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RACK1 and β -arrestin2 attenuate dimerization of PDE4 cAMP phosphodiesterase PDE4D5

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Abstract

PDE4 family cAMP-selective cyclic nucleotide phosphodiesterases are important in the regulation of cAMP abundance in numerous systems, and thereby play an important role in the regulation of PKA and EPAC activity and the phosphorylation of CREB. We have used the yeast 2-hybrid system to demonstrate recently that long PDE4 isoforms form homodimers, consistent with data obtained recently by structural studies. The long PDE4 isoform PDE4D5 interacts selectively with β -arrestin2, implicated in the regulation of G-protein-coupled receptors and other cell signalling components, and also with the β -propeller protein RACK1. In the present study, we use 2-hybrid approaches to demonstrate that RACK1 and β -arrestin2 inhibit the dimerization of PDE4D5. We also show that serine-to-alanine mutations at PKA and ERK1/2 phosphorylation sites on PDE4D5 detectably ablate dimerization. Conversely, phospho-mimic serine-to-aspartate mutations at the MK2 and oxidative stress kinase sites ablate dimerization. Analysis of PDE4D5 that is locked into the dimeric configuration by the formation of a *trans* disulfide bond between Ser261 and Ser602 shows that RACK1 interacts strongly with both the monomeric and dimeric forms, but that β -arrestin2 interacts exclusively with the monomeric form. This is consistent with the concept that β -arrestin2 can preferentially recruit the monomeric, or “open,” form of PDE4D5 to β_2 -adrenergic receptors, where it can regulate cAMP signalling.

Keywords

PDE4; PKA; ERK1/2; MK2; β_2 -adrenergic receptors; acrodysostosis

1. Introduction

Signal transduction pathways mediated by the second messenger cyclic AMP (cAMP) play a pivotal role in regulating numerous cellular processes, including inflammation and immunity, learning and memory, and cellular proliferation [1-4]. cAMP signalling in many

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cells is highly compartmentalized, where localized regulation of cAMP abundance, through its effectors PKA and Epac, can regulate targeted intracellular processes [5]. The breakdown of cAMP is mediated by 9 of the 11 members of the mammalian cyclic nucleotide phosphodiesterase (PDE) superfamily, each of which has a distinct pattern of expression, subcellular localization, and response to selective inhibitors [1-4].

Members of the PDE4 enzyme family are highly specific for cAMP and play a pivotal role in cell functioning. These enzymes are encoded by four genes in mammals (PDE4A/PDE4B/PDE4C/PDE4D), which in turn generate over 20 distinct isoforms through alternate mRNA splicing and the use of different promoters [2-4,6-8]. The various PDE4 isoforms can be sub-categorized as 'long' forms, such as the widely-found PDE4D5 isoform (Fig. 1), which possess both UCR1 and UCR2 regulatory domains, 'short' forms that lack UCR1, and 'super-short' forms that lack UCR1 and have a truncated UCR2 [2-4,6,8]. Each isoform has a distinct pattern of expression in tissues, including significant regional differences in expression in the CNS, suggesting that each has a distinct tissue or organismal function [9-18].

The cellular function of the various PDE4 isoforms also differ in their selective interaction with other cellular proteins, which in turn can have the potential to modify their enzymatic activity, location in cells, and interactions within other signalling components. For example, PDE4D5 [19] interacts selectively with β -arrestin2, implicated in the regulation of G-protein-coupled receptors and other cell signalling components [20-23], and also with the β -propeller protein RACK1 [24-30]. In contrast, the PDE4B1 isoform interacts selectively with the DISC1 protein, implicated in affective disorders and schizophrenia [31-34]. We and others have demonstrated that the interaction of β -arrestin2 with PDE4D5 recruits PDE4D5 to the β_2 -adrenergic receptor, where it serves to attenuate cAMP signalling [20-23]. Both RACK1 and β -arrestin interact more avidly with PDE4D5 than with any other PDE4 isoform that has been tested [24,35]. Using a combination of 2-hybrid, peptide display and co-immunoprecipitation techniques, we and our collaborators have shown that RACK1 interacts with the unique amino-terminal region of PDE4D5 and also separately with a region in the catalytic domain [24,25,28,30]. β -arrestin2 also interacts with both the unique amino-terminal region of PDE4D5 and the catalytic domain; however, the precise amino acids in PDE4D5 required for its interaction with β -arrestin2 differ significantly from those required for its interaction with RACK1 [28].

