Mammalian Cyclic Nucleotide Phosphodiesterases: Molecular Mechanisms and Physiological Functions

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Francis SH, Blount MA, Corbin JD. Mammalian Cyclic Nucleotide Phosphodiesterases: Molecular Mechanisms and Physiological Functions. Physiol Rev 91: 651–690, 2011; doi:10.1152/physrev.00030.2010.—The superfamily of cyclic nucleotide (cN) phosphodiesterases (PDEs) is comprised of 11 families of enzymes. PDEs break down cAMP and/or cGMP and are major determinants of cellular cN levels and, consequently, the actions of cN-signaling pathways. PDEs exhibit a range of catalytic efficiencies for breakdown of cAMP and/or cGMP and are regulated by myriad processes including phosphorylation, cN binding to allosteric GAF domains, changes in expression levels, interaction with regulatory or anchoring proteins, and reversible translocation among subcellular compartments. Selective PDE inhibitors are currently in clinical use for treatment of erectile dysfunction, pulmonary hypertension, intermittent claudication, and chronic pulmonary obstructive disease; many new inhibitors are being developed for treatment of these and other maladies. Recently reported x-ray crystallographic structures have defined features that provide for specificity for cAMP or cGMP in PDE catalytic sites or their GAF domains, as well as mechanisms involved in catalysis, oligomerization, autoinhibition, and interactions with inhibitors. In addition, major advances have been made in understanding the physiological impact and the biochemical basis for selective localization and/or recruitment of specific PDE isoenzymes to particular subcellular compartments. The many recent advances in understanding PDE structures, functions, and physiological actions are discussed in this review.
I. OVERVIEW OF THE 11 FAMILIES AND ISOENZYMES WITHIN FAMILIES OF MAMMALIAN CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

A diverse superfamily of cyclic nucleotide (cN) phosphodiesterases (PDEs) catalyzes hydrolysis of the cyclic phosphate bond in cAMP and cGMP to generate the products 5′-AMP and 5′-GMP, which are inactive in the respective cN-signaling pathways (Fig. 1). The catalytic activities of PDEs provide for breakdown of cNs over a spectrum of concentrations in all cells, and their varied regulatory mechanisms provide for integration and cross-talk with myriad signaling pathways. It has recently been established that particular PDEs are targeted to discrete compartments within cells where they control cN level and sculpt microenvironments for a variety of cN signalosomes that can contain cN-dependent protein kinases, Epacs (exchange proteins activated by cAMP), phosphoprotein phosphatases, and/or cN-gated cation channels (14, 30, 49, 58, 70, 104, 153, 158, 167, 170, 204, 299, 387, 391, 422, 423). Localization of some PDEs to specific areas of a cell is static, whereas that for others involves reversible recruitment (153). PDE activities are modulated in the short term and long term in response to a panoply of signals including hormones, neurotransmitters, cytokines, light, and oxidative influences.

Dysfunctions in PDE activities have been convincingly associated with asthma, erectile dysfunction (ED), chronic obstructive pulmonary disease (COPD), autoimmune diseases, hypertension, intermittent claudication, heart failure, schizophrenia, stroke, and depression. PDE inhibitors that are in clinical use include PDE5 inhibitors for treatment of ED and pulmonary hypertension (23, 55, 82, 97, 112, 119, 120, 242, 255, 284, 306, 359), PDE3 inhibitors for treatment of intermittent claudication and acute cardiac failure (180, 181, 242, 260), a PDE4 inhibitor for treatment of COPD (139), a nonspecific PDE inhibitor (theophylline) for treatment of asthma (22), and dipyridamole, a relatively nonspecific PDE inhibitor for prevention of blood clotting following stroke or heart valve replacement (51, 91). Many new PDE inhibitors are being developed to provide improved treatment of these and other maladies.

Despite more than 50 years of effort to define PDE functions, the field is still in its infancy due in large part to the complexity of this superfamily of enzymes, the spatial distribution and specificity of action of PDEs in different cell types, the varied controls on levels of PDE expression and activity, and our limited knowledge of structure/function relationships in these proteins. Major advances have been made on all of these fronts in just the last few years. This review focuses primarily on recent advances that define biochemical mechanisms associated with PDE actions, physiological functions and regulation of PDEs, and development of pharmacological agents that target PDEs.

A. Mechanisms for Lowering Cellular Cyclic Nucleotide Levels

PDE activities are modulated in coordination with adenyl cyclase (AC) and guanylyl cyclase (GC) activities through direct effectors and feedback pathways, thereby maintaining cAMP and cGMP levels within optimum ranges for responsiveness to signals. The main process for lowering active cN levels in cells is through catalytic breakdown by PDEs. However, the cN-binding properties of GAFs (named for cGMP-binding PDEs, Anabaena adenylyl cyclase, and Escherichia coli FhlA) in some PDEs can sequester cN (129, 198). Although cNs are extruded from some cells by certain multianion transporters, the impact of this process on lowering cN levels is suggested to be small (21, 64, 211). Other processes may lower cN levels in particular cells. cGMP in ovarian follicle somatic cells passes through gap junctions into oocytes, where it inhibits cAMP breakdown by PDE3A and increases cAMP to block meiosis (278). Thus total cN may be lowered by the 1) high-turnover catalytic action of PDEs, 2) extrusion of cN into the extracellular milieu, and/or 3) transit of cN from one cell type to another. The latter effect implies that cellular cN level and cN signaling can change even in the absence of altered cyclase or PDE activity. Pharmacological blockade of PDEs can frequently increase cellular cN levels in the absence of increased cyclase activity. The influences of these processes in determining cN level in the whole cell and specific cellular compartments vary among cells and thereby impact cN signaling.
B. Diversity of the Mammalian Phosphodiesterase Superfamily

Eleven families of mammalian PDEs (PDEs 1–11) are derived from 21 genes and classified based on amino acid sequences, regulatory properties, and catalytic characteristics (Fig. 2). PDEs share a conserved catalytic domain (C domain), but amino acid sequence outside this region differs markedly. Some genes encoding for PDEs have multiple promoters with distinct regulatory features, and myriad alternative mRNA splicing of PDE gene products contributes further to their molecular diversity (30, 70). Functional characteristics of PDE isoenzymes derived from a single gene can differ significantly since, despite retaining a common C domain, regulatory features may vary.

PDEs in a cell are invariably diverse and include both cAMP- and cGMP-hydrolyzing PDEs. PDEs in a given cell type frequently vary across species, which complicates understanding of their functions (2, 94, 311). Certain PDEs are highly specific for hydrolysis of cAMP (PDEs 4, 7, and 8) or cGMP (PDEs 5, 6 and 9), and others hydrolyze both cNs (PDEs 1, 2, 3, 10, and 11); isoenzymes within a dual-specificity family can differ significantly in preference for cAMP or cGMP as occurs among the PDE1 isoenzymes (30). PDEs 1, 2, 3, and 4 are expressed in many tissues, whereas others are more restricted. In most cells, PDE3 and PDE4 provide the major portion of cAMP-hydrolyzing activity. While they have discrete roles in many cells, their actions can overlap. The PDE6 family (PDE6αββγ and PDE6ααα′γ′γ′), which is required for light perception, is found primarily in retinal photoreceptors in unusually high abundance (~20 μM) (52, 80). PDE6 is also found in pineal gland and certain melanoma cells (25, 80). PDE5 is abundant in vascular and airway smooth muscle and platelets, but significant amounts also occur in cerebellar Purkinje cells, gastrointestinal epithelial cells, and endothelial cells (117, 344, 356, 436).

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PDEs 7, 8, 9, 10, and 11 are not widely expressed. However, PDEs low in abundance can still be central players in cN signaling as is evidenced in a number of recent reports. Moreover, these relatively obscure PDEs appear to be present in high concentrations in select cell populations and involved in critical functions therein.

FIG. 2. Depiction of domain organization of 11 mammalian PDE families. All mammalian PDEs share a conserved C domain located in the COOH-terminal portion of the protein. In GAF-containing PDEs, one or both GAFs can provide dimerization contacts. In addition, one of the GAFs in each of these proteins provides for allosteric cGMP binding (PDE2, PDE5, PDE6, PDE11), allosteric cAMP binding (PDE10), and regulation of catalytic site functions (PDE2, PDE5, PDE6). The encircled P indicates phosphorylation sites. The other families of PDEs have unique complements of various subdomains (UCR, NHR, PAS, membrane association) that contribute to regulation of activity.

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Members of the PDE7 family are low in abundance in most tissues, but PDE7A is high in many cells of the immune system (37), and PDE7B is relatively abundant in some other tissues (144). PDE8 is expressed at low levels in cardiomyocytes but is implicated in controlling a cAMP pool that is involved in regulating calcium movement and excitation-contraction coupling (288). PDE8A occurs in high levels in Leydig cells, where it plays an important role in luteinizing hormone (LH) control of testosterone synthesis (391). PDE8B is abundant in mouse adrenal fasciculata cells, where it modulates steroidogenesis in response to adrenocorticotropic hormone (ACTH) (382a), and it is also implicated in regulation of thyroid-stimulating hormone (TSH) levels, in adrenocortical disease (149), and in the insulin response of pancreatic islets to glucose (98). PDE10 is abundant in certain neuronal cells and is the target of medications being developed for treatment of several neuropsychiatric disorders (243, 244, 303, 332, 346–348, 378). PDE11 is found in few tissues, but PDE11 protein and mRNA are enriched in the ventral hippocampus, where it accounts for a significant portion of the cAMP-PDE hydrolyzing activity. PDE11 knockout mice exhibit behavioral changes that may relate to loss of this activity (187).

II. STRUCTURAL FEATURES OF PHOSPHODIESTERASES

A. Structures of Phosphodiesterases

Typically, PDEs have an NH$_2$-terminal regulatory domain (R domain) and a COOH-terminal C domain of ~250 amino acids (Fig. 2), but PDE4 also has regulatory features in the C domain (30, 50, 70, 157, 282). Identities in amino acid sequences among C domains range from 25–51%, but high sequence identity does not translate into functional similarities. PDEs 5, 6, and 11 are among the most similar in amino acid sequence, but their substrate preferences, catalytic rates, and substrate affinities vary markedly (30, 70, 282). Moreover, substrate preference does not translate into structural similarities; the x-ray crystallographic structure of PDE9, a cGMP-specific PDE, is more like that of the PDE4 family members, which are cAMP-specific PDEs, than that of other cGMP-specific PDEs (163).

Numerous types of protein domains that either provide or have the potential to provide regulatory control are appended to PDE C domains (Fig. 2); these include calcium/calmodulin-binding domains (PDE1 family), GAF domains (PDE2, PDE5, PDE6, PDE10, and PDE11), PAS (Per-ARNT-Sim) domains (PDE8), upstream conserved regulatory (UCR) domains (PDE4), and autoinhibitory domains. Interestingly, two of the major regulatory subdomains for PDEs (GAFs and UCR domains) are not present in other mammalian proteins. UCRs are appended to the NH$_2$ terminus of PDE4 isoenzymes (42) and are conserved in Caenorhabditis elegans (60) and Drosophila PDE4 homologs (85), but not in yeast or Dictyostelium. The UCRs directly regulate PDE4 functions (50, 154, 155, 297, 298) as well as interact with heterologous proteins (35, 68, 153, 206, 239, 269, 392).

In certain mammalian PDEs, GAFs participate in 1) heterologous protein-protein interactions, 2) protein-protein interactions within PDEs, and 3) cN binding (7, 230, 438). Some PDEs contain regions that provide for insertion into membranes: 1) COOH termini of PDE6 catalytic subunits are modified by isoprenylation, protein-protein interactions, and carboxymethylation (80). This introduces hydrophobic components that anchor PDE6 to the photoreceptor outer segments membranes, which is important for PDE6 regulation by transducin. 2) PDE3 contains two regions [NH$_2$-terminal hydrophobic regions (NHR1 and NHR2)] that provide for membrane association. Forms of PDE3A lacking NHR domains are largely cytosolic (89, 189, 340). 3) PDE4A1 contains a NH$_2$-terminal domain that interacts with phosphatidic acid and provides for membrane insertion (17, 50, 131, 153, 158, 297, 298). PDE regulatory subdomains are commonly connected to other portions of the protein by “linker regions” that communicate changes between portions of the PDE protein. Linker regions are not commonly described as functional “subdomains” but are frequently critical to regulation and function.

B. Oligomeric State of Phosphodiesterases

Mammalian PDEs that have been studied are homodimers with the exception of PDE1 and PDE6, which under physiological conditions are typically heterotetramers (30). In the presence of calcium, PDE1 is comprised of two calmodulin subunits that interact with two identical catalytic subunits (30, 355). PDE6 in rod (αβγγ) and cone (α′α′γγ′) outer segments is comprised of two catalytic subunits that form a dimer and two Py inhibitory subunits (80, 136, 216, 266, 267). PDE6 is also found in association with a 17-kDa PrBP/δ protein that interacts with the COOH-terminal prenyl groups, thereby dissociating PDE6 from the membrane.

The dimerization contacts of PDEs involve the NH$_2$-terminal region (PDEs 2, 4, 5, 6, and 10) and/or contacts in the C domains (PDEs 2, 3, 4, 8 and 11) (183, 184, 287, 427). In some PDEs, dimerization provides regulatory mechanisms such as phosphorylation, ligand binding, and autoinhibition. Studies of PDE4 were the first to establish dimerization as having an influence on the C domain (297, 298). Isolated C domains of PDEs are catalytically active, and some are monomers (103, 161–163, 168, 183, 184, 202, 327, 365, 398, 417, 418). In some cases, their kinetic char-
acteristics are indistinguishable from those of the holoenzyme, whereas in other instances there are differences (40, 103, 297, 298). Based on the x-ray crystal structure for a near full-length PDE2, Pandit et al. (287) have proposed that autoinhibition is provided by physical constraints imposed by dimer contacts in the C domains and that activation occurs by disruption of that interface (Fig. 3); this is discussed in greater detail in section IIIA. Recent insights derived from x-ray crystallographic studies (50) indicate that dimerization is also important for autoinhibition in PDE4, but the process differs from that in PDE2.

1. Dimerization of GAF-containing phosphodiesterases

Among most GAF-containing PDEs (PDEs 2, 5, 6, and 10), dimerization appears to significantly, if not exclusively, involve the GAF-containing sequence. However, most of these studies rely on use of isolated constructs of the PDEs (407, 437) and have obvious limitations. Martínez et al. (233) first reported the x-ray crystallographic structure of a mammalian GAF; this included the dimeric NH₂-terminal domain of PDE2 containing GAF-A which is involved in dimer formation and GAF-B which binds cGMP (233). However, in the PDE2 holoenzyme, both GAFs are involved in dimerization (233, 287). Subsequent studies with other PDE GAFs have revealed that there is no pattern for involvement of a GAF in dimerization and/or cN binding (Fig. 2) (137, 141, 142, 232, 333). Momers within GAF-containing PDEs are arranged in parallel; dimerization involves multiple regions and is uniquely mediated among PDEs (179, 182, 232, 233, 437). GAFs that appear to be involved in dimerization include GAF-A in PDEs 2, 5, and 10 (137, 142, 233) as well as GAF-B in PDEs 2, 5, and 10 (137, 437). In PDEs 2, 5, and 6, the GAF that appears to form the strongest dimer contacts does not bind cN, but GAF-B in PDE10 binds cAMP and dimerizes (137).

