protein is exposed to a temperature that elicits inactivation. In the present study, we used cells transfected with wild-type PDE4A5 that were either unchallenged or had been treated to achieve maximal phosphorylation by MK2 (10 μg/ml anisomycin, 60 min) or PKA (100 μM forskolin + 100 μM IBMX, 20 min) or both PKA and MK2 (anisomycin, 60 min, then forskolin + IBMX, 20 min). Lysates from these cells were incubated at 55 °C for the indicated times, then samples were taken and stored on ice prior to PDE assay analysis. Such treatment, at 55 °C, led to the first-order inactivation of PDE4A5 with a $t_{1/2}$ of 7.8 ± 0.4 min (mean ± S.D., n = 3 separate determinations) (Figure 4g). However, in anisomycin-challenged cells, MK2-phosphorylated PDE4A5 became more thermostable (Figure 4g), with a $t_{1/2}$ of 5.9 ± 0.3 min (mean ± S.D., n = 3 separate determinations). In marked contrast with this, PKA-phosphorylated PDE4A5 (forskolin/IBMX-treated cells; Figure 4g) exhibited an increase in thermostability, with a $t_{1/2}$ for thermal denaturation of 10.1 ± 0.9 min. However, multi-site MK2/PKA phosphorylation showed that MK2 phosphorylation of PDE4A5 overrode the stabilizing effect of PKA phosphorylation of PDE4A5, decreasing the thermostability of PDE4A5 to give a $t_{1/2}$ of 4.9 ± 0.5 min (Figure 4g). Note that when SB203580 was added together with anisomycin, when pre-treating the cells prior to forskolin/IBMX challenge, then no such decrease in PDE4A5 thermostability ensued ($t_{1/2}$ for denaturation of 11.5 ± 1.2 min; mean ± S.D., n = 3). These results are consistent with the notion that phosphorylation, by both PKA and MK2, alters the conformational status of PDE4A5. Furthermore, they suggest that phosphorylation by MK2 can override the thermostabilizing effect on PDE4A5 of PKA phosphorylation, which would be consistent with MK2 phosphorylation attenuating the ability of PKA phosphorylation to activate PDE4A5 (Figure 4d).

**Phosphorylation of PDE4A5 by MK2 reprogrammes the transience of the intracellular cAMP accumulation signature elicited by forskolin challenge**

Hormones and neurotransmitters that activate adenylate cyclase through receptors coupled to the G-protein G, yield a ‘signature’ transient rise in intracellular cAMP. One contributor to the transient cAMP rise is the uncoupling of stimulatory receptors from G, by β-arrestin-mediated desensitization. However, transient increases in intracellular cAMP accumulation occur even when adenylate cyclase is activated directly by forskolin and thus β-arrestin is not involved. Underpinning this important phenomenon is the PKA-mediated phosphorylation of PDE4 long forms, whose concomitant activation serves to drive down cAMP levels, thus providing a key component of the system controlling cellular desensitization to cAMP signalling [8]. Indeed, failure to activate PDE4 by either inhibition [8] or depletion [33] of PKA invariably confers a stable increase in cAMP levels, indicating the importance of PKA-mediated PDE4 phosphorylation and activation. This situation is exemplified in the present study in COS1 cells transfected to express the long PDE4A5 isoform, where it contributes >96% of total cAMP PDE activity. Challenge of these cells with forskolin causes a transient rise in cAMP (Figure 5a), whereas treatment with the PKA inhibitor KT5720, to ablate PKA phosphorylation of PDE4A5, elicits an elevated, sustained, increase in cAMP in response to forskolin challenge (Figure 5a).

In the present study, we show that pre-incubation of cells with anisomycin, in order to cause MK2 phosphorylation of PDE4A5, markedly reconfigures the transient effect of forskolin challenge on cAMP accumulation, so as to increase both its amplitude and duration of elevation (Figure 5a). This change is consistent with the degree to which anisomycin attenuates PKA activation of PDE4A5 (Figure 4d). This reprogramming of the cAMP transient was clearly due to the phosphorylation of PDE4A5 at Ser$^{97}$ as, in marked contrast with observations in cells expressing wild-type PDE4A5, no such anisomycin-elicited alteration in forskolin-stimulated cAMP accumulation was evident in cells transfected to express the S147A-PDE4A5 mutant, which is unable to be phosphorylated by MK2 (Figure 5b).

