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Cyclic AMP-specific phosphodiesterase, PDE8A1, is activated by protein kinase A-mediated phosphorylation

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ABSTRACT

The cyclic AMP-specific phosphodiesterase PDE8 has been shown to play a pivotal role in important processes such as steroidogenesis, T cell adhesion, regulation of heart beat and chemotaxis. However, no information exists on how the activity of this enzyme is regulated. We show that under elevated cAMP conditions, PKA acts to phosphorylate PDE8A on serine 359 and this action serves to enhance the activity of the enzyme. This is the first indication that PDE8 activity can be modulated by a kinase, and we propose that this mechanism forms a feedback loop that results in the restoration of basal cAMP levels.

1. Introduction

Cyclic-adenosine monophosphate (cAMP) is a ubiquitous second messenger that underpins a wide variety of important cellular functions. Although produced in response to stimulation of many different types of G-protein coupled receptors, cAMP signals can maintain specificity of receptor action by forming gradients inside cells that are shaped in space and time by pools of receptor associated phosphodiesterases, the only known superfamily of enzymes that can hydrolyze cAMP [1]. Dynamic cAMP gradients are then 'sampled' directly by localized cAMP effector proteins such as protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC) that act to trigger receptor specific functions. Work utilizing genetically encoded cAMP reporters has demonstrated that compartmentalisation and regulation of phosphodiesterases (PDEs) is crucial to underpin signal-specific responses [2]. PDEs are divided into 11 families and are characterized by their ability to hydrolyze either cAMP, cyclic guanine monophosphate (cGMP) or both cyclic nucleotides and by their modular structure [3]. Physiological roles for some of the better studied PDE families have been established, but the function of more recently discovered PDEs has been largely unexplored due to a lack of suitable selective pharmacological inhibitors.

Recently, there has been a surge in interest in the PDE8 family of PDEs due to their implication in steroidogenesis [4,5], T cell adhesion [6], lymphocyte chemotaxis [7] and excitation–contraction coupling [8]. Although important in all these cellular processes, nothing is known about how the activity of PDE8 is regulated. Sequence analysis of the full-length open reading frame of PDE8A has uncovered an N-terminal signaling motif known as a known as the Per, ARNT and Sim (PAS) domain [9]. PAS domains are known to direct protein–protein interactions and are likely to play a role in PDE8 regulation. Other interesting motifs that have been deduced from PDE8 sequences are a receiver (REC) domain, consensus sites for N-glycosylation, N-myristoylation, amidation and putative kinase substrate sites for PKC, casein kinase [10] and PKA [11]. It should be stressed that all of these sites are hypothetical and post-translation modification of PDE8 has never, before now, been observed. Using novel peptide array technology and phospho-site specific antibodies, we demonstrate that during times of elevated cAMP, PKA phosphorylates PDE8A on serine 359 and this event triggers the activation of the enzyme.

2. Materials and methods

2.1. Reagents

Forskolin, dipyridamole and 3-isobutyl-1-methylxanthine (IBMX) were dissolved in dimethyl sulfoxide (DMSO) and added

Abbreviations: PDE8, phosphodiesterase 8; PKA, protein kinase A; cAMP, cyclic-AMP; PI3K, phosphoinositide 3-kinase; DNAPK, DNA_dependent protein kinase; FSK, forskolin; EPAC, exchange protein directly activated by cAMP; cGMP, cyclic guanine monophosphate

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to cell media at a concentration of <0.1% DMSO. All were supplied by Sigma (UK). The following antibodies were used in this study, anti-FLAG (Cell Signalling technology, USA: Cat. No. 2368), anti-PKA phospho-substrate (Cell Signalling technology, USA: Cat. No. 9621) anti-CREB (Cell Signalling technology, USA: Cat. No. 9197) anti-phospho CREB (Cell Signalling technology, USA: Cat. No. 9198). In addition, anti-phospho PDE8A1 serine 359 antibody was custom made by AMS Biotechnology (Europe) in rabbits against a phosphorylated peptide corresponding to residues ^{354}DRRKG$_P$SLDVKA^{364}. Total PDE8A antibody was purchased from Scottish Biomedical, UK.