Dimerization by PDE GAFs mainly utilizes contacts in a single helical bundle, but amino acid sequences of these are poorly conserved among PDEs. Homodimer interactions in PDE2 and PDE10 largely involve this segment, but the contacts are entirely different (137, 233, 287). Importantly, these distinctions provide for the specificity in formation of PDE homodimers even when several GAF-containing PDEs are expressed in the same cell. Aside from the PDE6αβ heterodimer in rods, heterodimers of other GAF-containing PDEs have not been reported. In PDE6αβ, high affinity and selectivity for heterodimerization between the monomers requires a single α-helix (α1) (266). However, in the recently determined structure for GAF-A from PDE6α′, this helix is similarly positioned to that in GAF-B of PDE2, but it is a monomer (232).

The affinity with which PDE GAFs dimerize has only been studied in PDE5. Isolated GAF-A from PDE5 is a dimer with $K_D < 30$ nM, but GAF-B (alone or appended to the C domain) is sufficient for dimerization with $K_D$ of 1–20 pM (437). The sequence between GAF-A and GAF-B also contributes to overall dimer stability of constructs containing only GAF-B and the C domain (437). Deletion of 46 residues from the NH₂-terminal sequence of PDE5 GAF-B produces a monomer, but this could alter the GAF-B structure; therefore the effect cannot be attributed to direct contacts involving these amino acids. Another study of PDE5 GAF-A constructs (with cGMP bound) has identified a sequence of ~20 amino acids, located immediately NH₂-terminal to GAF-A, that weakly contributes to dimerization ($K_D \sim 100 \mu$M) (142). Caution must be used when considering affinities and interactions involving isolated domains; when placed in the context of the whole protein, influences among regions could enhance or weaken interactions.

2. Dimerization of other PDEs

Little is known about the dimerization status of PDEs 1, 3, 7, 8, or 9; studies suggest that PDE1 catalytic subunits are dimers even after introduction of an NH₂-terminal deletion that fully activates the enzyme and eliminates calmodulin stimulation (29, 355). The x-ray crystal structure of the refolded isolated C domain of PDE8 shows dimer contacts (402) that are similar to those in the

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**FIG. 3.** Depiction of cGMP-induced conformational changes in PDE2 that bring about activation. In the inactive state, the arrangement of the two C domains (shown in green) in the dimer block substrate access to the catalytic sites, and the H-loop in each subunit is folded into the respective catalytic pocket. cGMP binding to GAF-B in each subunit is proposed to elicit a conformational change in the linker helix that connects GAF-B and the C domain resulting in disruption of the dimer interface in the C domain. The H-loop is then free to exit the catalytic pocket, which allows for substrate access.
PDE4D2 C domain. Whether these provide for holoenzyme dimerization is not known.

Dimerization in PDE4 is mediated largely by UCR1 (and perhaps to some extent through contacts in the PDE4 C domain) (297, 298, 417). Dimerization in PDE4 isoenzymes is required for activation of long forms by PKA-mediated phosphorylation of UCR1 (298) or phosphatidic acid binding (17), and it contributes to stabilization of the conformation that provides for high-affinity rolipram binding (298). Recent structural work has advanced understanding of the influence of dimerization on PDE4 autoinhibition and inhibitor interactions (50, 155). These topics are discussed in section IIIB1.

C. Structure and Function of Phosphodiesterase Catalytic Sites

Initial insights into the interaction of cNs with PDE catalytic sites were based on the relative selectivity of those sites for cAMP or cGMP. Use of cN analogs better defined the spatial and shape constraints of PDE catalytic sites. Although cAMP and cGMP are almost identical in size (232 Å³), in solution they are in equilibrium between syn and anti conformations based on orientation of the ribose-cyclic phosphate rings at the N9 glycosidic bond (Fig. 4) (421); the distribution of cAMP and cGMP between the syn and anti configurations in solution is 30:70 and 95:5, respectively. Catalytic sites in PDEs 1, 2, and 10 bind cN in the syn configuration, whereas those in PDEs 3, 4, and 5 prefer the anti configuration. Appending chemical groups to N3 or C8 of the purine can shift the balance of the configurations, introduce bulk that interferes with binding, or add chemical influences that affect cN binding (28, 53, 375). Access to numerous x-ray crystal structures of PDE C domains brings greater insight into variations in the configuration of substrate versus product and in the orientation of the purine of cNs in dual-specificity PDEs.

The low level of most PDEs in mammalian tissues, the complex population of PDEs in a tissue, and the difficulty in purifying native PDEs to homogeneity have confounded efforts to more precisely define relationships between PDE structural features and function. This struggle continues unabated. Remarkable advances in understanding PDEs have been made thanks largely to the availability of high-quality recombinant PDE proteins whose functions can be studied directly or following mutagenesis. We also have the added luxury of studying the structures and functions of isolated R or C domains of these enzymes using both biochemical technologies and direct structural analysis. There are still many challenges including expression of high-quality PDEs that are suitable for biophysical studies, and appropriately relating findings in vitro to those in vivo.

1. Overall structure and conserved elements of catalytic domains

The x-ray crystallographic structures for the isolated C domains from nine PDE families (PDEs 1, 2, 3, 4, 5, 7, 8, 9, and 10) have been determined (160, 161, 168, 202, 215, 365, 398, 400, 402, 417, 418, 426). Multiple structures have been determined for several of these; some have been determined in the unliganded state and/or in complex with substrate/product/inhibitors.

PDE C domains contain 17 invariant amino acids; most are located in the catalytic pocket. Although most PDE holoenzymes are elongated, the C domains are globular and have a highly conserved topology (183, 427). The C domains are comprised of 15–17 α-helices (Fig. 5). Superimposition of the structures of isolated C domains of PDE5 (a cGMP-specific PDE), PDE4 (a cAMP-specific PDE), and PDE10 (a dual-specificity PDE) shows strong
similarities in the alignment of the backbone atoms with minor differences primarily at the extreme NH₂ terminus or COOH terminus (Fig. 5) (399). The catalytic site lies in a pocket (~10 Å deep) created by the juxtaposition of three subdomains of the protein, has a volume that ranges from 330 to 440 Å³, and exhibits a narrow aperture at the surface (183). It contains two major regions that relate to catalytic function: 1) a unique histidine-rich region that forms a binuclear metal-ion binding site where the catalytic hydroxyl is generated and catalysis occurs, and 2) a region that interacts with the cN purine. The metal ion-binding site will be discussed in greater detail below in section II.C.4.

2. The purine-binding pocket in the catalytic sites of PDEs

The region making contact with the purine of cNs or the ring structure of most inhibitors contains an invariant glutamine and a “hydrophobic pocket.” Based on x-ray crystal structures of a number of PDEs, the Os1 and Ne2 groups on the side chain of the invariant glutamine form one or two hydrogen bonds with the purine depending on the PDE (Fig. 6, A and B). The hydrophobic pocket is comprised of 1) a conserved aromatic residue on one side (phenylalanine in most PDEs) that forms a face-to-face \( \pi-\pi \) stacking interaction with the purine-like ring structures in cN substrates, 5’-nucleotide products or inhibitors, and 2) a cluster of aliphatic and/or hydrophobic residues on the other side. Together these form a “hydrophobic clamp” around the ring structures in substrates, products, or inhibitors. The importance of these interactions for binding substrate and inhibitors has been quantified in PDE5: mutation of Phe-520, the main aromatic residue in the clamp (P820A), produces losses in affinity for cGMP (60-fold), sildenafil (70-fold), vardenafil (450-fold), tadalafil (140-fold), and IBMX (90-fold). The other side of the purine (or rings of inhibitors) is surrounded by the side chain of another phenylalanine and aliphatic amino acids. In PDE5, effects of mutagenesis of the latter group are modest (440). A conserved tyrosine (Tyr-612) in this pocket contributes to the hydrophobicity; its substitution produces losses in affinities for cGMP (15-fold), vardenafil (120-fold), sildenafil (30-fold), and tadalafil (50-fold).

3. Interaction of cN substrates and products of hydrolysis with PDE catalytic domains

Until recently, there were no structures for PDEs in complex with the cN substrate. Consequently, it was proposed that products would serve as a guide to the substrate binding. This seems counterintuitive since 5’-nucleotides interact weakly with PDEs (\( K_i \sim 1–10 \) mM) compared with the affinities for substrates (\( K_m \sim 0.02–40 \) µM) (399). Wang and co-workers (399, 401) have determined the x-ray crystal structures of PDE4D2 (a cAMP-specific PDE) and PDE10 (dual-specificity PDE) utilizing C domains with WT sequence and those in which mutations of critical residues have rendered them largely inactive. The mutations introduced into PDE4D2 or PDE10A2 did not significantly alter the active site conformation compared with WT proteins, thereby validating insights regarding interaction of substrates with WT PDEs.

The structure of the inactive PDE4D2 mutant (D201N) in complex with cAMP shows significant differences to that of WT enzyme in complex with 5’-AMP; both product and substrate are in the anti configuration (28, 53, 401). The invariant glutamine (Gln-369) forms one hydrogen bond with adenine (N1) in cAMP compared with two bonds (at N1 and N6) in 5’-AMP. Oxygen appended to the phosphorous in 5’-AMP bridges the metal ions that provide the catalytic machinery, but the cyclic phosphate moiety in cAMP does not contact the metal ions. The fact that the cyclic phosphate moiety is not closely juxtaposed to the metal ions is surprising since current understanding of the PDE catalytic mechanism implies that the hydroxyl ion bridging the metal ions is in close proximity to the phosphorous to allow for insertion of that hydroxyl leading to breakage of the cyclic phosphate bond.

Wang et al. (399) have also determined eight structures of the PDE10 C domain, a dual-specificity PDE;
these include WT and mutant proteins that are largely inactive due to mutation of the two invariant aspartic acids. Results of this study have better defined contacts that provide for interaction with cAMP or cGMP, documented differences in these contacts versus those made with the products, and dissected the role of metals in enzyme structure/function (399). In contrast to PDE4D, the syn conformation of cAMP/cGMP binds to the PDE10 catalytic site albeit in different orientations, whereas products are bound in the anti configuration. This raises the question as to whether the initial syn product must be converted to the anti configuration prior to release. The difference in orientation of the cNs produces different hydrogen bonding patterns; the invariant glutamine (Gln-726) forms two hydrogen bonds with cAMP (N1 and N6) compared with one bond with cGMP (N7). The potential for a difference in the orientation of cNs in the site of this dual-specificity PDE may explain results of earlier studies on PDE3A (also a dual-specific PDE) in which it was concluded that both cNs interact with certain residues but that each contacts a unique subset of amino acids (433).

4. Metal ion content, coordination, and structural importance

A unique metal-binding site in PDEs that utilizes two metal ions with one of them being zinc was first suggested in studies with PDE5 (109, 116). However, a role for zinc in PDEs was not widely accepted until publication of the first x-ray crystallographic structure of a PDE4 C domain (417), which revealed a novel binuclear metal-ion binding site in which zinc was tightly bound in one site, i.e., the Me-1 site. Despite widespread acceptance that magnesium is required for PDE catalytic function, this is not a certainty since for most PDEs other metals support activity equally flexible in solution and sample multiple conformations, which thwarts efforts to trap them in a stable state for physical analysis. Many PDEs (and their functional domains) aggregate at concentrations required for biophysical techniques. A greater barrier has been experienced with the PDE6 family whose members cannot be expressed as active enzymes in numerous systems; this complication has resisted resolution until recently when Muradov et al. (265) reported expression of human cone PDE6 in rods of transgenic Xenopus laevis; the enzymatic characteristics of the recombinant enzyme are similar to those of native bovine PDE6C, but this system is tedious and the enzyme yield is low, which continues to restrict studies.
well or better. In almost all PDEs, a zinc ion is important for catalytic function; identity of the second metal ion has not been determined for any PDE, and the potential for regulation of metal-ion content/function of a PDE is rarely considered. However, PKA phosphorylation of PDE4 increases affinity for magnesium (Mg). Unphospho-PDE4 exhibits low-affinity ($K_{Mg} > 40$ mM) and high-affinity magnesium ($K_{Mg} \approx 0.06$ mM) sites for support of catalytic activity, which suggests two conformations (Fig. 7); $K_{Mg}$ of the low-affinity site is below what is considered to be physiological magnesium concentration (372). PKA phosphorylation of UCR1 converts this pattern to a single high-affinity species ($K_{Mg} \approx 0.06$ mM) (298). Long forms of PDE4A, -B, and -D have 3- to 12-fold higher affinity for magnesium than the respective isolated C domains, and appending UCR2 to the C domain restores high affinity for magnesium (318). It is plausible that divalent metal ion levels change within cellular compartments; if so, this is likely to impact PDE activities. The variation in affinity for magnesium among different forms of a PDE and the recent appreciation of the widespread compartmentalization of PDEs makes this a reasonable candidate for regulation.

Zinc occupies the Me-1 site in all x-ray crystallographic structures of isolated PDE C domains (Fig. 6, A and B) with the exception of that for PDE3 (183, 327, 427); the strong bias in the field is that magnesium occupies Me-2. However, many PDEs have higher affinity for manganese or cobalt than for magnesium in supporting catalytic activity, and for PDE9, $V_{max}$ with manganese is twice that for magnesium (163). Despite having similar coordination in the x-ray crystal structures, affinity for zinc appears to vary among PDEs. Crystals of PDE4D isolated C domain that have been formed in the presence of EDTA have zinc at Me-1. However, zinc can be removed from the PDE10 isolated C domain by EDTA within 30 min (399). In contrast, EDTA is relatively ineffective for removal of zinc from PDE6 holoenzyme where several days of exposure to a stronger chelator is required to remove zinc (140). Thus, on the basis of the presence of zinc in structures that have been determined for almost all mammalian PDEs and the biochemical evidence for tight binding of this metal ion, it seems likely that perhaps with the exception of PDE3 all mammalian PDEs will constitutively contain zinc in the Me-1 site under physiological conditions. In studies of PDE9, it has been concluded that, as isolated after purification, the most likely occupants of Me-1 and Me-2 are zinc and magnesium, respectively (163).