Macrophages play a key role in inflammatory events and are critical targets for the action of anti-inflammatory PDE4 inhibitor therapeutics [5]. As MK2 plays an important role in these cells and becomes activated during inflammation [2], we set out to determine whether MK2 activation could reprogramme the cAMP transience in primary macrophages. Cells invariably express a range of PDE4 isoforms and macrophages are no exception, expressing PDE4A5 and various other long isoforms [25,34]. These have conserved UCR1 and UCR2 regulatory regions with extremely similar catalytic units that exhibit identical $K_v$ values for cAMP. As they are all similarly activated by PKA [7], one might infer that phosphorylation by MK2 would similarly attenuate PKA activation of the various long PDE4 isoforms. Thus we set out to evaluate whether PDE4D3, a well-characterized long isoform, which is one of the predominant long PDE4 isoforms expressed in murine macrophages, is regulated by MK2 in a similar way [34]. As shown previously [7], challenge of COS1 cells with the adenylate cyclase activator forskolin increases intracellular cAMP levels, causing PKA to phosphorylate and thereby activate recombinant PDE4D3 expressed in these cells, as observed in the present study (Figure 5c). However, as with PDE4A5, we noted that PDE4D3 activation was markedly attenuated when the p38 pathway was activated with anisomycin (Figure 5c). However, such an effect was not evident when cells were transfected to express a form of PDE4D3 (S61A-PDE4D3) in which the serine target for phosphorylation by MK2 was mutated to alanine (Figure 5c). As with PDE4A5, the activity of S61A-PDE4D3 was similar to that of wild-type PDE4D3 (<10% difference; n = 3). Thus MK2 phosphorylation of the serine residue located in the conserved consensus motif found in UCR1 would seem likely to have a similar effect in attenuating the activation of all long PDE4 isoforms by PKA.

We thus set out to assess cAMP accumulation in primary murine macrophages from wild-type mice. In so doing we observed that, although forskolin caused a markedly transient increase in cAMP accumulation, anisomycin profoundly attenuated the transience of this response (Figure 5d). Such an effect of anisomycin was
clearly mediated by activation of the p38 MAPK pathway, as it was ablated by SB203580 (Figure 5e). As shown in COS1 cells overexpressing recombinant PDE4A5 (Figure 5a), the transience of cAMP accumulation in primary murine macrophages was clearly dependent upon PKA, as inhibition of this kinase with KT5720 ablated the transient pattern of cAMP accumulation (Figure 5e). We then wished to confirm whether the effect of anisomycin on cAMP accumulation was MK2-mediated using a null mouse model. The MK2-related kinase MK3, although invariably expressed at much lower levels than MK2, can substitute for MK2 in those cells where it is expressed upon MK2 knockdown. This prompted the generation of an MK2/MK3 double-null mouse line in order to assess MK2/3 signalling [35].

Using primary macrophages from MK2−/−/MK3−/− animals, we see that forskolin treatment elicits a transient increase in cAMP levels that is not only similar to that seen using macrophages from wild-type animals, but also singularly insensitive to alteration of PDE4A5, whose basis has been inferred from recent structural analysis with AIP functioning as a non-competitive inhibitory regulator of PDE4A5, which is thought to contribute to the molecular pathology caused no change in PDE4A5 activity.

MK2 phosphorylation of PDE4A5 attenuates its ability to be reversibly recruited into intracellular aggregates by chronic rolipram treatment

PDE4A5 and its human orthologue PDE4A4 are unique in being able to be reversibly recruited into cytosolic protein aggregates (foci) upon chronic treatment with the PDE4 selective inhibitor rolipram [23]. This process serves to remove PDE4A4/5 from functional partnerships with certain other proteins in cells and is also linked to the autophagy system by virtue of being dependent upon the scaffold/ubiquitin-binding protein p62 (also called SQSTM1) [22]. Such an ability of PDE4A4/5 to relocalize in cells in response to chronic rolipram treatment is ablated by anisomycin treatment [22] (Figure 6). However, we show in the present study that rolipram-induced foci formation of S147A-PDE4A5, the MK2 phospho-null form of PDE4A5, is insensitive to inhibition by anisomycin treatment (Figure 6). These observations suggest a molecular mechanism whereby MK2 phosphorylation of the regulatory UCR1 of PDE4A5 causes a conformational change that prevents PDE4A5 reversible recruitment to p62-containing intracellular aggregates (foci) in response to chronic rolipram treatment.

