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Binding of Tritiated Sildenafil, Tadalafil, or Vardenafil to the Phosphodiesterase-5 Catalytic Site Displays Potency, Specificity, Heterogeneity, and cGMP Stimulation

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ABSTRACT

Sildenafil, tadalafil, and vardenafil each competitively inhibit cGMP hydrolysis by phosphodiesterase-5 (PDE5), thereby fostering cGMP accumulation and relaxation of vascular smooth muscle. Biochemical potencies (affinities) of these compounds for PDE5 determined by IC_{50}, K_D (isotherm), K_D (dissociation rate), and K_D (½ EC_{50}), respectively, were the following: sildenafil (3.7 ± 1.4, 4.8 ± 0.80, 3.7 ± 0.29, and 11.7 ± 0.70 nM), tadalafil (1.8 ± 0.40, 2.4 ± 0.60, 1.9 ± 0.37, and 2.7 ± 0.25 nM); and vardenafil (0.091 ± 0.031, 0.38 ± 0.07, 0.27 ± 0.01, and 0.42 ± 0.10 nM). Thus, absolute potency values were similar for each inhibitor, and relative potencies were vardenafil ≫ tadalafil > sildenafil. Binding of each ^3H inhibitor to PDE5 was specific as determined by effects of unlabeled compounds. ^3H Inhibitors did not bind to isolated PDE5 regulatory domain. Close correlation of EC_{50} values using all three ^3H inhibitors competing against one another indicated that each occupies the same site on PDE5. Studies of sildenafil and vardenafil analogs demonstrated that higher potency of vardenafil is caused by differences in its double ring. Exchange-dissociation studies revealed two binding components for each inhibitor. Excess unlabeled inhibitor did not significantly affect ^3H inhibitor dissociation after infinite dilution, suggesting the absence of subunit-subunit cooperativity. cGMP addition increased binding affinity of [^3H]tadalafil or [^3H]vardenafil, an effect presumably mediated by cGMP binding to PDE5 allosteric sites, implying that either inhibitor potentiates its own binding to PDE5 in intact cells by elevating cGMP. Without inhibitor present, cGMP accumulation would stimulate cGMP degradation, but with inhibitor present, this negative feedback process would be blocked.

Phosphodiesterase-5 (PDE5) is 1 of 11 mammalian PDE families known to date (Francis et al., 2001). PDE5 is a cGMP-specific PDE and is abundant in most smooth muscle tissues as well as in platelets, gastrointestinal epithelial cells, and Purkinje cells of the cerebellum (Francis et al., 2001; Shimizu-Albergine et al., 2003). The enzyme was first identified, purified, and cloned in this laboratory (Lincoln et al., 1976; Francis et al., 1980; Thomas et al., 1990a; McAllister-Lucas et al., 1993). PDE5 is a homodimer, and each monomer is a chimeric protein that is composed of a regulatory domain and a catalytic domain (Corbin and Francis, 1999). The catalytic domain catalyzes the breakdown of cGMP to 5′-GMP, and the regulatory domain contains allosteric cGMP-binding sites and a phosphorylation site (Corbin and Francis, 1999). Two tandem homologous repeats of ~110 amino acids each in the regulatory domain are termed GAF domains (*a* and *b*) because of their presence in cGMP-binding cyclic nucleotide PDEs, Anabaena adenylyl cyclase, and the bacterial transcription factor FhlA (Thomas et al., 1990a; McAllister-Lucas et al., 1993; Aravind and Ponting, 1997). Isolated GAF *a* monomer binds cGMP with high affinity, but cGMP binding to GAF *b* has yet to be demonstrated (Liu et al., 2002). Allosteric binding of cGMP to PDE5 regulatory domain increases affinity of the catalytic site for cGMP, thereby stimulating the rate of cGMP hydrolysis (Thomas et al., 1990b; Corbin and Francis, 1999; Okada and Asakawa, 2002; Corbin et al., 2003; Mullershausen et al., 2003; Rybalkin et al., 2003). cGMP binding to the regulatory domain also stimulates phosphorylation of PDE5 at Ser-92 (bovine) by cGMP-dependent protein kinase in vitro and in vivo (Thomas et al., 1990b; Wyatt et al., 1998; Mullershausen et al., 2001; Murthy, 2001; Rybalkin et al., 2002). It is presumed that cGMP binding to the regulatory domain produces a conformational change in PDE5 that exposes Ser-92. The resulting phosphorylation of PDE5 increases affinity of the

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ABBREVIATIONS: PDE, cyclic nucleotide phosphodiesterase; GAF, mammalian cGMP-binding phosphodiesterase, *Anabaena* adenylyl cyclases, *Escherichia coli* FhlA; IBMX, 3-isobutyl-1-methylxanthine; KPM, 10 mM potassium phosphate, pH 6.8, containing 15 mM β-mercaptoethanol.

regulatory domain for cGMP and increases catalytic activity as well (Corbin et al., 2000). These effects suggest that PDE5 is critically involved in negative feedback regulation of cellular cGMP levels.

Several compounds that potently inhibit PDE5 have been synthesized recently, and three of these are now in clinical use for treatment of male erectile dysfunction. After sexual arousal, these inhibitors enhance accumulation of cGMP in the smooth muscle of the arteries supplying the penis and the sinusoids of the penile corpus cavernosum. Sildenafil (Viagra; Pfizer, New York, NY) was the first compound of this class to be marketed for the treatment of male erectile dysfunction. It also shows promise in the clinical treatment of ailments related to smooth muscle tissues, such as pulmonary hypertension (Weimann et al., 2000). Newer PDE5 inhibitors that have the same therapeutic mechanism as sildenafil, such as tadalafil (Cialis; Lilly-ICOS, Bothell, WA), and vardenafil (Levitra; Bayer Corporation, West Haven, CT), have also been approved for use in many countries. The availability of these high-affinity inhibitors provides significant new tools for studies of the PDE5 catalytic domain. This laboratory recently examined some characteristics of the catalytic domain and its regulation by investigating [^{3}H]sildenafil binding to the enzyme (Corbin et al., 2003). The structures of tadalafil and vardenafil differ significantly from that of sildenafil, and these three compounds have differing inhibitory potencies. Molecular contacts of the three inhibitors within the catalytic site of the PDE5 have recently been revealed by X-ray crystallography (Sung et al., 2003). In addition to [^{3}H]sildenafil, we have synthesized or acquired [^{3}H]tadalafil and [^{3}H]vardenafil. The availability of these compounds has allowed a thorough analysis of the interaction of these agents with PDE5, which is reported herein. These radiolabeled inhibitors have also permitted the most comprehensive, head-to-head comparison of potencies of these agents to bind to PDE5 using several approaches. Moreover, some novel features of the inhibitors and of PDE5 are uncovered using these approaches.

Materials and Methods

Materials. [^{3}H]cGMP and DEAE-Sephacel were purchased from Amersham Biosciences Inc. (Piscataway, NJ). 3-Isobutyl-1-methylxanthine (IBMX), histone type II-AS, *Crotalus atrox* snake venom, 5′-GMP, and cGMP were obtained from Sigma Chemical Co. (St. Louis, MO). His-tagged, full-length recombinant bovine PDE5 was isolated from infected Sf9 cells using nickel/nitrilotriacetic acid agarose (QIAGEN, Valencia, CA) as described previously (Corbin et al., 2003). Native bovine lung PDE5 was obtained and purified using Blue Sepharose described in an earlier report (Francis and Corbin, 1988; Thomas et al., 1990a). Sildenafil was purified from Viagra tablets by following the method established previously in this laboratory (Corbin et al., 2003). Purified sildenafil was submitted to Amersham Biosciences for radiolabeling with tritium. Tadalafil was synthesized according to Daugan (2000). After confirming the compound structure by mass spectrometry, tadalafil was submitted to Amersham Biosciences for radiolabeling with tritium. High-performance liquid chromatography results from Amersham indicated that [^{3}H]sildenafil was >98% pure, whereas the [^{3}H]tadalafil preparation was >99% pure. Vardenafil, [^{3}H]vardenafil, demethyl-vardenafil, and methyl-sildenafil were provided by Bayer AG (Wuppertal, Germany). All three ^{3}H inhibitors that had been stored for more than a year were subjected to Sephadex G-25 chromatography, which adsorbs PDE inhibitors and provides high resolution (Corbin et al., 2003; Francis et al., 2003). All three ^{3}H inhibitors were resolved in single peaks and coeluted with purified unlabeled inhibitors, suggesting that the ^{3}H inhibitors were unaltered after storage. Even so, it cannot be completely ruled out that the curvilinearity observed in the dissociation of ^{3}H inhibitors in Fig. 5 could be caused by slight structural heterogeneity of the inhibitors.

