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[^{3}H]Sildenafil Binding to Phosphodiesterase-5 Is Specific, Kinetically Heterogeneous, and Stimulated by cGMP

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ABSTRACT

Sildenafil (Viagra) potentiates penile erection by acting as a nonhydrolyzable analog of cGMP and competing with this nucleotide for catalysis by phosphodiesterase-5 (PDE5), but the characteristics of direct binding of radiolabeled sildenafil to PDE5 have not been determined. [^{3}H]Sildenafil binding to PDE5 was retained when filtered through nitrocellulose or glass-fiber membranes. Binding was inhibited by excess sildenafil, 2-(2-methylpyridin-4-yl)methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl)methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T-0156), 3-isobutyl-1-methylxanthine, EDTA, or cGMP, but not by cAMP or 5′-GMP. PDE5 was the only [^{3}H]sildenafil binding protein detected in human lung extract. Using purified recombinant PDE5, [^{3}H]sildenafil exchange dissociation yielded two components with $t_{1/2}$ values of 1 and 14 min and corresponding calculated K_D values of 12 and 0.83 nM, respectively. This implied the existence of two conformers of the PDE5 catalytic site. [^{3}H]Sildenafil binding isotherm of PDE5 indicated K_D was 8.3 to 13.3 nM, and low cGMP decreased the K_D to 4.8 nM but only slightly increased B_{max} to a maximum of 0.61 mol/mol-subunit. Results suggest that these effects occur via cGMP binding to the allosteric cGMP binding sites of PDE5. Results imply that by inhibiting PDE5 and thereby increasing cGMP, sildenafil accentuates its own binding affinity for PDE5, which further elevates cGMP. The data also indicate that after physiological elevation, cGMP may directly stimulate the catalytic site by binding to the allosteric cGMP-binding sites of PDE5, thus causing negative feedback on this pathway.

Phosphodiesterase-5 (PDE5), which specifically degrades cGMP, is the target of sildenafil (Viagra; Pfizer, New York, NY) in causing penile erection (Boolell et al., 1996; Corbin and Francis, 1999). PDE5 is present in high levels in smooth muscle tissues, but it is also present in platelets and other tissues (Francis et al., 1990, 2001). PDE5 is believed to contain a single catalytic domain and regulatory domain on each of two subunits. The catalytic domain is highly specific for cGMP, and it is homologous to those of the other family members of the superfamily of cyclic nucleotide phosphodiesterases (PDE) (McAllister-Lucas et al., 1993). The regulatory domain contains two cGMP phosphodiesterase-*Anabaena* adenylyl cyclase-*Escherichia coli* FhlA (GAF) domains (Thomas et al., 1990a; McAllister-Lucas et al., 1993; Aravind and Ponting, 1997), at least one of which functions as a highly selective allosteric site for cGMP binding (McAllister-Lucas et al., 1993; Liu et al., 2001b). Four other PDE families (PDE2, PDE6, PDE10, and PDE11) also possess GAF domains (Charbonneau, 1990; Fujishige et al., 1999; Soderling et al., 1999; Fawcett et al., 2000; Francis et al., 2001). In addition to PDE5, two other PDE families (PDE2 and PDE6) have been shown to bind cGMP (Yamazaki et al., 1980; Stroop and Beavo, 1991). Binding of cGMP to the GAF domains of PDE2 stimulates catalytic activity of this enzyme (Beavo et al., 1971). The regulatory domain in PDE5 is phosphorylated by cGMP-dependent protein kinase (PKG) at Ser-92 (bovine) both in vitro and in intact cells (Thomas et al., 1990b; Wyatt et al., 1998; Mullershausen et al., 2001; Murthy, 2001; Rybalkin et al., 2002). Regulation of phosphorylation of this site is substrate-directed because occupation of a GAF domain by cGMP is required for phosphorylation by

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ABBREVIATIONS: PDE5, phosphodiesterase-5; PDE, cyclic nucleotide phosphodiesterase; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; GAF, cGMP phosphodiesterase-*Anabaena* adenylyl cyclase-*Escherichia coli* FhlA; IBMX, 3-isobutyl-1-methylxanthine; KPM, potassium phosphate/β-mercaptoethanol; KP, potassium phosphate; T-0156, 2-(2-methylpyridin-4-yl)methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl)methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis.

PKG. Phosphorylation causes stimulation of both catalytic activity and cGMP binding to the GAF domain (Corbin et al., 2000; Francis et al., 2002). It has been proposed that these effects are responsible for negative feedback regulation of active cGMP levels in cells. For both the cGMP- and cAMP-signaling pathways, negative feedback regulation has emerged as a major function of PDEs. PDE3 and PDE4 have been reported to be activated by phosphorylation when cAMP is elevated in cells (Degerman et al., 1997; Conti, 2000). Likewise, elevation of cGMP has been shown to cause increased PDE5 activity that is associated with cGMP-sensitive phosphorylation (Wyatt et al., 1998; Mullershausen et al., 2001; Murthy, 2001). In each case, PDE phosphorylation correlates with increased degradation of cyclic nucleotide. Elevation of cGMP is required for phosphorylation of PDE5 by PKG or cAMP-dependent protein kinase (PKA), and this process stimulates degradation of cGMP as well as sequestration of cGMP in the GAF domain (Corbin et al., 2000), both of which represent negative feedback on the cGMP pathway. Negative feedback regulation of cGMP would be enhanced if cGMP binding to the GAF domain also directly stimulates the catalytic domain. This was predicted earlier from the principle of reciprocity (Weber, 1975; Francis et al., 1990; Thomas et al., 1990a). Binding of 3-isobutyl-1-methylxanthine (IBMX) or a similar ligand to the catalytic domain has been shown to stimulate binding of cGMP to a GAF domain, but direct evidence that binding of cGMP to a GAF domain stimulates the catalytic domain has been elusive. This has been caused in large part by difficulties in performing such studies with PDE5, in which both the catalytic domain and GAF domain possess high specificity for cGMP. Okada and Asakawa (2002) recently reported that cGMP stimulates PDE5 catalytic activity when measured using a fluorescent cGMP analog that is specific for the catalytic site of the enzyme. They suggested that this stimulation occurs through cGMP binding to the GAF domains.

