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# Inhibition of Cyclic GMP-Binding Cyclic GMP-Specific Phosphodiesterase (Type 5) by Sildenafil and Related Compounds

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## ABSTRACT

The cGMP-binding cGMP-specific phosphodiesterase (PDE5) degrades cGMP and regulates the intracellular level of cGMP in many tissues, including the smooth muscle of the corpus cavernosum of the penis. Sildenafil (Viagra), a specific PDE5 inhibitor, promotes penile erection by blocking the activity of PDE5, which causes cGMP to accumulate in the corpus cavernosum. In the present study, sildenafil, like other PDE5 inhibitors, stimulates cGMP binding to the allosteric sites of PDE5 by interacting at the catalytic site of this enzyme, but the drug does not compete with cGMP for binding at the allosteric sites. Both sildenafil and zaprinast are competitive inhibitors of PDE5, and double-inhibition analysis shows that these two inhibitors added together interact with the catalytic site of PDE5 in a mutually exclusive manner. After site-directed mutagenesis of each of 23 conserved amino acid residues in the catalytic domain of PDE5, the pattern of changes in the $IC_{50}$ values for sildenafil or UK-122764 is similar to that found for zaprinast. However, among the three inhibitors, sildenafil exhibits the most similar pattern of changes in the $IC_{50}$ to that found for the affinity of cGMP, implying similar interactions with the catalytic domain. This may explain in part the stronger inhibitory potency of sildenafil for wild-type PDE5 compared with the other inhibitors [sildenafil ($K_i$ = 1 nM) > UK-122764 ($K_i$ = 5 nM) > zaprinast ($K_i$ = 130 nM)]. The affinity of each of these inhibitors for PDE5 is much higher than that of cGMP itself ($K_m$ = 2000 nM). It is concluded that residues such as $Tyr^{602}$, $His^{607}$, $His^{643}$, and $Asp^{754}$ may form important interactions for sildenafil in PDE5, but because these amino acids are conserved in all mammalian PDEs, the selectivity and potency of sildenafil is likely to be provided by a nonconserved residue or residues in the PDE5 catalytic domain.

The molecular mechanisms governing contraction/relaxation of smooth muscle have been subjects of considerable research (for a review, see Somlyo and Somlyo, 1994). Smooth muscle tone is regulated by changes in cytosolic $[Ca^{2+}]$. The role of endothelial cell-mediated relaxation of vascular smooth muscle has been clearly established (Furchgott, 1984; Ignarro et al., 1987; Palmer et al., 1987), and the nitric oxide/cGMP system is an important component in the modulation of cellular $[Ca^{2+}]$ by mechanisms involving activation of cGMP-dependent protein kinase (Francis et al., 1988; Lincoln et al., 1994). Because cGMP is generated as the cellular second messenger that is coupled to relaxation, synthetic agents that alter the intracellular cGMP level may have significant clinical implications for conditions in which nitric oxide-mediated, cGMP-dependent relaxation is impaired. The high efficacy of the new therapeutic agent sildenafil (Viagra) to treat male impotence (Boolell et al., 1996; Jeremy et al., 1997; Ballard et al., 1998; Chuang et al., 1998; Moreland et al., 1998) is such an example.

Sildenafil is a selective and potent inhibitor of cGMP-binding cGMP-specific phosphodiesterase (PDE5), which catalyzes hydrolysis of cGMP. Consequently, this inhibition causes elevation of cellular cGMP. Bovine, rat, and human PDE5 cDNAs represent a single gene, PDE5A, that encodes proteins with a high degree of conservation (McAllister-Lucas et al., 1993; Kotera et al., 1997; Loughney et al., 1998; Stacey et al., 1998). Sequence alignment of human PDE5 with bovine and rat sequences reveals 95% and 93% identity, respectively, with the highest variations occurring near the extreme amino terminus. Distinct splice variants of PDE5 have been reported (Kotera et al., 1997; Loughney et al., 1998; Stacey et al., 1998), but the major features of these PDE5s, such as catalytic domain, cGMP-binding domain, and phosphorylation consensus site, are well conserved. PDE5 is very abundant in vascular smooth muscle cells and appears to play a significant role in modulating smooth muscle tone in general and penile corpus cavernosal smooth muscle tone in

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**ABBREVIATIONS:** PDE, 3′:5′-cyclic nucleotide phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine.

particular. The potential therapeutic applications of PDE5 inhibitors have been extensively reviewed (Sybertz et al., 1995; Czarniecki et al., 1996; Sekhar et al., 1996; Silver, 1996).

Using site-directed mutagenesis, we recently examined the contribution of 23 conserved amino acids in the catalytic domain of PDE5 to catalytic function and to inhibition by the classic PDE5 inhibitor zaprinast (Turko et al., 1998a). Sildenafil and its derivative, UK-122764, are structurally related to zaprinast. The objective of the present study was to develop an improved understanding of the molecular basis for substrate and inhibitor binding to PDE5 by using a set of structurally related PDE5 inhibitors and evaluating the inhibitor effects after changing single amino acids in the catalytic site of PDE5.

## Experimental Procedures

**Materials.** [$^3$H]cGMP was purchased from Amersham Life Science Inc. (Arlington Heights, IL). cGMP, *Crotalus atrox* snake venom, 3-isobutyl-1-methylxanthine (IBMX), and zaprinast were obtained from Sigma Chemical Co. (St. Louis, MO). Hydroxyapatite (HTP Gel) was from Bio-Rad (Hercules, CA). UK-122764 and sildenafil were provided by Pfizer Central Research (Sandwich, UK).

