cAMP-specific phosphodiesterase-4D5 (PDE4D5) provides a paradigm for understanding the unique non-redundant roles that PDE4 isoforms play in shaping compartmentalized cAMP cell signalling

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Abstract
The PDE4 (phosphodiesterase-4) enzyme family consists of a distinct array of N-terminal splice variant isoforms arising from four subfamily genes (4A, 4B, 4C and 4D). These all hydrolyse specifically the intracellular second messenger cAMP. Although identical in catalytic function, each isoform appears to serve a non-superfluous regulatory role. For example, a β-arrestin-sequestered subpopulation of the PDE4D5 isoform specifically regulates the phosphorylation of the β2-AR (β2-adrenergic receptor) by PKA (protein kinase A; also called cAMP-dependent protein kinase). This was elucidated by the use of novel technologies, including dominant-negative approaches, siRNA (small interfering RNA) knockdown and spot-immobilized peptide array analyses. Functional phenotypes uncovered using these methodologies have shown that β-arrestin-sequestered PDE4D5 shapes the spatial cAMP gradient around the membrane-bound β2-AR, regulating its phosphorylation by PKA and its ability to activate ERK (extracellular-signal-regulated kinase) through Gi in cardiomyocytes and HEK-293 (human embryonic kidney)-B2 cells. This approach has provided the very first identification of a non-redundant and specific role for a PDE isoform. The fact that phenotypes can be uncovered by displacing PDE4 isoforms from specific anchor sites using dominant-negative constructs and cell-permeable peptides points to novel means for developing therapeutics aimed at disrupting specifically sequestered PDE isoforms and even specifically sequestered subpopulations of individual isoforms.

Introduction
cAMP is a critical second messenger that can influence and regulate a vast array of cellular events vital for the response and control of signalling pathways to environmental cues [1–3]. It is now apparent that cAMP is spatially and temporarily localized within the cell, leading to compartmentalized signalling. Vital to the generation, preservation and regulation of these spatially distinct zones is the hydrolysis of cAMP to 5′-AMP by specific members of the cyclic nucleotide PDE (phosphodiesterase) superfamily, where members of the PDE4 family play a crucial regulatory role in many cell types and provide the paradigm for intracellular targeting [4–6].

Four genes comprise the PDE4 ensemble (4A, 4B, 4C and 4D), allowing the manufacture of over 20 variant PDE4 isoforms [5,7]. These share the ability to hydrolyse cAMP specifically, while containing domains and motifs allowing for regulation by multisite phosphorylation, involving, for example, PKA (protein kinase A; also called cAMP-dependent protein kinase), ERK (extracellular-signal-regulated kinase) and a kinase downstream of PI3K (phosphoinositide 3-kinase) [7–11], and the targeting of specific PDE4 isoforms to various signalling hubs, membranes and cytoskeletal structures within particular intracellular locales [5,6,12]. In this, the N-terminal region unique to each PDE4 isoform invariably plays a pivotal role [5,6,13]. PDE4 targeting to specific intracellular locales forms ‘sinks’ of cAMP [14] where the localized cAMP gradient can be shaped through the action of immobilized PDE4 [14–17]. This will be dynamically regulated by changes in PDE4 activity achieved through phosphorylation and the recruitment or release of PDE4 isoforms from spatially restricted sites within the cell.

The first insight into compartmentalized cAMP signalling came from studies performed in cardiomyocytes [17,18] where observations were made showing that PKA isoforms could be selectively activated by stimulation of different GPCRs (G-protein-coupled receptors) [19]. The field then opened up following pioneering work showing that PKA isoforms were targeted to discrete intracellular locales by a large family of anchor proteins [AKAPs (A-kinase-anchoring proteins; also called PKA-anchoring protein)] able to sense and act upon gradients of cAMP [1,2,12,20] and, subsequently,
the demonstration that cAMP gradient formation was underpinned by the targeting of PDE populations to distinct intracellular sites [14,21]. Indeed, AKAPs sequester many different signalling components, including PDE4 isoforms, which they use to sculpt localized regions of cAMP and thereby control the activity of PKA isoforms within that particular, spatially defined niche [2,20,22]. The discovery of PDE4 targeting [13,23] and sequestration of PKA through AKAPs [2,20,22] has now made compartmentalization part of the cAMP signalling canon in biological systems [6,20,24].

**PDE4D5 is the functionally relevant cAMP phosphodiesterase that controls β2-AR (β2-adrenergic receptor) phosphorylation by AKAP79-tethered PKA**

β2-AR activation of adenylate cyclase at the plasma membrane triggers phosphorylation of the β2-AR by a PKA subpopulation tethered to the receptor itself via AKAP79 [25,26]. In cardiomyocytes, this allows the β2-AR to switch its coupling from Gs activation of adenylate cyclase to the G𝑖 activation of ERK [12] (Figure 1). This G𝑖 switching event does occur in all β2-AR-expressing cells, but is a cell-type-specific phenomenon as activation of ERK by β2-AR agonists can occur through a variety of conduits including cAMP/B-Raf, cAMP/ERK phosphatase and through β-arrestin itself [12,27]. We have studied such a switching in cardiomyocytes and also in a HEK-293 (human embryonic kidney) cell line transfected to overexpress the β2-AR (HEK-293-B2 cells) where, unlike in ‘wild-type’ HEK-293 cells, G𝑖/ERK switching is the dominant process [12,28].

