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Phosphorylation of cyclic AMP phosphodiesterase-4A5 (PDE4A5) by MK2 (MAPKAPK2) attenuates its activation through PKA phosphorylation.

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Abbreviations: PDE, cyclic nucleotide phosphodiesterase; PDE4, cAMP specific phosphodiesterase-4; PKA, cyclic AMP activated protein kinase; AIP, Aryl Hydrocarbon Receptor Interacting Protein; DISC1, disrupted in schizophrenia 1; TNF, tumour necrosis factor; MAPK, mitogen activated protein kinase;

Abstract

Cyclic AMP phosphodiesterase-4 (PDE4) isoforms underpin compartmentalised 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 signalling cascade is a key signal transduction pathway involved in the control of cellular immune, inflammatory and stress responses. Here we show that phosphodiesterase-4A5 (PDE4A5) is phosphorylated at Ser147, within the regulatory UCR1 domain conserved amongst PDE4 long isoforms, by the p38 MAPK activated kinase, MK2 (MAPKAPK2). Phosphorylation by MK2, while not altering PDE4A5 activity, markedly attenuates PDE4A5 activation through phosphorylation by protein kinase A (PKA). This modification confers amplification of intracellular cAMP accumulation in response to adenylyl cyclase activation by attenuating a major desensitization system to cAMP. Such re-programming 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 and AIP, but not the UCR2-independent interacting scaffold protein, beta-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.

Mitogen-activated protein kinases (MAPKs) 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 extra-cellular stimuli such as growth factors, ultra-violet light, ionising 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 (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.

Cyclic AMP 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 phosphodiesterase (PDE) super-family [4]. Of these, selective inhibitors of the cAMP phosphodiesterase-4 (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 and asthma [5]. Four gene families (PDE4A/B/C/D) encode over 20 different isoforms that are expressed on a cell-type specific basis and appear to have non-redundant functional roles based upon siRNA-mediated knockdown, 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, RACK1 and AKAPs as well as receptors such as, p75NTR and the aryl hydrocarbon receptor interacting protein, AIP (XAP2, Ara9), which is a transcription factor [6]. Such specific sequestration events provide targeted cAMP degradation in cells, thereby contributing to both compartmentalised cAMP signalling and the therapeutic basis of PDE4 selective inhibitors [6]. Additionally, elevated cAMP levels cause PKA to phosphorylate, and thus activate, long PDE4 isoforms that have UCR1 and UCR2 regulatory regions, thereby providing a key part of the cellular desensitization system to cAMP [7, 8].

Here we identify cAMP specific phosphodiesterase-4A5 (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.

Methods

Cell culture – COS1 and HeLa cell lines were maintained at 37°C in an atmosphere of 5% CO₂, 95% air in complete growth medium containing Dulbecco's modified Eagle's medium supplemented with 0.1% penicillin/ streptomycin, glutamine and 10% foetal calf serum. Cell lines were maintained at 37°C in an atmosphere of 95% air and 5% CO₂. The cells were passaged when approximately 90% confluence was reached. Where indicated, anisomycin was added at 10 μ g/ml, SB203580 at 10 μ M, forskolin at 100 μ M and IBMX at 100 μ M.

DNA manipulation - Site-directed mutagenesis of plasmid DNA was carried out using the Stratagene[®] (La Jolla, CA, U.S.A.) QuikChange[®] Site-Directed Mutagenesis Kit, according to the manufacturer's instructions. The concentration of DNA samples was determined spectrophotometrically measuring the absorption at 260nm (A₂₆₀). Purified plasmid DNA was produced using the QIAGEN[®] (Crawley, U.K.) QIAprep[®] Kits and stored either at 4°C when eluted in 10mM Tris-Cl, pH 8.5 or at –20°C when eluted in sterile water.

Transfection – COS1 cells were transfected using either Polyfect® (Qiagen) or DEAE-dextran (Sigma) as described before by us [10, 11].

Small interfering RNAs (siRNAs) - the PolyFect® method of mammalian cell transfection, from QIAGEN® (Crawley, U.K.), was used for the transfection of COS1 cells with MK2 siRNA nucleotide from Santa Cruz Biotechnology Inc (Santa Cruz, CA, U.S.A.) as reported previously by San Miguel et al. [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 HEPES, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, pH7.5 with added protease inhibitors) and mixed at 4°C for 20 min. This was cleared by centrifugation before further procedures. Alternatively cells were washed in KHEM buffer before being scraped into KHEM with added protease inhibitors, lysis by freeze thaw and needle homogenisation and removal of insoluble material by centrifugation. Immunoblotting was done using various antisera as indicated. PDE4A specific antisera were as described before by us [13-15], being either specific to PDE4A5, being raised to its unique N-terminal region or specific to the PDE4A sub-family, 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 sub-families i.e. is a specific 'pan'-PDE4A antiserum.

