Review

Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown

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It is becoming increasingly apparent that spatial regulation of cell signalling processes is critical to normal cellular function. In this regard, cAMP signalling regulates many pivotal cellular processes and has provided the paradigm for signal compartmentalization. Recent advances show that isoforms of the cAMP-degrading phosphodiesterase-4 (PDE4) family are targeted to discrete signalling complexes. There they sculpt local cAMP gradients that can be detected by genetically encoded cAMP sensors, and gate the activation of spatially localized signalling through sequestered PKA and EPAC sub-populations. Genes for these important regulatory enzymes are linked to schizophrenia, stroke and asthma, thus indicating the therapeutic potential that selective inhibitors could have as anti-inflammatory, anti-depressant and cognitive enhancer agents.

Signalling in time and space

Cells are constantly bombarded by environmental cues, be they chemical, electrical or mechanical. These inputs are integrated to generate coordinated responses that are intimately connected with spatially discrete changes occurring within the three-dimensional cell envelope: functional outcomes therefore are defined in both time and space. Cell shape and intracellular organization confer defined properties on these myriad of signalling systems. Thus compartmentalization of signalling systems, a concept that not so long ago was deemed heretical, is now well accepted and subject to intense investigation. The identification of spatial constraints and cross-talk between signalling processes has not only revolutionized our understanding of biochemical and cellular processes, but has profound translational implications for developing novel therapeutics and diagnostics.

The discovery of cAMP transformed our understanding of cellular regulation by providing not only the 'second messenger' concept, but also the discovery of G-proteins, the G protein-coupled receptor (GPCR) superfamily and, importantly, the conceptual roots of compartmentalized signalling [1]. Indeed, the notion that intracellular signalling is compartmentalized originated in the early 1980s from work examining the functional consequence of adenylyl cyclase activation in cardiomyocytes via two different GPCRs, namely β-adrenergic and prostaglandin [2]. Whereas agonist occupancy of these receptors led to similar increases in 'global' cAMP levels, functional output was remarkably different, with only β-adrenergic stimulation coupling to increased force (inotropic) and rate (chronotropic) of contractions and, critically, activation of different protein kinase A (PKA) isoforms. This conundrum could be rationalized if cAMP signal transduction was compartmentalized in these cells. Since then, however, it has taken considerable effort by many investigators to uncover the critical machinery involved in achieving compartmentalization and to visualise cAMP gradients in living cells. This review discusses how targeted cAMP

Glossary

AKAP: (A-Kinase Anchor Proteins): a large family of PKA binding proteins that sequester PKA by binding to the dimerisation interface between the cAMP binding, regulatory R subunits.
EPAC: (exchange protein directly activated by cAMP): a cAMP effector protein that has either one (EPAC1) or two (EPAC2) cAMP binding domains and acts as a GTP exchange factor to activate the mini G-proteins RAP1 and RAP2.
ERK: (extracellular signal-regulated kinase): a downstream serine kinase activated by a pathway originating from activation of cell surface tyrosyl kinase growth factor receptors such as EGF and PDGF.
GEF: (GTP Exchange Factor): stimulates GDP/GTP exchange in mini G-proteins, causing their activation.
G-proteins: (GTP-binding protein): these proteins play pivotal roles in signalling processes by acting as OFF (GDP-bound) and ON (GTP-bound) switches that are able to self-inactivate by hydrolysing GTP to GDP.
GPCR: (G protein-coupled receptor): these receptors are found at the plasma membrane and have 7 transmembrane regions that allow agonist binding at the extracellular surface side to elicit a conformational change such that they now bind heterotrimeric G proteins at the intracellular surface side.
NTR: (N-terminal region): here used to describe the isoform-specific N-terminal region of particular PDE4 isoforms.
p75NTR: (p75 neurotrophin receptor): a TNF receptor superfamily member up-regulated after tissue injury that blocks fibrinolysis by down-regulating the serine protease, tissue plasminogen activator (tPA), and up-regulating plasminogen activator inhibitor-1 (PAI-1).
PDE: (cAMP phosphodiesterase): member of a large family of enzymes that hydrolyse either or both of the signalling cyclic nucleotides, cAMP and cGMP, and provide the sole means of inactivating these second messengers in cells.
PDE4: a four gene PDE subfamily whose members are selectively inhibited by the compound rolipram, are characterized by UCR1 and UCR2 regulatory domains and where isoforms have unique N-terminal regions.
PKA: (protein kinase A): a key effector system for cAMP signalling, which consists of an R_2C_2 dimer where 2 molecules cAMP bind each regulatory R subunit, activating the catalytic C subunits. The RI and RII subunits have particular functional significance regarding interaction with AKAPs and targeting.
UCR1: (upstream conserved region 1): a regulatory domain of around 55 amino acids that is uniquely found in long PDE4 isoforms, provides the site for stimulatory phosphorylation by PKA, and interacts with UCR2. Its amino terminal half is strikingly polar whereas its C-terminal half is hydrophobic.
UCR2: (upstream conserved region 2): a regulatory domain of around 80 amino acids that is found in all active PDE4 isoforms either in its entirety (long, short isoforms) or N-terminally truncated (super-short isoforms). It can interact with both UCR1 and the PDE4 catalytic unit and is very hydrophilic.

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degradation by specific phosphodiesterases (PDEs) underpins compartmentalized cAMP signalling in cells. It focuses on PDE4 isoforms where sequestered isoforms act to sculpt gradients of cAMP surrounding specific signalling complexes. In this way, the activation threshold can be regulated for signalling by cAMP detectors and downstream effectors in such spatially defined intracellular complexes. Thus, anchoring confers specific functional roles on PDE isoforms and targeted disruption of such events might provide a novel means for therapeutic intervention by displacing key isoforms from their functionally relevant site(s) in cells.

cAMP signalling and compartmentalisation

GPCR-mediated activation of adenylyl cyclases provides a source of cAMP generation at the cytosol surface of the plasma membrane [1,3]. This point source can be further delineated as adenylyl isoforms can be constrained to distinct regions within the plasma membrane. For example, calveolae and post-synaptic densities can organise adenylyl cyclases and GPCRs to form spatially and functionally discrete complexes within the two-dimensional surface of the plasma membrane [4].

