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CYCLIC–NUCLEOTIDE PHOSPHODIESTERASES IN THE CENTRAL NERVOUS SYSTEM

From Biology to Drug Discovery

Edited by
NICHOLAS J. BRANDON
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Cyclic-Nucleotide Phosphodiesterases in the Central Nervous System

From Biology to Drug Discovery

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Published by John Wiley & Sons, Inc., Hoboken, New Jersey

Published simultaneously in Canada

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Library of Congress Cataloging-in-Publication Data:

Cyclic-nucleotide phosphodiesterases in the central nervous system : from biology to drug discovery / edited by Nicholas J. Brandon, Anthony R. West.

p. ; cm.

Includes bibliographical references.

ISBN 978-0-470-56668-8 (cloth)

I. Brandon, Nicholas J., editor of compilation. II. West, Anthony R., 1970- editor of compilation [DNLM:1. 3’,5’-Cyclic-AMP Phosphodiesterases–metabolism. 2. 3’,5’-Cyclic-AMP Phosphodiesterases–therapeutic use. 3. Central Nervous System–physiology. 4. Central Nervous System Diseases–drug therapy. 5. Drug Discovery. QU 136]

QP370
612.8–2–dc23
2013042742

Preface

Cyclic-nucleotide phosphodiesterases (PDEs) are critically involved in the regulation of cellular processes at work from cell birth to death. PDEs are produced by and operate within all cells of the body, and their key role in dampening or redirecting cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) signaling cascades makes them essential for cell health. In both the brain and spinal cord, PDEs show intricate patterns of cellular localization, both regionally and at the subcellular level. Such an infrastructure undoubtedly contributes to the tremendous computational power needed for the effective execution of sensorimotor, cognitive, and affective functions. On the flipside, we are entering a period of time when diseases of the central nervous system (CNS), which disrupt these essential functions, will affect more and more of us. For reasons described in this book, it is now clear that PDEs have enormous potential as targets for new medicines. In this book we have brought together the expertise of leading researchers from both basic and applied sciences to highlight the beautiful biology of the diverse superfamily of PDEs, as well as the medical potential of targeting PDEs for the treatment of disorders of the CNS. Indeed, numerous applications for small-molecule inhibitors selective for specific PDE isoforms are being investigated for the treatment of CNS diseases, including schizophrenia, depression, Alzheimer's disease, Parkinson's disease, Huntington's disease, spinal cord injury, and others. Drug discovery for disorders of the CNS is exceptionally difficult, but undoubtedly, our understanding of PDE biology and PDE-based therapeutics will continue to evolve and hopefully lead to the

development of novel medicines of value for patients suffering from these devastating disorders.

We thank all of our wonderful colleagues who have contributed chapters to this book, as well as the numerous reviewers who have provided constructive criticism of its content. We hope that this work will render important insights into PDE biology and therapeutics that will inspire a new generation of researchers interested in this field.

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Chapter 1
Phosphodiesterases and Cyclic Nucleotide Signaling in the CNS

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Introduction

Discovery of PDEs, Historical Perspectives, and Progress in Understanding the Complexity of PDE Functions

Soon after the discovery of the second messenger cAMP by Sutherland and Rall [1], it was observed that cyclic nucleotides are unstable in tissue extracts. This observation paved the way for the identification of the enzymatic activities responsible for their destruction [1]. Sutherland and coworkers correctly attributed this activity to a Mg^{2+}-dependent, methylxanthine-inhibited enzyme that cleaves the cyclic nucleotide phosphodiester bond at the 3′-position, hence the name phosphodiesterase (PDE) (Figure 1.1). With the discovery of cGMP and the improvement of protein separation protocols [2], it also became apparent that multiple PDE isoforms with different affinities for cAMP and cGMP and sensitivity to inhibitors coexist in a cell (Figure 1.1). Only with the application of protein sequencing and molecular cloning techniques has it been realized that 21 genes code for PDEs in humans and that close to 100 proteins are derived from these genes, forming a highly heterogeneous superfamily of enzymes (Figures 1.1 and 1.2) [3].