Direct coordination of zinc in Me-1 involves two invariant histidines and two invariant aspartic acid residues (Fig. 6, A and B). Three of these (two histidines and one aspartic acid) reside in the “histidine-rich” segment of the PDE C-domain sequence; however, the other aspartic acid is located COOH terminal to this region. Coordination of metal ion in the Me-2 site utilizes one of the aspartic acids that is also involved in the Me-1 site along with five water molecules (183, 184, 427); direct contact with only one amino acid in Me-2 fits with a much lower affinity for metal in this site. For PDE9, different combinations of metal ions produce distinct properties and structural features (215) and indicate that an active PDE9 catalytic site can be generated by several combinations of divalent metal ions in Me-1 and Me-2, respectively. These include zinc-magnesium, manganese-magnesium, manganese-manganese, or magnesium-magnesium, but it appears that Me-1 is preferentially occupied by zinc or manganese.

Initially it was proposed that zinc plays a structural role by juxtaposing subdomains of the C domain to create the catalytic site; recent evidence argues against this. In PDE10A2, the residues that coordinate zinc are His-529, His-563, Asp-564, and Asp-674 (399). All of these contribute to the zinc-binding affinity, but the downstream aspartic acid (Asp-674) is key for binding zinc and maintaining catalytic activity (383). Wang et al. (399) generated WT PDE10 C domain along with two mutant proteins (D564A or D674A) and compared the proteins; the mutant proteins were three to four orders of magnitude less active than WT PDE10A2 and could be used to form crystals with cN as described in section H3. In the D674A mutant (unliganded or in complex with either cN), zinc is absent, although the Me-2 site is occupied. Since assays are conducted in the presence of magnesium, this indicates that magnesium, although bound, cannot support catalysis in PDE10. Similarly, PDE5 preparations that have been stripped of metal are inactive, but cN analogs and inhib-
itors can interact with the protein albeit with decreased affinity (75, 115). In the D564N PDE10A2, both metals are present, but the enzyme is still largely inactive. Moreover, superimposition of the structures of the WT and mutant PDE10 proteins (either in the unliganded state or in complex with cN or the products) reveals no significant differences.

Several conclusions can be derived from these studies: 1) the most critical amino acid for binding zinc and sustaining catalytic activity in PDE10 and perhaps in all mammalian PDEs is the downstream aspartic acid; 2) zinc does not play a critical role in assembling the segments of the C domain to form the catalytic pocket; 3) zinc is critical for maintaining a catalytically functional enzyme; 4) the presence of metal in Me-2 is not sufficient for support of catalysis; and 5) in PDE10, the aspartic acid (Asp-564) that bridges the two metal ions plays a critical role in catalytic function. However, in PDE5 holoenzyme, mutation of the homologous aspartic acid decreases catalytic activity by <10-fold (383). This may, like the different effects of EDTA on PDEs, reflect nuanced differences in the metal-binding sites.

However, in agreement with the results reported for PDE10, mutation of the downstream invariant aspartic acid in PDE5 abolishes catalytic activity (383). Notably, mutation of either of the invariant histidines in PDE5 (His-617 or His-653) that directly contacts zinc causes major loss of catalytic activity that can be overcome by high levels of manganese (but not magnesium) (116); however, manganese does not overcome the effect of the D764A mutation. Both the biochemical and structural studies emphasize the pivotal role of the downstream aspartic acid in PDE catalytic function. The results derived from mutation of either of the two histidines in PDE5 suggest that high manganese can fill the catalytic role of zinc in the Me-1 site or that mutation of either of these histidines introduces a physical perturbation in the metal-binding sites that can be overcome by a high level of manganese that perhaps occupies both Me-1 and Me-2. Zinc cannot be used in such studies due to nonspecific inhibition at >1 μM zinc (116).

Studies have been conducted based on the internuclear distances in the optimized geometries of the structures of PDE4 and PDE5 to ascertain whether the internuclear distances implicate the bridging nucleophile as a water molecule or a hydroxyl ion; a hydroxyl ion is indicated as the bridge (416). Moreover, the simulations and calculations have been performed with the assumption that Me-1 is always occupied by zinc, but with the possibility that Me-2 is occupied by either magnesium, manganese, or zinc. Similar analyses of the metal ion requirements of PDE9 have led these workers to conclude that in optimum catalytic conditions there is likely to be zinc in Me-1 and magnesium in Me-2 and that a hydroxyl bridges the metals (215).

5. Mediation of catalysis in PDEs

Studies of PDE6 support the interpretation that cGMP and magnesium bind independently to the catalytic site (357), a result that is now supported by kinetic studies and x-ray crystallographic structures of other PDEs (75, 215, 216) (Morris GZ, Ke H, Wang H, Francis SH, Corbin JD, and White H, unpublished results).

Generation of a nucleophilic hydroxyl ion appears to occur by the polarization of a solvent water molecule through interactions with the first conserved histidine in the histidine-rich sequence in the C domain and the two metal ions. Polarization and activation of this hydroxyl appears to be enhanced by the downstream aspartic acid described above since, where this has been studied, substitution of this residue produces inactive PDEs. The other invariant aspartic acid (immediately COOH-terminal to one of the invariant histidines that bind zinc) is likely to play a role in creation of this nucleophile, although the magnitude of this effect varies among PDEs (383, 399). However, in the co-complex of cNs and the PDE10A2 mutants described above, the metal ions are at least 4 Å from the cyclic phosphate moiety. The results demonstrate that we are still woefully ignorant of the molecular dynamics that approximate the attacking hydroxyl nucleophile that bridges the metals with the cyclic phosphate moiety that is its prey. The results suggest that cN binding to the PDE catalytic site is only the first step in the hydrolytic process and that subsequent adjustments must occur to bring the cyclic phosphate ring near to the catalytic machinery.

Only a few studies have investigated the catalytic mechanism of PDEs (215, 322, 357). Given the structural similarities in PDE catalytic sites that have thus far been determined, the basis for such diverse catalytic rates (>8,000-fold) is difficult to understand (30, 70). Liu et al. (215) have utilized crystals of the isolated C domain of PDE9 and a freeze-trapping technique to isolate intermediate species in catalysis for analysis by x-ray data collection. Multiple molecular species have been trapped, and contacts between the enzyme and the ligands have been defined. The forms of PDE9 that have been resolved include 1) unliganded enzyme (E); 2) the enzyme-cGMP complex (ES), which is present when cGMP is incubated with the enzyme in the absence of metal ions or in the presence of inhibitory zinc concentrations; 3) the enzyme-5*-GMP complex (EP) which is the immediate result of hydrolysis; and 4) the final enzyme-5'-GMP complex (E + P), which is found when active enzyme (in presence of metal ions) is incubated with cGMP or 5'-GMP, suggesting that it is the final species. The combined results indicate that dissociation of the 5'-GMP product (step 3) from PDE9 is the rate limiting step. Since cGMP and 5'-GMP bind to PDE9 in the anti conformation, an isomerization

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step to produce the product for release is not required (399).

The conformation and contacts of the ribose and phosphate moieties in the EP and E + P species differ: 1) in the EP complex, the ribose moiety retains the 3′-endo-4′-exo puckering that is found in the substrate, but in the E + P complex, this is changed to 3′-endo puckering; and 2) in the EP complex, the ribose and phosphate have well-defined electron densities, whereas in the E + P complex these are less well defined. Fewer contacts are consistent with weak product inhibition of PDE9, a characteristic that is true for all mammalian PDEs where this has been studied (399).

Additional insights into the catalytic process have been garnered from studies of PDE9. When cGMP binds to the catalytic site, its orientation and placement is characterized by 1) hydrogen bonding between the invariant glutamine and the C6 oxygen/N1 of guanine and 2) interactions between C1′, O4′, C4′, and C5′ with side chains of three hydrophobic residues, i.e., Phe-456, Met-365, and Ile-403. The homologous position for Met-365 in other PDEs is always occupied by a hydrophobic residue, which suggests the importance of this contact among all PDEs. The O2′ atom interacts with the Tyr-424 hydroxyl and O3′ atom through hydrogen bonding. Unlike the situation in PDE10, the axial and equatorial oxygens of the cyclic phosphate moiety coordinate with metals in Me-1 and Me-2, respectively. In this arrangement, the distance between the phosphorous and the catalytic hydroxyl that bridges Me-1 and Me-2 is estimated to be ~2.4 Å.

As expected based on early studies of PDE catalysis, insertion of the hydroxyl at the phosphorous results in 1) incorporation of the solvent-derived group into the product, 2) inversion of the appended oxygens (46, 127, 171), and 3) breakage of the O3′-phosphorous bond. His-252, the first histidine in the conserved histidine motif, appears to interact with the O3′ in both ES and EP and act as a general acid by donating a hydrogen that neutralizes the negative charge on O3′ upon transition from cGMP to 5′-GMP. Delivery of this proton to O3′ is purportedly fostered by a relay between His-252 and Glu-423. This report has provided valuable insights into the catalytic mechanism of PDEs, but there are still gaps in our understanding of this process.

6. Roles of conserved residues in catalytic function of mammalian PDEs

The importance of the conserved amino acids in the C domain is clear (183, 427). The invariant residues that coordinate the two metal ions are established; the invariant glutamine, conserved phenylalanine, and conserved tyrosine play critical roles in binding the substrate(s) in an orientation that facilitates hydrolysis by the catalytic machinery and are also important in binding PDE inhibitors. The impact of other conserved amino acids lining the catalytic site is modest (76, 406, 439, 440). The H-loop that adjoins the metal-binding site in the primary sequence of PDEs and begins with an invariant glycine has been implicated in determining interactions with substrates and inhibitors (63, 161, 163, 183, 287, 400, 403). In the x-ray crystallographic structure of the near full-length PDE2, insertion of the H-loop into the catalytic site is suggested to be a critical part of the autoinhibitory mechanism (Fig. 3) (287), and in the PDE5 C domain it assumes multiple conformations dictated largely by the inhibitor that is bound (183, 400, 403). The full implications of the role of the H-loop in PDE functions are yet to be determined, since its deletion in the isolated C domain of PDE5 causes minimal disruption in function (400).

The magnitude of changes in catalysis resulting from site-directed mutagenesis frequently differs among PDEs, a point that is cautionary to making global generalizations about function. Substitution of either of the metal-binding histidines or the downstream aspartic acid invariably produces profound loss in catalytic function. For other residues, the results are less consistent. Mutation of His-252 (H252A) in PDE9 produces total loss of catalytic activity, whereas mutation of the homologous histidine (His-603) in bovine PDE5 causes a 40-fold loss (215, 383). Mutation of PDE5 Glu-775 that is homologous to PDE9 Glu-423 which forms a hydrogen-bond relay with His-252 to foster catalysis produces only a threefold loss in k_cat (383). Likewise, mutation of Asp-644 (D644A) in bovine PDE5 holoenzyme causes an 8-fold reduction in k_cat (383) compared with >1,000-fold loss in activity when the homologous residue (Asp-564) in PDE10 is mutated (399). The mutational analysis in PDE5 has been performed in the holoenzyme in contrast to many results that are based on x-ray crystal structures and mutations in isolated C domains. A quantitative analysis of the function of amino acids implicated in catalysis by site-directed mutagenesis in other PDE holoenzymes would be useful in more fully evaluating their importance among PDEs.

A) THE FIXED SIDE CHAIN OF THE INVARIANT GLUTAMINE DOES NOT DETERMINE SUBSTRATE SELECTIVITY. The concept known as the “glutamine switch” that invokes the role of the invariant glutamine as the determinant of cAMP or cGMP specificity in PDEs has gained wide acceptance (426). While the role of the invariant glutamine in determining high-affinity interaction with substrates (and many inhibitors) is indisputable, the role of its anchored side chain in dictating cN selectivity through a “switching” orientation mechanism is not supported by structural or biochemical studies (163, 168, 398, 399, 439, 440). According to the glutamine switch hypothesis, in cAMP-specific or cGMP-specific PDEs, the invariant glutamine side chain is locked into one of two configurations by other residues. These “locked” orientations purportedly provide for selective formation of two hydrogen bonds with either
cAMP or cGMP and exclude interaction with the other cN. In dual-specificity PDEs, the proposal states that the side chain is free to rotate and bind either cN. Evidence that argues against the “glutamine switch” concept is as follows:

1) In PDE9, a cGMP-specific PDE, the side chain of the glutamine is not tethered in place (163).

2) In PDE2 and PDE10 (both dual-specificity PDEs), the side chain of the glutamine is locked in place through a hydrogen bond and therefore unable to rotate (168, 399).

3) The calculated loss in free energy of binding of cN substrate or inhibitors in PDE5 (cGMP-specific) or PDE11 (dual-specificity) following replacement of the glutamine indicates loss of one hydrogen bond (406, 439, 440). Catalytic rates in those mutants are like those of WT enzymes. In the x-ray co-crystal of PDE4D2 in complex with cAMP, there is only one hydrogen bond between the glutamine side chain and cAMP compared with the prediction of two bonds (401).

4) Replacement of the tethering amino acid in PDE5 (Gln-776) with alanine should allow for rotation of the Gln-817 side chain and improve affinity for cAMP, but it does not (439). Loss of affinity for the preferred cGMP, e.g., loss in PDE5 affinity for cGMP with the Q776A mutation, with no gain in affinity for cAMP does not support a role for the tethering of the invariant glutamine side chain in determining specificity.

B) THE CONCEPT OF A “SUBSTRATE-SPECIFICITY POCKET.” Ke’s group (399) has advanced the concept that interaction of PDEs with cN substrate(s) is dictated by a “substrate specificity pocket” or S-pocket that exhibits variations among PDEs (399). While retaining some shared features, the differences in these amino acids are proposed to provide for changes in the shape and size of the PDE catalytic pockets. This seems reasonable when considering PDE1 isoenzymes: the C domains of these enzymes share a high degree of similarity, but the affinities for cGMP versus cAMP vary by 20-fold (30).

III. MECHANISMS INVOLVED IN REGULATION OF PHOSPHODIESTERASES

PDEs are regulated through a variety of autoinhibitory mechanisms, activation schemes, and changes in protein expression. These are physiologically regulated in the developing and mature organism in response to hormonal, environmental, or pharmacological stimuli. Pathophysiological conditions also impact these mechanisms. In addition to biochemical and pharmacological studies, knockout mouse models have been designed with deletion of PDE1B (296), PDE1C (83), PDE3 isoenzymes (65, 234), PDE4 isoenzymes (8, 74, 176, 290), PDE7A (419), PDE7B (382a), PDE9 (92), PDE10A (346), PDE10A2 (326), or PDE11 isoenzymes (185). Combining insights from the behavioral and physiological characteristics of these animals with those based on pharmacological studies using selective PDE inhibitors continues to provide information regarding PDE functions.