Once cAMP is generated, it interacts with several cellular proteins, including PDEs that break cAMP down to 5′AMP, as well as effector proteins, primarily PKA and exchange protein activated by cAMP (EPAC), whose actions elicit a physiological response [5,6]. PKA is a heterotetramer consisting of 2 cAMP-binding regulatory (R) subunits, together with 2 catalytic (C) units. By contrast, EPAC1/2 are multi-domain proteins that include a discrete cAMP binding domain as well as a GEF domain that allows activation of the mini G-proteins, RAP1 and RAP2. Thus, cAMP binding to both PKA and EPAC triggers a conformational change that elicits a functional output.

PKA and EPAC are intrinsically soluble proteins, and sub-populations of each are found in the cytosol. However, for compartmentalisation to ensue, it is critical that sub-populations of these cAMP effectors are sequestered to specific intracellular complexes, whether they are located at membranes or in the cytosol, together with their downstream targets. The discovery of a family of anchor proteins, called AKAPs, was fundamental to our appreciation of this concept. AKAPs specifically sequester PKA [5,7] via a short α-helical structure that interacts with a groove formed by the N-terminal dimerization domain of PKA R-subunits. As PKA-RII provides the majority of membrane-associated PKA, whereas the majority of PKA-RI is cytosolic, it was originally assumed that only PKA-RII was able to bind AKAPs. We now know, however, that this is not the case. AKAPs with an additional interaction site have been identified; these proteins are either specific for PKA-RI or show dual specificity for PKA-RI and -RII [8]. This diverse AKAP family even includes cytosolic species.

Critically, a sub-population of PKA is spatially constrained within the cell through sequestration by AKAPs, together with functionally appropriate substrate proteins. This allows specific AKAPs to formulate spatially discrete signalling complexes responding to defined intracellular cAMP gradients. When PDEs are in these complexes or in close proximity, the coordinated action of such PDEs and adenylyl cyclases dictate the level and persistence of the cAMP signal at that particular spatial locale. Interestingly, some AKAPs can bind additional signalling proteins including other protein kinases, protein phosphatases, GTPases and adaptor proteins so as to provide further regulatory inputs into these compartmentalized complexes [5,7]. Indeed, some AKAPs can bind both GPCRs and adenylyl cyclases, thus directing PKA functionality to targets associated with cAMP production. Interestingly, mAKAP (muscle specific) can provide a dual output cAMP signalling system as a consequence of sequestering both EPAC and PKA [9]. Thus sequestered and spatially constrained PKA and EPAC sub-populations provide the machinery to interpret intracellular cAMP gradients.

The notion that small molecules can be compartmentalized in cells is a concept that some investigators have found difficult to come to terms with in the past because of the calculated rapid diffusion of cAMP in aqueous solution (130–700 $\mu m^2\ sec^{-1}$). Interestingly, however, many of the concepts related to compartmentalization of signalling arose from the development of optical probes that allowed Ca^{2+} gradients to be visualized in real time, in living cells [10]. Such ‘seeing is believing’ evidence has, until recently, eluded the cAMP signalling field. However, this has been solved recently (Figure 1) by the generation of a wide range of genetically encoded Fluorescence Resonance Energy Transfer (FRET) sensors, based upon both PKA and EPAC [11–13], allowing cAMP gradients to be visualized in living cells, in real time (Figure 1). Indeed, the use of such probes in cardiomyocytes enabled the first visualization of cAMP microdomains [13]. Genetic engineering has allowed not only the sensitivity of these sensors to be manipulated, but also for them to be targeted to specific intracellular sites and to specific signalling complexes within cells as well as in animals [12]. Additionally, a cAMP bio-sensor has been developed by engineering a cyclic nucleotide-gated ion channel, such that cAMP gradients can be detected at the cytosolic surface of the plasma membrane [14]. In addition, a FRET sensor based upon a PKA substrate motif has been used to detect compartmentalised PKA activity in cells [15]. This growing armory of bio-sensors now enables cAMP gradients to be dynamically detected in a wide range of intracellular locales and complexes.

How do such defined cAMP gradients arise? GPCR-stimulated adenylyl cyclases allow cAMP to be generated at point sources at the cytosol surface of the plasma membrane, thus potentially forming spatially discrete clouds of cAMP. However, there is also a growing appreciation that a bicarbonate- and Ca^{2+}-activated form of adenylyl cyclase is more widely expressed than originally thought, and can be found both in the cytosol and associated with various intracellular membranes [16]. Thus, as ‘time goes by’ after adenylyl cyclase activation, a cell would be expected to fill up uniformly with cAMP. So, what prevents such uniformity from occurring and is responsible for sculpting cAMP gradients in real time at discrete intracellular locales? A means of achieving this is essential to underpin compartmentalized cAMP signalling, by gating ‘access’ of cAMP to sequestered PKA and EPAC sub-populations. The answer to this question is provided by