Generation of GST and MBP fusion proteins – the E.coli strain JM109 was transformed with pGEX2T (Amersham biosciences) for the production of GST or transformed with pMALpC2 for the production of MBP. Clones encoding full-length rat PDE4A5 as a MBP fusion and AIP (XAP2) as a GST fusion were generated and purified as described previously [16].

In vitro phosphorylation – 1µg of the indicated MBP or GST fusion protein and a defined amount of recombinant protein kinase as per the information sheet (Upstate Tech., Dundee, U.K.) was diluted in 20mM MOPS; pH 7.2, 25mM β-glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate and 1mM DTT to a final volume of 40µl. 10µl of 1µCi/µl ³²P-ATP diluted in 75 mM MgCl₂ and 500 µM ATP was added to the target recombinant fusion protein and specific kinase mix. The reaction was incubated at 30°C for 10 min. The reaction was stopped by the addition of 12.5 µl of 5x SDS sample buffer. 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_{av} for 1min and 30µl of the sample subjected to SDS-PAGE and transferred to a nitro-cellulose membrane. The nitro-cellulose membranes were then air-dried and scanned using the Bio-Rad® (Hemel Hempstead, U.K.) Personal Molecular Imager® FX to resolve the radioactive regions utilising phosphor image screen technology. The Discovery Series™ Quantity One® 1-D Analysis Software, Version 4.4.0 was used to determine the densitometry of the radioactive regions and analysis. In all cases the extent of the phosphorylation was corrected for the immuno-reactive amount of target protein.

Metabolic labelling of PDE4A5 with [³²P] orthophosphate - COS1 cells were transfected with VSV-tagged PDE4A5. 48h 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% FCS and 20 mM HEPES pH7.4 with 100 mCi [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 β-glycerol phosphate, 20 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 2 mM ATP, 0.5 % NP-40 and containing 1x Roche® Diagnostics (Mannheim, Germany) protease inhibitor cocktail tablets). VSV-PDE4A5 was immunoprecipitated as described above and subjected to SDS-PAGE and visualised using a phosphorimager.

PDE and cAMP Assays - For determination of PDE activity, COS1 cells were transfected as described above and then homogenised in KHEM buffer (50 mM KCl, 10mM EGTA, 1.92 mM MgCl₂, 1 mM dithiothreitol, 50 mM HEPES, pH7.2). As before [16], in transfected cells

greater than 98% of the total PDE activity was due to the recombinant PDE4 construct. PDE activity was assayed using 1 μ M cAMP as substrate, as described previously [18]. Intracellular cAMP was determined by using the 'Cyclic AMP Competitive ELISA' kit (ThermoFisher/Pierce).

Pull-down assays and SDS-PAGE - These were carried out for as described in detail previously by us [11, 16, 19].

Protein Assay - The protein concentration of purified recombinant proteins or cell lysates (whole or sub-cellular fractions) was determined using bovine serum albumin (BSA) as a standard in a spectrophotometric assay with Bradford Reagent from Bio-Rad® (Hemel Hempstead, U.K.).

Macrophage analyses - MK2/3 null c57 black mice were a generous gift from M. Gaestel [20]. Bone Marrow Derived Macrophages were isolated from the femurs of adult c57 black 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 with 10% (vol/vol) FBS, penicillin (100 units/ml), streptomycin (100 μ g/ml), amphotericin B (0.25 μ g/ml), L-glutamine (5 mg/ml) and mouse recombinant macrophage 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-gradeplates. 24 hours after being replated, cells were stimulated.

PDE4A5 Foci analyses - HEK293 cells were grown in 6 well plates at approximately 70 % confluency overnight in DMEM media containing 10 % fetal bovine serum, 1 % penicillin/streptomycin and 1 % L-glutamine. Cells were transfected with plasmids encoding either wild-type or the MK2 null, S147A-PDE4A5-GFP mutant using the Polyfect (Qiagen) transfection protocol according to manufacturer's recommendation. After 48 h, cells were treated with either $\pm$ 5 μ M rolipram overnight followed by $\pm$ 10 μ g/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 (Fig. 1a) [16]. Its catalytic domain is highly conserved within the PDE4 family and its C-terminal domain is unique to the PDE4A sub-family and is found in all members from that sub-family [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] while its human orthologue (PDE4A4) is up-regulated in macrophages of patients with COPD [25]. Furthermore, PDE4A5 becomes inhibited upon binding the aryl hydrocarbon receptor regulatory protein, 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 $\emptyset$ -Xaa-Arg-Xaa-Xaa-Ser, where $\emptyset$ is a hydrophobic amino acid and Xaa is any amino acid. This residue is Ser147, which is located within the UCR1 regulatory domain and, specifically, the sequence, Leu-Tyr-Arg-Ser-Asp-Ser147 (Fig. 1a; Table 1). However, we also noted that another residue, namely Ser161 lies within a sub-optimal MK2 phosphorylation motif of Met-Ser-Arg-Asn-Ser-Ser161, which starts with a hydrophilic methionine residue rather than a hydrophobic amino acid, as required for MK2 action (Table 1).

