Phosphodiesterase Inhibitors: Factors That Influence Potency, Selectivity, and Action

Sharron H. Francis, Miles D. Houslay, and Marco Conti

Contents

S.H. Francis (✉)
Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615, USA
e-mail: sharron.francis@vanderbilt.edu

M.D. Houslay
Molecular Pharmacology Group, Institute of Neuroscience and Psychology, CMVLS, University of Glasgow, Glasgow G11 8QQ, Scotland, UK

M. Conti
Center for Reproductive Sciences, University of California, San Francisco, CA 94143-0556, USA

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Abstract Cyclic nucleotide phosphodiesterases (PDEs) are promising targets for pharmacological intervention. The presence of multiple PDE genes, diversity of the isoforms produced from each gene, selective tissue and cellular expression of the isoforms, compartmentation within cells, and an array of conformations of PDE proteins are some of the properties that challenge the development of drugs that target these enzymes. Nevertheless, many of the characteristics of PDEs are also viewed as unique opportunities to increase specificity and selectivity when designing novel compounds for certain therapeutic indications. This chapter provides a summary of the major concepts related to the design and use of PDE inhibitors. The overall structure and properties of the catalytic domain and conformations of PDEs are summarized in light of the most recent X-ray crystal structures. The distinctive properties of catalytic domains of different families as well as the technical challenges associated with probing PDE properties and their interactions with small molecules are discussed. The effect of posttranslational modifications and protein–protein interactions are additional factors to be considered when designing PDE inhibitors. PDE inhibitor interaction with other proteins needs to be taken into account and is also discussed.

Keywords Compartmentation · Cyclic AMP · Cyclic GMP · PDE1 · PDE2 · PDE3 · PDE4 · PDE5 · PDE6 · PDE7 · PDE8 · PDE9 · PDE10 · PDE11 · Phosphodiesterase · Phosphodiesterase inhibitors

1 PDE Superfamily

The mammalian superfamily of cyclic nucleotide (cN) phosphodiesterases (PDEs) is remarkably complex. It comprises 11 distinct families (PDEs 1–11) with protein products derived from 21 genes (Bender and Beavo 2006; Conti and Beavo 2007); some families are encoded by a single gene, whereas others are products of multiple genes, but there are alternative splice variants of the gene products in all the families except for PDE6. In several instances, multiple promoters that are differentially regulated influence expression of the PDE mRNA transcripts (Bender and Beavo 2006; Conti and Beavo 2007; Omori and Kotera 2007), and extensive alternative splicing of the mRNAs produces a vast array of protein products. It is now estimated that there are close to 100 different protein products of these genes, and these are distinguished by having different regulatory features, catalytic characteristics, tissue distributions, subcellular localizations, targeting to signaling complexes and sensitivities to PDE inhibitors (Fig. 1). The need for such a large array of PDE isoforms in controlling cN levels and maintaining appropriate cellular functions is still poorly understood. PDEs are typically in low abundance in cells but may be highly expressed in particular tissues or regions of cells for regulation of specific physiological effects (Castro et al. 2006; Cote 2006; Houslay 2010; Juilfs et al. 1997; MacFarland et al. 1991). Most cells contain multiple PDEs that have overlapping specificities and affinities for cAMP or cGMP. However, where studied, it seems clear that while there may be some degree of redundancy in function, each of the PDEs provides important regulatory control of cNs in a particular cell or region of

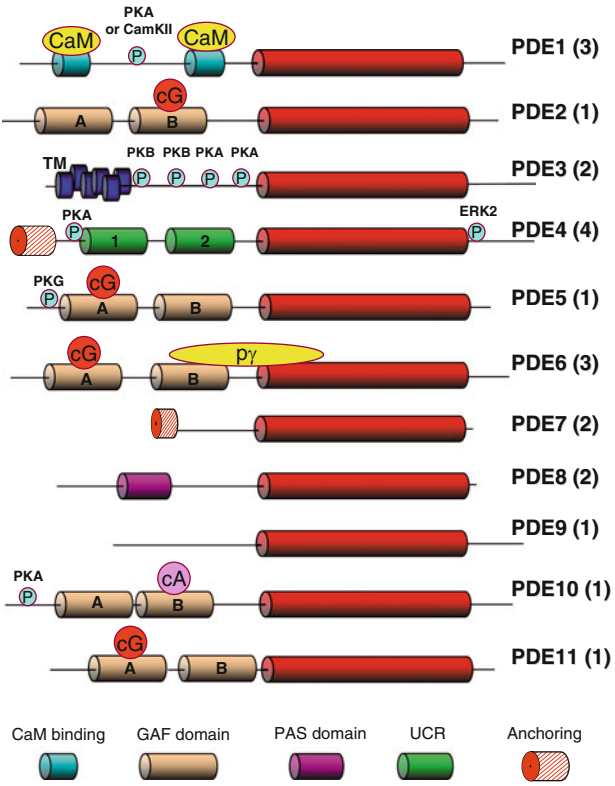

Fig. 1 Schematic representation of domain arrangment of the 11 mammalian PDE families. Family name is noted to the *right* of each structure, and the *number in parenthesis* denotes the number of genes composing the family. The conserved catalytic domain is represented as a *red cylinder*. Binding proteins are depicted in *yellow*. TM = transmembrane domain of PDE3; protein kinase (PKB, PKA, PKG, ERK, or CaMKII) phosphorylation sites are shown as a *teal ball* labeled with a P. Cyclic GMP binding to either GAF-A or GAF-B domains is marked by a *red ball* labeled cG, and cAMP binding to GAF-B in PDE10 is depicted as a *pink ball* labeled cA. Modified from Conti and Beavo (2007)

a cell. Some PDE families (PDEs 1–4) are widely expressed in mammalian tissues, but others (PDEs 5–11) occur in lower abundance or are expressed in fewer tissues. The PDE6 family appears to be the most restricted in distribution since it has only been found in the outer segments of photoreceptors, where it is in high concentration, and in the pineal gland (Cote 2006).

1.1 PDEs as Cellular Targets of Cyclic Nucleotides

The myriad forms of PDEs that serve as cellular targets of cNs and as major determinants of cN action exceed those of other targets such as that of cN-dependent

protein kinases, the cN-gated (CNG) channels and exchange-protein activated by cAMP (EPAC). All PDEs contain a conserved catalytic site that interacts with cNs and breaks them down into their respective 5′-nucleotides. Several PDEs (PDE 2, 5, 6, 10, and 11) contain cN-binding sites in their respective regulatory domains (Cote 2006; Gross-Langenhoff et al. 2006; Wu et al. 2004; Zoraghi et al. 2005; Handa et al. 2008; Martinez 2002a; Martinez 2002b; Martinez et al. 2008); these sites comprise ~120 amino acids known as GAFs [an acronym derived from the proteins in which these domains were originally identified, i.e., c**G**MP-binding PDEs, *Anabaena* **a**denylyl cyclases, and *Escherichia coli* transcription factor **F**hlA (Aravind and Ponting 1997)]. For PDE 2, 5, and 6, cN binding to one of these GAFs regulates catalytic site function, and it has been proposed that cN-binding GAFs in PDEs could act to sequester cNs under appropriate conditions (Bender and Beavo 2006; Conti and Beavo 2007; Corbin and Francis 1999; Gopal et al. 2001).

The respective cN-binding sites in GAFs are structurally and evolutionarily distinct from the PDE catalytic sites and cN-binding sites in the cN-dependent protein kinases, cN-regulated channels, the bacterial catabolite-gene activator protein (CAP), and EPACs (Bos 2006; Charbonneau 1990; Martinez et al. 2002a, b; Zoraghi et al. 2004). The regulatory (R) and catalytic (C) subunits of cAMP-dependent protein kinase (PKA), which combine to form the PKA holoenzymes, are derived from four and three genes, respectively. Any R subunit (RI or RII), which appear to exist as homodimers, can interact with any C subunit (Cα, Cβ, or Cγ) in mammalian tissues (Francis and Corbin 1999). EPACI and II, which are regulated by cAMP binding, are derived from two genes (Bos 2006), and cGMP-dependent protein kinases (PKGI and PKGII) are derived from two genes with alternative splicing of the PKGI mRNA to produce two isoenzymes (PKGIα and PKGIβ) (Uhler 1993; Wernet et al. 1989). PKGs appear to always exist as homodimers so it is predicted that there are only three PKGs in mammalian tissues. The exceptionally diverse characteristics of the PDEs provide excellent potential for development of selective inhibitors for these targets, but in most instances, their similarities continue to confound development of such inhibitors.

