Molecular Pharmacology Fast Forward. Published on August 22, 2006 as doi:10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

A 46-amino acid segment in phosphodiesterase-5 GAF-B domain provides for high vardenafil potency over sildenafil and tadalafil and is involved in PDE5 dimerization

Mitsi A. Blount, Roya Zoraghi, Hengming Ke, Emmanuel P. Bessay, Jackie D. Corbin, and Sharron H. Francis

From the Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN, USA 37232-0615 (MAB, RZ, EPB, JDC, and SHF) and Department of Biochemistry and Biophysics and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC USA 27599-7260 (HK)

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: PDE5 inhibitor affinity is influenced by regulatory domain GAF-B

Corresponding Author: Sharron H. Francis, 702 Light Hall, Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615. Tel: 615 322 4383; Fax: 615 343 3794; E-mail: sharron.francis@vanderbilt.edu

Text pages: 30

Tables: 2

Figures: 6

References: 40

Words in Abstract: 242

Words in Introduction: 745

Words in Discussion: 1,142

The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; PKG, cGMP-dependent protein kinase; GAF, mammalian cGMP-binding phosphodiesterase, *Anabaena* adenylyl cyclases, *Escherichia coli* FhlA; UCR, upstream conserved region; IBMX, 3-isobutyl-1-methylxanthine; KPM, 10 mM potassium phosphate (pH 6.8) containing 15 mM β-mercaptoethanol; KP, 10 mM potassium phosphate (pH 6.8)

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Phosphodiesterase-5 (PDE5) contains a catalytic domain (C domain) that hydrolyzes cGMP and a regulatory domain (R domain) that contains two GAFs (A and B) and a phosphorylation site for cyclic nucleotide-dependent protein kinases (cNPK). Binding of cGMP to GAF-A increases cNPK phosphorylation of PDE5 and improves catalytic-site affinity for cGMP or inhibitors. GAF-B contributes to dimerization of PDE5, inhibition of cGMP binding to GAF-A, and sequestration of the phosphorylation site. To probe potential PDE5 R domain effects on catalytic site affinity for certain inhibitors, four N-terminal truncation mutants were generated: PDE5Δ1-321 contained the sequence between GAF-A and -B, GAF-B, and C domain; PDE5Δ1-419 contained GAF-B and C domain; PDE5Δ1-465 contained the C-terminal portion of GAF-B and C domain; PDE5Δ1-534 contained only C domain. Truncated proteins with a complete GAF-B were dimers, but those lacking the N-terminal 46 amino acids of GAF-B were monomers, indicating that these residues are vital for GAF-B mediated PDE5 dimerization. K_ms of the mutants for cGMP were similar to that of full-length PDE5. All PDE5 constructs had similar affinities for 3-isobutyl-1-methylxanthine, sildenafil, tadalafil, and UK-122764, but mutants containing a complete GAF-B had 7- to 18-fold higher affinity for vardenafil-based compounds compared to those lacking a complete GAF-B. This indicated that the N-terminal 46-amino acids in GAF-B are required for high vardenafil potency. This is the first evidence that PDE5 R domain, and GAF-B in particular, influences affinity and selectivity of the catalytic site for certain classes of inhibitors.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Intracellular levels of cGMP are determined by the relative rates of synthesis by guanylyl cyclases and breakdown by cyclic nucleotide phosphodiesterases (PDEs) (Francis et al., 2001; Mullershausen et al., 2005; Rybalkin et al., 2003). PDE5, a cGMP-specific PDE, plays a prominent role in cGMP breakdown in numerous tissues including smooth muscle, platelets, gastrointestinal epithelial cells, and cerebellar Purkinje cells (Hanson et al., 1998; Rybalkin et al., 2003; Sopory et al., 2004; Wyatt et al., 1998). PDE5 is the target of erectile dysfunction medications, i.e., sildenafil (Viagra®), tadalafil (Cialis®), vardenafil (Levitra®), and udenafil (Zydena®); sildenafil (Revatio®) has recently been approved for treatment of pulmonary arterial hypertension (Ballard et al., 1998; Corbin and Francis, 1999; Galie et al., 2005; Jeremy et al., 1997; Kang et al., 2003; Kendirci et al., 2004; Rotella, 2003; Sebkhi et al., 2003; Turko et al., 1999). PDE5 is a homodimer; each monomer contains a regulatory (R) domain and a catalytic (C) domain that is highly conserved in all class I PDEs (Corbin and Francis, 1999; Francis et al., 2001). The R domain contains several functional subdomains including: 1) a consensus site for phosphorylation by cyclic nucleotide-dependent protein kinases; exposure of this site for phosphorylation is regulated by ligand binding; 2) two GAFs (A and B); and 3) dimerization contacts (Corbin and Francis, 1999; McAllister-Lucas et al., 1995; Thomas et al., 1992; Zoraghi et al., 2005). GAFs are composed of ~120 amino acids and named for the proteins in which they were first found, i.e., cGMP-binding PDEs, *Anabaena* adenylyl cyclases, and *Escherichia coli* (FhlA) (Aravind and Ponting, 1997; Martinez et al., 2002; Zoraghi et al., 2004). Although GAFs comprise a large family of protein modules, little is known about their functions or physical features. In some instances, GAFs have been shown to bind small ligands such as cGMP or cAMP (Francis et al.,

2002; Gross-Langenhoff et al., 2006; Martinez et al., 2005; Yamazaki et al., 1982). GAF-A in PDE5 binds cGMP with high affinity and contributes to dimerization. Allosteric cGMP binding in GAF-A increases affinity of the PDE5 catalytic site for the cGMP substrate or inhibitors and regulates exposure of the phosphorylation site (Blount et al., 2004; Rybalkin et al., 2003; Turko et al., 1998; Zoraghi et al., 2005). Dimerization of PDE5 occurs through multiple contacts within the R domain and both isolated GAF-A and GAF-B are dimers.

