

This Review is part of a thematic series on **Phosphodiesterases**, which includes the following articles:
Compartmentation of Cyclic Nucleotide Signaling in the Heart: The Role of Cyclic Nucleotide Phosphodiesterases
Overview of PDEs and Their Regulation
Regulation of Phosphodiesterase 3 and Inducible cAMP Early Repressor in the Heart

cAMP-Specific Phosphodiesterase-4 Enzymes in the Cardiovascular System: A Molecular Toolbox for Generating Compartmentalized cAMP Signaling

cAMP and cGMP Signaling Cross-Talk: Role of Phosphodiesterases and Implications for Cardiac Pathophysiology
PDE5 and Regulation of Vessel and Heart Function

David A. Kass, Editor

cAMP-Specific Phosphodiesterase-4 Enzymes in the Cardiovascular System A Molecular Toolbox for Generating Compartmentalized cAMP Signaling

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Abstract—Cyclic AMP regulates a vast number of distinct events in all cells. Early studies established that its hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) controlled both the magnitude and the duration of its influence. Recent evidence shows that PDEs also act as coincident detectors linking cyclic-nucleotide- and non-cyclic-nucleotide-based cellular signaling processes and are tethered with great selectivity to defined intracellular structures, thereby integrating and spatially restricting their cellular effects in time and space. Although 11 distinct families of PDEs have been defined, and cells invariably express numerous individual PDE enzymes, a large measure of our increased appreciation of the roles of these enzymes in regulating cyclic nucleotide signaling has come from studies on the PDE4 family. Four PDE4 genes encode more than 20 isoforms. Alternative mRNA splicing and the use of different promoters allows cells the possibility of expressing numerous PDE4 enzymes, each with unique amino-terminal-targeting and/or regulatory sequences. Dominant negative and small interfering RNA-mediated knockdown strategies have proven that particular isoforms can uniquely control specific cellular functions. Thus the protein kinase A phosphorylation status of the β_2 adrenoceptor and, thereby, its ability to switch its signaling to extracellular signal-regulated kinase activation, is uniquely regulated by PDE4D5 in cardiomyocytes. We describe how cardiomyocytes and vascular smooth muscle cells selectively vary both the expression and the catalytic activities of PDE4 isoforms to regulate their various functions and how altered regulation of these processes can influence the development, or resolution, of cardiovascular pathologies, such as heart failure, as well as various vasculopathies. (*Circ Res.* 2007;100:950-966.)

Key Words: phosphodiesterase-4 ■ cAMP ■ cardiomyocytes ■ vascular smooth muscle cells ■ compartmentation ■ β_2 adrenoceptor ■ β -arrestin

Cyclic AMP is used in cells as a second messenger to regulate a large number of key processes.¹⁻⁵ It can influence cell growth, differentiation and movement, for example, and regulates specialized actions unique to specific

cell types. In the case of cardiac myocytes, cAMP influences inotropic and chronotropic actions, in addition to influencing apoptosis and hypertrophy.^{6,7} In the vasculature, cAMP influences contraction/relaxation of blood vessel smooth

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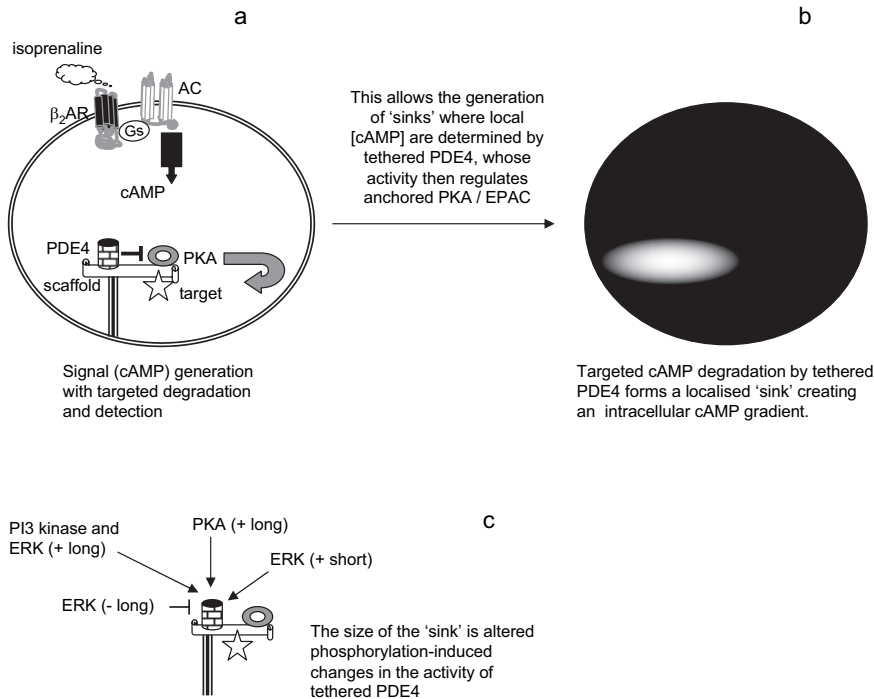


Figure 1. Compartmentalization of cAMP: barriers, sinks, holes. **a**, Schematic showing cAMP generation by plasma membrane–located adenylyl cyclase coupled to β_2AR -G_s plus PDE4 tethered to an AKAP scaffold, which generates a localized sink (white/grey) associated cAMP gradient. **b**, AKAP-tethered PDE4 activity gates the activation of AKAP-associated PKA and its action on tethered substrates, which include PDE4 long isoforms. **c**, Sink size depends on the tethered [PDE4] and activity regulation by inputs from other signaling pathways.

muscle cells (VSMCs), as well as their movement, proliferation, shape, and response to vascular trauma and hypoxia.^{8–11} Furthermore, various actions of cAMP can be altered in cardiovascular diseases such as heart failure and vascular stenosis.^{7,11}

How does cAMP exert such a myriad of sophisticated actions on particular cell types? Individual cells are complex entities whose components are intricately organized in 3D space. This extends to the machinery involved in the control of cAMP levels and the generation of specific responses at discrete intracellular loci.^{1,2,12–14}

cAMP is generated by adenylyl cyclase isoforms, the majority of which are embedded in the cell surface plasma membrane.¹⁵ These are activated by transmembrane receptors, such as the β -adrenergic receptor¹⁶ that, on agonist occupancy, couple to the stimulatory G protein G_s, thereby activating adenylyl cyclase. This confers cAMP generation to the cytosol surface of the plasma membrane, from which emanates a cloud of cAMP. However, because cells are polar, then different G_s-coupled receptors and adenylyl cyclase isoenzymes may be restricted to plasma membrane subdomains,^{15,17} so as to provide distinct “point sources” of cAMP generation at the plasma membrane. This offers potential for compartmentation (compartmentalization) of cAMP signaling in cells, a notion that was first mooted by Buxton and Brunton in milestone studies on cardiac myocytes¹⁸ and substantiated by others in such cells.^{19–26} However, as the free diffusion of cAMP is rapid (130 to 700 $\mu\text{m}^2 \text{sec}^{-1}$), the cell interior will quickly be equally distributed with cAMP. Furthermore, without any means of degrading cAMP then, after adenylyl cyclase activation, the cell interior would rapidly be saturated with cAMP. The ability to generate and shape cAMP gradients within the cell depends on the degradation of cAMP to 5'-AMP, which is achieved by cAMP

phosphodiesterases (PDEs).²⁷ Cytosol PDE activity would allow the formation of gradients of cAMP that depended on the source of cAMP generation by adenylyl cyclases located within subdomains of the plasma membrane. This adenylyl cyclase–PDE-dependent formation limits the potential for shaping gradients of cAMP in cells, thereby channeling cAMP signaling along specific conduits. To achieve this process, a further level of sophistication is engineered into the system, namely the ability to spatially restrict cAMP gradients at specific intracellular sites by targeting PDEs to specific intracellular sites and signaling complexes within cells.^{12,14,28–30}

Tethering of PDEs allows these enzymes to form and shape localized cAMP gradients that can be visualized with genetically encoded sensors.^{20,24,31,32} Up until recently the general notion was that tethered PDEs provided a barrier to free diffusion of cAMP from restricted microenvironments surrounding the site of generation at the plasma membrane. However, it is clear that such a model imposes severe limitations on the degree of control that can be exerted, not the least of which, that it confines spatial control to two compartments, namely those defined as being either inside or outside the PDE “barrier.” Recent experimental evidence, using siRNA knockdown of cAMP-specific PDE4, has suggested an additional scenario.³¹ This scenario envisages spatially confined populations of tethered PDEs generating localized “sinks” or “black holes,” down which cAMP “disappears” as it is converted into 5'-AMP (Figure 1). Spatially constrained PDE subpopulations coupled with free diffusion of cAMP will allow a myriad of localized gradients of cAMP to be generated and shaped in cells. Such a sophisticated system provides a means of generating a multitude of microenvironments in the cell interior that are under precise control of specific, tethered PDE subpopulations.

