Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, β -arrestin and RACK1, in cAMP-specific phosphodiesterase PDE4D5

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The cAMP-specific phosphodiesterase PDE4D5 can interact with the signalling scaffold proteins RACK (receptors for activated C-kinase) 1 and β -arrestin. Two-hybrid and co-immunoprecipitation analyses showed that RACK1 and β -arrestin interact with PDE4D5 in a mutually exclusive manner. Overlay studies with PDE4D5 scanning peptide array libraries showed that RACK1 and β -arrestin interact at overlapping sites within the unique N-terminal region of PDE4D5 and at distinct sites within the conserved PDE4 catalytic domain. Screening scanning alanine substitution peptide arrays, coupled with mutagenesis and truncation studies, allowed definition of RACK1 and β -arrestin interaction sites. Modelled on the PDE4D catalytic domain, these form distinct well-defined surface-exposed patches on helices-15–16, for RACK1, and helix-17 for β -arrestin. siRNA (small interfering RNA)-mediated knockdown of RACK1 in HEK-293 (human embryonic kidney) B2 cells increased β -arrestin-scaffolded PDE4D5 approx. 5-fold, increased PDE4D5 recruited

to the β_2 AR (β_2 -adrenergic receptor) upon isoproterenol challenge approx. 4-fold and severely attenuated (approx. 4–5 fold) both isoproterenol-stimulated PKA (protein kinase A) phosphorylation of the β_2 AR and activation of ERK (extracellular-signal-regulated kinase). The ability of a catalytically inactive form of PDE4D5 to exert a dominant negative effect in amplifying isoproterenol-stimulated ERK activation was ablated by a mutation that blocked the interaction of PDE4D5 with β -arrestin. In the present study, we show that the signalling scaffold proteins RACK1 and β -arrestin compete to sequester distinct ‘pools’ of PDE4D5. In this fashion, alterations in the level of RACK1 expression may act to modulate signal transduction mediated by the β_2 AR.

Key words: β_2 -adrenergic receptor, β -arrestin, cAMP-dependent protein kinase (PKA), cAMP-specific phosphodiesterase (PDE), RACK1.

INTRODUCTION

GPCRs (G-protein-coupled receptors), such as the β_2 AR (β_2 -adrenergic receptor), couple to the G-protein G_s and thereby activate adenylate cyclase [1–3]. Upon phosphorylation of β_2 AR by GRK2 (G-protein-coupled-receptor kinase 2), desensitization occurs and the consequential recruitment of cytosolic β -arrestins [2,4–6] blocks β_2 AR coupling to G_s . However, the β_2 AR can also be phosphorylated by PKA (protein kinase A), which switches its coupling to G_i , causing activation of ERK (extracellular-signal-regulated kinase) [5,7–10].

The sole means of degrading cAMP in cells is through PDE (phosphodiesterase) action [11–13]. There is much interest in the PDE4 (rolipram-inhibited cAMP-specific PDE) [14–17] family, as PDE4-selective inhibitors exert antidepressant, memory-enhancing, immunomodulatory and smooth-muscle relaxant activity in humans and mammals [14,16,18–22]. Four genes generate approx. 18 PDE4 isoforms, each characterized by a unique N-terminal region that determines its intracellular targeting [15,23–29].

PDE4 enzymes contain an as yet undefined binding site within their conserved catalytic unit that allows them to interact functionally with β -arrestins [5,8,30–33]. One functional consequence of this interaction is that β -arrestins serve to recruit PDE4 isoforms to the agonist-occupied GRK2-phosphorylated β_2 AR, where they lower cAMP levels in the local environment of the β_2 AR, reduce PKA activity and thereby regulate PKA phosphorylation of the β_2 AR. Through this action, the recruited PDE4 serves to regulate the ability of the β_2 AR to switch between the G_s -mediated activation of adenylate cyclase and the G_i -mediated activation of ERK2 signalling.

The PDE4D5 isoform exhibits preferential interaction with β -arrestins as its unique N-terminal region contains a β -arrestin-binding site in addition to that associated with the catalytic unit (Figure 1) [30]. However, unique among PDE4 isoforms identified to date, PDE4D5 can also bind to the widely expressed signalling scaffold protein RACK1 (receptors for activated C-kinase) [23,26]. The isoform-specific 88-amino-acid N-terminal region of PDE4D5 thus contains a RACK-binding site (RAID1),

Abbreviations used: β_2 AR, β_2 -adrenergic receptor; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; GRK2, G-protein-coupled-receptor kinase 2; GST, glutathione S-transferase; HEK, human embryonic kidney; PDE, phosphodiesterase; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; PDE4, rolipram-inhibited cAMP specific PDE; PKA, protein kinase A; RACK, receptors for activated C-kinase; RAID, RACK-binding site; siRNA, small interfering RNA; SEC, siRNA expression cassette.

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Figure 1 Location of sites for RACK1 and β -arrestin interaction on PDE4D5

A schematic of PDE4D5 with its unique N-terminal region, upstream conserved region 1 (UCR1), upstream conserved region 2 (UCR2), catalytic region and extreme C-terminal region. Indicated above the schematic are the sites of β -arrestin and RACK1 interaction identified prior to the present study. Below it are interaction sites arising from the present study.

extending from Asn²² to Leu³⁸ [23] and, as deduced from truncation studies, a β -arrestin binding site, extending from Met⁷⁰ to Cys⁸⁸ [30].

Since the unique N-terminal region of PDE4D5 provides sites for both RACK1 and β -arrestin binding, we set out in the present study to determine whether PDE4D5 could form a complex with both of these signalling scaffolds or if their binding to PDE4D5 would be mutually exclusive and potentially regulatory. Although previous studies indicate that these sites are discrete [23,30], it is unclear whether steric interactions would prevent simultaneous binding of RACK1 and β -arrestin to PDE4D5, especially as β -arrestin, presumably, straddles PDE4D5 by binding to both its N-terminal region and catalytic unit [30]. In the present study, we have used a novel peptide array approach, coupled with immunoprecipitation and two-hybrid studies, to identify and characterize binding sites for RACK1 and β -arrestin on PDE4D5, and demonstrate that RACK1 and β -arrestin bind in a mutually exclusive and regulatory fashion to PDE4D5.

EXPERIMENTAL

Materials

Polyclonal antisera specific to the PDE4D subfamily were used as described previously [8,30,31,34]. Antibodies for detection of the native and phosphorylated forms of ERK1/2 and for detection of phosphoserine PKA substrates were from Cell Signaling Technologies. A rabbit polyclonal antibody against the β_2 AR was from Santa Cruz Biotechnology. Mouse anti-FLAG-M2 monoclonal antibody, anti-FLAG-M2 antibody conjugated with agarose, Protein A beads, anti-VSV antibody, IgM-agarose, and an anti-tubulin antibody were from Sigma-Aldrich. An IgM monoclonal antibody against RACK1 was from BD Biosciences. Antisera detecting β -arrestin 1/2 were from Dr R. Lefkowitz (Howard Hughes Medical Institute, Duke University, Durham, NC, U.S.A.). HEK (human embryonic kidney)-293B2 cells [35], which stably express a GFP (green fluorescent protein)- and FLAG-tagged β_2 AR, were from Dr G. Milligan (Department of Biochemistry and Molecular Biology, University of Glasgow, Glasgow, U.K.).

Analysis of β -arrestin-RACK1-PDE4D5 interactions by two-hybrid assay

Analysis was performed using techniques described previously [26,30,36]. As 'bait', PDE4D5 cDNA (GenBank[®] AF012073) was cloned into the NotI site of pLEXAN. As 'prey', β -arrestin 1 (GenBank[®] BC003636), β -arrestin 2 (GenBank[®] BC007427) or

RACK1 (GenBank[®] M24194) were cloned into the NotI site of pGADN. In 'competition' experiments, RACK1 was expressed with only an N-terminal nuclear localization signal and with the 'bait' cloned into a pBridge derivative [37]. Filter β -galactosidase assays were performed in *Saccharomyces cerevisiae* strain L40.

Site-directed mutagenesis

This was performed by the circular mutagenesis method, as described previously [23,26]. All mutagenesis and deletion constructs were confirmed by DNA sequencing prior to use.

Mammalian cell expression constructs

Human PDE4D5 cDNA [34], with a C-terminal VSV epitope tag, was cloned into pcDNA3 (Invitrogen). pcDNA3NARB2FLAG, encoding wild-type, C-terminal FLAG-epitope-tagged human β -arrestin 2, has been described previously [30].

Cell culture

HEK-293 and HEK-293B2 cells were cultured as described previously [8,30,31]. Transfections were performed using Polyfect (Qiagen), according to the manufacturer's instructions.

Immunoprecipitation studies to evaluate the interaction between recombinant β -arrestin 2 and PDE4D5 constructs in transfected cells

Immunoprecipitation studies were performed as described previously [7,8,30,31]. In some instances, cells were transfected to express either VSV-tagged forms of PDE4D5 or a β -arrestin 2-GFP fusion protein.

Expression of fusion proteins in *Escherichia coli*

Full-length PDE4D5 was expressed as an N-terminal MBP (maltose-binding protein) fusion, and human β -arrestin 1 and β -arrestin 2 were expressed as N-terminal GST (glutathione S-transferase) fusion as described previously [26,30]. In some experiments, β -arrestin 1 was expressed as a C-terminal hexahistidine fusion protein [30]. Proteins were expressed and purified as described previously [26,30].