Kinetic properties [i.e., k_{cat}, K_m for cGMP, IC_{50} for sildenafil or 3-isobutyl-1-methylxanthine (IBMX)] of PDE5 isolated C domain resemble those of full-length PDE5 (Fink et al., 1999). In x-ray crystal structures of PDE5 isolated C domain, sildenafil and vardenafil have similar modes of binding despite a 10- to 40-fold difference in affinities (*in vitro* potencies) of full-length PDE5 for these inhibitors (Blount et al., 2004; Huai et al., 2004; Sung et al., 2003). Despite the apparent similarity in contacts between these inhibitors and PDE5 catalytic site, site-directed mutagenesis of several of these residues in the PDE5 holoenzyme produces selective losses in affinities for these inhibitors (Corbin et al., 2005; Zoraghi et al., 2006). This suggests that the x-ray co-crystallographic images derived from the isolated C domain may not contain or reveal structural nuances that impact inhibitor potencies in the PDE5 holoenzyme.

PDE5-selective inhibitors bind to the catalytic site and do not measurably interact with the allosteric cGMP-binding sites (Blount et al., 2004; Turko et al., 1999). However, the possibility that interaction of different classes of inhibitors with the catalytic site may be

selectively affected by the R domain has not been studied. To address this possibility, we hypothesized that the affinity of the PDE5 catalytic site for certain classes of inhibitors is influenced by features within the R domain. In addition, we consider the possibility that in some instances dimerization of PDE5 contributes to affinity and selectivity of the catalytic site for inhibitors. To test these ideas, four N-terminal truncation mutants of PDE5 were generated and tested for affinities of a collection of PDE inhibitors with varying structures [(IBMX, two sildenafil-based inhibitors (sildenafil and UK-122764), tadalafil, and three vardenafil-based inhibitors (vardenafil, demethyl-vardenafil, and BAY 51-1871)] (Fig. 1). The mutants were also analyzed for their quaternary structures. We report here that GAF-B in PDE5 R domain plays a key role in determining higher affinity and selectivity of PDE5 for vardenafil-based compounds versus sildenafil-based compounds or tadalafil, that a 46-amino acid segment in the N-terminal portion of GAF-B accounts for this effect, and that the same 46 amino acids are required for GAF-B mediated dimerization of PDE5.

Materials and Methods:

Materials

[^{3}H]cGMP and DEAE-Sephacel were purchased from Amersham Biosciences Inc. (Piscataway, NJ). IBMX, histone type II-AS, *Crotalus atrox* snake venom, 5'-GMP, and cGMP were obtained from Sigma Chemical Co. (St. Louis, MO). Full-length recombinant bovine PDE5 was isolated from infected Sf9 cells using Ni/NTA agarose (QIAGEN) as described previously (Blount et al., 2004). Sildenafil (Fig. 1) was purified from tablets of Viagra® following the method previously established in this laboratory (Corbin et al., 2003). Purified sildenafil was submitted to Amersham Biosciences Inc. for radiolabeling with tritium. Tadalafil (Fig. 1) was synthesized according to US Patent Number 6,140,329 (Inventor Alain Claude-Marie Daugan: ICOS Corporation, Bothell, Washington). After confirming the compound structure by mass spectrometry, tadalafil was submitted to Amersham Biosciences Inc. for radiolabeling with tritium. HPLC results from Amersham indicated that [^{3}H]sildenafil was > 98% pure while the [^{3}H]tadalafil preparation was > 99% pure. Vardenafil, [^{3}H]vardenafil, demethyl-vardenafil, and BAY 51-1871 (Fig. 1) were kindly provided by Bayer HealthCare A.G., Wuppertal, Germany. UK-122764 (Fig. 1) was generously provided by Pfizer Central Research, Sandwich, UK. All three ^{3}H inhibitors that had been stored for over a year were subjected to Sephadex G-25 chromatography, which adsorbs these PDE inhibitors and provides purification with high resolution (Corbin et al., 2003). All three ^{3}H inhibitors were resolved in single peaks and co-eluted with purified unlabeled inhibitors, suggesting that the ^{3}H inhibitors were unaltered after storage.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Constructs of PDE5

Full-length human PDE5A1 cDNA (Tanabe-Seiyaku Pharmaceutical Co. Ltd., Saitama, Japan) was the template used to generate His-tagged PDE5A1 truncation constructs (Fig. 2). Constructs were created by introduction of start and stop codons at the appropriate loci. Primers introduced *EcoRI* and *NotI* sites to the start and stop codons, respectively. The resulting PCR fragment was inserted into a pAcHLT-A vector (BD Pharmingen) that was linearized by digestion with *EcoRI* and *NotI*. The boundaries for GAF-A and GAF-B were selected based on homology with other GAF-containing PDEs (PDE2 and PDE6) because the precise boundaries of these modules are not known (Zoraghi et al., 2005). Mutants included: PDE5Δ1-321 containing the intervening sequence between GAF-A and -B, GAF-B, and the C domain; PDE5Δ1-419 containing a complete GAF-B and C domain but lacked the intervening sequence between GAF-A and -B; PDE5Δ1-465 containing the C-terminal portion of the GAF-B sequence and the C domain; isolated C domain (PDE5Δ1-534); full-length R domain (PDE5Δ540-875); and an isolated GAF-B construct containing flanking sequences on both sides of the module (T322-G539) (Fig. 2). The full-length isolated R-domain (PDE5Δ540-875) and isolated GAF-B mutants were prepared and characterized as previously described (Zoraghi et al., 2005).

