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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