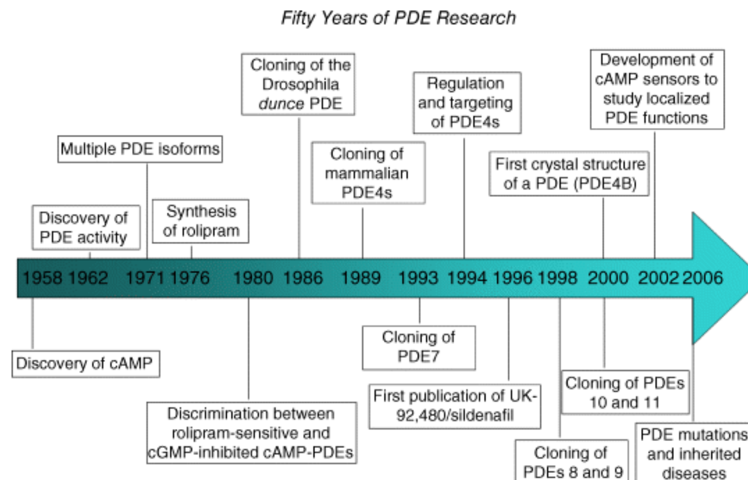

Figure 1.1 Timeline of the major discoveries related to the field of phosphodiesterases.

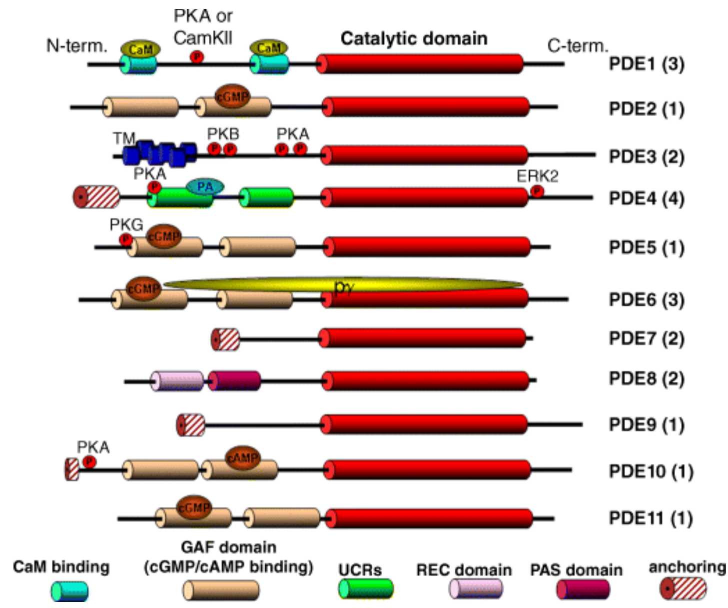

Figure 1.2 The domain organization of the different families of phosphodiesterases. Domains are depicted as "barrels" connected by "wires" indicating linker regions. Phosphorylation sites are shown as red circles with the respective kinase phosphorylating this site listed above. PDEs are composed of a C-terminal catalytic domain (shown in red) and distinct regulatory domains at the N-terminus. These include Ca^{2+}/calmodulin (CaM)-binding sites (PDE1), GAF domains that function as cAMP or cGMP sensors (PDE2, PDE5, PDE6, PDE10, and PDE11), the UCRs that include a phosphatidic acid (PA)-binding site in PDE4, and the PAS domain (PDE8). The inhibitory gamma subunit of PDE6 is indicated as a yellow ellipse. Domains functioning as targeting sequences by mediating membrane–protein or protein–protein interactions are indicated as red striated barrels and the transmembrane (TM) domains of PDE3 are indicated in blue. The number of PDE genes belonging to each PDE family is indicated in parentheses beside the PDE family name.