2.2. Immunocytochemistry

Endogenous phospho-PDE8A was visualized in HeLa cells using immunocytochemistry techniques. Briefly, media on cells was replaced with serum free DMEM containing 150 nM Mitotracker Red CMXRos, a mitochondrion-selective probe (Molecular Probes, Invitrogen), and induced with the addition of forskolin (100 μM) if required. Cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde, washed a further two times in tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris, pH 7.4) and permeabilized with three successive incubations with 0.1% Triton-X 100 in TBS. Cells were blocked in blocking buffer (10% of the appropriate serum, 2% BSA in TBS) for 2 h at room temperature. Incubation with the phospho-serine 359 PDE8A antibody at a dilution of 1:400 in a 1:1 solution of blocking buffer:TBS took place at room temperature for 2 h. Where blocking peptide was used, the peptide was incubated with diluted primary antibody for 1 h at room temperature before addition to the cells. Cells were washed with blocking solution three times prior to incubation with a 1:10 000 dilution of Alexa 488-conjugated F('ab)2 fragment IgG (Molecular Probes, Invitrogen). Washes with TBS were performed prior to mounting coverslips onto microscope slides with Immunomount ProLong Gold reagent with DAPI (Molecular Probes, Invitrogen) and visualized using a Zeiss Pascal laser-scanning confocal microscope with a Zeiss Plan-Apo 63 × 1.4 NA oil immersion objective.

2.3. Peptide array

Peptide libraries were produced by automatic SPOT synthesis and synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc-chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany) as previously described by us [12]. PKA phosphorylation of an immobilized library of PDE8 peptides was undertaken using purified PKA catalytic subunit (Promega). Recombinant kinase was diluted in phosphorylation buffer (100 mM Tris–HCl pH 7.5, 0.2 mM ATP, 10 mM $MgCl_2$, 30 mM β-mercaptoethanol, 20% (v/v) glycerol and 10 mM Calyculin A) and incubated with arrays at 30 °C for 30 min with shaking. Control experiments without addition of recombinant kinase were also done.

2.4. Phosphodiesterase assay and cellular transfection of PDE8A1

Phosphodiesterase activity was measured using a radioactive cAMP hydrolysis assay that has been described previously [13–15]. [8-^{3}H] adenosine cyclic-3′,5′-mono-phosphate was sourced from Amersham Biosciences (Little Chalfont, UK) and cyclic-3′,5′-mono-phosphate from Sigma. The substrate concentration used for PDE assays was 150 nM, and the specific PDE activity obtained was between 100–200 pmol cAMP/mg/ml. PDE activities were then normalized for expression of PDE8A1, and data was normalized to DMSO-treated PDE8A1 wild type activity. FLAG-tagged wild-type, Ser359Ala and Ser359Asp PDE8A1 constructs were transfected into COS7 cells (PDE assay) using Polyfect reagent (Qiagen). HEK293/HeLa cells were transfected, with the appropriate Flag-tagged PDE8A constructs (wildtype/dominant negative/S359A/S359D) for 48 h using Polyfect. For particular experiments, cells were pre-treated with KT5720 (4 μM) or H-89 (10 μM) for 20 min prior to treatment with forskolin (100 μM) for specific timepoints. Dipyridamole (100 μM) was added to the cell media after the transfection period. Cells were harvested in lysis buffer (25 mM Hepes/2.5 mM EDTA/50 mM NaC1/50 mM NaF/30 mM sodium pyrophosphate/10% glycerol/1% Triton X-100, pH 7.5, with addition of protease inhibitors). To confirm efficient phosphorylation of wild-type PDE8A1 after forskolin treatment, samples were also blotted using anti-phospho Ser359 antibody.

2.5. In vitro PKA phosphorylation of PDE8

Purified MBP-PDE8A1 (50 μg) was incubated with increasing amounts of the active catalytic unit of PKA (0.5, 6.2, 12.5 and 25 μg) in PKA phosphorylation buffer (20 mM Tris–HCl [pH7.5]; 100 mM NaCl; 5 mM $MgCl_2$; 1 mM DTT 0.2 mg/ml BSA) plus ATP (100 mM) for 1 h at 30 °C with agitation. The samples were run on an SDS–PAGE gel and immunoblotted with a PKA phospho-substrate antibody.