Phosphorylation of PDE4A5 by MK2 differentially affects co-immunoprecipitation with the scaffolding proteins AIP/XAP2, DISC1 and β-arrestin

AIP (XAP2) is able to sequester PDE4A5 [16] (Figure 1a) and this is thought to contribute to the molecular pathology of familial pituitary adenomas [36]. AIP shows specificity for PDE4A5 as it interacts with its isoform-specific N-terminal region [16]. However, for sequestration to occur, AIP has to interact with a second site on PDE4A5, namely one located within the conserved regulatory UCR2 domain (Figure 1a) [16]. A functional consequence of this interaction is a maximal reduction in the catalytic activity of PDE4A5 of approximately 60% without any change in its $K_m$ for cAMP [16]. This is consistent with AIP functioning as a non-competitive inhibitory regulator of PDE4A5, whose basis has been inferred from recent structural analyses of PDE4 UCR2 catalytic subunit constructs [29].

In the present study, we show that when COS1 cells, which do not natively express AIP, are transfected to express both PDE4A5 and AIP, then anisomycin challenge markedly reduces the ability of AIP to be co-immunoprecipitated with PDE4A5 (Figures 7a and 7b). Such an effect of anisomycin is ablated by SB203580 treatment and is not observed with the S147A-PDE4A5 mutant, which is unable to be phosphorylated by MK2 (Figure 7b). Thus MK2 phosphorylation of PDE4A5 attenuates its ability to be co-immunoprecipitated with AIP.

Note that the challenge of transfected COS1 cells with anisomycin did not elicit any phosphorylation of recombinant AIP and neither was recombinant AIP phosphorylated in resting cells labelled with $[^{33P}]$PiP, (results not shown). In addition, PKA phosphorylation of PDE4A5, achieved by challenging cells with forskolin/IBMX for 20 min, had no effect on the efficiency of PDE4A5 to co-immunoprecipitate with AIP, which was 94% ± 14% (mean ± S.D., n = 3) as compared with the control (100%). Furthermore, the simultaneous multisite phosphorylation of PDE4A5 by both MK2 and PKA elicited a similar reduction in the ability of AIP to co-immunopurify with PDE4A5 when compared with anisomycin alone (interaction 34 ± 11% of the control; mean ± S.D., n = 3). This further indicates that PKA phosphorylation of PDE4A5 has no role in modulating the PDE4A5–AIP interaction whether or not PDE4A5 is phosphorylated by MK2.

Ectopic expression of AIP with PDE4A5 generates a complex where AIP functionally inhibits the activity of PDE4A5 [16]. As MK2 phosphorylation of PDE4A5 causes PDE4A5 to be released from an inhibitor complex with AIP, we might expect that anisomycin treatment should increase PDE4A5 activity. Indeed, in cells transfected to express both PDE4A5 and AIP, then anisomycin challenge caused an increase in PDE4A5 activity in a manner that is ablated by SB203580 treatment (Figure 7c). This is consistent with MK2 phosphorylation of PDE4A5 reducing the interaction of inhibitory AIP with PDE4A5 in such dual-transfected cells. Indeed, in cells transfected to express PDE4A5 alone and not expressing endogenous AIP, anisomycin challenge caused no change in PDE4A5 activity.

The ability of MK2 phosphorylation to attenuate AIP co-immunoprecipitation with PDE4A5 does not reflect a general action in limiting interaction with all partner proteins as exemplified in the present study using the signalling scaffold protein β-arrestin, which can also sequester PDE4A5 [6]. Thus we show that triggering the MK2 phosphorylation of PDE4A5, by anisomycin challenge, does not alter the amount of PDE4A5 co-immunoprecipitating with β-arrestin (Figure 7d). This may be because, unlike AIP, β-arrestin does not interact with UCR2, but binds to a conserved site within the PDE4 catalytic unit [10].

We then set out to determine whether another protein that bound to PDE4A5 through UCR2 interaction might also be released as a consequence of PDE4A5 phosphorylation by MK2. The scaffold protein and schizophrenia risk factor DISC1 is an example of a PDE4A5 partner protein that interacts with UCR2 [11]. Indeed, we show in the present study that anisomycin challenge of cells co-expressing VSV–PDE4A5 and DISC1 led to a marked reduction (38 ± 7% reduction compared with the control; mean ± S.D., n = 3) in the amount of DISC1 co-immunoprecipitating with PDE4A5 (Figure 7e). This effect was not apparent when SB203580 was added together with anisomycin in order to ablate MK2 activation.