All constructs were confirmed by DNA sequencing before cotransfection into Sf9 cells with BaculoGold linear baculovirus DNA (BD Pharmingen) by the calcium phosphate method according to the protocol from BD Pharmingen. At five days post-infection, the co-transfection supernatant was collected, amplified three times in Sf9 cells, and then

used directly as viral stock for expression without additional purification of recombinant virus. Sf9 cells grown at 27°C in complete Grace's Insect medium with 10% fetal bovine serum and 10 µg/ml gentamicin (Sigma) in T-175 flasks (Corning) were infected with the optimum volume of viral stock determined experimentally. Sf9 cells were harvested at 72-96 h post-infection by centrifugation at 2000 rpm in a Beckman JA-10 rotor (Beckman Coulter, Inc., Fullerton, CA) for 10 min at 4°C. The pellet was dispersed with a pipette using 10 ml of lysis buffer [20 mM Tris (pH 8), 100 mM NaCl, and 15 mM β-mercaptoethanol] and homogenized twice for 4 sec 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 applied to a 0.9 x 3-cm Ni-NTA agarose column (QIAGEN) equilibrated with lysis buffer. The column was washed with 20 ml lysis buffer before eluting with 30 ml of this buffer containing 0.1 M imidazole. Fractions (2 ml) were collected; fractions were analyzed for cGMP PDE catalytic activity, protein content, and purity (using SDS-PAGE). Fractions exhibiting peak enzyme activity and protein purity were pooled and glycerol was added to a final concentration of 20%. Aliquots were quick-frozen in liquid nitrogen and stored at -70°C.

Isolated C Domain

One of the isolated C domain constructs (human E535-Q860) (Fig. 2) used in these studies was kindly provided by Dr. Hengming Ke, Univ. of North Carolina at Chapel Hill. The plasmid was transformed into *E. coli* strain BL21 (Codonplus) for overexpression. *E. coli* containing the vector was grown in enriched LB medium (10 g bacto-tryptone, 5 g bact-yeast extract, 10 g NaCl per liter with 0.4% glucose, and 100 mg AMP added after

autoclaving) at 37°C to A_{600} = 0.7, and then 0.1 mM isopropyl β-$_D$-thiogalactopyranoside was added for further growth at 15°C overnight. Cells were centrifuged at 4000 rpm in a Beckman JA-10 rotor for 10 min at 4°C. The resulting pellet from 3 L of Sf9 cells was dispersed with 4 ml extraction buffer [20 mM Tris (pH 8), 0.3 M NaCl, 15 mM imidazole, 1 mM β-mercaptoethanol, and one EDTA-free Complete® protease inhibitor cocktail tablet per 50 ml solution (Roche Applied Sciences, Indianapolis, IN)] by pipetting up and down. Cells were homogenized by sonication using a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT) by pulsing for 1 min, 4 times. After centrifugation at 9000 rpm in a Beckman JA-20 rotor for 20 min, the supernatant was applied to a Ni-NTA agarose column (0.9 x 3-cm) (QIAGEN) equilibrated with extraction buffer. The column was then washed with 150 ml buffer 1 [20 mM Tris (pH 8), 0.3 M NaCl, 15 mM imidazole, and 1 mM β-mercaptoethanol] followed by a 50 ml wash with buffer 2 [20 mM Tris (pH 8), 50 mM NaCl, 15 mM imidazole, and 1 mM β-mercaptoethanol]. The isolated C domain was eluted in 1 ml fractions from the Ni-NTA column with buffer containing 20 mM Tris (pH 8), 0.3 M NaCl, 150 mM imidazole, and 1 mM β-mercaptoethanol. Fractions were subjected to SDS-PAGE, protein, and PDE activity analyses. Fractions that contained the isolated C domain were pooled and quick-frozen in 20% glycerol.

A His-tagged isolated C domain of slightly different length (human E535 to N875) was also prepared by Sf9 expression using human PDE5 cDNA (Tanabe-Seiyaku Pharmaceutical Co. Ltd., Saitama, Japan) as a template. This construct was amplified by PCR using primers that introduce *EcoRI* and *NotI* restriction sites to the 5' and 3' ends,

respectively. The DNA was then transformed in DH5αBac cells and purified as outlined above. After being confirmed by sequencing, Sf9 cells (BD Pharmingen) were cotransfected with BaculoGold linear baculovirus DNA (BD Pharmingen) and isolated C domain DNA according to the protocol from BD Pharmingen. Virus was amplified and titered as outlined above. After purifying the cells and subjecting the lysate to Ni-NTA affinity chromatography as discussed above, the protein yield was low. To ensure that results obtained within this study with isolated C domain expressed in *E. coli* were not artifacts of the expression system, isolated C domain in crude lysate of Sf9 cells infected with this construct was used to verify the results.