Although PDEs were implicated early on in the control of intracellular levels of cAMP and cGMP and the termination of the neurotransmitter or hormonal signal, 30 additional years of research have been necessary to understand that PDEs are not simply housekeeping enzymes. The activity of PDEs is finely regulated by a myriad of regulatory loops and integrated in a complex fashion with the cyclic nucleotide signaling machinery and other signaling pathways. Blockade of PDE activity does not exclusively lead to an increase in cyclic nucleotides and a gain of function, as one would predict from the removal of cyclic nucleotide degradation. On the contrary, complex changes in cellular responses are associated with PDE inhibition, often causing loss of function, as documented by the phenotypes of natural mutations or engineered inactivation of the PDE genes [4–7]. These findings imply that PDEs and their regulation are indispensable to faithfully translate extracellular cues into appropriate biological responses. Indeed, in neurons as in other cells, the biological outcome of activation of a receptor is defined by the multiple dimensions of the cyclic nucleotide signal. This specificity of the response depends on the changes in concentration of the cyclic nucleotide, the time frame in which these changes occur, and the subcellular locale in which the nucleotides accumulate. Because cyclic nucleotide accumulation is dependent on the steady state of cAMP/cGMP production

as well as hydrolysis, degradation by PDEs is a major determining factor of all three dimensions of the cyclic nucleotide signal.

In spite of seemingly comparable enzymatic functions, each of the several PDEs expressed within a cell appears to serve unique roles. This view is paradoxical because it implies, as fittingly summarized by L.L. Brunton, that “Not all cAMP has access to all cellular PDEs” [8]. As an extension of this concept, a PDE may play critical functions in a cell even if it represents a minor fraction of the overall hydrolytic activity, a view with considerable impact on pharmacological strategies targeting PDEs. The discovery of macromolecular complexes involving PDEs has confirmed this concept and added a new dimension to the function of these enzymes in signaling. In those complexes in which they are associated with cyclic nucleotide targets, it is likely that PDEs play an essential role in controlling or limiting the access of cyclic nucleotides to their effectors. Since protein kinase A (PKA), protein kinase G (PKG), GTP exchange protein activated by cAMP (EPAC), and cyclic nucleotide-gated (CNG) channels are tethered to specific subcellular compartments, PDEs likely contribute to the compartmentalization of cyclic nucleotide signaling and to the spatial dimension of the signal. PDEs may also have scaffolding properties within these complexes, opening the possibility that PDEs serve functions beyond their catalytic activity and that a dynamic formation and dissolution of these complexes may contribute to the allosteric regulation of PDE activities.

The PDE Superfamily

After several more PDE genes were discovered through homology screening of nucleotide sequence databases between 1996 and 2000 (PDE8, PDE9, PDE10, and PDE11), the completion of the Human Genome Project in 2001 eventually established that there are 21 PDE genes in humans [9]. Orthologs of all 21 genes are encoded in the genomes of rats and mice and might be present in the same number in other mammals. Metazoan model organisms such as *Caenorhabditis elegans* or *Drosophila melanogaster* express orthologs of some, but usually not all of the mammalian PDEs [3]. Based upon their substrate specificities, kinetic properties, inhibitor sensitivities, and, ultimately, their sequence homology, the 21 mammalian PDE genes are subdivided into 11 PDE families, each consisting of 1 to a maximum of 4 genes (Table 1.1). Most PDE genes are expressed as a number of variants through the use of multiple promoters and alternative splicing. The PDE6 genes, with only 1 transcript per gene reported, and PDE9A, for which more than 20 putative variants have been proposed, represent the extremes in the number of variants generated from individual genes. Together, close to 100 PDE proteins are generated in mammals, each likely serving unique cellular functions.

Table 1.1 The Properties of the Mammalian PDE Genes

Chromosome Region[a]	Gene Symbol	Name/Aliases	Predominant CNS Distribution[b]	Disease Association[c]	References
2q32.1	*PDE1A*	Phosphodiesterase 1A, Ca^{2+}/calmodulin-dependent	Cortex	Schizophrenia	[10]
12q13	*PDE1B*	Phosphodiesterase 1B, Ca^{2+}/calmodulin-dependent	Striatum, hippocampus		
7p14	*PDE1C*	Phosphodiesterase 1C, Ca^{2+}/calmodulin-dependent	Cerebellum		
11q13.4	*PDE2A*	Phosphodiesterase 2A, cGMP-stimulated	Striatum, hippocampus, cerebellum		
12p12	*PDE3A*	Phosphodiesterase 3B, cGMP-inhibited	Striatum, hippocampus	RR and QT interval	[11]
11p15.1	*PDE3B*	Phosphodiesterase 3B, cGMP-inhibited	Hippocampus		
19p13.2	*PDE4A*	Phosphodiesterase 4A, cAMP-specific (*Drosophila dunce* homolog, DPDE2, PDE2)	Cortex, hippocampus		
1p31	*PDE4B*	Phosphodiesterase 4B, cAMP-specific (*Drosophila dunce* homolog, DPD4, PDE4)	Cortex, striatum, hippocampus	Schizophrenia, bipolar disorder, depression, alcohol responses, multiple sclerosis	[12–15]
19p13.1	*PDE4C*	Phosphodiesterase 4C, cAMP-specific (*Drosophila dunce* homolog, DPD1, PDE1)	Low level in CNS		