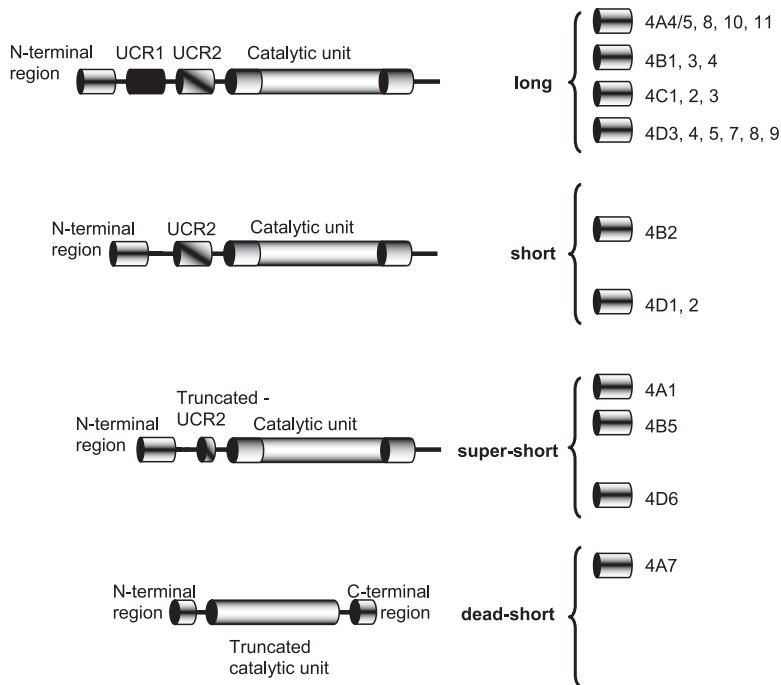


Figure 2. PDE4 isoform diversity: long, short, super-short and dead-short. Each isoform is defined by its unique N-terminal targeting region. Long isoforms have UCR1 and UCR2 domains; short isoforms lack UCR1; super-short isoforms have an N-terminally truncated UCR2; dead-short isoforms lack UCR1 and UCR2 and have an inactive catalytic unit that is both N- and C-terminally truncated. Current isoforms among the four *PDE4* genes are shown.

Each of these PDE subpopulations can be envisaged as regulating distinct cAMP-controlled processes through either protein kinase A (PKA) and its associated substrates or EPAC (exchange protein activated by cAMP) and its associated Rap1/2 effectors, with specific phenotypes therefore being associated with displacing specific PDEs from individual locales. Thus targeted PDEs are fundamental to the generation and control of compartmentalized cAMP signaling processes. These findings clearly underpin the complex nature of cAMP actions in the heart, where there are a plethora of distinct cAMP-regulated systems requiring distinct regulation of cAMP inputs that appear to be regulated by distinct PDEs. Such a system offers potential for sophisticated regulation by manipulating PDE tethering. Indeed, the cell type-specific expression of components of the proteins that form the cAMP signaling toolbox allows specific tailoring of the compartmentation of cAMP signaling. Additionally, it can be envisaged that alterations in either PDE isoform profile or tethering mode could contribute to certain pathologies.

There are 11 different PDE families, of which 8 encode a plethora of isoenzymes able to degrade cAMP.²⁷ The use of selective inhibitors,²⁷ small interfering RNA (siRNA)-mediated gene knockdown,³³ dominant negative constructs,^{33–35} and targeted gene knockouts^{36–41} has identified nonredundant, functional roles for an increasing number of PDEs.

Here we focus on the insight that investigation of the PDE4 family has provided into cAMP signal compartmentation, with special emphasis on cells of the cardiovascular system. Indeed, analysis of PDE4 enzymes has provided the paradigm for intracellular targeting of cAMP degradation³⁰ and highlights the fundamental role that individual PDE4 isoforms are poised to play in tailoring compartmentalized cAMP signaling. This, undoubtedly, is a key reason why

the complex 4 gene PDE4 family has been highly conserved through evolution.

“PDE4-ology”

Four genes (A/B/C/D) encode more than 20 different PDE4 isoforms through alternative mRNA splicing coupled to the use of different promoters.^{28,42,43}

PDE4A is located at Chr19p13.2, *PDE4B* at Chr1p31, *PDE4C* at Chr19p13.1, and *PDE4D* at Chr5q12. These genes span approximately 50 kb and comprise approximately 20 exons, the core catalytic unit of which is encoded by 7 exons.^{44–46} Additional exons encode regulatory regions and the N-terminal regions that uniquely identify individual isoforms. Various studies have linked the *PDE4D* gene to stroke⁴⁷ and to changes in bone mineral density⁴⁸; other studies have linked the *PDE4B* gene to schizophrenia.⁴⁹

Unique to the PDE4 family are the highly conserved regulatory regions upstream conserved region 1 (UCR1) and upstream conserved region 2 (UCR2).⁵⁰ Each is encoded by 3 separate exons, with UCR1 being formed from some 55 amino acids and UCR2 being formed by some 76 amino acids. PDE4 isoforms are subcategorized into 4 groups based on their UCR1/UCR2 complement. Thus “long” isoforms have UCR1 and UCR2, “short” isoforms lack UCR1, and “super-short” isoforms have just a truncated UCR2, whereas “dead-short” isoforms lack UCR1 and UCR2 and have an inactive catalytic unit that is both N- and C-terminally truncated (Figure 2).^{51,52} All *PDE4* genes encode long isoforms, although only certain *PDE4* genes encode types of short forms (Figure 2).

UCR1 and UCR2 likely interact in PDE4 long isoforms through ionic interactions⁵³ to form a regulatory module that directs the functional outcome of phosphorylation by PKA and extracellular signal-regulated kinase (ERK).^{54–61}

UCR1 is joined to UCR2 by LR1, a region that is encoded by 2 exons, is approximately 22/24 amino acids in length, and shows profound heterogeneity between subfamilies. LR2, which joins UCR2 to the catalytic unit, is encoded by a single exon and, as with LR1, shows no similarity between PDE4 subfamilies and varies in length between 10 and 28 aa. Their functional significance remains to be ascertained.

The final exon encodes part of the core catalytic unit as well as the C-terminal tail unique to each PDE4 subfamily. Indeed, the difference in primary sequence of this region has been exploited by us to make antisera specific to each PDE4 subfamily.

The most 5' isoform for each *PDE4* gene, seemingly, has dual 5' exons encoding its unique N-terminal region, whereas other isoforms have a single 5' exon encoding their unique N-terminal region. Specific promoters found immediately 5' to the N-terminal coding exon control the expression of individual isoforms.^{62–65} Such minimal PDE4 promoters appear to lack a canonical TATA box but contain CpG-rich islands and a series of perfect stimulating protein 1 (Sp1) consensus binding sites that drive basal promoter activity. Undoubtedly, regions 5' to this confer cell type-specific expression and further regulation.

It has also been shown that an alteration in the degree of histone acetylation of the PDE4D1/2 intronic promoter regulates the extent to which these variants are expressed in VSMCs.⁶⁶ Histone acetylation is among the numerous epigenetic factors that control expression of many genes,⁶⁷ and it will be interesting to determine whether other PDE4 isoforms are similarly regulated. Additional control of PDE4 isoform expression can occur through regulation of mRNA stability by cross-talk with the ERK pathway,⁶⁸ the action of which has been implicated in cardiac hypertrophy.⁶⁹

At a genomic level, the sequence of coding exons is highly conserved among species, indicating that the complexity of organization and plethora of PDE4 isoforms must provide a functional advantage to have survived evolutionary pressures in such an intact state. If changes in the PDE4 isoform and tethering-protein profiles change in pathological states, then this is likely to have a profound effect of compartmentation of cAMP signaling.

The Long, Short, and Super-Short of PDE4 Isoforms

UCR1 and UCR2 have a major functional role in regulating the activity of the PDE4 catalytic unit, particularly in integrating the effect of phosphorylation. This was first demonstrated for PKA,^{59,60} which phosphorylates the target serine within the conserved RRESF motif and causes activation of long isoforms from all 4 subfamilies.⁵⁸ Such activation contributes to the cellular desensitization system for cAMP⁷⁰ in cells in which long isoforms are expressed, such as cardiomyocytes²⁰ and VSMCs.⁶⁶

The mitogen-activated protein kinase ERK regulates numerous aspects of cardiomyocyte and VSMC functions, both in health and disease, including their hypertrophic responses.^{69,71–73} The catalytic unit of all PDE4 subfamilies, save for PDE4A, contains a serine within a consensus site (PQSP) that allows phosphorylation by ERK *in vivo*, altering

activity^{54,55,57,61} and expression.⁶⁸ As with all authentic ERK substrates, the catalytic unit of PDE4 enzymes contains both a KIM docking site (VxxKKxxxxxxLL), located on an exposed β -hairpin loop some 122 amino acids N-terminal to the target serine, and an ERK specificity motif (FQF), located on an exposed α -helix some 18 amino acids C-terminal to the target serine.⁵⁷ It is the presence or absence of UCR1/UCR2 that determines the functional outcome of ERK phosphorylation of PDE4, with long isoforms being inhibited, short isoforms being activated, and super-short isoforms being weakly inhibited. Thus cAMP signaling can be either positively or negatively coupled to ERK activation in specific intracellular locales dependent on the complement of short and long isoforms expressed. Such cross-talk can be reprogrammed by changes in the PDE4 isoform expression profile as seen in monocyte to macrophage differentiation,⁷⁴ and it will be of interest to see whether changes in cross-talk occurs in VSMC differentiation, where the PDE4 long/short profile changes.⁶⁶

Interestingly, ERK inhibition of PDE4 long isoforms can be negated by PKA phosphorylation.⁵⁵ This can lead to a situation where ERK-induced PDE4 inhibition can raise cAMP levels, causing PKA to become activated and phosphorylate the long PDE4, thereby ablating the inhibitory effect of ERK phosphorylation. Thus, as a consequence of ERK activation, long PDE4 isoforms may cycle through inhibition followed by activation, thereby causing either a transient, programmed rise in cAMP levels in their immediate locale or even oscillations.