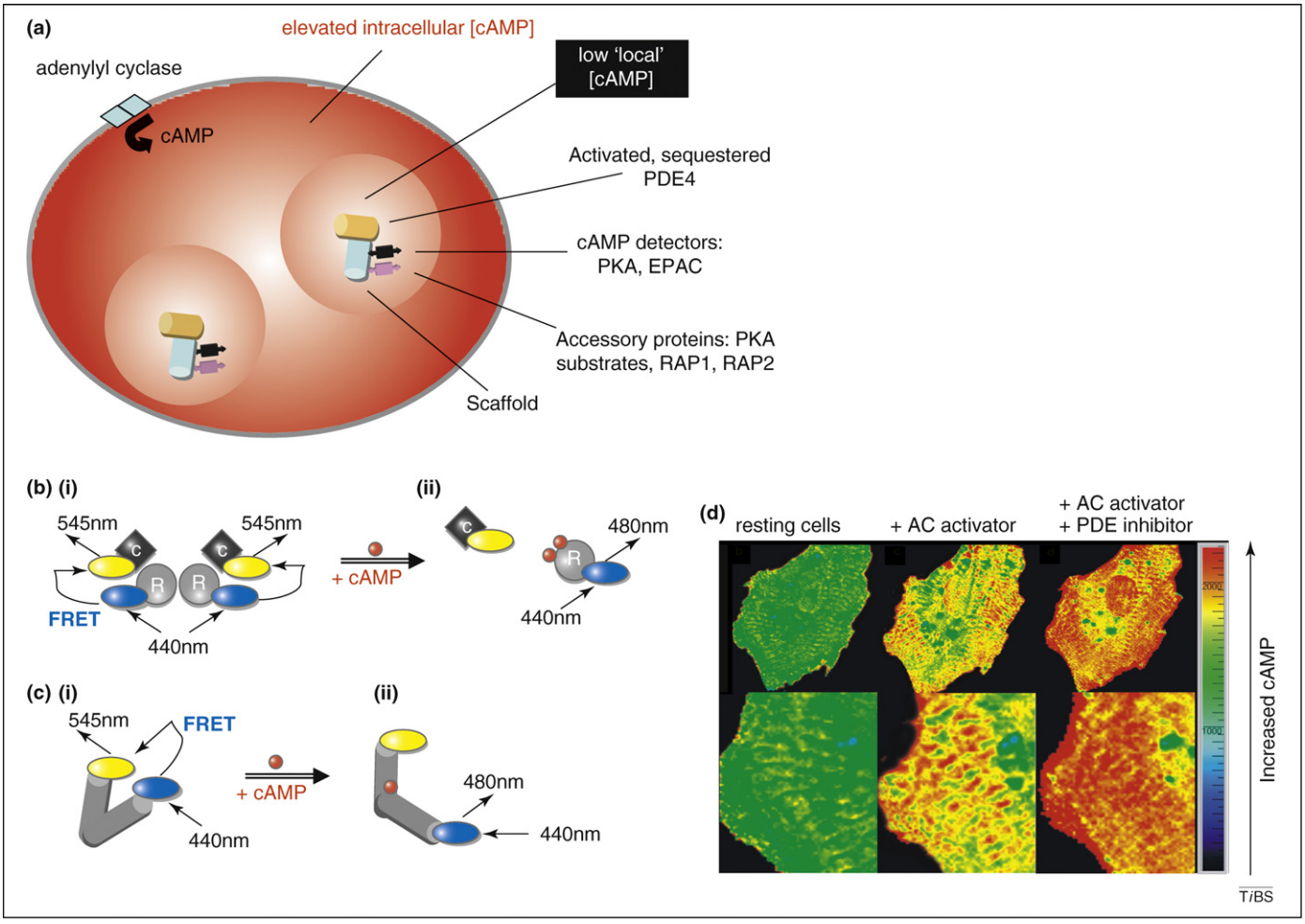

Figure 1. Intracellular cAMP gradients: detection and formation. The action of PDEs sequestered to specific complexes and membranes inside cells sculpts gradients around the complexes and, in doing so, gates the activation of associated cAMP effectors, such as PKA and EPAC. Such cAMP gradients can be detected in cells, such as cardiac myocytes, with genetically encoded sensors based upon either PKA or the cAMP binding domain of EPAC, which are engineered to exploit FRET for visualisation and quantification. (**a**) A schematic showing a cell with activated adenylyl cyclase activity generating intracellular cAMP (red), with active, sequestered PDE4 (orange) decreasing local cAMP levels and 'gating' activation of associated effectors, PKA and EPAC (black) on tethered downstream targets (pink). (**b**) A PKA-based FRET system for cAMP detection with CFP (blue) fused to PKA-R (R; gray) and YFP (yellow) fused to PKA-C (c; black). A standing FRET signal is present in the absence of cAMP (i). Binding of cAMP to PKA-R (CFP) causes release of PKA-C (YFP) and loss of FRET (ii). (**c**) An EPAC-based FRET system for cAMP detection with CFP and YFP fused to the opposite ends of the cAMP binding domain of EPAC. A standing FRET signal is present in the absence of cAMP (i). Binding of cAMP causes a conformational change that moves apart the N- and C-terminal regions of this domain leading to loss of FRET (ii). (**d**) A pseudocolour representation of cAMP gradients in cardiac myocytes expressing a PKA-based FRET sensor. Shown are cells under resting conditions as well as those stimulated with either the direct activator of membrane-bound adenylyl cyclase, forskolin, either alone or together with the non-selective PDE inhibitor, isobutyl methyl xanthine (IBMX). Adapted with permission [13].

PDEs, which provide the sole means of degrading cAMP in cells. Indeed, this recent appreciation of the fundamental role of PDEs in underpinning compartmentalized cAMP signalling (Figure 1) offers insight into the conundrum as to why there are so many cAMP-degrading PDEs encoded by mammalian genomes.

PDE-ology: a soccer team of enzyme families

Although 11 members make up the class I PDE super-family in mammals, the presence of multiple genes within various families, coupled with mRNA splicing, dramatically increase the number of PDE isoforms expressed [17,18]. PDE families show distinct kinetic and regulatory properties, with some specifically hydrolyzing cAMP (PDE4, 7, 8), some both cAMP and GMP (PDE1, 2, 3, 10, 11), and others just cGMP (PDE5, 6, 9). The structures of various PDE catalytic units have been solved recently; this work has given insight into the basis of selectivity of action of the various family-specific, active site-directed competitive inhibitors that have been developed over the years, and aided in developing species with increased potency and selectivity [19]. Selective inhibitors have been used both to gain functional insight into the role of particular PDEs and as therapeutics. Examples are the PDE5-specific inhibitor Viagra®, used to treat penile erectile dysfunction and infant pulmonary hypertension [20], the PDE3-specific inhibitor Cilostazol®, which treats intermittent claudication (cramping of the lower leg) [21,22] and PDE4-selective inhibitors, which are being developed to treat a variety of inflammatory diseases [23]. However, pivotal insight into the molecular mechanisms that underpin the role of PDEs in defining compartmentalization of cAMP signalling has arisen predominantly from recent studies on the cAMP-specific PDE4 family.