A. Autoinhibition

Most mammalian PDEs exhibit high- and low-activity states that relate to catalytic function and to affinities with which regulatory ligands are bound, ease with which the PDE is phosphorylated, and structural changes. In most instances, autoinhibition in PDEs involves/requires elements in the NH2-terminal portion, but some PDE families have regulatory features that are located in the C domain (18, 50, 155) or within the catalytic site itself (39, 40, 79). Catalytic activity of PDE6 isoenzymes appears to be largely mediated by the inhibitory Py rather than by an intrinsic autoinhibitory domain within the PDE6 catalytic monomer/dimer (80).

The characteristics of activation of PDEs differ, which implies variation in molecular events; activation can increase 1) the $V_{\text{max}}$ as occurs in PDEs 1, 2, 3, 4, 5, and 6; 2) affinity for substrate or inhibitors as occurs in PDEs 5 and 6; or 3) affinity for activators as occurs for PDEs 5 and 6 or for magnesium as occurs for PDE4 (30, 33, 117, 298, 377). Even among closely related PDEs, the structural elements that are directly involved in activation differ; allosteric cGMP binding in GAF-A of PDEs 5 and 6 modulate activation (80, 117, 142, 232, 257, 437), whereas cGMP binding in GAF-B activates PDE2 (233, 413). A change in $V_{\text{max}}$ alone implies that previously unavailable catalytic sites become catalytically active with no changes in the catalytic site structure. However, increased affinity for substrate, inhibitors, or metals implies that the catalytic site has undergone a conformational perturbation that alters interaction with these ligands.

B. Domains and Modifications That Regulate Phosphodiesterases

1. Calmodulin-binding phosphodiesterases (PDE1)

The PDE1 family members are the only mammalian PDEs that are regulated by calcium; this is an allosteric regulation through interaction with the calcium/calmodulin complex. As such, they provide an important mechanism for cross-talk between the calcium- and cN-signaling pathways. Functions of PDE1 family members are implicated in numerous physiological and pathophysiological processes.

A) SHORT-TERM REGULATION. PDE1 catalytic activity is largely determined by intracellular calcium level from specific pools of calcium that enter the cell, and physiological processes that lower intracellular calcium, such as...
elevation of cGMP or cAMP, blunt PDE1 action (108, 249). In some cells, PDE1 appears to hydrolyze a pool of cGMP other than that targeted by PDE5 (249). Each PDE1 monomer within the catalytic dimer contains two sites near the NH₂ terminus for binding calcium/calmodulin (Fig. 2); calcium/calmodulin binding increases Vₘₐₓ by as much as 10-fold with no effect on affinity for substrate (30, 355). The arrangement of monomers within the dimer or the molecular events that account for activation are not known. Affinity for calcium/calmodulin varies among the isoenzymes, but in the presence of excess calmodulin, activation occurs in the range of 0.3–3 µM calcium. An autoinhibitory domain resides between the calmodulin-binding sites, and either limited proteolysis or NH₂-terminal truncation of PDE1 activates catalysis, rendering it insensitive to calcium/calmodulin (29, 355). However, dimerization interactions appear to be COOH-terminal to this region (193, 355). PDE1 isoenzymes can be phosphorylated in vitro by PKA or calcium/calmodulin protein kinase, but the physiological relevance of this has not been established (138, 342).

B) CONTROL OF EXPRESSION. PDE1 is widely expressed in mammalian tissues (29, 341); the more recent reports of upregulation of PDE1 protein and activity will be noted herein. PDE1 in cardiomyocytes is implicated as having a role in cardiac hypertrophy and remodeling (249). In models of cardiac hypertrophy, PDE1A, which preferentially hydrolyzes cGMP, is upregulated in the hearts and cardiomyocytes isolated from these animals. A PDE1-selective inhibitor (IC86340) blunts the hypertrophic response. These results are highly relevant since PDE1 provides the majority of cGMP-hydrolyzing activity in the heart (259, 388, 389, 395) and acts to counter the cytoprotective effects of cGMP signaling through cGMP-dependent protein kinase (PKG).

The major PDE1 subfamily in vascular smooth muscle cells (VSMC) appears to be PDE1A. PDE1A1 is selectively upregulated by chronic exposure to nitrates (192) and may contribute to nitrate tolerance. PDE1C is upregulated in proliferating human VSMCs and atherosclerotic lesions, and a PDE1 inhibitor suppresses proliferation (312). PDE1C mRNA and protein are also upregulated in pulmonary arterial vessels from patients with idiopathic pulmonary hypertension compared with vessels from healthy individuals and is a possible drug target for blocking vascular remodeling in these patients (270, 329). PDE1A activity is increased in VSMCs in a mouse model of hypoxia, and chronic administration of a PDE1 inhibitor reverses the associated elevation of pulmonary artery pressure, remodeling of the small pulmonary blood vessels, and right heart hypertrophy (329). Moreover, expression of PDE1 differs depending on the phenotype of the VSMCs (312); cells exhibiting the “synthetic” phenotype (such as those in the neointima, atherosclerotic lesions, and cultured cells) express PDE1 in the nucleus, whereas those exhibiting the “contractile” phenotype (such as those located in the medial layer of a vessel) express PDE1 in the cytosol (273). Transgenic mice that overexpress constitutively active Goαs foster high AC activity and have increased PDE1 level (186). This is presumed to be mediated via a cAMP-mediated upregulation of PDE1 production. PDE1 activities in the ductus arteriosum are also increased during gestation, an effect that may reduce sensitivity to cAMP elevation elicited by PGE₂ (212). Induction of PDE1 activates occurs during the differentiation of monocytes to macrophages and is proposed to affect the phenotypic characteristics of the differentiated cell (31).

2. GAF-containing PDEs

A) STRUCTURAL FEATURES OF GAFS THAT PROVIDE FOR CYCLIC NUCLEOTIDE BINDING. X-ray structural data are now available for the cN-binding domains in PDE2A GAF-B, PDE5A GAF-A, PDE6C GAF-A, and GAF-B in PDE10 (137, 141, 142, 232, 233, 287, 401a). Overall topologies of the GAFs and contacts with cNs are similar despite some differences in the specifics and characteristics associated with stability or state of dimerization. The role of GAFs in dimerization in PDEs is discussed in section IIIB1. All of these studies have utilized an isolated GAF or a cGMP-binding GAF conjoined to another GAF, and influences present in PDE holoenzymes will be absent. That said, the results derived from these studies define many of the structure/function features in these proteins and lay the foundation for understanding their regulation. cGMP binding to PDEs 2, 5, and 6 has regulatory influence on catalytic functions, but cGMP is bound in GAF-A of PDE5 and PDE6 or in GAF-B in PDE2. It seems likely that conformational transitions that bring about these effects will differ.

cN binding to PDE GAFs produces a large conformational change (141, 142, 232); in the unbound form, the GAFs show different patterns of stability and conformations that may relate to their functional features (affinity for cGMP and/or selectivity for cAMP) (232, 401a). In the apo-form, most of the cN-binding GAFs appear to sample multiple conformations so that cN initially binds to a flexible pocket that subsequently becomes more ordered and stabilizes multiple contacts of the cN in the binding pocket; the cGMP-binding GAF-A in PDE6C exhibits greater homogeneity and stability than other PDE GAFs (141, 232). Amino acids that contribute to the structure/function of GAFs in PDEs have also been investigated by site-directed mutagenesis (135, 142, 164, 385, 413).

Each GAF is comprised of a six-stranded anti-parallel β-sheet that is sandwiched between a three-helix bundle and two short α-helices (Fig. 8A). In x-ray crystal structures of cN-bound GAFs, the cN is almost entirely buried in the site. Interactions with the cN ribose and phosphate are similar among GAFs, but the conformation (boat ver-
A conserved threonine in cGMP-binding GAFs forms a hydrogen bond with O2= (137, 141).

In PDE2, PDE5, and PDE6, cGMP binding to the respective GAF is known to be involved in regulation of enzyme functions and will be discussed herein. Although selective for cGMP, the discrimination between cGMP and cAMP and absolute affinities for cN vary widely (80, 117, 229, 384, 413). Affinities (K_D) for cGMP in binding to different states of PDE2, PDE5, and PDE6 are ~10–30 nM (413), ~10–200 nM (117, 198, 376), and 10 nM (80, 164), respectively. In PDE2, there is ~30-fold selectivity for cGMP, in PDE5, there is perhaps 1,000-fold selectivity for cGMP, and in PDE6, selectivity for cGMP exceeds 3,000,000-fold.

Three amino acids in cGMP-binding GAFs provide for strong interaction with the guanine (Fig. 8B) (232, 233, 413); these are not invariant, but the positions are occupied by chemically conserved residues that presumably make similar contacts. These include an Asp/Asn/Ser that can act as a hydrogen bond acceptor. In PDE2A GAF-B, this residue (Asp-439) forms a hydrogen bond with N1, and its backbone amide forms a hydrogen bond with the C6 oxygen (233). The homologous position in PDE5A GAF-A and PDE11A GAF-A (also a cGMP-binding GAF) is also occupied by aspartic acid, but in PDE6C GAF-A, asparagine (Asn-116) is in this position. In PDE6C GAF-A, Asn-116 forms two hydrogen bonds with the guanine. It has been proposed that differences in physical mobility of the Asp/Asn side chain at this position contribute to determining the selectivity between cGMP and cAMP among the GAFs in various PDEs (232). Given the range of cGMP/cAMP selectivity in these sites, there are likely to be additional influences. The second significant contact in PDE2A GAF-B involves Phe-438, which stacks with the guanine. The homologous position is occupied by a Phe in PDE5 GAF-A, PDE6A GAF-A, and PDE6B GAF-A and a leucine in PDE6C GAF-A. The third major contact (Thr-488 in PDE2A GAF-A) interacts with the N2 through a bridging water molecule, whereas Thr-172 in PDE6C GAF-A forms a direct bond.

PDE10 GAF-B binds cAMP with very high affinity and exhibits structural features similar to those of cGMP-binding GAFs with the major differences being in contacts with the purine (118). cAMP binds in the chair conformation, and the adenine is wedged in a hydrophobic pocket formed by Phe-304 and Val-385. Arginine-286 forms a hydrogen bond with N1 and N3 interacts with the side chains of Asp-357 and Thr-360 through hydrogen bonds mediated by water molecules (137). The conserved Thr (Thr-364) forms a hydrogen bond with O2’, which also hydrogen bonds with Asn-353 and Asp-357 through water. Oxygens surrounding the phosphorous interact with the Gln-383 side chain and the main chains of Ile-330, Ala-331, and Asn-353. cAMP binding to PDE10 holoenzyme or cGMP binding to PDE11 has not been shown to change enzyme function and will not be discussed further.

B) Regulation of PDE2. cGMP binding to GAF-B in PDE2 activates catalysis (27, 233). Based on the x-ray crystal structure of the near full-length PDE2 without...
cGMP bound, Pandit et al. (287) propose that dimerization holds the C domains in an orientation that restricts solvent exposure of the catalytic sites (Fig. 3). Comparison of the structure of the cGMP-bound form of the PDE2 R domain with that of the near-full length PDE2 suggests that cGMP binding induces a conformational change in GAF-B and the adjoining linker that connects it to the C domain. This linker sequence is an extension of an α-helix at the COOH terminus of GAF-B and is involved in contacts between monomers. Movement in the linker is proposed to alter C domain orientation and free the enzyme from autoinhibition (287). In the closed conformation of PDE2, the H-loop folds into the catalytic pocket where it forms a part of the C domain dimer interface. The H-loop must reorient during activation to allow substrate access. Ke and colleagues (161, 183, 184, 400) have long emphasized the importance of the H-loop in PDE catalytic site functions; the recent work reported by Pandit et al. (287) further validates their early insights. In PDE5, substitution of the glycine (Gly-659), which begins the H-loop, with alanine, which has greater rotational constraints, reduces catalytic activity and affinity for substrate by ~20-fold (400). This suggests that the H-loop and the flexibility for it to move around the invariant glycine may modulate access to the catalytic sites in GAF-containing PDEs.

PDE2 exhibits low cAMP catalytic activity even in the absence of cGMP binding to the allosteric site (363). This could be explained if 1) PDE2 is in equilibrium between the closed (inactive) and open (active) conformations even in the absence of cGMP occupation of GAF-B or 2) cAMP, which binds to GAF-B with reasonable affinity (K_D ~600 nM) (413), could bind to GAF-B and shift the equilibrium to the open active conformation.

C) REGULATION OF PDE ISOENZYMES. I) Short-term regulation. There appears to be at least two autoinhibitory domains in PDE5 (117). For regulation of catalytic site function, either allosteric cGMP binding at GAF-A or phosphorylation at Ser-102 (human PDE5A1) increases V_max and affinity for cGMP substrate or inhibitors (32, 38, 78, 117, 262, 263, 313, 415). Phosphorylation occurs in intact cells, is preferentially mediated by PKG, and is facilitated by allosteric cGMP binding or ligand occupation of the catalytic site (33, 263, 377, 415). The increases in V_max and substrate affinity (K_m) are approximately three- to fourfold in smooth muscle cell extracts, but upon storage, the enzyme becomes activated (without change in size) and unresponsive to regulation (314). Likewise, following purification and storage of PDE5, the activity increase in response to phosphorylation is modest (78). Binding of catalytic site ligands increases allosteric cGMP binding and induces a conformational change that is associated with increased affinity for that ligand (33, 39, 79, 117). This suggests that PDE5 assumes physically defined conformations that exhibit varied kinetic characteristics (33, 39, 79). The active state may be the more stable form, since it accumulates during storage or purification.

GAF-B in PDE5 is autoinhibitory for cGMP binding to GAF-A and consequently for activation of the catalytic site (437). Physical interaction of GAF-B and GAF-A has been detected in NMR studies (142). Constructs containing only GAF-A have higher affinity for cGMP (K_D ~30 nM) than that of the holoenzyme (K_D ~200 nM) or of truncated constructs containing both GAFs (K_D ~115 nM). Whether repression of cGMP-binding affinity requires the entire GAF-B and whether dimerization influences cGMP-binding affinity are unknown (437).