Isolated Regulatory Domain of PDE5. Residues Met1 to Glu539 of human PDE5 were amplified from the hPDE5 cDNA (courtesy of Tanabe Research Laboratories Inc., San Diego, CA). Using the forward primer RZMet1for (5′-GATATTGAATTCATGGAGCGGGCCGGCCCCAGCT-3′) and the reverse primer RZGlu539rev (5′-GATGATAGCGGCCGC***CTA***TCTCTTGTTTCTTCCTCTGCT-3′), containing EcoRI and NotI sites (underlined) and a stop codon (bold italic). The resulting PCR fragment (1649 base pairs) was cloned into pCR 2.1-Topo (Invitrogen, Carlsbad, CA) and verified by sequencing. The fragment was excised by digestion with EcoRI and NotI and was inserted into baculovirus transfer pAcHLT-A (BD PharMingen, San Diego, CA) digested with the same enzymes. The resulting plasmid was cotransfected with the BaculoGold baculovirus DNA (BD PharMingen) into Sf9 cells according to the manufacturer's instructions. The transfected cells were incubated at 27°C for 5 days. Afterward, 100 μl of collected culture medium was used to infect 2×10^7 freshly prepared Sf9 cells for viral amplification. The recombinant baculovirus was amplified two more times to obtain a high titer stock solution by infecting freshly seeded Sf9 cells. The infected cells were incubated at 27°C for 4 days before protein was harvested. Purification was carried out using nickel/nitrilotriacetic acid agarose as described previously (Corbin et al., 2003).

PDE Assays. PDE activity was determined using a modified method (Martins et al., 1982) as described previously (Gopal et al., 2001) with 0.4 μM [^{3}H]cGMP as substrate.

[^{3}H]cGMP-Binding Assay. The procedure was modified slightly from that described previously (Corbin et al., 2000). PDE5 or PDE5 (80 μl) isolated regulatory domain (4 nM final protein concentration in reaction mixture) was added to 2 ml of a mixture of 0.2 μM [^{3}H]cGMP, 10 mM potassium phosphate, pH 6.8, 25 mM 2-mercaptoethanol, and 0.2 mg/ml Type II-AS histone (Sigma). After 45 min at 4°C, the sample was filtered onto premoistened Millipore filters (pore size, 0.45 μm), which were then rinsed with 3 ml of 10 mM potassium phosphate, pH 6.8, and 25 mM β-mercaptoethanol, dried, and counted.

^{3}H Inhibitor Membrane Filtration-Binding Assay. Full-length bovine His-tagged PDE5 (80 μl) was added to 2 ml of a binding reaction mixture that contained 0.2 mg/ml histone IIA-S, various concentrations of ^{3}H inhibitor, and buffer that consisted of 10 mM potassium phosphate, pH 6.8, and 25 mM β-mercaptoethanol (KPM). Sticking of ^{3}H inhibitor to the sides of the test tube occurred when ^{3}H inhibitor was added in the absence of or before addition of histone. Histone also increased retention of PDE5 on the Millipore membranes. Binding reaction mixture containing the enzyme was incubated on ice or in a 30°C water bath for 45 min. Millipore nitrocellulose membranes (0.45 μm) were placed under house vacuum and prewetted with 1 ml of ice-cold 10 mM potassium phosphate, pH 6.8, that contained 0.1% Triton X-100. Next, 200 μl of 25% Triton X-100 at room temperature in KPM was added to the reaction tube. The entire contents of the tube were applied to the prewetted filter. The reaction tube was then washed with 3 ml of cold 0.1% Triton X-100 in 10 mM potassium phosphate, pH 6.8, and the wash was also applied to the filter. Filter membranes were removed, dried, and transferred to 6-ml scintillation vials. Nonaqueous scintillant (5 ml) was added to the tubes, which were then placed in a scintillation counter.

Statistical Analyses. All values are given as mean ± standard error of mean (S.E.M.) as determined by GraphPad Prism graphics software (GraphPad Software Inc., San Diego, CA). The software uses the following equation: S.E.M. = standard deviation/$n^{1/2}$, where standard deviation is determined as $[\Sigma(y_i - y_{mean})^2/(n - 1)]^{1/2}$. All S.E.M. values reported fit within a 95% confidence interval, which quantifies the precision of the mean.

Results

Inhibition of PDE5 Catalytic Activity. The concentration of inhibitor that produces 50% inhibition of PDE5 catalytic activity (IC_{50}) was determined for each of the inhibitors (sildenafil, tadalafil, and vardenafil) using 0.4 μM [^{3}H]cGMP as substrate (Fig. 1). The IC_{50} values were the following: sildenafil, 3.7 ± 1.4 nM (n = 4); tadalafil, 1.8 ± 0.4 nM (n = 7); and vardenafil, 0.091 ± 0.031 nM (n = 5). Similar values were obtained when using native bovine PDE5 (data not shown). These values agreed with the range of published IC_{50} values [sildenafil, 1–9 nM (Ballard et al., 1998; Turko et al., 1999; Corbin and Francis, 2002); tadalafil, 1–7 nM (Corbin et al., 2002; Gresser and Gleiter, 2002); and vardenafil, 0.1–0.8 nM (Saenz de Tejada et al., 2001; Gresser and Gleiter, 2002; Corbin et al., 2002)].

Stoichiometry of ^{3}H Inhibitor Binding to PDE5. The binding stoichiometry was determined for each inhibitor by dividing maximum binding (B_{max}, picomoles of ^{3}H inhibitor binding per milliliter of PDE5) obtained from GraphPad Prism graphics, by PDE5 enzyme concentration (picomoles of PDE5 subunit per milliliter of PDE5). PDE5 protein concentration was determined by amino acid analysis. Stoichiometry was corrected for 75% recovery of ^{3}H inhibitor binding to PDE5 using the vacuum filtration method as determined previously (Corbin et al., 2003). [^{3}H]Tadalafil bound to PDE5 with a stoichiometry of 0.68 ± 0.10 mol/subunit (n = 7), which was similar to the [^{3}H]vardenafil stoichiometry of 0.41 ± 0.05 mol/subunit (n = 8). These values compared well with the stoichiometry previously reported for [^{3}H]sildenafil of 0.61 ± 0.13 mol/subunit (Corbin et al., 2003). The [^{3}H]sildenafil binding stoichiometry was duplicated using the same enzyme preparation used to determine the [^{3}H]tadalafil and [^{3}H]vardenafil stoichiometry values calculated above.

Specificity for ^{3}H Inhibitor Binding to PDE5. The specificity of [^{3}H]sildenafil binding to the catalytic domain of PDE5 was presented in our previous report (Corbin et al., 2003). The specificities of [^{3}H]tadalafil and [^{3}H]vardenafil binding to PDE5 were determined by testing the effects of various unlabeled compounds using 4 nM ^{3}H inhibitor and recombinant bovine PDE5 (Fig. 2). A 240-fold excess of unlabeled sildenafil, tadalafil, or vardenafil abolished [^{3}H]tadalafil or [^{3}H]vardenafil binding. Addition of cAMP or 5′-GMP at 375,000-fold excess did not affect binding of either inhibitor. At 375,000-fold excess, cGMP reduced binding of either ^{3}H inhibitor by 40 to 60%. A 2400-fold excess of rolipram (a PDE4-specific inhibitor) or cilostamide (a PDE3-specific inhibitor) did not affect ^{3}H inhibitor binding. IBMX, a general, albeit weak, PDE inhibitor had a substantial inhibitory effect at 100,000-fold excess. The data suggested that binding of all three inhibitors is specific for the catalytic domain of PDE5 and that all three inhibitors compete for the same site.

Lack of Binding of Each of the ^{3}H Inhibitors to an Isolated Regulatory Domain of PDE5. Whereas [^{3}H]cGMP bound to the isolated regulatory domain of PDE5 nearly stoichiometrically, none of the ^{3}H inhibitors bound to this domain using the same assay conditions and concentration used in the studies of binding to full-length PDE5 (data not shown). Addition of a ~5-fold excess (0.96 μM) of unlabeled sildenafil, tadalafil, or vardenafil, which was in the range of 1000 times the K_D of each inhibitor for the catalytic domain, did not lower [^{3}H]cGMP binding to the regulatory domain (data not shown). In contrast, a 2500-fold (0.5 mM) excess of unlabeled cGMP, which was also approximately 1000 times the K_D of this ligand for the catalytic domain, abolished [^{3}H]cGMP binding to the regulatory domain. Together, these results indicated that inhibitor is specific for the PDE5 catalytic domain and does not bind to the regulatory domain under the conditions of the assays.