More recently it has been demonstrated⁶¹ that the N-terminal portion of the PDE catalytic unit (Ser239 in PDE4D3) can be phosphorylated by an unknown kinase that acts downstream of phosphatidylinositol 3-kinase and is activated by oxidative stress. Phosphorylation at this site alone in PDE4D3 has no effect on catalytic activity. However, oxidative stress also activates ERK and it is when PDE4D3 is phosphorylated both by ERK (Ser579) and the unknown kinase (Ser239) that the function of this kinase is uncovered as reprogramming the effect of inhibitory ERK phosphorylation to now cause activation. Indeed, this now mimics the “loss of UCR1,” seen in short isoforms, where ERK phosphorylation of the PDE4 catalytic unit confers activation.⁵⁷

This unknown kinase is activated by reactive oxygen (ROS), and it may be linked to stress-induced reprofiling evident in cardiovascular disease. Clearly it will be important to identify it.

The Motor in the Middle

The structure of the core PDE4 catalytic unit has been resolved.^{28,43,75} It is a compact structure of 17 α -helices folded into 3 subdomains. These subdomains come together to form a deep pocket containing the cAMP binding active site, which contains tightly bound Zn^{2+} and loosely bound Mg^{2+} , essential for catalytic activity. This pocket has a volume of 440 \AA^3 , which contains the 232 \AA^3 cAMP molecule. PDE4 activation by PKA phosphorylation is influenced by $[Mg^{2+}]$ and the dominant “connections” that hold Mg^{2+} and are links to amino acids on helices 10/11. These

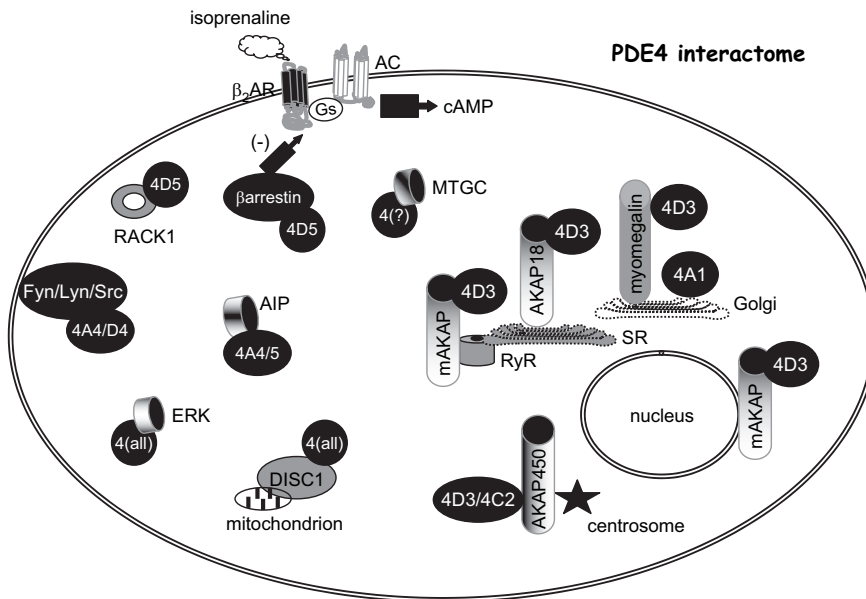


Figure 3. PDE4 interactome. Proteins currently known to interact with PDE4 isoforms to form signaling-specific signaling scaffold complexes that in certain instances are targeted to specific cellular regions. Such complexes underpin sink formation and the compartmentation of cAMP signaling.

connections, together with their connecting loop, fold over the surface of the catalytic center so as to create a “tweezer-like” motif that grips the Mg^{2+} . It is possible that UCR/UCR2 may direct actions to Mg^{2+} at the catalytic center via helices 10/11 or others that either interact directly with them or indirectly cause conformational changes in them.

Analyses of the PDE4 catalytic unit structure, proposed catalytic mechanism, binding of selective inhibitors and “inside-out” signaling where inhibitor binding might transmit changes to the molecule surface are discussed elsewhere.^{28,43,76}

Finding the Perfect Partner and Identifying Targeting “Zip Codes”

PDE isoforms play a pivotal role in creating and underpinning compartmentalized cAMP responses by generating gradients that are subsequently read and acted on by tethered PKA and EPAC subpopulations. This process, undoubtedly, explains the need for diversity among PDE isoforms and explains the importance as evidenced by the maintenance of such diversity against evolutionary pressures. Pivotal to this finding was the insight that the N-terminal regions unique to individual isoforms contain information that confers intracellular targeting.^{28,43,77} This targeting can take the form of targeting to specific subcellular membranes or to specific signaling complexes. In this way, unique gradients are generated that can be controlled by cross-talk with the ERK signaling pathway and by PKA activation of long isoforms.

The paradigm for this notion came from studies on the PDE4A1 super-short isoform.⁷⁷ Uniquely, PDE4A1 is exclusively membrane-associated and requires detergents to effect its release. PDE4A1 is uniquely characterized by its 25-aa N-terminal region, the removal of which generates a soluble, fully active species.^{77–82} Thus all of the information essential for membrane targeting is held within its unique N-terminal region. Consistent with this, chimeric species, made with various soluble, cytosolic proteins transformed them to

membrane-bound species that localized within cells as did PDE4A1.

A key feature of individual PDE4 isoforms is their ability to be targeted to specific sites/signaling complexes within cells, leading to the notion of a “PDE4 interactome” (Figure 3).

The N-terminal region of the long PDE4D5 isoform contains binding sites for the signaling scaffold proteins β-arrestin^{33,34,83,84} and RACK1,^{85–87} which we discuss in detail below.

The N-terminal regions of the long PDE4A4/5 and PDE4D4 isoforms contain proline-rich sequences that confer interaction with SH3-domains of certain proteins, such as the tyrosyl kinases Lyn, Fyn, and Src.^{88–91} Differences in specificity of interaction are seen between PDE4A4/5 and PDE4D4 because of different sequences surrounding their distinct proline- and arginine-rich sequences.

PDE4A4/5 can bind to the immunophilin AIP/XAP2/ARA9, which is known to interact with the aryl hydrocarbon receptor (AHR), a transcription factor required for normal cardiac development⁹²; AHR is upregulated in cardiomyopathy⁹³; and the genetic deletion of AHR leads to cardiac hypertrophy, hypertension, and fibrosis.⁹⁴ Aryl hydrocarbon receptor interacting protein (AIP) interacts not only with the PDE4A4/5 N-terminal region to give isoform specificity but also interacts with UCR2 to elicit an inhibitory effect on PDE4A4/5 activity.⁹⁵ This paradigm is a clear indication of how protein–protein interaction may regulate PDE4 catalytic activity. However, many PDE4-interacting proteins, such as RACK1, β-arrestin, and SH3 domain-containing proteins do not exert any profound effect on catalytic activity.

Interestingly, other proteins have been shown to interact with UCR2, namely the scaffold proteins, myomegalin,⁹⁶ myeloid translocation gene protein,⁹⁷ and DISC1.⁴⁹ However, it remains to be seen whether they interact with additional sites on PDE4 and whether they alter PDE4 activity.

The long PDE4D3 isoform interacts with the PKA anchor protein mAKAP,^{98–100} which is induced in cardiac hypertro-

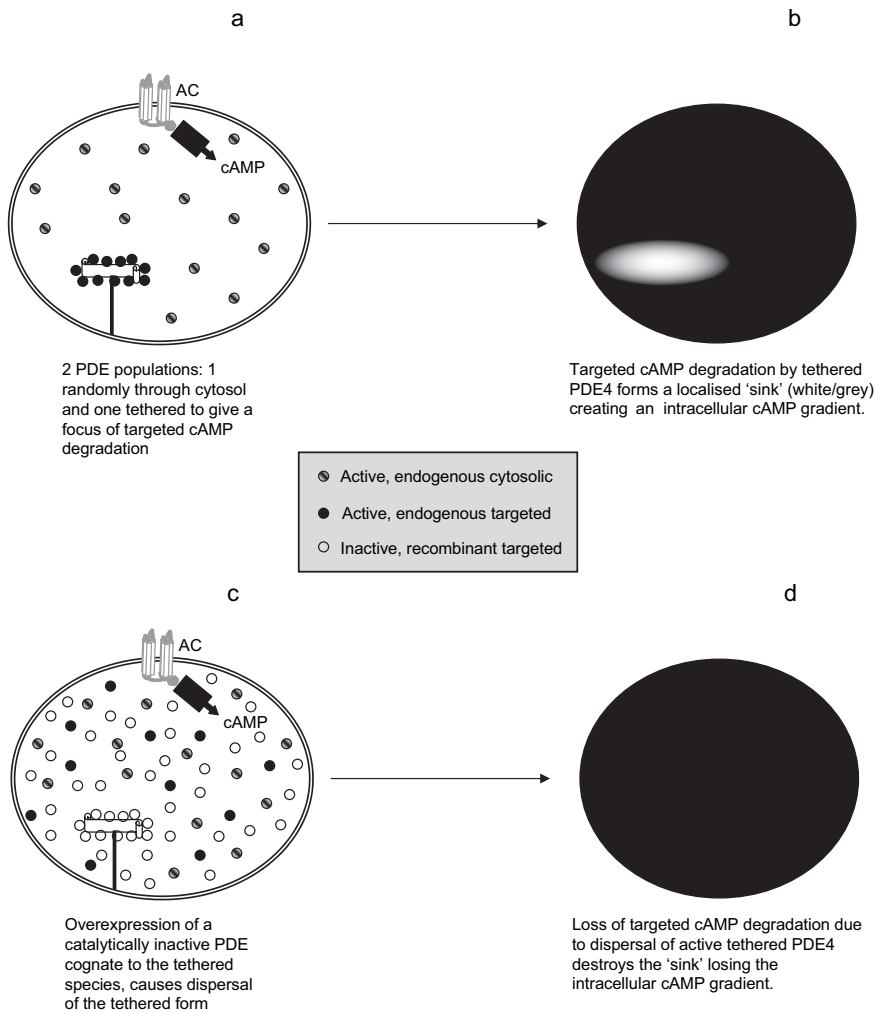


Figure 4. Ectopic expression of catalytically inactive PDE4 isoforms to uncover dominant negative functionality. **a**, Schematic of 2 PDE populations, 1 that is distributed randomly in the cytosol and 1 that is sequestered to a specific site. **b**, The sequestered PDE subpopulation will generate a sink/localized gradient. **c**, Overexpression of a catalytically inactive PDE that is cognate to the endogenously expressed, tethered species. This causes displacement of the endogenous active species such that it is randomly distributed in the cytosol. **d**, Loss of the active tethered species destroys the sink and localized gradient, conferring a dominant negative action on the catalytically inactive PDE. This will now allow PKA/EPAC subpopulations in the environment of the tethered inactive PDE to be activated.

phy¹⁰¹ and serves to relocate PDE4D3 to the perinuclear region of hypertrophic cardiac myocytes.⁹⁹ PKA phosphorylation of Ser13, within the unique N-terminal region of PDE4D3, increases interaction with mAKAP.⁹⁸ The activities of mAKAP-associated PDE4D3 and PKA are intertwined with PDE4D3 being phosphorylated at 2 sites by PKA,^{56,58–60} namely Ser54 in UCR1, causing activation, and Ser13, causing increased binding to mAKAP.