Setting out to determine if PDE4A5 could be phosphorylated by MK2, we first evaluated this *in vitro* using PDE4A5 purified as a N-terminal MBP fusion protein treated with purified, activated recombinant MK2 in the presence of [33 P]- γ -ATP (Fig. 1b). We showed that PDE4A5 is phosphorylated by MK2, but not by p38 MAPK (Fig. 1b).

The two potential MK2 phosphorylation sites of PDE4A5 were separately mutated to alanine with recombinant proteins purified as N-terminal MBP fusion proteins (Ser147Ala and Ser161Ala) and treated with purified, activated recombinant MK2 in the presence of [33 P]- γ -ATP. While the Ser147Ala-PDE4A5-MBP mutant was not phosphorylated by MK2, the Ser161Ala-PDE4A5-MBP mutant was (Fig. 1c). Indeed, wild-type PDE4A5-MBP could be phosphorylated stoichiometrically by MK2 (0.93 ± 0.11 mol P/mol; mean $\pm$ SD; $n=3$). Together, these data show that MK2 phosphorylates PDE4A5 on a single residue, namely Ser 147.

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 p38MAPK pathway [1], caused the time-dependent, stimulatory phosphorylation of both p38MAPK and MK2 (Fig. 2a, b) through a process ablated by the p38 MAPK specific inhibitor, SB203580 [1].

COS1 cells transfected to express vsv epitope-tagged PDE4A5 were labelled with 32 P and then challenged with either anisomycin or anisomycin + SB203580 prior to immunoprecipitation for analysis. Recombinant vsv-PDE4A5 was selectively immunoprecipitated from the cells using anti-vsv antisera coupled to Protein G Agarose (Fig. 2c; track 'IP') but not using Protein G agarose beads alone (Fig. 2c; track 'b'; beads only' control). Anisomycin challenge alone caused the time-dependent phosphorylation of PDE4A5 in these cells and this phosphorylation was ablated by SB203580 (Fig. 2c-e). Therefore activation of the p38MAPK pathway elicits the phosphorylation of PDE4A5 in COS1 cells. Comparison of the time course in which phosphorylation of MK2 occurs and the time course in which PDE4A5 phosphorylation occurs under anisomycin stimulation implies a role for MK2 in phosphorylation of PDE4A5 (Fig. 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 challenge at a similar level to wild type overexpressed PDE4A5 (Fig. 3a; delta NT). However, further N-terminal truncation, which removes UCR1, generated a construct that failed to become phosphorylated upon anisomycin challenge (Fig. 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 *in vitro* analyses above (Fig. 1b, c), anisomycin challenge of COS1 cells failed to elicit phosphorylation of the Ser147Ala-PDE4A5 mutant, but allowed phosphorylation of the Ser161Ala-PDE4A5 mutant (Fig. 3a). This is again consistent with Ser147 providing the sole site in PDE4A5 for phosphorylation by MK2.

Due to the lack of MK2-specific inhibitors then, in order to support the contention that MK2 can phosphorylate PDE4A5 in cells, we used a small interfering RNA (siRNA) strategy to knock down MK2 expression that others have previously proven 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 (Fig. 3a, b). This treatment neither affected p38MAPK expression levels nor its ability to become phosphorylated in anisomycin-challenged cells (Fig. 3c). However, it did ablate the ability of anisomycin challenge to elicit the phosphorylation of PDE4A5 in COS1 cells (Fig. 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, it plays a backup role to the dominant kinase, MK2 [27]. Whilst we readily detected MK2 in COS1 cells we singularly failed to identify MK3 using specific antisera (data not shown). This, coupled with the fact 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 types where MK3 is expressed it may also serve to phosphorylate PDE4A5.

Although anisomycin is routinely used as a robust reagent to activate the p38MAPK pathway [1] we also were able to demonstrate that both MK2 and PDE4A5 could be phosphorylated upon TNF- α challenge of cells in a manner ablated by SB203580 (Fig. 3d, e). TNF- α is known to activate the p38 MAPK pathway through TNF receptor 1 and the TRAF adaptor molecule. Such TNF- α induced phosphorylation was not evident in cells transfected with Ser147Ala-PDE4A5-vsv (data not shown).