Potencies (Affinities) for Binding of ^{3}H Inhibitors to PDE5. The concentration-dependence of ^{3}H inhibitor ([^{3}H]sildenafil, [^{3}H]tadalafil, or [^{3}H]vardenafil) binding to PDE5 is shown in Fig. 3. K_D values, obtained by using nonlinear regression analysis with GraphPad Prism software, were as follows: sildenafil, 4.8 ± 0.8 nM (n = 3); tadalafil, 2.4 ± 0.6 nM (n = 4); and vardenafil, 0.38 ± 0.07 nM (n = 5).

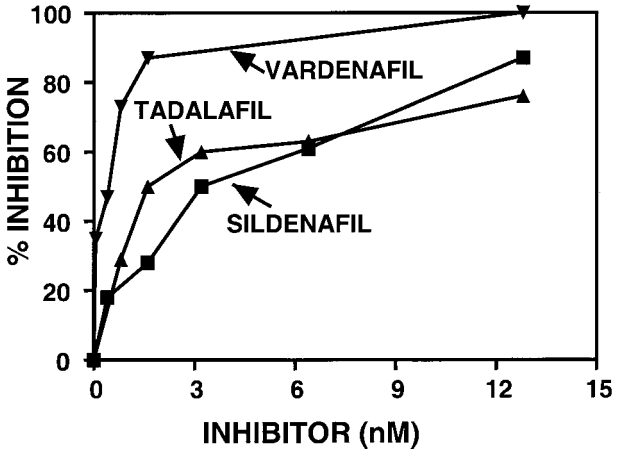


Fig. 1. Potency of inhibition of PDE catalytic activity by PDE5 inhibitors. PDE5 (10 μl; 0.113 nM final concentration in assay) was added to the PDE assay reaction mixture containing increasing concentrations of PDE5 inhibitors. PDE activity was determined in a 15-min incubation as described under *Materials and Methods* using 0.4 μM (final concentration) [^{3}H]cGMP as substrate. Data represent a typical experiment performed in triplicate.

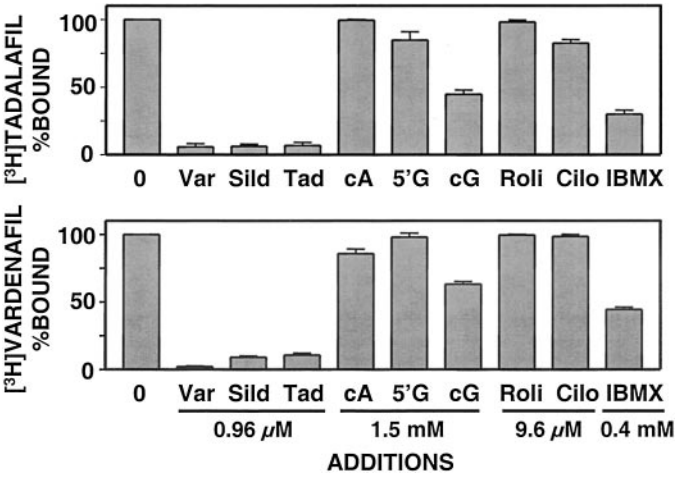


Fig. 2. Effects of nucleotides and inhibitors on binding of ^{3}H inhibitors to PDE5. PDE5 (0.7 nM final concentration in assay) was incubated in 2 ml of binding reaction mixture with 4 nM ^{3}H inhibitor and the following concentrations of competing compounds: unlabeled vardenafil (Var) = 0.96 μM, unlabeled sildenafil (Sild) = 0.96 μM, unlabeled tadalafil (Tad) = 0.96 μM, cAMP (cA) = 1.5 mM, 5′-GMP (5′G) = 1.5 mM, cGMP (cG) = 1.5 mM, rolipram (Roli) = 9.6 μM, cilostamide (Cilo) = 9.6 μM, and IBMX = 0.4 mM. All were filtered as described under *Materials and Methods*. Data represent three experiments, each performed in triplicate.

These values agreed well with the IC_{50} values reported above.

Potencies for sildenafil, tadalafil, and vardenafil were also determined by competition studies. For example, Fig. 4 shows the effect of increasing concentrations of unlabeled vardenafil on binding of 3 nM [^{3}H]tadalafil. The EC_{50} value was calculated from GraphPad Prism graphics software using a sigmoidal dose-response curve. Because EC_{50} values were determined using a ^{3}H inhibitor concentration at the approximate K_D value for PDE5, the Cheng and Prusoff/Chou equation (Cheng and Prusoff, 1973; Chou, 1974) could be applied to calculate the K_D from EC_{50} by dividing EC_{50} values by two (Table 1). It can be seen that ½ EC_{50} was in general agreement with the K_D or IC_{50} for each inhibitor, and the order of potency for the inhibitors was retained. The three ½ EC_{50} values for unlabeled inhibitor in competition with either [^{3}H]vardenafil, [^{3}H]sildenafil, or [^{3}H]tadalafil were similar. This suggested that the inhibitors compete for the same site on PDE5.

Potencies of Sildenafil and Vardenafil Analogs. Vardenafil has a ~40-fold higher affinity for PDE5 over sildenafil taken from IC_{50} values shown here. To determine which of the distinguishing molecular features of the two compounds determines this difference in potency, two analogs were synthesized. The first, demethyl-vardenafil, contained the [5,1-*f*][1,2]triazine ring of vardenafil and the appended methyl group of sildenafil. The second analog, methyl-sildenafil, contained the pyrazolo[4,3-*d*]pyrimidine ring of sildenafil and the appended ethyl group of vardenafil. The IC_{50} of each analog for PDE5 was determined using 0.4 μM [^{3}H]cGMP as substrate. These experiments yielded IC_{50} values of 0.14 ± 0.02 nM for demethyl-vardenafil and 8.90 ± 1.7 nM for methyl-sildenafil (Table 2). The EC_{50} for each of the analogs was determined using 0.5 nM [^{3}H]vardenafil. EC_{50} values were 0.88 ± 0.19 nM for demethyl-vardenafil and 72 ± 13 nM for methyl-sildenafil. K_D calculated from ½ EC_{50} was in general agreement with the IC_{50} for each analog (Table 2). The results indicated that the higher biochemical potency of vardenafil over sildenafil is caused by differences within the double rings of the two compounds.

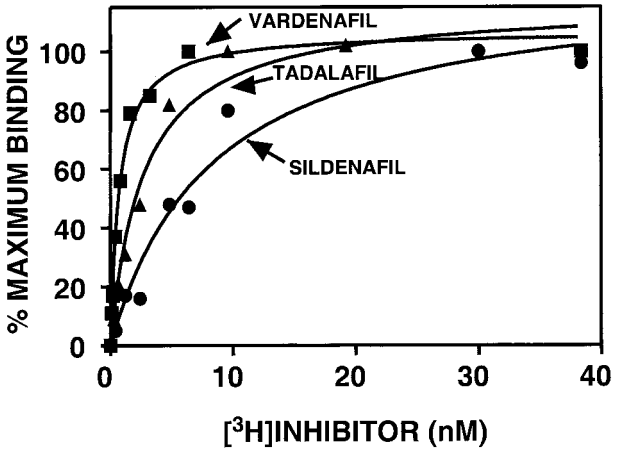


Fig. 3. Affinity of PDE5 for binding ^{3}H inhibitors. PDE5 (80 μl; 0.26 nM final concentration in assay) was incubated with increasing concentrations of ^{3}H inhibitors in 2 ml of binding reaction mixture containing 10 μM cGMP for 20 min on ice and then filtered as described under *Materials and Methods*. Data represent a typical experiment performed in triplicate.