Studies of the catalytic domain of PDE5 would be greatly enhanced by the availability of a specific, high-affinity, nonhydrolyzable PDE5 catalytic-site radioligand that binds with sufficient affinity to allow isolation of the ligand-enzyme complex. Properties of the catalytic site that are not directly dependent on catalytic activity could also be examined using such a ligand. Because of its known inhibitory potency (IC_{50} = 1 to 7 nM at 0.1 to 0.4 μM cGMP substrate concentrations) and specificity (Ballard et al., 1998; Corbin et al., 2000; Saenz de Tejada et al., 2001), sildenafil is an obvious choice for radiolabeling and pursuing such studies. Because of its high affinity, radiolabeled sildenafil is predicted to be retained by PDE5 after filtration through filter membranes. We have prepared this compound and used it as a probe for binding studies to explore previously unrecognized features of PDE5. This is the first report of direct binding of a cGMP analog to the catalytic site of this enzyme.

Materials and Methods

Materials

[γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences (Boston, MA). [^{3}H]cGMP, Sephadex G-25, and DEAE-Sephacel were from Amersham Biosciences Inc. (Piscataway, NJ). IBMX, histone IIA-S, *Crotalus atrox* snake venom, 5′-GMP, and cGMP were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine heart catalytic subunit of PKA (Flockhart and Corbin, 1984) and regulatory domain of PDE5 (Liu et al., 2001b) were purified to homogeneity as described earlier. Native bovine lung PDE5 was purified through the blue-Sepharose chromatography step as described previously (Francis and Corbin, 1988; Thomas et al., 1990a). After obtaining approval from the Committee for the Protection of Human Subjects at Vanderbilt University Medical Center, 2-g segments of lung were obtained from patients undergoing lung transplantation. Segments were excised under sterile conditions from the explanted lung and immediately frozen in liquid nitrogen. The PDE5-specific inhibitor T-0156 was a gift from Tanabe Seiyaku Co. Ltd. (Saitama, Japan).

Sildenafil and Radiolabeled Sildenafil

Two 50-mg tablets of Viagra (Pfizer) were placed into 100 ml of H_2O in a plastic beaker and broken into fine particles with a glass rod. The suspension and 20-ml rinse were placed into a 200-ml graduated cylinder with plastic covering and shaken manually for 15 min. Residual material was removed by centrifugation for 20 min at 27,000*g* at 4°C. Supernatant (120 ml) was applied to a 285-ml Sephadex G-25 (superfine) column equilibrated in deionized H_2O at 20°C. Nucleotides, PDE inhibitors, and other compounds with similar structures are known to adsorb to Sephadex G-25, and this resin has been used successfully to purify these compounds (Corbin et al., 1988). After the G-25 column was washed with 1.5 liters of H_2O, sildenafil was eluted with 500 ml of 1% formic acid. The sample was lyophilized, resuspended in H_2O, and relyophilized. The fluffy crystals were carefully collected from the lyophilization flask and used for experiments and for preparation of radiolabeled sildenafil. Recovery was >80%. A molar extinction coefficient (289 nm) of 13.8 ($\epsilon \times 10^3$) at pH 5.2 was determined using a sample of sildenafil kindly provided by Pfizer. The sample was either stored in crystalline form or in solution of 0.1% formic acid. The molecular weight of sildenafil (474 g/mol) was confirmed by positive-ion nanospray and matrix-assisted laser desorption ionization mass spectrometry, and no significant levels of impurities were detected. Sildenafil (6.7 mg) was purified as described above and was sent to Amersham Biosciences Inc. for tritium labeling. Stock solution was 6 Ci/mmol and 34 μM. Tritium label was expected on the methyl and propyl groups of the pyrazole ring.

His-Tagged PDE5

The full-length bovine PDE5 cDNA in BacPAK9 (BD Biosciences Clontech, Palo Alto, CA) purified by QIAGEN (Valencia, CA) was the starting material (Turko et al., 1996). The product was amplified using polymerase chain reaction to introduce *SfoI* on both the 5′ and 3′ end and subcloned into pFASTBAC HTc expression vector from Invitrogen (Carlsbad, CA). The vector has an rTEV protease site so that the tag and linker region can be cleaved. DNA was confirmed by sequencing before transformation and transfection into Sf9 cells.

Transformation of PDE5 Construct into DH10Bac for Transposition into the Bacmid

DNA (1.5 μg) was added to 100 μl of DH10Bac cells, and the mixture was incubated on ice for 30 min. The mixture was heat-shocked in a 42°C water bath for 45 s and then chilled on ice for 2 min. SOC medium (900 μl; 2 g of tryptone, 0.5 g of yeast extract, 8.6 mM NaCl, 10 mM $MgCl_2$, 10 mM Mg SO_4, and 2 mM glucose, in a volume of 100 ml of H_2O) was added, and the mixture was placed in a 37°C shaking incubator with medium agitation for 4 h. A 1:20 dilution of this mixture was made using SOC medium, and 100 μl was plated onto Luria agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal (Invitrogen), and 40 μg/ml isopropyl β-D-thiogalactoside. After a 24-h incubation, two white colonies were selected to set up a liquid culture consisting of 2 ml of Luria-Bertani medium supplemented with 50 μg/ml kanamycin, 7 μg/ml gentamicin, and 10 μg/ml tetracycline. The cultures were grown at 37°C shaking at 250 rpm for 36 h. The

bacmid DNA was prepared using procedures in the Instruction Manual for Bac-to-Bac Baculovirus Expression System (Invitrogen). Presence of high-molecular-weight bacmid DNA was confirmed with the use of agarose gel electrophoresis.

Transfection of Sf9 Cells with PDE5-Recombinant Bacmid DNA

We used a modified transfection procedure recommended by Invitrogen. The Sf9 cells used for transfection were grown in Grace's Insect medium (Invitrogen) supplemented with 10% FBS as well as L-glutamine. Cells (0.9 million) were seeded per well of a six-well plate and were allowed to attach for at least 1 h. Meanwhile, two solutions were prepared in 14-ml conical tubes. The first solution contained 5 μl of bacmid DNA diluted in 100 μl of Grace's Insect medium without any FBS or L-glutamine. CellFECTIN reagent (6 μl) (Invitrogen) was diluted in 100 μl Grace's Insect medium in the second solution. The solutions were combined, mixed, and incubated for 30 min at room temperature. The cells were washed twice with 2 ml of Grace's Insect medium. Grace's Insect medium (800 μl) was added to the tube containing the CellFECTIN and PDE5 bacmid DNA. The wash media was aspirated, and the transfection mixture was added to the cells. The cells were incubated for 5 h at 27°C. The 1-ml mixture was removed from the well, and 2 ml of fresh Grace's Insect medium containing 10% FBS and L-glutamine was added to the well. The cells were incubated for 72 h at 27°C. The virus was harvested after 72 h and amplified. Small-scale expression tests of the amplified virus were performed by infecting cells in a six-well plate with varying amounts of virus.