PDE Activity Assays

PDE activity assays were initiated by addition of the desired PDE5 construct to a reaction mixture (100 µl) containing 40 mM MOPS (pH 7), 0.8 mM EGTA, 15 mM magnesium acetate, 2 mg/ml bovine serum albumin (Sigma), [^{3}H]cGMP (Amersham) (80,000-150,000 cpm per assay tube) and various concentrations of unlabeled cGMP. Reaction mixtures were incubated at 30°C for 15 min or an appropriate time. A mixture (20 µl) with the following ingredients was added to terminate the reaction: 50 mM EDTA, 30 mM theophylline (Sigma), 10 mM cGMP, and 10 mM cAMP in 100 mM Tris (pH 7.5). Snake venom 5'-nucleotidase (Sigma) (200 µg) was added to the assay mixture and incubated for 10 min at 30°C. Assay samples were diluted in 1 ml of dilution solution (0.15 mM EDTA containing 100 µM each of adenosine and guanosine) and applied to QAE-Sephadex columns (8 × 33 mm) pre-equilibrated in 20 mM ammonium formate buffer (pH 7.4). Eluates were collected, aqueous scintillant was added (10 ml), and the

amount of [^{3}H]guanosine was measured in a scintillation counter. To determine IC_{50} for various inhibitors, the [^{3}H]cGMP substrate concentration was 0.4 μM; increasing concentrations of the respective inhibitors were diluted in the PDE assay mixture and then added to the assay. The K_m for each construct was determined using a range of cGMP concentrations. Unless indicated otherwise, PDE activity is expressed as pmol/min/ml enzyme in the reaction tube.

[^{3}H]cGMP Binding Assays

To measure allosteric cGMP binding, Millipore filter binding assays were conducted in a total volume of 50 μl reaction mixture that contained 10 mM potassium phosphate buffer (pH 6.8), 1 mM EDTA, 0.5 mg/ml histone type II-AS, 30 mM DL-dithiothreitol (DTT), 0.2 mM sildenafil and either 3 μM (for binding stoichoimetry determination) or 0.05 to 4 μM (for binding affinity determination) of [^{3}H]cGMP. The binding reaction was initiated by addition of enzyme and incubated on ice for 60 min. 1 ml cold KP buffer (10 mM potassium phosphate, pH 6.8) was added to each sample, and samples were filtered immediately onto pre-moistened Millipore HAWP filters (pore size 0.45 μm). Filters were then washed with 2 ml of cold KP buffer two times, dried, and counted using non-aqueous Ready Safe scintillation cocktail (Beckman). Counts bound to PDE5 were corrected by subtraction of nonspecific binding (+1 mM unlabeled cGMP). Blanks containing no PDE5 were run for each [^{3}H]cGMP concentration. [^{3}H]cGMP binding in PDE5 holoenzyme and isolated R domain indicated a stoichiometery and affinity for cGMP binding similar to those of full-length PDE5 (Zoraghi et al., 2005). There was no

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

significant cGMP binding detected in PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, isolated C domain, or isolated GAF-B module.

^{3}H Inhibitor Membrane Filtration Binding Assays

Full-length PDE5 or isolated C domain (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). ^{3}H inhibitor was added after addition of reaction mixture since it adhered to the test tube when added in the absence of or prior to addition of histone. Histone also increased retention of PDE5 on the Millipore filters. Binding reaction mixture containing the enzyme was incubated on ice for 45 min. Millipore nitrocellulose membranes (0.45 µm) were placed under house vacuum and prewetted with 1 ml cold 10 mM potassium phosphate (pH 6.8) (KP) that contained 0.1% Triton X-100. Next, 200 µl of room temperature 25% Triton X-100 in KPM was added to the reaction tube. The entire contents of the tube were quickly applied to the pre-wetted filter. The reaction tube was then rinsed with 3 ml ice-cold 0.1% Triton X-100 in KP, and the rinse was also applied to the filter. Filter membranes were removed, dried, and transferred to 6-ml scintillation vials. Non-aqueous scintillant (5 ml) was added to the vials which were then placed in a scintillation counter.

Determination of Stokes Radius

The Stokes radii of the PDE5 proteins were determined using a standardized Sephacryl S-200 gel filtration column (0.9 × 35 cm) equilibrated in a solution containing 20 mM Tris (pH 8) and 100 mM NaCl. Each sample applied to the column contained ~50 µg of one of the PDE5 constructs as well as 5 mg/ml cytochrome *c* (16.6 Å), and 2 mg/ml apoferritin (58.1 Å) (final volume 200 µl) as internal standards. The column was eluted with the same buffer, and fractions (0.8 ml) were collected. Elution positions for the constructs were determined by assaying PDE catalytic activity. Cytochrome *c* and apoferritin were located by absorbance at 400 nm. The column was standardized with protein standards of known Stokes radii: cytochrome *c* (16.6 Å), PKA C subunit (27 Å), ovalbumin (29 Å), bovine serum albumin (35 Å), PKGI-β (50 Å), and catalase (52 Å). Thyroglobulin was used to determine the void volume while [^{3}H]H_2O was used to determine the inclusion volume. Elution positions of the protein standards were used to generate a standard curve of $(-\log K_{av})^{1/2}$ versus Stokes radius as in equation 1.

$$K_{av} = \frac{\text{elution volume} - \text{void volume}}{\text{inclusion volume} - \text{void volume}} \qquad \text{(Eq. 1)}$$

The Stokes radius of each protein was determined from the standard curve.