II) Control of expression. PDE5 expression in tissues is modulated in response to a variety of stimuli, and overexpression is a likely culprit in many vascular maladies including hypertension, angina, diabetic angiopathy, and ED (173). PDE5 expression in corpus cavernosum (CC) increases in response to cN elevation (210), and in priapism, PDE5 levels are elevated (59). Oxidative stress in VSMC from CC increases PDE5 expression in response to 8-isoprostane F_2α, TNF-α, homocysteine and copper, or nicotine, which are all considered to be risk factors for ED (150 –152, 271). The increase in PDE5 protein expression is blocked by superoxide dismutase or catalase, which scavenges oxidants, apocynin, which blocks NADPH oxidase activity (the source of much of the superoxide), and sildenafil (151). Oxidative stress is also implicated in up-regulation of PDE5 mRNA and protein in lung and heart. PDE5 is widely expressed in neonatal lung, but at birth, expression decreases except for that in pulmonary VSMC (323). In a model of neonatal pulmonary hypertension (NPH), ventilation with 100% oxygen increases PDE5 expression and activity; this is reversed by administration of inhaled nitric oxide (iNO) or recombinant Cu/Zn superoxide dismutase (100, 101). In addition, PDE5 protein expression is increased in the failing heart under conditions of myocardial oxidative stress, pressure overload-induced left ventricular hypertrophy, and congestive heart failure (221). Selective inhibition of PDE5 protects ischemic/reperfusion-induced damage to the heart that is related to increased release of reactive oxygen species; PDE5 inhibitors blunt the damage incurred, suggesting that PDE5 protein and/or activity may be increased (47, 106, 200, 221, 319–321, 370).

D) REGULATION OF PDE ISOENZYMES. The physical presence of the inhibitor P_Ca at the catalytic site of PDE6 overwhelmingly controls its catalytic activity (80, 81, 256). P_Ca proteins block catalytic activity by forming a tight complex (K_D ~28 pM) with PDE6 catalytic subunits through contacts of a polycationic region with GAF-A and interaction with the catalytic site (10, 136, 264, 267). cGMP binding to PDE6 GAF-A enhances P_Ca binding (84). Light-activated transducin interacts with P_Ca to relieve its effect. In the x-ray crystallographic structure of GAF-A of PDE6α, a solvent-exposed segment that is enriched for
negatively charged and hydrophobic residues may be the interaction site for the Py polycationic region (232).

3. Regulation of PDE3 isoenzymes

A) SHORT-TERM REGULATION. PDE3A and PDE3B, which are products of separate genes, are widely expressed and involved in many processes. They are coexpressed in many cells but also have unique localizations (180, 213, 236, 286, 340, 364). These PDEs have similar affinities for cAMP and cGMP, but the rate of cGMP hydrolysis is ~10% that of cAMP; cGMP can compete with and inhibit cAMP breakdown at the catalytic site. Thus changes in cGMP level directly interfere with cAMP breakdown and thereby regulate cAMP signaling. PDE3B is activated in response to insulin, insulin-like growth factor I, or leptin (339, 434), which are compounds that negatively modulate cAMP, whereas cGMP is a inhibitor. When complexed with 14–3-3 proteins, phospho-PDE3B is resistant to dephosphorylation by phosphoprotein phosphatases and reversal of activation.

B) LONG-TERM REGULATION. Insulin increases expression of activities and protein of both PDE3B and PDE4D in 3T3-L1 adipocytes (Table 1) (281), and PDE3B protein is downregulated in adipocytes from animal models of diabetes and obesity (80, 371). Upregulation of PDE3B in response to insulin, dibutyryl cAMP, or dexamethasone involves actions of PKB and a PKA-mediated increase in phospho-CREB (93, 281), but the mechanism for this effect is not known. However, prolonged exposure of 3T3-L1 adipocytes to β-adrenergic agonists cause downregulation of PDE3B and upregulation of PDE4D (281).

4. Regulation of PDE4 isoenzymes

Years of studies have uncovered many of the complex processes involved in regulation of PDE4 (15–18, 44, 69, 74, 154–157, 175, 228, 275, 276, 297–299, 336). Recently, elegant studies using X-ray crystallography have provided a structural explanation for many earlier observations (50).

Four mechanisms for short-term regulation of the catalytic function of PDE4 isoenzymes include 1) autoinhibition by UCR2; 2) autoinhibition by a COOH-terminal domain; 3) modulation of activity by PKA phosphorylation, phosphatidic acid binding, or ERK phosphorylation; and 4) interaction with other proteins. The influences of these processes likely overlap.

A) AUTOINHIBITION BY UCR2. An autoinhibitory role for UCR2 in PDE4 was revealed when its removal was observed to increase catalytic activity (297, 298). Evidence indicates that UCR1 and UCR2 form a module that regulates catalytic function and oligomeric structure (26). PKA phosphorylation of a serine in UCR1 disrupts the UCR2 inhibitory influence and activates the enzyme (336).

Recent structural data suggest that PDE4 catalytic sites are regulated by a dual-gating mechanism involving direct interaction with a UCR2-derived helix or a helix derived from a COOH-terminal domain (Fig. 9) (50, 155). This suggests that dimeric PDE4 exists in at least three conformations: 1) an open conformation in which both catalytic sites within the dimer are active (Fig. 9A); 2) a conformation in which the catalytic sites are independently inhibited by contact with a helix from the COOH terminus of the same monomer (Fig. 9C), and 3) a conformation in which an α-helix derived from UCR2 (perhaps from the other subunit in the dimer) folds over the catalytic site to generate an asymmetric partially inhibited dimer (Fig. 9B). In the latter conformation, one site is capped and fully inactivated by UCR2, whereas activity of the other site is diminished, resulting in >50% inhibition when only one catalytic site is blocked. X-ray crystal structures show dimer contacts within the C domain, but solution biochemical studies indicate that UCR1 provides for dimerization (297, 417, 418).

The UCR2-derived α-helix binds in a groove across the active site where it makes multiple contacts. The sequence of this helix (−NQVSE[F/Y]ISXTFLD−) is identical in PDE4A-D except for a Phe/Tyr polymorphism shown in brackets in the sequence which is phenylalanine in PDE4D or tyrosine in PDE4A, PDE4B, and PDE4C, suggesting that this mechanism is shared among PDE4s (50, 155). Contacts are described using numbering for PDE4D7 (−193NQVSEFISNTLD203−): 1) Phe-201 inserts...

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### Table 1. Summary of changes in PDE activity and protein level after long-term treatment with insulin or isoproterenol

<table>
<thead>
<tr>
<th>PDE Activity or Protein</th>
<th>Insulin</th>
<th>Isoproterenol</th>
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<tr>
<td>PDE3 activity</td>
<td>+2.2-fold (46 h)</td>
<td>−30% (46 h)</td>
</tr>
<tr>
<td>PDE3 protein</td>
<td>+4-fold (46 h)</td>
<td>−40% (46 h)</td>
</tr>
<tr>
<td>PDE4 activity</td>
<td>+1.5-fold (46 h)</td>
<td>+2.5-fold (24 h)</td>
</tr>
<tr>
<td>PDE4 protein</td>
<td>+2-fold (46 h)</td>
<td>+2-fold (24 h)</td>
</tr>
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</table>

3T3-L1 cells were incubated with 100 nM insulin or 100 nM isoproterenol as indicated prior to measurement of phosphodiesterase (PDE) activity or PDE protein. +, Increase as compared with nonstimulated adipocytes; −, decrease as compared with nonstimulated adipocytes. [Adapted from Oknianska et al. (270), with permission from Elsevier.]
into the hydrophobic pocket in the catalytic site that is lined by Ile-542, Met-439, and Leu-485, and 2) Gln-192 hydrogen bonds with the backbone carbonyl of Asn-528. Phe-196 (or tyrosine in PDE4A-C) makes hydrophobic contacts. Autoinhibition by this helix is supported by the fact that an antibody generated against a portion of this sequence (193VSEFISNT200) activates PDE4D (209). The model proposed suggests that the interaction of only one UCR2 helix fully overlaps a catalytic site within the dimer. Access to the one remaining site may have positive pharmacokinetic implications (50). It is likely that UCR2-mediated inhibition is subject to many influences including PKA phosphorylation of UCR1, ERK phosphorylation of the C domain, and interaction with partner proteins. Insights derived from the Phe/Tyr polymorphism have recently directed development of PDE4 subfamily-selective inhibitors (50, 155) (discussed in sect. IVB4).

B) AUTOINHIBITION BY A C DOMAIN HELIX. Interaction between the PDE4 catalytic site and an α-helix (E6OHGQTEKFQFELIT⁸⁰⁵ in PDE4D) derived from the COOH terminus of that same monomer provides another autoinhibitory mechanism (Fig. 9) (50). The region where this helix is located is also involved in interactions with β-arrestin (19, 44, 289) or RACK1-scaffold proteins (43). ERK phosphorylation decreases catalytic activity of PDE4 enzymes containing UCR2 (227). In the recent structures, this phospho-serine has the potential to interact with an Arg in UCR2, which could stabilize the UCR2-capped autoinhibited state (50).

C) EFFECT OF ANCHORING/PARTNER PROTEINS. The list of proteins that interact with PDE4 isoenzymes continues to grow, and the impact of such complexes on catalytic function is complicated (44, 153). This now becomes more complex since some of these proteins interact with PDE4 in/near autoinhibitory regions. The immunophilin XAP2 interacts with the NH₂-terminal region of UCR2, resulting in inhibition of PDE4 catalytic activity and increased affinity for rolipram (45). This could be explained by XAP2 stabilization of the UCR2-capped conformation. Myomegalin interacts with the UCR2 NH₂ terminus, and in this complex, PDE4 is active (392); this interaction could stabilize the open active conformation (Fig. 9A).
DISC1 interacts with both the UCR2 and C domain of PDE4D, which decreases catalytic activity; elevation of cAMP disrupts this complex to activate catalysis (247, 269).

D) CONTROL OF EXPRESSION OF PDE4 ISOENZYMES. Many studies have addressed control of expression of PDE4 isoenzymes, but results differ most likely due to species differences and/or the model used (2, 154, 261, 368), and recent studies indicate differential regulation in expression of PDE4 isoenzymes (Table 2) (94, 281, 300). Elevation of cAMP is generally associated with upregulation of PDE4 isoenzymes (73, 154, 224, 281, 317, 379), but in neonatal mouse cardiomyocytes, expression of the PDE4A10 promoter is decreased in response to PKA activation through elevation of cAMP that is controlled by β-adrenergic coupled AC activity (238). This downregulation is thought to involve the transcription factor ICER and supports selective expression of PDE4 isoenzymes. In another report, cardiac hypertrophy induced by chronic pressure overload in a rodent model causes a decrease in the activities and protein levels of cAMP-hydrolyzing PDEs in the cardiomyocytes. PDE3 and PDE4 isoenzymes, the major cAMP-PDEs in cardiomyocytes, are decreased ~40 and 50%, respectively (2); PDE4A and PDE4B, but not PDE4D, are decreased. Repeated treatment of mice with antidepressants differentially changes expression of PDE4A, PDE4B, and PDE4D in regions of mouse brain, but the effects are not always in concert (Table 2) (94). Moreover, there are species differences in the PDE4 isoenzymes expressed in rodent brain regions and their responses to antidepressants; this may impact interpretation of results derived from effects of antidepressant effects on PDE4.

In studies of VSMC with either the synthetic or quiescent phenotype, cAMP elevation induces differential upregulation of PDE4D isoenzymes (379). In another study of aortic smooth muscle following angioplasty, expression and activity of PDE3A and PDE4B are increased, and fibroblast growth factor selectively increases expression of PDE4D in these cells (435). In human airway smooth muscle cells, there is regulation of selective expression of PDE4D isoenzymes at the transcriptional level (435), and hypoxia produces an upregulation of PDE4B2, PDE4A10/11, and PDE4D5 protein and activity without an increase in total PDE4 catalytic activity (248).

Temporal and developmental differences in expression and cellular distribution of PDE4D1 and PDE4D2 have been reported in Sertoli cells in response to follicle-stimulating hormone (FSH) (205). In white adipose tissue from FoxC2 transgenic mice, PDE4A activity and protein levels are decreased (134). This is primarily associated with loss of PDE4A5; PDE3 does not significantly change. Long-term insulin treatment of adipocytes elicits an increase in PDE4D protein and activity with no measurable change in PDE4A, -4B, or -4C, all of which are expressed in these cells (281). PDE3B activity and protein are also increased under these conditions. Long-term treatment with isoproterenol, a β-adrenergic agonist, also increases PDE4D protein and activity, but PDE3B activity and protein are lowered. Lastly, compartments containing the sarcoplasmic reticulum in cardiomyocytes from P3K-γ knockout mice have selective loss in PDE4, but not PDE3 (190).

5. SUMOylation of PDE4

Modification of a PDE by sumoylation [appending a SUMO group (small ubiquitin-related modifier)] occurs in the C domains of long members of the PDE4A and PDE4D families (207). Sumoylation does not alter basal catalytic rate, but activation by PKA phosphorylation in UCR1 is increased and inhibition by ERK phosphorylation is decreased; both effects require the presence of UCR1 (18, 146, 147, 336). In an overexpression system, PDE4 sumoylation translates into desensitization to β-adrenergic signaling.

C. Other Regulatory Effects Involving Phosphodiesterases

1. Migration of transducin in regulation of PDE6

Modulation of PDE6 activity in photoreceptors depends on light-mediated activation of transducin, the photoreceptor-specific G protein; Goα interacts with Py to relieve inhibition. In darkness, these proteins reside in outer segments of photoreceptor cells. At daytime light levels, ~90% of rod outer segment transducin translocates to the inner segments and synaptic terminals, but a critical threshold of transducin activation is required for this migration to occur (191, 354). Movement of Goα is rapid (t1/2 ~5 min) and exceeds that for Gβγ (t1/2 ~12.5 min). Transducin translocation decreases the amplification of the response to light through activation of PDE6 and

<table>
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<th>PDE4</th>
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<th>Cerebral Cortex</th>
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<tr>
<td></td>
<td>DMI</td>
<td>FLU</td>
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<tr>
<td>PDE4D3</td>
<td>† †</td>
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<td>PDE4B1/B3</td>
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<td>PDE4A1</td>
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†, Significant increase in expression; ††, significant decrease; †††, no significant change; DMI, desipramine (inhibits reuptake of norepinephrine and to a lesser extent serotonin); FLU, fluoxetine (serotonin uptake inhibitor); ROL, rolipram (PDE4-specific inhibitor). [Adapted from Dlaboga et al. (89), with permission from Elsevier.]
increases the range of light energies where rods can function. Prolonged light exposure increases the amount of activated transducin and extends the time in which transducin can dissociate from outer segment membranes, thereby allowing its migration (217).

When light intensity decreases, the transducin subunits undergo a slow reverse migration ($t_{1/2}$ ~ 2.5 h) (354). The rates of these translocations appear to be controlled by the rate of GTP hydrolysis on $G_{ot}$ (191). The same process controls transducin in cones (218), but even in bright light, cone transducin is rapidly inactivated so that the activated transducin level stays below the critical threshold for dissociation from the membrane and translocation.

2. Regulation of PDE7

Expression of PDE7B mRNA and protein in peripheral blood mononuclear cells from patients with chronic lymphocytic leukemia is greatly increased compared with cells from healthy individuals (428). PDE7B activity accounts for a greater portion of the cAMP-hydrolyzing activity in these cells, whereas levels of mRNA for PDEs 3B and 4D are reduced.