Expression and Purification of His-Tagged PDE5

Virus (2 ml) was added to 2.5 L Sf9 cells (1.3×10^6 cells) that were incubated in a spinner flask for 5 days at 37°C in a tissue-culture incubator. Cells were centrifuged at 2000 rpm in a Beckman JA-10 rotor (Beckman Coulter, Inc., Fullerton, CA) for 10 min at 4°C. Pellet was broken up with a pipette using 10 ml of lysis buffer (20 mM Tris, pH 8, containing 100 mM NaCl) and homogenized twice for 4 s using an Ultraturrax (Tekmar, Cincinnati, OH). After centrifugation at 9000 rpm in a Beckman JA-20 rotor for 20 min, the supernatant was collected and loaded on a 0.9 × 3-cm Ni-NTA agarose column (QIAGEN) equilibrated with lysis buffer. The column was washed with 20 ml of lysis buffer before eluting with 30 ml of lysis buffer containing 0.1 M imidazole. Fractions (2 ml) were collected and analyzed for cGMP PDE catalytic activity, protein (Bradford, 1976), and SDS-PAGE. Peak fractions of PDE activity were pooled, diluted 5-fold with H_2O, and loaded onto DEAE-Sephacel (0.9 × 4 cm) equilibrated in 10 mM potassium phosphate, pH 6.8, and 15 mM β-mercaptoethanol (KPM). After washing with 20 ml of KPM containing 50 mM NaCl, PDE5 was eluted with 60 ml of a linear NaCl gradient (50 to 300 mM) in KPM. Two-milliliter fractions were collected and analyzed for cGMP PDE catalytic activity, protein, and SDS-PAGE. Peak fractions were pooled, glycerol was added to a final concentration of 20%, and aliquots were quick-frozen in liquid nitrogen and stored at −70°C. His-tagged PDE5 was highly pure as indicated by protein staining after SDS-PAGE (Fig. 1). The enzyme had a specific enzyme activity of 7 μmol/min/mg, which compared favorably with our previously published value for native bovine PDE5 (5 μmol/min/mg) (Thomas et al., 1990a). His-tagged PDE5 was phosphorylated by catalytic subunit of PKA to a stoichiometry of 0.2 mol per subunit in 50 min at 20°C (Thomas et al., 1990b). Enzyme phosphorylation and catalytic properties verified structural and functional integrity of this PDE5 preparation.

PDE and PKG Assays

PDE activity was determined by a modified assay (Martins et al., 1982) as described previously (Gopal et al., 2001) using 0.4 μM [^{3}H]cGMP as substrate. PKG activity was determined in the presence of 2 μM cGMP as described previously (Gopal et al., 2001).

[^{3}H]Sildenafil Membrane Filtration Binding Assays

Method A. His-tagged PDE5 (80 μl) (final concentration in reaction, 0.77 nM) was added to 2 ml of 10 mM KPM containing 0.2 mg/ml histone IIA-S. Various concentrations of [^{3}H]sildenafil were added to the KPM-histone mixture before starting the reaction with enzyme. This order of addition prevented [^{3}H]sildenafil binding to the tube surface, which occurred when [^{3}H]sildenafil was added in absence of histone, and 0.2 mg/ml histone produced the optimum effect. It was also determined that histone had a marked effect to increase the retention of PDE5 on the membranes. Reactions were incubated in an ice-water bath for 20 min. To each, 200 μl of 25% Triton X-100 (2.2% final concentration) was added, and samples were rapidly filtered under house vacuum through Millipore nitrocellulose membranes (0.45 μm) that had been prewetted with 1.5 ml of cold 10 mM potassium phosphate, pH 6.8 (KP), containing 0.1% Triton X-100. Inclusion of Triton X-100 lowered blank values significantly. Tubes were each rinsed with 3 ml of the same buffer and filtered. The filter membranes were removed and placed into slots of a scintillation vial box for subsequent drying. The vial box was placed into a glassware dryer for 10 min, and dried papers were transferred to 6-ml scintillation vials. To this, 5 ml of nonaqueous scintillant was added, and papers were counted in a scintillation counter. Using purified [^{32}P]PDE5 prepared by phosphorylation in presence of cGMP, Mg[^{32}P]ATP and catalytic subunit of PKA followed by Seph-

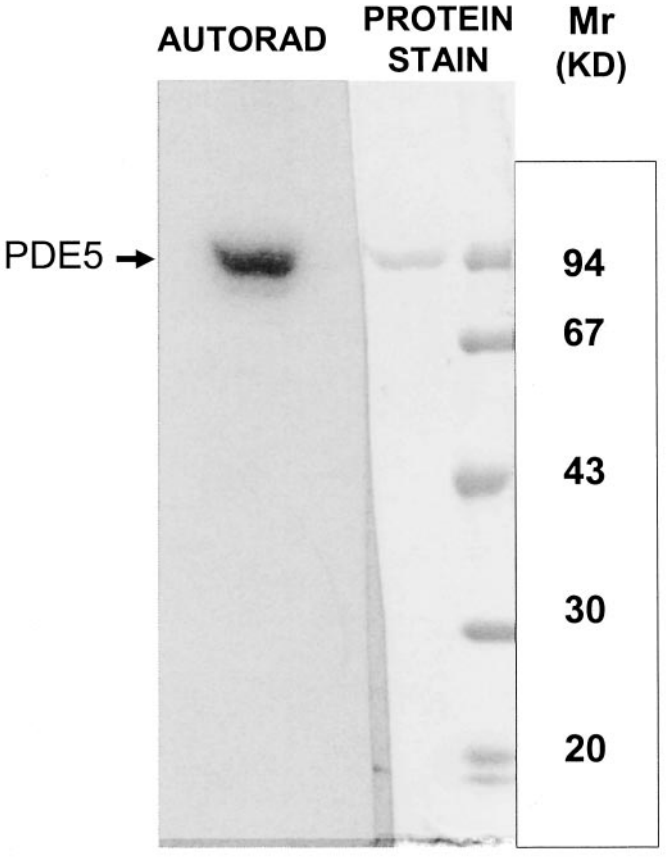

Fig. 1. Purified His-tagged PDE5. Enzyme was expressed and purified as described under *Materials and Methods*. Here, 200 μl of 4 μM enzyme (final concentration, 3.22 μM) was added to 48 μl of a mixture of 10 mM $MgCl_2$-0.1 mM [^{32}P]ATP (2000 cpm/pmol), 50 μM cGMP, 0.1 mM IBMX, and 26 μg/ml purified catalytic subunit of PKA for 50 min at 20°C. The mixture was chromatographed on Sephedex G-25 (0.9 × 13 cm) equilibrated in KPM. Fractions (0.5 ml) were collected, and 10 μl of peak protein fraction (fraction 8) was analyzed for ^{32}P incorporation by scintillation counting; 10 μl was applied to SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250 and developed overnight by autoradiography.

adex G-25 chromatography (Fig. 1) (Corbin et al., 2000), recovery of PDE5 by Millipore filters was determined to be 75%. Accordingly, [3H]sildenafil binding values were corrected for 25% loss of PDE5 through the filter.