The functions of PDE4 isoforms are also dynamically regulated through phosphorylation by kinases such as PKA, ERK1/2, MK2, and AMPK, as well as modification by ubiquitination and sumoylation [36-50]. The activity of all long PDE4 isoforms is increased by 2- to 6-fold upon PKA phosphorylation, and PKA phosphorylation also changes the ability of the enzyme to be inhibited by PDE4-selective inhibitors, such as rolipram [37-41]. In contrast, ERK1/2 phosphorylation attenuates PDE activity [44-47]. MK2 kinase serves to attenuate the degree of activation conferred by PKA phosphorylation and, in the case of PDE4D5, serves as a site for mono-ubiquitination by the β -arrestin-sequestered E3 ligase, Mdm3, which gates polyubiquitination of the PDE4D5 isoform-specific N-terminal region [48].

Selective pharmacologic inhibition of the PDE4s has anti-inflammatory, immunomodulatory and smooth-muscle relaxant properties (see [2-4] for a review). PDE4 inhibitors also produce antidepressant and memory-enhancing properties in humans [51-54] and rodents [55-60]. Currently, three PDE4-selective inhibitors, roflumilast, crisaborole and apremilast, have been developed for clinical use [61-68], and additional PDE4 inhibitors have been developed and tested for a variety of indications, including depression, schizophrenia and disorders of learning and memory [69,70]. All PDE4-selective inhibitors act at the catalytic sites of the PDE4 enzymes [71-74] and therefore act, at least in part, as competitive inhibitors of cAMP hydrolysis. Although there are over 20 PDE4 isoforms, the catalytic sites of these isoforms are extremely similar, which has greatly complicated the development of inhibitors selective for any individual isoform [73,74].

Long PDE4 isoforms, such as PDE4B1 and PDE4D5, have been demonstrated by a variety of assays, including yeast 2-hybrid and co-immunoprecipitation, to form homodimers [75-80]. Recently, the dimerization of long PDE4 isoforms has been greatly illuminated by structural and enzymatic studies [81]. Collectively, these approaches have shown that dimerization is mediated by an interaction of α -helical regions in the C-terminus of UCR1 with the N-terminus of UCR2, forming a tight 4-helix bundle [77,81]. In addition, there is a smaller, but nonetheless biochemically significant, interface between the two catalytic domains, mediated by electrostatic interactions between Asp463 and Arg499 (PDE4D5 coordinates; Asp 471 and Arg507 in PDE4B1; refs [80,81]).

The enzymatic and pharmacologic characteristics of the dimeric form are markedly different from those of the corresponding monomer. These differences are mediated by a specific α -helical domain in the C-terminal half of UCR1 and α -helical domain (3 discrete helices) in UCR2, which together associate in *trans* with the cAMP-hydrolytic catalytic domain [81]. As a result, the dimeric form has approximately a 50-fold lower specific activity for cAMP hydrolysis, as compared to the corresponding monomer [81]. In addition, these regions of UCR1, UCR2 and the catalytic domain create a high-affinity rolipram binding site (HARBS). The presence of HARBS therefore reflects a conformational state unique to long PDE4 isoforms; in contrast, short PDE4 isoforms, which lack UCR1, do not have HARBS [82-84]. The presence of HARBS in the long PDE4 isoforms has important pharmacologic potential, in that PDE4 inhibitors that interact with UCR1/2, as opposed to just the catalytic domain, would preferentially target long PDE4 isoforms. These “long-isoform selective” PDE4 inhibitors might therefore have a safety and/or efficacy profile distinct from the current generation of PDE4 inhibitors [81].