The allosteric cN-binding sites on PDEs 2, 5, 6, and 11 preferentially bind cGMP, although the sites on PDE2 can interact with cAMP with reasonable affinity (Martinez et al. 2002a, b; Wu et al. 2004; Zoraghi et al. 2005; Cote 2006); the allosteric cN-binding site in PDE10 tightly binds cAMP (Handa et al. 2008). When compared with other cN-binding sites, these sites are formed by a tight binding pocket; in PDEs 5 and 6, this pocket rigorously selects for cGMP versus cAMP and against substituents introduced in cGMP analogs (Huang et al. 2004; Martinez 2002; Thomas et al. 1992; Wu et al. 2004). These characteristics make them excellent targets for drugs that would impact the function of these PDEs. In PDEs 5 and 6, the allosteric site excludes PDE inhibitors. However, in PDE2, low concentrations of 3-isobutyl-1-methylxanthine (IBMX) or papaverine stimulate catalytic activity (Yamamoto et al. 1983), although it is now unclear whether this stimulation occurs through interaction with the allosteric cN-binding site or effects mediated via partial occupation of the catalytic site (Pandit et al. 2009). If this effect is mediated through the allosteric cN-binding site, it lends some promise for

development of compounds that selectively interact with PDE2 allosteric sites (Erneux et al. 1982; Yamamoto et al. 1983). Recent insights derived from X-ray crystallographic and NMR structures of several cN-binding GAFs have further defined topographical features that could lead to design of pharmacophores targeting these sites (Heikaus et al. 2008; Martinez et al. 2005; Pandit et al. 2009; Wang et al. 2011), and high throughput screening assays to identify compounds that might interact with these sites are currently being developed (see Demirbas et al. and Schultz et al. in Chapters 5 and 6, respectively, in this volume). Likewise, the *U*pstream *C*onserved *R*egions (UCRs), which profoundly impact catalytic functions in PDE4 isoforms, and the Rec and PAS subdomains in PDE8 are considered to be promising pharmacological targets for modulation of the catalytic activities of these PDEs.

1.2 Quaternary Structure of PDEs

PDEs exist as monomers, dimers, or higher oligomers, and in most instances, the contribution of this physical status to function, regulation, localization, and stability is poorly understood. Most PDEs appear to exist as homodimers except in the case of PDEs 1 and 6. Under physiological conditions, PDE1 is thought to occur as a heterotetramer comprising two identical catalytic subunits and two molecules of calmodulin. PDE6 isoforms in the outer segments of the photoreceptors also commonly exist as heterotetramers. The PDE6 in rod outer segments is composed of two closely related gene products (PDE6α and PDE6β) that form a catalytic heterodimer that is in complex with two small rod-specific inhibitory subunits (Pγ) to form an inactive heterotetramer (Cote 2006). The PDE6 in cone outer segments is also a heterotetramer that comprises two identical catalytic subunits (PDE6α′) and two cone-specific Pγ subunits.

The mechanisms employed for oligomerization of PDEs vary; some interact through their catalytic domains (Huai et al. 2003; Scapin et al. 2004), others make contacts through portions of their regulatory domains including GAFs or UCRs (Huai et al. 2003; Martinez 2002; Richter and Conti 2002; Zoraghi et al. 2005), and still others dimerize through contacts in both regions (Pandit et al. 2009). In all instances, the contacts and quaternary state appear to be very stable. For most PDEs, catalytic activity is retained in constructs that include only the catalytic domain; in several instances, these truncated constructs are monomeric indicating that oligomeric status is not required for catalytic function. For the isolated catalytic domains of some PDEs, many functional characteristics (k_{cat}, K_m, and IC_{50} values for certain inhibitors) are similar to those of the respective holoenzymes, but in others, there are important functional differences (Blount et al. 2006, 2007; Fink et al. 1999; Richter and Conti 2004; Saldou et al. 1998).

Almost all inhibitors that have been developed to date compete with cN substrate for access to the PDE catalytic sites. This region continues to be a major focus of medicinal chemists for development of inhibitors (Ke and Wang 2007b; Owen et al. 2009; Verhoest et al. 2009; Zhang 2006). The catalytic domain, which is conserved

among mammalian PDEs and comprises ~270 amino acids (Bender and Beavo 2006; Conti and Beavo 2007), is located toward the carboxyl-terminal portion of the PDEs and exhibits 24–51% amino acid sequence identity among the 11 families. X-ray crystal structures of the isolated catalytic domains have shown that the various PDEs share a similar overall topography that is comprised almost entirely of α-helices (16–17 helices), as well as sharing common structural features in the composition and conformation of the catalytic pockets (Ke and Wang 2007b; Zhang 2006). However, there are important differences among these proteins in the binding of cNs and inhibitors (Wang et al. 2005; Wang, Liu, Hou et al. 2007; Wang, Yan, Yang, Cai et al. 2008; Huai, Wang, Zhang et al. PNAS 2004; Huai, Liu, Francis, Corbin JBC 2004). Moreover, potent and selective inhibitors of PDE4 have recently been developed by dually targeting the catalytic sites of these enzymes together with regions outside the catalytic pocket itself (Burgin et al. 2010; Gurney et al. 2011).

1.3 Structural Characteristics

Divalent metal ions are required for catalytic function of all PDEs. In the X-ray crystallographic structures of PDEs, the catalytic pockets have been shown to contain a novel binuclear metal-binding site that is thought to contribute critical elements of the catalytic mechanism (Xu et al. 2000). Where it has been studied, the interactions of certain inhibitors with PDE catalytic sites can be influenced by the presence of these metal ions, but direct or indirect interaction with the metals does not appear to substantially impact potency (Chen et al. 2008; Corbin et al. 2003; Ke and Wang 2007b; Sung et al. 2003; Wang et al. 2008). However, it is reasonable that inhibitors could be designed that utilize contacts with one or more of the metal ions or that expel these cofactors, thereby eliminating catalytic activity. Since affinity of certain PDE4 long isoforms for various selective inhibitors and magnesium is sensitive to PKA phosphorylation of the regulatory UCR1 domain (Alvarez et al. 1995; Sette and Conti 1996b; Hoffmann et al. 1998), it seems likely that the metal ions bound to a particular PDE could also affect its interaction with certain inhibitors. Moreover, some inhibitors could influence metal occupancy of PDE catalytic sites (Wang et al. 2008), but this possibility has not yet been investigated.

Results of X-ray crystallographic studies of certain PDEs reveal that a hydroxyl derived from solvent water bridges the two metal ions in these catalytic sites. This arrangement provides for production of a highly nucleophilic hydroxyl that inserts at the phosphate in the cyclic phosphate ring of the cN and breaks the ring (Fig. 2) (Liu et al. 2008). These data are consistent with the results of early biochemical studies establishing that the hydroxyl inserted into the cyclic phosphate ring is derived from solvent water and that the stereochemistry of the product is consistent with this mechanism (Braumann et al. 1986; Burgers and Eckstein 1979; Jarvest et al. 1982; Walseth et al. 1983). In most instances, one of the metal sites is occupied by a zinc that is very tightly bound, and the other site is occupied by a more loosely

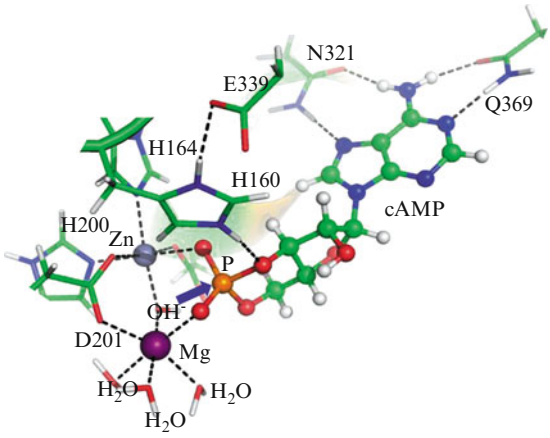

Fig. 2 Scheme proposed for mechanism of hydrolysis of cAMP by PDE4. The adenine (shown in *green* and *blue* at *upper right*) is coordinated through hydrogen bonds (*dashed lines*) to Gln369 and Asn321. The phosphorous in the cyclic phosphate ring is approximated to the hydroxyl ion that bridges the two metal ions [Zn (in *steel*) and Mg (in *purple*)], and the *blue arrow* indicates the attack of this hydroxyl at that phosphorous (shown in *orange*) to break the ring. Residues coordinating the metal ions are shown. The Glu339/His160 hydrogen-bond relay that fosters interaction of His160 with one of the oxygens (*red*) in the cyclic phosphate ring is shown. This figure was kindly provided by Prof. Chang-Guo Zhan, College of Pharmacy at the University of Kentucky

bound metal that is presumed to be either magnesium or manganese (Ke and Wang 2007b; Sung et al. 2003; Xu et al. 2000). The exact complement of metal ions that occupy this binuclear site has not been defined for any mammalian PDE since catalysis in various PDEs is preferentially supported by different metals including magnesium, manganese, cobalt, or zinc.

A zinc atom is clearly defined in most of the X-ray crystal structures of PDE catalytic domains and is present even when crystals are formed in the presence of chelators (Ke and Wang 2007b; Xu et al. 2000), but the metal ion occupying the second site has not been identified for any PDE. The X-ray structure of the refolded PDE3 catalytic domain is the only structure of a wild-type PDE protein determined thus far that lacks a zinc ion (Scapin et al. 2004). By direct chemical analysis, PDE6 has been shown to contain 3–4 zincs per dimer. Zinc is also critical for PDE6 catalytic function (He et al. 2000), but magnesium also stimulates PDE6 activity. Likewise, PDE5 binds ~6 zincs per dimer, and zinc at submicromolar levels support catalytic function, but manganese, cobalt, and magnesium at higher concentrations also support catalysis (Francis et al. 1994). The precise role of the respective metal ions in the catalytic function of each PDE is still not well understood. To exploit the effect of an inhibitor to interfere with the role of these metal ions in PDE functions, it will be critical to better define the particular metal complement that is important for catalytic function of a PDE isoform.