2.6. Cloning and purification of MBP-PDE8A1

Human flag-tagged PDE8A1 in the pCMV-2 plasmid was a gift from Professor Kenji Omori (Japan). The PDE8A1 open reading frame was cloned into a pMAL-c2x vector (NEB) using the following primers to incorporate the *SalI* and *XbaI* recognition sites. Forward primer: AATCTAGAATGGGCTGTGCCCCGA. reverse primer: AAGTCGACCTATTCAGGAGGTGGTC. BL21 competent cells (Invitrogen) were transformed and MBP fusion purified as described previously by us [13].

2.7. Site directed mutagenesis of PDE8A1

Site-directed mutagenesis was performed using the Quickchange kit (Stratagene) according to manufacturer's instructions. The following primers were used to create the required mutations. PDE8A1 dominant negative D726A mutant, forward primer: GCTGATTAAATGTGCTGCTGTGTCCAATCCCTGCC, reverse primer: GGCAGGGATTGGACACAGCAGCACATTTAATCAGC, PDE8A1 S359A mutant, forward primer: AAGACAGGAGAAAAGGCGCACTAGACGTCAAAGCT, reverse primer: AGCTTTGACGTCTAGTGCGCCTTTTCTCCTGTCTT, PDE8A1 S359D mutant, forward primer: CATAAAGACAGGAGAAAAGGCGATCTAGACGTCAAAGCTGTTGCC, reverse primer: GGCAACAGCTTTGACGTCTAGATCGCCTTTTCTCCTGTCTTTATG. All mutations were verified by sequencing.

3. Results

3.1. Delineation of a PKA site on PDE8A using peptide array technology

As it has been hypothesized that PDE8A may be phosphorylated by PKA, we incubated increasing amounts of purified MBP-PDE8A with a PKA assay mix containing active purified PKA catalytic unit. We then blotted the resulting proteins with an antibody that recognizes phospho-PKA substrates containing the sequence R-x-x-pS (where x is any amino-acid and pS is phospho-serine). Equivalent amounts of purified MBP-PDE8A were loaded (Fig. 1A lower panel), however, only samples containing active PKA catalytic unit were recognized by the phospho-PKA substrate antibody suggesting that PDE8A could indeed be directly phosphorylated by PKA.

Peptide array represents a novel method to look at post-translational modification of proteins. Recently we have used this method to delineate SUMOylation [13] and ubiquitination sites [16] on

PDE4 enzymes and PKA phosphorylation sites on phosphoinositide 3-kinase (PI3K) [17] and DNA_dependent protein kinase (DNAPK) [18]. As sequence analysis of PDE8A has three predicted PKA sites [11] (Fig. 1B), we decided to use peptide array to see if any or all of these sites could be phosphorylated by the kinase. Peptide arrays of overlapping 25-mer peptides, sequentially shifted by 5 amino acids and spanning the entire PDE8A sequence, were incubated with a PKA assay mix before detection of phosphorylation using a phosphorylation dependent antibody. Dark spots represent positive areas of phosphorylation whereas clear spots are negative for the modification by PKA (Fig. 1C). Doing this we observed that whilst no signal was observed for control PDE8A arrays (-PKA) or peptides containing putative PKA sites spanning residues ^{376}RRHSS^{380} and ^{454}RRLSG^{458} (data not shown), positive signals were obtained for spots containing ^{356}RKGSL^{360} (Fig. 1C). To ensure that the phosphate group was being added to serine 359 and that successful phosphorylation depended on the PKA consensus sequence, an identical experiment was carried out on immobilized peptides in which single or double alanine substitutions of the original peptide (^{351}K-^{375}R) were made (Fig. 1C, lower panel). Alanine substitution of either ^{359}S or the basic amino acids at ^{356}R, ^{357}K in the PKA consensus motif both severely attenuated phosphorylation. These data suggests that only one of the three possible PKA sites actually act as a substrate for PKA and that phosphorylation depends on the consensus ^{356}RKXS^{359}. As the sequences of PDE8A and PDE8B are relatively conserved around the region against which the phospho-PDE8A antibody was raised, we were keen to demonstrate the specificity of our anti-serum in detecting solely phospho-PDE8A. To this end, we constructed peptide arrays of the cognate PDE8A and PDE8B sequences either containing the phospho-serine, unphosphorylated serine and phospho-mimic serine to aspartate or glutamic acid substitution (Fig 1D). We probed the arrays with the phospho-PDE8A antibody and cross reactivity was seen only with the PDE8A sequence when a phospho-serine was present. These new data suggests the subtle differences in sequence between PDE8A and PDE8B in the vicinity of the PKA site is enough to ensure specific recognition of phosphorylated PDE8A by our phospho-PDE8A antibody.