Although our overall conceptualization of dimerization has deepened markedly with the recent publication of structural data, many questions remain. In particular, the potential effects of dimerization on the interaction of PDE4s with their “partner” proteins, such as that of PDE4D5 with RACK1 and β -arrestin2, have yet to be explored. In addition, it would be essential to assess the effects on dimerization of phosphorylation with PKA, ERK1/2 and MK2, each of which can affect the enzymatic and pharmacologic properties of PDE4 isoforms, including PDE4D5. In the present study, we used 2-hybrid approaches to address these questions. We show that RACK1 and β -arrestin2 both act to oppose PDE4D5 dimerization but differ very substantially in their ability to interact with the PDE4D5 when

“locked” into the dimeric form. We also show that selected mutations of PKA, ERK1/2, MK2 and oxidative stress kinase phosphorylation sites can affect dimerization.

2. Materials and Methods

2.1. Generation of PDE4D5 mutation constructs

A cDNA encoding an interdomain disulfide cross-linked PDE4D5 dimer was synthesized using the GeneArt process (Life Technologies, now ThermoFisher); the sequence was independently checked for accuracy by us by sequencing on both strands. This construct, called PDE4D5Cys, encoded human PDE4D5 (Genbank accession AF012073; ref [19]), but with codons optimized for expression in *S. cerevisiae* and with the following serine-to-cysteine mutations: S261C and S602C; in addition, all the existing PDE4D5 cysteines were mutated to alanine. A C-terminal VSV epitope “tag” [85] was added, as well as several restriction enzyme sites at each end, for ease in cloning. In addition, we used PDE4D5Cys as template to generate mutant constructs with the “single” mutations C261S or C602S (but also with all the pre-existing cysteines mutated to alanine). These mutants were called PDE4D5Cys261S and PDE4D5Cys602S, respectively. All mutations in PDE4D5 (i.e., wild-type) or PDE4D5Cys were generated by the circular mutagenesis method, using *Pfu* polymerase (Agilent Technologies) and were verified by sequencing prior to use.

2.2. Yeast 2-hybrid analyses

Yeast 2-hybrid techniques identical to those used previously by us were used to identify and analyze protein-protein interactions [24,28,35,76,80]. In all experiments, one of the two interacting components of the human PDE4D5 dimer was expressed as “bait”, as either in pLEXAN (a derivative of BTM116; refs. [24,86]) or in pBridge [87], as a *LexA* DNA-binding domain fusion. The second component of the dimer was expressed as “prey”, in pGADN (a derivative of pGAD.GH; ref. [86]), as a GAL4 activation-domain protein fusion. In some experiments, a third protein (i.e., either human RACK1 or human β -arrestin2) was also expressed, but as a native species and not as a fusion protein, using pBridge (i.e., pBridge expresses two proteins, one as a *LexA* DNA-binding domain fusion and the other with only a nuclear localization signal; ref. [87]). All proteins were targeted to the nucleus. In all figures, standards were added (the oncoproteins RAS and RAF1, ref. [88]). All interactions were evaluated in the *S. cerevisiae* strain L40 (ref. [88]) with our standard filter β -galactosidase assay [24,28,35,76,80]. For some experiments, a quantitative β -galactosidase assay was performed by the method of Guarente, using O-nitrophenyl- β -D-galactopyranoside as a substrate [89]. Except where indicated otherwise, all experiments shown here have been repeated at least twice, in at least two different yeast clones, with identical results.

