REVIEW ARTICLE

PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization

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cAMP is a second messenger that controls many key cellular functions. The only way to inactivate cAMP is to degrade it through the action of cAMP phosphodiesterases (PDEs). PDEs are thus poised to play a key regulatory role. PDE4 cAMP-specific phosphodiesterases appear to have specific functions with selective inhibitors serving as potent anti-inflammatory agents. The recent elucidation of the structure of the PDE4 catalytic unit allows for molecular insight into the mode of catalysis as well as substrate and inhibitor selectivity. The four PDE4 genes encode over 16 isoforms, each of which is characterized by a unique N-terminal region. PDE4 isoforms play a pivotal role in controlling functionally and spatially distinct pools of cAMP by virtue of their unique intracellular targeting. Targeting occurs by association with proteins, such as arrestins, SRC family tyrosyl kinases, A-kinase anchoring proteins ('AKAPs') and receptor for activated C kinase 1 ('RACK1'), and, in the case of isoform PDE4A1, by a specific interaction (TAPAS-1) with phosphatidic acid. PDE4 isoforms are 'designed' to be regulated by extracellular-signal-related protein kinase (ERK), which binds to anchor sites on the PDE4 catalytic domain that it phosphorylates. The upstream conserved region 1 (UCR1) and 2 (UCR2) modules that abut the PDE4 catalytic unit confer regulatory functions by orchestrating the functional outcome of phosphorylation by cAMP-dependent protein kinase ('PKA') and ERK. PDE4 enzymes stand at a crossroads that allows them to integrate various signalling pathways with that of cAMP in spatially distinct compartments.

Key words: arrestin, asthma, chronic obstructive pulmonary disease (COPD), phosphorylation, rolipram, targeting.

INTRODUCTION

cAMP is of pivotal importance in determining many aspects of cellular function [1,2]. It is generated at the cytosol surface of the plasma membrane through the action of adenylate cyclases, of which there is a large family. Increased levels of cAMP are translated into cellular responses through the action of cAMP-dependent protein kinase (PKA) [3,4]. However, in certain cells, cyclic-nucleotide-gated ion channels at the plasma membrane can also mediate cAMP signalling, as can cAMP-activated GTP-exchange factors [cAMP-GEFs or 'EPACs' (exchange proteins regulated by cAMP)], which regulate the small GTPase Rap1 [5,6].

It is now well recognized that cAMP signalling responses are compartmentalized. This originated from work done on cardiac myocytes, but has since been extended to a variety of other cell types and processes [2]. Indeed, recently, fluorescence-resonance-energy-transfer ('FRET')-based approaches have been used to visualize spatial and temporal gradients of cAMP in living cells [7,8]. In live cells, gradients of cAMP have also been inferred from sensing studies done by monitoring ion channels in heart [9] and using cAMP-gated ion channels [10,11].

Compartmentalization allows spatially distinct pools of PKA to be differentially activated. The basis of this is that various PKA isoforms are anchored at specific intracellular sites by proteins called A-kinase anchoring proteins (AKAPs) [3,4]. The cell-type-specific expression pattern of AKAPs, coupled with their distinct pattern of intracellular distribution, is presumed to allow discrete PKA populations to read and respond to gradients of cAMP in cells and modify localized target proteins. In this way compartmentalized effects can be achieved. Such spatial regulation has been elegantly supported using peptides able to disrupt AKAP-anchored PKA and hence ablate regulation of coupled target proteins [3,12].

The basis of such gradients will depend upon the activity and localization of both adenylate cyclases, which generate cAMP, and cAMP phosphodiesterases (PDEs), which degrade it [1]. There is a growing body of evidence that adenylate cyclases and receptors able to stimulate it are not evenly distributed across the cell-surface plasma membrane [2] and that PDEs are localized in distinct intracellular sites in order to tailor cAMP degradation [1,13,14].

Once cAMP is generated, the only way to inactivate it is to degrade it to 5′-AMP, through PDE action [1,15–17]. PDE activity is found not only in the cytosol, but also in a variety of membrane, nuclear and cytoskeletal locations [1,13,14]. Thus the spatial and temporal characteristics of cAMP gradients have been shown to be inherently dependent upon the action of

Abbreviations used: AKAP, A-kinase anchoring protein; β_2-AR, β_2-adrenoceptor; cAMP-GEFs, cAMP-activated GTP-exchange factors; COPD, chronic obstructive pulmonary disease; D440N (etc.) mutation, $Asp^{440} \rightarrow Asn$ (etc.); ERK, extracellular-signal-related protein kinase; FYVE, a domain named after the first letter of the first four proteins in which it was found (Fab1p, YOTB, Vac1p and EEA1); GPCR, G-protein-coupled receptor; GPK, G-protein-receptor kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; LR1 and LR2, linker regions 1 and 2; Lyn, an Src family protein-tyrosine kinase; mAKAP, muscle-selective AKAP; PA, phosphatidic acid; PDB, Protein Data Bank; PDE, phosphodiesterase; PGE_2, prostaglandin E_2; PKA, cAMP-dependent protein kinase; PS, phosphatidylserine; RACK1, receptor for activated C kinase 1; RAID1, RACK1 interaction domain; Rap1, a small GTPase; RASM, rat aortic smooth muscle; SH3, Src-homology 3; UCR, upstream conserved region; WD repeat, tryptophan/aspartate repeat; when referring to the Figures, the one-letter amino acid code is used.

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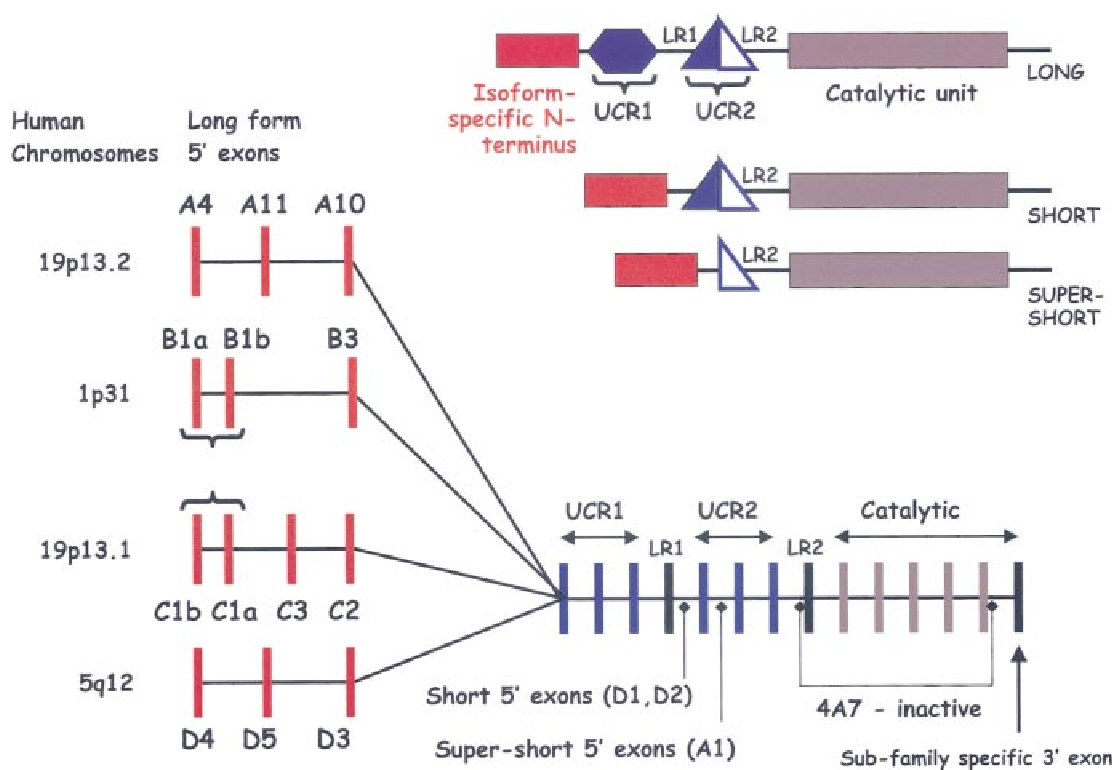

Figure 1 Schematic of the four-gene PDE4 enzyme family

The Figure shows the gene organization of four PDE4 subfamilies and their location on human chromosomes. The common structure of core regions forming the UCR1/2 and catalytic components is indicated. Certain unique N-terminal regions are encoded by two 5′ exons, which are indicated as 'a' and 'b'. Isoform subcategories, generated by alternative mRNA splicing, based upon presence of absence of UCR1/2 regions, are also shown schematically. The range of species interacting with PDE4 isoforms and presumed key sites of interaction is indicated.

anchored PDEs [7–9,11]. Indeed, localized PDE activity underpins compartmentalized cAMP signalling by shaping the asymmetric gradients of cAMP in cells that are subsequently 'read' by AKAP-bound PKA molecules.

There are 11 different PDE families, eight of which generate over 30 different isoforms able to hydrolyse cAMP [1,15–17]. Such conservation implies that this diversity has functional significance. One key facet of this undoubtedly relates to intracellular targeting, where specific PDE isoforms regulate particular sets of intracellular processes. Another is the ability of particular isoforms to be regulated by other signalling pathways. Thus PDEs play a pivotal role by controlling the spatial integrity of cAMP signalling and by integrating cellular information-transfer processes between various other intracellular signalling systems and that of cAMP and cGMP.

PDE4 cAMP phosphodiesterases exemplify these two important facets of PDE action. They exclusively hydrolyse cAMP and are clearly of physiological importance, since PDE4 selective inhibitors achieving 'chemical knockout' in animals exhibit potent anti-inflammatory actions. Indeed, there is currently much interest in deploying selective PDE4 inhibitors for the treatment of asthma, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis and cancer. Consistent with this, gene knockout studies have shown that PDE4B$^{-/-}$ mice exhibit a profound attenuation in the ability of lipopolysaccharide ('LPS') to generate tumour necrosis factor-α ('TNF-α') [18]. Such an effect was not evident in PDE4D$^{-/-}$ mice, indicating that there is little or no redundancy between PDE4 subfamilies [18]. Indeed, PDE4D$^{-/-}$ mice were uniquely characterized by an absence of a muscarinic cholinergic response in their airways, leading to loss of the airway hyper-reactivity as seen in asthma [19]. These data thus provide a basis underpinning the beneficial effects of PDE4-selective inhibitors in lung inflammatory diseases such as asthma and COPD. A number of excellent reviews cover the pharmacology of PDE4 inhibitors [20–31], an area that is outside the scope of this review. PDE4 inhibitors can also exert anti-depressant actions and allow for cognitive enhancement [32–34].

With hindsight it is apparent that the first PDE4 enzyme to be characterized biochemically as a cAMP-specific, cGMP-insensitive activity was a proteolysed form from liver membranes [35]. However, PDE4 enzymes were first recognized [36] as a distinct family, originally called 'PDE-IV', based upon their unique sensitivity to inhibition by rolipram [37], which now provides the archetypal example of a PDE4 selective inhibitor. The identification of the *dunce* gene in the fruitfly *Drosophila melanogaster* [38], the disruption of which leads to learning and memory defects, provided the first molecular cloning of not only a PDE4 enzyme, but of any PDE. The *dunce* PDE was then used to isolate a rat PDE4 (RD1; PDE4A1) [39]. Subsequent work then elegantly demonstrated the presence of four PDE4 genes in rat [40], which were later shown also in man [41], encoding four subfamilies (PDE4A, PDE4B, PDE4C and PDE4D).

MODULAR STRUCTURE OF PDE4 ISOFORMS

The four *PDE4* genes generate over 16 different isoforms, each of which is characterized by a unique N-terminal region (Figure 1). In most instances this is encoded by a single 5′ exon, although

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two such 5′ exons are involved in encoding that of PDE4C1 [42] and one of the *D. melanogaster* PDE4 isoforms [43]. The generation of these isoforms is under the control of distinct promoters, which, in the case of PDE4D1/2, PDE4A1, PDE4A4, PDE4A10, PDE4A11, PDE4B2 and PDE4D5, have been shown to lie immediately upstream of the 5′ exon that encodes their unique N-terminal region [44–46]. The four PDE4 subfamilies are each encoded by large complex genes of around 50 kb and consisting of 18+ exons. Despite this complexity, there is almost total conservation of exonic sequence between the human and murine *PDE4* genes – the only major differences identified to date being in the extreme 3′ coding region of the *PDE4A* gene. Furthermore, to date, there is no evidence for polymorphisms in coding regions. Such conservation, both in sequence and in the complex range of PDE4 isoforms, suggests that there has been a strong selective pressure during evolution to protect them from mutation and divergence. Thus individual PDE4 isoforms appear set to play key roles.