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 (Fig. 4a) or its sensitivity to inhibition by the archetypal PDE4-selective inhibitor, rolipram. Assayed using 1 μ M cAMP as substrate, rolipram IC_{50} values (mean $\pm$ SD, $n=3$) of 1.4 ± 0.5 μ M, for untreated cells, and 1.8 ± 0.4 μ M, 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 Ser140 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 adenylyl cyclase activator, forskolin (100 μ M) and the reversible, non-selective PDE inhibitor, IBMX (100 μ M) in order to increase intracellular cAMP and activate PKA as shown before by us [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, so as to phosphorylate PDE4A5 at Ser147, had no observable effect on the ability of PKA to phosphorylate PDE4A5 at Ser140 (Fig. 4b, c). We also noted that Ser147Ala-PDE4A5 was phosphorylated at Ser140 by PKA to a similar extent to wild-type PDE4A5 in cells challenged in a similar way (Fig. 4e). These data show that MK2 phosphorylation of PDE4A5 does not alter PKA phosphorylation of the enzyme.

However, in contrast to this, we observed that the ability of PKA phosphorylation to activate PDE4A5 was markedly attenuated upon pre-treatment of cells with anisomycin (Fig. 5d). When cells were simply challenged with forskolin/IBMX, then PDE4A5 activity increased to 281 ± 12 % of control (100%). However, when cells were pre-treated with anisomycin then subsequent forskolin/IBMX challenge increased PDE4A5 activity only some 174 ± 8 % of control (values are mean $n = 3 \pm$ SD; Fig. 4e). This action of anisomycin was not observed when the MK2 phospho-null Ser147Ala-PDE4A5 mutant was employed (Fig. 4d, f). 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 (detection with phospho-UCR1 antibody; data not shown) with its activity in untreated cells being identical to 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, then challenged with forskolin/IBMX caused a similar increase in activation to that seen in cells subjected to forskolin/IBMX challenge only (276 ± 14 %; mean $n = 3 \pm$ SD).

These data imply that, through actions on UCR1, MK2 phosphorylation alters PDE4A5 conformation to attenuate the degree of activation caused by PKA phosphorylation. While there is a wealth of structural data on the truncated PDE4 catalytic unit from all 4 PDE4 sub-families (see e.g. [28], the structure of full-length PDE4 species remains to be resolved. Nevertheless, recent structural data [29] strongly supports the notion [30] 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. 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 can be determined by following the $\log_{10}$ PDE4 activity remaining when the protein is exposed to a temperature that elicits inactivation. Here we used cells transfected with wild-type PDE4A5 that were either unchallenged or had been treated to achieve maximal phosphorylation by MK2 (10 $\mu\text{g/ml}$ anisomycin, 60 min) or PKA (100 μM forskolin + 100 μM IBMX, 20 min) or both PKA and MK2 (anisomycin, 60 min then forskolin + IBMX, 20min). Lysates from these cells were incubated at 55°C for the indicated times, whereupon 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 half-life of 7.8 ± 0.4 min (mean; $n=3$ separate determinations) (Fig. 4g). However, in anisomycin-challenged cells, MK2-phosphorylated PDE4A5 became more thermolabile (Fig. 5g), with a half-life of 5.9 ± 0.3 min (mean; $n=3$ separate determinations). In marked contrast to this, PKA phosphorylated PDE4A5 (forskolin/IBMX-treated cells; Fig. 4g) exhibited an increase in thermostability, with a half-life ($T_{0.5}$) for thermal denaturation of 10.1 ± 0.9 min. However, multi-site MK2/PKA phosphorylation showed that MK2 phosphorylation of PDE4A5 over-rode the stabilising effect of PKA phosphorylation of PDE4A5, decreasing the thermostability of PDE4A5 to give a $T_{0.5}$ of 4.9 ± 0.5 min (Fig. 4g). Note that when SB203580 was added together with anisomycin, in pre-treating the cells prior to forskolin/IBMX challenge, then no such decrease in PDE4A5 thermostability ensued ($T_{0.5}$ for denaturation of 11.5 ± 1.2 min; mean $\pm$ SD, $n=3$). These data 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 thermostabilising effect on PDE4A5 of PKA phosphorylation, which would be consistent with MK2 phosphorylation attenuating the ability of PKA phosphorylation to activate PDE4A5 (Fig. 4d).

Phosphorylation of PDE4A5 by MK2 re-programs the transience of the intracellular cAMP accumulation signature elicited by forskolin challenge.

Hormones and neurotransmitters that activate adenylyl cyclase through receptors coupled to the G-protein, G_s yield a 'signature' transient rise in intracellular cAMP. One contributor to the transient cAMP rise is the uncoupling of stimulatory receptors from G_s by β -arrestin-mediated desensitization. However, transient increases in intracellular cAMP accumulation occur even when adenylyl cyclase is directly activated 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 here 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 (Fig. 5a), while treatment with the PKA inhibitor KT5720, to ablate PKA phosphorylation of PDE4A5, elicits an elevated, sustained increase in cAMP in response to forskolin challenge (Fig. 5a).