SDS/PAGE and immunoblotting

SDS/PAGE and immunoblotting were performed as described previously [8,26,30].

SPOT synthesis of peptides and overlay experiments

Peptide libraries were produced by automatic SPOT synthesis [38]. They were synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments) [38,39]. The interaction of spotted peptides with GST and GST fusion proteins was determined by overlaying the cellulose membranes with 10 μ g/ml recombinant protein. Bound recombinant proteins were detected with specific rabbit antisera, and detection was performed with a secondary anti-rabbit antibody coupled with HRP (horseradish peroxidase) (1:2500 dilution; Dianova) as for immunoblotting. Where indicated, dual detection was performed at 680 and 800 nm using an Odyssey[®] infrared imager (LI-COR Biosciences). The anti-(β -arrestin) antisera, raised in rabbit, were detected using a goat anti-(rabbit IgG) antibody conjugated with

Alexa Fluor® 680 (Molecular Probes), and the anti-RACK1 monoclonal antibody was detected using rabbit anti-(mouse IgG) antibody conjugated with IRDye 800 (Rockland).

Generation and testing of siRNA (small interfering RNA) RACK1 knockdown constructs

The RACK1 mRNA sequence was scanned for appropriate 'target' regions for siRNA generation [40] using the Ambion program (http://www.ambion.com/techlib/tb/tb_506.html). All 'target' sequences were 21 nucleotides long and started with an AA dinucleotide, consistent with the observations of Elbashir et al. [41]. Sequences identified by this approach were then scanned against the GenBank® nucleotide database and only those that were unique to RACK1 were considered further. DNA oligonucleotides from four candidate 'target' sequences were synthesized and used to create SECs (siRNA expression cassettes) in which each of the 'target' sequences was expressed under the control of either of the U6 or H1 RNA Pol II promoter. Each of the eight SECs was then tested for its ability to knockdown RACK1 expression. For this purpose, COS7 cells were co-transfected, using Lipofectamine (Invitrogen), with the SEC and the plasmid pEGFP-RACK1, which encodes a fusion between enhanced GFP and the N-terminus of RACK1, and the effect of each SEC was scored as a reduction in GFP fluorescence. Four SECs consistently produced a greater than 90% reduction in GFP-RACK expression. These four SECs were cloned into the vector pSecPuro (Ambion) and the resulting constructs were tested for their ability to knockdown GFP-RACK1 in the same assay. All four cloned SECs produced a greater than 90% reduction in RACK1 expression and one, called pRACKSEC-H1-36, which consistently produced greater than 95% reduction in RACK1 expression, was used in all subsequent experiments.

Homology modeling

This was performed using Modeller software package version 7v7 [42]. Two models of the C-terminal parts of PDE4D (amino acids 324–677) were generated using structures of corresponding C-terminal stretches of PDE4B (PDB codes 1F0J [43] and 1XM6 [44] respectively). In addition, both models used the structure of catalytic domain of PDE4D, namely 1XOM [44], as a second template. In the case of the 1F0J template, the position of C-terminal helix-17 was taken from the symmetry-related molecule. This helix was then replaced by helix-17 from the 1XM6 structure (after structural superposition), as the latter was longer by five residues towards the C-terminus and contained a full stretch corresponding to PDE4D5 amino acids F⁶⁷⁰QFELTL⁶⁷⁶. The modelling procedure was repeated ten times in each instance with the model having the lowest objective function being selected. Finally, the catalytic domain (amino acids 324–647) was replaced by the corresponding one from the PDE4D structure (1XOM) and the C-terminal stretch 647–677 was energy-minimized.

RESULTS

RACK1 and β -arrestin bind in a mutually exclusive fashion to PDE4D5

RACK1 and β -arrestin 2 have been shown previously to interact with PDE4D5 [8,23,25,26,30,31] (Figure 1). In the present study we investigate whether they bind simultaneously to PDE4D5 or if their binding is mutually exclusive. Using a two-hybrid assay with PDE4D5 expressed as 'bait' and β -arrestin 2 as 'prey', we additionally expressed RACK1 as a 'competitor' (Figure 2a). This

showed that expression of the RACK1 'competitor' completely blocked the interaction between PDE4D5 and β -arrestin 2 (Figure 2a), indicating that RACK1 and β -arrestin 2 compete for binding to PDE4D5 rather than forming a heterotrimeric complex. Note that RACK1 was unable to interact directly with either β -arrestin 1 or β -arrestin 2 (Figure 2b).

We confirmed this result by examining immunopurified complexes from HEK-293B2 cells. Immunoprecipitates of endogenous β -arrestin contained endogenous PDE4D5, but not endogenous RACK1 (Figure 2c). Attempting to identify β -arrestin in RACK1 immunoprecipitates poses a fundamental difficulty, as β -arrestin migrates similarly to the Ig heavy chains from the immunoprecipitating antiserum. Thus we transfected HEK-293B2 cells with a GFP- β -arrestin 2 chimera, which is of higher molecular mass. This allowed PDE4D5 to be identified, but not GFP- β -arrestin 2 in immunoprecipitates of endogenous RACK1 (Figure 2d). In order to ascertain the distribution of RACK1- and β -arrestin-sequestered PDE4D5 in HEK-293B2 cells we undertook immunopurification of the endogenous pools of RACK1 and β -arrestin and immunoblotted each pool for PDE4D5. Of total PDE4D5 in HEK-293B2 cells, some $31 \pm 5\%$ was associated with RACK1 and $15 \pm 3\%$ with β -arrestin ($n=3$; values are means $\pm$ S.D.).

Note that densitometric analysis of endogenous β -arrestin immunoprecipitates did, as described previously by us [7], indicate a very small amount of associated PDE4D3 (results not shown). This comprised only 6–11% of the total β -arrestin-associated PDE4D ($n=3$; range). Our dominant-negative and siRNA-mediated knockout studies [7] clearly show that it is PDE4D5 and not PDE4D3 that is functionally important in mediating actions consequent upon β -arrestin recruitment to the β_2 AR. PDE4D3 appears to be sequestered to other scaffolds in these cells and, with the exception of this residual pool, is essentially unavailable for interaction with β -arrestin [11].

Peptide array analysis defines a binding site for RACK1 in the unique N-terminal region of PDE4D5

Previously, two-hybrid, pull-down and co-immunoprecipitation studies showed that RACK1 interacts with the unique N-terminal region of PDE4D5 (Figure 1) [23,26]. In the present study, we explored this interaction site further by using peptide array analysis, which provides a novel and powerful technology [38,39]. A library of overlapping peptides (25-mers), each shifted by 5 amino acids across the entire sequence of PDE4D5 was immobilized on cellulose membranes and probed with recombinant RACK1-GST. Dark spots signified positive interactions (Figure 3). Dramatically, the RACK1 probe interacted strongly with a single peptide (Figure 3; peptide 5) that contains all of the sequence, extending from Asn²² to Leu³⁸, constituting the proposed RACK1-binding site (RAID1) in PDE4D5 [23]. However, peptide 4 also contains this sequence while interacting considerably less effectively (Figure 3). This implies that amino acids within the sequence novel to peptide 5, namely E⁴¹KSKT⁴⁵ are additionally important for RACK1 binding to PDE4D5.

We thus set out to define the amino acids involved in forming RAID1 further. To do this we screened a family of peptides derived from a 25-mer parent peptide whose sequence reflected amino acids Asn²²–Thr⁴⁵ of PDE4D5. The 25 progeny of this parent peptide each had a single substitution, to alanine, of successive amino acids in the sequence to form a scanning peptide array (Figure 4a). This showed a striking absence or loss of binding upon substitution of Asn²², Pro²³, Trp²⁴, Asn²⁶, Val³⁰, Lys³¹, Leu³³, Arg³⁴, Glu³⁵, Asn³⁶, Leu³⁷, Glu⁴¹, Lys⁴² and Lys⁴⁴ (Figure 4a). Loss

Figure 2 RACK1 and β -arrestin interact with PDE4D5 in a mutually exclusive fashion

(a) Two-hybrid analysis with PDE4D5 as 'bait' and either β -arrestin 2 (β arr2) or RACK1 as 'prey'. Where indicated, RACK1 was additionally co-expressed as a 'competitor'. Blue indicates a positive interaction and pink a null interaction. Controls are vectors without inserts. (b) A two-hybrid assay was performed, as in (a). Also shown is the positive standard and negative ('empty' vector) control for this experiment ('standards'), which are both identical with those in (a). These data are typical of results obtained at least three times. (c) Immunoblots of endogenous PDE4D3, PDE4D5 and RACK1 from HEK-293B2 cell lysates (Ly) and β -arrestin immunoprecipitates (IP). (d) Using a RACK1-specific antibody, endogenous RACK1 was immunoprecipitated (IP) from HEK-293B2 cells transfected to express β -arrestin-GFP. Lysates (Ly) and RACK1 immunoprecipitates (IP) were immunoblotted for endogenous PDE4D3, PDE4D5 and transfected β -arrestin-GFP. No immunoreactive species were detected in control (Ctr) experiments using pre-immune antisera. These data are typical of experiments performed at least three times. (e) Various mutations in the EKFQFELTLEE motif of PDE4D5 (amino acids 668–678) were tested in two-hybrid assays for their ability to block the interaction of PDE4D5 with RACK1 and β -arrestin 2. A two-hybrid assay was performed, as in (a). Standards (not shown) were identical with those in (a). (f) Various C-terminal deletions of PDE4D5 were tested for their ability to interact with either RACK1 or β -arrestin 2. A two-hybrid assay was performed, as in (a). Standards (not shown) were identical with those in (a).

of RACK1 binding upon substitution of the charged amino acids in E⁴¹KSKT⁴⁵ explains why peptide 4 bound weakly to RACK1 compared with peptide 5 (Figure 3).