Functional PDE4 isoforms can be divided (Figure 1) into three major categories: long, short and super-short [14]. The long isoforms are characterized by two modules that are conserved in all 4 PDE4 subfamilies, namely upstream conserved regions 1 and 2 (UCR1 and UCR2). In contrast with long isoforms, the short forms lack UCR1, whilst super-short forms not only lack UCR1, but have a truncated UCR2 (Figure 1). UCR1 is formed from some 60 amino acids, whereas UCR2 is formed from some 80 amino acids. These two modules are joined together by linker region 1 (LR1), the composition of which (about 24 amino acids) differs in the four PDE4 subfamilies. UCR1 is joined to the catalytic unit by LR2, whose composition and size (10–28 amino acids) varies in the four PDE4 subfamilies. The catalytic unit then comprises some 315 amino acids, which form three distinct subdomains. Finally, there is a C-terminal region that is unique to each of the four subfamilies and whose role is, at present, ill-understood.

In addition to these isoforms, which all exhibit cAMP-specific PDE activity, the *PDE4A* gene encodes a curious isoform (PDE4A7; 2el) that lacks PDE activity [47]. This is the only known isoform to show alternative mRNA splicing at both the 5′ and 3′ ends. In the case of PDE4A7 this results in a unique N-terminal region of 32 amino acids attached to a truncated catalytic region lacking both UCR1 and UCR2. PDE4A7 also has a novel C-terminal region of 14 amino acids that is tagged on to the end of a C-terminally truncated catalytic unit (Figure 1). The two exons responsible for forming the unique portions of PDE4A7 have been located at the appropriate points in the *PDE4A* gene and have authentic splice sites. Thus PDE4A7 is a genuine splice variant and appears to be widely expressed, although its functional significance is enigmatic.

ROLE OF THE UCR1 AND UCR2 MODULES

Paired regulatory regions, positioned immediately N-terminal to the catalytic site, appear to be a central characteristic of the majority of known PDE families. Thus, for example, PDE1 has paired Ca^{2+}/calmodulin-binding domains, and PDE families 2, 5 and 6 have cGMP-binding GAF domains that exert regulatory effects on catalytic activity. GAF domains are also found in PDE10 and PDE11, although their functional role is, at present, unknown. The paired UCR1 and UCR2 modules were originally discovered and proposed to characterize PDE4 enzymes by Bolger and collaborators [41]. Recent functional studies [48–50] have now firmly established that UCR1 and UCR2 provide the molecular machinery that confers key regulatory functions on the PDE4 catalytic unit. This gives a key insight into why alternative mRNA splicing generates long, short and super-short species varying in their UCR1/2 complement and thus regulatory properties (Figure 1).

Interaction

Biochemical pull-down studies and two-hybrid analyses have shown that UCR1 interacts with UCR2 [48]. This occurs through the hydrophobic C-terminal portion of UCR1 interacting with the hydrophilic N-terminal region of UCR2. Key to this [48] is the interaction of two arginine residues in UCR1 (Arg^{98} and Arg^{100} in PDE4D3) with a cluster of negatively charged residues in UCR2 (Glu^{146}, Glu^{147} and Asp^{149} in PDE4D3). The UCR1/2 module has been suggested to interact with the catalytic unit via a contact involving UCR2 [51].

Activity regulation

The first indication that UCR1/2 played a regulatory role came from N-terminal truncation studies, where the removal of UCR2, in particular its N-terminal portion, led to an increase in PDE4 catalytic activity. This led to the notion [52] that UCR2 may exert a constitutive inhibitory effect on the activity of the PDE4 catalytic unit, which was first shown for PDE4D [51,52] and, more recently, also for PDE4A5 [53]. Intriguingly, it is this regulatory N-terminal portion of UCR2 that interacts with UCR1 and is also absent in PDE4 super-short isoforms.

PKA

Long PDE4 isoforms from all four subfamilies can be activated through PKA phosphorylation [50,54–57] of a single serine residue [50,56,58–61] found at the extreme N-terminal end of UCR1, within the PKA consensus, RRESF. In mammalian cells, such phosphorylation increases PDE4 activity by around 60 % for a variety of long isoforms [59]. However, a larger activation (2–3-fold) has been noted for PDE4D3 [50,56,58] and PDE4A4 [62], which may reflect either a regulatory role for their N-terminal regions or that they are subject to some additional modification that amplifies the stimulatory effect of PKA phosphorylation.

PKA activation can be mimicked by replacing the target serine residue with either an aspartate or glutamate residue activation [58]. Curiously, the target serine residue is adjacent to a glutamate residue that is conserved in all PDE4 subfamilies and which attenuates phosphorylation by PKA [58]. Mutation of this glutamate residue to a neutral amino acid also mimics activation by PKA phosphorylation [58], giving rise to the suggestion that this residue may be involved in an ion-pair interaction that holds the enzyme in a ‘low’ activity state. Thus activation of PDE4 long isoforms by PKA may be consequent on the disruption of this ion-pair by phosphorylation of the adjacent serine residue [58]. PKA phosphorylation of UCR1 is also believed to disrupt the interaction between UCR1 and UCR2 through a conformational change that is distinct from that which causes activation [48]. The suggested mechanism involves PKA phosphorylation, causing the disruption of an H-bond interaction involving the side-chain hydroxy group of the target serine residue. In the case of PDE4D3 this appears to give rise to a small increase in sensitivity to inhibition by rolipram [58,59].

Intriguingly, an antiserum generated to a peptide whose sequence reflects the C-terminal portion of UCR2 (VSEYISN-TFLDKQHEVEIPSPT: rat PDE4D3) effects activation of PDE4D3 to a level similar to that achieved through PKA phosphorylation [51]. As UCR2 is believed to exert a constitutive

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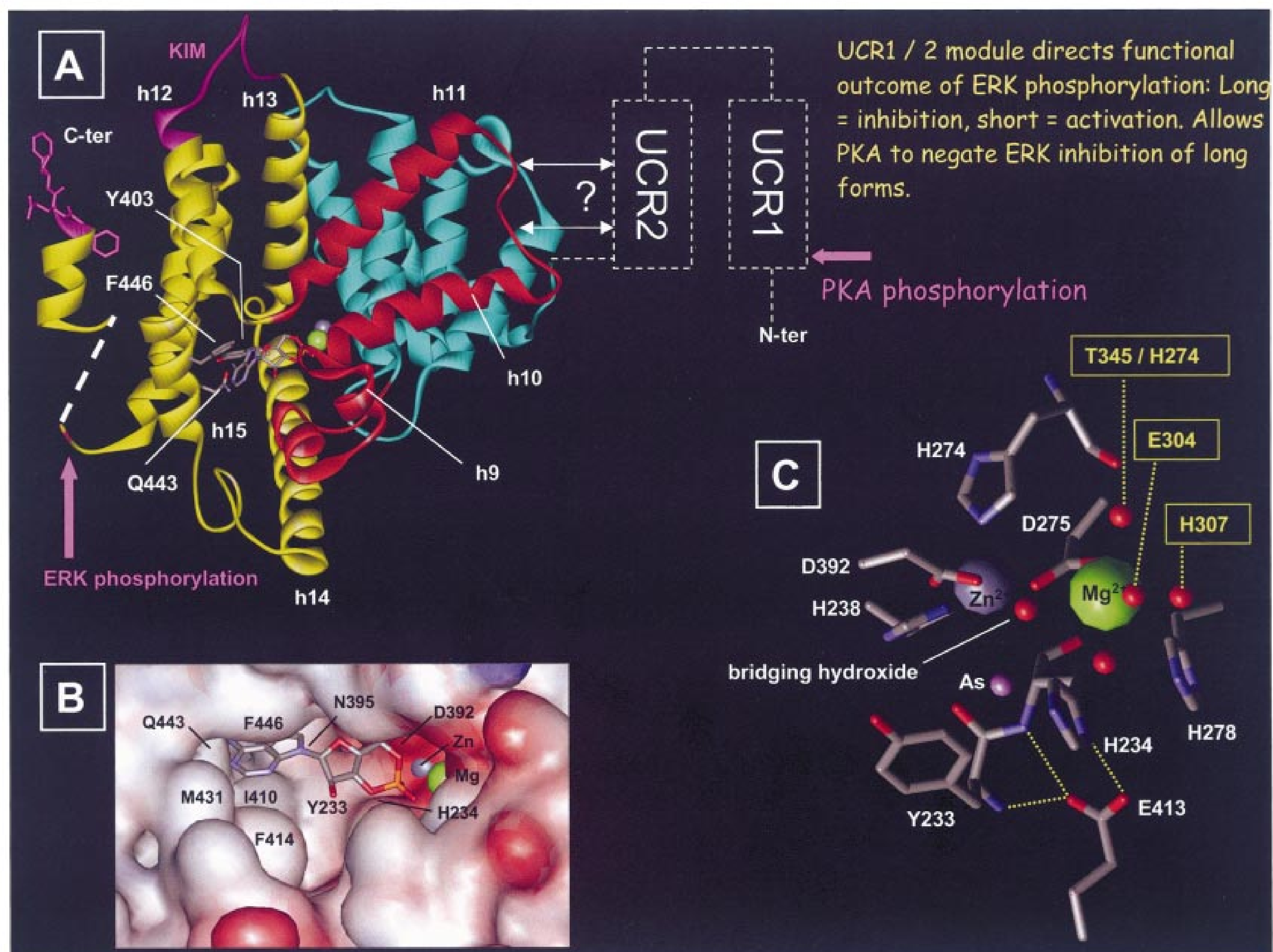

Figure 2 X-ray crystal structure of PDE4B2 core catalytic unit illustrating subdomains, ERK binding and specificity motifs, metal-ion environment and probable substrate-binding mode

(**A**) PDE4 enzymes are designed to be activated and regulated by ERK. The three-dimensional structure (1FOJ) of the PDE4B2 catalytic unit is shown with subdomains highlighted (blue, red and yellow). The docking (KIM), specificity (FQF) and target phosphorylation (S487) sites for ERK are shown in magenta. Folding of the C-terminal sequence containing the FQF motif is not defined, owing to disorder in the crystal structure. The schematic indicates the role of UCR1/2 modules in orchestrating the functional outcome of phosphorylation of the catalytic unit by ERK. It also highlights the role of the PKA phosphorylation site in UCR1 in negating the functional effect of inhibitory ERK phosphorylation of long isoforms and the importance of this in feedback regulation and reprogramming. (**B**) Detail of proposed binding mode for cAMP substrate. (**C**) Detail of metal-ion environment, including solvent ligands.

inhibitory function, activation by this antiserum and by PKA may be mediated by disrupting an interaction between UCR2 and the catalytic unit.

PKA phosphorylation also enhances the sensitivity of PDE4D3 and PDE4A4 to stimulation by Mg^{2+} [50,51,62,63], a bivalent cation that is essential for PDE activity and which is found in the catalytic site [64]. Thus phosphorylation by PKA may effect a conformational change in the UCR1/2 module that underpins a functional change in the catalytic site, as exemplified by this altered activity and sensitivity to Mg^{2+}.

PKA phosphorylation of PDE4 isoforms thus leads to activation, altered Mg^{2+}-sensitivity, altered rolipram-sensitivity and an ability to re-programme the functional output of ERK phosphorylation of PDE4 enzymes (see below). Intracellular targeting of specific PDE4 isoforms, coupled to PKA phosphorylation, also confers the control of distinct populations of PKA by interaction with either arrestins [65] or AKAPs [66,67]. PKA phosphorylation of PDE4s [63], as well as PDE3 [16,68,69], can thus provide part of the cellular desensitization mechanism to cAMP signalling.