Chromosome Region[a]	**Gene Symbol**	**Name/Aliases**	**Predominant CNS Distribution**[b]	**Disease Association**[c]	**References**
5q12	*PDE4D*	Phosphodiesterase 4D, cAMP-specific (*Drosophila dunce* homolog, DPD3, PDE3, STRK1)	Cortex, hippocampus, brain stem	Stroke susceptibility, asthma, sleep disorders, neuroticism, schizophrenia	[10,16–21]
4q25–q27	*PDE5A*	Phosphodiesterase 5A, cGMP-specific	Cerebellum, hippocampus		
5q31.2–q34	*PDE6A*	Phosphodiesterase 6A, cGMP-specific, rod, alpha	Retina	Retinitis pigmentosa	[5]
4p16.3	*PDE6B*	Phosphodiesterase 6B, cGMP-specific, rod, beta	Retina	Night blindness, congenital stationary type 3	[22]
10q24	*PDE6C*	Phosphodiesterase 6C, cGMP-specific, cone, alpha prime	Retina	Cone dystrophy	[7]
8q13–q22	*PDE7A*	Phosphodiesterase 7A, HCP1	Hippocampus		
6q23–q24	*PDE7B*	Phosphodiesterase 7B	Striatum, hippocampus		
15q25.3	*PDE8A*	Phosphodiesterase 8A	Cortex	Major depressive disorder	
5q13.3	*PDE8B*	Phosphodiesterase 8B	Striatum, hippocampus	Pigmented nodular adrenocortical disease	
21q22.3	*PDE9A*	Phosphodiesterase 9A	Cortex, cerebellum	Bipolar disorder	[23,24]
1q11	*PDE10A*	Phosphodiesterase 10A	Striatum	Autism	[25]
2q31.2	*PDE11A*	Phosphodiesterase 11A	Hippocampus CA1	Pigmented nodular adrenocortical disease	[26]

[a] Shown is the chromosomal localization of the PDE genes in humans.

[b] Expression data in human based on *in situ* hybridization; where not available, data from rat and mouse brain were used.

[c] The association with inherited diseases is based on published data, GWAS (http://gwas.nih.gov/) analysis, and OMIM (http://www.ncbi.nlm.nih.gov/omim) data.

Nomenclature

Due to the large number of PDE variants present in mammals, an initial classification based on regulatory properties and inhibitor sensitivities of newly discovered enzymes as well as their order of discovery soon became inadequate. It was subsequently replaced with a consensus nomenclature in which the first two letters indicate the species followed by the three letters “PDE”, an Arabic numeral indicating the PDE family, a letter indicating the gene within the PDE family, and finally another Arabic numeral indicating the precise PDE variant. For example, HsPDE4D3 identifies the species as *Homo sapiens*, the PDE family as 4, the gene as D, and the variant as 3. This nomenclature was widely adopted in 1994 [27]. For a complete list of PDE genes and variants as well as information regarding the nomenclature used before 1994, please see http://depts.washington.edu/pde/pde.html or Ref. [28].