3. Results

3.1. The PDE4D5 partners RACK1 and β -arrestin2 attenuate its dimerization

Given the extensive surfaces on PDE4D5 that appear to be necessary for dimerization [24,25,28,30], we felt that it was highly possible that bound RACK1 and/or β -arrestin2 would restrict access to these surfaces and thereby inhibit dimerization. Therefore, we tested

the ability of RACK1 and β -arrestin2 to block PDE4D5 dimerization, using a yeast 2-hybrid assay (Fig. 2). We have previously demonstrated that PDE4D5 dimerization can be detected by this assay and have used it to identify amino acids in UCR1, UCR2 and the catalytic region that are essential for dimerization, generating data that are highly consistent with those obtained by structural approaches [80]. Using this assay, we now show that co-expression of RACK1 completely blocks dimerization (Fig. 2, fifth row), while co-expression of β -arrestin2 significantly attenuates dimerization (Fig. 2, fourth row). These data suggest a potential functional role for RACK1 and/or β -arrestin2 in inhibiting the development of the dimeric form of PDE4D5.

3.2. Effect of mutations at phosphorylation sites on PDE4D5 dimerization

Numerous groups have studied the effects of phosphorylation on the enzymatic and pharmacologic properties of PDE4 isoforms; however, to date, there has been no published data on the effects of phosphorylation on dimerization. PKA phosphorylates a site (S54 in PDE4D3, S126 in PDE4D5 and S133 in PDE4B1; Fig. 1) in the motif QRRES located at the N-terminus of UCR2 [37-41]. ERK1/2 phosphorylates a site (S579 in PDE4D3, S651 in PDE4D5 and S659 in PDE4B1) located on the outer surface of the catalytic domain [44,46]. MK2 phosphorylates a serine (S61 in PDE4D3, S133 in PDE4D5 and S140 in PDD4B1) close to the PKA site, within UCR1 [48]. All 3 of these phosphorylation sites are located in highly flexible areas of the protein that are disordered in the crystal structure, suggesting that these regions are not essential for creation or maintenance of the dimer [81]. However, it is certainly possible that that phosphorylation of these sites might regulate dimerization in some way.

With these considerations in mind, we created both phospho-ablative (serine to alanine) and phospho-mimic (serine to aspartic acid) mutations at each of these phosphorylation sites in PDE4D5 and then tested their effect on dimerization with our yeast 2-hybrid assay. We felt that our *S. cerevisiae*-based system was a reasonably physiologic system for these studies, in that *S. cerevisiae* contains PKA, ERK and MK2 kinases that are expressed in vegetative yeast that have substrate specificities and enzymatic characteristics that are remarkably similar to their mammalian counterparts [90,91]. We therefore felt that our serine to alanine mutations had the ability to block biochemically-significant phosphorylation events by these kinases on PDE4D5 in *S. cerevisiae*, rather than solely affecting the charge or conformation of PDE4D5.

We first tested the effect of phospho-ablative mutations at the PKA and/or ERK1/2 sites (Fig. 3a for filter assay and Table 2 for quantitative assay). As part of these studies, we also tested mutations of the R123, R124 and E125 elements of the consensus PKA site. These experiments showed that mutations in any single amino acid had no major effect on dimerization, but that the S126A/S651A mutation significantly attenuated dimerization. The triple mutation R123A/R124A/S651A also attenuated the interaction (Fig. 3a and Table 2). Other mutations had more modest effects, although they produced some attenuation in the quantitative assay.

We then tested phospho-mimic mutations at the PKA and ERK1/2 sites and also at the putative oxidative stress kinase site in LR2 (S239 in PDE4D3, S311 in PDE4D5 and S319 in

PDE4B1; Fig. 1). We tested these mutants against both wild-type PDE4D5 and the R449D+DD-Quad mutant (the R499D+DD-Quad construct contains mutations in UCR1, UCR2 and the catalytic domains and ablates dimerization when present in both members of the dimer; ref. [80]). These experiments showed that none of the phospho-mimetic mutations affected dimerization when tested in a filter assay against wild-type PDE4D5 (Fig. 3b); however, some modest attenuation with the S311D oxidative stress kinase site mutation was seen in the quantitative assay (Table 2). The S311D mutation significantly reduced dimerization, both by itself and paired with the ERK1/2 site S651D mutation, when tested against the R449D+DD-Quad mutant (Fig. 3b). Given the previously-documented ablative effect of the R449D+DD-Quad mutant, this suggests that the S311D mutation has a modest, but reproducible, effect on dimerization that is augmented when it is paired with a separate ablative mutant. Intriguingly, in these experiments, the S126A/S651A mutant interacts with the R499D+DD-Quad mutant, which serves as a positive control for this mutant construct.