Heterogeneity of the PDE5 Catalytic Domain Revealed by ^{3}H Inhibitor Dissociation Kinetics. Exchange-dissociation kinetics of each of the ^{3}H inhibitors from PDE5 were examined. PDE5 was first saturated with ^{3}H inhibitor (30 nM), and aliquots were removed to determine ^{3}H inhibitor binding at 0 time. Unlabeled inhibitor (~33,000-fold excess) was then added to the reaction mixture, and aliquots were removed for filtration at various times to follow the time course of dissociation (exchange) of the radiolabeled inhibitor from the enzyme. Under these conditions, the enzyme remained saturated at all times with inhibitor. All three inhibitors exhibited nonlinear dissociation kinetics indicative of the presence of at least two rate components (Fig. 5A). In Fig. 5B, the *x*-axis was changed to emphasize the earlier time points. Assuming the presence of two components, when the line of the slower component was extrapolated to the *y*-axis, the calculated percentages of the two components were different for each inhibitor. Sildenafil, as reported previously, exhibited two equal components. The dissociation behavior of [^{3}H]tadalafil revealed 60% high-affinity (slow) and 40% low-affinity (fast) components. [^{3}H]Vardenafil dissociation exhibited 85% high-affinity and 15% low-affinity components. The overall rate of dissociation of [^{3}H]vardenafil was much slower than that of the other two inhibitors. After estimation of the $t_{1/2}$ for dissociation, the K_D of each inhibitor was calculated from the following equation: $K_D = 6.93 \times 10^{-7}$ M · s/$t_{1/2}$, where M = molar, s = seconds, and $t_{1/2}$ is measured in seconds. (Limbird, 1995). All exchange-dissociation experiments were performed three times with each ^{3}H inhibitor.

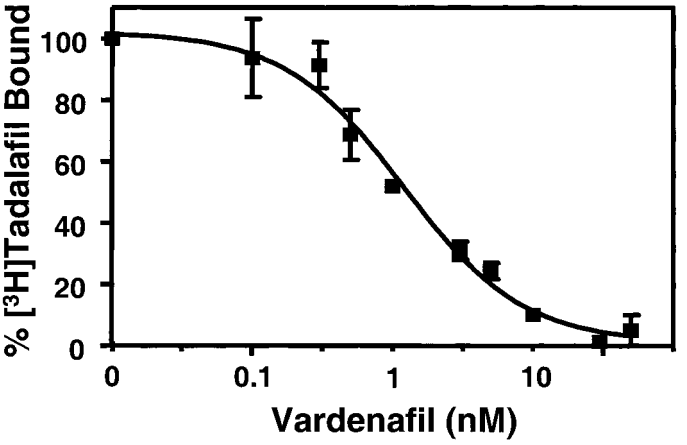


Fig. 4. Determination of EC_{50} for vardenafil. Increasing concentrations of unlabeled vardenafil were included in 2 ml of binding reaction mixture that contained 3 nM [^{3}H]tadalafil. PDE5 was then added (80 μl; 0.035 nM final concentration in assay). Filtration was performed as outlined under *Materials and Methods*. Data represent three experiments, each performed in triplicate.

TABLE 1

1/2 EC_{50} values for PDE5 inhibitors

Increasing concentrations of unlabeled inhibitor were added to 2 ml of binding reaction mixture that contained either 0.5 nM [^{3}H]vardenafil, 4 nM [^{3}H]sildenafil, or 3 nM [^{3}H]tadalafil. Filtration was performed as outlined under *Materials and Methods*. Based on Student's *t* tests, the three K_D values for each unlabeled inhibitor were not significantly different from each other.

	Tadalafil	Sildenafil	Vardenafil
	nM	*nM*	*nM*
[^{3}H]Vardenafil	3.16 ± 1.3	12.8 ± 1.1	0.42 ± 0.1
[^{3}H]Sildenafil	2.47 ± 0.3	10.6 ± 1.1	0.25 ± 0.1
[^{3}H]Tadalafil	2.5 ± 1.5	11.7 ± 2.2	0.59 ± 0.4

The resulting K_D values for the two [^{3}H]sildenafil components were 14.7 ± 2.3 and 0.7 ± 0.06 nM, for the two [^{3}H]tadalafil components were 9.3 ± 2.67 and 0.6 ± 0.00 nM, and for the two [^{3}H]vardenafil components were 6.0 ± 0.00 and 0.1 ± 0.01 nM. The geometric mean K_D values for each inhibitor (n = 3) were the following: sildenafil, 3.1 nM; tadalafil, 1.7 nM; and vardenafil, 0.32 nM. Each average K_D determined by this method was similar to IC_{50}, K_D obtained from isotherm, or K_D obtained from ½ EC_{50}. The similarity between IC_{50} values and average K_D values determined from dissociation rates of the respective inhibitors suggested that interaction of the inhibitor with both kinetic components contributes to inhibition of PDE5 catalytic activity.

In addition to the exchange-dissociation method used above, [^{3}H]tadalafil or [^{3}H]vardenafil dissociation from PDE5 was examined by infinite dilution. Dissociation of the respective radiolabeled inhibitor was determined in the absence and presence of excess unlabeled inhibitor after equilibrium binding and 80-fold dilution of the binding reaction. The pattern of [^{3}H]tadalafil dissociation (Fig. 6A) revealed two components either in the presence or absence of a 5000-fold excess of unlabeled tadalafil during dissociation. The lack of an effect of unlabeled tadalafil on the dissociation of [^{3}H]tadalafil from PDE5 suggested that even though PDE5 is dimeric, the catalytic domain in each of the respective monomers of the enzyme may not kinetically influence each other to a large degree. Likewise, the dissociation of [^{3}H]vardenafil after infinite dilution was not different from that in the presence of excess vardenafil, again suggesting that the PDE5 catalytic domains of the two monomers function independently (Fig. 6B).

Effect of cGMP on ^{3}H Inhibitor Binding. We recently reported that cGMP stimulates [^{3}H]sildenafil binding to the PDE5 catalytic domain at 4°C (Corbin et al., 2003). In addition to determining whether the same cGMP effect occurred with [^{3}H]vardenafil and [^{3}H]tadalafil, we also investigated if cGMP stimulates ^{3}H inhibitor binding at 30°C, which approaches physiological temperature. Increasing the temperature from 4° to 30°C had no effect or perhaps slightly inhibited sildenafil and tadalafil binding (data not shown). However, the increase in temperature increased vardenafil binding in the presence of cGMP, as is discussed below.

The effect of increasing cGMP concentrations on [^{3}H]vardenafil binding was carried out using 0.5 nM [^{3}H]vardenafil at both 4° and 30°C (Fig. 7). At 4°C, [^{3}H]vardenafil showed a 2.5-fold increase in binding at low levels of cGMP (1–50 μM), although this effect waned at higher cGMP concentrations. Repeating the experiment at 30°C with increasing cGMP produced a ~3.5-fold stimulation of [^{3}H]vardenafil binding to PDE5 at 30°C. The cGMP effect remained constant at moderate concentrations and waned slightly at very high cGMP concentration.

When binding using increasing concentrations of [^{3}H]vardenafil was performed at 30°C in the presence of constant 10 μM cGMP, the labeled compound bound to PDE5 with a slightly higher affinity than at 4°C (0.42 ± 0.06 nM, n = 3, versus 0.59 ± 0.02 nM, n = 3). [^{3}H]Vardenafil binding to PDE5 in the absence of cGMP at 30°C yielded a lower K_D than that found for [^{3}H]vardenafil binding at 4°C (0.74 ± 0.10 nM, n = 3, versus 2.19 ± 0.62 nM, n = 3) (Fig. 8, A and B).

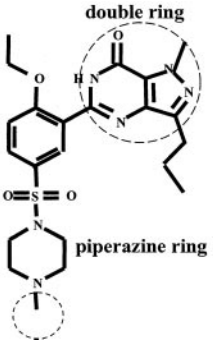
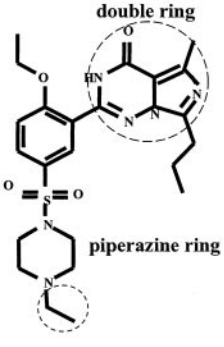
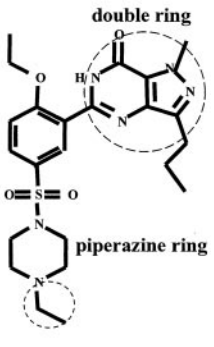
The addition of 10 μM cGMP to increasing concentrations of [^{3}H]vardenafil at 4°C decreased the K_D (0.74 ± 0.10 nM, n = 3, to 0.59 ± 0.02 nM, n = 3) while increasing the B_{max} for PDE5 (5.63 ± 0.38 to 6.58 ± 0.10 pmol/ml) (Fig. 8A). At 30°C, cGMP caused a 3.6-fold decrease in K_D from 2.19 ± 0.62 nM (n = 3) to 0.42 ± 0.06 nM (n = 3), whereas the B_{max} did not significantly change (4.93 ± 0.71 versus 5.25 ± 0.22 pmol/ml) (Fig. 8B).