Overall Protein Domain Arrangement

Despite their multitude and diversity, all PDEs share several structural and functional properties. One of the most obvious is their modular structure consisting of a relatively conserved catalytic domain located in the C-terminal half of the protein and N-terminal domains that are structurally diverse, but all function to regulate enzyme activity (Figure 1.2). The C-terminal catalytic domain contains all residues required for catalysis and determines the enzyme kinetics unique to each PDE subtype. The characteristic features of the N-terminal regulatory domains are highly conserved modules such as Ca^{2+}/calmodulin-binding domains (PDE1), GAF domains (cGMP-activated PDEs, adenylyl cyclase, and Fh1A; PDE2, PDE5, PDE6, PDE10, and PDE11), UCR domains (upstream conserved regions; PDE4), REC domains (receiver; PDE8), and PAS domains (period, aryl hydrocarbon receptor nuclear translocator (ARNT), and single minded; PDE8), as well as phosphorylation sites, which mediate the regulation of enzyme activity by posttranslational modifications and/or ligand binding. As a result of this modular structure, truncated PDEs encoding only the catalytic domain not only retain enzyme activity, but also exhibit kinetic properties and substrate specificity similar to those of the holoenzyme, whereas regulation of enzyme activity is lost or is altered compared to full-length proteins [29–31]. In most cases, the inhibitor sensitivity of the full-length proteins is also retained in the catalytic domain constructs. There are some exceptions, however. The catalytic domain of PDE4, for example, exhibits an affinity for the prototypical PDE4 inhibitor rolipram that is about 100-fold lower compared to full-length proteins, whereas the sensitivity toward structurally unrelated compounds is not affected by the truncation [30,31]. Thus, catalytic domain constructs may have limited value for the development of PDE4 inhibitors.

The regulatory domains, in turn, may function in a similarly independent manner. The GAF domains, for example, are found in PDEs, adenylyl cyclases, and the *Escherichia coli* Fh1A protein. Chimeras between the GAF domains of different PDEs (PDE5, PDE10, and PDE11) and the catalytic domain of the cyanobacterial cyaB1 adenylyl cyclase were found to be fully functional in that the PDE-GAF domains sense cyclic nucleotide levels and mediate activation of the catalytic domain of the cyclase [32,33]. This finding, together with the similar domain organization of all PDEs, led to the proposal that despite their structurally distinct N-termini, the mechanism by which the N-terminal domains regulate enzyme activity is perhaps conserved among all PDE subtypes. The functional properties of the different N-terminal regulatory domains are discussed in greater detail later in this chapter.

The unique combination of catalytic domain kinetics and regulatory domain properties defines a PDE family and is conserved among its members. The variants generated from a single PDE gene, in turn, contain with few exceptions the identical catalytic domain, but often possess variant-specific N-termini. These variants are divided into those that encode the entire regulatory module characteristic of a given PDE family, such as GAF domains or UCR domains, and those that encode only a portion of the regulatory module or lack it altogether. The latter include so-called short PDE4 variants, which contain only a portion of the UCR module, and PDE11 variants that lack GAF domains. Underlining the critical role of the regulatory domains, variants that encode the entire regulatory module exhibit similar mechanisms of regulation, whereas variants that lack part or all of the regulatory domains are either insensitive to ligand binding or posttranslational modification *per se* or respond differently (Figure 1.2) [3,34]. The extreme N-termini unique to individual PDE variants are encoded by variant-specific first exons (Figure 1.3) and often mediate subcellular targeting through protein–protein or protein–lipid interactions, thus allowing the cell to specifically target PDE variants to subcellular compartments [35].