Finally, we tested the effects of phospho-ablative and phospho-mimetic mutations at the MK2 kinase site. The phospho-ablative S133A mutation had minimal effect on dimerization (Fig. 3c, panel i), although it seemed to have some effect when paired with the R123A/R124A PKA site mutant in the other member of the dimer. However, the phospho-mimetic S133D mutant clearly blocked dimerization (Fig. 3c, panel ii). Intriguingly, this effect was reversed by phospho-mimetic mutations in the other member of the dimer (i.e., S126D and S133D, Fig. 3c, panel ii), which may act by their further increasing charge in this region of the dimer.

3.3 RACK1, but not β -arrestin2, interacts with dimeric PDE4D5

Given the high avidity that we see between the 2 components of the PDE4D5 dimer in our 2-hybrid analyses, it seems possible that PDE4D5 could exist much of the time in cells in the dimeric state. Therefore, it would be of interest to determine whether RACK1 and/or β -arrestin2 are capable of interacting with dimeric PDE4D5. To test this hypothesis, we created a construct, which we call PDE4D5Cys, that encodes PDE4D5 with the engineered mutations S261C and S602C (corresponding to S267C and S610C respectively in PDE4B1), and mutation of all existing cysteines to alanines. When expressed in cells, S-S bridges between the 2 engineered cysteines would generate a covalently-linked dimer. This approach was first used by Pandit's group for PDE4B1 and has been extensively validated by them [81]. In addition, we also generated constructs with the "single" mutations C261S or C602S (but also with all the preexisting cysteines mutated to alanine), to serve as comparisons. These mutants, called PDE4D5Cys261S and PDE4D5Cys602S, respectively, should not generate a covalently-linked dimer.

PDE4D5Cys interacted as avidly as wild-type PDE4D5 with RACK1, as shown by both filter (Fig. 4) and quantitative β -galactosidase assays (Table 3). When we tested the PDE4D5Cys261S and PDE4D5Cys602S constructs, the strength of the interaction, as measured in the quantitative assay, was lower than with PDE4D5Cys or wild-type PDE4D5; this presumably reflects a change in avidity related to the loss of dimerization; however, general changes in protein folding produced by the mutations cannot be excluded (Fig. 4 and Table 3). Significantly, β -arrestin2 showed no interaction with PDE4D5Cys, although it

interacted appropriately with wild-type PDE4D5 in parallel patches (Fig.4, right column). These data suggest strongly that β -arrestin2 preferentially interacts with the monomeric form of PDE4D5. However, somewhat inconsistently, β -arrestin2 did not interact with PDE4D5Cys261S or PDE4D5Cys602S; whether this reflects some residual dimerization in these mutants, or technical issues, is unclear.

4. Discussion

Recent structural data have greatly enlarged and deepened our understanding of the conformation and regulation of PDE4 enzymes, especially the role of the UCR1 and UCR2 domains in the long PDE4 isoforms [81]. The structural data in turn have built on prior interaction and mutagenesis studies that suggested an interaction between specific regions of UCR1 and UCR2, which appeared to form a module that in turn interacted with the catalytic domain [6,75-80]. They have also demonstrated conclusively, consistent with previous data [77-80], that long PDE4 isoforms can form dimers, with UCR1 and UCR2 being essential components of the dimeric structure [81]. The structural data also provide insights into the possible functional effects of PDE4D mutations that have been implicated in acrodysostosis, a complex disorder affecting bone formation, growth and the CNS [92-96]. Of the 16 different single amino acid acrodysostosis mutations that have been identified to date, 15 map to the interface between UCR1/2 and the catalytic domain, or the “hinge” region connecting the dimerization domain to UCR1/2 and the catalytic domains [81]. The 16th acrodysostosis mutation is at S133, the PKA catalytic site (ref. [95]; note that this and other genetic references use GenBank NM_001104631.1 for the mutation co-ordinates, with S133 in PDE4D5 being S190 in the GenBank entry).