The volume of the PDE catalytic sites has been estimated to be ~330–450 $Å^3$, and several structures of isolated catalytic domains in complex with either the

5′-nucleotide product or the selective inhibitors are now available (Ke and Wang 2007b; Wang et al. 2007; Xu et al. 2000, 2004). Interestingly, the orientation of substrate, inhibitor, or product can vary substantially among PDEs (Wang, Liu, Hou 2007). This may be due to the relatively large volume of the catalytic site compared to the size of the substrate/product or most inhibitors. However, this variation restricts the potential for generalizations from one structure to another. Recently, X-ray crystal structures of two PDEs containing either the near-full length regulatory domain (PDE2) (Pandit et al. 2009) or a portion thereof (PDE4) (Burgin et al. 2010) have been published. Both structures provide important new insights into regulatory domain functions, and the report on the PDE4 constructs reveals novel new approaches to inhibitor design and action (see below and in Gurney et al. in Chapter 7 in this volume). However, the X-ray crystal structure of PDE2 defines the structure in the absence of cGMP, that is, the lower activity state of the enzyme, and provides only a partial picture of the functional states of PDE2 (Pandit et al. 2009). In X-ray crystal structures of isolated catalytic domains in complex with various inhibitors, substrates, or catalytic products, hydrogen bonding with an invariant glutamine and hydrophobic stacking of the ring structure of the substrate/product/inhibitor with a conserved phenylalanine (in most PDEs) are common interactions (Fig. 2) (Ke and Wang 2007a, b; Xu et al. 2000, 2004). Another group of amino acids in the catalytic pocket forms a hydrophobic face that wedges the ring structure of the ligands against the conserved phenylalanine, thereby creating what has been termed a "hydrophobic clamp." These interactions occur for most PDEs when associated with a wide spectrum of inhibitors/products that vary significantly in affinities and chemical characteristics (Ke and Wang 2007b).

For several PDE holoenzymes, the energy contribution of amino acids in the catalytic pockets to the affinity for substrate or inhibitors have been quantified using site-directed mutagenesis (Burgin et al. 2010; Cheung et al. 1998; Jacobitz et al. 1996; Jin et al. 1992; Omburo et al. 1998; Turko et al. 1999; Wang et al. 2005; Zhang et al. 2002; Zoraghi et al. 2007). It is evident from the X-ray crystallography and mutagenesis studies that different inhibitors exploit novel features in and near the catalytic sites of the respective PDEs to enhance potency and selectivity (Ke and Wang 2007b; Sung et al. 2003; Wu et al. 2004). In some instances, enhanced specificity/potency is provided by sequence(s) well outside the catalytic domain (Blount et al. 2006; Burgin et al. 2010; McPhee et al. 1999; Omori and Kotera 2006; Richter and Conti 2004; Saldou et al. 1998), but currently insights into the mechanisms that provide for this are limited.

1.4 Functional Distinctions Among PDE Catalytic Sites

1.4.1 Catalytic Characteristics

Despite strong structural similarities among the PDE catalytic sites, PDEs 4, 7, and 8 are highly specific for hydrolysis of cAMP, PDEs 5, 6, and 9 are highly specific

for hydrolysis of cGMP, and others (PDEs 1, 2, 3, 10, and 11) hydrolyze both cNs. Moreover, functional features of closely related PDEs can differ substantially, a result that is encouraging for development of specific inhibitors for each family. Some PDEs that exhibit the highest identity in amino acid sequence, for example, PDEs 5 and 11 (catalytic domains have ~51% sequence identity) have very different selectivities for cAMP and cGMP (Bender and Beavo 2006; Omori and Kotera 2006, 2007). PDE5 exhibits ~100-fold greater affinity for cGMP than for cAMP, although both are hydrolyzed at ~equal rates (Francis and Corbin 2009). In contrast, PDE11 hydrolyzes both cNs with similar affinities and efficiencies. In addition, PDEs 5 and 11 exhibit very different affinities for potent PDE5 inhibitors currently in clinical use, that is, vardenafil, sildenafil, and tadalafil; potencies of these compounds for PDE5 compared to PDE11 differ by 7,000-, 950-, and 41-fold, respectively (Weeks et al. 2007).

In another example, the amino acid sequence identity of the catalytic domains of PDEs 5 and 6, both of which are highly specific for cGMP, are very similar (~42%), but the affinity of PDE6 catalytic site for cGMP (K_m ~ 14 μM), is seven times weaker than that of PDE5 (~2 μM), and the catalytic rate of PDE6 (~2,000 μmol/min/mg) exceeds that of PDE5 by ~1,000-fold (Cote 2006; Francis et al. 2006). Moreover, PDE6, like PDE5, is potently inhibited by sildenafil, vardenafil, and zaprinast, but tadalafil, a potent PDE5 inhibitor, is a weak inhibitor of PDE6 (Zhang 2006). These data imply that subtle differences in the topography and chemical characteristics of the active site can have profound effects on substrate preference, catalytic efficiency, and inhibitor potencies.

In some instances, there are even significant distinctions among catalytic sites of PDE isoforms within the same family. For example, PDE1A, PDE1B, and PDE1C are products of three separate genes with catalytic domains that share ~75% sequence homology but have quite different selectivity for cGMP (K_m = 1 μM for PDE1C2, 3 μM for PDE1B1, and 5 μM for PDE1A2), compared to that for cAMP (K_m = 1 μM, 24 μM, and 113 μM, respectively). Nevertheless, the maximum catalytic activities of these PDEs for breakdown of cGMP and cAMP are similar (Bender and Beavo 2006).

1.4.2 Potencies of Inhibitors Within PDE Families

The potency of an inhibitor for PDEs within a family, among splice variants within a subfamily, or between cytosolic and membrane-bound forms of the same PDE can also differ significantly. For example, vinpocetine, a selective PDE1 inhibitor, more potently blocks the catalytic activity of PDE1A and PDE1B than that of PDE1C (Yan et al. 1996). The inhibitory potency of IC86340, another selective PDE1 inhibitor, varies by ~7-fold among PDE1 isoforms; IC_{50} values are: PDE1C (0.06 μM), PDE1B (0.21 μM), PDE1A (0.44 μM) (Miller et al. 2009). Moreover, potency of another PDE1-selective inhibitor, SCH51866, for several alternative

splice variants of PDE1C differs by threefold: PDE1C1 (IC_{50} = 101 nM) versus PDE1C4/5 (IC_{50} = 36 nM) (Yan et al. 1996).

PDE4 inhibitors can also exhibit quite different potencies for the various members of this family. Cilomilast, a highly selective inhibitor of the PDE4 family, exhibits significantly higher potency (7- to 27-fold) toward PDE4D (IC_{50} ~ 12 nM) compared to that for PDE4A (IC_{50} ~ 115 nM), PDE4B (IC_{50} ~ 86 nM), or PDE4C (IC_{50} ~ 308 nM) (Torphy 1998). Inhibitors that show good selectivity for PDE4D over PDE4B have also been identified: NVP-ABE171 is 20-fold selective for PDE4D over PDE4B (Trifilieff et al. 2002) and CP-671305 is 95-fold selective for PDE4D over PDE4B (Kalgutkar et al. 2004; Zhang 2006). Moreover, D159687, a compound recently reported by investigators at deCode Genetics, is highly selective for PDE4D7 versus PDE4A1, PDE4B1, and PDE4C1 (93-, 54-, and 250-fold difference in potency, respectively) (Burgin et al. 2010). The structural subtleties that provide for these differences in such closely related enzymes are typically not fully understood; they could relate to differences in the catalytic site pocket or reflect the influence of interactions of the inhibitors with regions outside the catalytic pocket as occurs for the D159687 compound or interaction with protein binding partners and posttranslational modification (Burgin et al. 2010; Houslay 2001; Houslay and Adams 2003; Houslay et al. 2005, 2007). Exploiting these differences may open novel opportunities for the development of more selective inhibitors. However, each modest advance in devising a compound that shows selectivity among PDEs is just the beginning in the efforts to develop a compound with pharmacokinetic and biochemical features suitable for clinical use.

2 Factors That Impact Catalytic Site Function and Potency of Inhibitor Action

PDE catalytic site functions can be modified by many processes, including posttranslational modifications, change in the cellular milieu (pH or redox conditions), interaction with activators (e.g., calcium/calmodulin, cGMP, or phosphatidic acid), binding to other proteins, and exposure to drugs (Burgin et al. 2010; Houslay 2001; Houslay and Adams 2003, 2010; Houslay et al. 2005, 2007). The influence of these factors on the potencies of inhibitors for any PDE is not fully understood and effects identified for a particular inhibitor frequently do not apply to other inhibitors of that PDE family. Consequently, environmental or posttranslational effects on each inhibitor must be assessed independently. The state of oligomerization of a PDE may also be a determinant of the pharmacological properties of an inhibitor (Burgin et al. 2010; Richter and Conti 2004; Rybalkina et al. 2010). Moreover, it is clear that insights derived from structural studies of isolated catalytic domains of PDEs do not always reveal all of the important contact points that could be exploited in the design of new inhibitors (Blount et al. 2006; Burgin et al. 2010).