Determination of Sedimentation Coefficient

Each protein (~20 µg) was combined with two internal standards, aldolase (2 mg/ml), and cytochrome *c* (5 mg/ml) in a volume of 300 µl in 20 mM Tris (pH 8) and 100 mM NaCl and applied to a 13-ml linear 5-20% sucrose gradient containing 20 mM Tris (pH 8) and 100 mM NaCl. The gradients were centrifuged at 36,000 rpm in a Beckman SW 41 rotor for 36 h at 4°C and fractions (0.5 ml) were collected from the bottom of the tubes.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Cytochrome *c* has a sedimentation coefficient of 1.86 S and was located by absorbance at 400 nm. To detect aldolase (7.35 S), an aliquot (20 µl) of each fraction was submitted to SDS-PAGE (10%) analysis, stained with Coomassie Blue, and the intensity of the band was quantified using Metamorph software (Universal Imaging, Media, PA). The proteins of known $S_{20,w}$: cytochrome *c* (1.86 S), PKA C subunit (3.14 S), ovalbumin (3.5 S), bovine serum albumin (4.6 S), PKG Iβ (7.0 S), and catalase (11.3 S) were centrifuged using the same conditions and used as additional standards. To locate the peak of protein for each construct, PDE activity assays were performed as described above. The sedimentation coefficient of each protein was then determined by the distance migrated into the gradients compared with the position of the standards. The molecular mass of each protein was calculated using the experimentally determined Stokes radius and sedimentation coefficient using the Siegel and Monty equation (Siegel and Monty, 1966) below (Eq. 2):

$$M_r = \frac{6 \pi n \mathrm{N} a s}{(1 - v\rho)} \qquad \text{(Eq. 2)}$$

where N = Avogadro's number; n = the viscosity of medium, assumed to be 1; a = Stokes radius; s = sedimentation coefficient; ρ = density of the medium, assumed to be 1; and v = partial specific volume, assumed to be 0.725 ml/g. The predicted M_r was determined using Compute pI / Mw based on the amino acid composition. This was compared to the experimentally determined M_r of the proteins to establish if each recombinant protein was monomeric or dimeric.

Calculation of Free Energy of Binding

The Gibbs free energy change, ΔG, that occurs by association of a ligand with PDE5 is related to the equilibrium association constant for the interaction and was calculated using equation 3 (Gerstner et al., 1994):

$$\Delta G = -RT \ln K \qquad \text{(Eq. 3)}$$

Where *R* is the ideal gas constant (equal to 1.98×10^{-3} kcal/degree/mol), *T* is the temperature at which the assay was done (303°K), and $K = K_i$. K_i values were calculated from the experimentally determined IC_{50} values for the inhibitors using the equation (Eq. 4) (Cheng and Prusoff, 1973):

$$K_i = IC_{50} / 1 + [S]/K_m \qquad \text{(Eq. 4)}$$

High-affinity interaction is indicated when the ΔG is a large negative value. The contribution of a substituted amino acid side chain to the Gibbs free energy of binding in an enzyme-transition state complex was calculated from using equation 5 (Fersht, 1988):

$$\Delta\Delta G = \Delta G_{WT} - \Delta G_{mut} \qquad \text{(Eq. 5)}$$

ΔΔ*G* is the change in free energy of binding in enzyme-transition state complexes attributable to the substituted group.

Statistical Analyses

All values are given as mean ± standard error of mean (SEM) as determined by GraphPad Prism graphics software. The software uses the following equation: SEM = standard deviation/$n^{½}$, where standard deviation is determined as $[\sum (y_i - y_{mean})^2/(n-1)]^{½}$. All SEMs reported fit within a 95% confidence interval, which quantifies the precision of the mean.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Physical and Kinetic Characteristics of the PDE5 Constructs.

Full-length PDE5 and four truncation mutants were subjected to SDS-PAGE analysis. Migration of each of the proteins agreed with the predicted molecular weights based on amino acid composition and purity of each was > 90% (data not shown). To verify that the affinity of the catalytic site of the full-length PDE5 and all N-terminal truncation mutants of PDE5 for cGMP was not impaired, K_m for cGMP was determined as described in *Materials and Methods* in head-to-head experiments. K_m values for cGMP were similar among PDE5Δ1-321 (2.5 ± 1.8 μM), PDE5Δ1-419 (2.2 ± 2.2 μM), PDE5Δ1-465 (2.3 ± 1.4 μM), isolated C domain (Δ1-534) (2.5 ± 0.7 μM) and full-length PDE5 (2.5 ± 0.8 μM). These values were derived from two different preparations of all constructs and each was performed in triplicate (*n=4*). The K_m for full-length PDE5 was consistent with values previously determined in this laboratory (Thomas et al., 1990).

Effect of the R Domain in PDE5 Holoenzyme on Affinity of the Catalytic Site for Inhibitors.

IC_{50} values for seven PDE inhibitors representing four distinct chemical structures were determined in parallel for full-length PDE5 and the four N-terminal truncation mutants as outlined in *Materials and Methods* using 0.4 μM [^{3}H]cGMP as substrate (Table 1). The core ring structure of IBMX is a xanthine which closely resembles the guanine of cGMP (Fig. 1). The sildenafil-based compounds (sildenafil and UK-122764) contain a pyrazolopyrimidinone ring system, whereas the vardenafil-based compounds (vardenafil,

demethyl-vardenafil, and BAY 51-1871) contain an imidazotrazinone ring system; the heterocyclic ring systems in the sildenafil-based and vardenafil based compounds differ substantially from each other and from those of cGMP and IBMX. The ring system of tadalafil is different from those of all of the other compounds (Fig. 1). The structural differences in these core ring systems confer unique chemical characteristics on the compounds. The IC_{50} values of IBMX, a weak, non-specific PDE inhibitor, for full-length PDE5, PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, and isolated C domain (Δ1-534) were very similar (Table 1). The value for full-length PDE5 agreed with that in our previous report (Turko et al., 1999). The IC_{50} values of tadalafil for full-length PDE5, PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, and isolated C domain (Δ1-534) were also similar (Table 1). IC_{50} values for two sildenafil-based compounds (sildenafil and UK-122764) were also the same for all constructs, including the isolated C domain (Table 1). These results demonstrated that the PDE5 R domain does not significantly impact the potency of inhibition by IBMX, tadalafil, or sildenafil-based compounds.