We set out to assess whether the various amino acids identified as being important in conferring RACK1 interaction upon PDE4D5 from our peptide array analysis might also be important in allowing interaction between full-length PDE4D5 and RACK1. We have shown previously [23,26] that deletion of the unique N-terminal region of PDE4D5 (Met¹–Cys⁸⁸) ablates the interaction of PDE4D5 with RACK1 in two-hybrid and co-immunoprecipitation analyses. Thus, using these biochemical assays, we can expect to identify single amino acid mutations in the unique N-terminal region that disrupt interaction with RACK1. In a two-hybrid assay a good correlation with the scanning peptide

array analyses was observed. Thus loss or reduced interaction was observed upon alanine mutation of the core RAID1 [23] cluster of Asn²², Pro²³, Trp²⁴, Asn²⁶, its hydrophobic extension of Val³⁰, Leu³³ and Leu³⁷ plus the basic amino acid, Arg³⁴ and Asn³⁶ (Figure 4a). Note that in two-hybrid analyses we pairwise mutated Leu²⁹–Val³⁰ and Leu³⁷–Leu³⁸ to alanine. However, peptide array analysis indicated that Val³⁰ and Leu³⁷ are the species underpinning RACK1–PDE4D5 interaction (Figure 4a). In contrast with the alanine substitution peptide array analyses, the following mutations in full-length PDE4D5, namely K31A, E35A, E41A, K42A and K44A, did not ablate PDE4D5–RACK1 interaction in the two-hybrid assays (Figure 4a). It would appear that in full-length PDE4D5 any potential disruption at such sites is compensated for in some way.

Figure 3 Probing PDE4D5 peptide arrays for RACK1 and β -arrestin interaction sites

PDE4D5 is shown schematically as in Figure 1. Data show immobilized peptide 'spots' of overlapping 25-mer peptides each shifted along by five amino acids in the entire PDE4D5 sequence probed for interaction with either β -arrestin 2–GST or RACK1–GST and detection by immunoblotting. Positively interacting peptides generate dark spots whereas non-interacting peptide leave white (blank) spots. In all other sections of the array, spots were blank with either probe. Spot numbers relate to peptides in the scanned array and whose sequence is given in Table 1. The disposition of the peptides in PDE4D5 sequence are shown relative to identified domains in a schematic of PDE4D5, which indicates its isoform-specific N-terminal region, upstream conserved regions 1 and 2 (UCR1, UCR2), catalytic unit and PDE4D C-terminal region.

To evaluate PDE4D5–RACK1 interaction further we immuno-precipitated endogenous RACK1 from HEK-293B2 cells transfected to express either wild-type or various mutant forms of epitope-tagged PDE4D5 (Figure 4b). These experiments showed that PDE4D5 interaction with RACK1 was severely reduced or ablated using the N22A, W24A, L33D and R34A PDE4D5 mutants (Figure 4b).

Peptide array analysis defines a binding site for β -arrestin in the unique N-terminal region of PDE4D5

In order to define where β -arrestin 2 binds in the 88 amino acid unique N-terminal region of PDE4D5, we probed the PDE4D5 peptide library with recombinant β -arrestin 2–GST (Figure 3). The clear interaction (Figure 3) of β -arrestin with peptide 13 (Arg⁶¹–Ala⁸⁵) is consistent with our previous progressive truncation analyses that led us to infer that the region from Met⁷⁰–Cys⁸⁸ was involved in β -arrestin binding [30]. However, peptide array analysis indicated that β -arrestin was able to bind to amino acids covering a broad part of the PDE4D5 N-terminal region, extending from Thr¹¹–Ala⁸⁵ (Figure 3).

As this region encompasses RAID1 we set out to examine explicitly whether β -arrestin can compete with RACK1 to bind to the N-terminal region of PDE4D5. To do this we simultaneously probed peptides 3–6, which encompass RAID1, with equimolar amounts of β -arrestin and RACK1 (Figure 4c). Simultaneous detection of β -arrestin and RACK1 binding was performed using the Odyssey[®] system using mouse anti-RACK1 antibody and rabbit anti- β -arrestin antibody, each linked to distinct secondary antisera labelled with different wavelength probes. This independently identified (Figure 4c) β -arrestin 2 (green) and RACK1 (red) associated with specific peptide spots in single channel analyses. We then combined signals from both channels (overlay). Analysis using merged channels identified peptide spots with predominant or solely RACK1–PDE4D5 complexes (red), those with predominant or solely β -arrestin–PDE4D5 (green), those with mixed populations of RACK1–PDE4D5 and β -arrestin–PDE4D5 (yellow) and those not interacting with either giving a null result (black). This showed that peptides 4 and 5, which each encompass RAID1, contain sub-populations of peptides that are complexed with either β -arrestin or RACK1 (yellow), whereas peptides 3 and 6 contain solely β -arrestin–

PDE4D5 complexes, indicated as green spots in the overlay (combined) channel (Figure 4c).

As the RACK1 binding site is fully contained within the Asn²²–Thr⁴⁵ peptide, we set out to identify the sites of interaction of β -arrestin within this peptide (Figure 4d). We did this by screening a library of peptide progeny generated by alanine scanning substitution. β -Arrestin binding was either ablated in the case of L33A and R34A or severely attenuated with N26A, E27A, D28A, L29A and V30A (Figure 4d). Thus, β -arrestin and RACK1 both appear to require Leu³³ and Arg³⁴ for binding to this peptide (Figures 4a, 4d and 4e).

Again we used Odyssey[®] analysis to probe the Asn²²–Thr⁴⁵ alanine scanning peptide array simultaneously for the formation of both RACK1 and β -arrestin complexes (Figure 4e). Challenging this array with equimolar amounts of β -arrestin and RACK1 showed that the control (native) peptide spot contained a mixed population of peptide complexes involving both RACK1 and β -arrestin (Figure 4e; yellow). This method of analysis clearly identifies both Leu³³ and Arg³⁴ as common sites of interaction for both RACK1 and β -arrestin, as alanine substitution of either of these amino acids clearly ablated their binding (Figure 4e; black). Alanine substitution of Glu²⁷, Asp²⁸ and Leu²⁹ selectively compromised the binding of β -arrestin, leaving the binding of RACK1 to predominate (Figure 4e; red). Alanine substitution of Asn²², Pro²³, Trp²⁴, Asn²⁶, Val³⁰, Lys³¹, Asn³⁶, Leu³⁷, Glu⁴¹, Lys⁴² and Lys⁴⁴ selectively compromised the binding of RACK1, leaving the binding of β -arrestin to predominate (Figure 4e; green). Alanine substitution of a further set of amino acids (Figure 4e; yellow) did not appear to affect the binding of either β -arrestin or RACK1, with complexes of both found associated with peptides in these spots as seen in the native peptide.

We subsequently used immunoprecipitation studies to confirm that alanine mutation of Glu²⁷, Leu³³ and Arg³⁴ ablated the interaction between PDE4D5 and β -arrestin, whereas mutation of Leu²⁵ did not (Figure 4b).

The catalytic region of PDE4D5 contains an interaction site for β -arrestin

In the present study we set out to define the site within the PDE4D catalytic unit that interacts with β -arrestin 2 by probing the PDE4D5 peptide library with recombinant β -arrestin 2–GST

Figure 4 Binding of RACK1 and β -arrestin to sequential alanine-substituted versions of a RAID1-containing peptide

(a) The row of spots in the upper panel shows a peptide array based upon a 25 mer 'parent' peptide of sequence Asn²²–Thr⁴⁵ in PDE4D5. Ctr refers to the unmutated peptide and all other spots reflect peptide 'progeny' where indicated amino acids were sequentially and individually substituted for alanine. This array was probed with RACK1–GST and interaction was determined by immunoblotting with an anti-RACK1 antibody. The lower panel of coloured patches shows various mutant forms of PDE4D5, with the indicated amino acids substituted for alanine, probed for interaction with RACK1 in a two-hybrid assay (positive, blue; and null, pink). For substitution/mutation at specific residues, then red indicates abolition of binding using both assay procedures, whereas black arrows indicate binding is still apparent. Blue arrows indicate differences between the two assay procedures. **(b)** HEK-293B2 cells were transfected with either wild-type or the indicated mutant forms of VSV-epitope-tagged PDE4D5. Lysates and immunoprecipitates were immunoblotted as indicated. As β -arrestin migrates on SDS/PAGE identically with Ig heavy chain, we were unable to immunoblot for β -arrestin in immunoprecipitates. Equal protein loading was used in each track. Lysates contained 1/20 protein loading compared with the immunoprecipitates. **(c)** Odyssey[®] analysis of PDE4D5 peptides 3–6 (see Figure 3) challenged with equimolar (200 nM) β -arrestin 2–GST and RACK1–GST. β -Arrestin 2 (green) and RACK1 (red) binding were detected simultaneously, with dual binding (yellow) shown in the combined channels (Overlay). Null binding is black. Data are typical of those obtained using three separately synthesized arrays. **(d)** Peptide array and two-hybrid assays performed as in **(a)**, but β -arrestin 2 was used in the two screens instead of RACK1. **(e)** Performed as in **(c)** to probe β -arrestin 2–GST and RACK1–GST binding simultaneously, but with the alanine scanning peptide arrays used in both **(a)** and **(d)**. Data are typical of experiments performed at least three times. AA, amino acid.

