Cyclic GMP Phosphodiesterase-5: Target of Sildenafil*

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The advent of the medication, sildenafil,1 for treatment of male impotence has attracted widespread attention. This agent potently inhibits a cGMP-binding cGMP-specific phosphodiesterase (PDE5).2 PDE5 is particularly abundant in smooth muscle, which is enriched in other components of the cGMP signaling cascade. The characteristics of PDE5, its relationship to other PDEs, its role in cGMP signaling, and its involvement in the efficacious action of sildenafil on corpus cavernosum and vascular smooth muscle resulting in penile erection are the subjects of this review.

Cyclic GMP has emerged recently as a principal focus in signal transduction. Much of this attention has derived from the fact that most of the non-ly physiological effects of nitric oxide (Fig. 1) and all of the characterized effects of natriuretic peptides and guanylin are mediated by cGMP. In addition to the classical regulatory roles ascribed to cGMP such as stimulation of smooth muscle relaxation, neutrophil degranulation, inhibition of platelet aggregation, and initiation of visual signal transduction, numerous other physiological roles have recently been uncovered (1–10). Intracellular receptors for cGMP include cGMP-dependent protein kinases (PKG), cyclic nucleotide-gated channels, and cGMP-binding PDEs; cGMP may also cross-activate AMP pathways by binding to cAMP-binding sites on cAMP receptors such as cAMP-dependent protein kinases (PKA) (11). Tissue cGMP levels are determined by a balance between the activities of the guanylyl cyclases that catalyze formation of cGMP from GTP and the cyclic nucleotide PDEs that catalyze the breakdown of cGMP (Fig. 1). The combination of a stimulator of guanylyl cyclase and a cGMP PDE inhibitor such as sildenafil produces synergistic enhancement of tissue cGMP levels (12).

PDEs were first detected by Sutherland and co-workers (13, 14). The superfamily of PDEs is subdivided into two major classes, class I and class II (15), which have no recognizable sequence similarity. Class I includes all known mammalian PDEs and is comprised of at least 10 identified families that are products of separate genes (16–26). Some PDEs are highly specific for hydrolysis of cAMP (PDE4, PDE7, PDE8), some are highly cGMP-specific (PDE5, PDE6, PDE9), and some have mixed specificity (PDE1, PDE2, PDE3, PDE10). All of the characterized mammalian PDEs are dimeric, but the importance of the dimeric structure for function in each of the PDEs is unknown. Each PDE has a conserved catalytic domain of ~270 amino acids with a high degree of conservation (25–30%) of amino acid sequence among PDE families, which is located carboxyl-terminal to its regulatory domain. Activators of certain PDEs appear to relieve the influence of autoinhibitory domains located within the enzyme structures (27, 28).

PDEs cleave the cyclic nucleotide phosphodiester bond between the phosphorus and oxygen atoms at the 3'-position with inversion of configuration at the phosphorus atom (29, 30). This apparently results from an in-line nucleophilic attack by the OH of ionized H2O. It has been proposed that metals bound in the conserved metal binding motifs within PDEs facilitate the production of the attacking OH (31). The kinetic properties of catalysis are consistent with a random order mechanism with respect to cyclic nucleo-

dides and the divalent cation(s) that are required for catalysis (32). The catalytic domains of all known mammalian PDEs contain two sequences (HHXH_H(E/D)) arranged in tandem, each of which resembles the single Zn2+ binding site of metalloendoproteases such as thermolysin (31). PDE5 specifically binds Zn2+, and the catalytic activities of PDE4, PDE5, and PDE6 are supported by submicromolar concentrations of Zn2+ (31, 33). Whether each of the Zn2+ binding motifs binds Zn2+ independently or whether the two motifs interact to form a novel Zn2+ binding site is not known. The catalytic mechanism for cleaving phosphodiester bonds of cyclic nucleo-
tides by PDEs may be similar to that of certain proteases for cleaving the amide ester of peptides, but the presence of two Zn2+ motifs arranged in tandem in PDEs is unprecedented.

The group of Sutherland and Rall (34), in the late 1950s, was the first to realize that at least part of the mechanism(s) whereby caffeine enhanced the effect of glucagon, a stimulator of adenyl cyclase, on cAMP accumulation and glycolysis in liver involved inhibition of cAMP PDE activity. Since that time chemists have synthesized thousands of PDE inhibitors, including the widely used 3-isobutyl-1-methylxanthine (IBMX). Many of these compounds, as well as caffeine, are non-selective and inhibit many of the PDE families. One important advance in PDE research has been the discovery/design of family-specific inhibitors such as the PDE4 inhibitor rolipram and the PDE5 inhibitor sildenafil.