We show here 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 (Fig. 5a). This change is consistent with the degree to which anisomycin attenuates PKA activation of PDE4A5 (Fig. 4d). This re-programming of the cAMP transient was clearly due to the phosphorylation of PDE4A5 at Ser147 as, in marked contrast to 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 Ser147Ala-PDE4A5 mutant, which is unable to be phosphorylated by MK2 (Fig. 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 reprogram 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_m 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. We thus set out to evaluate whether PDE4D3, a well-characterised 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 with the adenylyl cyclase activator, forskolin increases intracellular cAMP levels causing PKA to phosphorylate and, thereby, activate recombinant PDE4D3 expressed in these cells, as observed here (Fig. 5c). However, as with PDE4A5, we noted that PDE4D3 activation was markedly attenuated when the p38 pathway was activated with anisomycin (Fig. 5c). However, such an effect was not evident when cells were transfected to express a form of PDE4D3 (Ser61Ala-PDE4D3) where the serine target for phosphorylation by MK2 was mutated to alanine (Fig. 5c). As with PDE4A5, the activity of Ser61Ala-PDE4D3 was similar to that of wild-type PDE4D3 (< 10% difference; $n=3$). Thus MK2 phosphorylation of the serine located in the conserved consensus motif found in UCR1 would seem likely to have a similar effect in attenuating 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 while forskolin caused a markedly transient increase in cAMP accumulation, anisomycin profoundly attenuated the transience of this response (Fig. 5d). Such an effect of anisomycin was clearly mediated by activation of the p38MAPK pathway as it was ablated by SB203580 (Fig. 5e). As shown in COS1 cells over-expressing recombinant PDE4A5 (Fig. 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 (Fig. 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 a double MK2/MK3 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 not only is similar to that seen using macrophages from wild-type animals but which is singularly insensitive to alteration upon anisomycin-challenge (Fig. 5d). This indicates a critical role for MK2/3 in regulating cAMP accumulation in primary cells. Macrophages from both wild-type and MK2(-)/MK3(-) animals had similar levels of p38 MAPK, which was similarly activated by anisomycin treatment (data not shown). *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 (SQSTM1) [22]. Such an ability of PDE4A4/5 to relocate in cells in response to chronic rolipram treatment is ablated by anisomycin treatment (Fig. 6; [22]). However, we show here that rolipram-induced foci formation

of Ser147Ala-PDE4A5, the MK2 phospho null form of PDE4A5, is insensitive to inhibition by anisomycin treatment (Fig. 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 scaffolding proteins AIP/XAP2, DISC1 and β arrestin.

AIP (XAP2) is able to sequester PDE4A5 [16] (Fig. 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 (Fig. 1a) [16]. A functional consequence of this interaction is a maximal reduction in the catalytic activity of PDE4A5 of around 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].

We show here 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 (Fig. 7a, b). Such an effect of anisomycin is ablated by SB203580 treatment and is not observed with the Ser147Ala-PDE4A5 mutant, which is unable to be phosphorylated by MK2 (Fig. 7b). Thus MK2 phosphorylation of PDE4A5 attenuates its ability to be co-immunoprecipitated with AIP.

Note that 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 ^{33}P -Pi (Data not shown). Also, 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 at 94% $\pm$ 14% (mean $\pm$ SD; $n = 3$) as compared to the control (100%). Furthermore, the simultaneous multi-site phosphorylation of PDE4A5 by both MK2 and PKA, elicited a similar reduction in the ability of AIP to co-immunoprecipitate with PDE4A5 when compared with anisomycin alone (interaction some 34 $\pm$ 11%; mean $\pm$ SD; $n = 3$ of the control (100%)). 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 inhibitory 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 (Fig. 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, then 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 here using the signalling scaffold protein β arrestin, which can also sequester PDE4A5 [6]. Thus we show here that triggering the MK2 phosphorylation of PDE4A5, by anisomycin challenge, does not alter the amount of PDE4A5 co-immunoprecipitating with β arrestin (Fig. 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 set out then to see if 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 here that anisomycin challenge of cells co-expressing vsv-PDE4A5 and DISC1 led to a marked reduction ($38 \pm 7\%$ reduction in control; mean $\pm$ SD, $n=3$) in the amount of DISC1 co-immunoprecipitating with PDE4A5 (Fig. 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 infection and tissue injury [1]. Indeed, it has been proposed that inhibition of p38 MAPK, and downstream kinases such as MK2, may be of potential therapeutic benefit in disease states characterised by excessive immune or inflammatory reactions, such as asthma, COPD, rheumatoid arthritis and psoriasis [37]. Similarly, inhibition of PDE4 activity elicits marked anti-inflammatory actions with PDE4 selective inhibitors being developed as therapeutic agents for treating inflammatory lung disease [5].

Here we uncover PDE4 as a node providing a direct regulatory connection between the p38MAP kinase 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 sub-families encode some 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 that, as well as being linked to familial pituitary adenomas [36], regulates the AHR, a protein activated by agents involved in promoting respiratory disease, namely tobacco smoke and TCCD [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 re-programming compartmentalised cAMP signalling involving this isoform [22, 23].