**DISCUSSION**

The p38 MAPK phosphorylation cascade plays a fundamental role in regulating the immune and inflammatory response to

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The direct phosphorylation of PDE4A5 at Ser147. This residue, ablated by both the p38 MAPK inhibitor SB203580 and by the activation and the phosphorylation of PDE4A5 at Ser147, within a MK2 consensus motif (Table 1). Furthermore, in intact cells, challenge with either anisomycin or TNFα causes MK2 activation and the phosphorylation of PDE4A5 at Ser147, which is ablated by both the p38 MAPK inhibitor SB203580 and by the siRNA-mediated knockdown of MK2 (Figure 2). MK2 thus elicits the direct phosphorylation of PDE4A5 at Ser147. This residue, and the associated MK2 consensus motif, is found in all PDE4 long isoforms, where they locate within the conserved regulatory UCR1 domain (human orthologue, PDE4A4). PDE4A5 is implicated in inflammatory lung disease, being up-regulated in COPD [25], and interacts with AIP, which, as well as being linked to familial pituitary adenomas [36], regulates AHR (aryl hydrocarbon receptor), a protein activated by agents involved in promoting respiratory disease, namely tobacco smoke and TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) [38]. Sleep deprivation also causes PDE4A5 up-regulation in the hippocampus, leading to cognitive deficits [24]. Furthermore, in complex with the autophagy-related scaffold protein p62, it is uniquely redistributed into foci in cells upon chronic challenge with certain PDE4 selective inhibitors, thereby reprogramming compartmentalized cAMP signalling involving this isoform [22,23].

In the present study, we uncover PDE4A5 as a node providing a direct regulatory connection between the p38 MAPK and cAMP signalling systems. In this, we show that activated MK2 can phosphorylate a conserved serine residue located within the regulatory UCR1 domain that defines PDE4 long isoforms [30]. The four PDE4 subfamilies encode ≥16 long isoforms [6] and, in order to provide a focus, we elected to analyse the long PDE4A5 isoform [9] (human orthologue, PDE4A4). PDE4A5 is implicated in inflammatory lung disease, being up-regulated in COPD [25], and interacts with AIP, which, as well as being linked to familial pituitary adenomas [36], regulates AHR (aryl hydrocarbon receptor), a protein activated by agents involved in promoting respiratory disease, namely tobacco smoke and TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) [38]. Sleep deprivation also causes PDE4A5 up-regulation in the hippocampus, leading to cognitive deficits [24]. Furthermore, in complex with the autophagy-related scaffold protein p62, it is uniquely redistributed into foci in cells upon chronic challenge with certain PDE4 selective inhibitors, thereby reprogramming compartmentalized cAMP signalling involving this isoform [22,23].

Figure 6  MK2 phosphorylation of PDE4A5 attenuates its ability to be reversibly recruited into intracellular aggregates by chronic rolipram treatment

COS1 cells were transfected with GFP-tagged forms of either wild-type (WT) PDE4A5 or S147A-PDE4A5. They were then incubated without any treatment or with rolipram (10 μM) for 12 h prior to treatment with or without anisomycin (10 μg/ml, 60 min) and subsequent fixation and detection of PDE4A4–GFP as shown in (a). Quantification of foci formation (arrows show examples of PDE4A5 foci) for such experiments and other control studies are shown in (b) (means ± S.D. for n = 3 separate experiments). Aniso, anisomycin; Roli, rolipram.
Figure 7  MK2 phosphorylation of PDE4A5 and its ability to be sequestered by partner proteins