**Site-Directed Mutagenesis.** cGB-8/14 clone encodes a full-length bovine lung PDE5 (Turko et al., 1996). The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) has been used to make point mutations in the cGB-8/14 clone in pBacPAK9 expression vector (Clontech, Palo Alto, CA) according to the protocol from Stratagene. Twenty-four single amino-acid mutants were generated: Y602A, Y602F, H602A, N604A, H607A, E632A, H643A, D644A, H647A, N652A, E672A, H674A, H675A, T713A, D714A, D754A, S756A, K760M, E775A, F776L, Q779A, G780A, D781A, and E783A. To avoid theoretically possible random mutations, the 1073-bp fragments containing the desired mutations were excised from cGB-8/14 using *KpnI*/*Bst*1107I digestion and resubcloned into the wild-type cGB-8/14 clone in the pBacPAK9 vector using the same restriction sites. *Escherichia coli* XL1-blue cells were used for all transformations. DNA fragments were purified by the freeze-squeeze method from agarose slices using SPIN-X centrifuge filter units (Costar, Cambridge, MA). DNA was purified from large-scale vector preparations using Qiagen Plasmid Maxi Kit according to the manufacturer's protocol (Qiagen Inc., Valencia, CA). All DNA segments subjected to mutagenesis and subcloning reactions were sequenced in their entirety to ensure the presence of the desired mutation and proper in-frame subcloning.

**Expression of Wild-Type and Mutant PDE5s.** Sf9 cells were cotransfected with *Bsu*36I-digested BacPAK6 viral DNA (Clontech, Palo Alto, CA) and one of the mutated cGB-8/14 clones in the pBacPAK9 expression vector by the lipofection method according to the protocol from Clontech. At 3 days postinfection, the cotransfection supernatant was collected, amplified twice in Sf9 cells, and then used directly as virus stock for expression without additional purification of recombinant viruses. High Five cells grown at 27°C in complete Grace's insect medium (Invitrogen, Carlsbad, CA) with 10% FBS and 10 mg/ml gentamycin in T-185 flasks were infected with 5 ml of virus stock/flask. The culture medium, which contained most of the PDE5, was harvested at 96 h postinfection.

**Purification of Wild-Type and Mutant PDE5s.** From 240 to 260 ml of the culture medium was fractionated by sequential ammonium sulfate precipitation at 4°C. The fraction precipitated by 25% to 40% saturation was resuspended in 30 ml of 10 mM sodium phosphate buffer, pH 7.2, and centrifuged at 48,000*g* for 30 min at 4°C. The supernatant was loaded onto a hydroxyapatite column (1.5 × 15