Method B. Reactions were carried out exactly as described under *Method A* (no Triton X-100 was added to the samples at the end of incubation). Samples were rapidly filtered under house vacuum using a Brandel Cell Harvester (Brandel, Gaithersburg, MD) with Whatman GF/B glass-fiber filters (Whatman, Clifton, NJ) that had been prewetted with 1.5 ml of cold KP containing 0.1% Triton X-100. After immobilization, filters were washed with 6 ml of cold KP buffer containing 0.1% Triton X-100. After washing, filters were removed for drying and counting as described under *Method A*. Using the same technique for calculating recovery of PDE5 in method A, recovery was 97% using method B, and the blank was at least 2 times lower using method B. Maximum [3H]sildenafil binding stoichiometry was 0.31 and 0.61 mol/PDE5 subunit using methods A and B, respectively. This finding suggested that some bound [3H]sildenafil was lost during filtration, particularly with method A.

Results

[3H]Sildenafil-Binding Assay. Highly purified His-tagged PDE5 was used for most of the experiments (Fig. 1). [3H]Sildenafil binding to PDE5 reached equilibration within 1 min of incubation at all concentrations tested, and using 30 nM [3H]sildenafil, binding was linear with increasing concentrations of PDE5 up to 23 nM (data not shown). Binding of [3H]sildenafil to PDE5 using method A required the presence of low ionic strength conditions (Fig. 2). Increasing ionic strength had less effect using method B, whereas 50 mM NaCl inhibited binding by only 40% (data not shown). In the absence of NaCl, the buffer (potassium phosphate) used for the assay was also inhibitory at concentrations greater than 10 mM. At 30 mM potassium phosphate, [3H]sildenafil binding was inhibited by 90% (data not shown) compared with binding obtained with 10 mM potassium phosphate, which yielded the same values as 5 mM potassium phosphate.

Specificity for [3H]Sildenafil Binding to PDE5. Specificity for [3H]sildenafil binding was examined by testing the effects of various compounds on 6 nM [3H]sildenafil binding to purified PDE5 (Fig. 3). Fourteen-fold excess of unlabeled sildenafil or the PDE5-specific inhibitor T-0156 (Tanabe Seiyaku) (Mochida et al., 2002) were strongly inhibitory for [3H]sildenafil binding, whereas 233,000-fold excess of either cAMP or 5′-GMP had no significant effect. The nonspecific PDE inhibitor IBMX (0.36 mM) inhibited binding by more than 90%. The 233,000-fold excess of cGMP was partially inhibitory, which may be explained by the relatively low affinity of cGMP for the catalytic site coupled with a balance between competition of cGMP with [3H]sildenafil at the catalytic site and stimulation of [3H]sildenafil binding by cGMP binding at the allosteric GAF domain (vide infra). Other unlabeled PDE inhibitors added at 14-fold excess, including vinpocetine (PDE1 inhibitor), *erythro*-9-(2-hydroxy-3-nonyl)adenine (PDE2 inhibitor), cilostamide (PDE3 inhibitor), or rolipram (PDE4 inhibitor), had no significant effect (data not shown). Combined data indicated that [3H]sildenafil binds specifically to the catalytic site of PDE5.

We reported earlier that sildenafil does not interact significantly with the GAF domains of PDE5 (Turko et al., 1999). This conclusion was derived from the finding that sildenafil, even at high concentrations, does not compete with [3H]cGMP for binding to the GAF domains of PDE5. Further proof of the lack of sildenafil binding to the GAF domains is our results from studies of [3H]sildenafil binding to the highly purified isolated regulatory domain of PDE5. This regulatory domain binds cGMP specifically and with high affinity (Liu et al., 2001b). However, at concentrations of this domain of 0.38 to 38 nM, and using method A, 12 nM [3H]sildenafil did not detectably bind to this domain (data not shown). This experiment included a positive control of [3H]sildenafil binding to intact PDE5 (0.77 nM) performed as described in Fig. 3.

We have demonstrated that PDE5 binds Zn^{2+} and that catalytic activity of this enzyme requires Zn^{2+} or other divalent cation (Francis et al., 1994). In Fig. 4, it can be seen that 10 mM EDTA strongly inhibits binding of 0.5 to 30 nM

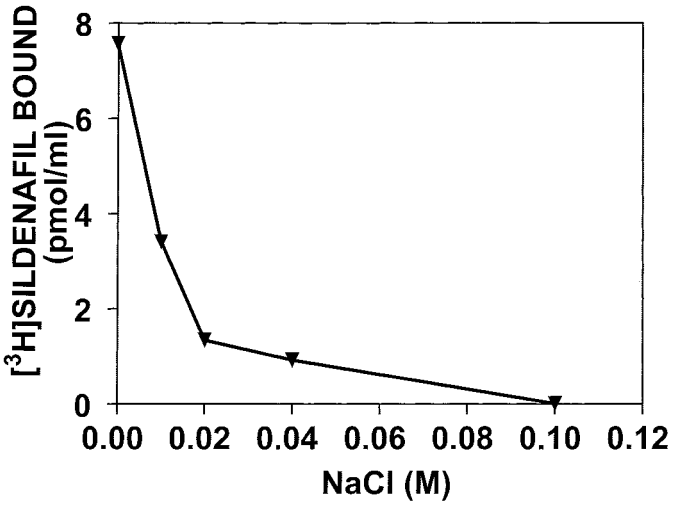

Fig. 2. Effect of NaCl on the [3H]sildenafil binding assay. Reactions were performed with His-tagged PDE5 using method A as described under *Materials and Methods,* except that the indicated concentration of NaCl was added to the reaction. The PDE5 concentration added was 20 pmol/ml (final assay concentration, 0.77 pmol/ml), and the final [3H]sildenafil concentration was 12 pmol/ml. Binding units are pmol/ml of added PDE5.