IV. ADVANCES IN DESIGN AND USE OF PHOSPHODIESTERASE INHIBITORS

A. Current and Emerging Clinical Uses of Phosphodiesterase Inhibitors

The therapeutic successes of PDE inhibitors include the use of 1) PDE3 inhibitors cilostazol (Pletal) and milrinone (Primacor) for treatment of intermittent claudication and acute heart failure, respectively; 2) PDE5 inhibitors [sildenafil (Viagra and Revatio), vardenafil (Levitra), and tadalfil (Cialis and Adcirca)] in treatment of ED and pulmonary hypertension; 3) PDE4 inhibitor roflumilast (Daxas) for treatment of chronic obstructive pulmonary disease; 4) theophylline, a nonspecific PDE inhibitor, for treatment of asthma; and 5) dipyridamole (Persantine), also a nonspecific PDE inhibitor, to decrease blood clotting following stroke or heart surgery. The approved uses of these medications have been extensively covered in other reviews and will not be fully addressed herein (55, 56, 82, 99, 108, 110, 111, 117, 119, 120, 123, 126, 259, 260). Milrinone is still used to treat acute heart failure, but increased mortality due to cardiac arrhythmias following chronic use has dampened enthusiasm for PDE3 inhibitors. Cilostazol (Pletal), a PDE3 inhibitor, is approved for treatment of symptoms of intermittent claudication associated with peripheral arterial occlusive disease (180, 181). Other potential applications of this medication include prevention of stroke, attenuation of restenosis of blood vessels following angioplasty (172, 203, 330, 334), treatment of vasospastic angina pectoris (404), promotion of cerebral vascular dilatation (34), and slowing progression of atherosclerosis in diabetics (250). There are some indications that milrinone may be useful in treatment of patients with NPH particularly when used in conjunction with iNO (62, 241).

2. Expanded uses for approved PDE5 inhibitors

The strong safety profile of the three PDE5 inhibitors that are currently marketed in most of the world has encouraged investigators to test the efficacy of these drugs in treatment of a number of ailments. In some instances, effects may relate to improved vascular health, but that does not appear to explain all of the benefits of these drugs.

A) NEUROLOGICAL INDICATIONS. 1) Cognition. In a study of memory consolidation in animal models, PDE5 inhibitors, like a number of other PDE inhibitors, improve cognitive function (194, 309, 310, 331, 386) and exhibit antidepressant effects (208). Vardenafil enhances early phase consolidation independent of its effects on cerebral vasculature (309, 310). Sildenafil also increases circadian adaptation to changes in exposure to light as occurs in transcontinental travel (5).

II) Stroke. In a model of stroke, sildenafil improves recovery of motor function and healing of experimentally induced neural lesions (429–432). In a model of middle cerebral artery occlusion, administration of a PDE5 inhibitor (PF-1) within 24 h of the insult with continued administration for 1 wk results in almost complete recovery of sensorimotor function (245). The authors hypothesize that improvement in recovery is due to vascular effects, perhaps involving microglial function. In combination, these studies support a potential role for PDE5 inhibitors in treatment of stroke.

B) MUSCULAR DYSTROPY. Preclinical studies in a model of Duchenne muscular dystrophy (mdx mice) show that PDE5 inhibitors blunt damage in skeletal muscle and improve stamina (11, 195). More recently, Adamo et al. (4) have shown that sildenafil treatment of aged mdx mice decreases the cardiomyopathy that is characteristic in these animals and rapidly reverses the cardiomyopathy once it is established. There is no effect of sildenafil on normal cardiac function in WT controls.
C) Neutral pulmonary hypertension. PDE inhibitors also show promise in treatment of NPH (20, 258). The only United States Food and Drug Administration (FDA)-approved drug for treatment of NPH is iNO (66), but some patients are unresponsive to iNO, or it can be difficult to wean them from dependency on the drug (165, 201). Evidence suggests that PDE activities impact pulmonary vascular tone in infants and changes following hyperoxic damage (62, 87, 100, 270, 329, 412). In some instances, use of a PDE5 inhibitor is helpful (274, 361). A few clinical trials for use of sildenafil in treating pulmonary maladies in infants have been completed (20, 258, 361). PDE5 inhibitors could be useful as a stand-alone medication or as an adjunct to other medications for NPH. If proven safe for use in neonates and children, these drugs would be a boon to treatment of NPH in developing countries where availability of inexpensive, orally administered drugs would provide a treatment option that is desperately needed.

D) Cardiovascular functions. There is mounting evidence that PDE5 inhibitors are atheroprotective (188), improve endothelial health (13, 304), and promote angiogenesis to treat ischemic cardiovascular diseases (47, 106, 200, 316, 319–321). In a model of postmyocardial infarction, sildenafil reduces infarct size, apoptosis, and cardiac hypertrophy as well as improves left ventricular function and survival (319). In a model of pressure overload, sildenafil blocks cardiac hypertrophy, dilatation, and fibrosis (370) and reverses established hypertrophy. These latter findings are controversial (225, 259, 388), but a clinical trial ("PDE-5 Inhibition to Improve Clinical Status and Exercise Capacity in Diastolic Heart Failure (RELAX)"") will soon assess the usefulness of PDE5 inhibitors in treating heart failure.

Physicians continue to evaluate clinical outcome when PDE5 inhibitors are used alone or in combination with other vasoactive medications (359). Prolonged exposure to PDE5 inhibitors may improve vascular functions; this possibility is supported by the fact that 46% of men whose ED is improved by daily tadalafil dosing for 1 yr have improved erectile function 4 wk after stopping treatment (291). The biological basis for this is not understood, but prolonged effects of PDE5 inhibitors have been previously reported (114, 251, 292); persistent effects could decrease dosing and medication exposure.

E) Other potential uses. Other possibilities for use of PDE5 inhibitors include treatment of Raynaud’s disease (54, 86, 118), suppression of other autoimmune diseases (48, 343), and relief of symptoms associated with cystic fibrosis (67, 222, 223). Potential treatment of genitourinary tract dysfunctions (benign prostate syndrome, overactive bladder, and urge incontinence) has garnered a lot of attention (324, 325, 362, 397, 425), as has treatment of Peyronie’s disease (128), premature ejaculation, and urethral relaxation for passage of kidney stones (132, 325). Some PDE5 inhibitors reportedly slow tumor cell growth (1, 268, 380).

B. Development of New Phosphodiesterase Inhibitors

The number of PDE inhibitors that are currently in development hold promise for new therapies. Depending on the target organ, a spectrum of potent and selective PDE inhibitors that incorporate different pharmacokinetic features are needed; these include drugs that are brain-penetrant, have fast or slow clearance, distribute preferentially to certain tissues, or can be administered as an aerosol.

1. PDE1 inhibitors

The need for potent selective PDE1 inhibitors is clear since PDE1 is linked to a role in the failing heart (389), cardiac hypertrophy (249), differentiation of monocytes in response to cytokines (31), VSMC proliferation (273, 311, 312), neurodegenerative diseases (244), and behavioral problems (185, 349). Vinpocetine, the best known PDE1 inhibitor, has been used clinically in some places but has poor cell penetration properties and a significant toxicity profile. Most PDE5-selective inhibitors also inhibit PDE1, which becomes problematic when high extra-cellular concentrations are used (388, 389). ICOS Corporation (now a part of Eli Lilly Corporation) has produced two selective and potent PDE1 inhibitors (IC229 and IC224 with IC50 values of 560 and 800 nM, respectively) (249, 388, 389).

2. PDE2 inhibitors

Potent selective inhibitors for the PDE2 family have not been available until recently. PDE2 is involved in aldosterone production (226), control of cGMP level in certain neurons (143), learning and enhancement of neuronal plasticity (309, 331, 386), modulation of cardiac L-type calcium current (104), control of specific cGMP pools in cardiomyocytes (58), blunting of anxiety (235), and impacting endothelial cell permeability (338, 366). Inhibition of PDE2 in any of these cells could result in elevation of cGMP, cAMP, or both cNs, thereby impacting both cAMP and cGMP signaling. For years, EHNA has been the most specific, albeit weak, PDE2 inhibitor available, but it also inhibits adenosine deaminase. Two potent and selective PDE2 inhibitors [BAY 60–7550 (2-(3,4-dimethoxybenzyl)-7-det-5-methylimidazo-[5,1-f][1,2,4]triazin-4(3H)-one) with an IC50 = 4.7 nM and PDP (9-6-phenyl-2-oxoex-3-yl)-2-(33,4-dimethoxybenzyl)-purine-6-one) with an IC50 = 0.6 nM] have been reported (41, 112, 338). Both compounds are being investigated for procognitive and antianxiolytic actions (235, 309).
3. PDE3 inhibitors

The involvement of PDE3 in the insulin- and leptin-signaling pathways and the association of these systems with the burgeoning problem of obesity and diabetes has renewed interest in development of compounds that block PDE3. To date, such compounds have not surfaced.

4. PDE4 inhibitors

PDE4 inhibitors have been reported to improve long-term memory (36, 309), reverse dietary-induced memory deficits (307), reverse the cholinergic deficit caused by scopolamine (308), and reduce symptoms associated with schizophrenia and depression (345). Development of clinically suitable PDE4 inhibitors has been hampered by side effects, which limit dosing and clinical efficacy.

Nycomed has recently received a positive recommendation in Europe for marketing of roflumilast (3-cyclopropylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide), a potent PDE4 inhibitor \((K_{IC50} < 1 \text{ nM})\) for use in treatment of COPD. Rofumilast (Daxas) is likely to be marketed by the end of 2010 (139, 315). It is a long-lasting, orally administered medication that blunts pulmonary inflammation, improves mucociliary function, and diminishes lung remodeling as well as pulmonary vascular remodeling and susceptibility to pulmonary hypertension. There is still a need for PDE4 inhibitors that are suitable for other uses. Aprremilast, a PDE4 inhibitor developed by Celgene Corporation, is currently in clinical trials for use in treatment of psoriasis and perhaps other inflammatory diseases (328).

PDE4 inhibitors fall into two categories: 1) “typical” competitive inhibitors that exhibit simple Michaelis-Menten kinetics and 2) “atypical” inhibitors such as rolipram, RS25344, and PMNPQ that block PDE4 catalytic action through interactions with two types of sites, i.e., high-affinity and low-affinity sites (318); the latter sites were given the names of high-affinity rolipram binding sites (HARBS) and low-affinity rolipram binding sites (LARBS) (169, 302), which vary in proportion among tissues.

For a PDE4 isoenzyme to exhibit HARBS, it must contain the C domain and UCR2 (169, 298, 302, 318). The recently resolved structural basis for HARBS and LARBS (50) reveals a new strategy for designing PDE4 inhibitors, including selective inhibitors for PDE4 subfamilies. In x-ray crystallographic studies of the PDE4 isolated C domain in complex with atypical PDE4 inhibitors, aromatic features of the inhibitors that impact potency when tested with full-length PDE4 isoenzymes extend from the catalytic pocket towards the solvent (50). Burgin et al. (50) reasoned that in the holoenzyme these components of the inhibitors might interact with residues contributed by UCR2, thereby increasing contacts and potencies. They have now shown that in the absence of inhibitor, an autoinhibitory UCR2 \(\alpha\)-helix lies over the catalytic site (see sect. IIIB4). When an atypical inhibitor binds to the catalytic site, an aromatic residue on that UCR2 \(\alpha\)-helix contacts the inhibitor and increases potency of interaction. In PDE4D, both phenylalaines in the UCR \(\alpha\)-helix stabilize interaction with atypical inhibitors. In PDE4A, -B, and -C, this effect is provided by the homologous phenylalanine and the tyrosine in the polymorphism.

The Phe/Tyr polymorphism in the UCR2 \(\alpha\)-helix alters interactions of PDE4 subfamilies with atypical inhibitors. This difference has now been exploited to design inhibitors that discriminate among PDE4 subfamilies. Several groups have recently reported potent selective PDE4B inhibitors that exhibit strong anti-inflammatory properties (272, 411).

5. PDE5 inhibitors

New PDE5 inhibitors are being sought to I) provide more choices in therapies for ED or pulmonary hypertension; 2) improve selectivity profiles for PDE5 versus other PDEs, which would reduce side effects and safety concerns; 3) improve pharmacokinetic properties or administration options that are desirable for emerging uses; and 4) develop drugs more suitable for unmet needs. Some drugs utilize a scaffold similar to that of sildenafil and vardenafil, whereas others (e.g., avanafil) have been developed based on novel scaffolds. Pfizer has recently reported novel compounds that are potent and selective PDE5 inhibitors (166, 283, 381). A collection of analogs of tadalafil have also been developed and tested for blocking tumor growth (1).

6. PDE7 inhibitors

Until recently, BRL 50481 [3-(N,N-dimethylsulfonamido)-4-methyl-nitrobenzene] has been the only PDE7-selective inhibitor available (352). More recently, two new potent PDE7A-selective inhibitors [SUN1817 (1-cyclohexyl-N-[3-fluoro-4-(4-methyl-1-piperazinyl)phenyl]-3-methyl-1H-tetraza[2,3-c]pyrazole-5-carboxamide methanesulfonate) with an IC\(_{50}\) value of 9 nM (130) and ASB16165 (1-cyclohexyl-N-[6-(4-hydroxy-1-piperidinyl)-3-pyridinyl]-3-methyl-1H-thieno[2,3-c]pyrazole-5-carboxamide monohydrate) with an IC\(_{50}\) value of 15 nM (178)] have been reported and are proving useful in cellular studies. Castano et al. (57) have designed a series of thioxoquinazoline derivatives that have submicromolar affinity for the PDE7A1 C domain versus micromolar affinity for PDE4D2. These compounds exhibit anti-inflammatory properties and are predicted to cross the blood-brain barrier.

7. PDE8 inhibitors

Until recently, the nonspecific inhibitor dipyridamole has been the most potent inhibitor of PDE8 isozymes, but the Pfizer group has recently produced a PDE8-selective inhibitor (PF-04957325), which is a zaprinast analog that
has IC$_{50}$ values of 0.3 and 0.7 nM for PDE8A and PDE8B, respectively, compared with IC$_{50}$ values for other PDEs of >1.5 µM (390).