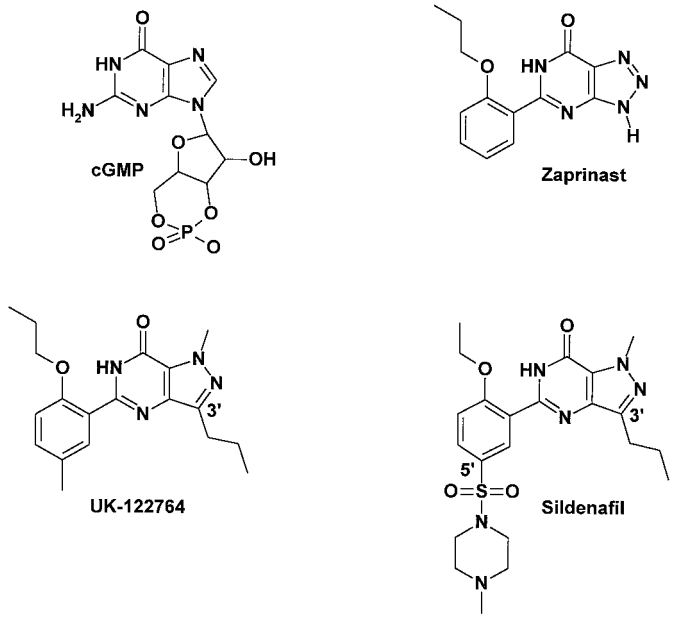


**Fig. 1.** Chemical structures of PDE5 substrate and inhibitors.

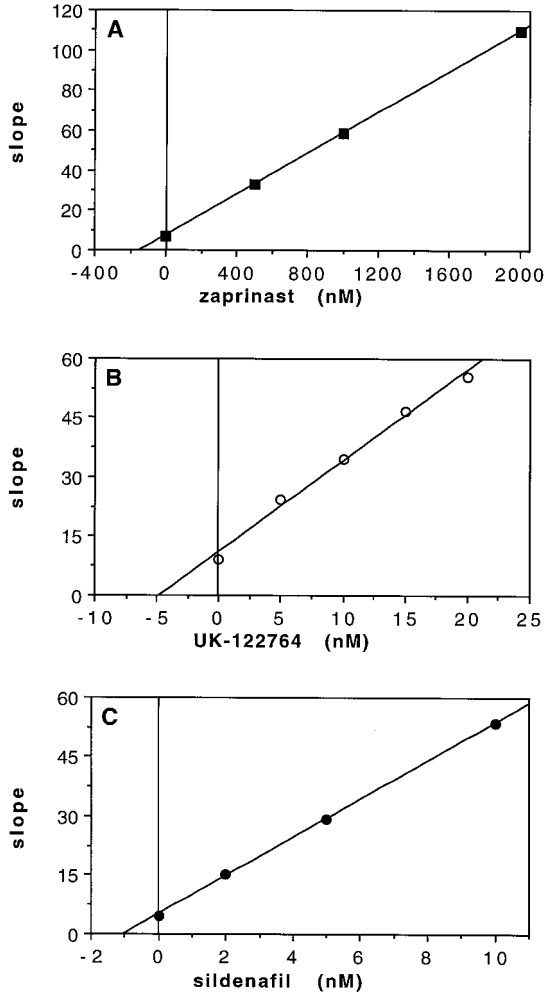


**Fig. 2.** Inhibition of PDE5 by zaprinast, UK-122764, and sildenafil. cGMP concentrations were 0.3 to 5.0 $\mu$M. The slopes from Lineweaver-Burk plots were replotted versus the corresponding inhibitor concentrations, and $K_i$ values of zaprinast (A), UK-122764 (B), and sildenafil (C) were determined from the point of intersection of each line with the horizontal axis.

cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.2. The column was washed with 100 ml of 70 mM sodium phosphate buffer, pH 7.2, and then eluted with 120 mM sodium phosphate buffer, pH 7.2, at a flow rate of 5 ml/h. The pool containing PDE5 activity was diluted with six volumes of ice-cold deionized water and concentrated to approximately 1 ml using an Amicon filtration cell equipped with a PM-30 membrane. All purification steps were performed at 4°C. The final preparation was stored in 20% glycerol at −70°C.

**Quantification of PDE5 Concentration.** Total protein concentrations were determined according to the method of Bradford (1976) using BSA as the standard. To determine the PDE5 protein concentration, the Coomassie Brilliant Blue-stained SDS-polyacrylamide gels of wild-type and mutant enzymes were scanned using an E-C Apparatus Corporation densitometer equipped with GS370 v.3.0 software from Hoeffer. The PDE5 protein concentration was calculated from the fraction of the PDE5 band multiplied by the total protein concentration as determined with use of the Bradford assay. To convert the PDE5 protein concentration into the molar PDE5 concentration, the value of the molecular weight of PDE5 of 98.5 kDa (calculated from the amino acid sequence of PDE5) was used.

**Catalytic Activity of PDE5.** PDE activity was measured using a modification of the assay procedure described previously (Thomas et al., 1990). Incubation mixtures contained 40 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.5, 0.5 mM EGTA, 15 mM magnesium acetate, 0.15 mg/ml BSA, 0.5 $\mu$M cGMP (unless otherwise stated), [$^3$H]cGMP (100,000–150,000 cpm/assay), and one of the PDE5 samples, in a total volume of 250 $\mu$l. Under these conditions, the reaction rate was approximately linear for more than 10 min for each of the wild-type and mutant proteins tested, and an incubation time of 10 min at 30°C was selected for all kinetic measurements. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. After cooling, 20 $\mu$l of 10 mg/ml *C. atrox* snake venom was added, followed by a 20-min incubation at 30°C. Nucleoside products were separated from unreacted nucleotides on columns of DEAE-Sephadex A-25 equilibrated with 20 mM Tris·HCl buffer, pH 7.5, and counted. In all studies, less than 15% of the total [$^3$H]cGMP was hydrolyzed during the reaction. To determine $IC_{50}$ values for UK-122764 or sildenafil, the PDE activity was assayed in duplicate in the presence of a wide range of inhibitor concentrations.

**Allosteric cGMP Binding.** The cGMP binding assay was conducted in a total volume of 60 $\mu$l containing 20 mM sodium phosphate buffer, pH 6.8, 2 mM EDTA, 25 mM $\beta$-mercaptoethanol, 0.5 $\mu$M [$^3$H]cGMP, and a range of different inhibitor concentrations. The reaction was initiated by the addition of an aliquot of enzyme. After a 30-min incubation on ice, assay mixtures were filtered onto premoistened Millipore HAWP filters (pore size, 0.45 $\mu$m), which were then rinsed four times with a total of 4 ml of cold 20 mM sodium phosphate buffer, pH 6.8, with 2 mM EDTA and then dried and

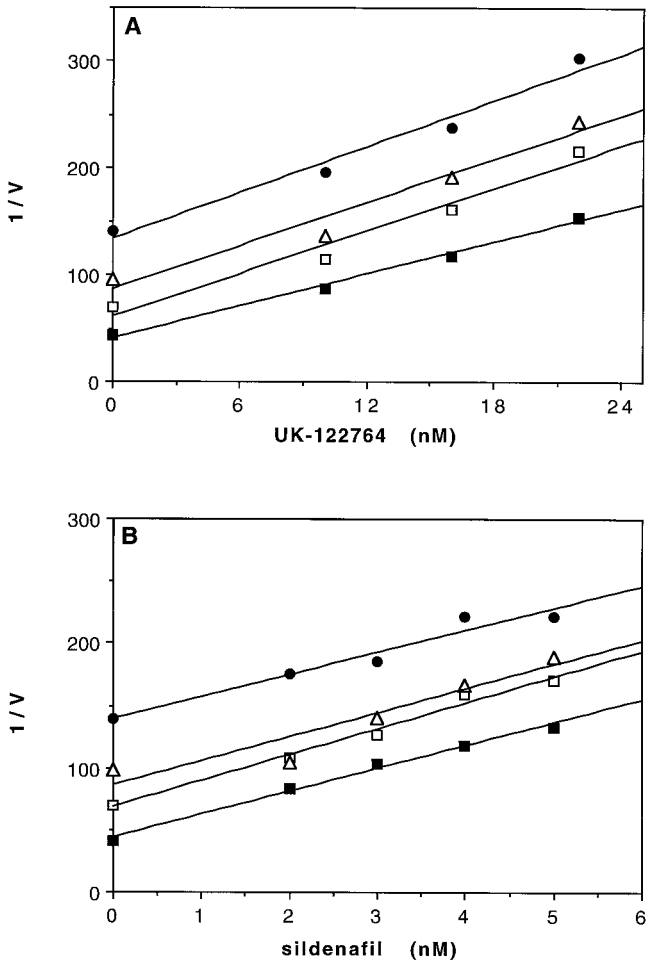


**Fig. 3.** Double-inhibition analysis shows the effect of 0 (■), 0.1 (□), 0.2 (△), and 0.4 (●) $\mu$M zaprinast on PDE5 inhibition by UK-122764 (A) or sildenafil (B) at 0.5 $\mu$M cGMP ([S] < $K_m$).