2.1 Comparison of Native and Recombinant Enzymes

A recurrent finding when investigating the properties of various PDEs is that the kinetic behavior of a recombinant, ectopically expressed enzyme does not entirely overlap with that of its endogenous native counterpart (Bolger et al. 1993; Livi et al. 1990; McPhee et al. 1999; Obernolte et al. 1993; Salanova et al. 1998; Saldou et al. 1998; Sullivan et al. 1994; Wang et al. 1997). The differences include affinity for substrate, catalytic efficiency, and inhibitor potencies (Obernolte et al. 1993; Salanova et al. 1998; Wang et al. 1997). Certainly, some differences are due to the fact that native PDE preparations are often heterogeneous in terms of enzyme composition (Obernolte et al. 1993). For many PDEs, the physicochemical properties of the various isoforms are not sufficiently different to allow for complete separation with even the most current chromatographic methods. More frequently, differences are associated with the heterologous expression system used (i.e., bacteria, yeast, insect cells, or mammalian cell lines) and the posttranslational modification of the recombinant proteins (Hoffmann et al. 1998; Saldou et al. 1998). Aberrant phosphorylations, different states of phosphorylation, or other modifications such as ubiquitination or proteolytic cleavage of overexpressed proteins have all been reported for heterologously expressed PDEs. Heterogeneity in the conformation of some recombinant proteins is an additional major variable. Overexpression of recombinant PDEs often produces proteins that are improperly folded, aggregated, or catalytically inactive (Rocque et al. 1997; Scapin et al. 2004; Wang et al. 1997). The presence of catalytic domains in different conformations due to posttranslational modification and interaction with partner proteins can confound the kinetics of inhibition of enzyme preparations with potencies reported for rolipram inhibition of PDE4 isoforms ranging from 0.01 to 10 μM (Hoffmann et al. 1998; Houslay and Adams 2003; Huston et al. 1996; Laliberte et al. 2000; McPhee et al. 1999; Rocque et al. 1997; Salanova et al. 1998; Tian et al. 1998). Proteolysis of overexpressed enzymes may also alter interaction with inhibitors without affecting catalytic activity (see below).

2.2 Truncated Versus Full-Length PDEs

An additional property to consider is whether truncated forms of PDEs recapitulate the properties of the full-length enzymes. Two examples involving PDEs 4 and 5 are provided to expand upon this concept. Rolipram, as well as some other compounds, inhibits isolated catalytic domains of PDE4s with potencies that are often 10- to 100-fold lower than those obtained with full-length enzyme (Jacobitz et al. 1996; Richter and Conti 2004; Tian et al. 1998). Progressive truncation at the amino terminus of PDE4B and PDE4A causes a parallel decrease in the affinity for rolipram (Jacobitz et al. 1996; Richter and Conti 2004; Rocque et al. 1997; Saldou et al. 1998). The potencies of three inhibitors (RS-25344, rolipram, and TVX 2706) for inhibition of the isolated catalytic domain of PDE4D are much weaker than for

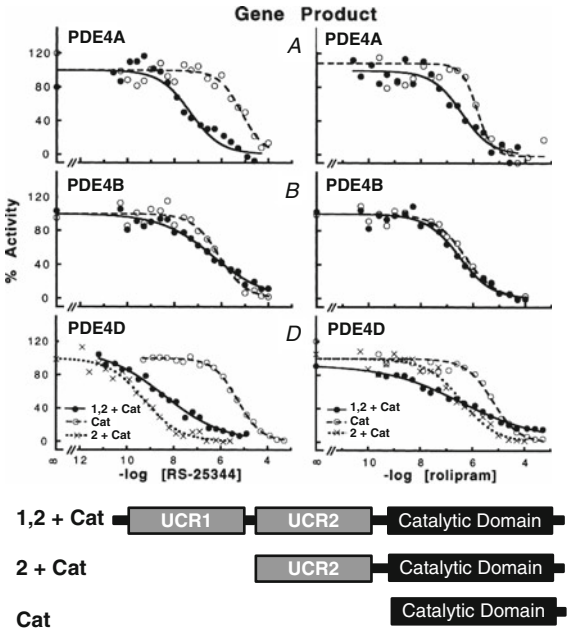

Fig. 3 Comparison of the potency of inhibition of cloned forms of PDE4 gene products with rolipram and RS-25344. Representative data showing the inhibition of PDE4A, 4B or 4D activity (*gene product A, B and D*, respectively) by RS-25344 (*left*) or rolipram (*right*). A cartoon model depicts the functional domains in the respective long (1,2 + Cat) forms of PDE4 proteins containing UCR1, UCR2, and the catalytic domain, the short (2 + Cat) forms of the enzymes containing UCR2 and the catalytic domain, as well as that of the isolated catalytic domain (Cat). Each data point represents the average of quadruplicates, and the curves are plotted as a percentage of the difference between the maximum and minimum rates of each enzyme. Reprinted from Saldou et al. (1998)

the full-length enzyme, but stronger potencies are exhibited in constructs where UCR2 is conjoined with the catalytic domain (Fig. 3) (Saldou et al. 1998). Likewise, Burgin and coworkers recently reported that two inhibitors (RS25344 and PMNPQ) are 10,000 times more potent toward inhibition of PDE4D7 than against the isolated PDE4 catalytic domain (Burgin et al. 2010). It is now clear that these truncations remove important regulatory/oligomerization domains in the holoenzyme that affect interdomain contacts and impact the conformation and/or functions of the catalytic domain (Burgin et al. 2010; Houslay 2001; Houslay and Adams 2003, 2010; Richter and Conti 2002). It has been observed that some variants of PDE4s, termed short forms, have lower affinity for rolipram and kinetic properties distinct from those of longer forms (see below). Since short and long forms are expressed in a tissue- and cell-specific manner, this must be considered when evaluating the potency and biological action of selected compounds.

Likewise, the inhibitory potency of the PDE5 inhibitor, vardenafil, for the PDE5 holoenzyme exceeds that of sildenafil by 10- to 40-fold (Blount et al. 2004, 2006).

The potency of sildenafil for inhibition of the isolated catalytic domain is essentially the same as that for the PDE5 holoenzyme, but the potency of vardenafil inhibition of the isolated catalytic domain is 10- to 40-times less than that for the holoenzyme. In fact, it has the same potency for inhibition of the isolated catalytic domain as that of sildenafil. In the X-ray crystal structures of the PDE5 catalytic domain in complex with the respective inhibitors, the contacts between each of the inhibitors and the catalytic site are very similar (Sung et al. 2003; Wang et al. 2006). The higher potency of vardenafil is retained in constructs of the catalytic domain conjoined with GAF-B indicating that in some instances structural features outside the catalytic domain contribute importantly to inhibitor potency and selectivity (Blount et al. 2006). Thus, comparing potencies of sildenafil and vardenafil, two closely related compounds, or predicting potencies using only the isolated catalytic domain would have missed important elements that contribute to the higher potency of vardenafil for the holoenzyme.

Given that many compound screens have been performed using truncated PDEs that lack their regulatory domains and because different PDE isoforms are characterized by subtle differences in conformation, this approach may have hindered identification of more selective compounds for certain PDE families and variants within PDE families.

2.3 Posttranslational Modification and Occupancy of Allosteric Sites

Posttranslational modification of a PDE may have profound effects on the potency of selected groups of compounds. This has been clearly demonstrated for both the PDE4 and the PDE5 families. PKA phosphorylation of PDE4 affects the potency of several compounds in a complex manner (Alvarez et al. 1995; Burgin et al. 2010; Hoffmann et al. 1998; Houslay and Adams 2003, 2010; Laliberte et al. 2002). PKA phosphorylation of long PDE4 isoforms, in general, increases the affinity for the prototypical inhibitor rolipram and alters the kinetics of this inhibition (Fig. 4). In the same vein, phosphorylation of PDE4D3 increases its sensitivity to inhibition by RS-25344 (~100-fold) and RS-33793 (~330-fold) and phosphorylation of PDE4A4 increases the potencies of (*R*)- and (*S*)-rolipram but does not affect the potencies of CDP-840 or SB-207499. Screening for compound potency with a phosphorylated PDE4 isoform is a strategy that has been adopted to identify compounds with high potency, but this has had little success in improving the therapeutic window of this class of compounds. Phosphorylation also modulates PDE5 enzyme functions, with detectable changes in the conformation of the enzyme (Bessay et al. 2008; Corbin et al. 2000). These changes in conformation, in turn, are associated with an increase in the affinity of PDE5 for substrate as well as for inhibitors, such as sildenafil. The concept has been proposed that when PDE5 inhibitor is present in cells, cGMP level and phosphorylation of PDE5 by PKG is increased, which in turn increases PDE5 inhibitor binding at the catalytic site, that

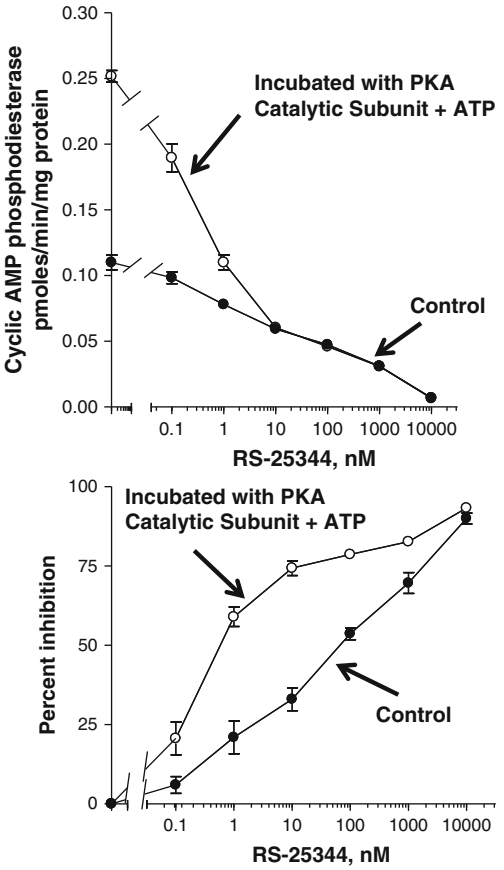

Fig. 4 Effect of phosphorylation on PDE4D3 sensitivity to inhibition by RS-25344. Phosphorylation causes a marked shift of the sensitivity of PDE4D3 to inhibition by RS25344. PDE4D3 was preincubated in the presence (*open circles*) or absence (*closed circles*) of the catalytic subunit of protein kinase and Mg/ATP. In *panel A*, the ordinate represents specific activity. In *panel B*, the ordinate represents percent inhibition calculated from the results shown in *panel A*. Values represent the value ± standard deviation of triplicate determinations. Data presented are from a representative experiment that was repeated three times with similar results. Reproduced from Alvarez et al. (1995)

is, a feed-forward effect on inhibitor binding and potency (Bessay et al. 2008; Corbin and Francis 1999; Francis and Corbin 2005; Francis et al. 2008). A similar regulatory loop likely applies to PDE4 isoforms that are phosphorylated by PKA, that is, inhibition of PDE4 would increase cAMP and activation of PKA, which would catalyze more extensive phosphorylation of PDE4 to produce higher affinity for certain inhibitors. Positive feedback loops, such as these for PDEs 4 and 5, will clearly impact the range of potency determined for compounds that are currently under development as well as their pharmacokinetic properties.