8. PDE9 inhibitors

A potent PDE9-selective inhibitor [BAY 73–6961 (1-(2-chlorophenyl)-6-{[(2R)-3,3,3-trifluoro-2-methylpropyl]-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidien-4-one]} with an IC$_{50}$ ~60 nM has been produced by Bayer (414). It has strong selectivity for PDE9 with much weaker potencies toward other PDEs: PDE1 and PDE11 (IC$_{50}$ values for PDE1 = 1,400 nM, PDE11 = 2,600 nM, and for PDEs 2A, 3B, 4B, 7B, 8A, and 10A >4000 nM) (414). Vardenafil inhibits PDE9 (IC$_{50}$ ~600–3,400 nM) with a much weaker potency than that for PDE5 (IC$_{50}$ ~0.1–0.4 nM) (75, 105). A series of brain-penetrant PDE9 inhibitors that increase cGMP levels in vivo have also recently been reported (384), as well as a new group of orally available, potent and selective PDE9 inhibitors with IC$_{50}$ values in the 10–40 nM range that are orally bioavailable (92).

9. PDE10 inhibitors

Particular interest has focused on PDE10 inhibitors for relief of dysfunctions associated with glutamatergic and dopaminergic pathways (133, 244, 303, 331, 332, 345, 347, 378). Studies using papaverine pointed to potential utility of PDE10 inhibitors in models of schizophrenia (243, 346) and are supported by results with drugs that are more potent and selective for PDE10. In preclinical studies of papaverine and MP-10 (2-quinoline), workers at Wyeth Research have demonstrated that in striatum these compounds affect the dopamine D1-direct and D2-indirect pathways and that inhibition of PDE10 is associated with increased phosphorylation of Ser-845 in the (+)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (133). Pfizer Global Research recently announced a class of brain-penetrant PDE10A inhibitors (393). One of these (PF-2545920 2-[4–1-methyl-4-pyridin-4-yl-hyrazol-3-yl-phenoxymethyl]-quinoline) is being considered for use in treatment of schizophrenia. Results of studies with a new class of inhibitors have also provided support for a role of PDE10 in schizophrenia and related disorders (332) as well as in neuroprotection in a model of Huntington’s disease (124). Biotie Therapies has reported another class of potent and selective PDE10A inhibitors (imidazo[1,5-alpyrido[3,2-e]pyrazines) (148). Development of effective PDE10 inhibitors is currently one of the most active areas of research in the PDE field and holds promise for treatment of a number of neurological dysfunctions.

V. FEEDBACK MECHANISMS INVOLVING PHOSPHODIESTERASES

Negative-feedback regulation is one of the most fundamental of biological regulatory processes and in mammals can occur at several levels. The most familiar of these is probably hormonal regulation at the level of the whole organism. Typically, a hormonal signal released by an organ is transported by the bloodstream to a target organ causing release of a chemical signal from that organ, which is then transported by the bloodstream to the organ that released the hormone and thereby inhibits further release. Such systems are vital for 1) maintaining homeostasis of organisms, 2) prevention of overstimulation of signaling pathways, and 3) reversal of signaling effects following decline of the original stimulus. Negative-feedback regulation occurs at other levels for the same general purposes. Most intracellular metabolic pathways and signaling cascades exhibit this type of regulation. This discussion centers on the influence of negative-feedback control within cN-signaling pathways mediated by PDEs.

cAMP and cGMP are signaling molecules that activate steps in intracellular signaling pathways. They are crucial for transmitting extracellular signals to intracellular targets. Extracellular signals may be hormonal (endocrine), paracrine (e.g., adenosine), environmental (e.g., chemicals for taste), nerve (e.g., catecholamines), or ionic (e.g., calcium). Cells exploit common mechanisms for generating negative feedback. Negative feedback may be initiated at steps within the cell such as altering functions of the signal receptor, G protein, the AC/GC enzymes, or at steps that dampen/terminate the signal such as the actions of the phospho-protein phosphatases or cN PDEs. This discussion emphasizes PDEs, which mediate negative feedback by numerous mechanisms. The most common mechanism for negative feedback involving PDEs is mass action; when cAMP or cGMP is elevated, this higher substrate concentration increases the activity of the PDE that degrades it. This feedback applies to all PDEs as long as cGMP or cAMP concentrations are near or below the $K_m$ for that cN. In addition, a specific cAMP or cGMP pathway is regulated by intricate concerts of PDE-mediated negative-feedback mechanisms that superimpose on and enhance the mass action effect. This comprehensive assortment of mechanisms underscores the critical importance of negative-feedback control in cell physiology.

The following discussion focuses on negative feedback involving lowering of the cN level by PDEs following a stimulus that increases the cN level. Emphasis is placed on PDE5 because this cGMP-hydrolyzing PDE has been studied the most with regard to negative-feedback regulation. Negative-feedback control of the cAMP-signaling pathway involving PDE3 and PDE4 will be compared, and it will be shown that they serve similar roles in this type of regulation, albeit with some interesting differences in mechanisms. As more studies are done, it is expected that other PDEs within the PDE superfamily will be involved in negative-feedback control of cN pathways in addition to their roles by mass action.
A. Negative-Feedback Regulation of the cGMP Pathway Mediated by PDE5

In tissues in which PDE5 is present at substantial levels, this enzyme plays a major role in negative-feedback regulation. As is typical for negative-feedback responses, elevation of cGMP in these tissues by signals such as nitrovasodilators is transient despite continued presence of the signal (196). This is largely accomplished by myriad effects involving PDE5, which act in concert to produce negative feedback on the cGMP level. Ten distinct, but overlapping, mechanisms (Fig. 10) have been delineated for PDE5 involvement in negative-feedback regulation of the cGMP pathway as outlined below.

Mechanism 1: mass action due to cGMP elevation

Mass action is probably the most underemphasized, yet among the most important, of all physiological regulatory processes. In the present instance, the Law of Mass Action dictates that the reaction rate of PDE5 degradation of cGMP is directly proportional to cGMP concentration. This means that when cGMP is elevated in cells, its rate of degradation increases, which amounts to a very simple negative-feedback process. However, like other enzymes, PDE5 obeys Michaelis-Menten kinetics so that mass action applies only when the cGMP level is near or below the \( K_m \) of the enzyme for cGMP. This appears to be the case for PDE5; its \( K_m \) for cGMP has been determined to be 2–5 \( \mu \text{M} \) (376), and the physiological range of total intracellular cGMP in VSMC is typically in the nanomolar range (129, 174).

Mechanism 2: cGMP binding to GAF-A in PDE5 stimulates its catalytic activity

By mass action, an elevated cGMP level increases allosteric binding of cGMP to GAF-A in the PDE5 R domain. Binding of cGMP increases the catalytic activity of the enzyme (280), thus lowering the cGMP level following its initial elevation.

Mechanism 3: interaction of cGMP with the catalytic site of PDE5 stimulates cGMP binding to GAF-A

As noted for mechanism 1, elevation of cGMP increases its interaction with the catalytic site of PDE5 by mass action. This interaction stimulates cGMP binding to GAF-A, which, as dictated by the Principle of Reciprocity (405) and as noted under mechanism 1, stimulates the PDE5 catalytic process to lower cGMP.

Mechanism 4: sequestration of cGMP by its binding to GAF-A

In some tissues the PDE5 level is quite high and sufficient to bind a significant proportion of the cellular cGMP. For example, in rabbit penile CC, the PDE5 level is 188 nM whilst basal level of cGMP is only 18 nM (129). Because of the high affinity of cGMP (\( K_D \sim 0.2 \mu \text{M} \)) for GAF-A of PDE5, most of the cellular cGMP is expected to be bound to this site and therefore sequestered away from its target proteins such as PKG. Thus, although its breakdown may not be stimulated by this process, cGMP is rendered “inactive,” which represents another negative-feedback control of cGMP signaling.

Mechanism 5: activation of PKG by cGMP elevation stimulates phosphorylation of PDE5

PKG phosphorylates numerous proteins and is an important mediator of cGMP action in many cells. Elevation of cGMP activates PKG and brings about phosphorylation and activation of the catalytic activity of one of its primary cellular substrates, i.e., PDE5. This reduces the level of cGMP following its elevation.

Mechanism 6: phosphorylation of PDE5 by PKG stimulates cGMP binding to GAF-A subdomain

As described under mechanism 5, cGMP elevation stimulates phosphorylation of PDE5. In addition to stim-
ulating the catalytic site for cGMP degradation, phosphor- 
ylation also stimulates cGMP binding to GAF-A. This al- 
losteric cGMP binding further activates breakdown of 
cGMP at the catalytic site as described under mechanism 2 and increases sequestration of cGMP as described un- 
der mechanism 4.

**Mechanism 7:** interaction of cGMP with the PDE5 
catalytic site stimulates its phosphorylation by PKG

According to mechanism 5, phosphorylation of 
PDE5 activates the catalytic site of PDE5. By the Principle 
of Reciprocity, it would be expected that interaction of 
cGMP with the catalytic site stimulates phosphorylation 
of PDE5. This is indeed the case and represents another 
negative-feedback mechanism since phosphorylation 
then stimulates catalytic breakdown of cGMP (32).

**Mechanism 8:** cGMP binding to GAF-A of PDE5 
stimulates its phosphorylation by PKG

With the use of the same logic applied under mecha- 
nism 7, there should be a reciprocal relationship between 
activation of allosteric cGMP binding to GAF-A by phos- 
phorylation and stimulation of phosphorylation by allosteric 
cGMP binding to GAF-A. This is borne out by experimental 
evidence, and since phosphorylation stimulates catalytic 
breakdown of cGMP as described under mechanism 6, 
elevation of cGMP would subsequently result in increased 
breakdown of this nucleotide by the combined effects.

**Mechanism 9:** slow activation of PDE5 by catalytic 
site binding

Prolonged incubation (12 h) of PDE5 with cGMP 
analogs such as sildenafil increases the affinity of the 
catalytic site for these ligands (39). This implies that 
occupation of the catalytic site by cGMP would cause 
tighter binding of this substrate to and thus greater activ- 
ity of this site. Unlike the more acute mechanisms of 
regulation described under mechanisms 1–9, this mecha- 
nism would increase breakdown of cGMP only after 
chronic elevation of this nucleotide.

**Mechanism 10:** upregulation of PDE5 level by 
cGMP elevation

Another potential chronic negative-feedback mecha- 
nism for reducing cGMP levels following elevation may be 
represented by increase in the actual amount of PDE5 
protein after prolonged cGMP elevation. This is suggested 
by the observation that long-term exposure of VSMC to 
8-BrcGMP causes an increase in PDE5 activity in extracts 
of the tissue (117).

A conformational change(s) in PDE5 may underlie 
activation of PDE5 for negative-feedback control of 
cGMP. The changes in PDE5 activities (catalytic activity, 
allosteric cGMP binding, and/or phosphorylation) respon- 
sible for most of the acute negative-feedback mechanisms 
controlling cGMP levels (described under mechanisms 2–9) could be explained by conformational change in the 
enzyme. Catalytic-site ligands, cGMP, and phosphoryla- 
tion cause a similar native PAGE gel-shift of PDE5 to 
lower mobility, which indicates a conformational and/or 
charge alteration in the enzyme (33, 79). Therefore, PDE5 
may exist in two conformations in cells: 1) a more active 
conformation that is either more elongated in shape 
and/or has more positive surface charge, or both, and 2) a 
less active form that is more compact in shape and/or has 
more negative surface charge, or both. Each modification 
could produce a very similar conformational change that 
relieves autoinhibition and activates the enzyme for each 
of the other modifications.

**B. Prediction of Positive Feedback**

(Feed-Forward) Control for PDE5 Inhibitors

The stimulation of phosphorylation and GAF-A bind- 
ing of cGMP caused by cGMP elevation (mechanisms 3 
and 7) implies that cGMP analogs such as Viagra, Levitra, 
and Cialis should cause the same effects. In fact, that is 
the case (33), suggesting that these drugs would stimulate 
their own affinities for PDE5 in men taking the pills. This 
would represent a positive-feedback process for the 
drugs, whereas the same mechanism mediates a negative- 
feedback process for the cGMP substrate. Exploitation of 
the endogenous negative-feedback mechanism by PDE5 
inhibitors to create a positive-feedback effect for these 
drugs should permit lower dosages of the drugs than 
would otherwise be predicted. These drugs should also 
block the cGMP-lowering negative-feedback process ini- 
tiated by cGMP elevation since they would occupy the 
catalytic site to prevent cGMP breakdown. Therefore, 
PDE5 inhibitors produce their therapeutic effects by raising 
cGMP levels and by blocking the negative-feedback 
process.

**C. Negative-Feedback Regulation of the cAMP**

Pathway Mediated by PDE3 and PDE4

In some respects PDE3 and PDE4 mediate negative 
feedback of the cAMP-signaling pathway by similar mecha- 
nisms as described for PDE5 in the cGMP-signaling path- 
way. In addition to the mass action route, PDE phos- 
phorylation is a prominent mechanism for this feedback 
control.

**1. PDE3**

In the 1970s several investigators reported that the 
“low K_m PDE” (PDE3) is activated by incubation of tis-
sues with hormones that elevate cAMP (197, 219, 220). It has subsequently been shown that these effects are mediated by activation of PDE3B by phosphorylation catalyzed by cAMP-activated PKA (88, 90, 339, 350). Proof that this reaction occurs in intact tissues was the finding that addition of PKA-specific cAMP analogs to intact cells lowers cAMP (122). Therefore, in some tissues, PDE3 plays a crucial role in negative-feedback regulation of the cAMP-signaling pathway.

2. PDE4

PKA-mediated phosphorylation of PDE4 activates the enzyme. In a thyroid cell line, TSH acutely increases cAMP, activates PKA, and stimulates PDE4D activity by PKA-catalyzed phosphorylation (337). This is believed to serve as negative-feedback regulation. Moreover, upregulation of certain PDE4 isoenzymes by chronic cAMP elevation occurs in some cells (72, 94, 281, 367). FSH treatment of Sertoli cells causes cAMP-induced increases in PDE4D protein and mRNA levels, resulting in desensitization to subsequent FSH treatment.

Although PDE3 and PDE4 preferentially hydrolyze cAMP and are among the most widely distributed and abundant PDEs, they are distributed unevenly in body tissues and within cells. It is expected that the enzyme that is present in a compartment will mediate negative-feedback control in that compartment, but in numerous tissues, these families have specific roles and do not appear to provide back-up protection for lack of activity in the other PDE family (89, 234). Although PDE3 and PDE4 are associated with a collection of signalosomes, rarely are members of both PDE families found in the same signalosome involving PKA, Epacs, or phosphoprotein phosphatases (190, 295). However, few pertinent experiments have been carried out to determine the relative contribution of each of these families when both are present.

Positive feedback control as described for PDE5 inhibitors (sect. VB) may also apply to PDE3 and PDE4. Phosphorylation of either enzyme by PKA after cAMP elevation increases the $V_{\text{max}}$ of their respective catalytic sites. This could engender greater interaction of their respective PDE inhibitors to facilitate their actions and potencies. Moreover, each inhibitor blocks the respective PDE catalytic site, which blocks the negative-feedback process, thus further facilitating the action(s) of the inhibitor.