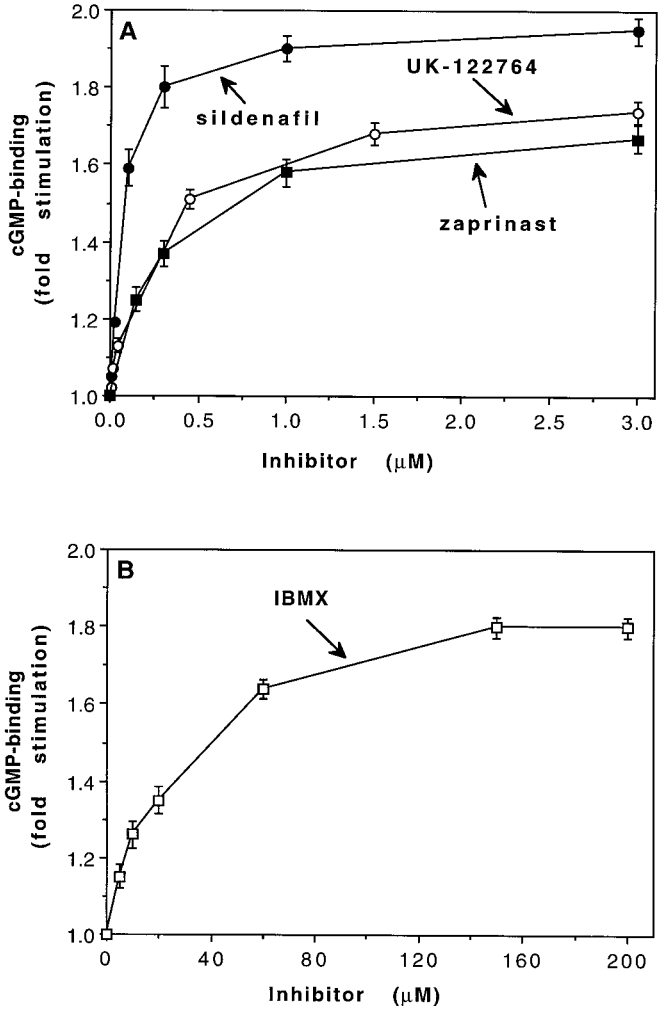


**Fig. 4.** Effect of PDE5 inhibitors on allosteric cGMP binding. [$^3$H]cGMP binding by the wild-type PDE5 was assessed using 0.5 $\mu$M [$^3$H]cGMP in a reaction mixture containing different concentrations of zaprinast (A), UK-122764 (A), sildenafil (A), or IBMX (B). The control binding without any inhibitor was taken as 1.0, and the values in the presence of different inhibitors were expressed as a fold-stimulation with respect to the corresponding inhibitor concentration. Data represent the mean ± S.E.M. of three determinations.

counted. The data were corrected by subtraction of nonspecific binding, which was defined as either the [$^3$H]cGMP bound in the absence of PDE5 or the [$^3$H]cGMP bound in the presence of a 100-fold excess of unlabeled cGMP. A similar 2 to 4% of nonspecific binding was obtained with each method.

**Other Methods.** SDS-polyacrylamide gel electrophoresis in 10% polyacrylamide gels and Western blot analysis with an antibody raised against a native PDE5 from bovine lung were performed as previously described (McAllister-Lucas et al., 1995).

## Results

**Rationale for Selecting Inhibitors.** Because classic PDE inhibitors are most commonly competitive inhibitors of the cyclic nucleotide substrates, a logical approach in designing PDE5 inhibitors is to use a heterocyclic nucleus that mimics the guanosine moiety of cGMP and to append substituents that could fill a space occupied by ribose and/or cyclic phosphate and reproduce the contribution of either or both of these two groups in substrate binding to the catalytic site (Sekhar et al., 1996). Zaprinast is a relatively selective inhibitor of PDE5 that has been extensively used to potentiate the effects of nitric oxide, nitrovasodilators, or atrial natriuretic factor in target cells where intracellular cGMP levels play an important role in regulating cellular function (Harris et al., 1989; McMahon et al., 1993; Ichinose et al., 1995). Structurally, UK-122764 and sildenafil are related to zaprinast and to cGMP (Fig. 1). The 3′ substituent in the pyrazolopyrimidone nucleus of UK-122764 or sildenafil may fill the space occupied by ribose, and the 5′ substituent on the phenyl ring of sildenafil could mimic a role of the cyclic phosphate in substrate binding. In addition to zaprinast, UK-122764 and sildenafil can be considered to represent a set of PDE5 inhibitors that sterically mimic the structure of cGMP.