2.4 Interaction with Other Proteins

For many years, PDE activity in cells was referred to either as soluble or particulate, but in most cases, there has been little understanding of either the importance or the molecular basis for this partitioning. In fact, members of the same PDE families could be found in both the cytosolic and the particulate fractions. Such complexes occur both at particular cell membranes and within the cytosolic fraction. More recently, it has become apparent that one reason for the diversity of PDEs in most tissues is to provide for their selective interaction with other proteins in signaling complexes allowing dynamic regulation of particular signaling processes and thus giving rise to the concept of "targeted cAMP degradation" (Houslay 2001, 2010). Indeed, it was initial studies in cardiac myocytes that provided a major building block for our understanding of compartmentalized cAMP signaling and its mechanistic implications (Corbin et al. 1977; Hayes et al. 1979; Steinberg and Brunton 2001; Zaccolo and Pozzan 2002).

Selective and diverse compartmentalization of a certain portion of the isoforms within a PDE family has now been well described for PDEs 1, 2, 3, 4, 5, 6, and 10. It seems highly likely that interaction of these PDEs with other proteins to form signaling complexes will affect inhibitor potencies. Indeed, this has been clearly demonstrated for various PDE4 partnerships, such as PDE4A4 with various SH3 domain containing proteins (McPhee 1999); PDE4D5 with RACK1 (Yarwood et al. 1999) and PDE4A4 with the aryl hydrocarbon interacting protein (AIP) (Bolger et al. 2003), where recent structural studies have afforded a potential molecular explanation for such actions (Burgin et al. 2010).

In addition, the determinants that dictate the proportion of a PDE family that is localized to particular intracellular domains or "free" in the cytosol are only beginning to be understood (Houslay 2010) and include competition by different scaffolds for binding specific PDEs and dynamic changes in association patterns that are triggered by phosphorylation (Collins et al. 2008; Murdoch et al. 2007) and ubiquitination (Li et al. 2009). Moreover, when "free" PDEs are translocated from the cytosol to a more restricted and defined locale, the impact on the cN metabolism in the cellular territory that was vacated has yet to be formally considered. Are there "pools/banks" of PDEs that are simply not needed in normal cellular functioning and await a call for action as needed in particular locales in the cell? Are these meaningful pools of PDE activity that should/could be targeted by inhibitors? The very different actions of inhibitors selective for different cAMP-hydrolyzing PDEs, such as PDEs1/2/3/4, were originally interpreted as reflecting actions related to the relative abundance of such enzymes in cells. However, it is now clear that functionally distinct pools of cAMP regulated by targeted PDEs is critical in controlling key cellular processes in cells. The most conclusive proof for a specific mechanism of subcellular targeting comes from studies on PDE4s (Houslay 2001, 2010). Critical to this concept was the discovery that PDE4A1, an enzyme that is entirely membrane bound, is generated by splicing a membrane-targeting domain located within its isoform-specific amino-terminal amino acid sequence to a segment of sequence

containing the regulatory and catalytic domains, that on its own is fully soluble, fully active, and common to all members of the PDE4A subfamily (Baillie et al. 2002; Huston et al. 2006; Scotland and Houslay 1995; Shakur et al. 1993; Smith et al. 1996).

Targeting of PDEs to particular regions of the cell is often dependent on interaction with other proteins. Myriad proteins have been identified that interact with the PDE4 family as a whole by binding to sites within core regions that are conserved across the entire family or are specific for particular PDE4s by binding to regions that are only common within specific subfamilies (Houslay 2001, 2010; Houslay et al. 2007). Such targeting allows particular PDE4 isoforms to associate with specific signaling complexes and control the local cAMP level. This targeting of specific PDEs underpins compartmentalized cAMP signaling, controlling the threshold and persistence for activation of PKA and EPAC, for example, within the environs of the complex containing the particular PDE that is sequestered there (see e.g., Huston et al. 2008). Indeed, the use of spatially constrained, genetically encoded cAMP reporters has allowed defined "pools" of cAMP to be detected and the role of certain sequestered PDEs to be shown as pivotal in establishing and maintaining cN gradients in cells (Mongillo et al. 2004, 2006; Penmatsa et al. 2010; Rich et al. 2001a, b, 2006; Zaccolo and Pozzan 2002).

PDE3A has also been shown to specifically form complexes with a variety of proteins including 14-3-3 proteins, plectin, brefeldin A-inhibited guanine nucleotide exchange proteins, and the cystic fibrosis transmembrane conductance regulator channel (CFTR); in some instances, these localizations have also been shown to control local pools of cAMP (Barnes et al. 2005; Mongillo et al. 2004; Penmatsa et al. 2010; Puxeddu et al. 2009; Tasken et al. 2001). Likewise, a portion of platelet PDE5 has been shown to form a complex with PKG and be selectively activated by elevation of cGMP, thereby regulating both local cGMP level and calcium transients (Wilson et al. 2008). The presence of PDE5 in cardiomyocytes is controversial (Lukowski et al. 2010; Vandeput et al. 2009). However, some investigators report it to be present at the z-bands along with PKG, and their results suggest that this localization of PDE5 is required for the antihypertrophic effects of sildenafil in the heart (Kass et al. 2007; Nagayama et al. 2008; Takimoto et al. 2005a, b). PDE7A1 and the C subunit of PKA have been shown to interact with high affinity ($K_D \sim 0.5$ nM), thereby blocking the catalytic activity of the C subunit, but the effect of this interaction on catalytic function/inhibitor sensitivity of the PDE is not known (Han et al. 2006).

Targeting a specific PDE isoform to a particular intracellular locale can be expected to confer a functional role on that isoform that is inherently associated with its unique spatial sequestration (Houslay 2010). This property cannot be gauged by the use of selective inhibitors, genetic ablation (Jin et al. 1999), or siRNA knockdown (Lynch et al. 2005) since each of these approaches will target both the sequestered and free populations of that PDE. Instead, the use of dominant-negative constructs has been successfully exploited to address this problem (Baillie et al. 2003; Lynch et al. 2005; McCahill et al. 2005). In this approach, a single point mutation that ablates catalytic activity while retaining overall structural integrity of the PDE is engineered. Overexpression of such a catalytically inactive PDE

construct in cells displaces the cognate, endogenous, active, and sequestered form from its site of action without affecting the activity of free populations of that PDE. In so doing, local cAMP or cGMP concentration around the complex involving the sequestered PDE will selectively rise, thereby generating a unique phenotypic signature that mimics inhibition of the sequestered PDE. It will be of great interest if either small molecules or peptidomimetics that disrupt specific PDE partnerships can be identified for therapeutic advantage. Certainly proof of principal has been garnered for this approach by the development of dominant negative constructs for PDE4 isoforms as elucidated above (Lynch et al. 2005) and use of cell-permeable peptides that duplicate the binding surface of one partner and thus disrupt specific PDE partnerships in intact cells (Murdoch et al. 2007; Smith et al. 2007).

The concept that PDEs interact with and are regulated by other proteins was first discovered from studies of the PDE1 family, which is regulated by reversible interaction with calmodulin, and the PDE6 family, which is located in photoreceptor rod and cone cells and regulated by interaction with the small inhibitory Pγ proteins and activated transducin. For other PDE families, appreciation of this type of regulation is relatively new. Consequently, the focus in studying protein interactions involving most PDEs has been devoted to identifying protein partnerships, defining modes of interaction, and appreciating the functional significance of such interactions. The paradigm for PDE partner proteins that profoundly regulate PDE catalytic site functions is well illustrated by control of PDE6 cN-hydrolyzing activity through the direct interaction of its catalytic subunits with its inhibitory Pγ-subunits and the effect of transducin in the activated state to bind to PDE6γ in that complex, thereby relieving the PDE6γ inhibitory effects (Bender and Beavo 2006). Elegant biochemical and structural studies have recently allowed the molecular basis of this to be determined (Barren et al. 2009; Zhang et al. 2009). Two distinct types of interactions between Pγ and PDE6 catalytic subunits that provide for the potent inhibition of PDE6 catalysis have been proposed. One set of interactions involves direct contact of the carboxyl-terminal residues of Pγ with the PDE6 catalytic pocket, thereby blocking cGMP entry. The second set of interactions involves binding of other regions of Pγ to the PDE6 catalytic subunit, so as to attenuate catalytic activity in an allosteric manner. Thus, activation of PDE6 by the GTP-bound form of transducin apparently requires interaction with the carboxyl-terminus of PDE6γ as well as additional regions of PDE6γ to relieve the inhibitory constraint on the PDE6 catalytic subunits. Proteins that interact with members of other PDE families may also employ the strategy of multiple contacts that diversely influence enzymatic activity, specific localization, and functional features; such complexities should always be considered.