PDE4-ology

PDE4s are highly conserved over evolution, being found in *Drosophila melanogaster* and *Caenorhabditis elegans;* iso-

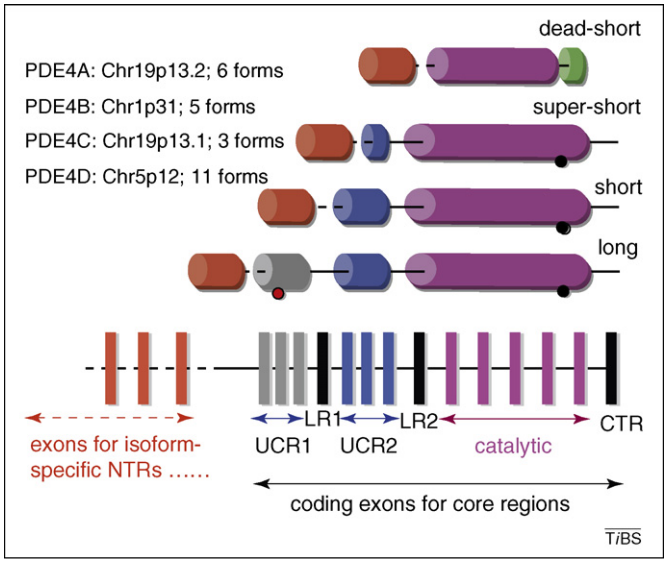

Figure 2. The long and short of PDE4 isoforms. Four PDE4 genes encode a panel of PDE4 isoforms that are categorised as long, short, super-short and dead-short, as indicated. Different splicing mechanisms endow these isoforms with different combinations of the UCR1 (grey) and UCR2 (blue) regulatory components. Sites for PKA-mediated phosphorylation of UCR1 (red circle) and ERK-mediated phosphorylation of the catalytic unit (black circle) are shown. LR1, Linker Region 1 and LR2, Linker Region 2, are stretches of sequence that differ markedly in all four PDE4 subfamilies. CTR, C-terminal region is a stretch of sequence that differs markedly in all four PDE4 subfamilies. NTR (red), is the N-terminal region encoded by distinct exon(s) and defines each isoform. The chromosome localisation of all four genes is shown, together with the known number of isoforms generated by each gene.

forms in man and mouse exhibit near identical sequences. Four genes (*PDE4A/B/C/D*) encode over 20 distinct PDE4 isoforms (Figure 2) as a result of mRNA splicing and the use of distinct promoters [17,24,25]. Each PDE4 sub-family has a highly conserved catalytic unit consisting of 17 α-helices organized into 3 sub-domains, at the junction of which is found a deep binding pocket for cAMP [19,24,26]. This substrate binding site also contains binding sites for Zn^{2+} and Mg^{2+}, which are essential for catalysis.

So, why are there so many PDE4 isoforms when they each appear to do exactly the same thing, namely hydrolyse cAMP with identical K_m and similar V_{max} values? Conservation of multiplicity and sequence among species implies importance for individual isoforms. Indeed, dominant negative and knockdown strategies have recently shown [27,28] that particular PDE4 isoforms have unique, non-redundant functional roles. Furthermore, particular PDE4 isoforms are subject to different regulatory influences, such as phosphorylation [25], ubiquitylation [29], and activity changes induced by interacting proteins [30].

Initial insight into the functional importance of PDE4s came from studies showing that *dunc*, a gene associated with learning and memory in D. *melanogaster*, encodes a family of PDE4 isoforms [31]. This insight relates to the appreciation that PDE4-selective inhibitors were originally shown to act as anti-depressants and cognitive enhancers [32]. Indeed, very recent work demonstrates [33] that sleep disturbances result in up-regulation of a specific PDE4A isoform, causing marked cognitive deficits correctable by PDE4 inhibitor treatment. Furthermore, *PDE4B* has been linked to schizophrenia [34,35], consistent with observations that PDE4 inhibitors have anti-psychotic potential, and animals deficient in PDE4B display an anti-psychotic profile [36].

The wider importance of the PDE4 family can be inferred from linkage and related studies that show association of *PDE4D* with ischemic stroke [37], asthma [38], osteoporosis [39] and prostate cancer [40]. Currently, PDE4 inhibitors are being developed to treat chronic obstructive pulmonary disease (COPD), asthma and other inflammatory conditions [23].

Designed for targeting: PDE4 cAMP specific phosphodiesterases

Individual PDE4 isoforms are characterized (Figure 2) by a unique N-terminal region (NTR) encoded by 1 or 2 exons under the control of a specific promoter [17,25]. An overwhelming body of evidence now points to the importance of these unique identifiers in the intracellular targeting and functional significance of individual PDE4 isoforms. Indeed, these unique NTRs allow particular PDE4 isoforms to be sequestered by specific protein partners. However, targeting can also be directed to lipid bilayers, as seen with PDE4A1, whose investigation has provided the paradigm for our appreciation of the functional role of isoform-specific NTRs [41]. Sequestered PDE4 isoforms thus sculpt their immediate cAMP gradient and, in so doing, control the activation of tethered cAMP effectors within the complex.

In recent years, much has been learned regarding the breadth of species interacting with PDE4 isoforms (Figure 3) based upon motif searches, 2-hybrid screens, proto-arrays, proteomic studies and intuition! Mapping PDE4–target protein interaction sites, using truncation, site-specific mutagenesis and, more recently, scanning peptide arrays [42], has aided in authenticating complexes and determining functional significance.

PDE4 isoforms bind scaffold proteins such as AKAP6 [43], AKAP7 [44], AKAP9 [45], β-arrestin [46], receptor for activated protein kinase C (RACK1) [47], myomegalin [48], nuclear element-like protein 1 (NDEL1) [49], spectrin [50], and disrupted in schizophrenia (DISC1) [35], as well as chaperones like the aryl hydrocarbon receptor interacting protein (AIP) [30]. They can also be sequestered to receptors, such as the p75 neurotrophin receptor (p75NTR) [51] and the β_1-adrenoceptor [52], as well as kinases such as ERK [53] and those from the SRC tyrosyl kinase family [54,55]. Channel regulation is also evident with PDE4s sequestered directly to the cardiac ryanodine (RyR2) receptor [56] and indirectly to both the cystic fibrosis transmembrane conductance regulator via the PDZ-containing scaffold protein, SHANK2 [57], and to the cardiac I_{Ks} potassium channel via AKAP9 [58]. Indeed, it is even thought that the cAMP detector, EPAC might directly sequester PDE4 [9].