**Determination of $K_i$ Values for Inhibition of PDE5 Activity.** To determine the $K_i$ value for each inhibitor, the slopes of individual double-reciprocal plots generated from initial rates of cGMP hydrolysis using varying concentrations of cGMP and inhibitor are replotted as a function of the corresponding concentration of inhibitor (Fig. 2). The point of intersection of each line with the horizontal axis gives $-K_i$ values. The $K_i$ values determined using the indicated conditions are 130 nM for zaprinast (Fig. 2A), 5 nM for UK-122764 (Fig. 2B), and 1 nM for sildenafil (Fig. 2C). These values for recombinant PDE5 from bovine lung are consistent with published values ($K_i$ = 2 nM for sildenafil, $K_i$ = 250 nM for zaprinast) for PDE5 from human corpus cavernosum smooth muscle cells (Moreland et al., 1998).

**Double-Inhibition Analysis.** Double-inhibition analysis has been used to further characterize the mechanism of PDE5 inhibition by sildenafil, UK-122764, and zaprinast. This method tests whether two inhibitors are either mutually exclusive or bind at different subsites within the substrate-binding pocket. The initial rates of cGMP hydrolysis at a fixed subsaturating concentration of cGMP ([S] $< K_m$) have been examined over a range of concentrations of two inhibitors (Fig. 3). The patterns for UK-122764 (Fig. 3A) and sildenafil (Fig. 3B) inhibition in the presence of different concentrations of zaprinast are clearly parallel. The parallel lines provide a measure of the unfavorable interaction between two inhibitors, which in this instance approaches infinity. These results indicate that both pairs of inhibitors, UK-122764/zaprinast and sildenafil/zaprinast, are mutually exclusive inhibitors of PDE5, implying that no ternary complex, such as enzyme-inhibitor$_1$-inhibitor$_2$, is formed.

**Stimulation of Allosteric cGMP Binding.** PDE5 exhibits allosteric cGMP-binding sites that are distinct from the site of cGMP hydrolysis. The cGMP-binding assay measures binding of cGMP to the allosteric sites and does not detect binding to the catalytic site (Francis et al., 1980). From the earliest studies of PDE5, it has been evident that PDE5 inhibitors such as IBMX or dipyridamole enhance [$^3$H]cGMP binding activity 2- to 4-fold under assay conditions in which no hydrolysis of [$^3$H]cGMP is observed (Hamet and Coquil, 1978; Francis et al., 1980; Thomas et al., 1990). Some cGMP analogs such as $N^2$-hexyl-cGMP that are specific for the catalytic site also stimulate [$^3$H]cGMP binding activity under the same conditions (Francis et al., 1980). It is believed that the PDE inhibitors and cGMP analogs stimulate [$^3$H]cGMP binding to the allosteric sites by binding to the catalytic site of the enzyme. Because sildenafil possesses high affinity for the PDE5 catalytic site ($K_i$ = 1 nM) and, presumably, mimics the cGMP structure, the question arises as to whether sildenafil could also interact with the cGMP-binding allosteric sites of PDE5. Figure 4A shows that zaprinast, UK-122764, and sildenafil stimulate [$^3$H]cGMP binding by approximately 2-fold. IBMX exhibits the same 2-fold effect (Fig. 4B) and is used in the present study as a positive control. The fact that neither of these catalytic site inhibitors competes for [$^3$H]cGMP binding to the allosteric sites of PDE5 indicates that despite their structural similarity to cGMP, these inhibitors do not interact significantly with the allosteric sites.

**Rationale for Mutagenesis.** The rationale for using the selected catalytic domain mutants is based partly on our previous observation that the amino acid sequence of the PDE5 between residues 602 and 783 is important for substrate selectivity, catalytic function, and inhibitor potency (Turko et al., 1998a,b). Figure 5 shows the amino acid sequences of PDE5 within the conserved catalytic domain that were targeted in the present study. Single conserved amino

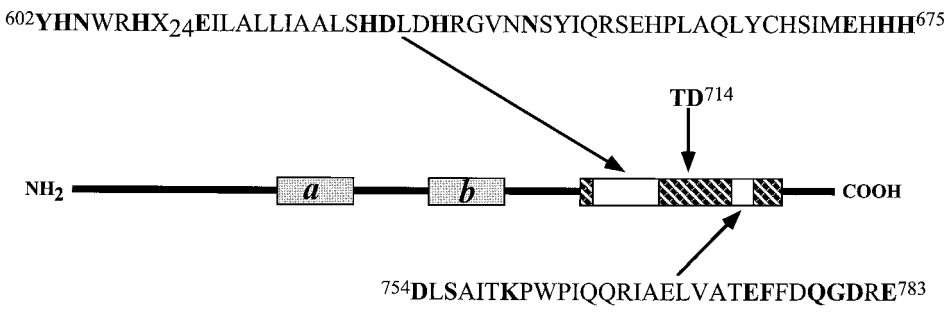


**Fig. 5.** Functional domains of PDE5 and amino acid sequences selected for mutagenesis. Allosteric cGMP-binding sites (a and b) are indicated in gray boxes. Catalytic domain is cross-hatched. White boxes within the catalytic domain represent sequences subjected to site-directed mutagenesis. The conserved amino acid residues that were individually changed in the current study are shown in bold. The location of invariant TD-dyad is indicated by an arrow. The numbers on the left and right indicate the position in the PDE5 sequence.

acids have been substituted to examine the involvement of functional groups for sildenafil and UK-122764 binding. A number of kinetic properties ($K_m$ for cGMP, $k_{cat}$, and $IC_{50}$ for zaprinast) of these mutant PDE5s have been reported previously (Turko et al., 1998a).