As shown in Fig. 9, cGMP also stimulated binding of 3 nM [^{3}H]tadalafil at 4°C, and the effect was maximal at ~25 μM cGMP. The stimulatory effect waned at higher cGMP concentrations in a manner similar to the cGMP effect on vardenafil binding at 4°C. The addition of 10 μM cGMP to increasing concentrations of [^{3}H]tadalafil at 4°C decreased K_D ~2-fold from 3.7 ± 0.39 nM (n = 3) to 1.74 ± 0.05 nM (n = 3),

TABLE 2

IC_{50} and 1/2 EC_{50} values for PDE5 inhibitor analogs

Structures of analogs are shown with differences encircled. IC_{50} values were determined by adding PDE5 (10 μl; 0.11 nM final concentration) to PDE assay reaction mixture containing increasing concentrations of the analogs. PDE activity was determined in a 15-min incubation as described under *Materials and Methods* using 0.4 μM (final concentration) [^{3}H]cGMP as substrate. EC_{50} values were determined by adding increasing concentrations of unlabeled inhibitor analog to 2 ml of binding reaction mixture that contained 0.5 nM [^{3}H]vardenafil. Filtration was performed as outlined under *Materials and Methods*. Student's t tests indicate that IC_{50} and K_D values for methyl-sildenafil were significantly different ($p < 0.05$) from the IC_{50} and K_D values for demethyl-vardenafil, vardenafil, and sildenafil.

	Sildenafil	Vardenafil	Demethyl-Vardenafil	Methyl- Sildenafil
				
IC_{50} (nM)	3.7 ± 1.4 (n=4)	0.17 ± 0.04 (n=7)	0.14 ± 0.02 (n=4)	8.90 ± 1.7 (n=3)
½EC_{50} (nM)	2.47 ± 0.3 (n=3)	1.0 ± 0.26 (n=5)	0.44 ± 0.10 (n=4)	36 ± 6.5 (n=3)

whereas the B_{max} was 4.97 ± 0.14 and 5.95 ± 0.19 pmol/ml, respectively (Fig. 10).

The combined results suggested that [^{3}H]vardenafil, but not [^{3}H]sildenafil or [^{3}H]tadalafil, binds to PDE5 with higher affinity at 30°C than at 4°C. The affinities of all three inhibitors are increased by the presence of cGMP, whereas maximum binding of each inhibitor is increased only slightly by cGMP.

Discussion

[^{3}H]Sildenafil binding to PDE5 is specific for the catalytic site of PDE5 (Corbin et al., 2003). The present report demonstrates that [^{3}H]tadalafil and [^{3}H]vardenafil are also specific for binding to the catalytic site. Binding of each of the three ^{3}H inhibitors was inhibited by catalytic site-selective agents and by unlabeled sildenafil, tadalafil, or vardenafil, suggesting that binding of each inhibitor is restricted to the catalytic domain and that all three inhibitors also bind to the same catalytic site. The stoichiometry of each ^{3}H inhibitor binding approached 1 mol/PDE5 subunit, which was consistent with inhibitor binding specifically to the catalytic site and also was indicative of one catalytic site per PDE5 monomer.

The isolated regulatory domain of PDE5 did not bind ^{3}H inhibitor using the same binding assay used for PDE5 holoenzyme even though the regulatory domain bound [^{3}H]cGMP nearly stoichiometrically. In addition, unlabeled sildenafil, tadalafil, or vardenafil did not compete with [^{3}H]cGMP for binding to the regulatory domain, confirming that these inhibitors do not bind to the regulatory domain. K_D values determined by binding isotherms, EC_{50}, or exchange-dissociation agreed with IC_{50} of each inhibitor, again supporting the conclusion that the PDE5-specific inhibitors interact exclusively with the catalytic site of PDE5. Because cGMP-binding sites in the PDE5 regulatory and catalytic domains are evolutionarily and biochemically distinct, this result was not surprising.

This laboratory has used membrane vacuum filtration to measure [^{3}H]cGMP binding (Francis and Corbin, 1988), ^{65}Zn binding (Francis et al., 1994), and [^{3}H]sildenafil binding (Corbin et al., 2003). This assay was modified slightly for specific [^{3}H]tadalafil and [^{3}H]vardenafil binding to PDE5. All three ^{3}H inhibitor binding assays produced high recoveries and yielded nearly 1 mol/subunit binding. Radiolabeled rolipram binding to PDE4 has been reported (Schneider et al., 1986; Torphy et al., 1992); however, the stoichiometry of

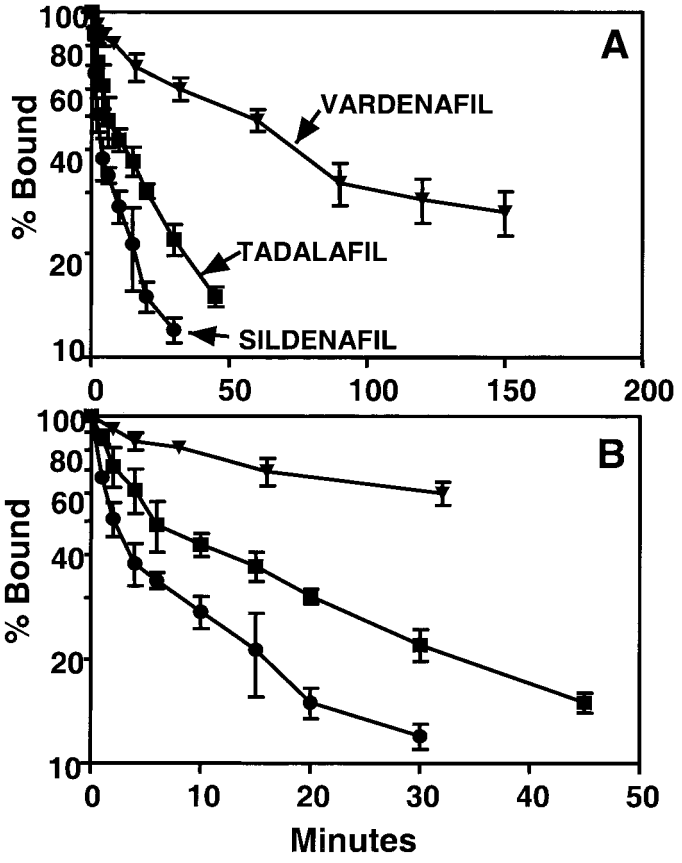


Fig. 5. Exchange-dissociation of ^{3}H inhibitor from PDE5. PDE5 (0.35 nM final concentration in assay) was added to 4.5 ml of binding reaction mixture containing ^{3}H inhibitor (30 nM final concentration). Then, to determine the zero time point, a 520-μl aliquot of this mixture was filtered as described under *Materials and Methods*. Next, 30 μl of a 1 mM solution of the corresponding unlabeled inhibitor was added to the remaining incubating binding reaction mixture at 4°C. Aliquots were removed and filtered by the same procedure at the indicated time points. A represents a longer time course, whereas B shows a shorter time course to emphasize curvilinear kinetics. Data represent three experiments, each performed in triplicate.