3.2. Characterization of a phospho serine 359 antibody

To investigate whether PDE8A is a PKA substrate in a cellular context, we commissioned a phospho-site specific antibody to serine 359. The antibody recognized a band corresponding to FLAG-PDE8A only after cells were stimulated with the adenylyl cyclase activator forskolin (FSK) (Fig 2A). This band was significantly ($P < 0.05$, Student's T test, $n = 3$) reduced following pre-treatment of the cells with the PKA inhibitors H89 and KT5720 (Fig 2A). The phosphorylation of another PKA substrate, CREB, was also equally diminished following KT5720 pre-treatment (Fig. 2G). Additionally, the antibody did not recognize PDE8A following forskolin treatment if serine 359 was mutated to either alanine or aspartic acid (Fig 2B), verifying the specificity of the antibody for phosphorylation of a single site. The antibody could also be used to detect phosphorylation of both exogenous and endogenous PDE8A, as samples isolated from untransfected HEK293 cells gave

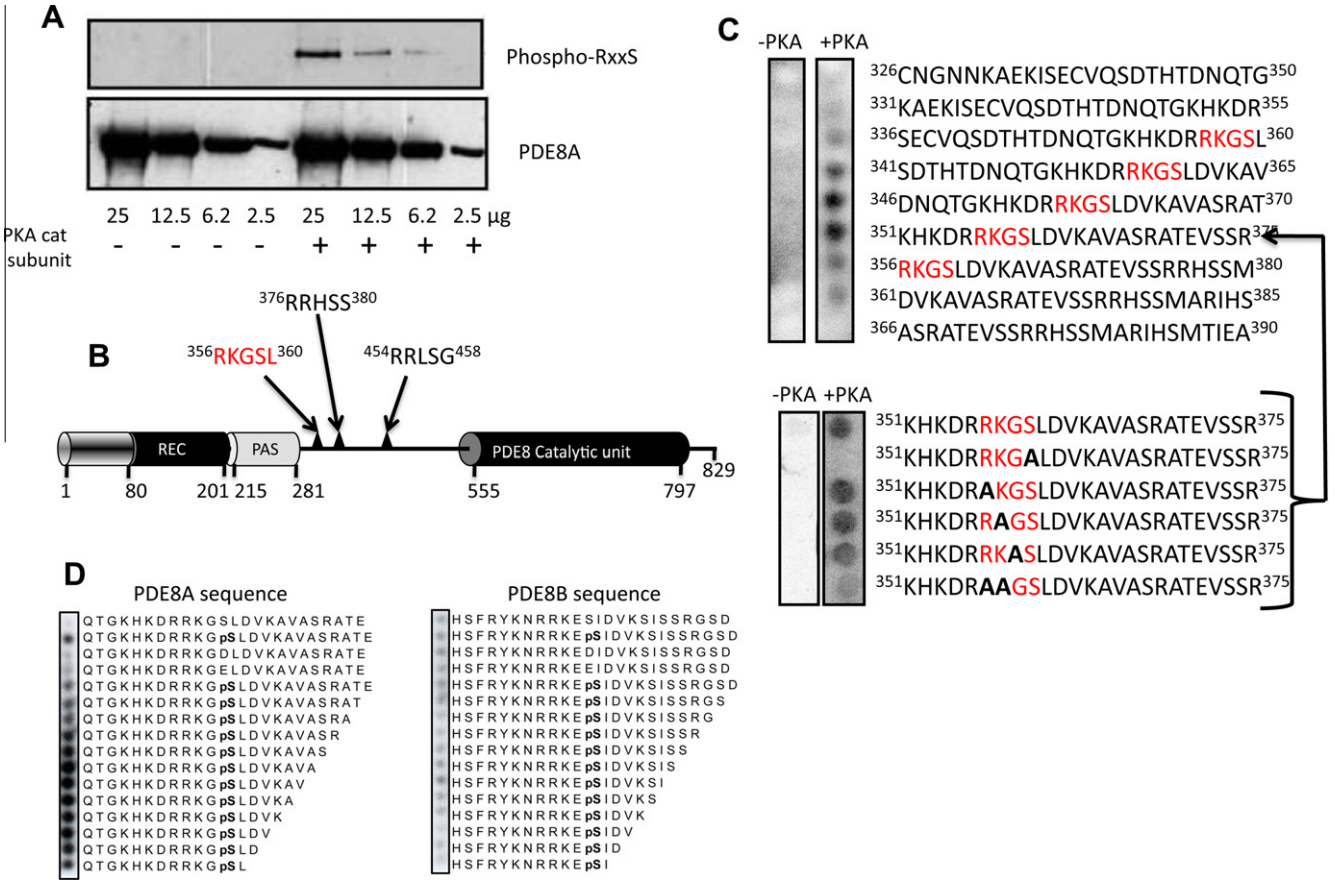

Fig. 1. PKA phosphorylates PDE8A1 on serine 359. (A) Purified MBP-PDE8A1 was phosphorylated in vitro using the catalytic subunit of PKA and blotted with a phospho-PKA substrate antibody. (B) A schematic diagram indicating the three putative PKA sites on PDE8A. (C) Peptide array technology combined with in vitro phosphorylation was used to show that serine 359 is the PKA site on PDE8A1. (D) Peptide arrays of the cognate PDE8A and PDE8B sequences either containing the phospho-serine, unphosphorylated serine and phospho-mimic serine to aspartate or glutamic acid substitution were probed with the phospho-PDE8A antibody.