Precise modulation of PDE function in cells is critical for maintaining cyclic nucleotide levels within a narrow rate-limiting range of concentrations. Increases in cGMP of 2–4-fold above the basal level will usually produce a maximum physiological response. There are three general schemes by which PDEs are regulated: (a) regulation by substrate availability, such as by stimulation of PDE activity by mass action after elevation of cyclic nucleotide levels or by alteration in the rate of hydrolysis of one cyclic nucleotide because of competition by another, which can occur with any of the dual specificity PDEs (e.g. PDE1, PDE2, PDE3); (b) regulation by extracellular signals that alter intracellular signaling (e.g. phosphorylation events, Ca2+, phosphatidic acid, inositol phosphates, protein-protein interactions, etc.) resulting, for example, in stimulation of PDE3 activity by insulin (18), stimulation of PDE6 activity by photons through the transducin system (35), which alters PDE6 interaction with this enzyme, or stimulation of PDE1 activity by increased interaction with Ca2+/calmodulin; (c) feedback regulation, such as by phosphorylation of PDE1, PDE3, or PDE4 catalyzed by PKA after cAMP elevation (17, 18, 36, 37), by allosteric cGMP binding to PDE2 to promote breakdown of cAMP or cGMP after cGMP elevation, or by modulation of PDE protein levels, such as the desensitization that occurs by increased concentrations of PDE3 or PDE4 following chronic exposure of cells to cAMP-elevat-
ing agents (17, 38) or by developmentally related changes in PDE5 content. Other factors that could influence any of the three schemes outlined above are cellular compartmentalization of PDEs (19) effected by covalent modifications such as prenylation or by specific targeting sequences in the PDE primary structure and perhaps translocation of PDEs between compartments within a cell.

Within the PDE superfamily, four (PDE2, PDE5, PDE6, and PDE10) of the 10 families contain highly cGMP-specific allosteric (non-catalytic) cGMP-binding sites in addition to a catalytic site of varying substrate specificity. Each of the monomers of these di-
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**Properties of PDE5**

The first recognized cGMP-binding PDE was discovered in our laboratory as a cGMP-binding protein in lung tissue during a search for cyclic nucleotide-binding proteins other than cyclic nucleotide-dependent protein kinases (42). By DEAE-cellulose chromatography, this protein appeared as a “peak 1” cGMP-binding protein, which was shown to be PKG. The peak 1 protein possessed both cGMP-binding as well as a distinct cGMP-specific PDE catalytic activity (43), and it was subsequently named PDE5. Davis and Kuo (44) also described a cGMP-specific PDE activity in lung tissue, and Hamet and Coquil (45) characterized a cGMP-binding protein that was separated from a “peak 2” cGMP-binding protein, containing the two Zn$^{2+}$-binding motifs A and B (52). Substitution of either of the invariant aspartic acid residues (Asp-714, Asp-754) further downstream in these motifs A and B (52) demonstrates the presence of conserved Zn$^{2+}$-binding motifs (HX$_3$HX$_2$(E/D)) in PDEs and their involvement in catalysis was first demonstrated using PDE5 (31) (Fig. 2). Site-directed mutagenesis confirms the catalytic importance of each residue of these motifs A and B (52). Substitution of either of the invariant aspartic acid residues (Asp-714, Asp-754) further downstream in the sequence is also highly deleterious, and each of these residues may participate in the catalytic process perhaps as a catalytic base or as a coordinating ligand for a required metal. The most dramatic increases in $K_m$ for cGMP are caused by site-directed mutagenesis of Tyr-602 and Glu-775. These two residues could form part of the cGMP-binding pocket of the catalytic site of PDE5. Because some mutations affecting $K_m$ and $V_{	ext{cat}}$ are juxtaposed in the primary sequence, the cGMP-binding pocket and catalytic machinery are likely to involve overlapping subdomains within the catalytic domain of PDE5. All of the components required for catalytic activity of PDE5 are contained within a single monomeric catalytic domain.