The regulation of PDEs by protein–protein interactions has been explicitly and elegantly addressed in studies performed with PDE4, where a large number of binding partners have been identified (Houslay 2010). It is already appreciated from phosphorylation studies of PDE4 isoforms (described above) that conformational changes induced by phosphorylation are associated with altered activity and sensitivity to some inhibitors (Conti et al. 2003; Houslay and Adams 2003; Houslay et al. 2005). Four PDE4 genes (A/B/C/D) encode around 25 isoforms that are characterized by isoform-specific amino-terminal regions. These are then grouped based

upon the presence of regulatory UCR domains (UCR1 and UCR2), with long forms having both UCRs, short forms having UCR2, super-short forms having a truncated UCR2 and the catalytically inactive, dead-short isoforms having neither (Bolger et al. 2006; Conti and Beavo 2007; Houslay 2001, 2010).

While the structure of the catalytic domains from all four PDE4 subfamilies is known (Ke and Wang 2007a), until very recently nothing has been known about the structure of the UCR regulatory domains or interactions of UCRs with the catalytic domain. A recent report documents the X-ray crystal structure of UCR2, a helical structure that can bind adjacent to and over the PDE4 catalytic pocket, thereby gating cN substrate access (Burgin et al. 2010). This gives a structural basis to the previous biochemical evidence and proposal that UCR2 provides an autoinhibitory domain (Lim et al. 1999) and for previous observations that PDE4 enzymes can adopt distinct conformational states that vary in sensitivity to certain inhibitors (Houslay 2001; Houslay and Adams 2003; Houslay et al. 2005). Burgin et al. used information derived from a collection of X-ray crystal structures of PDE4 isoenzymes, molecular modeling, site-directed mutagenesis, and systematic synthetic chemistry to produce the novel small molecule inhibitors of PDE4D. These are bifunctional compounds that form contacts with both the catalytic site and the UCR2, thereby locking the enzyme in the inactive state; the double set of contacts increases points of interaction with PDE4, thereby enhancing inhibitory potency (Burgin et al. 2010; Houslay and Adams 2010).

The diverse interactions of various inhibitors with PDE4 are exemplified by RS25344 and roflumilast. RS25344 interacts with the gating sequence, which stabilizes the UCR2-capped state (Burgin et al. 2010), whereas roflumilast, which has recently been approved in Europe for treatment of chronic obstructive pulmonary disease (COPD) (see Tenor et al. 2011), occupies the uncapped catalytic pocket and interacts minimally with residues in the UCR. These discoveries provide the beginnings of insight into the complex inhibition kinetics of compounds, such as rolipram, that may have different affinity for the catalytic pocket in the UCR2-uncapped and UCR2-capped states. This discovery also offers a molecular explanation (Burgin et al. 2010) for the reported observations that PKA phosphorylation alters the inhibitor sensitivity (Hoffmann et al. 1998; Sette and Conti 1996a), for example, by stabilizing an uncapped, activated state. Some of the new compounds produce only partial inhibition (80–90%) of catalytic activity, which may contribute to their actions in the biological setting. The proximity and arrangement of regulatory domains of other PDEs with respect to their catalytic sites as well as their mechanisms of activation/autoinhibition will likely dictate whether this innovative approach to drug design is useful for other PDEs or restricted to the PDE4 family.

The recent structural insights for UCR2 interaction with the PDE4 catalytic unit (Burgin et al. 2010) will undoubtedly stimulate new lines of research and drug discovery relating to PDE4. However, it is also likely to provide a stimulus to understanding of the PDE superfamily as a whole, where paired regulatory regions, for example, GAFs, located amino-terminal to the catalytic domain are common. Solution of the X-ray crystallographic structure of the PDE2 holoenzyme has suggested a different mechanism of regulation of catalytic activity (Pandit et al.

2009). Thus, questions still awaiting an answer include whether distinct mechanisms of regulation of the catalytic domain are present in PDEs, how these interactions might be altered by either protein–protein interaction, phosphorylation, or other modifications, the physiological significance of this and how this all might be exploited to generate novel therapeutics. Also, despite the historical importance of PDE1 in understanding calcium/calmodulin activation of cAMP and cGMP degradation (Bender and Beavo 2006; Goraya and Cooper 2005), there are still no structural insights into the mechanisms whereby the calcium/calmodulin complex interacts with the paired binding domains in PDE1 to elicit activation of this enzyme.

2.5 Exposure to Drugs

PDEs 5 and 4 provide two examples of PDEs that exhibit different affinities for family-selective inhibitors that are impacted by the history of exposure of these proteins to inhibitors.

PDE5 holoenzyme exhibits biphasic affinity for interaction with vardenafil, sildenafil, and tadalafil, that is, a low-affinity site and a high-affinity site, although the inhibitors interact only with the PDE5 catalytic site. Prolonged exposure of PDE5 holoenzyme to these inhibitors converts the kinetics of the low-affinity interaction to that of the high-affinity site (Fig. 5) (Blount et al. 2007; Rybalkina et al. 2010). This shift in affinity for the inhibitors indicates a functional transition that is associated with a physical change in the protein following inhibitor binding. It does not occur in the isolated catalytic domain, but when GAF-B is appended to the catalytic domain, the shift in affinity is like that observed in the holoenzyme. Thus, the affinity of PDE5 for these inhibitors is mediated by cGMP-dependent and cGMP-independent mechanisms, the latter being an effect of the inhibitor itself. Recently, it has been suggested that aggregation of PDE5 fostered by high concentrations of the enzyme in particular tissues may also impact affinity for certain inhibitors (Rybalkina et al. 2010). These observations emphasize once again the importance of not only examining inhibitor potencies within the context of PDE holoenzymes but also with consideration of the molecular status and concentration of the PDE within local environs in the cell.

Interaction of PDE4 isoenzymes with the inhibitor rolipram and related compounds (such as RS-25344) has also uncovered both high- and low-affinity states (Alvarez et al. 1995; Souness and Rao 1997). It should be noted that this is not a generalized property, as inhibitors derived from other classes of compounds, for example, piclamilast, do not share this property and behave as simple competitive inhibitors of PDE4s (Tian et al. 1998; Zhao et al. 2003). This complexity of rolipram interactions with PDE4s was originally uncovered by measurements of isotopically labeled rolipram binding to the two conformation states termed *H*igh-*A*ffinity *R*olipram-*B*inding *S*ites (HARBS) and *L*ow-*A*ffinity *R*olipram-*B*inding *S*ites (LARBS) (Houslay and Adams 2003; Jacobitz et al. 1996; Rocque et al. 1997; Souness and Rao 1997; Tian et al. 1998). Both have been thought to reflect

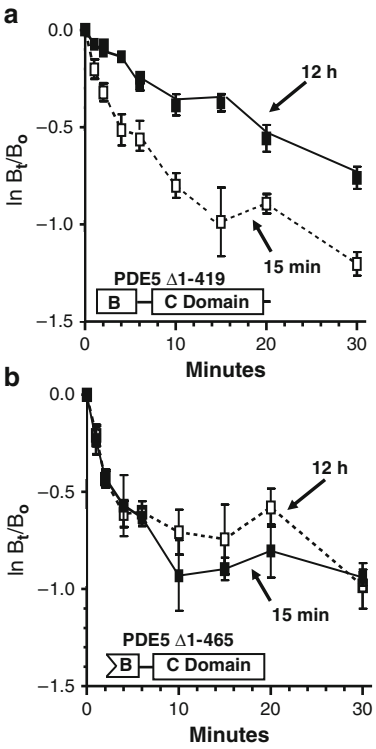

Fig. 5 Exchange-dissociation of [^{3}H]tadalafil from PDE5Δ1-419 but not PDE5Δ1-465 is affected by time of preincubation with the inhibitor. PDE5Δ1-419 (*top*) or PDE5Δ1-465 (*bottom*) (80 μl; 0.035 nM final) was added to 4.5 ml of binding reaction mixture containing [^{3}H]tadalafil (30 nM final concentration) after being preincubated for either 15 min or 12 h at 4°C. The zero time point (B_o) was determined as described in Blount et al. (2007). Following 15-min or 12-h preincubation of the respective constructs with [^{3}H]tadalafil, the stoichiometry of inhibitor binding (B_o) was not significantly different. Approximately 30 μl of a 1 mM tadalafil solution was then added to the remaining incubating binding reaction mixture at 4°C. Aliquots were removed at various times (B_t) and filtered by the same procedure at the indicated time points

characteristics of apparently different conformations of the same catalytic site; however, results of recent X-ray crystal structures of various constructs of PDE4 isoforms in complex with certain inhibitors (described above) reveal that the high-affinity interaction of some inhibitors results from direct contacts with the catalytic pocket as well as with UCR2 (Burgin et al. 2010; Houslay and Adams 2010).