Mapping sites of interaction between proteins has, traditionally, been an arduous process involving truncation and mutation approaches. However, we have recently pioneered peptide array technology as a rapid means of ascertaining interaction sites.⁸⁵ Here a recombinant interactor protein is used to probe a library of overlapping, immobilized 25-mer peptides that scan the entire sequence of a particular protein. This allows rapid identification of regions that may contribute to the protein–protein binding. An interacting peptide was used as a template to generate a library of progeny where individual amino acids in the 25-mer parent are replaced by alanine, for example, in a corollary to scanning mutagenesis. Thus amino acids of putative importance to binding can be identified and used to direct mutagenesis approaches using intact proteins in 2-hybrid, pull-down, coimmunoprecipitation, and colocalization approaches. Where structural infor-

mation is also available, this can additionally be used to identify surface residues that likely form a binding site, thereby further facilitating mutagenesis strategies.

Cellular Function and Phenotype Conferred by PDE4D5 Association With β -Arrestin

Although great strides have been made in determining the location and mode of targeting of various PDE4 isoforms, the exact cellular function attributable to the targeting of individual PDE4 isoforms is now just beginning to be appreciated with the deployment of novel technologies. Paramount to this progress has been our development of the “dominant negative” approach.^{33–35,85} This took advantage of knowledge of the 3D structure of the PDE4 catalytic unit⁷⁵ to formulate a catalytic mechanism²⁸ and identify key amino acids in the putative cAMP binding pocket that would be essential for allowing hydrolysis of the phosphodiester bond of cAMP. The mutation of a key aspartate residue sufficed to render PDE4 isoforms from all 4 subfamilies catalytically inactive.³⁵ Thus overexpression of catalytically inactive isoforms in cells would not be expected to generate an overt phenotype unless they acted to replace/displace their cognate, native functional isoform from the site its tethered to in a cell, thereby removing the sink mechanism and allowing for an increase in

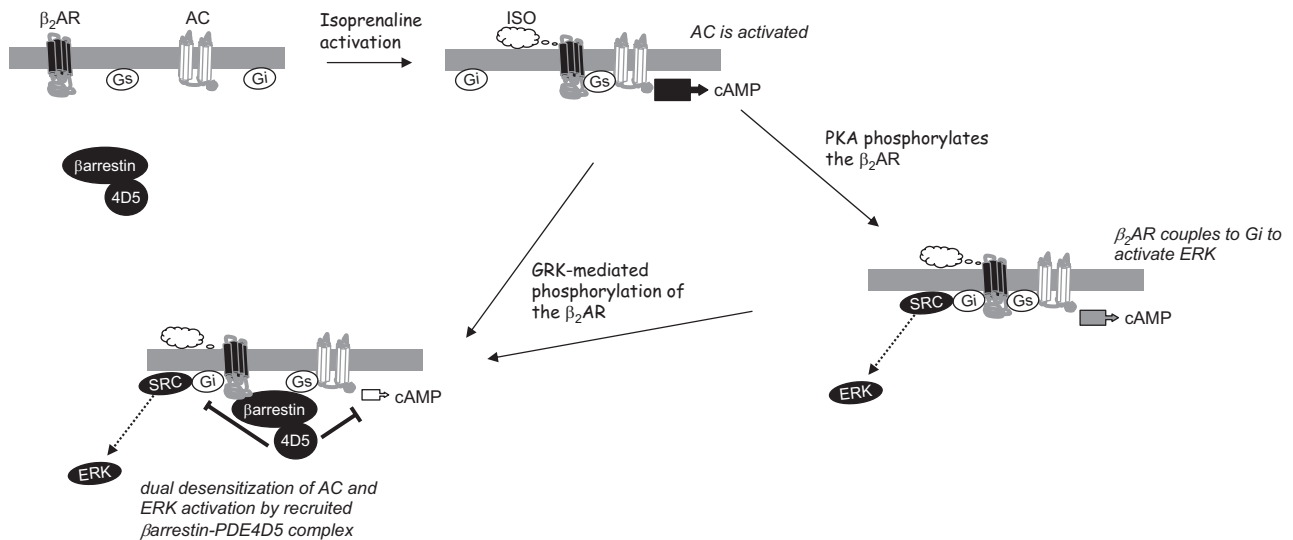


Figure 5. Dual desensitization of β_2 AR signaling through recruitment of β -arrestin/PDE4D5 complex. Isoprenaline (ISO) stimulation of the β_2 AR causes activation of adenylyl cyclase and increases cAMP levels. This activates PKA-RII tethered to the receptor by AKAP79, causing PKA phosphorylation of the β_2 AR, which can now couple to G_i to activate ERK. G_s activation allows G protein coupled receptor kinase (GRK) recruitment to, and phosphorylation of, the β_2 AR. This signals recruitment of a β -arrestin-bound PDE4D5 complex to the β_2 AR, which confers dual desensitization; (1) β -arrestin attenuates β_2 AR coupling to G_s ; and (2) PDE4D5 forms a localized cAMP sink, causing deactivation of PKA-RII tethered to the β_2 AR by AKAP79 with loss of PKA-phosphorylated β_2 AR and ERK activation.

localized cAMP levels (Figure 4). Such an approach does not require any understanding of the nature of the anchor for the PDE4 isoform. Of course, if the anchor and mode of anchoring were known, then the catalytically inactive PDE4 isoform could be suitably mutated, so that it were unable to bind the anchor, which would lead to a loss of phenotype and its ability to exert a the dominant negative action.⁸⁵ The paradigm for this approach came from studies on PDE4D5,¹⁰² leading to the first demonstration that a single PDE4 isoform can express a unique functional role in cells.³³ Thus PDE4D5 bound to the multifunction adapter protein β -arrestin allows for the dynamic movement of active PDE4D5 from the cytosol to the β_2 adrenoceptor (β_2 AR) after agonist challenge (Figure 5). This generates a localized sink for cAMP adjacent to the β_2 AR that controls the activity of a PKA subpopulation tethered to this receptor by AKAP79.³³ Both of these actions were unequivocally shown to be β -arrestin dependent as they were not apparent in β -arrestin-1/2 double knockout mouse embryo fibroblasts (MEFs) but could be reconstituted in such cells on ectopic reintroduction of β -arrestin-1 using an adenoviral vector.⁸⁴

In various cell types including cardiomyocytes PKA phosphorylation of the β_2 AR partially attenuates coupling to G_s and switches on coupling of the phosphorylated β_2 AR to the guanine nucleotide regulatory protein, G_i (Figure 5).^{12,103} Through this process, the PKA phosphorylated β_2 AR elicits the SRC-dependent activation of ERK.

Recruitment of the β -arrestin-PDE4 complex serves to orchestrate a dual desensitization event. Thus the recruited β -arrestin physically interdicts signaling between receptor and G_s , initiating a reduction in adenylyl cyclase activation and a subsequent decrease in cAMP production. However, simultaneously, β -arrestin-bound PDE4D5 provides a localized sink for cAMP degradation, which acts to downregulate β_2 AR associated PKA activity and, thereby, dampen signal-

ing to ERK through G_i . Such a regulatory function has been demonstrated in neonatal cardiomyocytes as well as model cell lines.^{12,34,83,103,104}

It has also been demonstrated that cAMP pools generated by stimulation of different G_s coupled receptors in rat ventricular myocytes are shaped by the differential coupling of each receptor type to different PDE families.¹⁹ However, the extent to which G protein-coupled receptor-specific cAMP “pools” are influenced by recruited PDE4D5 in complex with β -arrestin remains to be ascertained.