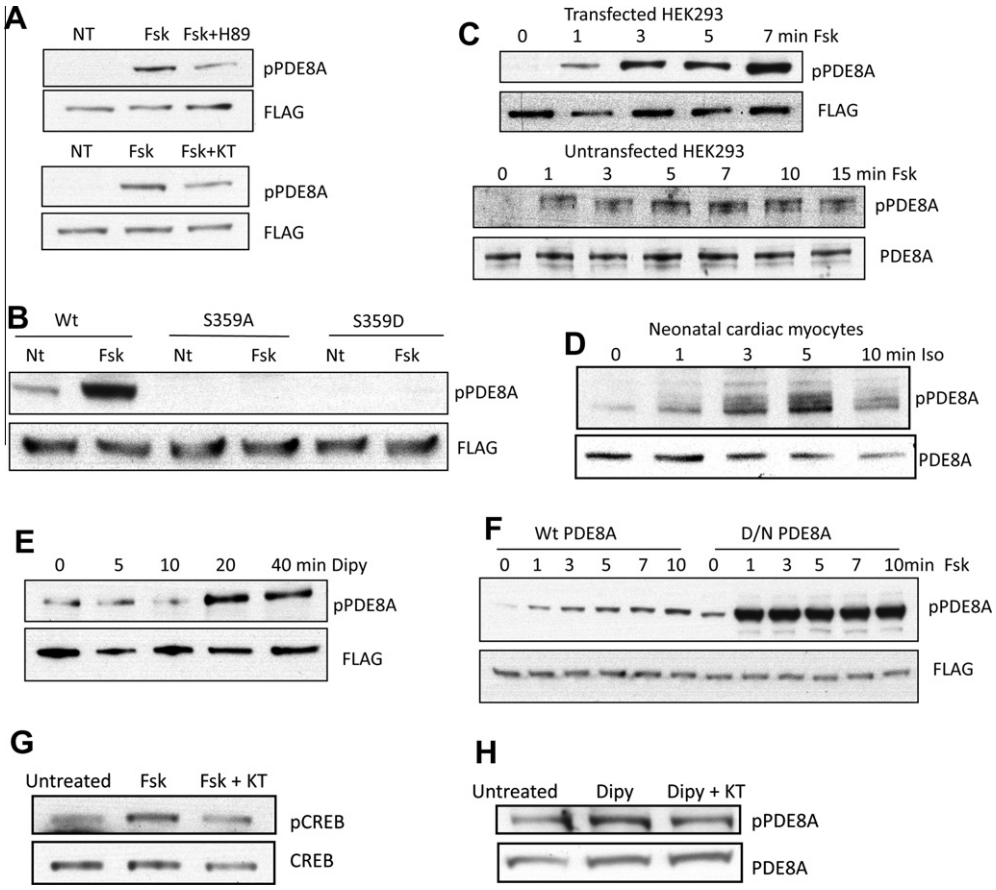

Fig. 2. PDE8A1 is phosphorylated by PKA in cells. (A) Using a phospho-serine 359 specific antibody, the phosphorylation of PDE8A1 was shown to be PKA dependent as pre-treatment with PKA inhibitors H89 (10 μM) and KT5720 (4 μM) reduced phosphorylation levels. (B) The phosphorylation of transfected and endogenous PDE8A1 was triggered by the adenylyl cyclase activator, forskolin (100 μM) for 5 min. (C) Mutation of serine 359 completely blocked PDE8A1 phosphorylation by PKA. (D) The phosphorylation of endogenous PDE8A1 was observed in cardiac myocytes following treatment with isoprenaline (10 μM). (E) The PDE8 inhibitor, dipyridimole (50 μM) induced PDE8A1 phosphorylation. (F) A dominant negative, catalytically dead form of PDE8A1 was more readily phosphorylated by PKA following forskolin (100 μM) treatment. (G) The induction of phospho-CREB following forskolin treatment (100 μM for 5 min) is partially attenuated by KT5720. (H) Increases in PDE8A1 phosphorylation triggered by dipyridimole (50 μM for 20 min) were attenuated by KT5720.

an increasing signal at the correct molecular weight in response to forskolin (Fig. 2C, lower panel). The signal was obviously reduced compared with protein from cells transfected with FLAG-PDE8A (Fig. 2C, upper panel), but in both cases, temporal increases in PDE8A phosphorylation were observed. Increases in PKA phosphorylation of PDE8 could also be detected in cellular lysates isolated from neonatal cardiac myocytes following a timecourse of isoprenaline stimulation (Fig. 2D). Finally, inhibition of PDE8 activity using either pharmacological means via dipyridimole (Fig. 2E) or a catalytically inactive version of PDE8A (Fig. 2F) resulted in an increase in PDE8A phosphorylation compared with wild type PDE8A. Inhibition of PKA by KT570 diminished the increase in PDE8A phosphorylation induced by dipyridimole (Fig. 2H). Such increases in phosphorylation could be seen under basal cAMP concentrations (Fig. 2E and F, compare zero