The molecular factors that determine whether the HARBS or LARBS form predominates for a particular inhibitor have not been unequivocally identified, nor has it been established whether these states are interconvertible, as occurs when inhibitor binding to the PDE5 catalytic site shifts the low-affinity site to the high-affinity site. The chemical characteristics of the inhibitor, the contacts that it forms with the catalytic site and/or other regions of the PDE, as well as the oligomerization state, degree of phosphorylation, or metal-ion occupancy of the catalytic center

have all been implicated in stabilizing these dual states (Laliberte et al. 2000). A consistent finding is that the high-affinity conformation is usually reduced in truncated PDE4 enzymes, indicating that the amino terminus of PDE4 exerts important constraints on the conformation of the catalytic domain (Conti et al. 2003). Whether these sites can be flexibly interconverted or are "fixed" in perpetuity will impact considerations regarding future design of PDE inhibitors targeting these PDEs. The dual-affinity state of PDE4s has been used in the PDE4 field as a guiding criterion to predict therapeutic windows of PDE4 inhibitors under development with respect to undesirable side effects of PDE4 inhibitors that have been associated with HARBS. It seems likely that isoforms of other PDE families will also have multiple conformations that exhibit varied affinities for particular inhibitors.

3 Design of PDE Inhibitors

3.1 Inhibitor Design

Emerging appreciation of the many factors that contribute to the physiologically relevant action of PDEs has expanded options for development of new inhibitors. Medicinal chemists are now setting goals for design of a spectrum of inhibitors that selectively target either particular PDE catalytic sites, individual allosteric sites such as the GAFs in PDEs 2, 5, 6, 10, or 11, UCRs in PDE4, a combination of the catalytic sites and regulatory domains, or sites that provide for PDE interactions with proteins/lipids to localize the PDE to particular regions of the cell (Burgin et al. 2010; Keravis and Lugnier 2010; Verhoest et al. 2009). Even with the availability of a number of clinically approved inhibitors that are selective and potent for a particular PDE family, for example, PDE5, development of a different class of inhibitors may be needed to address specific pharmacokinetic needs in optimizing use in diverse medical regimens, for example, compounds with improved bioavailability, slowed clearance, improved stability, brain penetration, etc. (Owen et al. 2009). Moreover, new classes of inhibitors that block only a portion of a particular PDE activity may have merit that has not been appreciated previously (Burgin et al. 2010).

Traditionally, medicinal chemists have generated compounds that directly compete with cN substrate for access to the catalytic sites of PDEs. This has primarily been achieved by systematically modifying a chemical scaffold derived from compounds known to interact with or inhibit PDE catalytic activity. The trial-and-error approach using myriad derivatives of a lead scaffold has successfully produced numerous potent and selective PDE inhibitors with diverse structural characteristics; many of these compounds incorporate the purine of the cNs as the basic scaffold with the goal of developing inhibitors that are substrate mimics but that also include additional elements to enhance affinity and selectivity for a particular group of PDEs (Fig. 6). However, selective inhibitors for most PDE families are still not commercially available despite the great need for their use in biochemical investigations and clinical settings.

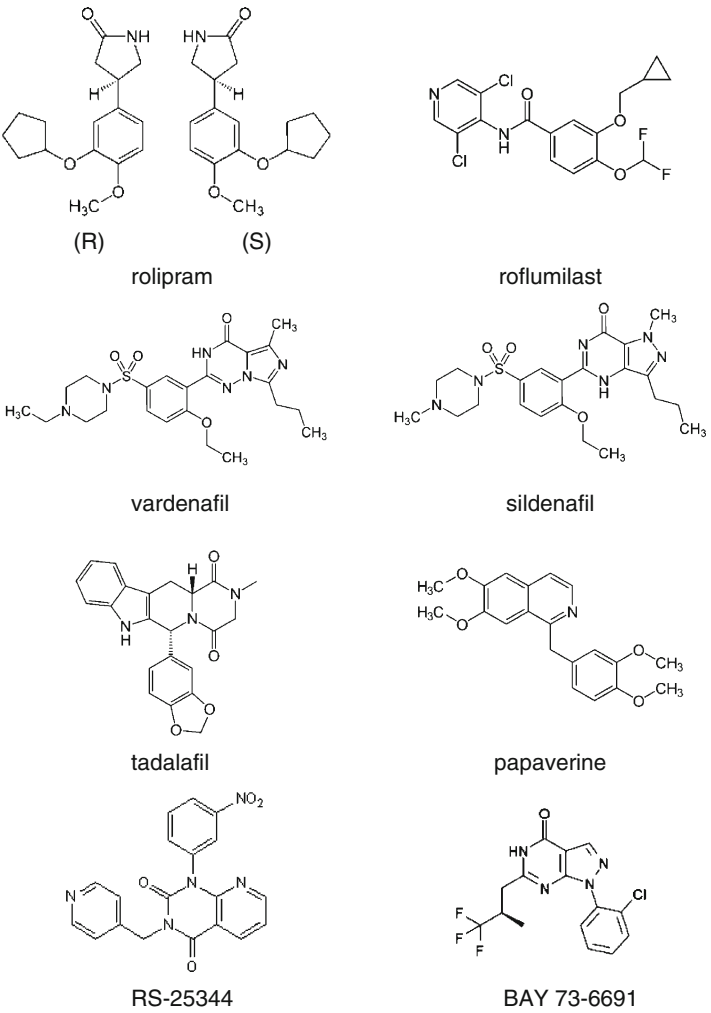

Fig. 6 Structure of the most common nonselective or family-specific PDE inhibitors. Rolipram, Roflumilast and RS25344 are PDE4-selective. The two enantiomers of rolipram [(*R*)-(−)-rolipram (the more potent enantiomer of rolipram) and (*S*)-(+)-rolipram (the less potent enantiomer of rolipram)] are indicated as (*R*) and (*S*), respectively. Vardenafil, sildenafil, and tadalafil are PDE5-selective, although vardenafil and sildenafil are potent inhibitors of PDE6. BAY 73-6691 is selective for PDE9. Papaverine is nonselective but has high affinity for PDE10

Guided by insights derived from X-ray crystal structures of various PDE catalytic sites as well as GAFs and UCR domains, chemists are now systematically exploiting features that provide for novel drug designs exhibiting greater specificity and potency. Structural information that is available for many isolated catalytic domains reveals new insights into specific topographical features of particular catalytic sites. Much effort is now being devoted to utilization of this new information for

development of more selective inhibitors, for example, development of inhibitors that selectively target the catalytic sites of the respective PDE4 isoforms, which have clear clinical implications. Although this approach has been slow to produce better inhibitors, some progress is being made as evidenced by the recent emergence of a specific, a potent PDE10 inhibitor (Verhoest et al. 2009), an improved PDE5 inhibitor (Owen et al. 2009), and new PDE4 inhibitors (Burgin et al. 2010).

3.2 Inhibitor Selectivity and Potency

The goals involved in generating desirable PDE inhibitors include selectivity and potency. Selectivity is a key feature in most instances; typically, a compound that exhibits potency for one PDE that is 50- to 100-fold greater than potency of inhibition for other PDEs is deemed to be a "specific" inhibitor. However, this measure is not always rigorously applied in the literature. Although sildenafil and vardenafil are commonly referred to as PDE5-selective or PDE5-specific, they also potently inhibit PDE6 (Zhang et al. 2005). This close overlap in function confounds use of these compounds in studies involving visual transduction or pineal functions. The restriction of PDE6 expression to photoreceptor cells and the pineal gland minimizes the issue surrounding this lack of selectivity between these two families, but it should be kept in mind for studies involving neuronal tissues or other tissues, where PDE6 might be expressed (Bazhin et al. 2010). Likewise, even when an inhibitor meets the criteria of being defined as "specific" for one PDE versus another, partial inhibition of the other PDE may come into play in the physiological setting (Lukowski et al. 2010).

Potency, while generally desirable, may have reasonable limitations. A potent inhibitor is likely to require lower doses to achieve the desired effect thus translating into lower drug exposure for the patient and lesser chance for unanticipated reactions. However, to inhibit a meaningful portion of the activity of a PDE in a tissue, there will have to be sufficient circulating inhibitor to block a significant portion of that activity. It has been conjectured that high-affinity interaction between an inhibitor and a PDE could foster increased concentration of the inhibitor in the cell until the PDE is saturated, that is, the PDE would simply soak up the inhibitor as long as high-affinity sites are available (Francis et al. 2008). In these cases, the absolute concentration of the PDE in a particular cell would determine the amount of the high-affinity inhibitor localized to the cellular milieu.

4 Interactions of PDE Inhibitors with Multiple PDEs or Non-PDE Proteins

New drugs are typically designed to selectively interact with one targeted protein, and PDE inhibitors are no exception. However, the molecular scaffold of many PDE inhibitors mimics the purine in cAMP and cGMP. Given the number of

cellular proteins that interact with purine-containing compounds, cross-reaction is always a consideration, and detection of such unintended interactions can be challenging. Moreover, PDE inhibitors whose structures or components of these structures that do not mimic the purine in cNs have also been found to interact with non-PDE cellular proteins. When possible, employing several PDE inhibitors with different structures in a study significantly strengthens the validity of the interpretations. Concepts based on biochemical results associated with use of one inhibitor may need to be revisited if other interactions are subsequently found (Ohshiro et al. 2008; Taniguchi et al. 2006; Thompson 1991).