**Expression and Purification of Wild-Type and Mutant PDE5s.** All PDE5s have been expressed in High Five cells. The levels of expression of the mutants are comparable to that of the wild-type enzyme (data not shown) and are in the range of 1 to 6 mg of PDE5/100 ml of culture (Turko et al., 1998a). The wild-type and mutant PDE5s have been partially purified (approximately 5–20% purity) from culture medium using ammonium sulfate precipitation and hydroxyapatite chromatography as described in *Experimental Procedures*. Figure 6 shows a Western blot analysis of partially purified mutants obtained after the hydroxyapatite column step. On SDS-polyacrylamide gel electrophoresis, all mutated PDE5s migrate with essentially the same mobility as that of the wild-type enzyme. This indicates that all mutated PDE5s are expressed as full-length proteins.

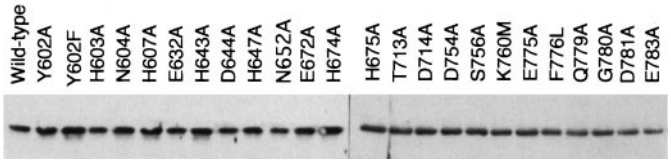


**Fig. 6.** Western blots of partially purified wild-type and mutant PDE5s. Samples after hydroxyapatite step of purification (see *Experimental Procedures*) containing approximately 0.5 $\mu$g of PDE5 were applied to each lane. Immunoblotting was performed using antibodies raised against native lung PDE5 as described earlier (McAllister-Lucas et al., 1995). The identity of the protein preparation is indicated above each lane.

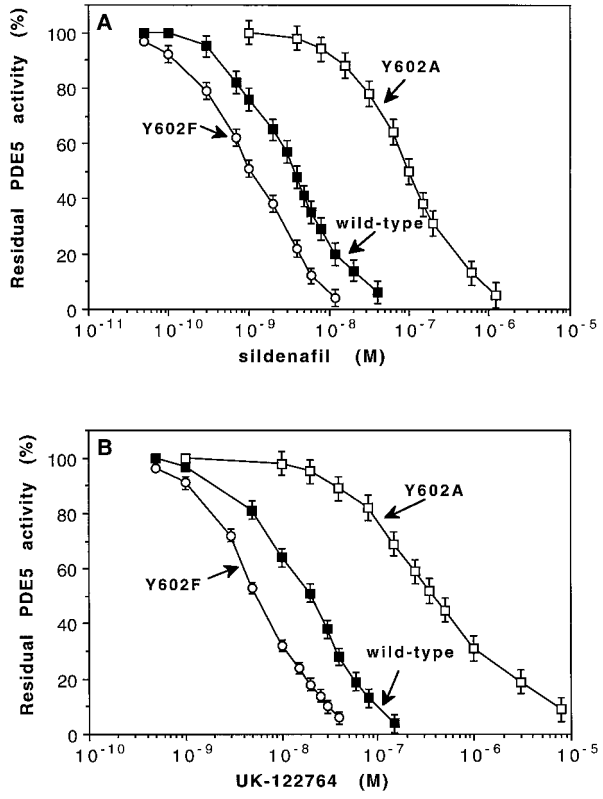


**Fig. 7.** Inhibition of cGMP-hydrolyzing activity of PDE5 and $Tyr^{602}$ mutants by sildenafil (A) or UK-122764 (B). Experiments were conducted at a substrate concentration of 0.5 $\mu$M as described in *Experimental Procedures*. Data represent the mean ± S.E.M. of three determinations.

**$IC_{50}$ Determinations.** $IC_{50}$ values for UK-122764 and sildenafil (Fig. 7 and Table 1) have been determined at a substrate concentration of 0.5 $\mu$M cGMP, which is in all cases lower than the $K_m$ values for these mutants (Turko et al., 1998a). To facilitate comparisons between different PDE5s, relative $IC_{50}$ values (Table 1) have been calculated as the $IC_{50(mutant)}$ value divided by the $IC_{50\ (wild\text{-}type)}$ value. Relative $IC_{50}$ values allow the potency of a particular inhibitor for each PDE5 mutant to be compared with the potency for wild-type enzyme. A 10-fold increase in $IC_{50}$ value yields a change in the free energy of inhibitor binding ($\Delta\Delta G_{binding}$) of approximately 1.4 kcal/mol (Andrews, 1986; Beltman et al., 1995). Because this value is near the lower limit for loss of a hydrogen bond formed by a charged residue, such as that which could be formed by His, Glu, or Asp residues (Table 1), the changes of $IC_{50}$ values for mutation of these residues less than 10-fold are considered to be insufficient to propose an essential inhibitor binding contact by these residues (Fersht et al., 1985). The only mutation that significantly impairs PDE5 binding of UK-122764 is Y602A, which results in a 22-fold increase in the $IC_{50}$ value (Fig. 7). The changes found for all other mutants are not sufficient to suggest an essential role for these residues in UK-122764 binding. The greatest increase in the $IC_{50}$ value for sildenafil (25-fold) is also found for the Y602A mutant (Fig. 7). Four other mutants (H607A, H643A, D754A, and E775A) exhibit moderate increases (10- to 13-fold) in the $IC_{50}$ value. The values expected for $\Delta\Delta G_{binding}$ are maximum values that may also include loss of binding energy due to small perturbations of the overall conformation of the enzyme. Therefore, the role of the residues whose substitution leads to moderate changes of $IC_{50}$ cannot be interpreted unambiguously. Any of these four residues, or any combination of them, could directly interact with sildenafil. Alternatively, they may be involved only in maintaining the general arrangement of the catalytic site.