time point samples) suggesting that the intrinsic activity of the enzyme protects itself from ''inappropriate'' PKA phosphorylation when cAMP is low. Conversely, conditions of high cAMP, promoted PKA phosphoylation of wild type PDE8A that was more robust and reached its peak earlier in the catalytically dead form (Fig. 2F) when compared with wild type. Such findings suggest that the antibody we have developed is a useful tool to detect PKA dependent phosphorylation of a single site on PDE8A at serine 359 and that under basal conditions, this site is protected by the enzyme's catalytic activity, which acts to dampen localized PKA activity.

3.3. Visualisation of PDE8A phosphorylation in HELA cells

As the phospho-serine 359 antibody had been effective in detecting endogenous levels of PDE8 phosphorylation using western blotting (Fig. 2), we decided to determine whether we could visualize this in cells using immuno-cytochemical methods. Little endogenous phospho-PDE8A could be detected under basal cAMP conditions (Fig. 3), however a strong signal was observed following 3 min of forskolin treatment and this was still evident after 10 min. Phosphorylation of PDE8 appeared to occur throughout the cell, being particularly evident in the cytoplasm and nucleus but with no obvious plasma-membrane staining. Very little signal for phospho-PDE8 was observed in control experiments where the antibody had been pre-incubated with the peptide against which it was raised. This further confirmed the specificity of the antibody.

3.4. PKA phosphorylation activates PDE8A

Phosphorylation and activation of phosphodiesterase enzymes by PKA provides a feedback loop where increased cAMP stimulates phosphodiesterase activity to reduce levels of the second messenger back to basal levels following activation of a Gs-coupled receptor. This type of regulation has been shown for PDE4 and PDE3 [19] and we were interested to determine whether PDE8A activity was similarly affected. Lysates isolated from HEK293 cells that had