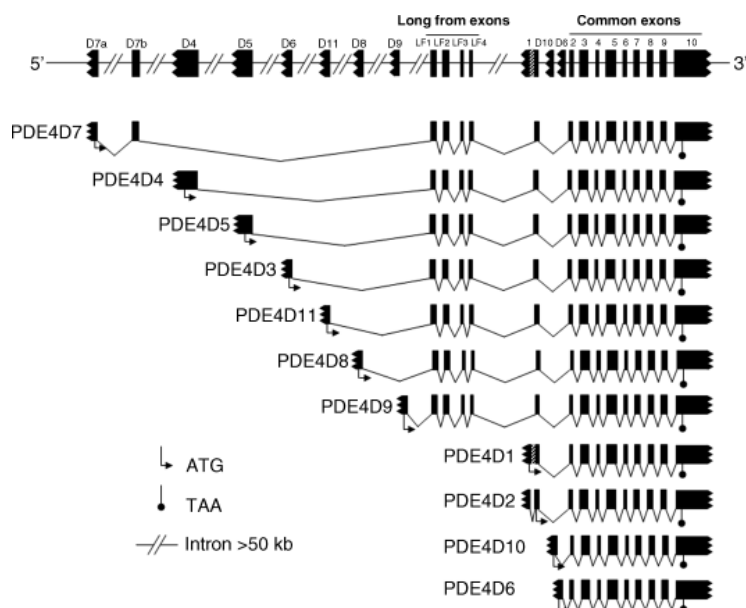

Figure 1.3 Structure of the PDE4D locus. Schematic representation of the structure of the mammalian PDE4D locus (top) and the encoded mRNAs. Exons are presented as filled bars, introns are drawn as solid lines, and a noncoding exon is depicted as a striated box. Indicated are variant-specific first exons, long form (LF) exons shared by all so-called long PDE4 isoforms, and common exons shared by most PDE4D variants. The scheme is not drawn to scale.

Although the mechanisms of regulation of PDE activity have been described biochemically in great detail, structural aspects of enzyme regulation had remained elusive (see Chapter 6 for a detailed discussion). Pandit et al. [36] recently provided a major breakthrough on the question of how modification of the N-terminal domains by posttranslational modifications and/or ligand binding exerts its effect on the conformation of the distal catalytic domain, thereby controlling PDE activity. The authors crystallized a PDE2A that, although lacking some N- and C-terminal sequence of the holoenzyme, contains the critical components of the PDE2 structure: a tandem set of GAF domains linked to the C-terminal catalytic domain. PDE2A crystallized as a linear structure that extends along a GAF-A/GAF-B/catalytic domain axis. The enzyme

forms head-to-head dimers with the dimer interface spanning the entire length of the molecule with interactions between the GAF-A and GAF-A, GAF-B and GAF-B, and between the two catalytic domains of the individual monomers. When the GAF domains are unoccupied, the substrate-binding pockets of the two catalytic domains are packed against each other, essentially closing off access to the substrate. As a mechanism for the allosteric activation of PDE2, the authors propose that cGMP binding to the GAF-B domain results in a reorientation of the linker regions connecting GAF-B and the catalytic domain, which in turn leads to a disruption of the dimer interface between the two catalytic domains, thus promoting an “open” conformation of the enzyme that allows substrate access. Both the general organization of the PDE2 structure and the mechanism of PDE activation proposed by Pandit et al. [36] are in agreement with many structure–function relationships observed in other PDEs. Most PDEs have been reported to form homo- or heterodimers [29,34,37–41], and critical dimerization domains were identified in the N-terminal domains. The catalytic domains also retain some affinity as evidenced by the fact that several PDE catalytic domains form dimers in purified protein preparations as well as in crystal structures [42–44]. In addition, most PDEs also possess the elongated structure described for PDE2 as indicated by their high frictional ratios [39,45,46]. Electron microscopic images of PDE5 and PDE6 show an elongated structure highly similar to the atomic structure of PDE2, with points of contact between the GAF-As, the GAF-Bs, and the catalytic domains of the individual monomers [47,48]. Dimerization mediated by the N-terminal domains of PDE2 plays a critical role in stacking the substrate-binding sites at the catalytic domain against each other, thus preventing substrate access. This is in agreement with the observation that N-terminal domains in various PDEs exert an inhibitory constraint on the active site, which can be uncovered through deletion mutagenesis or proteolytic digest of full-length enzyme [49,50]. Taken together, these similarities suggest that the atomic structure of PDE2 might represent a model for a general organization of PDEs and a mechanism of enzyme activation.

However, Burgin et al. [51] recently suggested an alternative mechanism of how inhibitory constraint and regulation of enzyme activity is achieved in PDE4. In crystal structures of a truncated PDE4, substrate access to the catalytic site is prevented not by stacking of the catalytic domains against one another, as proposed for PDE2 [36], but by direct binding of a helix in the regulatory UCR2 domains to the substrate-binding pocket in the catalytic domain. PDE4 variants are divided into so-called long forms that contain the complete UCR1/2 module and short forms that lack UCR1 but still contain all or a portion of UCR2. The constructs crystallized by Burgin et al. lack UCR1 and encode only a portion of UCR2, thus encoding a PDE4 that resembles short forms. There are significant structural and functional differences between long and short forms including oligomerization, enzyme activation, and inhibitor sensitivity [40]. Thus, it remains to be determined whether the mechanism of enzyme inhibition/activation proposed by Burgin et al. [51] reflects properties of all PDE4 isoforms or whether this model describes properties inherent only to short PDE4 isoforms, whereas long isoforms are regulated differently. If the former is the case, PDE4 regulation of enzyme activity would be different from models described for PDE2, PDE5, and PDE6. This in turn would suggest that distinct modes of regulating PDE activity evolved for the different PDE families.