To further probe the function of Tyr-602 in UK-122764 and sildenafil binding, the Tyr-602 has also been replaced with phenylalanine. The phenylalanine residue could mimic a potential hydrophobic contribution of tyrosine such as participation in stacking interactions, but it does not contain a group that provides for hydrogen bonding. The Y602F mutant has increased sensitivity to inhibition by each of the two inhibitors (Fig. 7). The $IC_{50}$ values are 4-fold lower than that for the wild-type enzyme (Table 1). This strongly supports the conclusion that $Tyr^{602}$ in the catalytic site of PDE5 stacks with the pyrazolopyrimidone nucleus of UK-122764 or sildenafil.

## Discussion

Now that PDE5 has been identified as a major drug target for treating male impotence (Boolell et al., 1996), as well as other disease processes (Braner et al., 1993; Clarke et al., 1994), this protein can be used for structure-based approaches in drug design. This requires a careful definition of the topography of the PDE5 substrate site, which is the molecular target of sildenafil and other experimental drugs. Kinetic analysis of PDE5 inhibition in combination with scanning mutagenesis yields valuable information, particularly in the absence of a solved tertiary structure. Recently,

site-directed mutagenesis of the catalytic domain of PDE5 has revealed that despite the competitive nature of PDE5 inhibition by zaprinast, the residues important for substrate and inhibitor binding in the catalytic site of PDE5 are not identical. $Tyr^{602}$ and $Glu^{775}$ have been shown to be critical for binding the substrate, but $Asp^{754}$ and $Gly^{780}$ are critical for interaction with zaprinast (Turko et al., 1998a).

The results of the present study demonstrate that zaprinast, UK-122764, and sildenafil interact with the catalytic site of PDE5 in a mutually exclusive manner and that these compounds do not interact with the allosteric cGMP-binding sites of PDE5 to any appreciable extent. The latter observation is consistent with our proposal that the allosteric cGMP-binding sites and the catalytic site of PDE5 represent two different families of cyclic nucleotide-binding sites with different structural requirements for cyclic nucleotide binding (Turko et al., 1998a). It follows that the allosteric cGMP-binding sites of PDE2 and PDE6 also are not likely to interact with sildenafil. The functional significance of these allosteric sites is not yet completely understood in each case, but because these sites are structurally and functionally unique, they represent potential targets for highly selective pharmacological agents.

The present study was further designed to identify some of the important amino acid residues for UK-122764 and sildenafil binding to PDE5. The inhibition of 24 PDE5 catalytic domain mutants by UK-122764 and sildenafil has been characterized. Only point mutations of residues conserved in mammalian PDEs were studied to compare the effects with those already studied regarding their interaction with cGMP and zaprinast. Because these residues are conserved, it is likely that the selectivity and potency of sildenafil are also at least partially due to contribution or contributions from a nonconserved residue or residues in the PDE5 catalytic domain. The potency with which each of these inhibitors blocks catalysis was greatly diminished in the Y602A mutant only. The cGMP-binding activity of this mutant was also decreased ($K_m$ = 65 $\mu$M) in comparison with wild-type PDE5 ($K_m$ = 2 $\mu$M), but $k_{cat}$ values (4.27 $s^{-1}$ for wild-type and 1.12 $s^{-1}$ for Y602A mutant) were similar (Turko et al., 1998a). These results indicate that alteration of $Tyr^{602}$ affects mainly substrate and inhibitor binding, not catalysis. The substrate kinetic parameters for Y602F mutant ($K_m$ = 2 $\mu$M, $k_{cat}$ = 4.12 $s^{-1}$) were quite similar to those for wild-type enzyme (Turko et al., 1998a). Furthermore, the potency of inhibition by sildenafil or UK-122764 was even greater for this mutant. Cumulatively, the data suggest that $Tyr^{602}$ in the catalytic site of PDE5 is involved in a stacking interaction with substrate or inhibitors used in this study. In the case of sildenafil, it is interesting that E775A mutant exhibits an 11-fold change in $IC_{50}$ value. This moderate change could be relevant to the current discussion because the same $Glu^{775}$ mutation weakened cGMP binding to the catalytic site by 35-fold ($K_m$ = 70 $\mu$M for E775A). Importantly, E775A mutant is also catalytically competent with $k_{cat}$ = 1.53 $s^{-1}$ (Turko et al., 1998a), so the changes in $K_m$ and $IC_{50}$ values must be attributed to substrate and inhibitor binding. Using the present mutagenesis data together with those already published (Turko et al., 1998a), the inhibitors can be arranged in the order sildenafil > UK-122764 > zaprinast in terms of their inhibitory potencies and the degree with which they mimic the interactions of cGMP in the catalytic site of PDE5. The latter property may explain in part the high affinity of sildenafil for PDE5.