ERK

The ERK MAP kinase signalling pathway provides a pivotal route whereby various growth factors and hormones exert key effects on cell growth and survival. The third subdomain of the catalytic unit of all PDE4 subfamilies, but not that of PDE4A, contains a single, ERK consensus motif (Pro-Xaa-Ser-Pro). The serine residue in this site can be phosphorylated, both *in vitro* and *in vivo*, by ERK [49,70,71]. Studies on transcription factors phosphorylated by either ERK or c-Jun N-terminal kinase (JNK) has identified authentic *in vivo* substrates as having kinase docking sites [72]. A common docking site ('KIM') is utilized by both ERK and JNK and takes the form ${}^{L}/_{V}XX^{R}/_{K}{}^{R}/_{K}X_{3\text{–}6}LX^{L}/_{S}$.

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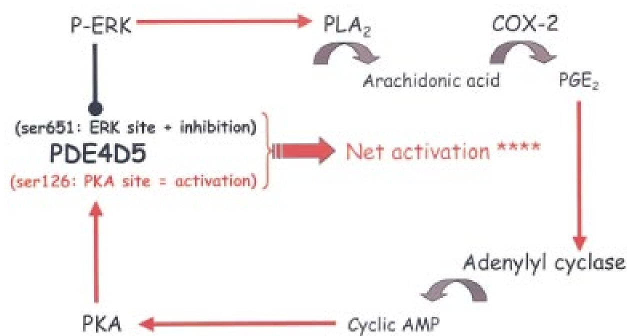

Figure 3 Schematic showing how the activation of ERK in smooth-muscle cells leads to multi-site phosphorylation of the PDE4D5 long isoform

Whilst ERK activation causes a direct inhibitory phosphorylation action of PDE4D5, it also activates an autocrine pathway that allows PKA to rapidly phosphorylate PDE4D5. This autocrine effect on PKA reprogrammes ERK from causing net inhibition to net activation of PDE4D5.

This is located some 120–150 residues N-terminal to the target serine. Specificity for ERK, rather than JNK, is achieved by the FXF motif, located some 5–30 residues C-terminal to the target serine residue [72]. Subdomain 3 in the PDE4 catalytic domain contains functional KIM and FQF sites [49] (Figure 2), with the KIM motif being well presented on an exposed β-hairpin loop and the FQF motif on an exposed α-helix. These allow ERK to dock on to PDE4 to elicit its efficient phosphorylation in intact cells [49].

ERK phosphorylation of PDE4 long isoforms leads to inhibition under conditions where the serine target is the sole residue phosphorylated [49,70,71]. Consistent with this, mutation of the ERK target serine residue to aspartate yields an enzyme with an activity decreased similarly to that of the ERK-phosphorylated wild-type enzyme ($\approx 60+\%$ inhibition). In intact cells, ERK-mediated inhibitory phosphorylation of PDE4 long isoforms can cause an increase in (localized) cAMP levels, allowing activation of PKA. This can elicit the phosphorylation of the target serine residue in the UCR1 of PDE4 long isoforms, leading to the ablation of the inhibitory effect of ERK phosphorylation on long PDE4 isoforms. This provides a novel feedback regulatory system [71,73]. Thus a key feature of ERK-mediated inhibition of PDE4 is its transience, as it can be rapidly overturned by the subsequent action of PKA. The kinetics of transience will depend upon parameters that influence the activation of (localized) PKA as well as dephosphorylation by phosphatases. One can then expect that the nature of ERK-mediated inhibition of PDE4 isoforms will depend critically on the cell type, the nature of the prevailing stimuli and temporal window over which analyses are made [73].

This is highlighted by the intriguing reprogramming (Figure 3) of the effect of ERK activation on the activity of the long PDE4D5 isoform in human aortic smooth-muscle cells [55]. In these cells ERK causes the autocrine generation of prostaglandin E_2 (PGE_2) through activation of PKA_2 and the production of arachidonic acid. Thus the rapidly generated PGE_2 stimulates adenylate cyclase, increasing cAMP levels, and so causes PKA to phosphorylate PDE4D5 in an ERK-dependent fashion. The magnitude and rapidity of the activation of PKA militate against any resolution of ERK-mediated inhibition of PDE4D5. However, such an effect was clearly uncovered when either phospholipase A_2 or cyclo-oxygenase ('COX') inhibitors were added to prevent PGE_2 generation [55]. Such a reprogramming is also likely to account for the ERK-mediated activation of the long PDE4D3 isoform seen in rat aortic smooth-muscle (RASM) cells [74] where, indeed, PDE4D3 underwent a mobility shift that uniquely characterizes the phosphorylation of this isoform by PKA [50,56,59]. Whether a similar mechanism underpins the ability of interleukin 3 (IL-3) and IL-4 to increase PDE4 activity in myeloid cells through an ERK-mediated process [75], or because ERK-activated short forms predominate in such cells (see above), remains to be seen. Interestingly, it has been suggested [74] that ERK activation in RASM cells allows a small fraction of PDE4D3 to be released from membrane fractions to the cytosol. This suggests that phosphorylation (by ERK or PKA or both?) may affect the anchoring of at least a subpopulation of PDE4D3, perhaps by modifying the conformation of the UCR1/2 and unique N-terminal regions of this isoform.

It is the paired UCR1/2 modules of long isoforms that programme inhibition consequent upon phosphorylation of the catalytic unit by ERK, with the activity of the 'naked' catalytic unit, free of the UCR1/2 modules, being little affected [49]. This action is ablated by PKA phosphorylation of UCR1, which disrupts the interaction between UCR1 and UCR2. Thus the UCR1/2 module orchestrates and integrates the consequences of phosphorylation. The lack of UCR1 in short forms might have suggested that they would be inhibited in a PKA-independent fashion. Not so, for the lone UCR2 in PDE4 short forms programmes ERK phosphorylation to cause their activation [49,70]. In this way we have the mirror image to cAMP inhibiting ERK activation through the Raf-1 isoform and activating ERK through the B-Raf isoform [73]. Specific positive and negative coupling of cAMP and ERK signalling can thus be achieved on a cell-type-specific basis through the selective expression of different PDE4 and Raf isoforms [73]. Many cells express both sets of PDE4 and Raf isoforms, leading to the notion that distinct patterns of intracellular targeting may create signalling modules that control spatially distinct subpopulations of ERK. The discovery of these regulatory actions of ERK and PKA have, for the first time, given a functional rationale for the generation of long and short splice variants by *PDE4* genes.

Targeting

Recent evidence suggests that UCR2 may also have a role in intracellular targeting. The first evidence for this came from two-hybrid analyses done on PDE4D3, which identified myomegalin as a potential binding partner [76]. Myomegalin is a large scaffold protein (260 kDa) that has several splice variants and is localized to the Golgi/centrosomal region. It has a domain akin to that in dynactin/centractin, which binds activator protein-1 ('AP-1'), a helix–loop–helix domain, an Src-homology 3 (SH3)-like domain and a leucine-zipper region, and has been found in heart, skeletal muscle and COS-7 cells [76]. The insolubility of full-length myomegalin has provided formidable technical difficulties in probing interactions and functional analyses. However, truncation studies have shown that the extreme C-terminal 550 amino acids of myomegalin interact with the N-terminal portion of UCR2 and confer targeting to the perinuclear Golgi/centrosomal region [76]. Nevertheless, it remains to be seen as to whether other PDE4 isoforms can interact with myomegalin.

As myomegalin interacts within a subregion of UCR2 that has an established regulatory function, it will be interesting to see whether myomegalin can alter PDE4 activity and whether PKA phosphorylation can affect myomegalin interaction. Additionally, the interaction of a protein such as myomegalin with UCR2 might also affect the functional response of PDE4 enzymes to ERK phosphorylation, as PKA phosphorylation of UCR1 in

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long isoforms nullifies the inhibitory effect of ERK phosphorylation of long isoforms [73]. Indeed, treatment of RASM cells with PMA and forskolin together, and not alone, led to the release of particulate PDE4D3 [74]. This highly intriguing observation suggests that, at least in these cells, multisite phosphorylation of PDE4D3 may cause re-targeting. Given that PDE4D3 interacts with AKAP450 [67] and myomegalin [76] through its UCR2 region, then it seems possible that multisite phosphorylation may serve to release PDE4 from such anchor sites. Such receptor-mediated dynamic redistribution of PDE4 enzymes offers the exciting possibility of re-shaping spatial and temporal gradients in cells.

Evidence for a role of UCR2 in intracellular targeting has come from truncation and deletion studies done on the PDE4A5 long isoform [53]. Expressed in COS-7 cells, PDE4A5 is found not only in the cytosol, but associated both with ruffles at the cell periphery/cortical region and at a discrete perinuclear localization [53,77]. Targeting to the cell periphery is attributable to two distinct sites located within the unique N-terminal region [53], one of which is an SH3 interaction site that confers interaction with Src family protein-tyrosine kinases such as Lyn [53,78,79]. However, perinuclear localization is attributable to the N-terminal portion of UCR2 [53], although the identity of the anchor protein is unknown. Of course this could be myomegalin, which is expressed in COS-7 cells [76], the cells for which these analyses were done.

If UCR2 has a role in intracellular targeting, then this might mean that super-short isoforms, which lack the N-terminal portion of UCR2, may be the sole subclass where targeting is exclusively due to their isoform-specific N-terminal region. Indeed, this has been shown conclusively for PDE4A1 [80–83].

Activation by negatively charged phospholipids

Phosphatidic acid (PA) is an important signalling lipid [84]. However, little is known about how PA specifically interacts with signalling proteins. No module akin to the pleckstrin homology ('PH'), to the FYVE domains involved in selectively binding 3-phosphorylated phosphoinositides or to the C2 domains that are able to bind certain phospholipid molecules has been recognized [85,86] until recently [80] [the FYVE domain was named after the first letter of the first four proteins in which it was found (**F**ab1p, **YOTB**, **V**ac1p and **E**EA1)].

Various studies have shown that PDE4 enzyme activity could be increased by the addition of PA to assays [87–91]. PA causes a marked (0.7-3-fold) activation of various long PDE4 isoforms, but fails to affect short and super-short isoforms [87,91]. Similar activation was seen with the acidic phospholipid phosphatidylserine (PS), but not with neutral phospholipids. Thus the stimulatory action of PA and PS is likely to be charge mediated, like the effects of PA and PS on Raf-1 [92]. UCR1, found exclusively in long forms, is an amphipathic (helical?) structure with an N-terminal polar half carrying a net positive charge and a strikingly apolar C-terminal half, making it ideally suited to interact with PA/PS. Indeed, it is through UCR1 that PKA causes activation of long forms [93], implying a similar mechanism for activation. This is certainly consistent with observations that the stimulatory effects of PKA phosphorylation of PDE4D3 and PA interaction are mutually exclusive and that each causes identical levels of activation and increased sensitivity to activation by Mg^{2+} [91]. Interestingly, antisera [87] raised to a peptide whose sequence (IHDVDHPGVSNQFLINTNSE) reflected the interhelix-7/8 and helix-8 region of the PDE4 catalytic unit ablated activation by PA. This might indicate that the C-terminal portion of UCR2 in the UCR1/2 module interacts with the interhelix-7/8 and helix-8 region of the catalytic unit. The interhelix-7/8 region contains several of the key conserved metal-binding residues, whereas the short helix 8 is well exposed. Thus antisera to the interhelix-7/8 and helix-8 region might negate activation [87] by mimicking the effect of the binding of the C-terminal portion of UCR2. Binding of antibody to an exposed helix 8 might suppress activation by affecting the metal centres in the catalytic site. Thus antisera to the C-terminal portion of UCR2 might activate PDE4 [51] by exposing the interhelix-7/8 and helix-8 region. It would thus be intriguing to evaluate whether antisera to the interhelix-7/8 and helix-8 region ablated activation of PDE4 long isoforms by PKA, and to determine if PA, like PKA phosphorylation of UCR1, leads to ablation of inhibitory ERK phosphorylation of long isoforms [73].

Thus it seems that the appropriate placing of a negative charge in this locus, either by phosphorylation of the serine target, in the RRESF motif, or by acidic phospholipid binding, engenders a similar conformational change, resulting in PDE4 activation.