Table 1 Scanning peptide array analysis of PDE4D5 with RACK1 and β -arrestin probes

Amino acid sequence of sequential 25-mer PDE4D5 peptides, as probed in Figure 3, is given with the residue span in the PDE4D5 sequence (residues) plus peptide number (peptide) in analysis. Identification of interaction with either RACK1 (R bind) or β -arrestin (β A bind), as ascertained from overlay assays, is shown as no binding (N), and weak (+), medium (++) and strong (+++), based upon intensity of the spots visualized as in Figure 3. All other peptides, which completed the scan of PDE4D5 sequence, failed to interact with either RACK1 or β -arrestin.

25-Mer peptide sequence	Residues	Peptide	R bind	β AR bind
MAQQTSPDTLTVPEDNPHCPNPWL	1–25	peptide 1	N	N
SPDTLTVPEDNPHCPNPWLNEDLV	6–30	peptide 2	N	N
TVPEVDNPHCPNPWLNEDLVKSLRE	11–35	peptide 3	N	+++
DNPCHCPNPWLNEDLVKSLRENLLQH	16–40	peptide 4	+	+++
ENKSPWLNEDLVKSLRENLLQHEKSKT	21–45	peptide 5	+++	+++
NEDLVKSLRENLLQHEKSKTARKSV	26–50	peptide 6	N	+++
KSLRENLLQHEKSKTARKSVSPKLS	31–55	peptide 7	N	++
NLLQHEKSKTARKSVSPKLSPVIS	36–60	peptide 8	N	++
PKSPKLSPVISPVISPRNRRM	41–65	peptide 9	N	+++
ARKSVSPKLSPVISPRNRRMLSSN	46–70	peptide 10	N	+++
SPKLSPVISPRNRRMLSSNIPKQR	51–75	peptide 11	N	+
PVISPRNRRMLSSNIPKQRRFTVA	56–80	peptide 12	N	+
RNSPRNRRMLSSNIPKQRRFTVA	61–85	peptide 13	N	+
LLRRMLSSNIPKQRRFTVAHTCFD	66–90	peptide 14	N	N
LLSNIPKQRRFTVAHTCFDVGNGT	71–95	peptide 15	N	N
RRGMEISPMCDKHNASVEKSVQVGF	586–610	peptide 118	N	N
EISPMCDKHNASVEKSVQVGFIDYIV	591–615	peptide 119	N	N
CDKHNASVEKSVQVGFIDYIVHPLWE	596–620	peptide 120	+	N
ASVEKSVQVGFIDYIVHPLWETWADL	601–625	peptide 121	++	N
SVQVGFIDYIVHPLWETWADLVHPDA	606–630	peptide 122	++	N
IDYIVHPLWETWADLVHPDAQDILD	611–635	peptide 123	++	N
HPLWETWADLVHPDAQDILDTLEDN	616–640	peptide 124	+++	N
TWADLVHPDAQDILDTLEDNREWYQ	621–645	peptide 125	+++	N
VHPDAQDILDTLEDNREWYQSTIPQ	626–650	peptide 126	++	N
QDILDTLEDNREWYQSTIPQSPSPA	631–655	peptide 127	+	N
TLEDNREWYQSTIPQSPSPAPDDPE	636–660	peptide 128	++	N
REWYQSTIPQSPSPAPDDPEEGRQG	641–665	peptide 129	+++	N
APDDPEEGRQGQTEKFQFELTLED	655–679	peptide 130	N	+++
EEGRQGQTEKFQFELTLEDGEDSDT	660–684	peptide 131	N	+++
GQTEKFQFELTLEDGEDSTEKDSG	665–689	peptide 132	N	+++
FOFELTLEDGEDSTEKDSGSQVEE	670–694	peptide 133	N	+
TLEDGEDSTEKDSGSQVEEDTSCS	675–699	peptide 134	N	N
GESDTEKDSGSQVEEDTSCSDSKTL	680–704	peptide 135	N	N

(Figure 3). This probe interacts with four, sequentially located, peptides (Figure 3; peptides 130–133) in the PDE4D5 array whose sequence spans Ala⁶⁵⁵–Glu⁶⁹⁴ of PDE4D5 (Figure 3). These peptides all contain the E⁶⁶⁸KFQFELTLEE⁶⁷⁸ motif, which is conserved in all PDE4 proteins.

We also used two-hybrid analysis to obtain independent confirmation that residues within the E⁶⁶⁸KFQFELTLEE⁶⁷⁸ motif contribute to PDE4D5– β -arrestin 2 interaction. Thus the F670A:Q671A:F672A-PDE4D5 and L674A:T675A:L676A-PDE4D5 mutants failed to interact with β -arrestin 2, whereas the E668A:K669A-PDE4D5, E673A-PDE4D5 and E677A:E678A:D679A-PDE4D5 mutants still interacted with β -arrestin 2 (Figure 2e). However, all of these PDE4D5 mutants bound RACK1 (Figure 2e).

In order to gain further insight into the interaction of β -arrestin with the PDE4D catalytic unit, we used scanning alanine substitution of a peptide representing amino acids Glu⁶⁶⁰–Glu⁶⁸⁵ in PDE4D5 (Figure 5a). Substitution, with alanine, of any one of Phe⁶⁷⁰, Phe⁶⁷², Leu⁶⁷⁴ or Leu⁶⁷⁶ led either to ablation or severe attenuation of the interaction of β -arrestin with PDE4D5 (Figure 5a). This shows remarkable agreement with the two-

hybrid analyses where various alanine mutants of PDE4D5 in this region were assessed for their ability to bind to β -arrestin (Figure 2e).

The catalytic region of PDE4D5 contains an interacting site for RACK1

Probing the PDE4D5 peptide array library with RACK1–GST not only identified the established N-terminal binding site, but, additionally, identified a novel RACK1-binding site associated with the PDE4D5 catalytic unit (Figure 3). Indeed, RACK1 interacted with ten sequential peptides in this region, namely 120–129 (Figure 3). RACK1 did not appear to interact uniformly with all of these, as indicated by different intensities of the spots (Figure 3). This suggests that RACK1 interacts at multiple sites within the PDE4D5 catalytic unit, extending over a region spanning Cys⁵⁹⁶–Gly⁶⁶⁵.

Scanning alanine substitution analysis identified key amino acids involved in RACK1 binding to the PDE4D catalytic unit (Figure 5b). Amino acids in three peptides, spanning the region from Ala⁶⁰¹–Gly⁶⁶⁵ in PDE4D5 and whose substitution led to either ablation or marked reduction in RACK1 interaction were Gly⁶⁰⁹, Phe⁶¹⁰ and Asp⁶¹² in helix-15a of the catalytic unit; His⁶¹⁶, Pro⁶¹⁷, Glu⁶²⁰, Thr⁶²¹ and Trp⁶²² in helix 15b; Ile⁶³³, Thr⁶³⁶, Arg⁶⁴¹, Trp⁶⁴³ and Tyr⁶⁴⁴ in helix-16; and Asp⁶⁵⁷ and Asp⁶⁵⁸ in the flexible linker between helices-16 and -17 (Figure 5b).

That the binding sites for RACK1 and β -arrestin 2 in the catalytic unit abut, rather than overlap, is clearly evident from Odyssey[®] analysis of peptides 125–132, which span the region Thr⁶²¹–Gly⁶⁸⁹ in PDE4D5 (Figure 5c). In both single channel and overlay analysis this shows exclusive RACK1 binding (red) to peptides 125–129 and exclusive β -arrestin 2 binding (green) to peptides 130–132 (Figure 5c).

We used two-hybrid analysis to obtain independent confirmation for a novel RACK1 interaction site in the PDE4D catalytic unit. Thus C-terminal truncation of PDE4D5, to Gly⁶⁶², which removes a region that includes the E⁶⁶⁸KFQFELTLEE⁶⁷⁸ motif, ablated β -arrestin binding but did not affect RACK1 binding (Figure 2f). In contrast, C-terminal truncation to Lys⁵¹³ also ablated RACK1 binding to PDE4D5 (Figure 2f).

Consequences of the siRNA-mediated knockdown of RACK1 on PDE4D5– β -arrestin complex functioning in HEK-293B2 cells

Using a specific siRNA we achieved the efficient (> 90%) knockdown of RACK1 in HEK-293B2 cells as detected by immunoblotting (Figure 6a). This procedure had no discernible effect upon β -arrestin, PDE4D3, PDE4D5 and tubulin expression (Figure 6a). Similarly, knockdown of β -arrestin had no discernible effect upon RACK1, PDE4D3, PDE4D5 and tubulin expression (Figure 6a), and scrambled siRNA had no effect on expression of any of these proteins (< 5%).