(a) COS1 cells were co-transfected to express VSV–PDE4A5 and FLAG–AIP, then cells were untreated (Ctr), treated with anisomycin (10 μg/ml) (Aniso) or treated with both anisomycin (10 μg/ml) and SB203580 (10 μM) (SB) for 60 min. After this, lysates were generated and split for immunopurification (IP) of either AIP or PDE4A5, and each immunoprecipitate was then immunoblotted for both AIP (FLAG) and PDE4A5 (VSV). (b) Quantification of AIP pulled-down with either immunopurified wild-type (WT) PDE4A5 or immunopurified mutant VSV–S147A-PDE4 as in (a); treatments are anisomycin, SB203580 and anisomycin plus SB203580. Results are means ± S.D. (n = 3). (c) COS1 cells were co-transfected with VSV–PDE4A5 and FLAG–AIP, then either untreated (Ctr) or challenged for 60 min with either anisomycin alone or anisomycin plus SB203580. After this, VSV–PDE4A5 was immunopurified from lysates and the cAMP PDE activity was determined for equal immunoreactive amounts of VSV–PDE4A5 isolated from each of the cell lysates. The cAMP PDE activity is expressed relative to that observed for control cells that had not been treated with either anisomycin or SB203580; results are means ± S.D. (n = 3). (d) Cells were co-transfected with VSV–PDE4A5 and β-arrestin 2 and then either treated (Aniso) or not (Control) with anisomycin for 60 min. Equal amounts of immunopurified β-arrestin 2 were then immunoblotted for either VSV–PDE4A5 or for β-arrestin 2 as indicated. Ig heavy chain (Ig H-chain) was used to allow for the resolution of β-arrestin, as discussed previously [10]. (e) Cells were co-transfected with VSV–PDE4A5 and DISC1 and then either treated (Aniso) or not (Control) with anisomycin for 60 min. Equal amounts of immunopurified DISC1 were then immunoblotted for either VSV–PDE4A5 or for DISC1 as indicated. All immunobots shown are typical of blotting experiments performed at least three times. (f) Schematic representation of the key functional changes occurring in long PDE4 isoforms as a consequence of phosphorylation of the UCR1–UCR2 module by MK2. The diminished sequestration by DISC1 is likely to be a pan effect, as all subfamilies bind this scaffold, whereas the effect on AIP is specific to PDE4A4/5, as the unique N-terminal regions of this isoform is also required for AIP binding.
PDE4A5, the profound transience of this response was abolished. Instead, a steady-state level of accumulation was attained that slowly declined (Figure 5). This is consistent with previous studies performed on the PDE4D3 long form [8], which led to the appreciation that stimulatory PKA phosphorylation of long PDE4 isoforms accelerates cAMP degradation, thereby facilitating a ‘signature’ transient accumulation of cAMP upon adenylate cyclase activation [33].

We then set out to assess whether this occurred in native cells and chose to use macrophages, as they play a key role in inflammatory events, are critical targets for the action of anti-inflammatory PDE4 inhibitor therapeutics [5] and it is known that, during inflammation, MK2 becomes activated in these cells [2]. These cells express various PDE4 long isoforms, including PDE4A5 and PDE4D3, which we have shown in the present study are similarly regulated upon phosphorylation by PKA and MK2 (Figures 4 and 5). As with the model system, adenylate cyclase activation caused a marked increase in cAMP accumulation in murine primary macrophages whose transience was profoundly attenuated upon activation of the p38 signalling pathway with anisomycin (Figure 5). This effect was evidently MK2/3-mediated, as it was not observed in macrophages from double-null MK2−/−/MK3−/− animals, which retained a profoundly transient response to adenylate cyclase activation even in the face of anisomycin challenge (Figure 5).

Thus the novel regulatory system we have identified clearly has importance in a relevant primary cell type.

Proteins that interact with PDE4 isoforms often bind at multiple sites, where one site invariably lies within the N-terminal region that defines each isoform and so confers specificity, as is seen with AIP, DISC1, RACK1 and β-arrestin, for example [6]. However, for certain partner proteins, such as DISC1 and AIP, UCR2 provides an additional binding site [6]. Not only do UCR2 and UCR1 interact with each other to form a module able to regulate the functioning of the catalytic unit, but also this interaction is altered by PKA-mediated phosphorylation of UCR1 [17,31,32]. Interestingly, although MK2 phosphorylation of PDE4A5 attenuates its ability to interact with DISC1 and AIP, it does not attenuate its ability to sequester β-arrestin, a partner whose binding does not involve UCR2 [6] (Figure 5). This indicates that MK2 phosphorylation alters the conformation of the UCR1–UCR2 module not only to reprogramme the functional consequences of PKA phosphorylation, but also to reprogramme partnerships with interacting proteins that utilize this module for binding (Figure 7e).