SIGNALLING COMPLEXES INVOLVING PDE4S

A seminal feature of PDE4 isoforms is their ability to target to specific intracellular sites. Thereby they may form part of spatially constrained signalling modules that generate and monitor cAMP gradients in cells and integrate actions with other signalling systems. Selective expression of both PDE4 isoforms and their anchor proteins will occur the tailoring of cAMP signalling in a cell-type-specific fashion.

Arrestin

The arrestin gene family consists of four known members; β-arrestin1 and β-arrestin2, plus two visual arrestins [94]. These serve as signalling scaffold proteins that, crucially, play a key role in cellular desensitization mechanisms by uncoupling G-protein-coupled receptors (GPCRs) from their cellular effectors [94]. The paradigm for this is the β_2-adrenoceptor (β-AR), which, upon agonist occupancy, couples to G_s to effect activation of adenylate cyclase and the concomitant generation of cAMP. This process is rapidly desensitized when G-protein-receptor kinases (GRK) phosphorylate the β-AR, allowing recruitment of cytosolic β-arrestin to be to the phosphorylated β-AR and thus its uncoupling from G_s.

Recently it has been shown that β-arrestins form a complex with PDE4 enzymes, thereby providing a means of delivering an enzyme degrading cAMP to the site of active cAMP synthesis in the plasma membrane in an agonist-dependent fashion (Figure 4) [65]. All three categories of PDE4 isoforms from all four subfamilies can interact with β-arrestin1/2, implying that a common region in the PDE4 catalytic unit provides a binding site for β-arrestins [65]. Thus challenge of cells with a β-agonist has been shown to cause recruitment of a PDE4–arrestin complex to the β_2-AR. The functional significance of this was clearly shown when a catalytically inactive PDE4 was overexpressed in cells such that it displaced active PDE4 from interaction with β-arrestin. This inactive 'dominant-negative' form of PDE4 is unable to facilitate localized cAMP degradation, allowing β-agonists to achieve a much higher activation of plasma-membrane PKA than is seen when active PDE4 is delivered there under normal conditions. This event emphasizes the gain of function seen in targeting and thus concentrating an enzyme to a specific intracellular site, in this case the β_2-AR, as only a very small fraction of the total cellular PDE4 is recruited. The interaction between PDE4 isoforms and arrestin, which allows for the agonist-stimulated recruitment of a cAMP-specific

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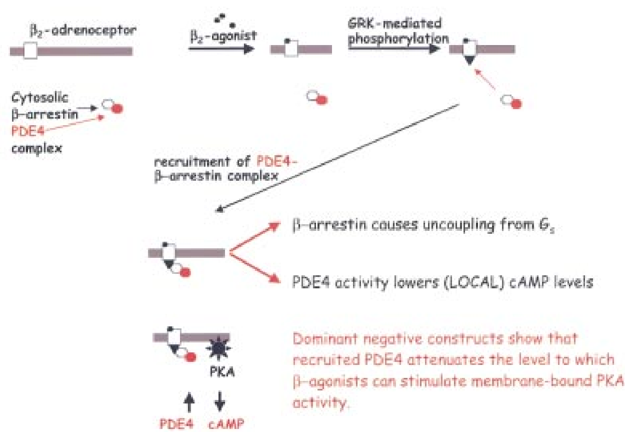

Figure 4 New insights into cellular desensitization mechanisms: agonist-dependent recruitment of a β-arrestin–PDE4 complex to the β_2-adrenoceptor

β-Arrestin can be found in a complex with PDE4 in cells that is recruited to the β_2-adrenoceptor, when it is phosphorylated by GRKs upon agonist occupancy. This allows β-arrestin to uncouple the β_2-adrenoceptor from G-proteins as well as delivering a new pool of PDE4 to the site of cAMP synthesis. Overexpression of catalytically inactive PDE4 has a dominant-negative effect and leads to heightened activation of membrane, but not cytosolic, PKA on β_2-adrenoceptor addition.

PDE to the β-AR, provides a key and hitherto unrecognized component of the cellular desensitization mechanism for GPCRs that stimulate adenylate cyclase.

AKAPs

These are a large family of structurally unrelated proteins that can bind to the dimerization interface of the RII regulatory (cAMP binding) subunit of PKA [3,12,95,96]. High-affinity interaction is achieved by insertion of an amphipathic helix from the AKAP into a hydrophobic pocket formed at the dimerization interface of the PKA R-subunit [97]. AKAPs are associated with targeting to specific intracellular sites and can act as signalling scaffolds for various proteins in addition to PKA-RII. In this way they are able to detect intracellular gradients of cAMP and generate a compartmentalized response on PKA targets that are either associated with them or in their near vicinity.

The ability of a PDE to interact with an AKAP offers the potential for controlling PKA activity in this module. Indeed, the long PDE4D3 isoform can bind both muscle-selective AKAP (mAKAP) [66] and AKAP450 [67]. With mAKAP a site distinct from the PKA R-subunit interaction site binds to the unique N-terminal region of PDE4D3. With AKAP450 interaction is through UCR2, indicating that other long PDE4 isoforms may also bind this AKAP. Indeed, if AKAP450 binds to the regulatory UCR2 module, it may affect PDE activity and regulation by PKA and ERK phosphorylation. Certainly, PKA phosphorylation of PDE4D3 is facilitated by mAKAP interaction, presumably by provision of localized PKA. However, it is also possible that the binding of mAKAP causes a conformational change in PDE4D3 that facilitates its phosphorylation by PKA.

A key feature of this interaction is that the association of PDE4D3 with mAKAP–PKA provides a signalling complex with a unique negative feedback control. Thus, as cAMP levels rise and mAKAP-associated PKA is activated, it will phosphorylate, and thus activate, PDE4D3. This action amplifies the ability of PDE4D3 to lower cAMP levels, hence facilitating the de-activation of mAKAP-bound PKA and then the dephosphorylation of PDE4D3. In such a manner the system will reset itself, showing that the PDE4D3–mAKAP–PKA module is a spatially localized self-regulatory system [66].

Intriguingly, as mAKAP is specifically induced in cardiac myocytes under hypertrophic conditions, this causes the dynamic redistribution of PDE4D3 from the cytosol into a perinuclear compartment [66]. Such spatial reprogramming of a major PDE in these cells might contribute to altered cellular functioning seen consequent to heart failure and post-myocardial-infarction processes.

Receptor for activated C kinase 1 (RACK1)

The signalling scaffold protein RACK1 is formed from seven tryptophan/aspartate (WD) repeats that are believed to allow it to form a seven-bladed β-propeller structure akin to that seen in G-protein β-subunits [98]. It was originally shown [99] to serve as a binding protein for protein kinase C; however, a wide variety of proteins, such as Src, integrin β-subunits and γ-aminobutyric acid ('GABA') receptors, can interact with RACK1 [98], including the long PDE4D5 isoform [100,101]. PDE4D5 binds to RACK1 through a helical domain, termed RAID1 (RACK1 interaction domain) located within its unique N-terminal region [101]. This is formed from a repeat of hydrophobic amino acids, which provide a ridge along one face of a helix that has an essential NPW cluster at its head. RAID1 interacts with a site on RACK1 that is formed primarily from WD repeats 5, 6, 7 [102] and which has been proposed [98,101] to form a bifurcated trough that can accommodate RAID1. In this way interaction is similar to that seen for the interaction between the Gβ- and Gα-subunits. However, as yet the functional consequences of PDE4D5 and RACK1 interaction are not known. Binding does not markedly affect PDE4D5 activity, although it does cause a small change in sensitivity to inhibition by rolipram. The most probable role is that recruited PDE4D5 controls cAMP levels and in the vicinity of the complex and thus regulates the susceptibility of RACK1-associated proteins to phosphorylation by PKA.

Src, Lyn and Fyn

These protein-tyrosine kinases play pivotal roles in controlling a wide variety of cellular processes. Their SH3 domains bind proteins with proline-containing motifs that have PXXP at their core [103]. Such motifs are found in the N-terminal regions of PDE4A4/5 and PDE4D4, allowing them to interact with these protein-tyrosine kinases (Figure 1) [53,78,79,104]. There is clear specificity for particular SH3 domains, and differences between the specificity shown by PDE4A5 and PDE4D4, which are, undoubtedly, attributable to the distinct N-terminal regions of these isoforms.

One role of this interaction is in intracellular targeting. Thus membrane-associated PDE4A5 is found at the cell periphery, where it is localized to ruffles, and also at a discrete perinuclear localization [53]. Deletion or disruption of the SH3-domain-interacting site on PDE4A5 prevents PDE4A5 being constrained within ruffles at the cell margin, thus allowing it to distribute uniformly through the cell margin. Functional insight into this targeting has come from studies [77] done on apoptotic cells where PDE4A5 undergoes a similar loss of fidelity of targeting to ruffles. For, in apoptosis, the caspase-3 cleavage of PDE4A5 at Asp^{72} removes the SH3-domain targeting site [77], whilst leaving the two other membrane anchoring sites intact [53]. Considering that this occurs through caspase 3, an 'executioner' of apoptosis, it might be that this action facilitates apoptosis, perhaps by removing PDE4A5 from a functionally relevant site in the cell.

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Indeed, overexpressing PDE4A5 in these cells attenuated apoptosis, whilst overexpression of a differently targeted PDE4 of the same subfamily, did not [77]. Thus localization of PDE4A5 at the correct site within the cell margin may promote cell survival by controlling compartmentalized cAMP levels. Indeed, PDE4A5 is activated through the phosphoinositide 3-kinase cell-survival pathway, although the mechanism that underpins this has yet to be identified [105].

PDE4A4, the human homologue of PDE4A5, contains an additional site for interaction with SH3 domains [78]. This is located to LR2, the region that joins UCR2 to the catalytic unit. LR2 is encoded by a single exon (exon-9) in *PDE4A*, which is hypervariable amongst species and, in humans, encodes an insertion of a ten-amino-acid proline-and-arginine-rich sequence [106]. The binding of Lyn and Src to this site has a unique action in that it markedly enhances the sensitivity of PDE4A4 to inhibition by rolipram [78]. This action is discriminatory, being observed using the (*R*)-(−)- but not (*S*)-(+)-rolipram enantiomers, and not by certain other inhibitors such as Ariflo®. (SB-207499, SmithKline Beecham). It also leads to a subtle change in the kinetics of rolipram inhibition from simple to partial competitive, perhaps signifying an alteration in the positioning of rolipram in the large trench that forms the catalytic site, perhaps from a distal localization to one adjacent to the bound bivalent-metal ion.

It has been suggested [23,107] that a PDE4 isoform showing such a 'high affinity' for rolipram inhibition in the central nervous system may provide the target for emetic/nausea side-effects seen with certain PDE4 inhibitors such as rolipram.

PA

The super-short form PDE4A1 is entirely membrane-associated [80,81,108] and serves as the paradigm for the notion that the N-terminal regions of PDE4 isoforms could confer intracellular targeting [82]. Indeed, if the N-terminal portion of UCR2 can also play a role in intracellular targeting [53,67,76], then it is only the super-short isoforms whose targeting will be directed exclusively by their isoform-specific N-terminal regions.

The PDE4A1 N-terminal region consists of two helices separated by a mobile hinge region where primary membrane association is directed by helix-2 [83]. Uniquely to date, this takes the form of insertion into lipid bilayers rather than binding a protein anchor [80]. The primary membrane-association module is formed by a pair of adjacent tryptophan residues (Trp^{19}-Trp^{20}), the efficiency of which is enhanced by the Leu^{16}-Val^{17} pairing, allowing bilayer insertion within 5–10 ms. This process is absolutely dependent on the presence of micromolar concentrations of free Ca^{2+}. Asp^{21}, located immediately adjacent to the Trp^{19}-Trp^{20} pairing, provides the Ca^{2+}-operated molecular switch that gates insertion. Presumably Ca^{2+} interacts with Asp^{21} and serves to orientate the helix in order to organize polar residues at one surface and facilitate the presentation of a hydrophobic surface for bilayer insertion at the opposing side.