In cardiac myocytes, PDE4D5 is preferentially associated with β -arrestin and selectively recruited to the β_2 AR on agonist challenge, despite the fact that PDE4D5 expression was some 5 times lower than that of PDE4D3.^{34,83} As discussed below, PDE4D5 interacts preferentially with β -arrestin because of an additional binding site unique to this isoform.^{83,85}

In cardiac myocytes, chemical ablation of PDE4 activity by the specific inhibitor, rolipram enhances both PKA phosphorylation of the β_2 AR and the switching of its signaling to ERK activation.^{34,83} However, rolipram inhibits all PDE4 isoforms similarly and so cannot identify control by any one PDE4 isoform. That selective silencing of all PDE4D isoforms by siRNA-mediated knockdown mimicked such actions of rolipram³³ identifies the importance of this subfamily in modulating β_2 AR signaling but gives no insight into which particular isoform and whether targeting is required. Isoform-specific, siRNA-mediated knockdown subsequently identified PDE4D5 as the relevant species.³³ However, although this technological approach indicates the role of PDE4D5, it gives no insight into whether the entire cellular PDE4D5 pool is of importance or whether a subpopulation is important, namely one that is tethered specifically to β -arrestin. Such an analysis demanded a new technological approach, and for this we used overexpression of a catalyti-

cally inactive PDE4D5 (Asp556Ala), which was introduced into cardiac myocytes by adenoviral-mediated gene delivery.³⁴ This catalytically inactive PDE4D5, when overexpressed, serves to displace endogenous PDE4D5 from β -arrestin and prevent agonist-mediated delivery of active PDE4D5 to the β_2 AR, providing a dominant negative action.^{33,34,85} Dominant negative PDE4D5 amplifies PKA activity at the plasma membrane but not in the cytoplasm. This mimics the phenotype engendered by treatment with either rolipram or PDE4D5 knockdown. Final verification that the PDE4D5 phenotype resulted from its preferential association with β -arrestin resulted from the demonstration that a discrete mutation in the N-terminus of catalytically inactive PDE4D5 construct, made so as to compromise its ability to bind β -arrestin; prevented such a species from displacing endogenous active PDE4D5 from β -arrestin; and failed to elicit a dominant negative effect.⁸⁵ This dominant negative approach, undertaken on cardiac myocytes, provided the first indication that a cellular phenotype could be assigned to an individual PDE isoform.

Clearly, a dominant negative strategy provides a means of dissecting out functional roles for anchored subpopulations of PDE4 isoforms that cannot be determined using either active site-directed inhibitors or siRNA knockdown. The identification of small molecules that disrupt targeting of specifically anchored PDE4 isoforms may provide for novel therapeutic agents that are not plagued by the various side effects seen with active site-directed PDE4 inhibitors. Such targeting disruptors can be expected to allow diminution of PDE4 activity at a highly specific spatial locale.

Molecular Determinants Mediating PDE4D5– β -Arrestin Interaction

Pull-down and 2-hybrid analyses showed that members from all 4 PDE4 families could bind β -arrestin.⁸⁴ Further investigations showed that this was attributable to the presence of a common site within the highly conserved catalytic unit of the enzyme.⁸³ Pull-down studies using truncated PDE4D5 constructs coupled with 2-hybrid analyses went on to demonstrate that the PDE4D5 isoform interacted preferentially with β -arrestin because, in addition to the binding site for β -arrestin in its catalytic region, PDE4D5 has an additional binding site in its unique N-terminal region.⁸³ These two sites on PDE4D5 interact with distinct sites on β -arrestin. Thus the common interaction site in the PDE4 catalytic unit binds to the N-domain of β -arrestin, whereas the site unique to PDE4D5 binds to the C-domain of β -arrestin. Such 2-point interaction allows PDE4D5 to straddle β -arrestin. This underpins the preferential association of PDE4D5 with β -arrestin, which is pivotal in conferring its precise functional action in regulating the PKA phosphorylation status of the β_2 AR.

PDE4D5 can also form a complex with the WD repeat scaffold protein RACK1. This interaction is unique to PDE4D5 because, as with β -arrestin, it involves a binding site located in the PDE4D5-unique N-terminal region.^{86,87} Indeed, overlapping binding sites for both β -arrestin and RACK1 in the unique N-terminal region of PDE4D5 coupled to distinct second sites of interaction on the catalytic unit

confers their mutually exclusive binding to PDE4D5.⁸⁵ Thus β -arrestin and RACK1 independently sequester PDE4D5, ensuring fidelity of signaling through these distinct scaffold proteins.

Insight into the location and nature of the binding sites for β -arrestin and RACK1 on PDE4D5 was garnered using peptide array technology (Figure 6).⁸⁵ This allowed us to demonstrate that both RACK1 and β -arrestin to PDE4D5 could bind to amino acids between residues 22 and 45 within the N-terminal portion of PDE4D5. Using alanine substitution arrays to scan this region, specific amino acids were identified as involved in determining the binding of either β -arrestin (E27, D28, L29) or RACK1 (N22, P23, W24, V30, K31), exclusively, or were found in common as important for the binding of each of these signaling scaffold proteins (L33, R34). Analysis of the 3D structure of this portion of the PDE4D5 N-terminal region showed these residues to be surface exposed and that the concomitant binding of both β -arrestin and RACK1 to PDE4D5 was not possibly attributable to the proximal and overlapping nature of their respective binding sites. Thus in any one cell, there are likely to be specific, spatially distinct subpopulations of PDE4D5 because of the association of PDE4D5 with scaffolding proteins such as β -arrestin and RACK1.

Peptide array analysis also facilitated resolution of the amino acids that contribute to the common β -arrestin binding region within the conserved catalytic unit of all PDE4s.⁸⁵ The amino acids identified (F670, F672, L674, and L676) are all surface exposed and located on helix-17, which appears to be attached to the compared core catalytic unit by a mobile hinge region (Figure 6).⁸⁵ Interestingly, 2 of the amino acids implicated (F670, F672) in β -arrestin binding also form part of the ERK specificity/docking binding motif on PDE4 enzymes,⁵⁷ which would preclude PDE4 isoforms from binding directly to both β -arrestin and ERK. As ERK can phosphorylate and deactivate PDE4 long forms, it is tempting to speculate that by preventing ERK docking to PDE4D5, β -arrestin-bound PDE4D5 ensures only activated enzyme is found in this complex and so recruited to the β_2 AR on agonist activation.

This work highlights the power of peptide array technology for the rapid and informative definition of protein–protein interactions.

Cardiomyocyte PDE4s

PDE4B and PDE4D variants are expressed in rodent, murine, and human cardiomyocytes,¹⁰⁵ and selective PDE4 inhibitors have been suggested to have small effects on Ca^{2+} currents and contractility.^{62,106–108} In particular, it has been suggested that PDE4 inhibitors, although promoting isoprenaline-induced inotropic responses, have little effect alone.^{106,109} Intriguingly, much analysis of this effect has been performed using diazepam, which has been suggested to function through PDE4 inhibition,¹⁰⁹ although any possible action on the “novel” cAMP-hydrolyzing PDE7/8/10/11 forms has yet to be assessed. Indeed, PDE4 inhibitors, which have potential therapeutic use in treating sepsis, improve cardiac contractility in endotoxemia,¹¹⁰ suggesting that they may have a role for treating critically ill patients.

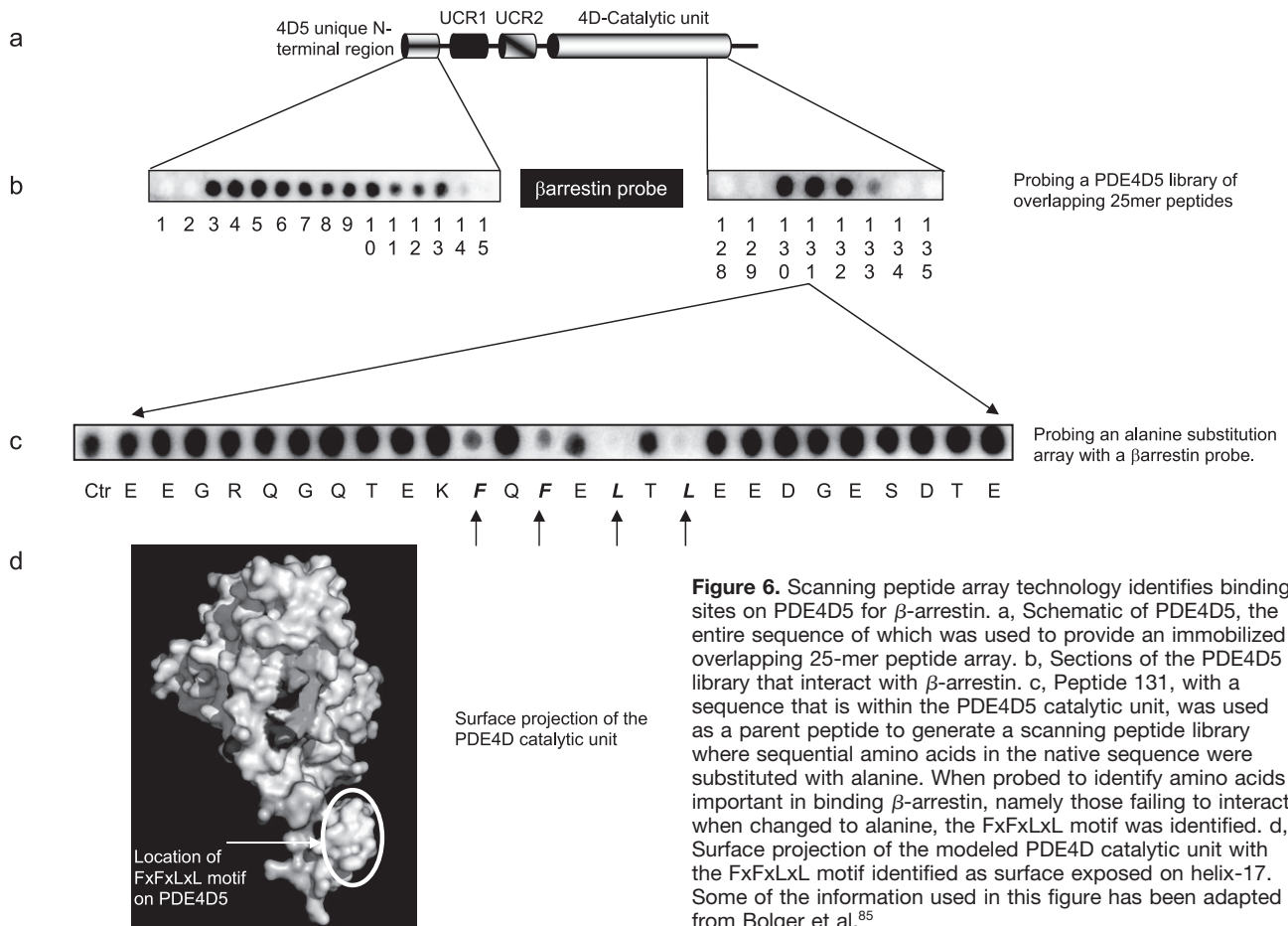


Figure 6. Scanning peptide array technology identifies binding sites on PDE4D5 for β -arrestin. a, Schematic of PDE4D5, the entire sequence of which was used to provide an immobilized overlapping 25-mer peptide array. b, Sections of the PDE4D5 library that interact with β -arrestin. c, Peptide 131, with a sequence that is within the PDE4D5 catalytic unit, was used as a parent peptide to generate a scanning peptide library where sequential amino acids in the native sequence were substituted with alanine. When probed to identify amino acids important in binding β -arrestin, namely those failing to interact when changed to alanine, the FxFxLxL motif was identified. d, Surface projection of the modeled PDE4D catalytic unit with the FxFxLxL motif identified as surface exposed on helix-17. Some of the information used in this figure has been adapted from Bolger et al.⁸⁵