In order to determine if RACK1 knockdown affected the level of PDE4D5 associated with β -arrestin, we selectively immunoprecipitated β -arrestin from HEK-293B2 cell lysates and immunoblotted for PDE4D5 (Figure 6b). RACK1 knockdown increased the level of β -arrestin-complexed PDE4D5 approx. 5-fold (Figure 6b and 6c). No PDE4D5 was seen in β -arrestin immunoprecipitates from HEK-293B2 cells subjected to β -arrestin knockdown (Figure 6b).

Treatment of HEK-293B2 cells with isoproterenol elicits the rapid time-dependent translocation of β -arrestin-bound PDE4D5 to the β_2 AR [5,8,30,31], as seen in PDE4D5 immunoblots of β_2 AR immunoprecipitates (Figure 6d). However, RACK1 knockdown markedly increased (approx. 4-fold) the amount of

Figure 5 Defining the binding of RACK1 and β -arrestin to the PDE4D catalytic unit using scanning peptide arrays

(a) Scanning Glu⁶⁶⁰–Glu⁶⁸⁵ in PDE4D5 for interaction with β -arrestin–GST. Anti-(β -arrestin) antisera was used for detection; all other conditions were as in Figure 4(a). Native peptide (Ctr) plus progeny with the indicated residue substituted for alanine. (b) Scanning of Ala⁶⁰¹–Glu⁶⁶⁵ in PDE4D5 for interaction with RACK1–GST. Three peptides were used to cover this region (Ctr) with conditions as in Figure 4(a). The indicated residues were substituted for alanine, unless the indicated amino acid was alanine in native PDE4D5, when it was substituted for aspartate. Mutation of amino acids coloured red indicate null interaction (< 10 %), whereas those coloured blue indicate reductions > 50 % based upon densitometric analysis of spots for $n = 3$ experiments. (c) Odyssey[®] analysis of PDE4D5 peptides 125–132 (see Figure 3) incubated with equimolar (200 nM) β -arrestin 2–GST and RACK1–GST. Detection was as in Figure 4(c). Data are typical of experiments performed at least three times.

PDE4D5 associating with the β_2 AR after 5 min of exposure to isoproterenol (Figures 6d and 6e).

siRNA knockdown of RACK1 attenuates the ability of isoproterenol to elicit the PKA phosphorylation of the β_2 AR and activate ERK in HEK-293 cells

Isoproterenol challenge of HEK-293B2 cells causes the time-dependent phosphorylation of the β_2 AR by AKAP79-tethered PKA [7]. As shown in Figures 7(a) and 7(b) siRNA-mediated RACK1 knockdown reduced (approx. 4-fold) isoproterenol-stimulated phosphorylation of the β_2 AR by PKA.

In HEK-293B2 cells, PKA phosphorylation of the β_2 AR allows it to switch its coupling from G_s to G_i and thereby activate ERK [7,8,30]. This action is desensitized via the β -arrestin-mediated delivery of PDE4D5, which attenuates PKA phosphorylation of the β_2 AR [7]. As shown in Figures 7(c) and 7(d) RACK1 knockdown clearly attenuates (4–5 fold) isoproterenol activation of ERK. However, RACK1 knockdown did not cause any change in ERK activation by isoproterenol when cells were additionally challenged with the PDE4-selective inhibitor rolipram (Figure 7e). This argues against any PDE4-independent effect of RACK1 knockdown on ERK activation by isoproterenol in HEK-293B2 cells.

PDE4D5 must bind to β -arrestin for it to modulate isoproterenol-stimulated ERK activation

As observed previously [8], overexpression of a catalytically inactive PDE4D5 (D556A-PDE4D5), whilst still able to be co-immunoprecipitated with β -arrestin (Figure 7f), provides a dominant-negative function in displacing active endogenous PDE4D5 from complex with β -arrestin, thereby facilitating the PKA-mediated phosphorylation of the β_2 AR and profoundly increasing the ability of isoproterenol to activate ERK in HEK-293B2 (Figure 7e).

Using information derived from the analyses described above, we set out to determine whether the dominant-negative action of D556A-PDE4D5 could be compromised if its ability to bind β -arrestin was disrupted. To do this we generated the E27A mutation in D556A-PDE4D5 on the basis that E27A mutation would negate the binding of β -arrestin but not that of RACK1 (Figures 3 and 4). Consistent with this, E27A:D556A-PDE4D5 did not co-immunoprecipitate with β -arrestin (Figure 7f) although it did with RACK1 (Figure 4d). E27A:D556A-PDE4D5 singularly failed to exert a dominant-negative effect in promoting isoproterenol-stimulated ERK activation (Figure 7g). Indeed, in cells overexpressing E27A:D556A-PDE4D5, the ability of isoproterenol to activate ERK was reduced compared with that

Figure 6 siRNA-mediated knockdown of RACK1 and β -arrestin on PDE4D5 recruitment to the β_2 AR

(a) HEK-293B2 cells were subject to siRNA knockdown of either β -arrestin, RACK1 or with scrambled siRNA. Lysates were immunoblotted for endogenous RACK1, β -arrestin, tubulin, PDE4D3 and PDE4D5, as indicated. (b) Cells were treated with siRNA as above and immunoprecipitates generated from lysates (Ly) using either a β -arrestin-specific antiserum (IP) or with non-specific serum (Ctr). Note that loading blots cannot be performed for β -arrestin as it co-migrates with Ig heavy-chain. Upper panel shows immunoblots, and lower panel shows a quantitative analysis of three independent experiments. (c) As in (b), except that RACK1 immunoprecipitates were probed for PDE4D. (d) Cells were treated with siRNA as in (a) and then treated with isoproterenol (10 μ M) for the indicated times. The β_2 AR was selectively immunoprecipitated (IP) and probed for PDE4D5. (e) A quantitative analysis of three independent experiments performed as in (d). Data are means $\pm$ S.D.

observed in control cells (Figure 7g). In these various experiments, the degree of expression of both D556A-PDE4D5 and E27A:D556A-PDE4D5 was similar, as detected by immunoblotting for their VSV epitope tag, and had no discernible effect on ERK expression (Figure 7g).

DISCUSSION

PDE4D5 has a unique 88-amino-acid N-terminal region that is able to interact with the signalling scaffold proteins RACK1 and β -arrestin (Figure 1) [5,8,23,26,30,31]. In the present study we show that RACK1 and β -arrestin bind to PDE4D5 in a mutually exclusive manner (Figure 2). Using a novel peptide array approach, we suggest that this is because RACK1 and β -arrestin interact with overlapping sites within the unique N-terminal region of PDE4D5. Furthermore, they also bind to distinct sites within the third sub-domain of the PDE4D catalytic region. In so doing they can be expected to straddle PDE4D5, which may serve to sterically interdict mutual interaction with PDE4D5 further.

Scanning N²²PWxNxxxVxxLRxNLxxxEKxK⁴⁴ (where x is not a key amino

acid) as providing the RACK1 interaction domain, RAID1 in the unique N-terminal region of PDE4D5 (Figure 4a). These data were remarkably consistent with that from the two-hybrid approach. Nevertheless, the underlined amino acids appeared not to be essential for RACK1 to bind full-length PDE4D5 in two-hybrid studies (Figure 4a). This may be due to conformational differences between the folding of the full-length protein and the 25-mer peptides folding and/or the presence of additional compensating binding sites in the intact protein. In this regard, note that the sensitivity of assays used in the present study to detect binding of full-length proteins requires that RACK1 binds both the N-terminal and catalytic sites of PDE4D5 to obtain a positive result [30]. Nevertheless, these data clearly indicate that scanning substitution peptide array analysis provides a rapid and effective means of highlighting amino acids that are potentially involved in protein-protein interactions.

The power of peptide array analysis, compared with simple truncation, is evident in analyses of β -arrestin with the N-terminal portion of PDE4D5 (Figure 3). Previously, two-hybrid assay analysis of progressive N-terminal truncations [30] indicated that the last 18 or so amino acids of the unique N-terminal region of PDE4D5 were involved in β -arrestin binding. However,

Figure 7 siRNA-mediated knockdown of RACK1 on β_2 AR phosphorylation by PKA and activation of ERK in HEK-293B2 cells

(a) Cells were transfected with either scrambled siRNA or siRNA specific for RACK1 prior to challenge with isoproterenol ($10 \mu\text{M}$ for 5 min). Equal amounts of cell lysate were immunoblotted for RACK1, total β_2 AR, PKA-phosphorylated form of the β_2 AR, β -arrestin and PDE4D. (b) Quantification of the PKA phosphorylation status of the β_2 AR upon isoproterenol ($10 \mu\text{M}$) challenge (5 min) of cells after prior transfection with either scrambled siRNA or siRNA specific for RACK1 in three separate experiments performed as in (a). (c) As in (a), but lysates were immunoblotted for total ERK and phospho-ERK. (d) Quantification of ERK phosphorylation ($n = 3$). (e) Cells treated with either RACK1 siRNA or scrambled siRNA with subsequent challenge using isoproterenol plus the PDE4 selective inhibitor, rolipram ($10 \mu\text{M}$). Lysates were immunoblotted for total ERK and phospho-ERK (pERK). (f) Cells were transfected with VSV-epitope-tagged versions of wild-type PDE4D5 (wt), D556A-PDE4D5 (DN) or E26A:D556A-PDE4D5 (E27A-DN). β -Arrestin immunoprecipitates (IP) were generated from cell lysates (Ly) and both were immunoblotted with a VSV-specific antibody to detect recombinant PDE4D5. (g) Cells were transfected as in (f) and then challenged for the indicated times with isoproterenol ($10 \mu\text{M}$). Equal amounts of lysate were immunoblotted to identify VSV-tagged PDE4D, total ERK and phosphorylated ERK. Data are typical of experiments performed at least three times. Data are means $\pm$ S.D.