This module shows a profound selectively for interaction with PA rather than any other negatively charged species. Such selectivity could be due to spatial constraints. However, it has been suggested [80] that a charge neutralization domain may provide the PA selectivity module on one surface of helix-2. This would be formed by the conjunction of $Asp^{21}(-1)$, $Ca^{2+}(+2)$, PA(-2) and Lys($+1$), where specificity for PA is because it is the only acidic phospholipid having a suitable, net -2 charge at physiological pH. Certainly, mutation of either Asp^{21} or Lys^{24} to neutral amino acids ablates PA selectivity. Thus helix-2 contains the molecular machinery, called 'TAPAS-1' (tryptophan anchoring PA selective domain 1), which allows Ca^{2+}-gated membrane insertion with selectivity for PA.

Interestingly then, bilayer association of PDE4A1 is a reflection of the 'memory' that it has once been exposed to the elevated levels of Ca^{2+} that characterize activation by various cellular stimuli. Given that PDE4A1 appears to be localized in brain [39,108,109] and that PDE4 inhibitors can serve as cognitive enhancers [32,33], it may be that such an event has a role in imprinting/long-term potentiation/learning processes in brain, where raised Ca^{2+} levels cause membrane insertion of PDE4A1 and thus alter defined spatio-temporal aspects of cAMP signalling.

CATALYTIC UNIT

A deeper understanding of how PDE4 isoforms interact with proteins, are regulated by phosphorylation and of the mechanisms governing the enzyme's catalytic activity, requires detailed structural information at the molecular level. Such information is not yet available for the protein in its entirety. However, a significant advance has been made through the X-ray crystal determination of the structure of an active PDE4B core catalytic unit [64], formed from residues 152–528 of PDE4B2 and whose sequence is highly conserved throughout the PDE4 family. Whilst this crystal structure [Brookhaven Protein Data Bank (PDB) accession number 1FOJ] lacks the UCR1 and UCR2 regulatory regions it, nevertheless, provides key insights into the structure of the enzyme's catalytic site.

The substrate-binding site

The 1FOJ crystal structure unveils the PDE4 catalytic unit as a compact structure of 17 α-helices folded into three subdomains (Figure 2A). Two key metal-ion-binding sites are found at the junction of the three subdomains with a deep substrate-binding cleft extending from these sites through subdomain-3. The deeper set of the two metal ions (Me1) is tightly bound by four direct ligand interactions involving H238 (His^{238}), H274 (His^{274}), D275 (Asp^{275}) and D392 (Asp^{392}) (Figure 2B). A solvent molecule completes the fifth position of a trigonal bipyramidal co-ordination shell for this ion and bridges to the second metal ion (Me2). Me2 is bound close to the Me1 centre, but is less deeply situated in the substrate-binding pocket and less tightly engaged by the protein. Indeed, Asp^{275}, which acts as a bridging ligand between the two metal centres, provides the only direct protein–ligand interaction with Me2. The remaining five sites of a distorted octahedral co-ordination shell for Me2 are filled by solvent molecules, which form a hydrogen-bonded network to adjacent protein residues [H274 (His^{274}), E304 (Glu^{304}), H307 (His^{307}), T345 (Thr^{345}) and D392 (Asp^{392})]. The solvent molecule shared between Me2 and Me1 is most appropriately formulated as a bridging hydroxide ligand [110]. This ligand may play the key role of attacking nucleophile in the cyclophosphodiester hydrolysis mechanism. A Zn^{2+} ion provides the Me1 centre on the basis of biochemical evidence and the high affinity that the enzyme exhibits for this metal [111,112]. The Me2 ion is less tightly held than Me1, and occupancy was variable in crystals grown from solutions lacking added metal ions. However, it is highly occupied when Mn^{2+}, Mg^{2+} or Zn^{2+} ions are added [64] and, although a number of metal ions are known to support PDE4 activity [113], the physiologically relevant ion is considered to be Mg^{2+}.

The enzyme's catalytic centre thus comprises a binuclear motif with a 'tightly gripped' Zn^{2+} ion coupled to a 'loosely held' Mg^{2+} ion. Apart from the bridging hydroxide and aspartate ligands,

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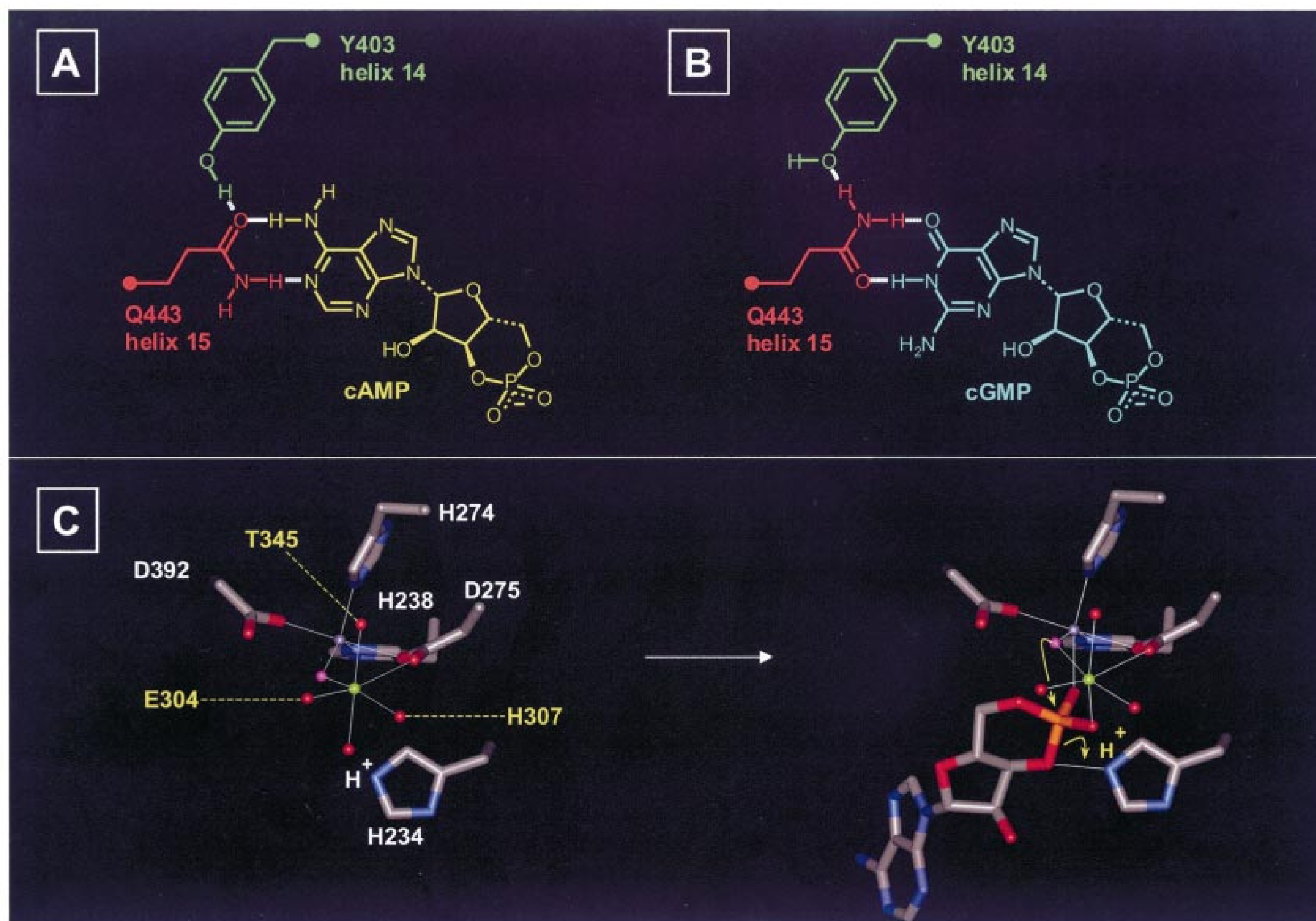

Figure 5 The basis of substrate recognition and catalytic mechanism of PDE4B

(**A**) Proposed hydrogen-bonded interaction between cAMP (yellow), Q443 (red) and Y403 (green) for substrate bound in the PDE4 catalytic site. (**B**) In principle a 180° rotation of the Q443 terminal amide (red) could still accommodate a hydrogen-bonded interaction with Y403 (green) and ought to present an appropriate motif for binding the guanine base of cGMP (blue). (**C**) Schematic illustration of cAMP-hydrolysis mechanism. Co-ordination of the cyclic phosphate may occur with displacement of a solvent ligand from the Mg^{2+} centre and bridging between the two metal ions. The bridging hydroxide ligand (magenta) attacks the phosphorus rearside to the scissile P–O$^{3'}$ bond. H234 is poised to protonate O-3′ as this bond is cleaved.

the dominant connections holding the Mg^{2+} ion in place are hydrogen-bonded interactions from water ligands to E304 (Glu^{304}) and H307 (His^{307}) on helix 10 and to T345 (Thr^{345}) at the junction between helices 11 and 12. In this way the Mg^{2+} centre is held between the N- and C-terminal ends of helices 10 and 11 respectively. These helices, and their connecting loop, fold over the surface of the core catalytic unit to create a 'tweezer-like' motif that grips the Mg^{2+} ion (Figure 2A). Structural changes arising from phosphorylation or protein complexation of the N-terminal regulatory region might thus be relayed into the catalytic centre via helices 10 and 11 to the Mg^{2+}-ion-binding residues. In particular, the exposed loop connecting helices 10 and 11 might, conceivably, be poised to engage an N-terminal regulatory region such as UCR2. Significantly, while regulatory changes mediated via UCR1/2 can alter the enzyme activity (V_{max}), they exert little influence on substrate affinity and specificity [50,58]. Moreover, long and short splice variants exhibit similar substrate K_m values, and N- and/or C-terminal deletion has little effect on this as well [114–118]. On the other hand, metal-ion affinity is known to be sensitive to PKA phosphorylation of UCR1 [50]. Structural changes in UCR1/2 might, then, be relayed via the helix 10/11 motif and modulate enzymic activity by altering sensitivity to Mg^{2+} and/or exerting leverage on the bound ion. Thus PDE4 catalytic efficiency is likely to be sensitive to the precise geometrical arrangement of the bimetal motif and its bridging hydroxide ion.

A number of important questions need to be addressed with regard to PDE4 catalytic function that have a bearing on the development of selective PDE4 inhibitors of therapeutic utility. These include: how does cAMP substrate bind to the protein? what is the basis of cAMP versus cGMP discrimination by the enzyme? and how does the substrate-binding pocket differ from that in other PDE families? The acquisition of a crystal structure for the complexed enzyme with either a hydrolysis-resistant substrate analogue or catalytically crippled protein would help in addressing these issues. In their absence, computer docking studies have been performed to try to rationalize cAMP substrate binding [64,119]. With the cyclic phosphodiester positioned close to the metal centres, the adenine base docks into the largely hydrophobic distal end of the binding pocket (Figure 2). When the substrate is docked in an *anti* conformation, Q443 (Gln^{443}) appears to be well disposed to engage the adenine N-1 and 6-NH_2

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Table 1 Phosphodiesterase activity and K_m values for various catalytic unit mutants of PDE4A4

Adapted from [120] ([a]) and [122] ([b]) with ([c]) $K_{m,cGMP} > 100\ \mu M$ (cGMP activity < 0.05 nmol · min^{-1} · mg^{-1}) and ([d]) $K_{m,cGMP}$ $11.4 \pm 0.2\ \mu M$ (cGMP activity 1.00 ± 0.1 nmol · min^{-1} · mg^{-1}). PDE activities were measured in cell homogenates at 1 μM cAMP and 1 μM cGMP concentrations. Abbreviation: IOWT, increase over wild-type.