4.1 Interaction with Multiple PDEs

Recently, consideration has been given to the potential clinical advantages of combining administration of compounds that are selective for different PDEs, utilizing compounds that inhibit two PDEs, that is, dual-selective compounds, or use of more nonselective compounds that inhibit different PDEs (Giembycz 2006). For instance, inhibitors that block either PDE5 or PDE1 appear to impede pathological remodeling in vascular smooth muscle and cardiac tissue, although the relative roles of these PDEs is yet to be determined (Kang et al. 2003; Kass et al. 2007; Kim et al. 2001; Miller et al. 2009; Rybalkin et al. 1997, 2003; Takimoto et al. 2005a; Zhu and Strada 2007). However, the potential to combine selective inhibitors for both PDE families as a therapy to block the remodeling merits further study. The rationale for the use of either dual-selective or nonselective inhibitors is that relatively low concentration of such an inhibitor could partially block several PDEs, for example, PDE5/PDE1, PDE4/PDE1, PDE4/PDE3, etc. in a given tissue, to produce significant increases in cNs. In contrast to the concept described above of nearly absolute specificity of a particular PDE to control cAMP or cGMP in a particular locale, this approach assumes that at least in some situations multiple PDEs work jointly to affect cN concentrations. The hypothesis suggests that moderate blockage of several PDEs might produce modest, but physiologically relevant, changes in cN without incurring the adverse effects commonly associated with more marked changes in cN in either the target or the nontarget tissues when a specific inhibitor more completely blocks a particular PDE (Giembycz 2006). This is conceptually similar to the potential advantages of partial inhibition of a particular PDE described above (Burgin et al. 2010).

4.2 Interaction with Other Proteins in the cN-Signaling Pathway

Use of compounds designed to block PDE action as well as activating other proteins in the cN-signaling pathway is also worthy of consideration. cN analogs commonly function not only as activators of particular cN-dependent protein kinases, cN-gated channels, or EPACs, but also as inhibitors of certain PDEs; many cN analogs are

highly resistant to breakdown by PDEs (Beltman et al. 1995; Butt et al. 1995; Poppe et al. 2008; Sekhar et al. 1996). Low doses of such compounds would be predicted to work on two target proteins to synergistically foster increased cN signaling through that pathway. Examples include YC-1 and BAY 41-2272, both of which are NO-independent activators of soluble guanylyl cyclase and act to elevate cGMP in platelets, inhibit platelet aggregation, and lower blood pressure (Friebe et al. 1998; Galle et al. 1999; Mullershausen et al. 2004; Stasch and Hobbs 2009); each also acts as a PDE inhibitor. Thus, the biological actions result from the simultaneous increase in cGMP production and inhibition of cGMP breakdown. The combined effects of such compounds at two points in a pathway would foster a synergistic increase in cN signaling and potentially produce meaningful changes in signaling even at low ligand concentrations.

4.3 Interaction with Other Proteins

Interaction of PDE inhibitors with proteins other than PDEs is a serious problem that is quite commonly ignored by investigators. However, this oversight compromises many studies since many investigators accept the hyperbole asserting that compounds are truly "specific and selective" for the targeted PDE.

The use of zaprinast, which has long been touted as a selective inhibitor for PDE5/PDE6, is a prime example of such a problem. The interpretation of the results of the vast majority of studies where zaprinast has been used attributes its effects entirely to inhibition of PDE5. Its action to also inhibit PDE1 with reasonable potency is largely ignored. However, zaprinast has recently been shown to be a potent agonist for GPR35 (EC_{50} as low as 16 nM), a widely expressed receptor for kynurenic acid (Ohshiro et al. 2008; Taniguchi et al. 2006). In contrast to the widely and erroneously accepted action of zaprinast to exclusively inhibit PDE5 (IC_{50} ~ 130 nM), promote elevation of cNs and lower calcium, its action on GPR35 inhibits adenylyl cyclase activity and induces intracellular calcium mobilization. Notably, these effects are not mimicked by two potent PDE5/PDE6 inhibitors that structurally differ from zaprinast. In referring to the earlier studies, the authors of the report note "it may be necessary to repeat those experiments (*done only with zaprinast*) with different structural classes of selective PDE inhibitors."

Theophylline, a natural compound and one of the first PDE inhibitors to be discovered, is still in wide use for investigational purposes and various clinical treatments; its biological effects are mediated by actions as an antagonist of A_1-adenosine receptors or as a nonspecific inhibitor of PDEs. Relatively high plasma concentrations of theophylline (10–20 mg/L, which translates to ~50–100 μM) are required to elicit significant bronchodilation (Barnes 2006). Based on IC_{50} values of theophylline for several PDEs, it is entirely possible that this level could significantly diminish PDE activity, thereby promoting cN elevation (Butcher and Sutherland 1962; Dent and Rabe 1996), and it is well accepted that the relationship between the extracellular concentration of a PDE inhibitor and its intracellular

concentration cannot be accurately predicted (Thompson 1991). Evidence derived from studies of airway smooth muscle indicates that effects of theophylline in that tissue are mediated through inhibition of PDEs 3–5 to cause increases in cAMP and cGMP and activation of the respective signaling pathways (Cortijo et al. 1993; Rabe et al. 1995). Effects of theophylline to blunt airway inflammation in COPD occur below 10 mg/ml plasma (Hirano et al. 2006; Kobayashi et al. 2004) and are therefore unlikely to act through PDE inhibition.

Cilostazol is somewhat selective as an inhibitor of the PDE3 family (IC_{50} ~ 0.5 μM) and is marketed for treatment of intermittent claudication (Kambayashi et al. 2003); it also reportedly inhibits PDE5 with nine-fold lower affinity (IC_{50} ~ 4.4 μM) (Sudo et al. 2000) and antagonizes adenosine uptake at clinically relevant concentrations (3 μM). The inhibitory effect of cilostazol for both PDE3 and adenosine uptake was not appreciated initially, but now this dual effect is suggested to be a significant factor in the overall safety and efficacy of cilostazol compared to PDE3 inhibitors that do not block adenosine uptake (Kambayashi et al. 2006). Since the clinically relevant concentration of cilostazol is also in the range of its IC_{50} for PDE5, it is plausible that inhibition of PDE5 may account for a portion of cilostazol's vasodilatory and antiaggregatory effects on vascular smooth muscle and platelets, respectively, where PDE5 is abundant.

Dipyridamole, a PDE inhibitor that is approved for clinical use for prevention of ischemic events following stroke, also has dual actions; it is a somewhat nonselective inhibitor of PDEs (PDE5 IC_{50} ~ 0.9 μM, PDE7 IC_{50} ~ 0.6 μM, PDE8 IC_{50} ~ 9 μM, PDE10 IC_{50} ~ 1.0 μM, and PDE11 IC_{50} ~ 0.4 μM) (Beavo and Brunton 2002) and a relatively potent inhibitor of adenosine uptake (Klabunde 1983; Schaper 2005). Although the antiaggregatory effects of dipyridamole in platelets are largely attributed to its action to block adenosine uptake, its concentration in plasma (1.7 μg/L or ~3.5 μM) is sufficient to block PDE5 as well (Serebruany et al. 2009); the combined actions of this drug would decrease platelet aggregation.

Although the effects of the PDE5 inhibitors, vardenafil, sildenafil, and tadalafil are thought to be mediated exclusively through inhibition of PDE5, differences in the effects of vardenafil versus those of sildenafil and tadalafil in rabbit pulmonary arteries and human platelets have been reported (Toque et al. 2008). The evidence in this report suggests that vardenafil, in addition to acting as a PDE5 inhibitor in these tissues, may also block store-operated calcium channels. This possibility warrants further study. A recent report indicates that *in vitro* sildenafil activates several carbonic anhydrase isoenzymes (hCA I, hCA VB and hCA VI) with K_a values in the range of 1–7 μM (Abdulkadir Coban et al. 2009); this effect may be mediated by the piperazine moiety in sildenafil since other carbonic anhydrases activators contain a piperazine moiety. Whether this effect impacts the pharmacological actions of sildenafil is not known, but since plasma sildenafil (bound and free) in a typical patient approaches 1 μM, the possibility should not be entirely dismissed. This effect emphasizes that a variety of molecular features of PDE inhibitors may contribute to unanticipated interactions. Recently, Jeon et al. reported that the anti-inflammatory action of vinpocetine, which has traditionally been considered to be a PDE1-selective inhibitor, proceeds through a PDE-independent

pathway (Jeon et al. 2010). Finally, EHNA, a selective and weak inhibitor of PDE2, also inhibits adenosine deaminase (Mery et al. 1995), which complicates its use in biochemical studies.

5 Concluding Remarks

Major advances have been made in recent years in defining the physical and chemical characteristics of the catalytic sites of PDEs and identifying improved strategies for development of potent and selective inhibitors of the various PDE families. X-ray crystallographic structures of the catalytic domains of numerous PDEs has allowed for precise spatial considerations for future design of more selective inhibitors. More recently, the X-ray crystal structures of regulatory domains of these proteins have become available and provide new opportunities for development of PDE inhibitors. The clinical success of the PDE5 inhibitors has been a major advance in understanding the medical implications of these types of drugs. Despite the availability of some selective PDE inhibitors, it is clear that great caution is warranted when using these compounds. There are now well-documented examples in which a PDE inhibitor that was considered to specifically target a particular PDE has mechanisms of action that are unrelated to inhibition of PDE activity. In addition, interpretation of results based on the concentration of a "selective" inhibitor that is applied extracellularly is particularly problematic since it is not possible to discern the concentration of that compound in the intracellular milieu or in particular cellular compartments where it might reach concentrations sufficient for inhibition of other PDEs. Use of several inhibitors and a variety of approaches is important to validate interpretation of results in such studies. Innovative strategies are needed to generate new inhibitors that are selective for specific PDE families and their subfamilies since these compounds are greatly needed for investigational purposes that will hopefully lead to clinical use.

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