peptide array studies, confirmed by point mutation analysis in pull-downs and two-hybrid studies, show that β -arrestin 2 interacts over an extended surface of the PDE4D5 unique N-terminal region (Figures 1 and 3). We might then expect that progressive N-terminal truncation of PDE4D5 will probably yield constructs with gradually diminishing affinities for β -arrestin 2. Indeed, peptide array analysis clearly shows that RACK1 and β -arrestin compete for binding to two peptides encompassing a region bounded by amino acids 16–45 in PDE4D5 (Figure 4c). Scanning arrays of a peptide encompassing amino acids 22–45 in PDE4D5 clearly identified substitution of either Leu³³ or Arg³⁴ with alanine as ablating the interaction of both β -arrestin 2 and RACK1 (Figures 4a, 4d and 4e), suggesting these two amino acids form part of the binding sites for both β -arrestin 2 and RACK1 on PDE4D5. Consistent with this, alanine mutation of these amino acids ablated PDE4D5- β -arrestin 2 interaction in both two-hybrid and pull-down studies (Figures 4a and 4b). Scanning array analysis of a peptide encompassing amino acids 22–45 in PDE4D5 indicated additional amino acids, namely those within a motif of N²⁶EDLVxxLR³⁴ (where x is not a key amino

acid), as also being important for PDE4D5- β -arrestin interaction (Figure 4d). Interestingly, previous N-terminal truncation of this sub-region did not ablate PDE4D5- β -arrestin interaction in either two-hybrid or immunoprecipitation studies [30]. Thus mutation of certain amino acids in this sub-region may trigger extensive changes in the conformation of the N-terminal portion of PDE4D5 as a whole, such that β -arrestin-binding site there is ablated whilst its deletion does not.

Previous truncation analyses indicated [30] that β -arrestin bound to a site within the C-terminal 83 amino acids of PDE4D5. As the catalytic unit is highly conserved between PDE4 sub-families, whereas the extreme C-terminus is not, we inferred the binding site for β -arrestin might be located between amino acids 662–683 of PDE4D5 [30]. Our peptide array analyses indicate that β -arrestin 2 interacts with the catalytic unit of PDE4D5 in a sequence that spans Ala⁶⁵⁵–Glu⁶⁹⁴ (Figure 3). This region contains the E⁶⁶⁸KFQFELTLEE⁶⁷⁸ motif that is conserved in all PDE4 sub-families. Consistent with this region being involved in β -arrestin binding, we noted that β -arrestin failed to bind peptide 129, which lacks the Leu-Glu-Glu component of this motif and

Figure 8 Structural representation of binding sites for RACK1 and β -arrestin on the PDE4D structural models

(a and b) Two different perspectives of the structural model of the 324–677 stretch of the PDE4D generated, as described in the Experimental section, using 1F0J PDE4B structure as a template. Amino acids whose substitution with alanine leads to loss of RACK1 interaction signal are shown in red, and those where substitution leads to a clear reduction in interaction signal are shown in blue. Amino acids whose substitution with alanine leads to loss of β -arrestin interaction signal are shown in yellow, the flexible linker between helices-16 and 17 is shown in pink, and the 1XOM inhibitor cilomilast is shown in spacefill representation in green. (c) The structural model of the 324–677 stretch of the PDE4D generated using 1XM6 PDE4B structure as a template. Colour-coding as above. These structural Figures were created using the PyMOL program (<http://pymol.sourceforge.net/>).

showed considerably reduced binding to peptide 133, which lacks the Glu-Lys component of this motif (Figure 5c). Scanning alanine substitution arrays (Figure 5a), confirmed by two-hybrid analysis (Figure 2e), identified Phe⁶⁷⁰, Phe⁶⁷², Leu⁶⁷⁴ and Leu⁶⁷⁶, which are located in sub-domain 3 of the PDE4 catalytic unit, as being critically involved in this interaction.

Peptide array analysis allowed an entirely novel interaction site for RACK1 to be identified, which is located within the common PDE4D catalytic unit (Figure 3). This site is crucial as its deletion by C-terminal truncation prevented PDE4D5 from interacting with RACK1 in a two-hybrid assay (Figure 2f). Unlike the highly localized interaction site for RACK1 found within the PDE4D5 unique N-terminal region, that associated with the PDE4D5 catalytic unit is more extensive, spanning a region from around Gly⁶⁰⁹ in helix-15a through to Asp⁶⁵⁸ in the flexible linker region between helices-16–17 (Figure 5b). Within this region, alanine substitution of a number of amino acids led to loss of RACK1 interaction. Thus RACK1, like β -arrestin, binds the PDE4D catalytic unit where these sites abut each other (Figures 1 and 5c). This contrasts with the unique N-terminal region of PDE4D5, where their binding sites overlap (Figures 1 and 4c).

The crystal structure of the isolated PDE4B and PDE4D catalytic regions is well known [43–49]. We show in the present study that RACK1 binds to sub-domain 3 and involves helices-15a, -15b and -16 as well as the flexible linker region between helices-16 and -17. The majority of the amino acids identified as involved in RACK1 binding form a well-defined surface-exposed patch (red/blue surface; Figures 8a and b). Also evident are

two smaller surface exposed patches located to the side of this (red/blue surface; Figure 8a). The larger surface patch stretches from inside the catalytic pocket over some 20 Å (1 Å = 0.1 nm) along the length of the combined helix-15/16 surface. Within the catalytic pocket Phe⁶¹⁰ forms part of the purine-binding region, whereas Gly⁶⁰⁹ is located at the rim (Figure 8b). Should RACK1 interact directly with the aromatic ring face of Phe⁶¹⁰ this would block substrate binding. This is unlikely, as RACK1 does not alter PDE4D5 catalytic activity [26]. However, it may be that RACK1 binds across Gly⁶⁰⁹ and the edge of the Phe⁶¹⁰ side chain without occupying or occluding the substrate-binding site. Such an intimate interaction of RACK1 close to the catalytic site might be expected to exert a functional effect. Indeed, this might explain why RACK1 alters PDE4D5 inhibition by rolipram [26]. If RACK1 binds across a surface extending from Thr⁶²¹ to Arg⁶⁴¹ and, simultaneously, wraps around Phe⁶¹⁰, this would require a sharp bend in the RACK1 surface in the region that contacts Gly⁶⁰⁹. Thus the G609A substitution might be expected to compromise severely the ability of RACK1 to do this. Three of the surface-exposed amino acids in this patch are also involved in contacts between helices-15–16 [43–49]. Asp⁶¹², on helix-15, forms a salt bridge with Arg⁶⁴¹ on helix-16, whereas His⁶¹⁶, on helix-15, forms a salt bridge with Glu⁶³⁸ on helix-16. Mutation of any of these could, potentially, perturb RACK1 binding by upsetting inter-helix alignments. Although H616A substitution diminished RACK1 interaction, alanine substitution of Glu⁶³⁸, to which His⁶¹⁶ ion pairs on helix-16, did not (Figure 5b). This suggests that His⁶¹⁶ may be directly implicated in the association

of PDE4D5 with RACK1 rather than simply serving a structural role in aligning helices-15 and -16 in order to allow RACK1 binding.

We also observed ablation of interaction upon P617A substitution (Figure 5b). As Pro⁶¹⁷ is surface-exposed (Figures 8a and 8b) and has no direct interactions with amino acids in the PDE4D catalytic unit it may be involved in direct surface contact between RACK1 and PDE4D5. Additionally, Pro⁶¹⁷ is associated with a pronounced bend in helix-15 caused by the unique inability of Pro⁶¹⁷ to hydrogen-bond to the peptide carbonyl group in the P-4 position of the helix (Tyr⁶¹³). P617A mutation might allow for a hydrogen-bond between helix amino acids at positions 613 and 617, thereby straightening helix-15. This would affect its alignment with helix-16, inducing a wide-ranging conformational change.

Asp⁶⁵⁷ and Asp⁶⁵⁸, implicated in the interaction of PDE4D5 with RACK1 (Figure 5b), lie beyond the C-terminal end of helix-16. Detailed structural information for this region of the enzyme is not available, but it is likely that they lie in a region of flexible loop (Figures 8a and b) as noted below.