In contrast, the IC_{50}s of the three vardenafil-based compounds for the proteins of different lengths differed markedly. The IC_{50} of vardenafil for full-length PDE5 (0.3 ± 0.1 nM) was similar to the previously reported value (Blount et al., 2004). PDE5Δ1-321 and PDE5Δ1-419 had IC_{50} values for vardenafil that were very similar to that for full-length PDE5 (0.2 ± 0.1 nM and 0.4 ± 0.1 nM, respectively) (Table 1). However, the shorter constructs that lacked a complete GAF-B, i.e., PDE5Δ1-465 and the isolated C domain (Δ1-534), yielded ~10-fold higher IC_{50} values (2.7 ± 0.5 nM and 2.9 ± 0.3 nM,

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

respectively) (Table 1). The ~10-fold difference between the IC_{50} of vardenafil for full-length PDE5 compared with that for the isolated C domain is also illustrated in Fig. 3.

IC_{50} values for two other vardenafil-based compounds were also determined in head-to-head experiments. The first compound, demethyl-vardenafil, contained the [5,1-f][1,2]triazine ring of vardenafil and the appended methyl group of sildenafil (Fig. 1). Previous studies established that demethyl-vardenafil inhibits full-length PDE5 with the same potency as does vardenafil (Corbin et al., 2004); IC_{50} values (0.3 ± 0.1 nM) determined here agreed with those previous findings. Demethyl-vardenafil had a similar IC_{50} (0.6 ± 0.2 nM) for PDE5Δ1-321 and PDE5Δ1-419 (0.4 ± 0.1 nM). However, PDE5Δ1-465 and the isolated C domain (Δ1-534), both of which lacked an intact GAF-B, had ~10-fold higher IC_{50} values (5.6 ± 1.2 and 3.2 ± 0.5 nM, respectively).

BAY 51-1871 also contains the [5,1-f][1,2]triazine ring of vardenafil but a carbonyl group replaces the methyl group (Fig. 1). The IC_{50} values determined for full-length PDE5, PDE5Δ1-321, and PDE5Δ1-419 for BAY 51-1871 were all similar (0.4 ± 0.3, 0.5 ± 0.1, and 0.4 ± 0.2 nM, respectively). In contrast, the IC_{50} values for PDE5Δ1-465 and the isolated C domain (Δ1-534) were 3.1 ± 0.8 and 2.9 ± 0.4 nM, respectively.

Full-Length PDE5 Has Higher Binding Affinity for [^{3}H]Vardenafil Than Does PDE5 Isolated C Domain.

The affinity of vardenafil for full-length PDE5 and isolated C domain was examined via direct binding studies using [^{3}H]vardenafil and the membrane-filtration binding assay

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

described in *Materials and Methods.* Maximum [^{3}H]vardenafil binding to both proteins was ~0.5 mol/ mol PDE5 monomer. The specificity of [^{3}H]vardenafil binding was validated by testing the effects of various unlabeled PDE inhibitors. This included a 10,000-fold excess of heterocyclic inhibitors that are selective for PDE5 (vardenafil, sildenafil, tadalafil) as well as other heterocyclic inhibitors that are selective for other PDEs [rolipram (PDE4), milrinone (PDE3), EHNA (PDE2), or vinpocetine (PDE1)]. High concentrations of rolipram, milrinone, EHNA, or vinpocetine did not affect [^{3}H]vardenafil binding (3 nM), whereas unlabeled vardenafil, sildenafil, or tadalafil blocked over 90% of the binding (data not shown). A 100,000-fold excess of either cAMP, 5'-GMP, or IBMX had no effect on [^{3}H]vardenafil binding to either isolated C domain or full-length PDE5 (data not shown).

The K_D of [^{3}H]vardenafil was determined for isolated C domain and full-length PDE5 by performing a binding isotherm with increasing concentrations of [^{3}H]vardenafil in the absence of cGMP in a head-to-head manner. The K_D obtained for full-length PDE5 was 1.1 ± 0.2 nM (*n=3*), a value similar to that previously determined in this laboratory (Blount et al., 2004). The K_D of vardenafil for isolated C domain was 7.3 ± 3.4 nM (*n=3*). This ~7-fold increase in K_D of isolated C domain for vardenafil was similar to the ~10-fold increase in the IC_{50} of vardenafil described above.

Vardenafil Affinity of PDE5Δ1-465 or Isolated PDE5 C Domain Is Not Affected by Addition of Either Excess Isolated R Domain or Isolated GAF-B.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

The possibility that addition of isolated R domain or GAF-B module to the isolated C domain could restore high affinity of the catalytic site for vardenafil was tested. Approximately 160-fold (6.4 nM) excess purified recombinant isolated R domain (PDE5Δ540-875) or ~245-fold (10 nM) excess purified isolated GAF-B (PDE5ΔT322-G539) was preincubated with 0.04 nM isolated C domain for 45 min on ice. Aliquots of these mixtures were then added to tubes containing the PDE catalytic assay mixture with either no vardenafil or varying concentrations of vardenafil. Identical assays using full-length PDE5 were performed alongside in a head-to-head manner (Fig. 4). IC_{50} values of vardenafil for full-length PDE5 and isolated C domain were 1.5 ± 0.1 nM and 9.6 ± 1.7 nM, respectively. Addition of either isolated GAF-B module (IC_{50} = 9.1 ± 1.0 nM) or isolated R domain (IC_{50} = 9.2 ± 1.1 nM) did not alter vardenafil potency. Addition of excess R domain or isolated GAF-B module also did not affect catalytic activity of either protein (data not shown). Integrity of the isolated R domain was verified by demonstrating that the protein is dimeric and binds cGMP with high affinity, high specificity, and > 0.5 mol/mol binding stoichiometry.