Here we show that purified MK2 can phosphorylate purified PDE4A5 at Ser147 (Fig. 1), which lies within a MK2 consensus motif (Table 1). Furthermore, in intact cells, challenge with either anisomycin or TNF- α 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 (Fig. 2). MK2 thus elicits the direct phosphorylation of PDE4A5 at Ser147. This residue, and associated MK2 consensus motif, is found in all PDE4 long isoforms, where they locate within the conserved regulatory UCR1 domain, which extends from Ser135 to Asn195 in PDE4A5. UCR1 characterises long isoforms from all four PDE4 sub-families [39] and performs a major regulatory role in transducing the effect of stimulatory phosphorylation by PKA [7]. This allows PDE4 long isoforms such as PDE4A5, which is phosphorylated at Ser140 by PKA [7], to provide a feedback regulatory system that forms a key part of the cellular desensitization system for cAMP signalling [8]. Their PKA-mediated activation thus imposes a transient increase in cAMP accumulation in cell subsequent to adenylyl cyclase activation (see Fig. 5; [8, 33]). However, in marked contrast to the stimulatory effect of phosphorylation by PKA, we show that MK2 phosphorylation of PDE4A5 has no direct effect on PDE4A5 activity alone but, rather, it attenuates the degree to which PDE4A5 becomes activated upon phosphorylation by PKA (Fig. 4d). Thus MK2 phosphorylation of PDE4 long isoforms serves to re-programme a major function of PDE4 long isoforms in underpinning cellular desensitization to cAMP. This it does by diminishing the magnitude of the extent to which PDE4 long isoforms become activated by PKA phosphorylation when cAMP levels rise in cells, thereby altering the normally transient increase in cAMP seen upon adenylyl cyclase activation to a larger and more sustained response (Fig. 5). Consistent with this we observed that the activation of PDE4D3 by PKA phosphorylation was similarly attenuated by MK2 phosphorylation that, of itself, did not

alter the activity of PDE4D3 (Fig. 5c). In order to examine whether MK2 phosphorylation of PDE4 long isoforms could alter the cellular cAMP signature we first examined a model system using COS1 cells ectopically expressing PDE4A5 so as to achieve levels of expression where >98% of the total cAMP PDE activity was due to this recombinant enzyme. Challenging these cells with forskolin allowed us to observe a characteristic, markedly transient rise in cAMP (Fig. 5). However, when cells were treated with the PKA inhibitor, KT5720 to prevent phosphorylation and activation of PDE4A5, the profound transience of this response was abolished. Instead, a steady state level of accumulation was attained that slowly declined (Fig. 5). This is consistent with previous studies performed on the PDE4D3 long form [8], which led to the appreciation [33], that stimulatory PKA phosphorylation of long PDE4 isoforms accelerates cAMP degradation, thereby facilitating a 'signature' transient accumulation of cAMP upon adenylyl cyclase activation. 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 here are similarly regulated upon phosphorylation by PKA and MK2 (Fig. 4, 5). As with the model system, adenylyl 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 (Fig. 5). This effect was evidently MK2/3 mediated as it was not observed in macrophages from double MK2(-)/MK3(-) animals, which retained a profoundly transient response to adenylyl cyclase activation even in the face of anisomycin challenge (Fig. 5). Thus the novel regulatory system we have identified clearly has import 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]. UCR2 and UCR1 not only interact with each other to form a module able to regulate the functioning of the catalytic unit but this interaction is altered by PKA-mediated phosphorylation of UCR1 [17, 31, 32]. Interestingly, while 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 (Fig. 5; [6]). This indicates that MK2 phosphorylation alters the conformation of the UCR1/UCR2 module not only to re-program the functional consequences of PKA phosphorylation but also to re-program partnerships with interacting proteins that utilise this module for binding (Fig. 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 [29] support this concept, indicating that UCR2 can not only dock to the PDE4 catalytic unit but that a part of UCR2 is able to fold across and occlude access to the cAMP binding site itself, thereby provided a molecular basis for inhibition [29]. Here we show that AIP-sequestered PDE4A5 becomes activated upon MK2 phosphorylation, presumably due to the release of the inhibitory AIP from PDE4A5 (Fig. 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 to 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 we show here 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 (Fig. 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.

Here we have uncovered a novel means of selectively regulating PDE4 long isoforms through phosphorylation by MK2. Our data are consistent with MK2 phosphorylation triggering a conformational change in UCR1 that reprograms 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 (Fig. 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 can be profoundly influenced by the magnitude and duration of the cAMP response [3, 42]. Our data show that the transient elevation in cAMP levels, which is a signature response of adenylyl cyclase activation, is not only influenced by stimulatory PKA phosphorylation of PDE4 long forms but that such a can be profoundly re-modelled by MK2 phosphorylation. Thus MK2 phosphorylation of PDE4 long isoforms may impact on cAMP signalling by re-programming 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 signaling in cells[6].