Although there is no structural information available for the Phe⁶⁷⁰–Leu⁶⁷⁶ region of PDE4D, two crystal structures for a truncated PDE4B2 enzyme, namely 1F0J [43] and 1XM6 [44], extend as far as the region corresponding to amino acids 672–677 in PDE4D5 respectively. As amino acids 324–677, in PDE4D5, show 85 % sequence identity with the corresponding region in PDE4B, we built a structural model of PDE4D that used the PDE4B structures as templates (Figure 8). Both templates taken together indicated that the sequence corresponding to E⁶⁶⁰EGRQGQTEKFQFELTLE⁶⁷⁷ in PDE4D5 formed an α -helix-containing structure (helix-17) that is separated from helix-16 by a flexible linker (pink surface; Figure 8). However, the position of helix-17, with respect to the catalytic domain, is entirely different in these two templates. In the 1F0J structure, helix-17 makes no direct packing contacts with the core catalytic domain of its parent protein molecule, but is packed against an adjacent symmetry-related molecule in the crystallographic unit cell [43]. In contrast, in the 1XM6 structure, helix-17 is folded across the mouth of the catalytic pocket, trapping the PDE4 inhibitor mesopram within [44].

Consequently, we constructed two models based upon each of the 1F0J (Figures 8a and b) and 1XM6 (Figure 8c) structures. In the model based upon 1F0J (Figures 8a and 8b), helix-17 is conformationally mobile and does not interact with the catalytic domain. The amino acids involved in β -arrestin binding, namely Phe⁶⁷⁰, Phe⁶⁷², Leu⁶⁷⁴ and Leu⁶⁷⁶ (FxFxLxL motif; where x is not a key amino acid) form a coherent semi-circular patch on an exposed surface located upon one side of the C-terminal part of helix-17 (yellow surface; Figures 8a and 8b). It is therefore likely that this part of the surface of helix-17 physically interacts with β -arrestin and any flexibility in the region between helix-16 and helix-17 may aid in accommodating the binding of β -arrestin to both this location and within the unique N-terminal region of PDE4D5. On the other hand, the PDE4D model based upon the 1XM6 structure (Figure 8c) presents, at least in principle, the ability of helix-17 to fold across the opening of the catalytic site. In this arrangement, Leu⁶⁷⁴, which is key for β -arrestin binding (Figure 5a), is entirely packed against the catalytic domain (Figure 8c). Additionally, helix-17, together with the flexible linker region that connects it to helix-16, partially occludes the surface implicated in RACK1 interaction (Figure 8c).

It seems likely that helix-17 is conformationally mobile as it is connected to helix-16 via a 13-amino-acid long flexible linker (Figure 5b; Figures 8a–8c; pink surface) [43,44]. Consequently, its positions in both models may not reflect exactly that adopted

in either a β -arrestin–PDE4D5 complex, where an additional β -arrestin-binding site is involved, nor in complexes of β -arrestin with PDE4 isoforms that interact solely within the catalytic domain. Thus these models represent two different and possible, but not exclusive, scenarios. However, we consider that the PDE4D model based upon the 1XM6 structure may well be discounted, as the folding of helix-17 across the opening of the catalytic site is likely to generate a catalytically inactive species that can neither bind to RACK1 nor to β -arrestin. In contrast, the PDE4D model based upon 1F0J clearly shows surface-exposed sites for both RACK1 and β -arrestin binding (Figures 8a and 8b). Whatever the precise folding of helix-17, it is apparent that PDE4D5 straddles each of these scaffolding proteins in a similar manner. Their association is mediated by interaction at overlapping sites within the unique N-terminal region of PDE4D5 supplemented by interactions with distinct, but proximal, surfaces of the core PDE4 catalytic unit. Both sets of interactions probably conspire to interdict simultaneous binding of RACK1 and β -arrestin, explaining the competition for their binding to PDE4D5 as demonstrated in the present study.

Immunoprecipitation of the entire pools of RACK1 and β -arrestin identified approx. 31 % of the total PDE4D5 associating with RACK1 and approx. 15 % associating with β -arrestin in HEK-293B2 cells. This suggests that additional proteins act to sequester PDE4D5 in these cells. Indeed, PDE4D5 may effect multiple roles in cells each of which is associated with its ability to interact with distinct scaffolding proteins. Alterations in the endogenous levels of particular scaffolds may redistribute PDE4D5 among other scaffolding proteins with consequential effect on their signalling. We demonstrate this through the targeted knockdown of RACK1 in HEK-293B2 cells, which leads to a profound increase in PDE4D5 found associated with β -arrestin (Figure 6). Such an increase in β -arrestin-associated PDE4D5 cannot simply be due to competing out residual PDE4D3-bound β -arrestin, as the amount of PDE4D5 associating with β -arrestin consequent upon RACK1 knockdown increases some 5-fold (Figure 6b) whereas PDE4D3 forms some 6–11 % of total PDE4D associated with β -arrestin in these cells. Our observations indicate that in normal cells the endogenous pool of β -arrestin is not saturated with PDE4D5.

Such a redistribution of PDE4D5 upon RACK1 knockdown has a functional consequence in that it increases the amount of PDE4D5 that is delivered to the β_2 AR, in complex with β -arrestin, upon challenge of cells with isoproterenol (Figure 6). The enlarged pool of PDE4D5 delivered to the β_2 AR acts to attenuate the PKA phosphorylation of the β_2 AR (Figure 7). This has functional consequences for β_2 AR signalling as PKA phosphorylation of the β_2 AR causes it to switch its coupling from the G_s-mediated activation of adenylate cyclase to the G_i-mediated activation of ERK [10]. Thus the attenuated PKA phosphorylation of the β_2 AR leads to a consequential, marked reduction in the ability of isoproterenol to activate ERK (Figure 7). Consistent with this action being mediated by enhanced recruitment of PDE4D5, we noted that RACK1 knockdown failed to reduce the ability of isoproterenol to activate ERK in HEK-293B2 cells treated with rolipram, which acts to inhibit PDE4D5 (Figure 7e).

The ability of PDE4D5 to attenuate the PKA phosphorylation of the β_2 AR and the switching of its signalling to ERK is dependent upon PDE4D5 being recruited to the β_2 AR in complex with β -arrestin [7,8]. Such an inhibitory constraint supplied by endogenous PDE4D5 can be relieved by overexpressing a catalytically inactive D556A-PDE4D5 mutant [8]. This provides a dominant-negative function in displacing active endogenous PDE4D5 from complex with β -arrestin, thereby facilitating PKA phosphorylation of the β_2 AR [8]. However, based upon our

peptide array data, if we mutate catalytically inactive PDE4D5 (E27A:D556A-PDE4D5) such that it cannot bind β -arrestin but can still bind to RACK1, then this species is now unable to exert a dominant-negative effect on β_2 AR phosphorylation by PKA and signalling to ERK through G_i (Figure 7). These results demonstrate that our peptide array and mutational analyses can be successfully exploited to generate PDE4D5 mutants that identify functional consequences associated with selective PDE4D5 targeting.

The present study exemplifies the power of analysing protein-protein interactions through peptide arrays. It has allowed the identification and definition of novel binding sites in PDE4D5 for both RACK1 and β -arrestin and to generate mutants of PDE4D5 that selectively interact with these proteins. We show that the knockdown of a scaffolding protein for PDE4D5, namely RACK1, has functional consequences that relate to another PDE4D5 scaffolding protein, namely β -arrestin. Thus changes in expression levels and binding properties of a particular PDE4D5 scaffolding protein may have far-reaching consequences. This can ensue through redistribution of released PDE4D5 between other proteins that sequester PDE4D5 provided that binding is not already saturated. Our results thus highlight an additional degree of functional complexity associated with the PDE4 enzyme family in instances where specific isoforms are tethered to arrays of functional scaffolds in cells. PDE4 isoforms are poised to play pivotal roles in underpinning the compartmentalization of cAMP signalling in cells. The identification and characterization of these partnerships may have potential for understanding the role of PDE4 isoforms in molecular pathologies as well as in providing novel means of therapeutic intervention.