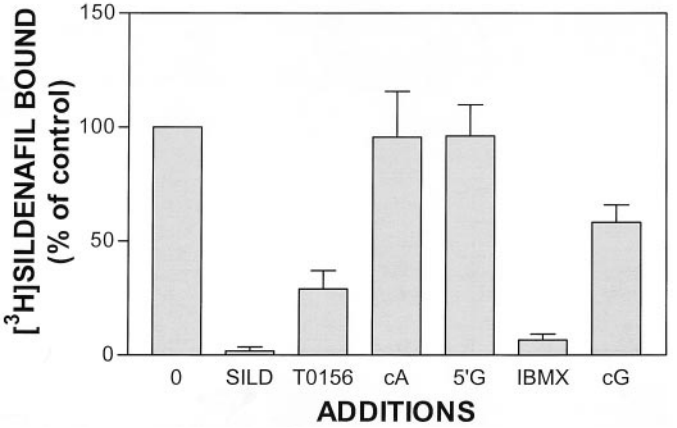

Fig. 3. Effects of nucleotides and unlabeled sildenafil on [3H]sildenafil binding to PDE5. Reactions were performed with His-tagged PDE5 using method A as described under *Materials and Methods*. The PDE5 concentration added was 20 pmol/ml (final assay concentration, 0.77 pmol/ml), and the final [3H]sildenafil concentration was 6 pmol/ml. Binding units are pmol/ml added PDE5. Concentrations of competing compounds: unlabeled sildenafil (SILD) = 0.083 μM (n = 9), T-0156 (PDE5 inhibitor) = 0.083 μM (n = 6), cAMP (cA) = 1.4 mM (n = 9), 5′-GMP (5′G) = 1.4 mM (n = 9), IBMX = 0.36 mM (n = 6), and cGMP (cG) = 1.4 mM (n = 6).

[^{3}H]sildenafil, suggesting that divalent metal is not only necessary for PDE5 catalysis but also required for the structural integrity of the catalytic site. Because PDE5 catalytic activity is known to require a divalent cation, these results also further supported the catalytic-site specificity of [^{3}H]sildenafil binding to PDE5.

[^{3}H]Sildenafil Binding in Crude Extract of Human Lung. Lung tissue is known to be a rich source of PDE5 (Francis et al., 2001). To seek additional proof for the specificity of [^{3}H]sildenafil binding to PDE5, a supernatant fraction of human lung homogenate was prepared. Fractions were analyzed for PDE5-specific PDE activity and for [^{3}H]sildenafil binding activity. It can be seen in Fig. 5 that these two activities coeluted, and no other significant peak of [^{3}H]sildenafil-binding activity was detected. The high resolution of proteins by this procedure was indicated by the elution position of endogenous PKGIα, which was used as marker in the experiment. The NaCl concentration at which the PDE5 peak eluted was only 0.04 M lower than that at which the PKG peak eluted. Only trace binding activity was detected in the flow-through/wash fraction and in a 20-ml 800 mM NaCl wash fraction collected after termination of the linear NaCl gradient. When the amount of PDE5 in the peak fraction was calculated using the specific enzyme activity of PDE5 at 0.4 μM cGMP as substrate (0.4 μmol/min/mg), a value of 12 nM was obtained. This was approximately double that obtained by direct [^{3}H]sildenafil binding activity (5 to 6 nM) from the left ordinate. This would be expected because maximum binding stoichiometry of [^{3}H]sildenafil using method A for purified PDE5 was 0.35 mol/mol, suggesting the loss of some bound [^{3}H]sildenafil during filtration. Therefore, quantification of PDE5 by PDE activity and [^{3}H]sildenafil binding activity yielded very similar values, suggesting the absence or very low levels of inhibitors or activators of the binding assay after DEAE chromatography. Estimation of PDE5 content in rabbit corpus cavernosum using both PDE activity and cGMP binding activity yielded values that were similar to each other (Gopal et al., 2001). The combined results suggested that [^{3}H]sildenafil binding activity can be used as a new method to identify and quantify PDE5 in crude systems. The results also imply that in human lung tissue, which has a high content of smooth muscle, sildenafil has strong selectivity for PDE5 over any other protein, including other PDEs.

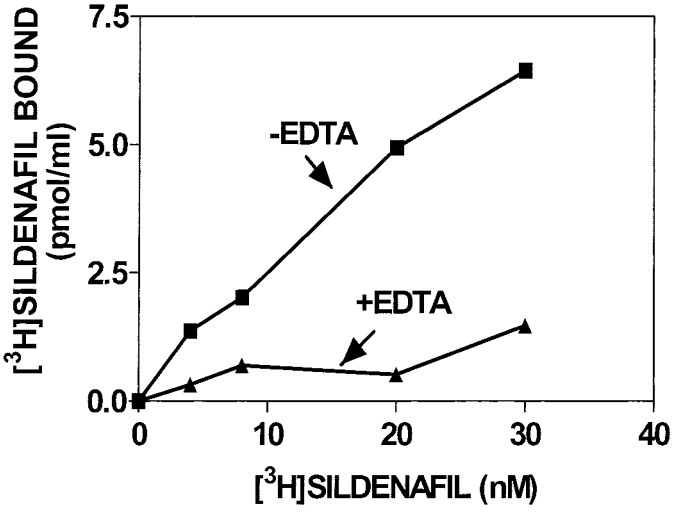

Fig. 4. Effect of EDTA on [^{3}H]sildenafil binding to PDE5. Experiment was performed ±10 mM EDTA using His-tagged PDE5 in a manner similar to that used in Fig. 3, except that the [^{3}H]sildenafil concentration varied between 4 and 30 pmol/ml.

[^{3}H]Sildenafil Binding Affinity. Dependence of [^{3}H]sildenafil concentration on binding to PDE5 in the absence of cGMP using His-tagged PDE5 is shown in Fig. 6. Using nonlinear regression analysis with Prism graphics, the K_D was calculated to be 13.3 ± 3.0 nM (n = 18). Scatchard plot of the same data (Fig. 6, inset) revealed K_D = 11.7 ± 1.8 nM. The goodness-of-fit value (R^2) for nonlinear regression of the isotherm shown in Fig. 6 and linear regression of the Scatchard plot was only 0.83 and 0.84, respectively. Therefore, the presence of more than one component of [^{3}H]sildenafil binding could not be ruled out. The K_D value obtained using binding assay methods A and B (vide infra) in the absence of cGMP was similar. Similar results were also obtained using either the peak fraction of crude human bovine lung PDE5 (Fig. 5) or partially purified native bovine PDE5 (data not shown).