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Figure Legends

Figure 1. MK2 phosphorylates PDE4A5 *in vitro*.

(a) This shows 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, LR2), catalytic domain and sub-family specific C-terminal domain. The sites for phosphorylation by PKA (Ser140) and MK2 (Ser147) are shown. Also shown are the binding sites for AIP (XAP2), which binds at both UCR2 and the N-terminal region; with DISC1, which binds at both UCR2 and the catalytic unit and with β arrestin, which binds at the catalytic unit only. (b) shows an autoradiograph (upper panel) and loading immunoblot for PDE4A5 (lower panel) from *in vitro* phosphorylation experiments with 32 P-ATP and either no added kinase (Ctr) or purified MK2 or purified p38 MAPK together with purified MBP-PDE4A5. This shows a typical experiment of one done 3 times. (c) shows quantification of such experiments using the indicated protein kinases and either wild-type MBP-PDE4A5 (WT) or the Ser147Ala (S147A) or Ser161Ala (S161A) mutants of MBP-PDE4A5. Data show means $\pm$ SD ($n = 3$) 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.

Figure 2. MK2 phosphorylates PDE4A5 *in vivo*.

(a) show immunoblots of cell lysates done to assess activation of MK2 and p38 MAPK in resting cells (Ctr), those treated with anisomycin (10 μ g/ml) (A) and those treated with both anisomycin (10 μ g/ml) and SB203580 (10 μ M) (A + S). (b) shows the time-course of phosphorylation of both p38 MAPK and MK2 in cells treated with anisomycin (10 μ g/ml) for the indicated times. (c) shows the phosphorylation of VSV-tagged recombinant PDE4A5 expressed ectopically in COS1 cells labelled with 32 P-Pi by phosphorimager analysis. Cells were subjected to challenge (60 min) with either anisomycin (A) alone or together with SB203580 (S) or with no additions (Ctr), as indicated. PDE4A5 was immunopurified and then subjected to SDS-PAGE with, subsequent, phosphorimager analysis (upper panel) and immunoblot analysis (vsv epitope) for 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 μ g/ml) for the indicated times and samples 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)) where data are means $\pm$ SD, $n=3$.

Figure 3. MK2 phosphorylates PDE4A5 at Ser147 *in vivo*.

(a) COS1 cells were transfected to express either the indicated wild-type and mutant forms of vsv-PDE4A5, loaded with 32 P-Pi and challenged for 60 min with anisomycin (10 μ g/ml) 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 scrambled control siRNA (ctr siRNA) prior to transfection with wild-type vsv-PDE4A5, treatment with anisomycin and phosphorimager analysis as with the others. Data are means $\pm$ SD, $n=3$. (b) shows an 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) shows an 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) shows lysates of cells challenged for the indicated times with TNF- α (10 ng/ml) prior to immunoblotting

for either total MK2 or p-MK2. (e) shows the phosphorylation of VSV-tagged recombinant PDE4A5 expressed ectopically in cells labelled with ^{32}P -Pi by phosphorimager analysis. Cells were subjected to challenge (20 min) with either TNF- α (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 vsv-PDE4A5 loading control (lower panel). All immunoblots shown are typical of ones done at least 3 times.

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

(a) This shows the 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; data are means $\pm$ SD, $n=3$. (b) Uses a phospho-UCR1 antisera [7] to assess the PKA phosphorylation status of the Ser residue in the conserved PKA motif located in the conserved UCR1 of all PDE4 long isoforms, which is Ser140 in PDE4A5. Here vsv-PDE4A5 was first immunopurified from COS1 cells treated for the indicated times with forskolin (100 μM) plus IBMX (100 μM) that either had (anisomycin; open squares) or had not (control; solid circles) been pre-treated for 60 min with anisomycin (10 $\mu\text{g/ml}$) and this immunoblotted using phospho-UCR1 antisera; data are means $\pm$ SD, $n=3$. (c) shows a 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 done 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 circles) or had not (Control; solid circles) been pre-treated for 60 min with anisomycin (10 $\mu\text{g/ml}$); data are means $\pm$ SD, $n=3$. (e) As in (b) but using the Ser147A mutant form of vsv-PDE4A5; data are means $\pm$ SD, $n=3$. (f) As in (d) but using the Ser147A mutant form of vsv-PDE4A5; data are means $\pm$ SD, $n=3$. (g) COS1 cells, transfected to express PDE4A5, were either not treated or treated, as indicated, with either anisomycin (10 $\mu\text{g/ml}$; 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 $^{\circ}\text{C}$ and samples removed at the indicated times and immediately flash cooled on ice. These were then taken for assay of cAMP PDE4 activity and data plotted in semi-log fashion as Log % of PDE4 activity remaining (no heat treatment taken as 100%) versus time. This shows a typical example of an experiment performed 3 times. All immunoblots shown are typical of ones done at least 3 times.