Protein	cAMP activity (nmol · min^{-1} · mg^{-1})	K_m (μM)
HSPDE4A4B wild-type[a,c]	4.00 ± 0.40	3.2 ± 0.3
HSPDE4A4B Asp^{440} → Asn[a,d]	1.25 ± 0.20	16.4 ± 1.8
HSPDE4A4B Asp^{440} → Ala[a]	1.80 ± 0.20	6.9 ± 0.4
HSPDE4A4B Pro^{595} → Ile[a]	0.20 ± 0.05	24.4 ± 2.1
HSPDE4A4B Tyr^{602} → Phe[b]	0.10 ± 0.02	9-fold IOWT
HSPDE4A4B Trp^{605} → Phe[a]	1.10 ± 0.20	3.8 ± 0.2
HSPDE4A4B Trp^{605} → Gln[a]	0.10 ± 0.02	–
HSPDE4A4B Trp^{605} → Tyr[a]	< 0.05	–
HSPDE4A4B Trp^{605} → Ile[a]	< 0.05	–
HSPDE4A4B Trp^{605} → Val[a]	< 0.05	–

centres through hydrogen bonds, while the aromatic side chain of F446 (Phe^{446}) is suitably positioned to stack against the purine bicycle. Interestingly Q443 (Gln^{443}) is strictly conserved throughout the PDE superfamily, and F446 (Phe^{446}) conserved in all save PDE11A, which has a tryptophan residue at this position. Other amino acids lining the distal end and sides of the putative substrate-binding pocket are Y233 (Tyr^{233}), M347 (Met^{347}), L393 (Leu^{393}), N395 (Asn^{395}), P396 (Pro^{396}), Y403 (Tyr^{403}), W406 (Trp^{406}), I410 (Ile^{410}) and F414 (Phe^{414}).

The hydrogen-bond acceptor–donor character of the adenine N-1 and 6-NH_2 positions is reversed in guanine. However, in principle, the PDE4 substrate-binding pocket might accommodate cGMP by a 180° rotation of the Q443 (Gln^{443}) terminal amide function. Interestingly, the 1FOJ structure reveals a hydrogen-bonded interaction between the phenolic hydroxyl of Y403 (Tyr^{403}) and the Q443 terminal carbonyl group (Figure 5). This interaction might serve to control the presentation of the Q443 side chain and have bearing on the substrate selectivity of PDE4. However, were hydrogen-bonding between Q443 and Y403 to be reversed, as shown in Figure 4, rotation of the Q443 side chain might still be facilitated to allow recognition of cGMP as substrate.

Is the energy difference between the two complexed rotameric states (Figure 5) sufficient then to explain the cAMP specificity of the PDE4 enzyme family? Mutagenesis studies have been performed in order to try and unravel the determinants of PDE4 substrate specificity. In one study with PDE4A, residues conserved throughout the cAMP-hydrolysing PDEs, but which are variant in the cGMP-specific PDEs, were mutated [120]. Significant increases in K_m for cAMP were observed with D440N (Asp^{440} → Asn) and P595I mutations (Table 1), the cognate residues in PDE4B2 being D241 (Asp^{241}) and P396 (Pro^{396}). P396 (PDE4B) lies on the sequence connecting helices 13 and 14, which provide dominant secondary-structure elements defining the purine-binding end of the catalytic pocket and are orthogonally orientated with respect to one another (Figure 6). Helix 15, which lies antiparallel to helix 13, contributes the key Q443 and F446 residues. The P595I mutation in PDE4A caused a 7-fold increase in substrate K_m and a 95 % reduction in catalytic activity. The prolyl ring of the corresponding residue (P396) in PDE4B2 is packed against Y403, the residue which hydrogen-bonds to the key purine ring-scanning Q443 side chain (Figure 6) and is adjacent to N395, a residue that hydrogen-bonds to Y233

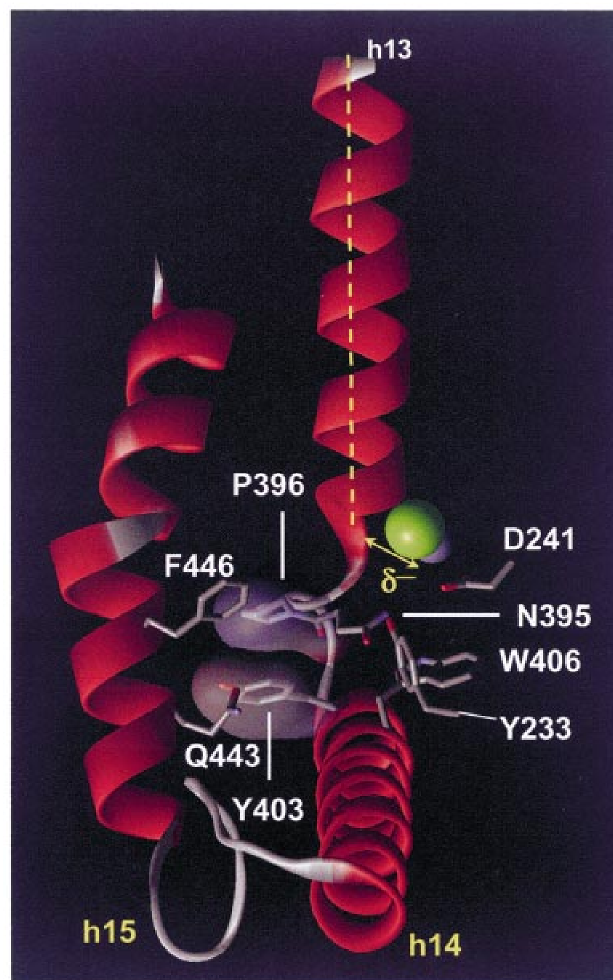

Figure 6 Structural detail of the distal region of the PDE4B2 binding pocket

Some key amino acids that define the distal region of the binding pocket are Y233, D241, N395, Y403, W406, Q443 and F446. D241 may establish a charge-dipole opposition with the axial dipole of helix 13. This may make an important contribution to the structure of the distal binding pocket by positioning the loop connecting helices 13 and 14.

to form part of the surface of the substrate-binding pocket. The location of P396 is then consistent with it exerting a defining influence over the purine-binding end of the catalytic pocket.

The D440N mutation in PDE4A [120] exhibited similar catalytic activities for both cAMP and cGMP hydrolysis, coupled with a 4-fold increase in K_m for cAMP and a greater-than-10-fold decrease in the K_m for cGMP. The cognate mutation (D333N) in PDE4D3 causes a similar effect [121]. Such a mutation, therefore, confers dual substrate specificity upon PDE4. Interestingly, the corresponding residue at this position in cGMP-specific PDE5 is asparagine. So how might an aspartate-to-asparagine mutation at this position cause such a dramatic change in the substrate specificity? The PDE4B crystal structure reveals that the cognate residue, D241, lies behind the surface of the substrate-binding pocket and should not make direct contact with substrate (Figure 6). Here the D241 carboxylate is positioned proximal to the amide NH_2 of N395 in the wall of the substrate-binding pocket. However the distance between the opposing carboxyl oxygen and amide nitrogen in these residues [0.34 nm (3.4 Å)], together

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with their geometrical arrangement, suggests that they may not be optimally positioned for a hydrogen-bonded interaction. The asparagine side chain in the mutant protein is isosteric with the natural aspartate residue in PDE4 and, in principle, could maintain such a hydrogen-bonded link to N395, if one exists with D241. This suggests that the impact of the aspartate-to-asparagine mutation may not be due to its direct influence on the wall of the substrate binding pocket through N395. The D241 side chain is also located proximal to the C-terminal end of helix 13, which contributes one of the Zn^{2+}-binding ligands (D392), in addition to defining the purine-binding end of the catalytic pocket through the loop to helix 14. This raises the possibility that charge-dipole opposition between the D241 carboxylate and axial dipole of helix 13 influences the structure of the PDE4 catalytic pocket. Aspartate-to-asparagine mutation might permit relaxation of helix 13, thus influencing the important connecting loop to helix 14. Subtle structural changes resulting from such relaxation could alter the energy difference between the rotameric complexes of Q443 with cAMP and cGMP (Figure 6) sufficiently to abolish substrate selectivity.

Mutational analysis of residues in the PDE4A catalytic pocket [120] have shown that replacement of W605 (W406 in PDE4B2; Figure 6), by aliphatic amino acids or tyrosine, caused a dramatic reduction (80-fold) in catalytic activity, whilst replacement with phenylalanine resulted in a much less severe reduction (4-fold). Why should there be such a striking difference in catalytic activity of the W406F and W406Y mutants? The side chain of W406 projects from helix 14 behind the hydrogen-bonded N395–Y233 residues and is orientated towards D241. The indolic nitrogen is a potential hydrogen-bond-donor site but, at 0.41 nm (4.1 Å) from the nearest carboxylate oxygen, is spaced too far to make such a contact with D241. The replacement of this tryptophan by tyrosine, however, might introduce such a hydrogen bond between D241 and the phenolic hydroxy group – a group that is lacking in the W406F mutant. The formation of such a hydrogen bond in the W406Y protein may, then, lead to perturbation of the D241 residue and contribute to the activity difference between the mutant W406Y and W406F enzymes. Although most of the W406 side chain is buried behind the surface of the catalytic pocket and cannot make direct contact with the substrate, it is clear from these functional studies [120] that W406 plays an important role. The corresponding residue at this position in PDE5 is isoleucine, and there are a number of other significant differences between PDE4 and PDE5 at the distal end of the substrate-binding pocket. Thus the cognate residues of D241, N395, P396, Y403 and W406 in PDE4 are respectively asparagine, alanine, isoleucine, glutamine and isoleucine in PDE5. It is likely that several differences between the enzymes in this region of the protein together underpin the substrate selectivity of PDE4 and PDE5.

Q443 is strictly conserved across all the PDE families and the only variation to F446 is seen in PDE11A, where the cognate tryptophan residue should also be capable of π-stacking with the substrate's purine ring. These residues are likely to be important for substrate binding in all PDE isoforms. Thus the conserved distal glutamine may fulfil a purine-ring-scanning function throughout the PDE superfamily. If this is the case, then factors controlling its presentation and interaction with substrate must differ between isoforms. Most notably Y403, which may control presentation of the Q443 side chain in PDE4B2, is not conserved outside of the PDE4 family and, indeed, a puzzling diversity is seen in the range of residues at this position across different PDE families. Thus the corresponding residue is histidine in both PDE1 and PDE3, threonine in both PDE2 and PDE10, glutamine in both PDE5 and PDE6, serine in both PDE 7 and PDE11, cysteine in PDE8 and alanine in PDE9. While several of these residues retain hydrogen-bonding capability, their potential hydrogen-bond-donor–acceptor sites are necessarily positioned at widely differing distances from the main chain. Hydrogen-bonded interaction with the conserved distal glutamine residue is unlikely to be retained in all of these cases, particularly with the shorter side chains. Thus removal of the rotational constraint on the glutamine side chain might contribute to the dual cAMP/cGMP-hydrolysing capacity of some PDE isoforms. However, in the cGMP-specific PDE9 isoform, where rotational constraint on the glutamine side chain might have been anticipated, the position corresponding to Y403 of PDE4B2 is occupied by alanine, which clearly cannot exert hydrogen-bonded control over the presentation of the glutamine side chain. The hydrogen bonding between Y403 and Q443 side chains in the PDE4B structure suggests that tyrosine-to-phenylalanine mutation at position 403 might remove the rotational constraint on the Q443 amide, and thus the substrate selectivity of such a mutant would be interesting to assess. The Y602F mutation has, however, been shown [122] to interfere with substrate binding in PDE4A4, where it causes a 9-fold increase in K_m for cAMP. However, no assessment was made to evaluate whether this mutation enhances cGMP binding or confers cGMP-hydrolysing capacity.

Although there are some differences between the PDE isoforms at the distal end of the proposed catalytic pocket, the residues that ligate the metal ions are absolutely conserved across all the isoforms. The details of how the substrate interacts with these centres and the hydrolytic mechanism are not fully clear, but it is likely that a solvent molecule co-ordinated to one or both of the metal centres serves as the attacking nucleophile. The bridging hydroxide ligand (Figure 5C) may well fulfil this role, because it is co-ordinated on the accessible face of the binuclear motif from which the phosphate must approach the metal centres. It is likely that co-ordination of one or both of the unsubstituted phosphate oxygen atoms serves to position the substrate and stabilize the transition state. Indeed, of the five solvent ligands in the Mg^{2+} co-ordination shell, one in particular, appears to be weakly networked into the protein structure. This ligand, which lies on the side of the ion proximal to H234 and H278 (Figures 2C and 5C), is most exposed to approach from the substrate-binding pocket and appears to be vulnerable to displacement by one of the phosphate oxygen atoms. The second unsubstituted phosphate oxygen atom may bridge to the Zn^{2+} ion. To do so, however, it would require some reorganization of the ion's co-ordination shell, from 5-co-ordinate to 6-co-ordinate. It is unclear whether the four protein ligands to the Zn^{2+} ion could accommodate such a movement, but, with or without this additional ligand interaction, the bridging hydroxide ligand between the two metal centres appears to be well disposed for attack on the phosphorus centre rearside to the cyclophosphodiester's scissile P–O bond. An important feature of the docked substrate model [64] is that the substrate's 3′-O is proximal to the N^{ϵ} centre of H234 and thus ideally situated for protonation during attack of the nucleophile and cleavage of the $O^{3'}$–P bond. In the 1FOJ crystal structure the H234 side chain is organized by packing interactions with the side chains of H278 and Y233, as well as by a hydrogen bond from its N^{δ} centre to the carboxylate of E413. The latter residue completes a hydrogen-bonded network to the backbone amide groups of H234 and Y233. These four amino acids (Y233, H234, H278 and E413) are strictly conserved through all PDE isoforms with the sole exception of PDE9A, where the residue corresponding to Y233 is phenylalanine. The mechanism, as summarized in Figure 5(C), is formulated on the assumption that the substrate's phosphate bridges between the two metal centres.