Dimerization also appears to provide many new insights into the enzymology and pharmacology of the long PDE4 isoforms. The dimeric form appears to exist as a “closed” or less-active conformation of the enzyme, with a specific activity for cAMP hydrolysis of dimeric PDE4B1 being roughly 50-fold lower than the corresponding monomeric form [81]. In addition, dimerization clearly affects the ability of long PDE4 isoforms to be inhibited by many PDE4-selective inhibitors, including the prototypical PDE4 inhibitor rolipram. This is because UCR1, UCR2 and the catalytic domain are all necessary for the formation of a HARBS, while the isolated catalytic region (e.g., seen in short isoforms) cannot form a HARBS [82-84]. These insights expand and modify prior models of PDE4 active site conformation [71-74,97,98] and are highly likely to stimulate the identification of novel “long-form specific” PDE4 inhibitors.

Members of the PDE4 family modulate major physiological processes, including immunity/inflammation, learning and memory, and the cell cycle [2-5,64]. In these functions, specific PDE4 isoforms act as network nodes between diverse signalling systems, largely through their ability to undergo phosphorylation [2-5,64]. Their regulation also reflects the ability of specific PDE4 isoforms to be targeted to specific intracellular locations through protein-protein interactions. Although the regulation of PDE4 isoforms by phosphorylation and by protein-protein interactions has been studied extensively, to date there has been little data on how these processes might affect PDE4 dimerization.

In the present study, we demonstrate a potential role for PKA, ERK1/2 and MK2 phosphorylation in PDE4 dimerization. In particular, blocking phosphorylation at both the PKA and ERK1/2 phosphorylation sites ablated dimerization; mutations of each individual site had only modest effect (Fig. 2 and Table 1). Because both the PKA and ERK1/2 sites are located in disordered regions of the dimer [81], the precise mechanism of how PKA-ERK1/2 phosphorylation might promote dimerization is uncertain; however, it is likely that phosphorylation at these sites would affect the conformation of the dimer and thereby push the equilibrium towards the dimeric form. In contrast, our analysis of phospho-mimetic mutations at the MK2 and stress oxidation kinase sites suggests that their action would be to promote the monomeric form.

We have also assayed the effects on dimerization of two well-characterized protein-protein interactions that involve PDE4D5, specifically RACK1 and β -arrestin2. Using our assay, both RACK1 and β -arrestin2 strongly attenuate the dimerization of PDE4D5 (Fig. 2). Given the high avidity and multiple sites of interaction between PDE4D5 and both of these proteins [20-22,24-30], it is perhaps not surprising that they would have such an effect. However, since our prior studies have shown that both RACK1 and β -arrestin2 largely interact with the unique N-terminal and C-terminal regions of PDE4D5 [21,24,25,28,30], which are unstructured in the dimer [81], it is unlikely that they act to directly restrict interaction at the UCR1/UCR2/catalytic or catalytic/catalytic interfaces that mediate dimerization. Instead, they presumably have indirect effects, possibly by sequestering the monomeric protein and thereby preventing it from forming a dimer, or by affecting its conformation in other ways. Preventing the dimerization of PDE4D5 could have multiple possible functional roles, such as increasing the enzymatic activity of PDE4D5 in certain cellular contexts, or targeting monomeric PDE4D5 to specific subcellular compartments.