Although several studies have described altered cardiomyocyte PDE3A expression in certain types of heart disease,^{111,112} no studies have systematically addressed whether the PDE4 expression profile is altered in heart failure. Given the distinct spatial and temporal regulation of cAMP levels afforded by distinct PDE4 subtypes, their altered expression in heart disease would be predicted to, potentially, markedly alter cardiac function. In contrast, significant advances have been made relative to the manner by which these enzymes allow compartmentalized cAMP signaling in cardiomyocytes. Recent elegant molecular studies have determined that individual PDE4 variants can be tethered to distinct sarcoplasmic reticulum (SR) proteins, allowing them to either directly or indirectly control SR function.^{12,19,20,22,25,31,34,38,108,113} Indeed, tethering of PDE4 variants to specific signaling complexes likely represents the molecular basis for much of the selective actions seen with PDE4 inhibitors, compared with PDE2 and PDE3-selective inhibitors in cardiomyocytes. Moreover, it is possible that loss of tethering for certain PDE4D variants may lead to cardiac failure, as seen in PDE4D-null mice and, perhaps, in humans (see below).³⁸

PDE4B and PDE4D localize to sarcomeric M- and Z-line structures, respectively, in neonatal rat ventricular cardiomyocytes. Consistent with this, in fully differentiated adult cardiomyocytes, PDE4 activity is high at the transverse (T) tubule/SR junctional space of cardiomyocytes, the area regulating excitation-contraction coupling.^{25,114} Notwithstand-

ing that cardiomyocytes express several long PDE4D isoforms (PDE4D3, PDE4D5, PDE4D8, PDE4D9), to date most studies have limited their analysis to how PDE4D3, and PDE4D5 tethering contributes to compartmented cAMP signaling in cardiomyocytes. Thus PDE4D3 associates with the ryanodine receptor 2 (RyR2)³⁸ and A-kinase anchoring proteins (AKAPs)^{99,115,116} in cardiomyocytes, so as to spatially and temporally regulate cAMP. PDE4D5 is the key isoform interacting with β -arrestin,^{33,34,83,85} which allows it to preferentially regulate β_2 AR signaling^{12,34} but can also interact with the signaling scaffold protein, RACK1.^{85-87,117,118}

PDE4D-RyR2 Interaction

PDE4D3 can integrate into an SR-associated RyR-based complex (RyR2, cardiac, RyR2). RyR2 is a tetrameric SR Ca²⁺ channel that represents the dominant Ca²⁺-release channel in cardiomyocytes.^{119,120} Kinetics of RyR2 channel opening is complex, with the open probability state being stabilized by several factors including cytosolic Ca²⁺, cyclic ADP-ribose (cADPR), caffeine, and phosphorylation by several protein kinases including PKA and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII).^{121,122} The closed state of RyR2 is stabilized by binding of 4 subunits of the \approx 12-kDa FK506-binding protein FKBP12.6. In addition to FKBP12.6, several other proteins interact with the RyR2 within a large macromolecular signaling complex in cardiomyocytes, which may include PKA, CaMKII, PP1, PP2A,

mAKAP, spinophilin, PR130, sorcin, triadin, junctin, calsequestrin, and Homer.¹²³

Until very recently, analyses of PDE4D- and PDE4B-null mice had revealed no significant pathological role for these enzymes. Indeed, most of the attention had focused on either PDE3B within a phosphatidylinositol 3-kinase γ complex or on PDE3A, the more abundant isoform expressed in cardiomyocytes.^{124,125} However, recently, PDE4D-null mice were reported³⁸ to display a very late, age-dependent, cardiac phenotype composed of a progressive cardiomyopathy and an increased incidence of exercise-induced arrhythmias. This phenotype is similar to that reported when a RyR2 defect in patients produces heart failure and sudden cardiac death.¹²⁶ At a functional level, the PDE4D-null mouse phenotype was associated with RyR2 hyperphosphorylation and a reduced capacity of hyperphosphorylated RyR2 to gate Ca^{2+} . It was suggested that hyperphosphorylation of RyR2 was attributable to the absence of PDE4D within the RyR2-based macromolecular complex of PDE4D-null animals.^{38,127} The authors suggested that, because the phenotype was suppressed in mice engineered to lack one of the potential PKA phosphorylation sites within RyR2 (S2808) and because less PDE4D3 was associated with RyR2 in human myocardium from heart failure patients, their findings were consistent with the hypothesis that PDE4D deficiency may contribute to heart failure and arrhythmias by promoting defective regulation of the RyR2 channel in humans. In addition, the authors also speculated that prolonged PDE4 inhibitor use might predispose patients to unexpected cardiac events. In this context, because no fewer than 70 individual mutations that alter the biophysical properties of the RyR2 have been described, because phosphorylation of the RyR2 is catalyzed by numerous kinases in addition to PKA, and because PDE4D activity and targeting are also each dynamically regulated by multiple factors, it is likely that further work in human cardiomyocytes will be required to fully assess the significance of the effect in humans. Additional work is needed to explore this proposal in studies using animals with different genetic backgrounds and, importantly, with conditional knockouts, so as to exclude any phenotypic input resulting from loss of PDE4D during development. Also, because the PDE4D knockout was generated by deletion of a catalytic exon such an approach might lead to the generation of truncated proteins that could interact with signaling complexes to exert dominant negative actions independent of loss of PDE4 activity. In this context, it should be noted that no significant cardiac side effects have been reported in clinical trials of PDE4 inhibitors designed for use in treating chronic obstructive pulmonary disease or asthma in humans, and no cardiac toxicology has been reported in animal studies performed using various PDE4-selective inhibitors in development.^{43,128–130} Thus it is important to extend studies of PDE4 action in the heart to understand fully its role in health and disease.

PDE4D3–mAKAP

In addition to regulating the acute contractile functions of the heart, PDE4 enzymes have also been shown to have potential in regulating the trophic responses of cAMP in cardiomyocytes. For example, binding of PDE4D3 to mAKAP in

neonatal hypertrophic ventricular cardiomyocytes targets this PDE4 variant to the perinuclear region and allows it to regulate cAMP levels within this locale.⁹⁹ Thus, PDE4D3 association with mAKAP, a striated muscle-specific AKAP scaffold tethered to nuclear membranes, was shown to promote more efficient control of PKA-mediated phosphorylation of several proteins, including PDE4D3 itself, in cardiomyocytes.⁹⁹ In this context, further studies will be required to determine whether other PDE4 isoforms can interact with either mAKAP or other cardiomyocyte AKAPs and contribute to compartmentalized signaling in these cells.

Although little is known concerning the number of PDE4 variants that interact with cardiomyocyte AKAPs, recent studies have identified some of the proteins that also populate the mAKAP signaling complex. Indeed, the EPAC, a cAMP-activated Rap-GEF,⁴ as well as ERK5, may be found together in certain complexes.¹⁰⁰ Interestingly, the presence of both PKA and EPAC within such a complex may represent a situation in which local cAMP concentrations can differentially control the activity of 2 cAMP effectors with distinct sensitivities to activation by cAMP. Indeed, whereas cAMP activation of mAKAP-associated PKA served to phosphorylate and thereby activate PDE4D3, reducing local cAMP concentrations, maximal activation of mAKAP-associated ERK5 served to suppress PDE4D3 activity, thus allowing for activation of EPAC. The mechanism by which ERK-mediated phosphorylation inhibits PDE4D3 involves phosphorylation of this long isoform at Ser579.^{54,55,57}

Although it has been suggested that PDE4D3 represents the adaptor protein that recruits EPAC1 to the mAKAP complex,¹⁰⁰ this has yet to be established. Indeed, PDE4D3 interacts with mAKAP via its unique 18-aa N-terminal domain.⁹⁹ Thus, unless EPAC also interacts with this small PDE4D3-specific domain, which seems unlikely as it might then be expected to compete with mAKAP for binding to PDE4D3, we would expect that EPAC binds to a different site. Indeed, this appears to be the case as we (M. Houslay, H. Bos, M. Lynch, G. Baillie, unpublished results, 2006) can show that there is a common binding site on PDE4 isoforms for EPAC. Thus various other PDE4 isoforms may also be able to recruit EPAC to their site of anchorage within the cell. If multiple PDE4 isoforms were able to recruit EPAC to such signaling complexes, this would further increase the need to determine whether the PDE4 isoform expression profile is impacted in heart failure.