Catalytic Site Properties and Interaction with the Substrates

PDEs are divalent metal ion-dependent enzymes and share with other metal-dependent phosphohydrolases an HD(X2)H(X4)N motif, which defines residues forming the metal ion-binding site. Much progress has been made in understanding

the structure and properties of the catalytic domains since crystal structures have become available in 2000 [43]. The catalytic domain consists of 16 α-helices folded into a compact structure. Whereas the sequence homology of the catalytic domains can be as low as 25% for members of different PDE families, the three-dimensional structure of the catalytic domain aligns all residues that are invariant or semiconserved among all PDEs to form the substrate-binding pocket. These residues include an invariant glutamine that forms hydrogen bonds with the 1- and/or 6-positions of the cyclic nucleotides, several residues that form a “hydrophobic clamp” that anchors the purine ring, and residues that form two metal ion-binding sites, termed M1 and M2, which are positioned at the bottom of the substrate-binding pocket. Based on biochemical data and X-ray diffraction, M1 is likely occupied by Zn^{2+}, whereas M2 is occupied by Mg^{2+} or Mn^{2+} in the native enzyme. The two metal ions function to activate the substrate phosphate and to coordinate a water molecule that acts as a nucleophile in the PDE reaction. Recent studies suggest that the water molecule coordinated by the metal ions may partially dissociate into a hydroxide ion [52–55], and that this hydroxide acts as the nucleophile on the phosphorus, eventually becoming part of the outgoing phosphate, whereas a nearby strictly conserved histidine assists with the protonation of the O3′ group.

Defining the first atomic structure of any PDE, Xu et al. [43] reported the crystal structure of the catalytic domain of PDE4B in 2000. Since then, crystal structures for the catalytic domain of most PDE families have been reported, providing further insight into the distinct mechanisms of inhibitor binding and substrate specificity [42,43,56–64].

On the basis of their substrate specificity, the 11 PDE families can be divided into three groups. PDE4, PDE7, and PDE8 selectively hydrolyze cAMP, whereas PDE5, PDE6, and PDE9 are selective for cGMP hydrolysis. The remaining PDEs (PDE1, PDE2, PDE3, PDE10, and PDE11) bind and hydrolyze both cyclic nucleotides with varying efficiency. An invariant glutamine residue that is conserved among all PDEs and that was shown to form hydrogen bonds with either AMP or GMP in crystal structures has been proposed as the major determinant of PDE substrate specificity. As both cyclic nucleotides were thought to bind to the substrate-binding pocket in the same conformation and the hydrogen-bonding character of the 1- and 6-positions of adenine and guanine is essentially reversed, a so-called glutamine switch mechanism was previously proposed to determine PDE substrate specificity [43,63]. According to this model, the amide group of the invariant glutamine can rotate by 180° to accommodate binding of either cAMP or cGMP. It was assumed that PDEs in which additional residues limit the mobility of the invariant glutamine are selective for one of the cyclic nucleotides, whereas PDEs that allow a free rotation of the invariant glutamine could bind both. However, several recent findings suggest that this cannot be the only mechanism that determines substrate specificity among PDEs. Mutation of this invariant glutamine in PDE5A, for example, did reduce affinity of the enzyme for its physiological substrate cGMP but did not enhance binding of cAMP [65]. In addition, mutation of an aspartic acid residue conserved among the cAMP-PDEs ablates the substrate specificity of PDE4 isoenzymes, suggesting that this residue represents an additional evolutionary conserved component of substrate specificity [66,67]. Most important are recent studies by Wang et al. [61,68] that demonstrate that PDE10 binds the substrates cAMP and cGMP in *syn*-conformation, whereas the PDE reaction products AMP and GMP dock in *anti*-conformation in crystal structures. This suggests that previous reports, which relied on the analysis of cocrystals of various PDEs with AMP or GMP, might not reflect the binding of substrates in the