Finally, we have investigated the avidity of RACK1 and β -arrestin2 for the “closed” or obligate-dimer conformation of PDE4D5. For these experiments, we adopted the approach of Pandit's group for PDE4B1 [81] and constructed a mutant form of PDE4D5 in which the dimer was stabilized by the addition of a disulfide bond (Fig. 4). RACK1 interacts avidly with this “closed” conformation of PDE4D5, which is not entirely surprising, given its high avidity and selectivity for PDE4D5 and the extensive regions on PDE4D5 that can interact with RACK1. However, to our surprise, β -arrestin2 did not detectably interact with the “closed” conformation (Fig. 4). This result could provide novel insight into the physiological mechanism of the PDE4D5- β -arrestin2 interaction, in which β -arrestin2 serves to recruit PDE4D5 to the ligand-occupied, GRK2-phosphorylated state of the β_2 -adrenergic receptor and thereby down-regulate cAMP signalling [20,22]. Since the major function of this recruitment is to move PDE4 enzymatic activity close to the β_2 -adrenergic receptor, it would be logical that β -arrestin2 preferentially recruit the monomeric, or “open,” form of PDE4D5, as this has much higher (50-fold greater, as measured for PDE4B1; ref. [81]) catalytic activity. Therefore, the preferential interaction of β -arrestin2 with the monomeric form would maximize its physiologic function.

5. Conclusion

In conclusion, we have provided evidence that the behaviour of PDE4 long forms is likely to be inherently distinct from that of PDE4 short forms as a direct consequence of their differential ability to dimerize. We have also provided evidence for possible regulation of the dimer-monomer equilibrium by phosphorylation and by interaction with RACK1 and β -arrestin2. It is likely that these effects contribute to the distinct physiological roles fulfilled by long and short PDE4 isoforms.

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Highlights

1. We use 2-hybrid approaches to demonstrate that RACK1 and β -arrestin2 inhibit the dimerization of PDE4D5.
2. We also show that serine-to-alanine mutations at PKA and ERK1/2 phosphorylation sites on PDE4D5 detectably ablate dimerization. Conversely, phospho-mimic serine-to-aspartate mutations at the MK2 and oxidative stress kinase sites ablate dimerization.
3. Analysis of PDE4D5 that is locked into the dimeric configuration by the formation of a trans disulfide bond between Ser261 and Ser602 shows that RACK1 interacts strongly with both the monomeric and dimeric forms, but that β -arrestin2 interacts exclusively with the monomeric form. This is consistent with the concept that β -arrestin2 can preferentially recruit the monomeric, or “open,” form of PDE4D5 to β 2-adrenergic receptors, where it can regulate cAMP signalling.

Fig. 1.

Domain organization of PDE4D5.

Like all “long” PDE4 isoforms, PDE4D5 contains UCR1, UCR2, and catalytic domains, which are separated by the unstructured LR1 and LR2 regions. The 88 amino acid unique N-terminal region (N-term) of PDE4D5 is also indicated, along with the C-terminus (C-term) and regions required for its interaction with RACK1 and β-arrestin2. The locations of PKA, ERK1/2, MK2, and oxidative stress kinase sites, as well as the S261C and S602C mutations, are also shown.

Fig. 2.

PDE4 dimerization in living cells is blocked by both RACK1 and β -arrestin2.

Yeast 2-hybrid experiments were performed using PDE4D5 as both “bait” and “prey”. For “bait,” PDE4D5 was expressed in *S. cerevisiae* as a *LexA* fusion. For “prey,” PDE4D5 was expressed as a *GAL4* fusion. Yeast cells in any given row contained the same “bait” and those in any given column contained the same “prey.” Cells in the indicated patches also expressed β -arrestin2 or RACK1. Positive interactions, assessed with a filter β -galactosidase assay, produce blue patches, while negative interactions produce pink patches. Controls are vectors alone (vector 1: pLexan; vector2: pBridge). Standards are the oncoproteins RAS^{V12} and RAF1. This figure shows data typical of experiments performed at least 3 times.

a

b

Fig. 3.

Effects of phosphorylation site mutants on dimerization.

Yeast 2-hybrid experiments were performed as in Fig 2. All panels show data typical of experiments performed at least 3 times.