Although the importance of a particular isoform for a particular functionality can be gauged through knockdown by specific siRNA, such studies can only determine whether the entire pool of that isoform is important, but not whether the scaffolded, sub-population is critical. To address this question, a dominant negative approach has been formulated [27,28]. In this approach, alanine substitution of an aspartate located deep within the catalytic

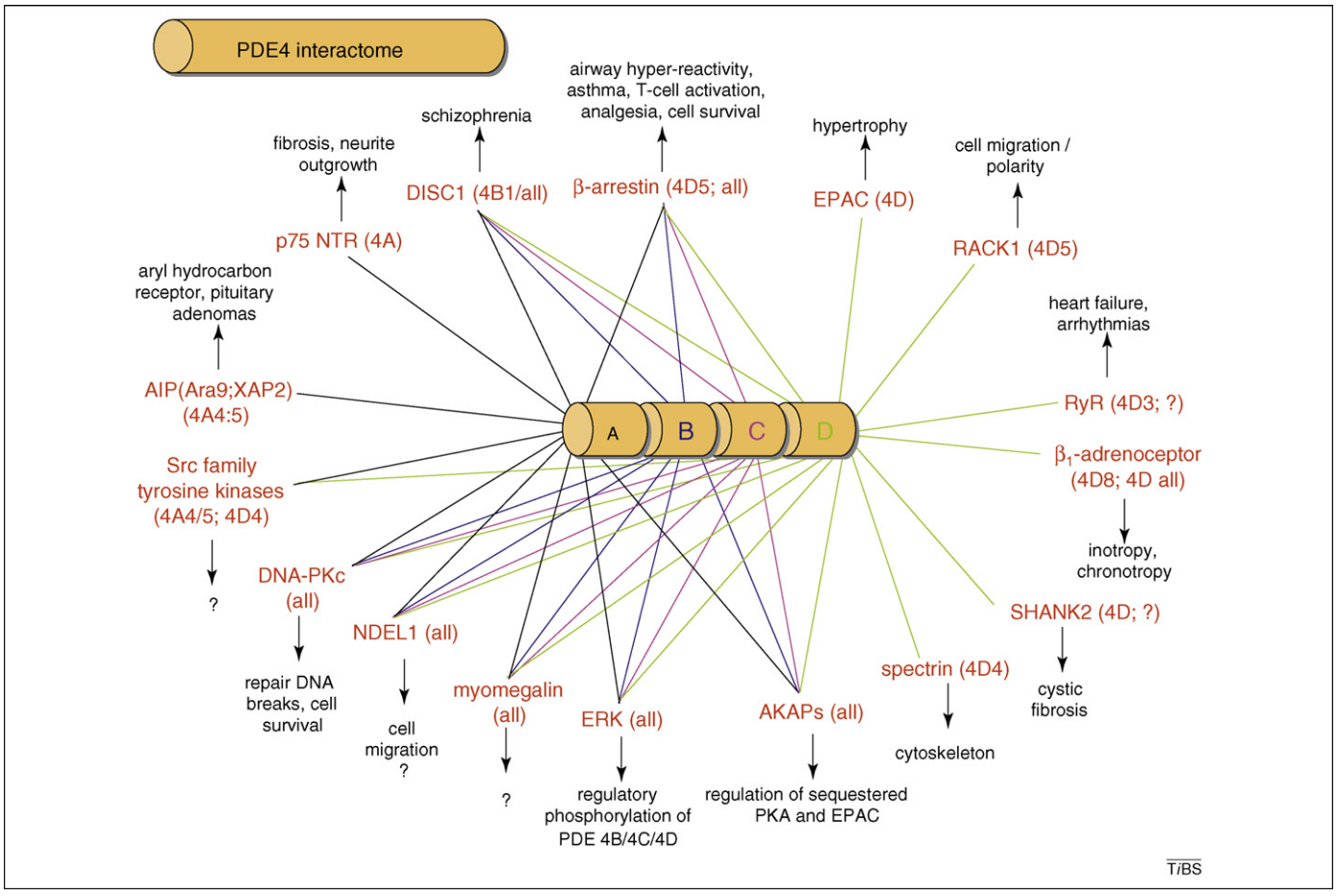

Figure 3. The PDE4 interactome. The currently known range of proteins able to interact directly with various PDE4 isoforms from the four PDE4 sub-families (PDE4A, B, C, D). Known or proposed functional role of such complexes are indicated; in the absence of a function, a question mark is listed.

pocket causes inactivation without affecting overall conformation. Overexpression of an inactivated PDE4 isoform displaces the cognate, endogenous, active isoform from scaffolds that sequester it, thereby sensitizing sensors in the complex to cAMP action. Thus, a dominant negative phenotype will only arise if the displaced endogenous species is involved in functional protein–protein partnerships. Engineering the catalytically inactive isoform to destroy its binding site for a specific partner protein will then define the phenotype associated with a particular partnership.

A paradigm for intracellular targeting of PDEs: PDE4A1

PDE4A1 is a brain-specific isoform with a unique 25-amino-acid NTR consisting of two α-helical domains separated by a flexible hinge (Figure 4). PDE4A1 is entirely membrane-associated and preferentially located at the Golgi and its associated vesicles. The deletion of its unique NTR generates an entirely soluble, cytosolic species that is folded correctly, and is more active than the native form [59]. These observations provided the paradigm for targeting of specific PDEs and for identifying a role for isoform-specific NTRs. Indeed, all of the information for PDE4A1 targeting is encapsulated within its NTR, as fusion of this region with various cytosolic proteins confers on them a membrane location identical to that of PDE4A1 (Figure 4). Furthermore, discrete mutations in the NTR ablate PDE4A1 membrane targeting [41,60]. Ca^{2+} signalling provides the trigger for PDE4A1 membrane association: Ca^{2+} binding to Asp21 triggers a conformational change that allows the Trp19–Trp20 pairing to insert in the bilayer, conferring long-term 'memory' of elevated [Ca^{2+}] (Figure 4). Intriguingly, in binding to Asp6, subsequent increases in [Ca^{2+}] allow the dynamic, reversible redistribution of PDE4A1 to phosphatidic acid (PA)-rich membrane regions, driven by a PA-preferring binding domain in helix-2 (TAPAS1) [41,60] (Figure 4). Despite this detailed knowledge of membrane association, the specific functional role of PDE4A1 remains unclear. Recent studies, however, identify PDE4A1 as up-regulated and driving proliferation in malignant gliomas, indicating that PDE4A1 could be an important therapeutic target [61].