Dissociation behavior of PDE5-bound [^{3}H]sildenafil was examined in the absence of cGMP and in the presence of excess unlabeled sildenafil (Fig. 7). PDE5 was first saturated with 30 nM [^{3}H]sildenafil using the conditions described in Fig. 6. Then an exchange of [^{3}H]sildenafil was initiated by the addition of 117-fold excess of unlabeled sildenafil. The time course of exchange indicated the presence of two components with $t_{1/2}$ of 14 and 1.0 min, respectively. The slow component extrapolated back to the y-axis at approximately the 50% mark, indicating that the two components were present in approximately equal amounts. Assuming a diffusion-limited association of [^{3}H]sildenafil to PDE5 and using the equation $K_D = 6.93 \times 10^{-7}$ M·s/$t_{1/2}$, K_{D1} was 0.83 nM and

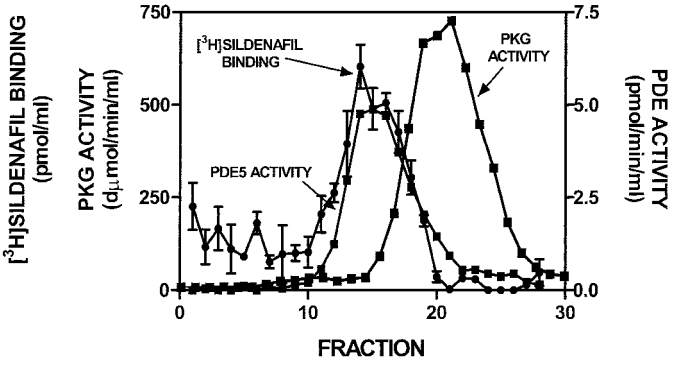

Fig. 5. DEAE-Sephacel chromatography of crude human lung extract. Human lung (5 g) was thawed, cut into small pieces, and homogenized in 80 ml of 2.5 mM potassium phosphate, pH 6.8, containing 0.25 mM EDTA, 0.025 mM IBMX, 4 mM β-mercaptoethanol, and 1 mM $CaCl_2$ with use of a Cuisinart apparatus (high speed) using 3 bursts lasting 15 s each at 4°C. After centrifugation in a Beckman JA-20 rotor (Beckman Coulter) for 30 min at 10,000g, 72 ml of supernatant was applied to a DEAE-Sephacel column (0.9 × 10 cm) equilibrated in KPM, and the column was washed with 20 ml of KPM containing 20 mM NaCl and 1 mM $CaCl_2$. The column was developed with a linear (20 to 280 mM) NaCl gradient in KPM containing 1 mM $CaCl_2$. Fractions (1.8 ml) were collected and analyzed (substrate = 0.4 μM [^{3}H]cGMP) for PDE activity ±40 pmol/ml sildenafil to calculate PDE5-specific PDE activity. A second peak of cGMP PDE activity eluted just after PDE5, and this activity, which was not inhibited by sildenafil, accounted for approximately 35% of the total cGMP PDE activity in the profile. [^{3}H]Sildenafil binding activity was measured using 400-μl aliquots with use of method A as described under *Materials and Methods,* except the final concentration of [^{3}H]sildenafil was 16 nM. PKG activity was determined in the presence of 2 μM cGMP as described under *Materials and Methods*.

K_{D2} was 12 nM. The average of these two K_D values was calculated to be 3.1 nM, which was similar to the K_D value of 4.8 nM determined using direct binding of [3H]sildenafil in the presence of cGMP in Fig. 9. The finding of two components of [3H]sildenafil binding using [3H]sildenafil dissociation behavior suggested caution in interpreting the results of Fig. 6, in which a single [3H]sildenafil binding component in PDE5 was assumed for the calculation of the binding properties.

Effect of cGMP on [3H]Sildenafil Binding. The high selectivity of sildenafil for the catalytic site over the GAF domains of PDE5 (Turko et al., 1999) permitted studies of the effects of cGMP binding to the GAF domains on the catalytic site. Using method B with 5 nM [3H]sildenafil in the binding reaction, we examined the effect of cGMP concentration on [3H]sildenafil binding to PDE5 (Fig. 8). Experiments were done in the absence of MgATP and protein kinases so that effects of phosphorylation could be avoided. Low concentrations of cGMP (<10 μM) caused progressive increases in [3H]sildenafil binding, and the stimulatory effect waned at concentrations greater than 10 μM cGMP. Concentrations greater than 1 mM cGMP were inhibitory for [3H]sildenafil binding (Fig. 3). It is suggested that low concentrations of cGMP bind to the allosteric GAF domains and cause direct stimulation of [3H]sildenafil binding to the PDE5 catalytic site. Higher concentrations of cGMP bind both to the GAF domains and to the catalytic site. This latter effect causes a progressive decrease in [3H]sildenafil binding at >10 μM cGMP. At very high cGMP, only the inhibitory effect would be observed because [3H]sildenafil binding would be strongly inhibited by competition. The results were generally consistent with the relative affinity of cGMP for the GAF domains (K_D = 0.2 μM) and catalytic site (K_m = 5 μM) of PDE5, particularly considering that the sildenafil concentration in the reaction was lower than the optimum concentration for stimulating cGMP binding to the GAF domains. The results of Fig. 8 were reproduced using method A for filtration (data not shown), and 2- to 5-fold stimulations by cGMP were obtained in six experiments using both methods.

To investigate the mechanism of the stimulatory effect of cGMP on [3H]sildenafil binding, the optimum stimulatory concentration of cGMP (10 μM) observed in Fig. 8 was tested using various concentrations of [3H]sildenafil in the method B binding reaction (Fig. 9). It was calculated from results of three separate experiments, each done in triplicate, that the average [3H]sildenafil binding affinity increased from K_D = 8.3 ± 1.8 nM in the absence of cGMP to K_D = 4.8 ± 0.8 nM in the presence of cGMP, whereas the B_{max} increased only slightly from 10.6 ± 0.7 to 15.5 ± 1.2 pmol/ml. Using 64 nM [3H]sildenafil in the presence of cGMP, the stoichiometry of binding was calculated to be 0.61 ± 0.13 mol [3H]sildenafil bound per mol PDE5 subunit (n = 3).