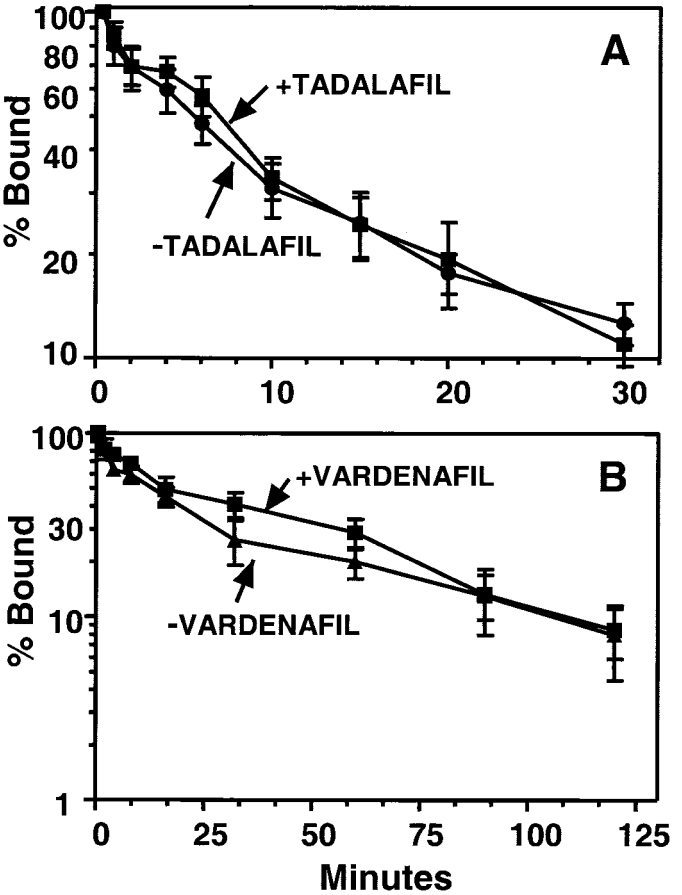


Fig. 6. Dissociation of ^{3}H inhibitors from PDE5 after infinite dilution. PDE5 (76 μl, 0.32 nM final concentration in assay) was added to 360 μl of binding reaction mixture containing a final concentration of 2 nM [^{3}H]tadalafil (A) or 0.5 nM [^{3}H]vardenafil (B). After incubating for 1 h on ice, 35 ml of 0.2 mg/ml histone AII-S in the absence or presence of 10 μM of the respective unlabeled inhibitor was added to dilute the binding reaction mixture 80-fold. Filtration was performed at the indicated time points by the procedure outlined under *Materials and Methods*. Data represent three experiments, each performed in triplicate.

binding in those studies was less than 0.01 mol/subunit using membrane filtration.

IC_{50} values of sildenafil, tadalafil, and vardenafil determined here in head-to-head assays using bovine PDE5 were in the same range as IC_{50} values reported in the literature using human PDE5 (Table 3) (Corbin and Francis, 2002; Corbin et al., 2002). Therefore, results are similar using recombinant PDE5, native PDE5, or PDE5 from different mammalian species. Whereas IC_{50} is the classic method of determining potency (affinity) of PDE inhibitors, measurement of binding strength, or K_D, is a more direct method of determining potency and it also provides a measure of stoichiometry of ligand binding. This report determined the potencies for sildenafil, tadalafil, and vardenafil using four separate head-to-head methods. IC_{50} measurements yielded a potency ratio of 1:2:41, K_D (binding isotherm) yielded a ratio of 1:2:13, K_D (½ EC_{50}) yielded a ratio of 1:5:26, and K_D (exchange-dissociation) yielded a ratio of 1:2:14 for sildenafil, tadalafil, and vardenafil, respectively (Table 3). This investigation represents the most comprehensive examination of the absolute and relative potencies of these drugs.

Dissociation rates of inhibitors from PDE5 correlated with potencies determined by IC_{50} or isotherm K_D, i.e., the slower the rate, the higher the potency. However, the faster dissociation rate of tadalafil from PDE5 compared with that of vardenafil may be unexpected in view of the longer lasting clinical effects of tadalafil. These clinical differences of tadalafil may be caused by pharmacokinetic considerations such as slower intestinal absorption or slower degradation by the liver, rather than by different biochemical properties.

In comparing the distinctive chemical structures of sildenafil and vardenafil, two major differences are evident: 1) a methyl group is appended to the piperazine ring of sildenafil, whereas the same ring in vardenafil has an appended ethyl group, and 2) a nitrogen atom is present in the 7-position of the double ring of sildenafil, but it is not present in the ring of vardenafil, although vardenafil contains a nitrogen atom in the 5-position, which is absent in sildenafil. To resolve which of these structural differences of the compounds determines potency, two analogs were synthesized: demethyl-vardenafil (analog of vardenafil containing the appended methyl group of sildenafil) and methyl-sildenafil (analog of sildenafil containing the appended ethyl group of vardenafil). Demethyl-vardenafil and vardenafil had almost identical IC_{50} values, whereas methyl-sildenafil had 52-times higher $IC_{50,}$ which was similar to the IC_{50} of sildenafil. K_D values obtained from EC_{50} experiments using both analogs also indicated that methyl-sildenafil had much lower potency than either of the other two analogs. From these results, the higher biochemical potency of vardenafil compared with sildenafil is caused by differences within the double rings of the two compounds. The crystal structure of the PDE5 catalytic domain containing either sildenafil or vardenafil was reported recently (Sung et al., 2003); however, the resolution of the crystal structure was not sufficient to identify distinct interactions of either of these two inhibitors with the enzyme. The difference in the double ring of vardenafil, compared with sildenafil, may possibly allow for a stronger interaction between the compound and one or more of the amino acids (e.g., Tyr-612, Val-782, Phe-820, Leu-785, and Gln-817) that

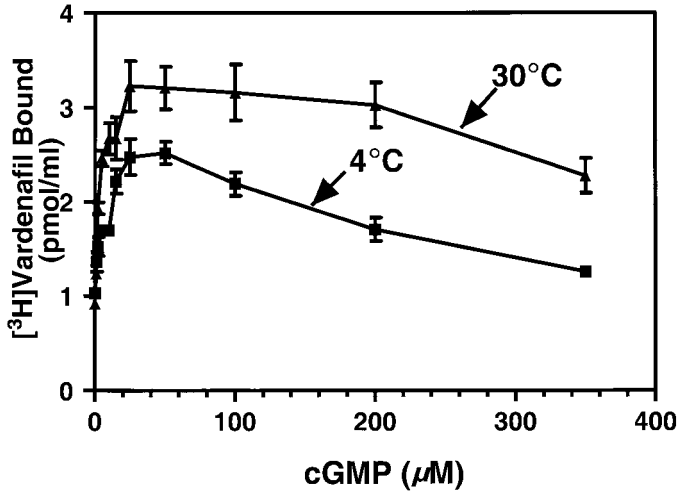


Fig. 7. Effect of cGMP on [^{3}H]vardenafil binding at 4° and 30°C. PDE5 (80 μl, 0.07 nM final concentration in assay) was added to 2 ml of binding reaction mixture containing 0.5 nM [^{3}H]vardenafil and 0 to 350 μM cGMP and incubated for 45 min on ice or 20 min in a 30°C water bath. Filtration was performed as outlined under *Materials and Methods*. Units indicate picomoles of inhibitor per milliliter. Data represent three experiments, each performed in triplicate.

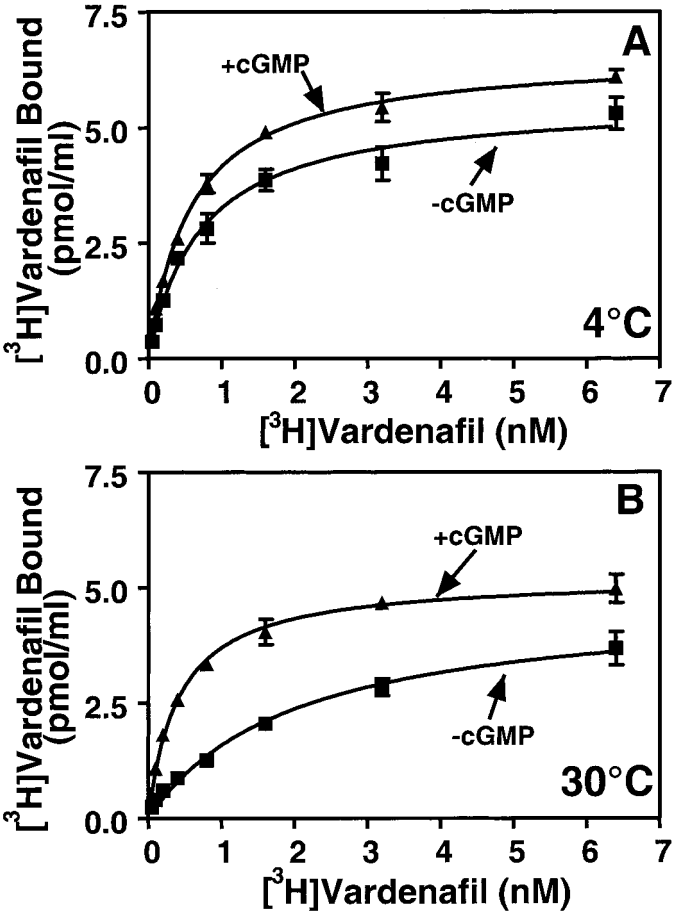


Fig. 8. Effect of temperature on cGMP stimulation of [^{3}H]vardenafil binding using varying concentrations of [^{3}H]vardenafil. PDE5 (80 μl, 0.07 nM final concentration in assay) was added to 2 ml of binding reaction mixture containing 0.05 to 6.4 nM [^{3}H]vardenafil in the absence and presence of 10 μM cGMP and incubated for 45 min on ice (A) or for 20 min in a 30°C water bath (B). Binding was performed as outlined under *Materials and Methods*. Units indicate picomoles of inhibitor per milliliter of PDE5 added to the reaction. Data represent three experiments, each performed in triplicate.

could be important for binding of the double ring of the inhibitor to human PDE5 (Sung et al., 2003). In addition, the position of the nitrogen atom in the vardenafil double ring may impart a change in an atom or group of this molecule that provides contact with a residue that is not contacted by sildenafil or that provides an indirect contact resulting from change in the electron distribution in the double ring.