Physical Properties of N-Terminal Truncation Mutants of PDE5.

Molecular mass of each of the four N-terminal truncation mutants was calculated as described in *Materials and Methods* using the experimentally determined Stokes radius and sedimentation coefficient for each. When PDE5Δ1-321 was chromatographed on a standardized gel filtration column, the protein eluted at a position similar to that of the internal standard apoferritin (Fig. 5A), and had a calculated Stokes radius of 46.9 ± 0.46 Å (Table 2). Upon sucrose density gradient centrifugation, PDE5Δ1-321 sedimented

near the internal standard aldolase (Fig. 6A) and had a calculated sedimentation coefficient of 5.83 ± 0.15 S (Table 2). Using the Siegel and Monty equation, the calculated molecular mass of PDE5Δ1-321 was determined to be 113 kDa (Table 2); this value was approximately twice that of the predicted molecular mass of a monomer of this construct (63.7 kDa) based on amino acid composition, indicating that this mutant was a dimer.

PDE5Δ1-419 eluted from the standardized gel filtration column at a position near that for apoferritin (Fig. 5B). The Stokes radius for this construct was calculated to be 44.7 ± 1.45 Å (Table 2). Upon sucrose density centrifugation, this protein was located near aldolase (Fig. 6B) and had a sedimentation coefficient of 5.13 ± 0.09 S (Table 2). The final molecular mass was determined to be 94.8 kDa (Table 2). This mass was almost double the predicted molecular weight (52.4 kDa), indicating that this protein was also a dimer.

Elution position of PDE5Δ1-465 from the standardized gel filtration column was near that of the internal standard cytochrome *c* (Fig. 5C), and the Stokes radius was determined to be 34.8 ± 2.03 Å (Table 2). This protein sedimented near the position of internal standard cytochrome *c* and had a sedimentation coefficient of 3.03 ± 0.175 S (Fig. 6C). Together, the data determined that the molecular mass of the protein was 43.6 kDa (Table 2). The predicted molecular mass (47.2 kDa) was similar to the calculated value, indicating that PDE5Δ1-465 was a monomer.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

By gel filtration chromatography, isolated C domain eluted near cytochrome *c* (Fig. 5D) and the Stokes radius was calculated to be 24.5 ± 0.51 Å (Table 2). By sucrose density gradient centrifugation, this protein sedimented near standard cytochrome *c* (Fig. 6D) and had a sedimentation coefficient of 2.6 ± 0.15 S (Table 2). The M_r for isolated C domain was determined to be 26.8 kDa (Table 2), which was similar to the predicted molecular mass (37.7 kDa), indicating that this construct was monomeric.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The N-terminal truncation mutants of PDE5 used in this study contained all of the essential structural requirements for hydrolytic function and had affinities for cGMP like that of full-length PDE5. These results indicated that interactions of sildenafil-based inhibitors, tadalafil, or IBMX with PDE5 are located exclusively within the C domain. Surprisingly, a portion of the high-affinity inhibition of PDE5 by the three vardenafil-based compounds requires the R domain. Direct determination of the K_D for [^{3}H]vardenafil binding supported the results ascertained using IC_{50} values. Other high-affinity PDE5 inhibitors with structures dissimilar to vardenafil are currently unavailable for testing; therefore it cannot be concluded that the effect of GAF-B applies to high-affinity inhibitors per se or if this is a unique property associated with the novel structure of the heterocyclic rings of vardenafil based-structures. Taken together, the data show that a significant portion of the high-affinity interaction of vardenafil with the PDE5 catalytic site requires an intact GAF-B within the R domain.

These findings are the first to show that the affinity and selectivity of the enzyme for different classes of inhibitors is significantly influenced by R domain features. The differential impact of the R domain on the affinity of the PDE5 catalytic site for different classes of inhibitors may not be unique to PDE5. R domains of many PDEs vary markedly in length among variants within a given PDE family, therefore potencies of a particular class of inhibitor may vary significantly among these proteins, and isolated C domains should be used cautiously in screening for inhibitor potency. This is supported

by studies of the PDE4 family in which N-terminal truncations of PDE4 holoenzyme revealed important influences of the UCR2 domain in high-affinity rolipram binding to the PDE4 catalytic site (Richter and Conti, 2004). The results emphasize the need for careful analysis of structural differences in PDEs that could potentially impact potencies of different classes of PDE inhibitors.

These results along with those from a number of other recent reports also emphasize the limitations in conclusions drawn from co-crystal structures of the isolated C domain of PDEs and their inhibitors (Corbin et al., 2005; Zoraghi et al., 2006). The co-crystal structures of PDE5 isolated C domain with either sildenafil or vardenafil suggested that these two bind in the same manner, i.e., make the same contacts with the same amino acids in the catalytic site binding pocket. However, the dramatic difference in the affinity of vardenafil binding to the isolated C domain versus the full-length PDE5 (shown herein) demonstrates that, at least for vardenafil, some contacts that contribute to inhibitor potency in the two forms of the enzyme differ substantially. These conclusions are supported by studies using site-directed mutagenesis of either Gln817 or Tyr612, in the catalytic site of PDE5 (Corbin et al., 2005; Zoraghi et al., 2006). According to the x-ray crystal structures, the contacts between Gln817 or Tyr612 with vardenafil and sildenafil appear to be identical. However, substitution of either of these residues by an alanine causes a significantly greater loss in affinity for inhibition by vardenafil than for sildenafil; the loss in affinity for vardenafil and sildenafil for the Q817A mutant is ~600-fold and ~50-fold, respectively. These data support the interpretation that the mode of binding for these inhibitors differs significantly.