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Inhibitors

It is now generally accepted that the catalytic unit of PDE4 isoforms can adopt at least two distinct conformational states that can be identified by their very different affinities for interaction with rolipram. These are the so-called 'low-affinity rolipram-binding state' ('LARBS'), which exhibits IC_{50} values in the 0.1–1.0 μM region, and the so-called 'high-affinity rolipram-binding state' ('HARBS'), which exhibits IC_{50} values in the 1–50 nM region. Although initially thought to represent two distinct binding sites, there is now a wealth of experimental data showing that high- and low-affinity rolipram binding involves a single site in two conformationally distinct forms of a PDE4 enzyme and that modifications, such as interaction with other proteins and phosphorylation, can achieve switching between these states.

The molecular mechanisms that underpin such conformational switching remain to be elucidated and, indeed, it is quite possible that a number of routes are able to serve as triggers. However, involvement of the bivalent-metal-ion centres has been implicated [123,124] from studies showing that Mg^{2+}-deficient and Mg^{2+}-replete PDE4 states had very different affinities for rolipram. Of course a Mg^{2+}-deficient enzyme will be inactive and thus will not provide a source of active PDE4 with low affinity for rolipram that is commonly seen. Furthermore, at physiological Mg^{2+} concentrations, PDE4 isoforms are likely to be saturated with this ion. Nevertheless, such studies suggest that alterations in the Mg^{2+}-chelating residues and the helices that contain them, may well provide a mechanism through which alterations in conformation change rolipram binding affinity. It is important then to try to appreciate the modes through which rolipram interacts with PDE4. Ideally structures are needed which represent examples of high- and low-affinity interactions. A start to this has come from studies on a low-affinity catalytic-site crystal structure of the PDE4D core catalytic domain complexed with rolipram [64]. In this structure, the rolipram catechol, a potential metal-co-ordinating motif, is not orientated towards the metal centres, but, rather, engages the distal purine-scanning glutamine residue (Q443 in PDE4B). Both the Me1 and Me2 sites in this complex were shown with Zn^{2+} occupancy, which might conceivably influence the mode of rolipram binding. In addition to this, very recently the structure of the PDE4D catalytic unit complexed with a structurally different catechol ether inhibitor, namely zardaverine, has been described [125]. Reassuringly, zardaverine binds in the same manner as rolipram, to a PDE4 formulated with Zn^{2+} and Mg^{2+} in the Me1 and Me2 sites, respectively. Thus the finding that two structurally different catechol ethers adopt the same bound orientation suggests that a well defined catechol-binding mode exists across many of the PDE4 inhibitors, which might extend to 7-methoxybenzofuran, 7-methoxybenzimidazole and 8-methoxyquinoline inhibitors (Figure 7A). We show here then docked models of PDE4B with (*R*)-rolipram and zardaverine (Figure 7) that illustrate the catechol ether binding mode disclosed by the PDE4D inhibitor-complexed crystal structures. In both cases the structure of the unliganded PDE4B protein (Brookhaven PDB 1FOJ) is strongly preserved, with only minor side-chain rotations. Note that the structures of the inhibitor-complexed enzyme show a very different placement of rolipram from published work of simple theoretical docking studies [119] on the PDE4B structure that were carried out without inclusion of the enzyme's metal centres.

An important feature of rolipram binding to PDE4 (Figure 7) is the formation of hydrogen bonds to the terminal side-chain amide NH_2 of Q443, which is straddled by the catechol ether. Thus the methoxy methyl group of rolipram fits into a tight corner of the binding pocket as defined by N395, L402, Y403, W406 and T407 (PDE4B2). The limited space in this region of the binding pocket probably accounts for the requirement for a small alkoxy group at this position, which is seen throughout the catechol ether PDE4 inhibitors. The cyclopentyl group fills a larger basin-shaped subsite near to the opening of the binding-site cleft. This subsite is defined by the side chains of M411, F414, M431, S442 and Q443. Stacking of the F446 side chain with the rolipram aromatic ring is another strong feature of the catechol binding mode. The rolipram lactam ring projects towards both Me1 and Me2, but, apparently, makes no direct interaction with these centres or their proximal residues in the complexed PDE4D crystal structure. In the unliganded PDE4B2 structure an arsenate ion, introduced from the crystallization buffer solution, is located in the substrate-binding pocket close to the enzyme's bimetal motif (Figure 2C). It is likely that the arsenate approximates to the position of the phosphate group of the cAMP substrate, although the geometrical organization of the metal ligands in the 1FOJ structure suggests that the arsenate does not form part of the inner co-ordination shell of either metal. In the absence of the arsenate ion, modelling suggests that the (*R*)-rolipram lactam carbonyl group might be able to engage one of the water ligands attached to the Mg^{2+} ion with a hydrogen bond. Such an interaction would be dependent on the inhibitor's stereochemistry, would be sensitive to occupancy of the Me2 site and might also, potentially, be sensitive to movement of the Mg^{2+} centre induced by regulatory complexation of the enzyme with other proteins. Thus structural changes induced by protein complexation of PDE4 could be relayed into the Mg^{2+} centre to cause movement of Mg^{2+} and its associated solvent ligands, which might either establish or abolish an interaction with the rolipram lactam carbonyl group. The formation or abolition of such an interaction would provide an attractive molecular explanation for the difference between the high- and low-affinity rolipram-binding states of PDE4.

The crystal structure of PDE4D complexed with zardaverine reveals a similar inhibitor binding mode to rolipram [125]. The compound adopts a co-planar conformation for its two rings, with the catechol ether ring stacking against F469 (equivalent to F446 in PDE4B; Figure 7) and the ether oxygen atoms engaging the purine-scanning glutamine residue with hydrogen bonds. The difluoromethoxy group of zardaverine takes the position of the rolipram methoxy group. The zardaverine methoxy group approximately occupies the position of the cyclopentyloxy substituent of rolipram. The bound position of zardaverine is likely, however, to be influenced by the presence of a dimethylarsenate ion introduced from sodium cacodylate in the crystallization medium. The arsenate is located proximal to the bimetal catalytic centre in a similar position to that seen in the unliganded PDE4B 1FOJ crystal structure (Figure 2C). It may be that, under physiological conditions, the arsenate site is occupied by phosphate, which, presumably, would be displaced by substrate binding. The pyridazinone ring nitrogens of zardaverine are located within hydrogen-bonding distance of the arsenate in the crystal structure, raising questions about the phosphate-dependence of inhibitor binding for compounds like zardaverine that have potential hydrogen-bonding motifs at similar positions. On the other hand, if the arsenate site is unoccupied when zardaverine binds to the protein under physiological conditions, then its bound position is likely to differ somewhat from that disclosed in the crystal structure.

The alteration in binding of certain inhibitors induced by protein complexation with PDE4 also raises the interesting possibility of a reciprocal regulation of protein complexation caused by ligand binding in the catalytic pocket. Thus, con-

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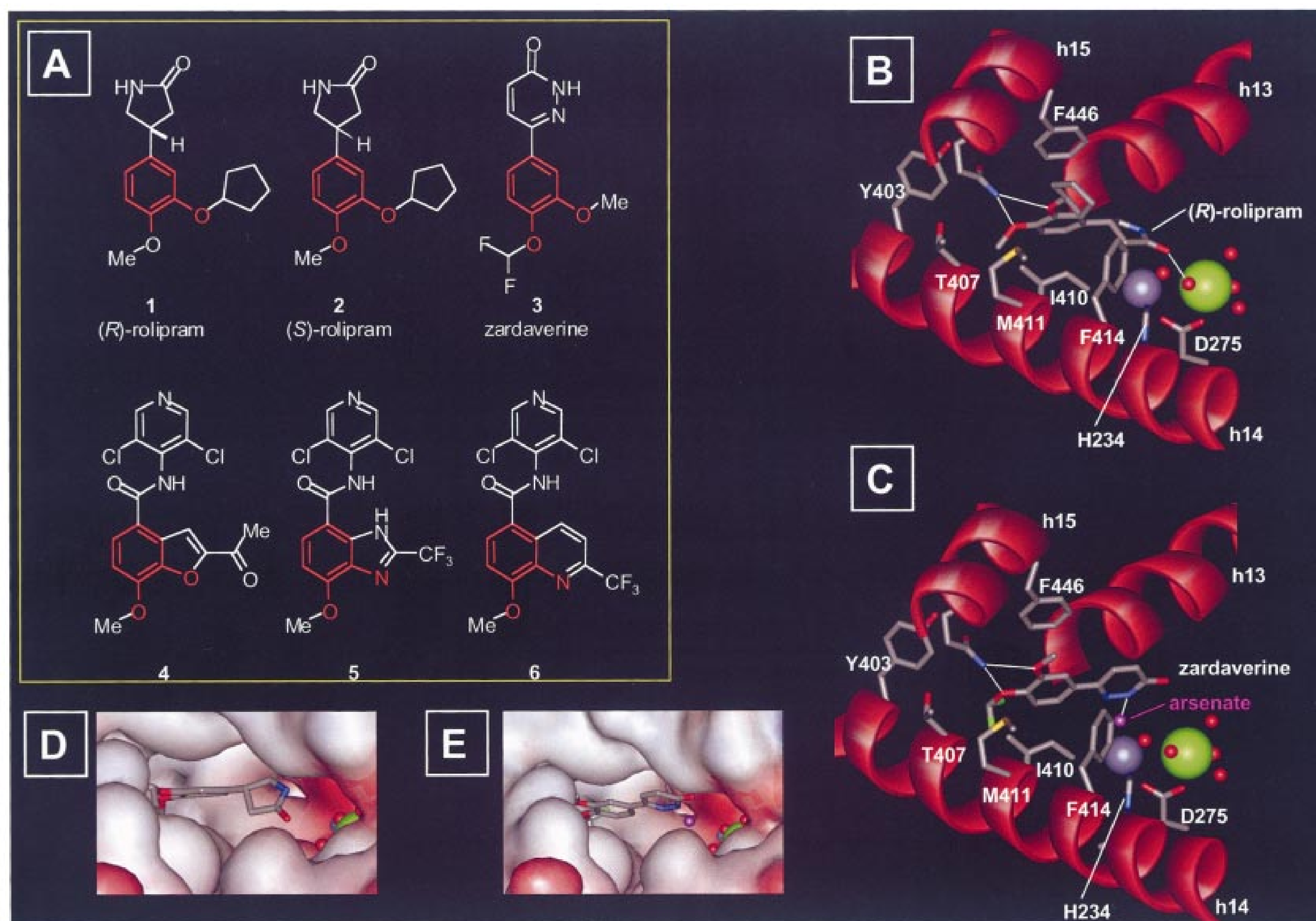

Figure 7 PDE4 inhibitors and binding modes for rolipram and zardaverine recently disclosed from X-ray crystal structures of complexes with PDE4D

(**A**) The catechol ether motif is a prominent structural feature of many PDE4 inhibitors. The binding mode disclosed for rolipram and zardaverine may extend to inhibitors with surrogate catechol ether units such as structures **4**–**6**. (**B** and **D**) Superimposition of (*R*)-rolipram (**1**) into the 1FOJ PDE4B crystal structure – illustrating the binding mode for this inhibitor disclosed in its complex with PDE4D. (**C** and **E**) Superimposition of zardaverine (**3**) into the 1FOJ PDE4B crystal structure – illustrating the binding mode for this inhibitor disclosed in its complex with PDE4D.