A spatially constrained functional complex: β-arrestin-mediated PDE4D5 sequestration and trafficking

Adrenaline, in binding to β-adrenoceptors, causes a rapid but transient rise in cAMP [1]. One factor contributing to the transience is β-arrestin, a cytosolic protein that is dynamically recruited to the agonist-occupied β-adrenoceptor so as to interdict receptor coupling to G_s, thereby desensitizing the activation of adenylyl cyclase. However, a novel facet of this desensitization system is that β-arrestin can sequester PDE4D5, thereby delivering an active cAMP-degrading system to the site of cAMP synthesis [28,46]. The non-redundant role of β-arrestin-sequestered PDE4D5 in this process was gauged in a variety of ways. Firstly, a dominant negative approach, which employed

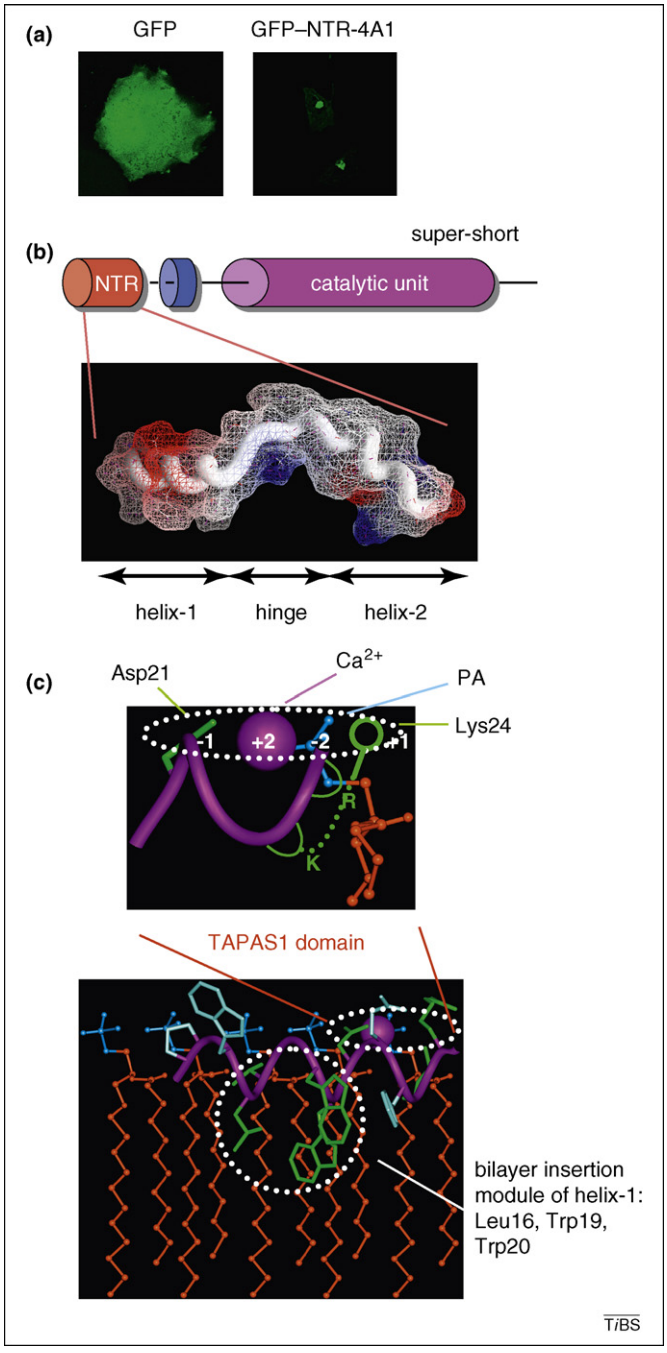

Figure 4. Membrane-targeting of PDE4A1. Studies on PDE4A1 provided the paradigm for intracellular targeting of PDEs and insight into the functional role of the isoform-specific NTR in targeting. This figure shows how this 25 amino acid region contains all the information necessary for intracellular targeting of PDE4A1 through fusing it to GFP, which normally distributes throughout cells, whereas the GFP–NTR-PDE4A1 chimera is targeted to the Golgi. The unique NTR of PDE4A1 consists of 2 helical regions separated by a mobile hinge region. Membrane association is triggered by the binding of Ca^{2+} to Asp21, which elicits a conformational change in helix-2 that allows 2 tryptophan residues (Trp19; Trp20) and a leucine (Leu16) in helix-2 to insert in the membrane. (**a**) The intracellular distribution of GFP compared to fusion of GFP to the unique NTR of PDE4A1. Figure adapted with permission [60]. (**b**) The ^{1}H-NMR-derived structure of the 25 amino acid membrane-targeting NTR of PDE4A1. This consists of 2 alpha-helical regions, helix-1 (amino acids 1-7) and helix-2 (amino acids 14-25) separated by a mobile hinge region. Helix-2 contains the core membrane association (insertion) region [60,62], whereas helix-1 provides a regulatory domain where Ca^{2+} dynamically regulates trafficking of membrane-bound PDE4A1 from the Golgi to phosphatidic acid-rich regions in the cell [41]. Figure adapted with permission [62]. (**c**) Modelling of the membrane insertion unit within helix-2 of the PDE4A1 NTR. This consists of two tryptophan residues (Trp19; Trp20) and a leucine residue (Leu16) that locate within the lipid bilayer. The trigger for this insertion is Ca^{2+} (purple ball) binding to an aspartate group (Asp21) on the aqueous facing surface of this helix, which lies within a phosphatidic acid-preferring domain called TAPAS1. Figure adapted with permission [62].

the catalytically inactive Asp556Ala-PDE4D5 mutant, was used [27,28]. Secondly, peptide array analyses allowed the generation of membrane-permeable peptides to disrupt β-arrestin-sequestered PDE4D5 complexes; thirdly PDE4D5 mutants unable to bind β-arrestin were utilized [42,62]. Recruited PDE4D5 specifically decreases sub-plasma membrane cAMP concentrations, and, in the case of the β_2-adrenoceptor, inhibits its phosphorylation by a specific PKA-RII sub-population tethered to it by AKAP5. Thus β-arrestin-sequestered PDE4D5 desensitizes activation of a defined PKA sub-population, whose functional role is to phosphorylate the β_2-adrenoceptor and switch signalling from activation of adenylyl cyclase to ERK activation.