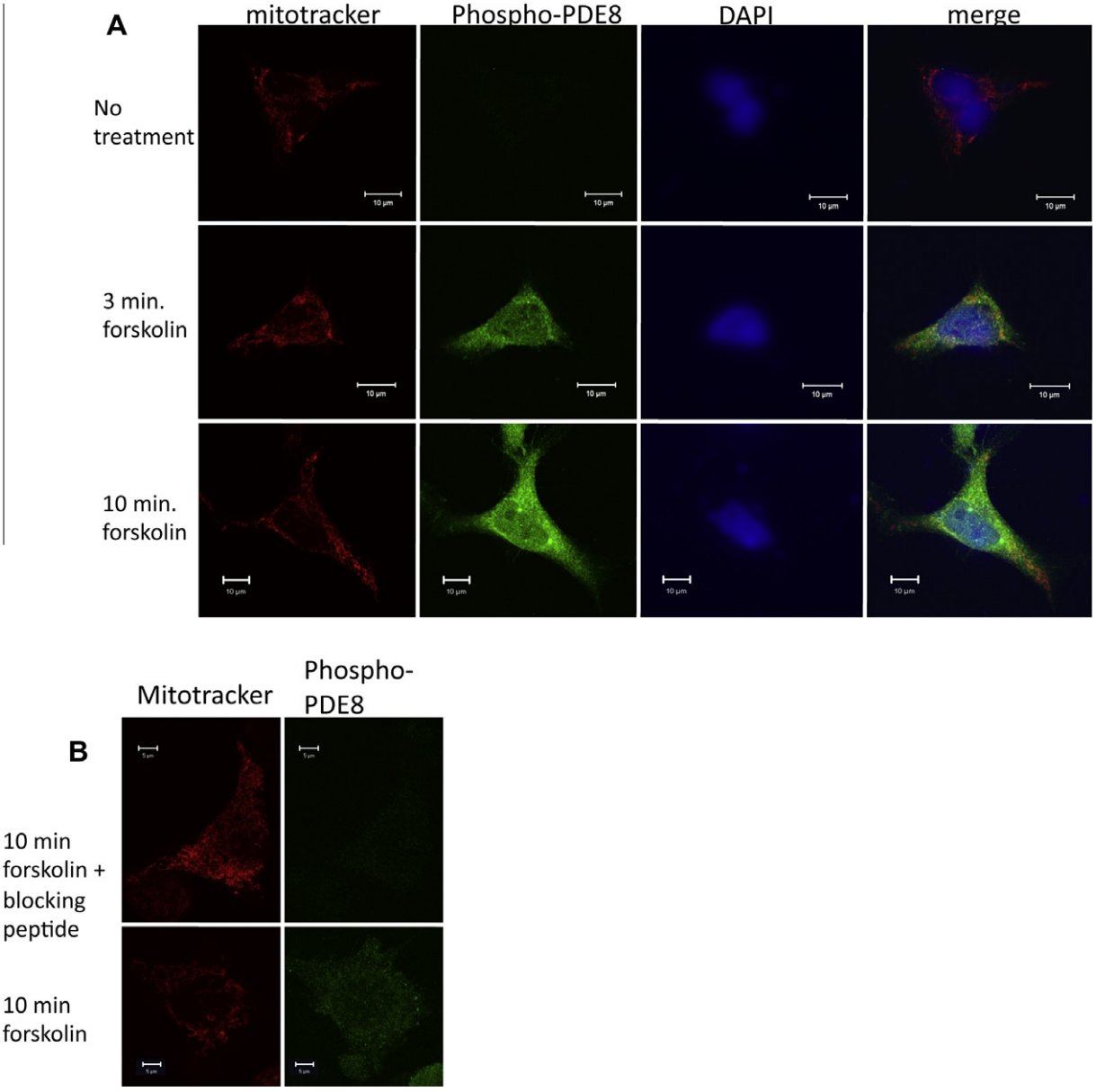

Fig. 3. Visualisation of PDE8A phosphorylation in HELA cells. (A) The PKA phosphorylation of PDE8A was induced by forskolin. (B) The phospho-peptide against which the phospho-serine 359 antibody was raised blocks the signal triggered by forskolin.

been transfected with either PDE8A wild type or PDE8A mutants containing the substitutions S359A/S359D were tested for PDE activity and PKA phosphorylation of PDE8A (Fig. 4). Wild type PDE8A activity was significantly stimulated following forskolin treatment (ANOVA, $P = 0.003$) whereas the S359A mutant was not suggesting that PKA phosphorylation at that site resulted in the activation of the enzyme. Interestingly, substitution of serine 359 with a negatively charged aspartic acid residue (to mimic phosphorylation) produced an activation that was similar in magnitude and significance to FSK treatment (ANOVA, $P = 0.004$). There was also a significant difference between forskolin-stimulated WT PDE8A activity versus DMSO-stimulated S359A PDE8A activity (ANOVA, $P = 0.04$) and also a significant difference between forskolin-stimulated WT PDE8A activity and forskolin-stimulated S359A PDE8A activity (ANOVA, $P = 0.03$). These data suggest that PKA phosphorylation of PDE8A on serine 359 activates the enzyme.