It has also been demonstrated that in binding to PDE4A5, AIP acts as a non-competitive inhibitor [16]. This may well ensue through its interaction with UCR2, as Conti and colleagues showed that antisera directed to this module could alter PDE4 activity and suggested that UCR2 could exert an inhibitory function [32]. Indeed, recent structural studies support this concept, indicating that UCR2 can not only dock to the PDE4 catalytic unit, but also that a part of UCR2 is able to fold across and occlude access to the cAMP-binding site itself, thereby providing a molecular basis for inhibition [29]. In the present study we show that AIP-sequestered PDE4A5 becomes activated upon MK2 phosphorylation, presumably due to the release of the inhibitory AIP from PDE4A5 (Figure 5d). Thus inflammatory mediator activation of MK2 in the phosphorylating PDE4A5 can be expected to relieve any AIP-mediated inhibitory constraint placed upon this isoform. Given that PDE4 inhibitors exert a potent anti-inflammatory effect [5], it may that any such activation of PDE4A5 could potentiate pro-inflammatory cell function by enhancing degradation of local cAMP. This may be of importance in the macrophages of COPD patients where PDE4A4, the human orthologue of PDE4A5, is selectively up-regulated [25].

There is currently a great interest in autophagy, a process of recycling intracellular organelles and modified proteins, especially with regard to its links with cell death, neurological disease processes involving protein aggregates and cancer [40]. Critical to this process is p62 (SQSTM1), a multi-domain scaffold protein that sequesters proteins that are either misfolded or have adopted a particular conformation or become ubiquitinated, to either form intracellular aggregates or, in binding to LC3, elicits membrane encapsulation of complexes to form autophagosomes. Rolipram and certain other PDE4 selective inhibitors cause only PDE4A5 and its human orthologue PDE4A4 to be reversibly recruited to intracellular aggregates [23] that lack an encapsulating membrane, in a process that depends upon p62 and leads to the sequestration of these isoforms away from functionally important partner proteins [22]. This process can be inhibited by activation of the p38 MAPK pathway [22], and in the present study we show that the underlying mechanism for this is the phosphorylation of PDE4A5 by MK2, whose ensuing conformational change in the UCR1–UCR2 module presumably underpins this (Figure 6). Given the current interest in PDE4 inhibitors as anti-inflammatory therapeutics, such observations suggest that the cellular response to PDE4 therapeutics that have PDE4A4/5 aggregate-forming properties is likely to differ in cells, dependent upon whether the p38/MK2 pathway is activated or not.

In the present study, we have uncovered a novel means of selectively regulating PDE4 long isoforms through phosphorylation by MK2. Our results are consistent with MK2 phosphorylation triggering a conformational change in UCR1 that reprogrammes both the magnitude of the response to stimulatory PKA phosphorylation, the association of proteins whose binding involves UCR2 and the ability of certain PDE4 inhibitors to recruit PDE4A4/5 to intracellular aggregates (Figure 7e). These observations may be of particular importance, as the p38 MAPK/MK2 pathway is activated in the inflammatory response, where PDE4 activity has been shown to have a regulatory role based on the utility of PDE4 selective inhibitors to act as potent anti-inflammatory agents [5] and from targeted gene-knockout studies [41]. The biological output of cAMP signalling through PKA and EPAC (exchange protein directly activated by cAMP) can be profoundly influenced by the magnitude and duration of the cAMP response [3,42]. Our results show not only that the transient elevation in cAMP levels, which is a signature response of adenylate cyclase activation, is influenced by stimulatory PKA phosphorylation of PDE4 long forms, but also that such a response can be profoundly remodelled by MK2 phosphorylation. Thus MK2 phosphorylation of PDE4 long isoforms may have an impact on cAMP signalling by reprogramming the PKA-driven desensitization response and disrupting protein partnerships. Thus MK2 phosphorylation of PDE4 long isoforms may exert both temporal and spatial influences on cAMP signalling in cells [6].

**AUTHOR CONTRIBUTION**

Kirsty MacKenzie, Derek Wallace and Elaine Hill as co-first authors provided major contributions to experimental work and to project development, and assisted in writing and revising the manuscript. Diana Anthony contributed foci analysis. David Henderson performed bioinformatic analyses and assisted in siRNA analyses. Daniel Houslay directed the project, provided a major contribution in experimental design and analyses, and wrote and revised the manuscript.
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