Phenotypic Modulation of VSMCs

VSMCs can exist in 2 distinct phenotypes, contractile/quiescent (herein contractile) or synthetic/activated (synthetic).^{131,132} In healthy blood vessels *in vivo*, contractile VSMCs have low proliferative and migratory indexes, express contractile proteins, and contract or dilate in response to numerous hormonal or biophysical demands. In contrast, in culture, or *in situ* following vascular insult, synthetic VSMCs have a higher proliferative and migratory index, express fewer contractile proteins, and release extracellular matrix (ECM) proteins. Synthetic VSMCs do not directly maintain vascular tone, but rather, during developmental vasculogenesis and angiogenesis or in response to vascular injury or insult in the

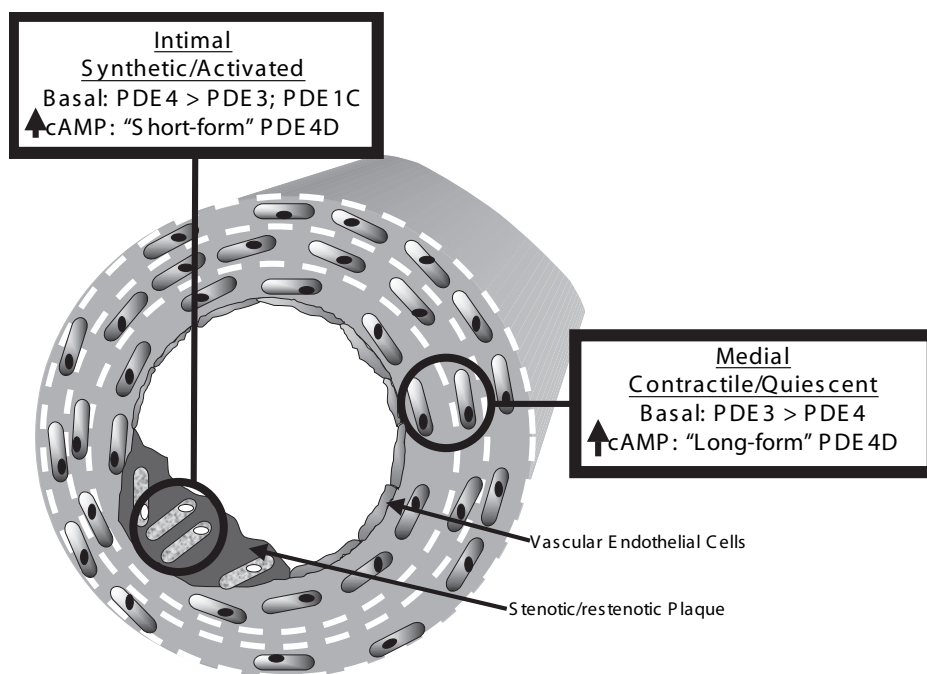


Figure 7. Differential expression of PDE4 and PDE3 in contractile and synthetic VSMCs. Contractile VSMCs populate the medial layer of arteries, whereas synthetic VSMCs accumulate in the intimal layer in response to vascular insult. Although PDE3 and PDE4 activities are roughly equivalent in contractile VSMCs, a dramatic downregulation of *PDE3A* expression in synthetic VSMCs markedly increases the dependence of these cells on PDE4 for cAMP hydrolysis. In human VSMCs, PDE1C may also contribute to cAMP hydrolysis. Prolonged increases in cAMP in contractile VSMCs increases the level of expression of PDE4D1/2 long-form variants in these cells, whereas a similar treatment markedly upregulates expression of the PDE4D1 and PDE4D2 short forms in synthetic VSMCs.

adult, they maintain vascular structural integrity.^{131–133} Substantial evidence indicates that both acute local effects, such as changes in VSMC–vascular endothelium communications, as well as longer-term genetic and epigenetic effects contribute to such phenotypic switches. These concepts and how they impact the maintenance of blood vessel structural and functional homeostatic integration have been reviewed elsewhere.⁶⁷

VSMC Phenotypic Modulation and PDE3/PDE4 Activity Ratios

Contractile VSMCs isolated from several distinct rat or human arteries express one variant each of PDE4A and PDE4B, as well as numerous PDE4D gene-derived variants including PDE4D3, PDE4D5, PDE4D7, PDE4D8, and PDE4D9.¹⁰⁵ Selective immunoprecipitation of PDE4A, PDE4B, or PDE4D enzymes from these cells identifies PDE4D as representing the dominant catalytic activity.^{68,134} Although both contractile and synthetic VSMCs use PDE3 and PDE4 to hydrolyze cAMP, the individual gene family variants expressed in these cells, as well as their relative proportions, can differ in VSMCs isolated from different blood vessels within a given species, or in the same blood vessel between species.¹⁰⁵ Indeed, whereas cAMP hydrolysis by PDE3 is dominant in rat and human aortic contractile VSMCs, PDE4 activity surpasses PDE3 activity in the synthetic VSMC phenotype (Figure 7).^{66,135–137} Although a phenotypic modulation-based regulation of the PDE3/PDE4 activity ratios has been reported in all rat and human VSMCs thus far studied, further work will be needed to establish the generality of this event to VSMCs from all vascular beds. Although the molecular basis for the reduced level of PDE3 activity in rat or human synthetic VSMCs is dependent on a marked reduction in PDE3A, the underlying mechanisms, and the factors that limit changes in PDE3B, or PDE4

expressed in these cells, during the phenotypic switch is currently unclear. However, it is apparent that the reduction in PDE3A expression in synthetic VSMCs represents another example in which synthetic VSMCs effectively reduces sensitivity to regulation through cGMP-dependent mechanisms. Indeed, it has been established that several cGMP-sensitive enzymes, including protein kinase G (PKG) and NO-sensitive guanylyl cyclases (sGCs), are downregulated in synthetic VSMCs compared with their contractile counterpart.¹³⁸ In this context, it may be that PDE3B levels do not become reduced in synthetic VSMCs because PDE3B is significantly less sensitive to cGMP-mediated inhibition than PDE3A.¹³⁹ PDE4 would be excluded from this regulatory scheme, being insensitive to physiological [cGMP]. Consistent with the proposition that synthetic VSMCs are somewhat more “cAMP-centric” than their contractile equivalent is the observation that the cAMP-hydrolyzing PDE1C is upregulated in human synthetic VSMCs and inhibition of its induction impacts VSMC proliferation.^{140,141}

With respect to the mechanism(s) that regulate these changes and render the synthetic VSMCs more dependent on PDE4-mediated regulation of cAMP signaling, it may be relevant that inducible cAMP early repressor (ICER), a transcriptional repressor, was recently shown^{111,112} to reduce PDE3A, but not PDE3B nor PDE4, expression in cardiomyocytes in response to certain rodent models of heart failure. Although a role for ICER in regulating CREB-mediated expression of some gene products has been reported in rodent synthetic VSMCs,^{142,143} further studies will be required to assess the involvement of this mechanism in the PDE3/PDE4 switch observed during the VSMC phenotype switch. In addition, although currently untested, it may be possible that changes in histone acetylation of the PDE3A promoter is reduced in synthetic VSMCs,⁶⁶ an effect known to reduce transcription of several genes during the phenotypic switch.⁶⁷

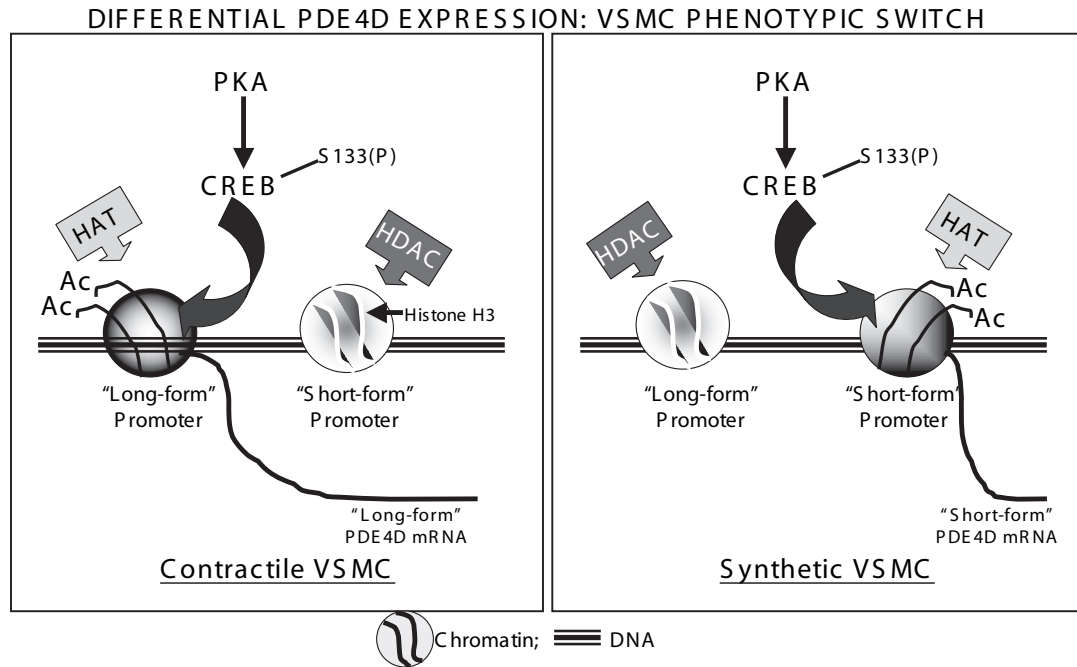


Figure 8. Differential regulation of basal PDE4D expression and of cAMP-induced upregulated expression of PDE4D in contractile and synthetic VSMCs. A dynamic balance between histone acetylation (Ac) by histone acetyl transferases (HAT) and histone deacetylation by histone deacetylases (HDAC) during the phenotypic switch of VSMCs from contractile to a synthetic phenotypes allows induction of different PDE4D variants in these cells in response to prolonged increases in cAMP.