The interpretation that binding of [3H]sildenafil to the catalytic site of PDE5 is stimulated by cGMP binding to the

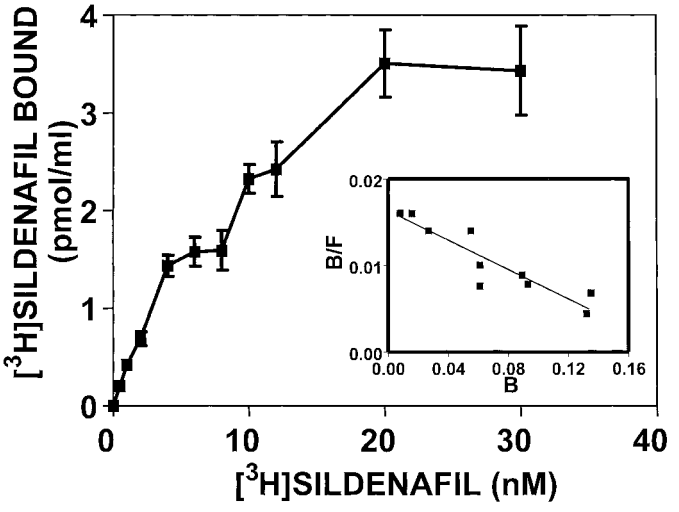

Fig. 6. Effect of [3H]sildenafil concentration on binding to PDE5. Conditions were as those described in Fig. 3 using method A. PDE5 concentration added was 20 pmol/ml (final assay concentration, 0.77 pmol/ml), and [3H]sildenafil concentration varied between 0.5 and 30 pmol/ml. Binding units are pmol/ml added PDE5. Values are mean ± S.E.M. for 18 determinations. Prism graphics (nonlinear regression) were used to calculate K_D values. Scatchard plot of the data are shown in the inset. B, bound; F, free.

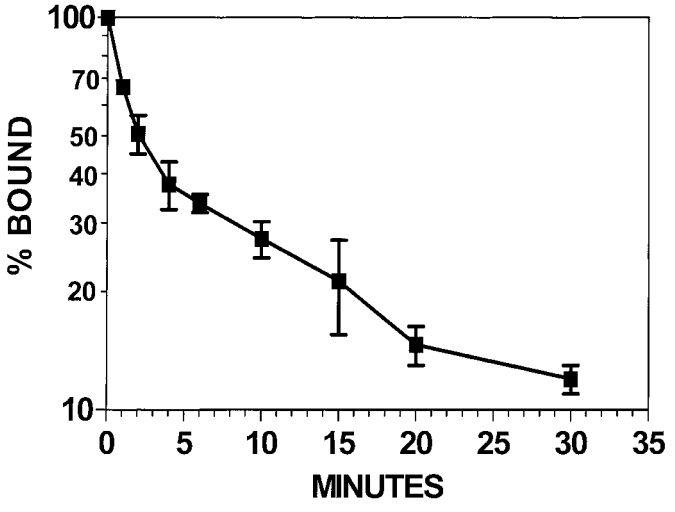

Fig. 7. [3H]sildenafil exchange dissociation from PDE5. His-tagged PDE5 (950 μl; ~1 nM final concentration) was added to 4500 μl KPM-0.2 mg/ml histone IIA-S containing 30 pmol/ml [3H]sildenafil for 10 min at 0°C. For the zero time sample, a 550-μl aliquot was added to 200 μl of 25% Triton X-100 and filtered as described in method A. Then, 5 μl of 0.6 μmol/ml unlabeled sildenafil was added to the remaining reaction mixture. Aliquots (550 μl) were removed at the times indicated and pipetted into 200 μl of 25% Triton X-100 and filtered as described in method A.

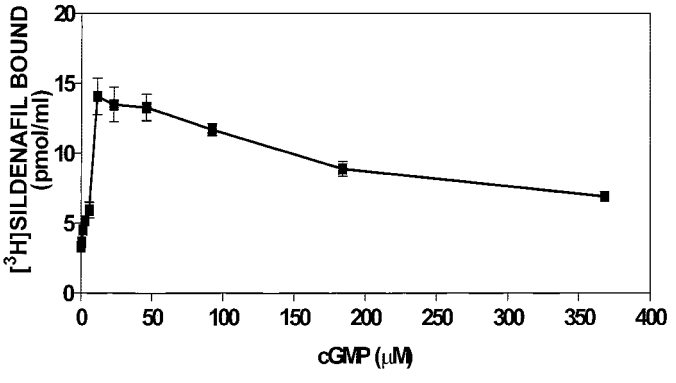

Fig. 8. Effect of cGMP concentration on [3H]sildenafil binding to PDE5. Conditions were as those described under *Materials and Methods* except that cGMP varied between 0 and 368 nmol/ml and [3H]sildenafil was 5 pmol/ml. His-tagged PDE5 final concentration was 1.5 pmol/ml (added concentration, 40 pmol/ml). Units are pmol/ml added PDE5. Values are mean ± S.E.M. of triplicates in a single experiment. The experiment was performed three times with similar results. Filtration was performed as described for method B.

allosteric sites of the enzyme predicts that the binding of the natural ligand cGMP to the catalytic site would be stimulated by cGMP binding to the allosteric sites. Such studies would be difficult because cGMP interacts at both types of sites simultaneously, and direct measurement of cGMP binding to the catalytic site has not been achieved to date. However, a recent report (Okada and Asakawa, 2002) asserting that cGMP binding at the allosteric sites stimulates catalytic activity is consistent with our interpretation. Two other laboratories have also drawn the same conclusion using different approaches (D. Koesling and J. Beavo, personal communications).

Discussion

The binding assay methods presented in this report are specific for [^{3}H]sildenafil binding to the catalytic site of PDE5. It is suggested that the procedures described here could be used to study the binding of other PDE inhibitors to PDE5 and to study PDE inhibitor binding to other PDEs. It is extremely unlikely that [^{3}H]sildenafil binds to the PDE5 regulatory domain in these assays for several reasons: 1) binding is inhibited by catalytic site-selective agents; 2) stoichiometry of [^{3}H]sildenafil binding is <1 mol/PDE5 subunit; 3) [^{3}H]sildenafil does not bind to the isolated regulatory domain, which binds [^{3}H]cGMP stoichiometrically; 4) [^{3}H]sildenafil K_D value for binding is approximately the same as sildenafil IC_{50} for catalysis, which should approximate K_i because the assays were done at a cGMP substrate concentration considerably below that of K_m; 5) up to 1000 nM sildenafil does not compete with [^{3}H]cGMP binding to the regulatory domain (Turko et al., 1999); and 6) there is no sequence homology between PDE5 catalytic and regulatory domains (McAllister-Lucas et al., 1993). This represents the first study of direct binding of a ligand to the catalytic site of PDE5. Previously, characteristics of the catalytic site were investigated by measurement of the catalytic activity of the enzyme. Because sildenafil is a competitive inhibitor of PDE5 catalysis (Ballard et al., 1998), it represents a nonhydrolyzable analog for the catalytic site of the enzyme. The present studies reveal some properties of the catalytic site of PDE5 not seen before: 1) a divalent cation is required not only for catalytic activity but also for structural integrity of the catalytic site; 2) the catalytic site exhibits heterogeneity: two components (slow and fast dissociation or high and low affinity) of [^{3}H]sildenafil binding were clearly revealed by studies of [^{3}H]sildenafil dissociation behavior. This heterogeneity could be caused by the existence of more than one conformer of the catalytic site of PDE5 (Francis et al., 1998). It seems plausible that PDE2 (Beavo et al., 1971) and PDE5 undergo similar conformational changes in response to cGMP to increase catalytic site function; and 3) binding of cGMP at the allosteric GAF domains stimulates the binding of ligand to the catalytic site independently of PDE5 phosphorylation.