ceivably, the binding of some ligands in the catalytic pocket might stabilize the protein in an active conformation required for particular protein–protein associations in the N-terminal portion of the molecule. The helix 10-helix 11 'tweezer' motif might possibly serve to relay structural changes from the catalytic pocket and the Mg^{2+} centre to the N-terminal portion of the enzyme. Interestingly, the catalytic-site dimerization surface [125] implies involvement of the helix 10/11 tweezer motif and this might, conceivably, have an important impact on the catalytic activity of the enzyme itself, since it is this motif which grips the Mg^{2+} centre. Thus if the involvement of UCR2 in dimerization (see above) is in fact mediated by interaction at this site, then we have identified a putative structural and functional relay system that couples the regulatory N-terminal regions to the catalytic unit, where we might expect to see changes in activity and in Mg^{2+}-sensitivity ensuing, as we do for PKA-mediated phosphorylation of UCR1 [50]. In addition to this, it has recently been shown (R. Terry, D. R. Adams and M. D. Houslay, unpublished work) that (*R*)-rolipram and certain other PDE4 inhibitors can induce a profound intracellular redistribution of PDE4A4. This redistribution, which is not seen with (*S*)-rolipram, may be mediated by altered protein–protein interactions of the enzyme. Interestingly, a point mutation of one of the key residues in the helix 10/11 tweezer motif, which grips the Mg^{2+} ion, H506N (H307N in PDE4B), abolishes the rolipram-induced redistribution of PDE4A4. Inhibitor binding to the catalytic centre and protein complexation of the enzyme may therefore reciprocally influence each others action in certain instances. This concept is potentially of importance for the development of PDE4 inhibitors, since it may provide a route for seemingly cAMP-independent actions of certain PDE4 inhibitors consequent on the inhibitor-induced intracellular relocalization of certain PDE4 isoforms and associated proteins. It remains to be seen as to whether such 'additional' properties associated with rolipram and certain other PDE4 inhibitors may underpin side effects, such as emesis (vomiting), or provide additional therapeutically beneficial actions.

Oligomerization

Various studies on purified and recombinant enzymes suggested that PDE4 enzymes have the potential to oligomerize and that this primarily involves a region within the C-terminal catalytic portion [126–128]. The identity of this site has come very recently

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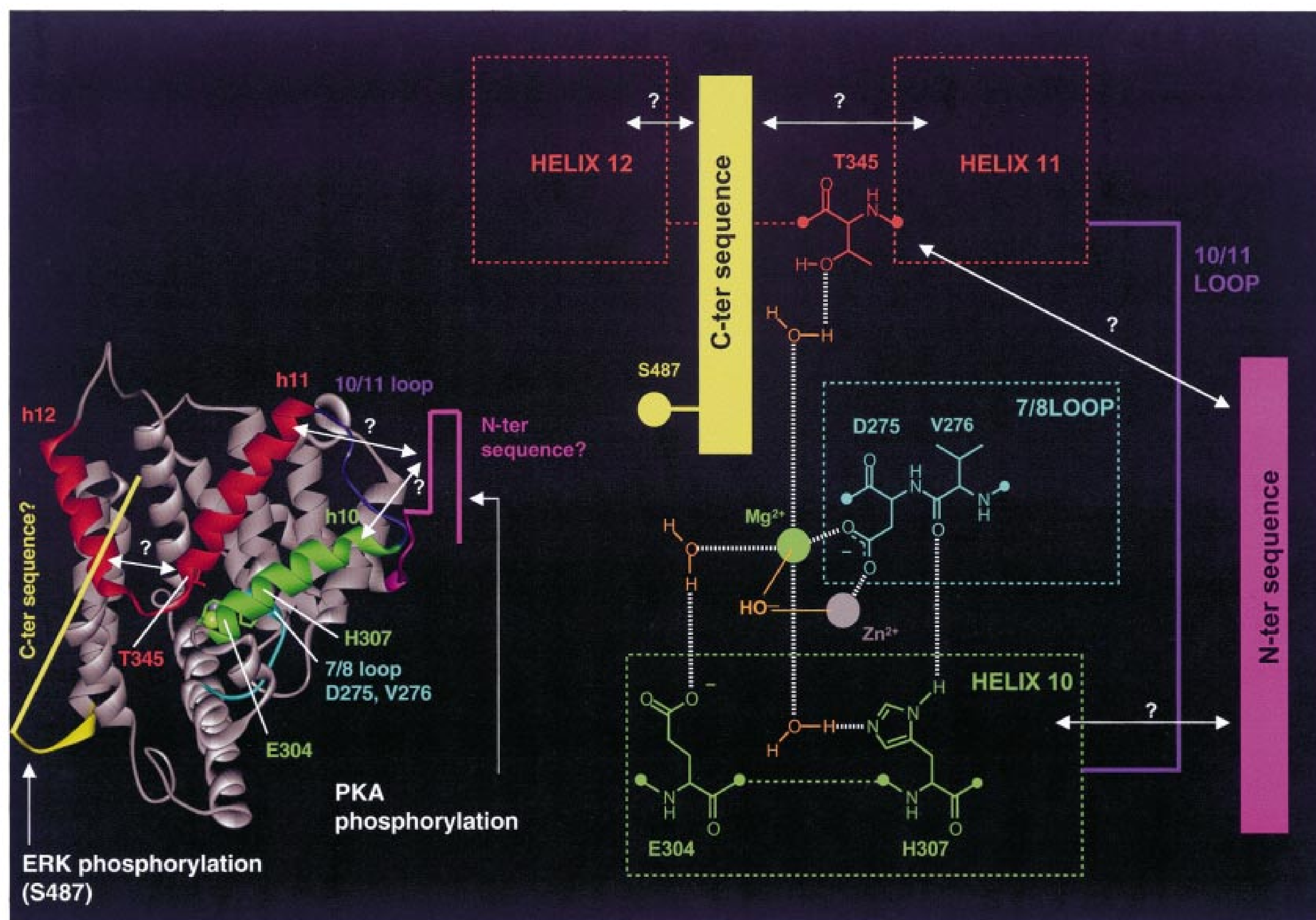

Figure 8 Regulatory hypothesis for co-operative control of PDE4 activity mediated by N-terminal and C-terminal phosphorylation

(1) Changes mediated at the N-terminal UCR1/2 regions of the protein are relayed into the catalytic centre via the helix 10/11 tweezer motif. (2) The C-terminal peptide segment extending from S487 folds across the protein to interact with helix 12 and/or helix 11 and/or the adjoining loop. (3) Phosphorylation of S487 is relayed into the catalytic centre by movement in the C-terminal peptide section affecting helices 11/12 and the adjoining loop. (4) Both conformational relays (from N and C termini) meet at Mg^{2+} through the network of hydrogen bonds to water ligands – via T345 from loop 11/12 and via H307 and E304 from helix 10. (5) Catalytic activity is modulated by movement of the Mg^{2+} centre.

from elucidation of the crystal structure of PDE4D, followed by biochemical studies encompassing structure-based mutation [125]. This has unequivocally demonstrated the presence of a dimerization interface within the core catalytic unit involving residues on helices 9, 10 and 11. Intriguingly, studies [127–129] have also indicated that the N-terminal portion of UCR2 may influence the formation of PDE4 homo-oligomers, although there is no evidence showing that the isolated N-terminal portion of UCR2, or even UCR2 alone, can oligomerize. Indeed, analyses of oligomerization [127,128] indicate that this is a relatively low-affinity interaction, with the K_d of monomer/n-mer complex formation being of the order of 10–20 μM, and that this affinity is reduced upon removal of UCR2. Thus among the conformational changes that UCR2 effects, it may control accessibility of a surface contained within the catalytic unit, which has the potential for allowing dimerization.

Certainly hetero-oligomerization appears not to occur in cells, as extensive analyses have shown that enzymes from different PDE4 subfamilies can be selectively immunoprecipitated from a variety of cell types (see, e.g., [105,130]). Indeed, it would appear to make little teleological sense for hetero-oligomers of PDE4 isoforms to form, as this would immediately lead to similar intracellular targeting of all PDE4 isoforms in a cell, which clearly does not occur. Of course, gel-filtration analyses can be expected to show PDE4 isoforms with sizes above those of their monomeric units, as these can be expected to co-purify with various interacting proteins. This can even affect PDE4 species purified from baculovirus cells, where recombinant PDE4B has been shown to co-purify as a complex with heat-shock protein 70 ('HSP70') [128].

PDE4 enzymes have an extremely high specific activity and are present at vanishingly low molar concentrations in cells. Thus, given the relatively low affinity for dimer formation, it seems extremely unlikely that oligomerization will be significant physiologically.

Phosphorylation

As described above, subdomain 3 of the catalytic unit provides a site for interaction with ERK, namely KIM and FQF docking and specificity sites as well as single target serine site for phosphorylation in all PDE4 subfamilies save that of PDE4A [49].

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The structural basis for the functional regulation of PDE4 caused by ERK phosphorylation of this site and how this is modulated by the UCR1/2 modules and by the phosphorylation status of UCR1 remains to be defined. The ERK phosphorylation site is located distant from the site of attachment for the N-terminal regions (Figure 2A) and does not appear to be positioned for direct interaction with this region of the protein. One possible mechanism that might account for co-operative N- and C-terminal regulation could be envisaged if ERK phosphorylation alters folding of the C-terminal sequence across helix 12 of the catalytic domain. One of the key Mg^{2+}-gripping residues, T345 (PDE4B), is located at the junction between helices 11 and 12. Other important Mg^{2+}-binding residues are E304 and H307 (PDE4B) located on helix 10. A key question, then, is whether structural changes in these two regions are relayed into the Mg^{2+} ion via helix 12 from the C-terminal portion of the catalytic unit and perhaps through the helix 10/11 tweezer motif from UCR1/2. In principle, both conformational relays could meet at the Mg^{2+} ion to co-operatively reposition this centre and thus control the enzyme's catalytic efficiency (Figure 8).

Intriguingly, it has also been suggested that PDE4A4/5 can be activated by stimulation of the phosphoinositide 3-kinase pathway in pre-adipocytes [105] and in human monocytes [130]; however, the kinases involved and the molecular basis of these changes have yet to be elucidated. Additionally, ligation of the T-cell receptor has been suggested to cause tyrosine-phosphorylation of PDE4B2. Molecular analyses of these further regulatory modifications to the enzyme can be expected to give important new insights into the functioning and regulation of PDE4 isoforms.

CONCLUSIONS

PDE4 enzymes have attracted great interest because of the possibility of deploying selective inhibitors of these enzymes as therapeutic agents in a number of major disease areas [20–31]. The true complexity of this enzyme family has yet to be uncovered [13,14]. However, it is clear that they sit at a pivotal crossroad where they underpin not only compartmentalized cAMP signalling, but also serve in networks that integrate responses with other major signalling pathways. The provision of a large family of isoforms allows such networks to be formulated in a cell-type specific fashion. The importance of individual PDE4 isoforms can be deduced from the extremely high level of conservation of their primary sequence and promoter structure between species. The recent crystal structures of unliganded PDE4B and PDE4D complexed to rolipram and zardaverine have provided the first clear insights into the substrate-binding site environment of the enzyme and its interaction with inhibitors. The observed binding mode can probably be extended to numerous other PDE4 inhibitors and will, undoubtedly, aid in the generation of novel selective inhibitors of potential therapeutic importance. Many key questions, however, remain – not least the functional role of specific PDE4 isoforms in particular cells, the extent of interacting partner proteins and the structural basis of catalytic site regulation by the N-terminal regulatory regions. The further appreciation of this enzyme family can be expected to give key insights into cellular signalling processes, as well as in aiding the development of more effective therapeutic agents and unravelling the molecular pathology of certain disease states.

The work carried out in our laboratory is supported by grants from the Medical Research Council (G8604010) and the European Union (QLG2-CT-2001-02278; QLK3-CT-2002-02149).

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Received 31 October 2002/13 November 2002; accepted 22 November 2002
Published as BJ Immediate Publication 22 November 2002, DOI 10.1042/BJ20021698

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