4. Discussion

As cAMP is a ubiquitous second messenger that can be synthesized to evoke cellular reaction to the activation of a plethora of membrane associated receptors, specificity of receptor action must be underpinned by discrete compartmentalisation of signaling intermediates within the cAMP signaling system [20]. One method by which cells rapidly control cAMP dynamics is by regulation of the activity and localization of cAMP-specific phosphodiesterases via post-translational modification. Phosphorylation of enzymes from the phosphodiesterase 4 family by specific kinases can activate [21], inhibit [22] or modify the outcome of a pre-existing phosphorylation by a different kinase [22]. PDE4 enzymes can also be modified by SUMO to enhance activation following PKA phosphorylation [13] and by ubiquitin to promote a complex with the scaffolding protein βarrestin [16] that leads to a more efficient desensitization of the β-adrenergic receptor. It has also been

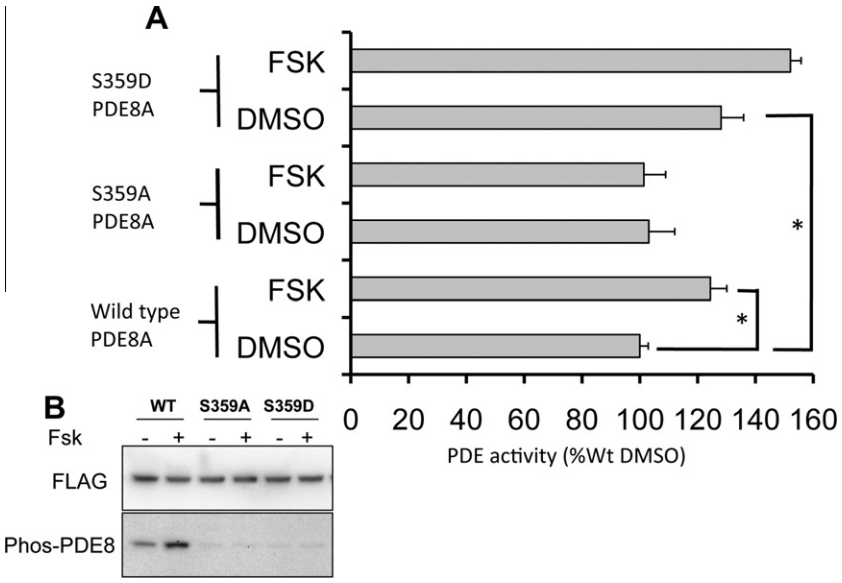

Fig. 4. PDE8 activity is significantly enhanced following PKA phosphorylation. (A) Forskolin (100 μM for 10 min) treatment significantly enhanced PDE8A activity and this was recapitulated with the phospho-mimic mutant S359D. * denotes significant changes as calculated using ANOVA analysis. See Section 3. (B) Samples used in (A) were monitored for PKA phosphorylation.

established that PKA can phosphorylate and activate PDE5 [23] and PDE3 isoforms [24]. So although it is known that cells can upregulate PDE protein expression to combat chronic increases in cAMP [25,26], almost instant feedback or feed forward regulation of cAMP can be achieved via modification of existing levels of PDE.

Interest in the PDE8 family increases as new and important roles for these enzymes are found. It is clear from recent work that PDE8 activity is fundamental to processes such as steroidogenesis [5] and excitation–contraction coupling [8], however little information exists on the molecular mechanisms that regulate fine control of PDE8 activity. Here we demonstrate for the first time that as with PDE3, PDE4 and PDE5, PDE8 can be phosphorylated and activated by PKA and this action serves to enhance enzyme activity at times of elevated cAMP. Surprisingly, this modification does not occur in regions that are thought to be important for PDE8 regulation, namely the REG or PAS domains [9–11] (see Fig. 1), however, this represents the first report of a post-translation modification of PDE8. In discovering this novel point of cAMP control, we have developed a novel antibody that can detect the phosphorylation of PDE8 in cells and we hope that use of this biological tool will facilitate a better understanding of the mechanisms underpinning PDE8 function.

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