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REFERENCES

- Hall, R. A. (2004) β -Adrenergic receptors and their interacting proteins. *Semin. Cell. Dev. Biol.* **15**, 281–288
- Lefkowitz, R. J. and Shenoy, S. K. (2005) Transduction of receptor signals by β -arrestins. *Science* **308**, 512–517
- Wong, W. and Scott, J. D. (2004) AKAP signalling complexes: focal points in space and time. *Nat. Rev. Mol. Cell. Biol.* **5**, 959–970
- Penela, P., Ribas, C. and Mayor, Jr, F. (2003) Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell. Signalling* **15**, 973–981
- Baillie, G. S. and Houslay, M. D. (2005) Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes. *Curr. Opin. Cell. Biol.* **17**, 129–134
- Luttrell, L. M. (2005) Composition and function of G protein-coupled receptor signalosomes controlling mitogen-activated protein kinase activity. *J. Mol. Neurosci.* **26**, 253–264
- Lynch, M. J., Baillie, G. S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., van Hecke, G. and Houslay, M. D. (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with β -arrestin to control the protein kinase A/AKAP79-mediated switching of the β_2 -adrenergic receptor to activation of ERK in HEK-293B2 cells. *J. Biol. Chem.* **280**, 33178–33189
- Baillie, G. S., Sood, A., McPhee, I., Gall, I., Perry, S. J., Lefkowitz, R. J. and Houslay, M. D. (2003) β -arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from G_s to G_i . *Proc. Natl. Acad. Sci. U.S.A.* **100**, 940–945
- Daaka, Y., Luttrell, L. M. and Lefkowitz, R. J. (1997) Switching of the coupling of the β_2 -adrenergic receptor to different G proteins by protein kinase A. *Nature (London)* **390**, 88–91
- Lefkowitz, R. J., Pierce, K. L. and Luttrell, L. M. (2002) Dancing with different partners: protein kinase A phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. *Mol. Pharmacol.* **62**, 971–974
- Beavo, J. A. and Brunton, L. L. (2002) Cyclic nucleotide research – still expanding after half a century. *Nat. Rev. Mol. Cell Biol.* **3**, 710–718
- Francis, S. H., Turko, I. V. and Corbin, J. D. (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog. Nucleic Acid Res. Mol. Biol.* **65**, 1–52
- Maurice, D. H., Palmer, D., Tilley, D. G., Dunkerley, H. A., Netherton, S. J., Raymond, D. R., Elbatary, H. S. and Jimmo, S. L. (2003) Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. *Mol. Pharmacol.* **64**, 533–546
- Conti, M., Richter, W., Mehats, C., Livera, G., Park, J. Y. and Jin, C. (2003) Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J. Biol. Chem.* **278**, 5493–5496
- Houslay, M. D. and Adams, D. R. (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem. J.* **370**, 1–18
- Houslay, M. D., Schafer, P. and Zhang, K. Y. (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov. Today* **10**, 1503–1519
- Houslay, M. D., Sullivan, M. and Bolger, G. B. (1998) The multienzyme PDE4 cyclic adenosine monophosphate-specific phosphodiesterase family: intracellular targeting, regulation, and selective inhibition by compounds exerting anti-inflammatory and anti-depressant actions. *Adv. Pharmacol.* **44**, 225–342
- Giembycz, M. A. (2005) Life after PDE4: overcoming adverse events with dual-specificity phosphodiesterase inhibitors. *Curr. Opin. Pharmacol.* **5**, 238–244
- Huang, Z., Ducharme, Y., Macdonald, D. and Robichaud, A. (2001) The next generation of PDE4 inhibitors. *Curr. Opin. Chem. Biol.* **5**, 432–438
- Jeffery, P. (2005) Phosphodiesterase 4-selective inhibition: novel therapy for the inflammation of COPD. *Pulm. Pharmacol. Ther.* **18**, 9–17
- O'Donnell, J. M. and Zhang, H. T. (2004) Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). *Trends Pharmacol. Sci.* **25**, 158–163
- Renau, T. E. (2004) The potential of phosphodiesterase 4 inhibitors for the treatment of depression: opportunities and challenges. *Curr. Opin. Invest. Drugs* **5**, 34–39
- Bolger, G. B., McCahill, A., Yarwood, S. J., Steele, M. R., Warwicker, J. and Houslay, M. D. (2002) Delineation of RAID1, the RACK1 interaction domain located within the unique N-terminal region of the cAMP-specific phosphodiesterase, PDE4D5. *BMC Biochem.* **3**, 24
- McCahill, A., Warwicker, J., Bolger, G. B., Houslay, M. D. and Yarwood, S. J. (2002) The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. *Mol. Pharmacol.* **62**, 1261–1273
- Steele, M. R., McCahill, A., Thompson, D. S., MacKenzie, C., Isaacs, N. W., Houslay, M. D. and Bolger, G. B. (2001) Identification of a surface on the β -propeller protein RACK1 that interacts with the cAMP-specific phosphodiesterase PDE4D5. *Cell. Signalling* **13**, 507–513
- Yarwood, S. J., Steele, M. R., Scotland, G., Houslay, M. D. and Bolger, G. B. (1999) The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. *J. Biol. Chem.* **274**, 14909–14917
- McPhee, I., Yarwood, S. J., Scotland, G., Huston, E., Beard, M. B., Ross, A. H., Houslay, E. S. and Houslay, M. D. (1999) Association with the SRC family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B. Consequences for rolipram inhibition. *J. Biol. Chem.* **274**, 11796–11810
- Tasken, K. A., Collas, P., Kemmer, W. A., Witczak, O., Conti, M. and Tasken, K. (2001) Phosphodiesterase 4D and protein kinase A type II constitute a signaling unit in the centrosomal area. *J. Biol. Chem.* **276**, 21999–22002
- Baillie, G. S., Huston, E., Scotland, G., Hodgkin, M., Gall, I., Peden, A. H., MacKenzie, C., Houslay, E. S., Currie, R., Pettitt, T. R. et al. (2002) TAPAS-1, a novel microdomain within the unique N-terminal region of the PDE4A1 cAMP-specific phosphodiesterase that allows rapid, Ca^{2+} -triggered membrane association with selectivity for interaction with phosphatidic acid. *J. Biol. Chem.* **277**, 28298–28309
- Bolger, G. B., McCahill, A., Huston, E., Cheung, Y. F., McSorley, T., Baillie, G. S. and Houslay, M. D. (2003) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with β -arrestins. *J. Biol. Chem.* **278**, 49230–49238
- Perry, S. J., Baillie, G. S., Kohout, T. A., McPhee, I., Magiera, M. M., Ang, K. L., Miller, W. E., McLean, A. J., Conti, M., Houslay, M. D. and Lefkowitz, R. J. (2002) Targeting of cyclic AMP degradation to β_2 -adrenergic receptors by β -arrestins. *Science* **298**, 834–836
- Abrahamsen, H., Baillie, G., Ngai, J., Vang, T., Nika, K., Ruppelt, A., Mustelin, T., Zaccolo, M., Houslay, M. and Tasken, K. (2004) TCR- and CD28-mediated recruitment of phosphodiesterase 4 to lipid rafts potentiates TCR signaling. *J. Immunol.* **173**, 4847–4858
- Bradaia, A., Berton, F., Ferrari, S. and Luscher, C. (2005) β -Arrestin 2, interacting with phosphodiesterase 4, regulates synaptic release probability and presynaptic inhibition by opioids. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 3034–3039
- Bolger, G. B., Erdogan, S., Jones, R. E., Loughney, K., Scotland, G., Hoffmann, R., Wilkinson, I., Farrell, C. and Houslay, M. D. (1997) Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. *Biochem. J.* **328**, 539–548

- 35 McLean, A. J. and Milligan, G. (2000) Ligand regulation of green fluorescent protein-tagged forms of the human β_1 - and β_2 -adrenoceptors; comparisons with the unmodified receptors. *Br. J. Pharmacol.* **130**, 1825–1832
- 36 Bolger, G. B., Peden, A. H., Steele, M. R., MacKenzie, C., McEwan, D. G., Wallace, D. A., Huston, E., Baillie, G. S. and Houslay, M. D. (2003) Attenuation of the activity of the cAMP-specific phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2. *J. Biol. Chem.* **278**, 33351–33363
- 37 Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J. and Egly, J. M. (1997) A conditionally expressed third partner stabilizes or prevents the formation of a transcriptional activator in a three-hybrid system. *J. Biol. Chem.* **272**, 22995–22999
- 38 Kramer, A. and Schneider-Mergener, J. (1998) Synthesis and screening of peptide libraries on continuous cellulose membrane supports. *Methods Mol. Biol.* **87**, 25–39
- 39 Frank, R. (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports—principles and applications. *J. Immunol. Methods* **267**, 13–26
- 40 Yuan, B., Latek, R., Hossbach, M., Tuschl, T. and Lewitter, F. (2004) siRNA Selection Server: an automated siRNA oligonucleotide prediction server. *Nucleic Acids Res.* **32**, W130–W134
- 41 Elbashir, S. M., Harborth, J., Weber, K. and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26**, 199–213
- 42 Sali, A. and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815
- 43 Xu, R. X., Hassell, A. M., Vanderwall, D., Lambert, M. H., Holmes, W. D., Luther, M. A., Rocque, W. J., Milburn, M. V., Zhao, Y., Ke, H. and Nolte, R. T. (2000) Atomic structure of PDE4: insights into phosphodiesterase mechanism and specificity. *Science* **288**, 1822–1825
- 44 Card, G. L., England, B. P., Suzuki, Y., Fong, D., Powell, B., Lee, B., Luu, C., Tabrizi, M., Gillette, S., Ibrahim, P. N. et al. (2004) Structural basis for the activity of drugs that inhibit phosphodiesterases. *Structure* **12**, 2233–2247
- 45 Lee, M. E., Markowitz, J., Lee, J. O. and Lee, H. (2002) Crystal structure of phosphodiesterase 4D and inhibitor complex(1). *FEBS Lett.* **530**, 53–58
- 46 Xu, R. X., Rocque, W. J., Lambert, M. H., Vanderwall, D. E., Luther, M. A. and Nolte, R. T. (2004) Crystal structures of the catalytic domain of phosphodiesterase 4B complexed with AMP, 8-Br-AMP, and rolipram. *J. Mol. Biol.* **337**, 355–365
- 47 Huai, Q., Colicelli, J. and Ke, H. (2003) The crystal structure of AMP-bound PDE4 suggests a mechanism for phosphodiesterase catalysis. *Biochemistry* **42**, 13220–13226
- 48 Zhang, K. Y., Card, G. L., Suzuki, Y., Artis, D. R., Fong, D., Gillette, S., Hsieh, D., Neiman, J., West, B. L., Zhang, C. et al. (2004) A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. *Mol. Cell.* **15**, 279–286
- 49 Huai, Q., Wang, H., Sun, Y., Kim, H. Y., Liu, Y. and Ke, H. (2003) Three-dimensional structures of PDE4D in complex with roliprams and implication on inhibitor selectivity. *Structure* **11**, 865–873

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