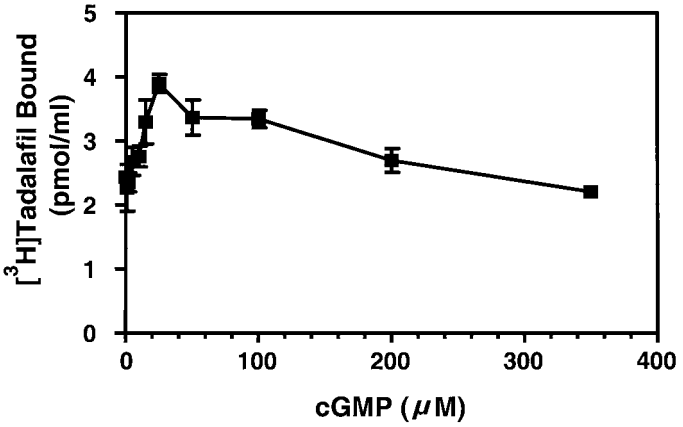


Fig. 9. Effect of cGMP on [^{3}H]tadalafil binding. PDE5 (80 μl, 0.07 nM final concentration) was added to 2 ml of binding reaction mixture containing 3 nM [^{3}H]tadalafil and 0 to 350 μM cGMP. The mixtures were then incubated for 45 min on ice. Filtration was performed as outlined under *Materials and Methods*. Units indicate picomoles of inhibitor per milliliter of PDE5 added to the reaction. Data represent three experiments, each performed in triplicate.

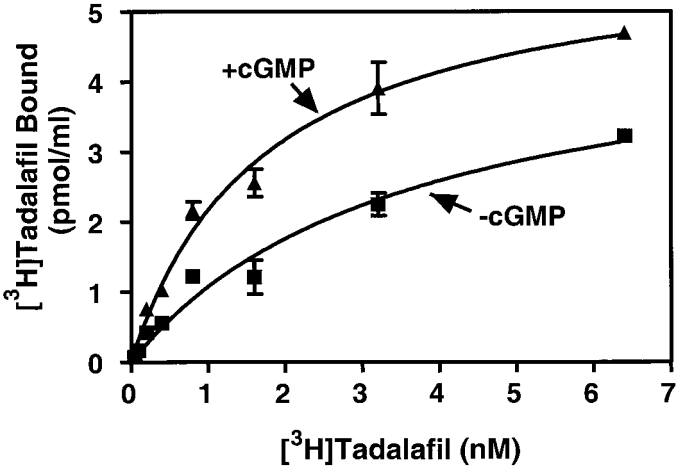


Fig. 10. Effect of cGMP on [^{3}H]tadalafil binding using varying concentrations of [^{3}H]tadalafil. PDE5 (80 μl, 0.07 nM final concentration in assay) was added to 2 ml of binding reaction mixture containing 0.05 to 6.4 nM [^{3}H]tadalafil in the absence and presence of 10 μM cGMP, and the mixture was incubated for 45 min on ice. Filtration was performed as outlined under *Materials and Methods*. Units indicate picomoles of inhibitor per milliliter of PDE5 added to the reaction. Data represent three experiments, each performed in triplicate.

Exchange-dissociation experiments using each of the three ^{3}H inhibitors revealed curvilinear dissociation kinetics, suggesting the presence of two or more catalytic site components. There was an apparent link between inhibitor potency and percentage of high-affinity (slow) component of binding. This could mean that 1) the three ^{3}H inhibitors selected differently for binding to two preexisting populations of PDE5 having different affinities; 2) the inhibitors had different potencies for promoting conversion of one population into another; or 3) a combination of both mechanisms. Dissociation of ^{3}H inhibitor induced by infinite dilution also displayed heterogeneous kinetics. One possible explanation for the presence of two or more components of ^{3}H inhibitor dissociation is that PDE5 exists in different conformations (Francis et al., 1998). PDE2 (Manganiello et al., 1990) and PDE4 (Laliberte et al., 2000) also demonstrated kinetic heterogeneity, which was interpreted to represent different enzyme conformations. The present report is the first to extensively demonstrate that the PDE5 catalytic site exhibits more than one kinetic state, but whether or not it was caused by the presence of different PDE5 conformations remains to be proved. It cannot be ruled out that PDE5 undergoes partial modification during preparation, which could explain the heterogeneity observed, although the presence of two components is observed in different preparations of recombinant PDE5 and native PDE5. Regardless, caution must now be used in interpreting binding isotherm K_D values that assume the presence of a single component in the calculation (Corbin et al., 2003).

Cooperativity of inhibitor binding to PDE5 might occur if binding of inhibitors to the catalytic site of one of the two subunits affects binding to the other subunit. However, inhibitor dissociation after infinite dilution in the absence and presence of excess unlabeled inhibitor indicated that this is not the case, at least under the conditions used for the experiment.

Whereas the molecular mechanism for stimulation of PDE5 catalytic activity by cGMP binding to the regulatory domain is unknown, it is suggested that cGMP binding to this domain relieves PDE5 of an autoinhibitory constraint, at which point filling of the catalytic site at subsaturating substrate levels of cGMP is facilitated, increasing catalytic activity. This negative feedback mechanism promotes rapid degradation of cGMP within the cell. This negative feedback could be problematic for individuals with erectile dysfunction who are unable to maintain the high level of cGMP in the corpus cavernosum for the extended time that is required to achieve and maintain penile erection. This potential deficiency is apparently overcome by the presence of nonhydro-

TABLE 3

Head-to-head comparison of PDE5-specific inhibitor potencies (affinities)

Student's *t* tests indicated that K_D values for each unlabeled inhibitors were significantly different from each other with the exception of the K_D value of sildenafil obtained from 1/2 EC_{50}, which was significantly different ($p < 0.05$) from all other K_D and IC_{50} values for sildenafil. The IC_{50} value for vardenafil was significantly different ($p < 0.05$) from all K_D values for vardenafil.

	IC_{50}	K_D from Isotherm	K_D from 1/2 EC_{50}	K_D from Exchange-Dissociation Average	IC_{50} from Literature
	nM	*nM*	*nM*	*nM*	*nM*
Sildenafil	3.7 ± 1.4	4.8 ± 0.8	11.6 ± 0.7	3.1 ± 0.29	1–9
Tadalafil	1.8 ± 0.4	2.4 ± 0.6	2.72 ± 0.3	1.7 ± 0.37	1–7
Vardenafil	0.091 ± 0.031	0.38 ± 0.07	0.42 ± 0.1	0.32 ± 0.01	0.1–0.8

lyzable PDE5 inhibitors that are specific for the catalytic site. The inhibitors may increase cGMP levels by blocking the negative feedback process while simultaneously increasing cGMP levels by competition.

Because cGMP stimulates binding of [^{3}H]tadalafil and [^{3}H]vardenafil, as well as [^{3}H]sildenafil, to the PDE5 catalytic site, cGMP stimulation is not inhibitor-specific. Thus, cGMP stimulation should also lower the level of drug that can be administered to cause smooth cell relaxation, which is desirable to minimize side effects and safety concerns.

A relatively high concentration (1–25 μM) of cGMP was required to stimulate maximal binding of both [^{3}H]tadalafil and [^{3}H]vardenafil. This concentration was unusually high considering that previous results indicated that the K_D for cGMP binding to the GAF domains is 0.2 μM (Thomas et al., 1990b). The apparent discrepancy could be caused by the different assay conditions used for measuring binding affinities of [^{3}H]cGMP and ^{3}H inhibitors. On the other hand, although it has been shown so far that cGMP binds only to a high-affinity GAF *a* site (Liu et al., 2002), the high concentrations of cGMP required to stimulate ^{3}H inhibitor binding to PDE5 suggests additional binding to a lower affinity GAF *b* site, which could lead to increased catalytic site affinity for ligands.