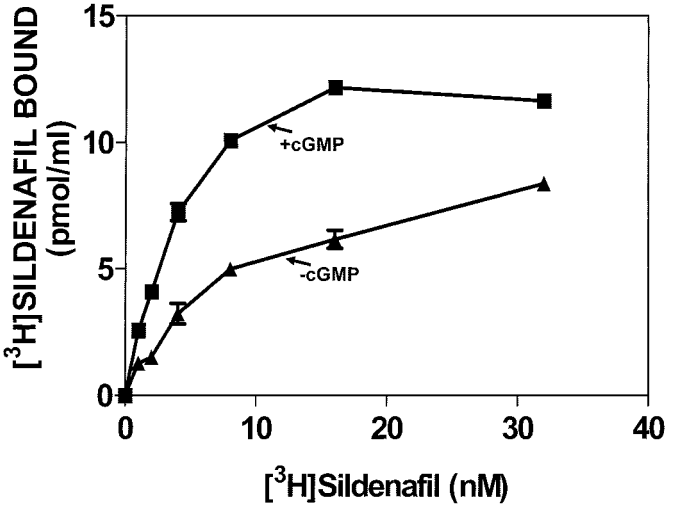

Fig. 9. Effect of [^{3}H]sildenafil concentration on cGMP stimulation of binding. Conditions were as those in Fig. 8 using 1.5 pmol/ml as the final concentration of PDE5 (added concentration, 40 pmol/ml). Units are pmol/ml added PDE5. Reactions were performed in the absence and presence of 10 nmol/ml cGMP. Filtration was performed as described for method B. Values are mean ± S.E.M. of triplicates in a single experiment. The experiment was done three times with similar results. Prism graphics (nonlinear regression) were used to calculate K_D and B_{max} for [^{3}H]sildenafil binding.

Direct stimulation of the PDE5 catalytic site by allosteric cGMP binding was predicted from the principle of reciprocity (Weber, 1975) and adds yet another mechanism to our proposed scheme for negative feedback control of cGMP levels in tissues (Corbin et al., 2000). These mechanisms have all been described by this laboratory and now include: 1) increased PDE5 catalytic activity caused by mass action of elevated cGMP; 2) increased cGMP binding to PDE5 allosteric sites caused by mass action of elevated cGMP; 3) increased PDE5 catalytic activity caused by phosphorylation and activation of PDE5 by activated PKG (Wyatt et al., 1998; Corbin et al., 2000; Mullershausen et al., 2001; Murthy, 2001; Rybalkin et al., 2002); 4) increased cGMP binding to PDE5 allosteric sites caused by this phosphorylation, which sequesters cGMP (Corbin et al., 2000); and 5) as shown here, phosphorylation-independent stimulation of the PDE5 catalytic site by allosteric cGMP binding to the enzyme. The existence of an array of mechanisms for negative feedback control of cGMP levels suggests that cells require judicious dampening and rapid termination of the cGMP signaling pathway. The narrow 2- to 4-fold window for cGMP regulation of cell processes (Jiang et al., 1992) could be partly responsible for the evolution of these mechanisms.

The molecular mechanism for the stimulation of PDE5 catalytic activity by allosteric cGMP binding is unknown. The enzyme is presumably in an autoinhibited state that is relieved when cGMP binds. Stimulation by cGMP binding causes a substantial increase in ligand binding affinity of the catalytic site, but we cannot rule out a small effect on the capacity of this site as well. The affinity (K_D = 3.0 to 4.8 nM) of [^{3}H]sildenafil for binding to PDE5 in the presence of cGMP reported here compares favorably with values found in the literature (Ballard et al., 1998; Corbin et al., 2000; Saenz de Tejada et al., 2001) for IC_{50} of sildenafil (IC_{50} = 1 to 7 nM) for inhibiting PDE5 catalytic activity. The cGMP stimulation of [^{3}H]sildenafil binding could be caused by conformational changes known to be induced in PDE5 by cGMP binding to the GAF domains (Francis et al., 1998).

To our knowledge, PDE5 is the first PDE shown to bind a catalytic site ligand nearly stoichiometrically by membrane filtration. The only other PDE to be studied by radiolabeled ligand binding is PDE4, which binds tritiated rolipram and its derivatives (Schneider et al., 1986; Torphy et al., 1992; Liu et al., 2001a). However, tritiated rolipram has been re-

ported to bind to PDE4 with a stoichiometry of less than 0.01 mol/subunit using membrane filters, although nearly stoichiometric rolipram binding can be obtained using PDE4 in an antibody-based scintillation proximity assay (Liu et al., 2001a). Whether or not the use of conditions shown here for PDE5 could be used to increase the retention of other radioligand-bound PDEs, including PDE4, on membrane filters should be examined.

Although we suggest that the GAF domains serve a negative feedback function in the cGMP pathway in normal physiology, they serve a quite different pharmacological role. Our results indicate that cGMP binding to the PDE5 GAF domains stimulates binding of sildenafil to the catalytic site. Although this implies that after in vivo elevation of cGMP by sildenafil administration, increased binding of cGMP to the GAF domains would stimulate cGMP breakdown, this should not occur to any large extent because sildenafil would simultaneously occupy the catalytic site. Thus, it would be expected that increased cGMP binding to the GAF domains caused by sildenafil addition should cause further increase in sildenafil binding to the catalytic site, which should cause further increase in cGMP. This represents a positive feedback effect on sildenafil action, meaning that the inhibition of cGMP degradation by sildenafil interaction with the PDE5 catalytic site should stimulate further sildenafil binding to this site, resulting in the potentiation of cGMP elevation. This should also occur with PDE5 inhibitors other than sildenafil. Because these inhibitors have very high binding affinities for PDE5, the possibility should be considered that such inhibitors could be effectively concentrated within cells containing high levels of PDE5 and could be cleared from these cells relatively slowly if they are not metabolized. These considerations could theoretically have strong relevance to the efficacy and duration of effects of Viagra and other PDE5 inhibitors that are used for treating patients.

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