D. Possibility of Tachyphylaxis Due to Activation of Negative-Feedback Mechanisms Involving PDE5

Tachyphylaxis is defined as “a rapid decrease in the response to a drug after repeated doses over a short period of time.” So far, tachyphylaxis has not been observed during clinical tests of PDE5 inhibitors (114, 251, 291, 292). Tachyphylaxis produced by cAMP-elevating drugs that do not target PDEs is well-documented. A commonly known example is the treatment of asthma with $\beta$-adrenergic drugs. This condition could be brought about at least in part by effects on steps in the cAMP-signaling pathway that are not related to PDE actions, e.g., the level of the $\beta$-adrenergic receptor. However, studies are needed to quantify contributions by negative-feedback mechanisms involving cAMP-hydrolyzing PDEs such as PDE3 and PDE4.

VI. NONREdundant ROLES FOR PHOSPHODIESTERASES

The synthesis of cAMP and cGMP and action of the respective signaling pathways in selective cellular compartments has long been established. It is generally accepted that cyclases localized in different regions of the cell account for generating specific pools of cN. Likewise, cN-dependent protein kinases, which are major mediators of cN action, are in some instances anchored in certain regions of a cell as well as being found in the cytosol. The physiological importance of particular PDEs and the specific localization or dynamic relocalization of different PDEs has only recently been appreciated (58, 104, 153, 158, 159, 204, 387). However, particular PDEs and their specific locations within cells are key to physiological function. Moreover, the phenotypes of animals that have been rendered null for one PDE family/subfamily indicate that other PDEs cannot completely supplant the absent enzyme (44, 70, 74, 89, 185, 186, 300, 348, 364).

A. Physiological Implication for Localization and Function of cAMP-Hydrolyzing Phosphodiesterase Isoenzymes

With the use of cAMP sensors, microdomains of concentrated cAMP can be visualized to specific subcellular locations (424). Although most ACs are membrane-bound, this alone cannot explain the compartmentalized cAMP gradients. Evidence suggests that creation of cAMP microdomains requires fastidiously compartmentalized and anchored pools of PDEs that tightly regulate cN action at specific points of interest in the cell, and cAMP-hydrolyzing PDEs that have different affinities for cAMP within the same cell may be involved in physiological responses at different levels of intracellular cAMP (6, 96, 391). PDE localization to subcellular compartments can occur by various mechanisms including recruitment to lipid rafts, AKAPs, or other anchoring proteins like $\beta$-arrestin (6, 95, 340, 374). Indeed, members of the same PDE family located in different regions of the cell have been shown to
be selectively modulated by actions of single or different agonists (6, 410).

Increasing PDE activity near the stimulated AC rapidly reduces cAMP level in that area and dampsens PKA activation (24). cAMP-sensitive cellular processes that are mediated by PKA must function within a narrow range of cAMP concentration since a two- to threefold increase in cAMP produces maximum physiological response in most tissues (107, 353). Co-compartmentalization of the signaling proteins allows for certain PDEs to restrict cAMP access to specific pools of PKA near the target of interest and efficiently modify a limited number of downstream targets. This is certainly advantageous in regulating distinct functional effects in the heart where the role of PDE-mediated compartmentalization of cAMP has been well studied.

Several cAMP-degrading PDEs are expressed in the heart (PDE1, PDE2, PDE3, PDE4, and PDE8) (224); however, in cardiomyocytes, PDE4 and PDE3 account for the majority of cAMP degradation, whereas PDE1 and PDE2 degrade a minor fraction of intracellular cAMP (252). Despite the presence of substantial PDE3 activity, PDE4 primarily regulates cellular cAMP following stimulation of the β-adrenoceptor (β-AR) (252). Moreover, PDE2 activity, which is low in cardiomyocytes, is dramatically increased by β-AR agonists (253). The surprising sensitivity of PDE2 and PDE4 activities, but not that of PDE3, to the catecholamine-mediated rise in cAMP may be explained by their specific localizations in cardiomyocytes. Immunohistochromy shows that PDE2 and PDE4 are localized at the sarcomeric Z line suggestive of anchoring to subcellular structures, whereas PDE3 is associated with multiple pools of cAMP (252, 253). Live-cell imaging reveals a variable increase in cAMP due to PDE3 and PDE4 inhibition in ventricular myocytes, confirming the existence of distinct subcellular compartments (204). Assuming PDE localization correlates to function, one could hypothesize that PDE3 regulates cAMP-mediated cardiac contractility while PDE4 regulates β-AR-sensitive cAMP microdomains. With the use of a FRET-based sensor in the presence of IBMX, decay of cytosolic cAMP is delayed in myocytes indicating that PDE1 or PDE2 degrades cytosolic cAMP (204). This may also explain why PDE1 and/or PDE2 but not PDE3 or PDE4 regulate intracellular cAMP in response to prostaglandin E2 receptor stimulation (301). These experiments demonstrate that localization of specific PDEs to certain cellular compartments is more crucial to regulating cAMP level than the amount of the PDE isoform expressed in a particular cell. An example of the importance of PDE-mediated compartmentalization is observed in transgenic mice that specifically express human neuronal AC8 in the heart. Studies have found that these mice are protected from Ca2+ overload due to increased compartmentalization of cAMP resulting from rearrangement of PDE4 and PDE1 within the cardiomyocyte (121). Live-cell imaging confirms that changes in cAMP depend on a combination of Ca2+-stimulated AC8 activity and PDE4 activity (409).

Localization of PDEs is mediated by various mechanisms. The unique NH2 termini of PDE2A2 and PDE2A3 provide for their targeted localization to particulate cellular fractions. Several studies have shown that differential localization of PDE2 results in compartmentalization of cAMP and regulation of cellular functions (102, 277, 279, 305, 420). Posttranslational modification of PDEs may also contribute to cellular localization. PKA-mediated phosphorylation of PDE10A2 changes localization of the enzyme from the Golgi to the cytosol (190). Charyach et al. (61) have found that PDE10A2 must be palmitoylated to target this enzyme to the membrane (190). Currently, most PDEs appear to be localized through protein-protein interaction with a targeting protein such as immunophilin XAP2, DISC1, SH3 domain-containing proteins, myomegalin, Ndel1, Shank2, β-arrestin, RACKs, or AKAPs (282).

A majority of PDE4 compartmentalization and regulation of cAMP relies on AKAP-mediated localization (240). Association of particular PDE4 isoenzymes with specific AKAPs allows for tight regulation of cAMP levels by AKAPs. For example, PDE4 that can block AKAP-anchored PKA from activation at basal levels (239). AKAP149 (mitochondria) binds to PDE4A, AKAP95 (perinucleus) binds to PDE4A, and muscle AKAP (mAKAP) (perinucleus) binds to PDE4D3 (239). AKAP450, which anchors to the Golgi, interacts with PDE4A and AKAP7 and forms a complex with PDE4D to regulate osmotic water permeability (358). PDE7A has also been shown to interact with an AKAP, MTG (myeloid translocation gene) in the Golgi to form a regulatory module in the T cell (12). PDE3A has been shown to complex with another AKAP, BIG (BFA-inhibited GEP), which likely contributes to the cAMP-mediated regulation of ADP-ribosylation factors (293).

Whereas PDE-AKAP interactions form localized cellular pools of cAMP, interaction with other proteins can result in a dynamic compartmentalization of cAMP. Studies examining the PDE4-β-arrestin complex reveal that PDE4 can redistribute in the cell. PDE4D3 and PDE4D5 form stable complexes with β-arrestin, which under basal conditions are located in the cytosol. Addition of catecholamines results in accumulation of PDE4-β-arrestin complex at the membrane domains that contain activated β-ARs and ACs. Subsequently, the recruited PDE4 regulates the agonist-induced PKA activity at the plasma membrane (19).

PDE-mediated compartmentalization of cAMP may explain the need for having multiple PDE isoenzymes expressed in the same tissue. Studies of transgenic animals in which specific PDEs have been ablated as well as siRNA technology have advanced our understanding in the nonredundancy of PDE functions. Female PDE3A
knockout (KO) mice are sterile despite normal ovarian structure, folliculogenesis, ovulation, and mating behavior (234). PDE3B KO mice, while fertile, have altered regulation of energy homeostasis and display signs of insulin resistance (65). PDE4D KO mice have retarded growth, reduced viability, and low female fertility (177), whereas PDE4B KO mice produce significantly less TNF-α in response to lipopolysaccharide stimulation (176), and PDE4A KO mice have a normal phenotype (351). In all cases, the action of the ablated isoenzyme is not compensated by other PDEs, indicating that expression of multiple PDEs in a tissue is key for physiological functions unique to each.

In T cells, stimulation of the T-cell antigen receptor (TCR) increases intracellular cAMP, which causes increased interleukin-2 production and inhibition of T-cell proliferation; creation of tightly regulated pools of cAMP is critical to maintaining T-cell functions (373). PDE4A, PDE4B, and PDE4D are expressed in inflammatory cells, which creates difficulties in investigating the actions of these isoenzymes. Based on studies using PDE4A-, PDE4B-, and PDE4D-deficient mice, Ariga et al. (8) have determined that PDE4D and PDE4B, but not PDE4A, are necessary for neutrophil recruitment to the lung. Moreover, while PDE4D and PDE4B have similar functions, loss of one isoenzyme is not compensated by the remaining isoenzyme (8).

Nonredundancy among PDE4 isoenzymes is likely explained by their respective subcellular localizations. In transfected Jurkat cells, PDE4B2 associates with lipid rafts and enhances interleukin-2 production following TCR stimulation (9). PDE4A4 and PDE4D1/PDE4D2 are also recruited to lipid rafts following TCR stimulation and are complexed with β-arrestin (3). Several proteins involved in proximal TCR signaling, e.g., AC and PKA, are also localized to lipid rafts (254). In addition, PDE4 isoenzymes can complex with various AKAPs expressed in T cells (373). The heart has two β-AR receptors, β1-AR and β2-AR, and stimulation of either produces distinguishable outcomes. Different PDE4D variants complex with these receptors (299). PDE4D8 association with β1-AR controls basal cAMP around the receptor, whereas PDE4D5, complexed with β-arrestin, is recruited to β2-AR after the receptor is stimulated. The finding that PDE4D8, rather than PDE4D3 or PDE4D9, is the dominant PDE isoenzyme incorporated into a PKA-based signaling complex in VSMCs provides further evidence that the PDE4 splice variants have specific functions in the heart (294).

PDE4-mediated compartmentalization of cAMP in the T cell is both spatially and temporally regulated. Stimulation of TCR increases PDE4A and PDE4D transcription and function within 5 days, whereas PDE4B expression and activity is greater after 24 h (290). This suggests that PDE4B activity is critical to the immediate inflammatory response of T cells, whereas PDE4D activity is involved in chronic augmentation of T-cell function. The temporal compartmentalization of cAMP in T cells by these PDE4 isoenzymes is further proof that tissue-specific expression of multiple PDE isoforms is not redundant but is often significant to cellular function.

### B. Physiological Implication for Localization and Function of cGMP-Hydrolyzing Phosphodiesterase Isoenzymes

The role of compartmentation in cGMP signaling, while less extensively studied than that for cAMP signaling, is increasingly appreciated. In some cells, cGMP synthesized by NO-GC or particulate GCs (pGC) can produce different effects since these GCs commonly reside in different compartments and both activate cGMP signaling (125, 246, 369, 382, 408). It seems likely that other proteins involved in this pathway are also residents of these regions. This organization would provide for modulation of cGMP elevation and termination of the signal through PDE hydrolytic activity and sequestration of cGMP in certain PDE allosteric sites. Under normal physiological functioning, the catalytic action of regionally located PDEs limits the spread of cGMP beyond certain boundaries, thereby restricting its effect to specific compartments. When nonspecific PDE inhibitors block PDE action across the cell or when cGMP synthesis is excessive, normal boundaries are breached, and cGMP spills out across the entire cell.

Elevation of cGMP in platelets is well known to inhibit platelet aggregation (236, 396). Platelets contain three cGMP-hydrolyzing PDEs, i.e., PDE2, PDE3, and abundant amounts of PDE5. It has recently been reported that a fraction of platelet PDE5 is localized to a complex composed of PKGIβ, IP3R1, and IRAG (410). Elevation of cGMP and activation of PKG results in phosphorylation/activation of PDE5 only in this complex, thereby affecting intracellular calcium in certain compartments. This suggests that discrete subpopulations of PDE5 control certain cGMP pools. If so, changes in cGMP in response to NO (or PDE5 inhibitors) can selectively impact calcium mobilization and its proaggregatory effects. However, the extensive studies of platelet cGMP and PDE5 function by Koelsling’s group indicate that a significant portion of platelet PDE5 is activated and phosphorylated in response to NO donors or PDE5 inhibitors and in this activated state modulates global cGMP (196, 262, 263). The explanation for the apparent disparity in studies of platelet PDE5 is unclear.

Moreover, cross-talk between cGMP and cAMP interaction with PDEs localized to different portions of a cell can have significant impact. Increased cGMP level in particular cells results in greater competition of cGMP for the PDE3 catalytic site, which is a dual-specificity PDE (236,
237). PDE3 has similar affinities for cGMP and cAMP, but cGMP is hydrolyzed at ~10% of the rate for cAMP (89). Consequently, cGMP competes with cAMP for the PDE3 active site, resulting in less cAMP breakdown and increased cAMP accumulation (408). This process is thought to be important in platelets where both cNs counter aggregation and is implicated in the effects of cAMP and cGMP on endothelial permeability (366). Although PDE2 is present in platelets and can hydrolyze both cNs, its action apparently does not affect this process, suggesting that it is physically unavailable to the cGMP/cAMP involved in disaggregation. In cardiomyocytes, cGMP level near the plasma membrane is controlled by the synthetic action of the pGC in response to ANP and the hydrolytic action of PDE2. In contrast, the cGMP level in the cytosol is primarily controlled by the synthetic action of NO-GC and hydrolytic actions of PDE2 and PDE5 (88).

VII. CONCLUDING REMARKS

In the last decade, mammalian cN PDEs have proven to be a worthy target for pharmaceutical intervention in treatment of a number of maladies including ED, COPD, intermittent claudication, and pulmonary hypertension. In the last 5 years, there has been an incredible advance in knowledge of the structure/function/regulation of these enzymes; this lays solid groundwork for future investigators who strive to exploit this information for understanding various biological processes and development of new medications targeting PDEs. We are only at the initial edge of understanding the potential for pharmacological interventions involving the PDE superfamily. Enormous intellectual and financial resources have been and continue to be devoted to development of new PDE inhibitors that are selective for particular PDE families or variants within a family in an attempt to provide relief from many maladies that currently have few treatment options. It is an exciting time for those interested in determining the molecular basis for PDE actions and for those interested in developing new therapies.

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