Each of the inhibitors exhibits a much stronger interaction with the catalytic site of PDE5 than does cGMP. Presumably, in addition to molecular mimicking of cGMP, these inhibitors exploit novel interactions within the catalytic site, thereby optimizing binding compared with cGMP binding. To discuss

TABLE 1

Potency of sildenafil and UK-122764 inhibition of wild-type and catalytic domain mutants of PDE5

All $IC_{50}$ values were measured using 0.5 $\mu$M cGMP as substrate. Number of experiments *(n)* is shown in parentheses. $IC_{50}$ values are mean ± S.E.M. for $n \geq 3$ or mean ± range for $n$ = 2. Relative $IC_{50}$ values is $IC_{50(mutant)}/IC_{50(wild\text{-}type)}$ for each PDE5.

| PDE-5 | $IC_{50}$ | | Relative $IC_{50}$ | | |
|---|---|---|---|---|---|
| | UK-122764 | Sildenafil | UK-122764 | Sildenafil | Zaprinast[a] |
| | *nM* | *nM* | | | |
| Wild-type | 20 ± 2 (4) | 4 ± 0.3 (6) | 1 | 1 | 1 |
| Y602A | 410 ± 15 (3) | 100 ± 6 (3) | 21 | 25 | 7 |
| Y602F | 5 ± 1 (3) | 1 ± 0.2 (3) | 0.25 | 0.25 | 0.25 |
| H603A | 118 ± 11 (3) | 27 ± 3 (3) | 6 | 7 | 6 |
| N604A | 28 ± 2 (2) | 12 ± 3 (2) | 1 | 3 | 3 |
| H607A | 85 ± 8 (3) | 53 ± 7 (3) | 4 | 13 | 3 |
| E632A | 20 ± 3 (2) | 4 ± 0.5 (2) | 1 | 1 | 2 |
| H643A | 155 ± 12 (3) | 40 ± 3 (3) | 8 | 10 | 8 |
| D644A | 35 ± 3 (3) | 8 ± 1 (2) | 2 | 2 | 4 |
| H647A | 48 ± 3 (3) | 19 ± 2 (3) | 2 | 5 | 10 |
| N652A | 20 ± 5 (2) | 3 ± 0.5 (2) | 1 | 1 | 1 |
| E672A | 86 ± 6 (3) | 30 ± 6 (3) | 4 | 8 | 1 |
| H674A | 27 ± 3 (2) | 11 ± 1 (2) | 1 | 3 | 14 |
| H675A | 20 ± 1 (2) | 3 ± 0.5 (2) | 1 | 1 | 1 |
| T713A | 20 ± 4 (2) | 4 ± 0.5 (2) | 1 | 1 | 1 |
| D714A | 20 ± 4 (2) | 7 ± 0.6 (2) | 1 | 2 | 3 |
| D754A | 144 ± 16 (3) | 53 ± 6 (3) | 7 | 13 | 43 |
| S756A | 20 ± 3 (2) | 5 ± 0.5 (2) | 1 | 1 | 2 |
| K760M | 20 ± 5 (2) | 5 ± 0.5 (2) | 1 | 1 | 3 |
| E775A | 63 ± 4 (3) | 43 ± 4 (3) | 3 | 11 | 4 |
| F776L | 20 ± 4 (2) | 5 ± 0.2 (2) | 1 | 1 | 2 |
| Q779A | 20 ± 3 (2) | 4 ± 0.5 (2) | 1 | 1 | 4 |
| G780A | 66 ± 3 (3) | 15 ± 1 (2) | 3 | 4 | 33 |
| D781A | 20 ± 2 (2) | 5 ± 0.5 (2) | 1 | 1 | 3 |
| E783A | 20 ± 4 (2) | 5 ± 0.3 (2) | 1 | 1 | 5 |

[a] $IC_{50}$ values for zaprinast were recently published (Turko et al., 1998a) and shown in this table for comparison.

this point, it is necessary to consider the chemical structures of the inhibitors. The chemical structure of zaprinast resembles the structure of the guanine base of cGMP with an additional hydrophobic moiety at C2. There is no substituent in the zaprinast structure that is comparable to the cyclic phosphate-ribose moiety of cGMP (Fig. 1). Thus, two factors likely contribute to the higher affinity of zaprinast binding ($K_i$ = 130 nM) compared with cGMP ($K_m$ = 2 $\mu$M): an additional hydrophobic interaction and an overall less electronegative structure. Further analysis of the chemical structures of UK-122764 ($K_i$ = 5 nM) and sildenafil ($K_i$ = 1 nM) suggests that to achieve maximum affinity, removal of the negatively charged cyclic phosphate-ribose moiety is not sufficient; it is apparently necessary that the space occupied by this charged moiety be filled with an uncharged substituent that can form additional contact or contacts with one or more surrounding amino acid residues. Presumably, the 3′ substituent in UK-122764 or sildenafil could play the same role as does ribose in cGMP, and the 5′ substituent in sildenafil could occupy the same space as does the cyclic phosphate.

Although the negatively charged cyclic phosphate group is required for hydrolysis of cyclic nucleotides by PDEs, the observed affinity for cGMP may reflect a balance between interactions that favor high-affinity binding and those that discriminate against an excessively high-affinity cGMP binding. The fact that zaprinast, UK-122764, and sildenafil are all less electronegative than cGMP but bind more tightly to PDE5 is consistent with the possibility that the negatively charged cyclic phosphate group may restrict the cGMP-binding affinity in the catalytic site of PDE5. The results also have interesting applications in defining the molecular mechanism of cAMP versus cGMP selectivity in the catalytic sites of different PDEs. Because the cyclic phosphate-ribose moieties of cAMP and cGMP are identical, this group cannot account for selectivity for either cyclic nucleotide. In fact, if there were a strong charge interaction with the cyclic phosphate group, it could actually reduce the capacity for cyclic nucleotide selectivity. Variability in the potency of such an interaction may explain in part the dual activity of some PDEs for cAMP and cGMP compared with higher cyclic nucleotide selectivity of other PDEs. Ultimately, the experimental and theoretical observations made in the present study should define more clearly the nature of the substrate recognition process used by PDEs and provide useful information for design of new types of PDE inhibitors.

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