Rather than simply providing yet another example of differences between contractile and synthetic VSMCs, we believe that the altered PDE3/PDE4 activity ratio between these cells may be physiologically and therapeutically important. Thus, although it is generally accepted that PDE3 inhibitors are more effective at relaxing contractile VSMCs than PDE4 inhibitors, the increased dependence of synthetic VSMCs on PDE4 may afford selectivity in the regulation of cAMP-mediated events in synthetic VSMCs using PDE4-selective inhibitors.¹⁰⁵ Indeed, although a PDE3 inhibitor (cilostamide) reduces accumulation of intimal (synthetic) VSMCs in a rat model of restenotic injury,¹⁴⁴ given the potential proarrhythmic potential of these compounds, it is unlikely that they could be used safely to reduce postangioplasty restenosis in humans.¹⁴⁵ In contrast, because PDE4 inhibitors have generally been found to have only very modest effects on the heart, selective PDE4 inhibitors could prove useful in this therapeutic arena. Also, because synthetic human VSMCs may also express the dual cAMP/cGMP-hydrolyzing PDE1C, inhibitors of this enzyme in combination with PDE4 inhibitors could also be a very powerful combination in these situations.

Distinct PDE4D Variants Coordinate Desensitization to Prolonged cAMP Signaling in Contractile and Synthetic VSMCs

The transition of rat and human VSMCs from a contractile to a synthetic phenotype is accompanied by a major shift in the ratio between total PDE3 and PDE4 activities (Figure 7). Synthetic and contractile VSMCs both express approximately equal PDE4 activity. In addition, short-term (<30 minutes) treatment of these cells with cAMP-elevating agents causes

the PKA-mediated phosphorylation and activation of the expressed PDE4D long isoforms.^{68,134} In contrast, the mechanisms by which these cells use PDE4 to desensitize the effects of prolonged challenges with cAMP-elevating agents (>1 hour) are markedly different regarding which PDE4D isoforms are upregulated (Figure 8).⁶⁶ Indeed, a greater increase in PDE4 expression is seen in synthetic VSMCs. Thus, prolonged cAMP elevation *in vivo* initiates a program involving cAMP-PKA-CREB/CRE, but not EPAC, that stimulates increased expression of the dominant PDE4D variants present in these cells before challenge. Consistent with a role for increased transcription and translation, pharmacological inhibition of such processes abolished the increases. Further work is needed to analyze fully changes in PDE4 isoform profile in these cells by such treatments.

Interestingly, and of potential therapeutic relevance, when synthetic VSMCs were similarly treated with cAMP-elevating agents, there was no evidence that the levels of the dominant PDE4D variants expressed in these cells were increased. In contrast, prolonged incubations of synthetic VSMCs caused a marked PKA/CREB/CRE signaling cascade-mediated induction of 2 PDE4D short-form variants (PDE4D1 and PDE4D2).⁶⁶ Again, there was no obvious role for EPAC-Rap signaling in this effect. PDE4D1/2 short-form enzymes were not expressed in either contractile or synthetic VSMCs before treatment and were not induced in contractile VSMCs following similar treatments. As was the case in contractile VSMCs, a role for increased transcription and translation of these short-form PDE4D variants was evident in synthetic VSMCs.

Reversible posttranslational modifications of histones, including ADP ribosylation, methylation, glycosylation, phos-

phorylation, and acetylation regulates whether individual promoter sequences can bind transcription factors and be “turned on.”⁶⁷ In general, increased levels of histone acetylation within promoter sequences correlate with increased gene expression. Marked advances in our understanding of the role of histone acetylation in regulating gene expression have recently been made, and this process serves as an important factor regulating the differential expression of certain genes during the process of VSMC phenotypic modulation. Interestingly, hyperacetylation of the intronic promoter regulating PDE4D1/2 expression in synthetic VSMCs is consistent with the idea that histone acetylation is involved in determining which PDE4D isoforms are upregulated in response to cAMP elevation (Figure 8).

Significance of a Distinct PDE4D Expression Pattern in Contractile and Synthetic VSMCs

Several physiological and therapeutically important consequences may flow from the differences by which contractile and synthetic VSMCs react to prolonged increases in cAMP. Thus, differences in the magnitude of the cAMP-induced increase in PDE4 activity in contractile and synthetic VSMCs may indicate that PDE4D upregulation plays a less significant role in desensitizing contractile VSMCs to prolonged actions of cAMP than in synthetic VSMCs. Also, these data are consistent with the idea that synthetic VSMCs might be much more dependent on increased PDE4D to desensitize the effects of prolonged cAMP elevation, than the contractile cells. Although neither of these hypotheses has yet been formally tested, they are internally consistent with the idea that PDE4 inhibitors might more markedly influence cAMP-mediated signaling in synthetic than contractile VSMCs. Of course, should this be the case, one would further suggest that PDE4 selective, and perhaps even PDE4D-selective inhibitors, would have more marked effects in neointimal VSMCs in stenotic lesions, which are by definition synthetic, than in the contractile VSMCs resident within the medial muscular layers of the artery. Because most attempts to use cAMP-elevating agents to reduce intimal hyperplasia have been hampered by effects of these agents on systemic blood pressure, PDE4-selective agents may prove useful in obviating this limitation.

Several reports^{54,55,57,68,134} have shown that the differential impact of ERK-mediated phosphorylation of distinct PDE4D variants provides a powerful mechanism through which activation of a mitogen-activated protein kinase–signaling cascade can integrate with cAMP-mediated effects in cells, including cardiomyocytes and VSMCs. Indeed, because ERK-mediated phosphorylation^{54,55,57} of PDE4D long-form variants can either activate or inhibit these enzymes, whereas the PDE4D short forms are always activated by this event, it is reasonable to propose that the interactions between cAMP-elevating agents and growth factors that activate ERK will be different in these cells. For example, we suggest that because ERK-mediated phosphorylation of PDE4D1 activates this enzyme, physiologically relevant VSMC trophic factors, such as PDGF or angiotensin II, which activate ERK, might reduce the antiproliferative actions of cAMP-elevating agents in synthetic VSMCs. Clearly, further work is required to assess

the functional impact of these events. Because activation of ERK signaling by PDGF or angiotensin II can also regulate PDE4D expression through effects dependent on messenger RNA stabilization,⁶⁸ it is clear that the levels of cross-talk are complex.

The Future

Exploiting Tethered PDE4

The plethora of PDE4 isoforms confers specific functional attributes that relate to (1) targeting to specific intracellular complexes and membranes so as to exert control by shaping local cAMP gradients and (2) regulation by phosphorylation. These indicate that specific PDE4 isoforms will have particular functional roles. We have provided the paradigm for this in the interaction of PDE4D5 with β -arrestin. This delineates how recruitment and redistribution of a particular PDE4 isoform confers a specific functional role.

Active site-directed inhibitors cannot discriminate between PDE4 isoforms with a subfamily as they have identical catalytic sites. Indeed, even between subfamilies the similarity of active sites militates against effective selective inhibitors being developed. To demonstrate that specific isoforms can have particular functional roles novel approaches and technologies are required. We have pioneered this in generating catalytically inactive isoforms whose overexpression in cells acts to displace the cognate endogenous, active isoform from its functionally relevant scaffold(s).^{12,33,34,110} This so-called dominant negative approach can be used to determine the function attributable to tethering of specific isoforms. Additionally, the use of siRNA targeted to specific isoforms allows us to uncover functional attribute(s). However, unlike the dominant negative approach, this does not inform directly on functions attributable to tethering. Such approaches have identified specific nonredundant functional roles for specific PDE4 isoforms, which is consistent with observations from PDE4 subfamily knockout analyses.^{36,37}

Cardiomyocytes

The PDE4D5/ β -arrestin, PDE4D3/mAKAP, PDE4D3/EPAC, and PDE4D3/RyR complexes have been identified in cardiomyocytes. However, as cardiomyocytes express a range of other PDE4 isoforms, it is likely that these represent a fraction of PDE4 complexes in these cells. It is, as yet, unknown whether the PDE4 isoform profile is altered in heart disease. However, should this be the case, it may become possible to identify individual molecular complexes that more strongly influence cardiomyocyte dysfunctions and for which therapeutic strategies may be developed.

Vascular Smooth Muscle Cells

Virtually all cAMP-dependent pharmacological agents used to treat heart conditions have effects on the ability of contractile VSMCs to control blood pressure. Indeed, several cAMP-dependent agents, that might be useful to inhibit VSMC proliferation and migration in the context of restenosis, alter blood pressure by influencing the functions of contractile VSMCs. Clearly, advances in our understanding of how cardiomyocytes and contractile or synthetic VSMCs regulate cAMP-mediated effects will be required to achieve

cell-type-selective therapeutic effects. We suggest that recent advances in our understanding of PDE4-mediated regulation of cAMP-signaling in these distinct cell types may be important. Thus, the marked increase in the PDE4/PDE3 activity ratio that occurs during the phenotypic switch of VSMCs may allow PDE4 inhibitors to selectively affect synthetic VSMC functions compared with those in contractile VSMCs. In addition, because synthetic VSMCs markedly induce PDE4D to desensitize the impact of prolonged cAMP-signaling, selective PDE4 inhibitors may prolong the effects of cAMP-elevating agents in these cells compared with those seen in contractile VSMCs. Similarly, the observation that PDE4D3 is anchored via an AKAP in cardiomyocytes, but not in contractile nor synthetic VSMCs, may afford some measure of selectivity if, as proposed above, noncatalytic domain-based strategies of PDE4 inhibition become feasible.

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Disclosures

None.

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