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References

Aravind L and Ponting CP (1997) The GAF domain: an evolutionary link between diverse phototransducing proteins. *Trends Biochem Sci* **22:**458–459.
Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, and Naylor AM (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol* **159:**2164–2171.
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.
Chou T (1974) Relationships between inhibition constants and fractional inhibition in enzyme-catalyzed reactions with different numbers of reactants, different reaction mechanisms and different types and mechanisms of inhibition. *Mol Pharmacol* **10:**235–247.
Corbin JD, Blount MA, Weeks JL 2nd, Beasley A, Kuhn KP, Ho YS, Saidi LF, Hurley JH, Kotera J, and Francis SH (2003) [^{3}H]Sildenafil binding to phosphodiesterase-5 is specific, kinetically heterogeneous and stimulated by cGMP. *Mol Pharmacol* **63:**1364–1372.
Corbin JD and Francis SH (1999) Cyclic GMP phosphodiesterase-5: target of sildenafil. *J Biol Chem* **274:**13729–13732.
Corbin JD and Francis SH (2002) Pharmacology of phosphodiesterase-5 inhibitors. *Int J Clin Pract* **56:**453–459.
Corbin JD, Francis SH, and Webb DJ (2002) Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology* **60:**4–11.
Corbin JD, Turko IV, Beasley A, and Francis SH (2000) Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur J Biochem* **267:**2760–2767.
Daugan AC-M (2000), inventor, ICOS Corporation, assignee. Use of cGMP-phosphodiesterase inhibitors in methods and compositions to treat impotence. U.S. patent 6,140,329. 2000 Oct 31.
Francis SH, Colbran JL, McAllister-Lucas LM, and Corbin JD (1994) Zinc interactions and conserved motifs of the cGMP-binding cGMP- specific phosphodiesterase suggest that it is a zinc hydrolase. *J Biol Chem* **269:**22477–22480.
Francis SH and Corbin JD (1988) Purification of cGMP-binding protein phosphodiesterase from rat lung. *Methods Enzymol* **159:**722–729.
Francis SH, Chu DM, Thomas MK, Beasley A, Grimes K, Busch JL, Turko IV, Haik TL, and Corbin JD (1998) Ligand-induced conformational changes in cyclic nucleotide phosphodiesterases and cyclic nucleotide-dependent protein kinases. *Methods* **14:**81–92.
Francis SH, Lincoln TM, and Corbin JD (1980) Characterization of a novel cGMP binding protein from rat lung. *J Biol Chem* **255:**620–626.
Francis SH, Sekhar KR, Rouse AB, Grimes KA, and Corbin JD (2003) Single step isolation of sildenafil from commercially available Viagra tablets. *Int J Impot Res* **15:**369–372.
Francis SH, Turko IV, and Corbin JD (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog Nucleic Acid Res Mol Biol* **65:**1–52.
Gopal VK, Francis SH, and Corbin JD (2001) Allosteric sites of phosphodiesterase-5 (PDE5). A potential role in negative feedback regulation of cGMP signaling in corpus cavernosum. *Eur J Biochem* **268:**3304–3312.
Gresser U and Gleiter CH (2002) Erectile dysfunction: comparison of efficacy and side effects of the PDE-5 inhibitors sildenafil, vardenafil and tadalafil—review of the literature. *Eur J Med Res* **7:**435–446.
Laliberte F, Han Y, Govindarajan A, Giroux A, Liu S, Bobechko B, Lario P, Bartlett A, Gorseth E, Gresser M, et al. (2000) Conformational difference between PDE4 apoenzyme and holoenzyme. *Biochemistry* **39:**6449–6458.
Lincoln TM, Hall CL, Park CR, and Corbin JD (1976) Guanosine 3′:5′-cyclic monophosphate binding proteins in rat tissues. *Proc Natl Acad Sci USA* **73:**2559–2563.
Limbird LE (1995) *Cell Surface Receptors: A Short Course on Theory and Methods*, 2nd ed, Kluwer Academic Publishers, Boston.
Liu L, Underwood T, Li H, Pamukcu R, and Thompson WJ (2002) Specific cGMP binding by the cGMP binding domains of cGMP-binding cGMP specific phosphodiesterase. *Cell Signal* **14:**45–51.
Manganiello VC, Smith CJ, Degerman E, and Belfrage P (1990) Cyclic GMP-inhibited cyclic nucleotide phosphodiesterases, in *Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action* (Beavo J and Houslay M eds) pp 87–109, John Wiley and Sons, New York.
Martins TJ, Mumby MC, and Beavo JA (1982) Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J Biol Chem* **257:**1973–1979.
McAllister-Lucas LM, Sonnenburg WK, Kadlecek A, Seger D, LeTrong H, Colbran JL, Thomas MK, Walsh KA, Francis SH, Corbin JD, et al. (1993) The structure of a bovine lung cGMP-binding, cGMP-specific phosphodiesterase deduced from a cDNA clone. *J Biol Chem* **268:**22863–22873.
Mullershausen F, Friebe A, Feil R, Thompson WJ, Hofmann F, and Koesling D (2003) Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. *J Cell Biol* **160:**719–727.
Mullershausen F, Russwurm M, Thompson WJ, Liu L, Koesling D, and Friebe A (2001) Rapid nitric oxide-induced desensitization of the cGMP response is caused by increased activity of phosphodiesterase type 5 paralleled by phosphorylation of the enzyme. *J Cell Biol* **155:**271–278.
Murthy KS (2001) Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle. *Biochem J* **360:**199–208.
Okada D and Asakawa S (2002) Allosteric activation of cGMP-specific, cGMP-binding phosphodiesterase (PDE5) by cGMP. *Biochemistry* **41:**9672–9679.
Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, and Beavo JA (2002) Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J Biol Chem* **277:**3310–3317.
Rybalkin SD, Rybalkina IG, Shimizu-Albergine M, Tang XB, and Beavo JA (2003) PDE5 is converted to an activated state upon cGMP binding to the GAF A domain. *EMBO (Eur Mol Biol Organ) J* **22:**469–478.
Saenz de Tejada I, Angulo J, Cuevas P, Fernandez A, Moncada I, Allona A, Lledo E, Korschen HG, Niewohner U, Haning H, et al. (2001) The phosphodiesterase inhibitory selectivity and the in vitro and in vivo potency of the new PDE5 inhibitor vardenafil. *Int J Impot Res* **13:**282–290.
Schneider HH, Schmiechen R, Brezinski M, and Seidler J (1986) Stereospecific binding of the antidepressant rolipram to brain protein structures. *Eur J Pharmacol* **127:**105–115.
Shimizu-Albergine M, Rybalkin SD, Rybalkina IG, Feil R, Wolfsgruber W, Hofmann F, and Beavo JA (2003) Individual cerebellar Purkinje cells express different cGMP phosphodiesterases (PDEs): in vivo phosphorylation of cGMP-specific PDE (PDE5) as an indicator of cGMP-dependent protein kinase (PKG) activation. *J Neurosci* **23:**6452–6459.
Sung BJ, Yeon Hwang K, Ho Jeon Y, Lee JI, Heo YS, Hwan Kim J, Moon J, Min Yoon J, Hyun YL, Kim E, et al. (2003) Structure of the catalytic domain of human phosphodiesterase 5 with bound drug molecules. *Nature (Lond)* **425:**98–102.
Thomas MK, Francis SH, and Corbin JD (1990a) Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase. *J Biol Chem* **265:**14964–14970.
Thomas MK, Francis SH, and Corbin JD (1990b) Substrate- and kinase-directed regulation of phosphorylation of a cGMP-binding phosphodiesterase by cGMP. *J Biol Chem* **265:**14971–14978.
Torphy TJ, Livi GP, Balcarek JM, White JR, Chilton FH, and Undem BJ (1992) Therapeutic potential of isozyme-selective phosphodiesterase inhibitors in the treatment of asthma. *Adv Second Messenger Phosphoprotein Res* **25:**289–305.
Turko IV, Ballard SA, Francis SH, and Corbin JD (1999) Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. *Mol Pharmacol* **56:**124–130.
Weimann J, Ullrich R, Hromi J, Fujino Y, Clark MW, Bloch KD, and Zapol WM (2000) Sildenafil is a pulmonary vasodilator in awake lambs with acute pulmonary hypertension. *Anesthesiology* **92:**1702–1712.
Wyatt TA, Naftilan AJ, Francis SH, and Corbin JD (1998) ANF elicits phosphorylation of the cGMP phosphodiesterase in vascular smooth muscle cells. *Am J Physiol* **274:**H448–H455.

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