Occupation of the allosteric cGMP-binding sites of PDE5 is required for specific phosphorylation of Ser-92 by PKG or PKA, and occupation of the binding sites is also associated with an increase in the Stokes radius of the enzyme, implying that a conformational change occurs (53). A direct effect of cGMP binding to the allosteric sites on cGMP breakdown at the catalytic site has not been demonstrated, although the principle of reciprocity (binding of cGMP at the catalytic site stimulates binding at the allosteric sites) dictates that there should be an effect (54, 55). The stimulatory effect of cGMP analogs specific for the catalytic site on cGMP binding to the allosteric site(s) of PDE5 suggests that interaction of cGMP with the catalytic site precedes cGMP binding to the allosteric site(s) (43, 51). This implies that upon cGMP elevation in cells, cGMP breakdown at the catalytic site would increase because of mass action (increased substrate availability). This increased cGMP interaction at the catalytic site would enhance cGMP binding at the allosteric sites, thus increasing phosphorylation of the enzyme to promote further increases in cGMP breakdown. Although experimental results are consistent with such a sequence of events, this pathway has not been proven unequivocally in broken cell systems. However, rapid phosphorylation of PDE5, which is associated with increased PDE activity, occurs in intact tissues in response to stimulation by atrial natriuretic factor and may be caused by PKG action (56). This process could represent negative-feedback regulation of cGMP levels in cells. PKA can also phosphorylate PDE5 in vitro, albeit at about 10% the rate at which PKG catalyzes this reaction; whether or not this occurs in vivo is uncertain because the concomitant elevation of cGMP and cAMP would be required to expose Ser-92 and activate PKA, respectively. Burns et al. (57) have reported that a partially purified PDE5 from guinea pig lung is activated when phosphorylated by PKA. PDE5 may also be regulated by other low molecular weight factors, and these could alter the effects of phosphorylation (58). As is the case for PDE4, PDE5 may also be subject to long term regulation through changes in enzyme concentration in some cell types (59–61).

The $K_d$ of PDE5 for binding cGMP in the allosteric sites is $-0.2 \mu M$ (47). The presence of two kinetically distinct allosteric cGMP-binding sites in PDE5 was first suggested by the curvilinear pattern of cGMP dissociation from the enzyme. Studies using site-directed mutagenesis confirm the presence of two sites and indicate that the binding of cGMP to each allosteric site (Fig. 2) could involve a NKXX$_3$D motif (39, 62), which resembles that used by G proteins for binding GTP (63). The conserved sequence of the allosteric cyclic nucleotide-binding sites in PDE2, PDE5, PDE6, and PDE10 is evolutionarily distinct from that of the family containing allosteric or catalytic sites are indicated in yellow.

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5. T. Hamet, unpublished results.
6. T. Fink, unpublished results.
Penile Erection and PDE5 Inhibition

Arterial blood flows into the sinusoidal spaces of the corpus cavernosum and corpus spongiosum of the penis and exits via the postcavernous venules (Fig. 4) (66). The arteries supplying the sinusoids, as well as the sinusoids themselves, contain a layer of smooth muscle cells in their walls. Relaxation of these smooth muscle cells falls because of a decrease in synthesis coupled with the ongoing degradation of cGMP by PDE5. These muscle cells return to the more contracted state, and the penis becomes more flaccid because of the reduced amount of blood in the corpora. Alteration in either psychological, hormonal, neurological, vascular, or cavernosal factors can cause some degree of erectile dysfunction, which affects about 50% of men in the age range of 40–70 years (68).

The major intracellular receptors for cGMP, i.e. PKG and PDE5, are abundant in vascular smooth muscle cells, including those of the penis. Because cGMP elevation is known to relax vascular smooth muscle, there has been keen interest in developing oral inhibitors of PDE5 that would block cGMP degradation. It was anticipated that such agents could be useful in treating hypertension or angina. To this end, PDE5 has been used to screen for potentially potent and selective PDE5 inhibitors (65). The active compound, sildenafil, was apparently selected from such an initial screening (69). Sildenafil is a potent and reversible inhibitor of the PDE5 (IC_{50} ~4 nM), and it is highly selective for this PDE when compared with other known PDE families (64). In initial clinical trials with sildenafil to determine its efficacy in treating angina, men reported that the drug enhanced the penile erectile response. The reason that sildenafil acts more specifically on penile blood flow and less well on the general circulation, which would affect systemic blood pressure, is not clear at this time. One possibility is that sexual stimulation, which is necessary for sildenafil effectiveness, causes a rather specific release of nitric oxide in the penis, which would produce a large increase in cGMP synthesis mainly in this tissue. A PDE5 inhibitor would block cGMP breakdown and therefore act synergistically with nitric oxide to elevate cGMP and
cause penile smooth muscle relaxation. This synergistic effect, which is likely to occur to some extent in vascular tissues throughout the body, is the reason given that patients being treated with nitroglycerin should avoid the use of sildenafil. Regardless, sildenafil so far has been a successful oral treatment for male impotence and may prove effective for some female sexual dysfunctions as well. The most common side effects (flushing, headaches, dyspepsia, and slight lowering of blood pressure) that are associated with sildenafil therapy are also likely to result from PDE5 inhibition. Transient aberrations in vision are thought to be caused by inhibition of the retinal PDE6 family; compared with other PDE families this family is closely related to PDE5, and the inhibition of PDE5 by sildenafil is 10-fold weaker than that for PDE5 (64). It is anticipated that even more potent and specific PDE5 inhibitors or effectors of the other components of the cGMP pathway will be developed in the future to treat erectile dysfunction as well as other maladies that involve smooth muscle function.

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