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

COS1 cells were transfected with either wild-type PDE4A5-vsv (a) or Ser147Ala-PDE4A5-vsv (b) or (c) then, as indicated, either wild-type or the Ser61Ala mutant form of PDE4D3 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. In (a) and (b) transfected cells were challenged with forskolin (100 μM) and samples taken at the indicated time for determination of intracellular cAMP, which is expressed relative to basal levels in untreated cells (100%) with data shown as means $\pm$ SD ($n=3$ separate experiments). Where indicated cells were pre-treated with either anisomycin (10 $\mu\text{g/ml}$; 60 min pre-incubation) or the PKA inhibitor, KT5720 (4 μM ; 20 min pre-incubation). In (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%). In (d) and (e) primary murine macrophages from either wild-type or double knockout MK2(-/-)MK3(-/-) (KO) animals were incubated with combinations of

forskolin (100 μ M), anisomycin (10 μ g/ml; 60 min pre-incubation) and KT5720 (4 μ M; 20 min pre-incubation) prior to harvesting at the indicated times for assessment of intracellular cAMP levels, which are expressed here relative to the maximal levels achieved in each case (100%). Data shows means $\pm$ SD for 3 separate experiments (n=3), with individual cAMP determinations done in triplicate.

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 PDE4A5 or Ser147Ala-PDE4A5. They were then incubated without any treatment or with rolipram (10 μ M) for 12h prior to treatment with or without anisomycin (10 μ g/ml; 60 min) and subsequent fixation and detection of PDE4A4-GFP as shown here in (a). Quantification of foci formation for such experiments and other control studies are shown in (b) (means $\pm$ SD for n=3 separate experiments).

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 either untreated (Ctr), treated with anisomycin (10 μ g/ml) (A) or treated with both anisomycin (10 μ g/ml) and SB203580 (10 μ M) (A + S) 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 PDE4A5 or immunopurified mutant VSV-Ser147A-PDE4 (M) as in (a) with data as means $\pm$ SD, n=3 and where treatments are anisomycin (A), SB203580 (S) and anisomycin plus SB203580 (A + S). (c) COS1 cells were co-transfected with VSV-PDE4A5 and FLAG-AIP, they were then either untreated (Ctr) or challenged for 60 min with either anisomycin alone (A) or anisomycin plus SB203580 (A + S). After this VSV-PDE4A5 was immunopurified from lysates and the cAMP PDE activity determined for equal immunoreactive amounts of vsv-PDE4A5 isolated from each of the cell lysates. This is expressed here relative to that observed for control cells that had not been treated with either anisomycin or SB203580 with data as means $\pm$ SD, n=3. (d) cells were co-transfected with vsv-PDE4A5 and β arrestin2 and then either treated (A) or not (Ctr) with anisomycin for 60min. Equal amounts of immunopurified β arrestin2 were then immunoblotted for either vsv-PDE4A5 or for β arrestin2 as indicated. (e) cells were co-transfected with vsv-PDE4A5 and DISC1 and then either treated (A) or not (Ctr) with anisomycin for 60min. Equal amounts of immunopurified DISC1 were then immunoblotted for either vsv-PDE4A5 or for DISC1 as indicated. All immunoblots shown are typical of ones done at least 3 times (f) schematic representation of the key functional changes occurring in long PDE4 isoforms as a consequence of phosphorylation of the UCR1/2 module by MK2. The diminished sequestration by DISC1 is likely to be a pan effect as all subfamilies bind this scaffold, whilst the effect on AIP is specific to PDE4A4/5 as the unique N-terminal regions of this isoform is also required for AIP binding.

Table 1. Consensus motif for phosphorylation by MK2

This table lists the amino acid sequence surrounding the serine target for phosphorylation (position '0') by MK2 for known MK2 substrates together with the recognised consensus phosphorylation motif. Also shown are the sequences surrounding Ser147 and Ser161 in PDE4A5.

Consensus	X	X	Hyd	X	R	X	X	S	X	X
<i>Substrates</i>	-7	-6	-5	-4	-3	-2	-1	0	+1	+2
TSC2	T	A	L	Y	K	S	L	S	V	P
SRF	R	G	L	K	R	S	L	S	E	N
eEF2	P	P	L	L	R	P	L	S	E	N
5LO	C	S	L	E	R	Q	L	S	L	E
HSP27	R	A	L	S	R	Q	L	S	S	G
CREB	E	I	L	S	R	R	P	S	Y	R
LSP1	P	K	I	D	R	T	E	S	I	E
Nogo-B	V	A	P	E	R	Q	P	S	W	D
PDE4A5 S147	S	F	L	Y	R	S	D	S	D	Y
PDE4A5 S161	K	T	<u>M</u>	S	R	N	S	S	V	A

Fig. 1

Figure 2

Figure 3

Figure 4

Figure 6

Figure 7