In most instances, partner proteins bind at least two sites on PDE4 isoforms. The β-arrestin N-domain interacts with a 'common' site located within the conserved PDE4 catalytic unit, with specificity conferred by a second binding site within the PDE4D5 NTR where the β-arrestin C-domain binds [42,62,63] (Figure 5). The fidelity of this partnership is further enhanced by transient MDM2-mediated ubiquitylation of PDE4D5 initiated upon β_2-adrenoceptor activation [29]. Here a priming mono-ubiquitylation triggers a transient cascade of polyubiquitylation at 3 sites within the PDE4D5 NTR, thereby enhancing binding to a non-ubiquitylated β-arrestin sub-population.

Interestingly, the signalling scaffold protein RACK1, originally discovered as a PKC binding protein, but now known to interact with a wide variety of other signalling proteins, also binds PDE4D5 specifically [47]. Again, binding involves two sites on PDE4D5, one within its NTR and another within its catalytic unit (Figure 5). Although these sites are distinct from those through which β-arrestin binds, sites in the NTR overlap such that PDE4D5 cannot bind both β-arrestin and RACK1 at the same time [42,62]. This finding illustrates an important concept, namely scaffold fidelity. If a PDE4 could simultaneously bind >1 scaffold, then it would act as an adaptor/bridge, forming aggregates within the cell. Studies on various PDE4 partnerships indicate that a given PDE4 probably binds only one scaffold at a time, thus forming discrete signalling modules around which it sculpts cAMP gradients, and gates activation of the associated effector, PKA or EPAC.

The β-arrestin–PDE4D5 partnership and its dynamic recruitment to the β_2-adrenoceptor thus exemplifies, in some molecular detail, components of a highly compartmentalized cAMP signalling system as well as the non-redundancy of the PDE4D5 isoform.

Multi-site phosphorylation of PDE4 confers dynamic regulation on signalosomes

Long PDE4 isoforms contain highly conserved domains, called UCR1 and UCR2 (for upstream conserved regions), located between their NTR and catalytic unit (Figure 2). By contrast, short isoforms lack UCR1, super-short isoforms have only a truncated UCR2, and dead-short isoforms lack both UCR1 and UCR2 and are catalytically inactive due to

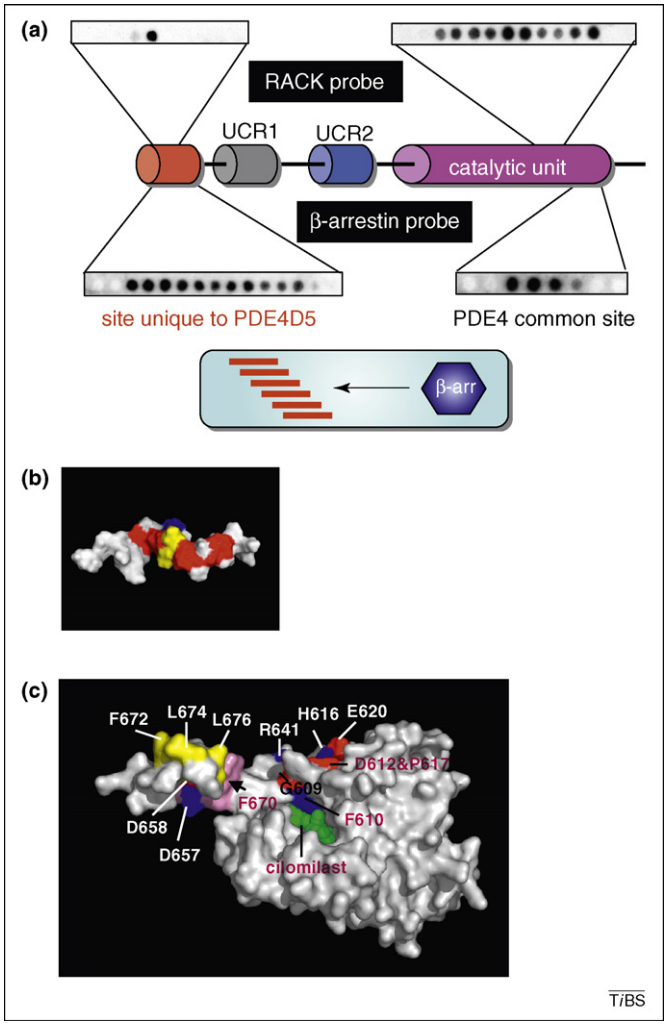

Figure 5. Mapping the interaction sites on PDE4D5 for β-arrestin and RACK1. Spot-immobilized peptide arrays provide a novel and powerful technology for identifying binding sites that determine protein–protein interactions. Libraries of overlapping peptides (25 mers), each shifted by 5 amino acids across the entire sequence of the target protein, are produced by automatic SPOT synthesis on continuous cellulose membrane supports. They are then probed with the purified partner protein whose binding to particular spots can then be identified, for example, with specific antisera. This is exemplified here for a peptide array of PDE4D5 probed with, separately, RACK1 and β-arrestin. Insight into which specific amino acids contribute to the binding site can be achieved by generating a family of peptides derived from a 25-mer parent peptide whose progeny have a single substitution, to alanine (or asparate), of successive amino acids in the sequence to form a scanning peptide array. Loss of binding in any progeny highlights the potential importance of the amino acid substituted in that peptide. Knowledge of the 3-dimensional structure of a protein can then be used to determine the location of the identified residues and which are surface exposed and, therefore, most likely to present part of an interaction surface. This technique has been used successfully to identify a number of interaction sites between PDE4 isoforms and their partner proteins. However, it is not infallible and certain sites are not detected, such as the binding of ERK to its KIM on a β-hairpin loop on the PDE4 catalytic unit [42,44,49,51,63,69]. (**a**) Segments of PDE4D5 spot-immobilised peptide arrays probed with either RACK1 or β-arrestin, identifying distinct binding sites within the unique NTR of PDE4D5 as well as the PDE4D common catalytic unit. The box shows schematic of how overlapping 25-mer peptides, each displaced 5 amino acids in sequence, are interrogated with β-arrestin (or RACK1) to identify those that interact, seen above as dark spots. Figure adapted with permission [42]. (**b**) Shows the NMR-derived structure of a region of the PDE4D5 NTR where the entire RACK1 binding site lies, delineating regions where RACK1 (red) and β-arrestin (blue) alone bind and a region where they both bind (yellow), ensuring exclusivity of interaction. Figure adapted with permission [62]. (**c**) The structure of the PDE4D catalytic unit is shown with the PDE4-selective, reversible, competitive inhibitor cilomilast [23] (green) located in the catalytic pocket, the common binding site for RACK1 (red, blue) and the common binding site for β-arrestin (yellow) and a mobile hinge region (pink). Figure adapted with permission [42].