3a. Effects of phospho-ablative mutations at the PKA and ERK1/2 sites. The S126A/S651A mutation, which blocks phosphorylation at both PKA and ERK1/2 sites, significantly attenuated dimerization. See also Table 1.

3b. Effects of phospho-mimic mutations at the PKA, oxidative stress kinase and ERK1/2 sites. The S311D oxidative stress kinase site mutation, either alone or paired with the ERK1/2 site mutation, significantly attenuated dimerization. See also Table 2.

3c. Effects of mutations at the MK2 site. The S133A MK2 kinase site phospho-ablative mutation blocked dimerization when paired with the R123A/R124A mutation (Panel i). The S133D MK2 kinase site phospho-mimetic mutation blocked dimerization with wild-type PDE4D5 and also when paired with several other phospho-mimic mutations (Panel ii).

Fig. 4.

β-arrestin2 interacts preferentially with the monomeric form of PDE4D5.

The PDE4D5Cys construct, which “locks” PDE4D5 into the dimeric state, or the putative dimerization-deficient mutants C261S or C602S, or unmutated PDE4D5, respectively, were expressed in *S. cerevisiae* as LexA fusions (rows) and tested for their ability to interact with RACK1 or β-arrestin2 (β-Arr), expressed as GAL4 fusions (columns). See also Table 3. This figure shows data typical of experiments performed at least 2 times.

Table 1

Quantitative β -galactosidase assay of the effect of PDE4D5 phospho-ablative mutants on dimerization. The mutations tested are the same as in Fig. 3a. t-tests were performed using the wild-type (W.T.) PDE4D5 value as the standard. Note that a few mutations (e.g., E125A) produced an increase in β -galactosidase activity; the significance of this is unclear.

BAIT	PREY	Mean beta-gal activity	SD, beta-gal activity	T-test, v W.T./W.T.
R123A,R124A	PDE4D5 W.T.	12.422	0.934	0.03684
E125A	PDE4D5 W.T.	26.527	0.906	0.00673
S126A	PDE4D5 W.T.	4.393	0.073	0.00130
R123A,R124A,S651A	PDE4D5 W.T.	2.709	0.311	0.00055
E125A,S651A	PDE4D5 W.T.	61.261	1.227	0.00017
S126A,S651A	PDE4D5 W.T.	1.053	0.076	0.00046
S651A	PDE4D5 W.T.	14.121	1.208	0.08460
PDE4D5 W.T.	PDE4D5 W.T.	12.996	0.681	

Quantitative β -galactosidase assay of the effect of selected PDE4D5 phospho-mimic mutants on dimerization. The mutations tested are a subset of those tested in Fig. 3b. t-tests were performed using the wild-type PDE4D5 value (with either vector or wild-type PDE4D5) as the standard.

Table 2

PREY	BAIT	Mean beta-gal activity	SD, beta-gal activity	T-test, v W.T./Vector	T-test, v W.T./W.T.
PDE4D5 W.T.	PDE4D5 W.T.	23.717	0.03391	0.00029	
PDE4D5 S311D	PDE4D5 W.T.	15.119	0.14160	0.00005	0.00288
PDE4D5 S651D	PDE4D5 W.T.	28.637	1.58536	0.00073	0.02788
PDE4D5 S311D/S651D	PDE4D5 W.T.	15.317	0.37413	0.00056	0.00056
PDE4D5 W.T.	Vector	1.475	0.05350		0.00029

Table 3

Quantitative β -galactosidase assay of the interaction between RACK1 and various PDE4D5 mutants. The mutations tested are the same as in Fig. 4. “Vector” refers to no GAL4 fusion and “both vectors” refers to no *LexA* or GAL4 fusion. t-tests were performed using the “both vector” value as the standard.

	Mean β -gal activity	SD, β -gal activity	<i>t</i> -test
Cys	16.863	3.660	0.008
Cys261	7.194	1.065	0.004
Cys602	1.160	0.091	0.016
No mutant	19.612	3.353	0.005
Vector	0.041	0.490	0.698
Both vectors	0.220	0.094	