β-Arrestin is a cytosolic scaffolding protein that is recruited to the GRK (GPCR kinase)-phosphorylated β2-AR at the plasma membrane upon agonist stimulation. β-Arrestin induces uncoupling of the agonist signal by sterically blocking coupling of the receptor with G𝑖. Subsequent to this, it also initiates the internalization of agonist-stimulated receptor [29].

Three PDE4 isoforms provide the major PDE4 activity within HEK-293-B2 cells, namely PDE4B2, PDE4D3 and PDE4D5. Potentially, PDE4 isoforms from all four sub-families can bind to the signalling scaffold β-arrestin as they can a distinct binding site within the conserved PDE4 catalytic unit [30–32]. However, in cells expressing PDE4D5, it is this isoform that is found preferentially associated with β-arrestin [32]. Indeed, decisively, it is PDE4D5 that forms the predominant complex with β-arrestin and is recruited to the β2-AR upon agonist challenge in HEK-293-B2 cells and cardiomyocytes. Such a dynamic recruitment places β-arrestin-sequestered PDE4D5 as providing the functional key in the hydrolysis of cAMP localized to the immediate environs of the β2-AR [12,28,31–33]. In so doing, recruited PDE4D5 regulates intimately the activity of the PKA subpopulation that is tethered to the β2-AR by AKAP79 [12] (Figure 1).

The functional implication of this β-arrestin/PDE4-mediated translocation has been elucidated using a novel approach that encompassed developing, for the first time for PDE enzymes, an engineered ‘dominant-negative’ system (Figure 2). This technique involved exploiting information on the three-dimensional structure of the PDE4 catalytic unit [34] to generate, by a single, discrete mutation within the catalytic pocket, a catalytically inert PDE4 mutant [28,35]. The rationale for such a dominant-negative approach involves the overexpression of a defined catalytically inactive PDE4 isoform such that it will compete out the cognate, endogenous active isoform for its binding site(s) in cells [35]. In so doing, this can be expected to cause an increase in cAMP levels that is localized to the site of the displaced active PDE4 isoform, as shown to occur when deployed in cardiomyocytes [21] and wild-type HEK-293 cells [14].
This approach (Figure 2) allows the analysis of functional phenotypes via ablation of anchoring of endogenous active PDE4 species to specific intracellular locales and thus gives information based solely on spatial considerations, only generating phenotypes through disruption of sequestration of the cognate endogenously expressed isoform. This contrasts with the siRNA (small interfering RNA)-mediated knockdown approach that we also pioneered for studying PDE action, in this instance by knockdown of specific PDE4 isoforms and PDE4 subfamilies [26,35].

In both cardiomyocytes and HEK-293-B2 cells, overexpression of catalytically inactive PDE4D5 gave a unique phenotype. This induced a rise in PKA phosphorylation status of the β2-AR, whereas dominant-negative forms of PDE4B2 and PDE4D3 did not [28]. This supports the notion that β-arrestin-sequestered PDE4D5 uniquely regulates the phosphorylation status of the β2-AR through AKAP79-tethered PKA. Confirming this, only the siRNA-mediated knockdown of PDE4D5 augmented PKA phosphorylation of the β2-AR [26]. These approaches (Figure 2) have provided the first identification of a functional phenotype attributable to a single PDE isoform.

The N- and C-domains of β-arrestin interact preferentially with PDE4D5

All PDE4 isoforms have the potential to bind β-arrestins via a conserved site on helix-17 in their conserved catalytic unit [31,32]. However, we have shown that PDE4D5, the prevailing PDE4 isoform sequestered to β-arrestin in a variety of cell types, contains an additional binding site located within its isoform-specific N-terminal region [31,32]. These sites were initially identified by truncation analyses but detailed information was rapidly and effectively garnered employing a scanning peptide array approach (Figure 2). In this, a library of overlapping 25-mer peptides that covered the entire PDE4D5 sequence were spot-immobilized on cellulose membranes and probed with purified recombinant β-arrestin in order to identify sites of interaction [31]. This approach can give rapid information on the location of binding sites that can be used to drive confirmatory mutagenesis and truncation studies [31,33]. It works best when a binding site is formed from the juxtaposition of small regions of significant binding potential in their own right in a manner that is akin to linear epitopes recognized by antibodies.