severe C-terminal truncation [25]. UCR1 and UCR2 interact dynamically [64] to determine the functional outcome of PDE4 phosphorylation. By example, PKA phosphorylates UCR1, thereby activating long forms and providing them with a critical role in the cellular cAMP desensitization process [17,25]. UCR1 and UCR2 also determine the functional outcome of ERK-mediated phosphorylation of the PDE4 catalytic unit, conferring inhibition upon long isoforms, activation of short isoforms and a null-effect on super-short forms [53].

PKA phosphorylation of AKAP-sequestered PDE4 long isoforms provides a negative feedback system where elevated cAMP concentrations trigger PKA to phosphorylate and activate PDE4, driving down local cAMP levels and resetting PKA activity [43]. A more complex system is associated with AKAP6, which sequesters the long PDE4D3 isoform together with both PKA and ERK to form two coupled cAMP-dependent feedback loops focused on PDE4 and coordinated within the spatial context of the AKAP [9]. This complex, which regulates cardiac hypertrophy, has two other key features. Firstly, through PDE4D3, it also sequesters EPAC, making it an AKAP with dual cAMP signalling outputs. Secondly, PDE4D3 has a second site for phosphorylation by PKA, lying within the PDE4D3 NTR, which confers enhanced association with AKAP6 [65]. Conversely, PKA phosphorylation of PDE4D3 NTR releases it from complex with NDEL1 [49]. Thus, PDE4 phosphorylation can dynamically change partnerships with certain scaffolds, causing redistribution of specific PDE4 isoforms, and re-programming cAMP gradients within specific spatial locales.

Additional examples of PDE4 signalling complexes

Contributing further to our understanding of cAMP signal compartmentalisation in cardiomyocytes is the discovery that PDE4D8 can be selectively recruited to interact directly with the β_1-adrenoceptor but not the β_2-adrenoceptor [52]. It has long been appreciated that, although these receptors are expressed in cardiomyocytes and activate adenylyl cyclase, they exert very different phenotypic actions, with β_1-adrenoceptors regulating contraction and β_2-adrenoceptors regulating survival. We now appreciate that different PDE4 species, namely PDE4D8 for the β_1-adrenoceptor [52] and β-arrestin-sequestered PDE4D5 for the β_2-adrenoceptor [28], aid in channelling cAMP originating from occupancy of these receptors to activate distinct downstream targets and shape distinct gradients in cardiac myocytes [66,67]. In addition to these actions and those associated with mAKAP in the heart, PDE4D isoforms also interact with the ryanodine receptor, the major intracellular Ca^{2+}-release channel, to control its regulation by PKA [56].

Until recently, it was unknown how p75NTR, which is up-regulated after tissue injury, blocks fibrinolysis and promotes scarring. However, it now appears that by specifically recruiting PDE4A5 (or the PDE4A4 human ortholog), local cAMP levels are lowered to enhance p75NTR signalling, resulting in decreased extracellular proteolytic activity by down-regulating tissue plasminogen activator and up-regulating plasminogen activator inhibitor-1 [51]. PDE4A5 also uniquely binds and is inhibited by the co-chaperone AIP [30]. Interestingly, mutant AIP proteins,

which are associated with familial and sporadic pituitary adenomas, no longer interact with PDE4A5, thereby pointing to a functional link for this interaction [68].

Convergent genetic and biochemical studies have recently linked *PDE4B* with the major debilitating disease, schizophrenia [34,35]. These studies include linkage analyses and identification of polymorphisms, along with the observation that isoforms from all PDE4 families can be directly sequestered [69] to the scaffold protein, DISC1, whose gene is strongly implicated in the etiology and pathophysiology of schizophrenia. The functional role of such sequestered PDE4 is unknown, although PKA action can selectively and dynamically release PDE4 isoforms from various DISC1 isoforms.

Concluding remarks

Compartmentalised cAMP signalling has come of age, with the appreciation that a diverse array of PDEs provides a set of components that can be differentially sequestered by specific signalling complexes. Sequestered PDEs sculpt localized cAMP gradients, allow input from other signalling pathways and contribute to setting the 'gate' for activation of associated effector systems, as well as protecting them from inappropriate activation by fluctuations in basal cAMP levels. Thus, changing the amount and activation status of PDEs associated with signalling scaffolds provide important regulatory mechanisms.

The growing number of identified scaffolds that sequester PDE4 isoforms is a clear indication of the importance of targeting of these enzymes. Notwithstanding this, we hardly know anything about the precise function of the 25 + identified PDE4 isoforms or whether the entire cohort of PDE4 isoforms is known. Certainly, from the purported links of *PDE4B1* with schizophrenia [35], *PDE4D7* with stroke [70] and *PDE4D4* with prostate cancer [71], there is a particular need to understand phenotypes associated with these isoforms. Another major need is to define the proteome of particular scaffolds that sequester PDE4 isoforms. For example, scaffolds such as β-arrestin [72] and DISC1 [73] can purportedly bind so many proteins that it is impossible that they could bind them simultaneously. We can thus envisage sub-populations of such scaffolds, each having a defined partner cohort. What are these, what function do they have, and what is the proteome of the PDE4-sequestered sub-population? In this regard, PDE4D5 specifically binds a non-ubiquitylated β-arrestin sub-population with whom its interaction is actually enhanced when it becomes ubiquitylated by the sequestered E3 liase, MDM2 [29]. Might then the inappropriate interaction between particular PDE4 isoforms and scaffolds underpin disease states, and could targeting the displacement of specific PDE4 isoforms from particular scaffolds generate more effective PDE4-based therapeutics? These are just some of the critical questions that need to be addressed.

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