We also used this approach to identify the sites on β-arrestin that interact with PDE4D5 by probing β-arrestin arrays with purified PDE4D3, to identify the sites interacting with the PDE4D catalytic unit, and with purified PDE4D5 to identify also any additional sites interacting with its unique N-terminal region. This identified the C-domain of β-arrestin interacting with the unique N-terminal region of PDE4D5 and the PDE4 catalytic region with the β-arrestin N-domain [33].

This methodology can also give insight into the amino acids that form binding sites (Figure 2). In a manner akin to alanine scanning mutagenesis, the interacting peptides can be used as a template to generate progeny that ‘scan’ across the sequence with alanine substitution. This identified Lys27 and Arg26 as critical in allowing specific binding of β-arrestin to the PDE4 catalytic unit [33]. Similarly, a stretch of five amino acids (Leu215 to Lys220) in the β-arrestin C-domain proved crucial in allowing preferential binding of PDE4D5. Information from these studies was utilized in functional studies employing MEFs (mouse embryo fibroblasts) from double-knockout β-arrestin1−/−:β-arrestin2−/− mice where transfection allowed mutant forms of recombinant β-arrestin2 to be analysed free from any input of endogenous β-arrestin. Significantly, all the mutant forms of β-arrestin2 in either the N-terminus or C-terminus that ablated PDE4D5 interaction negated the inhibitory effect seen for wild-type β-arrestin2 on isoprenaline-stimulated PKA phosphorylation of the β2-AR.

PDE4D5 interacts with β-arrestin and RACK1 (receptors for activated C-kinase 1) in a mutually exclusive manner

RACK1 is a 36-kDa scaffold protein initially identified as an anchoring protein for PKC (protein kinase C) that shows relative homology to the G-protein β-subunit and is predicted to form a β-propeller structure involving seven WD repeats [36]. It has been suggested to sequester various proteins such as Gβγ, Ras-GAP (GTPase-activating protein), c-Src tyrosine kinase, Dynamin-1 and the β-subunit of integrins [36]. RACK1 interacts specifically with PDE4D5 and not other PDE4 isoforms due to the presence of a specific binding site within the isoform-specific N-terminal region of PDE4D5 [26,31,37–39].

Interestingly, RACK1 acts as an ‘inhibitor’ of PDE4D5–β-arrestin2 interaction when expressed as a ‘competitor’ in yeast two-hybrid studies, suggesting a mutually exclusive relationship between RACK1 and β-arrestin2 for binding PDE4D5 that was confirmed in pull-down studies from mammalian cells [31]. Scanning peptide array analyses identified the β-arrestin2-binding site to overlap the RACK1-binding site within the unique N-terminal region of PDE4D5 [31]. Furthermore, the binding sites for RACK1 and β-arrestin2 juxtaposed each other on the PDE4D5 catalytic unit. Thus both RACK1 and β-arrestin2 appear to straddle PDE4D5 and have an overlapping binding site, making their interaction with this isoform mutually exclusive [31].

This fastidious interplay between β-arrestin2 and RACK1 over interaction with PDE4D5 has a functional correlate as uncovered upon siRNA knockdown of endogenous species in HEK-293-B2 cells. Consistent with mutually exclusive interaction, siRNA knockdown of RACK1 increased dramatically the amount of PDE4D5 sequestered to β-arrestin2, indicating a dynamic interaction between scaffolds in the ability to bind PDE4D5. Knockdown of RACK1, however, did not alter the total levels of PDE4D5 expression, indicating that RACK1-sequestered PDE4D5 does not include a sensor for controlling PDE4D5 levels. Thus levels of PDE4D5 are not compensated for upon knockdown of RACK1.
the RACK1 scaffold, which leads to increased binding to β-arrestin. RACK1 knockdown leads to an indirect phenotypic change in that the increased sequestration of PDE4D5 to β-arrestin and, thereby, increased agonist-dependent delivery of PDE4D5 to the β2-AR serves to attenuate further the ability of isoprenaline to elicit the PKA phosphorylation of this receptor [26]. RACK1 and β-arrestin thus compete to sequester distinct pools of PDE4D5.

The exclusive nature of PDE4D5 pairing to these scaffolding molecules is likely to have broad and significant functional implications for β2-AR signalling. Indeed, chronic exposure to β-agonists in asthma is likely to cause up-regulation of PDE4D5, due to its cAMP-sensitive promoter region [40], facilitating desensitization of β2-AR functioning. This may be therapeutically approachable by disrupting PDE4D5 interaction with β-arrestin.

**Conclusions**

Our studies have revealed distinct functional facets attributable to PDE4D5, a prevalent enzyme in cAMP signalling in many cells. This has been achieved through the utilization of dominant-negative and siRNA strategies together with novel peptide array analyses. We have deciphered how PDE4D5 may correlate to prevalent scaffold proteins such as β-arrestin and RACK1. We highlight that PDE4D5 plays a non-redundant and functionally significant role in its interaction and functioning. This may be therapeutically approachable by disrupting PDE4D5 interaction with β-arrestin.

**References**


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