The present studies reveal that all or some of the amino acid sequence extending from Glu420 through Gly466 is critical for potent inhibition by vardenafil-based compounds. This finding adds to the growing evidence that different regions of the PDE5 R domain influence catalytic function . Several regulatory functions including phosphorylation and ligand binding that are contained in R domains have been shown to impact the catalytic sites of many PDEs. However, there is no known mechanism by which this 46-amino acid segment in PDE5 impacts the catalytic site. This stretch of amino acids could provide 1) direct contacts with vardenafil-based compounds that contribute to more optimal positioning of these inhibitors in the catalytic binding pocket, 2) structural features for the formation of a stable GAF-B structure that could in turn directly affect catalytic site function or make contact with the inhibitor, 3) dimerization contacts that impact conformation and affinity of the catalytic site for vardenafil-based compounds, or 4) a combination of these. The reported evidence herein provides new insights into the 10-fold higher potency of vardenafil over sildenafil and generates new considerations pertaining to the design and evaluation of potency of future inhibitors.

The present results expand appreciation for the role of the R domain and GAF-B in PDE5 function. The region containing GAF-B and its flanking amino acids modulates the affinity of cGMP binding by GAF-A and also regulates the cGMP-dependent exposure of Ser102 (human PDE5) for phosphorylation by cNPK (Zoraghi et al., 2004). Some evidence suggests that cGMP binds to the GAF-B domain with low affinity, but this is yet to be proven (McAllister-Lucas et al., 1995; Turko et al., 1998); however, it should be

noted that low-affinity cGMP binding might not be detected by methods that have been used. The present finding that high potency of PDE5 inhibition by vardenafil-based compounds requires a portion of the sequence in the GAF-B domain is the first proven effect of GAF-B on the catalytic domain of PDE5. Taken together, the results support and emphasize the complex and central role of GAF-B in PDE5 function.

Previous results from this laboratory and those contained in this manuscript strongly suggest that GAF-B participates in dimerization of PDE5. The studies herein reveal that the same 46-amino acid segment in the N-terminal portion of GAF-B that is required for high vardenafil potency is also required for the GAF-B-mediated PDE5 dimerization. Although this correlation could be coincidental, the possibility that enhanced vardenafil affinity requires PDE5 dimerization should be considered. The critical segment of 46 amino acids may be directly involved in contacts providing for dimerization or they may be required for the structural integrity of PDE5 GAF-B that can in turn form a dimer interface involving this or other areas in GAF-B. Recent experiments with PDE4 demonstrated that disruption of an α-helix in the R domain of that enzyme disrupts dimerization (Richter and Conti, 2004). A computational program (TMHMM Server v. 2.0) did not predict an α-helix in this 46-amino acid segment of PDE5 and this segment of sequence lacks other distinguishing features such as a high degree of hydrophobicity or charge.

The mechanism by which GAF-B influences potent selectivity for certain classes of PDE5 inhibitors is also unknown. The increase in the Gibbs free energy of binding

provided by GAF-B (~1.8 kcal/mol) is less than that provided by an additional H-bond (2.5 – 4 kcal/mol), but nevertheless it is a substantial increase. Since the holoenzyme is the pharmacological target, rational drug design of new PDE inhibitors must fully appreciate that subtle differences between PDE holoenzymes and their isolated C domains can profoundly affect inhibitor potency and selectivity.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements:

We especially thank Drs. Kenji Omori and Jun Kotera of Tanabe-Seiyaku Pharmaceutical Co. Ltd. (Saitama, JAPAN) for kindly providing human PDE5A1 cDNA and Dr. Hengming Ke for providing the PDE5 isolated C domain construct in an *E. coli* vector. We thank Bayer Healthcare AG, Wuppertal Germany for providing vardenafil, [^{3}H]vardenafil, demethyl-vardenafil, and BAY 51-1871 for these studies and Pfizer Central Research in Sandwich, UK for providing UK-122764. We also thank Dr. Raja Konjeti for synthesizing tadalafil. Many reagents were obtained via the Vanderbilt University Diabetes Center Core facilities with the aid of Scott Wright, Kris Ellis and Kennetra Price. DNA sequencing was performed by the Vanderbilt Ingram Canter Center Sequencing Core facility.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

References

Aravind L and Ponting CP (1997) The GAF domain: an evolutionary link between diverse phototransducing proteins. *Trends Biochem Sci* **22**(12):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**(6):2164-2171.

Blount MA, Beasley A, Zoraghi R, Sekhar KR, Bessay EP, Francis SH and Corbin JD (2004) Binding of tritiated sildenafil, tadalafil, or vardenafil to the phosphodiesterase-5 catalytic site displays potency, specificity, heterogeneity, and cGMP stimulation. *Mol Pharmacol* **66**(1):144-152.

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**(23):3099-3108.

Corbin J, Francis S and Zoraghi R (2005) Tyrosine-612 in PDE5 contributes to higher affinity for vardenafil over sildenafil. *Int J Impot Res*.

Corbin JD, Beasley A, Blount MA and Francis SH (2004) Vardenafil: structural basis for higher potency over sildenafil in inhibiting cGMP-specific phosphodiesterase-5 (PDE5). *Neurochem Int* **45**(6):859-863.

Corbin JD, Blount MA, Weeks JL, 2nd, Beasley A, Kuhn KP, Ho YS, Saidi LF, Hurley JH, Kotera J and Francis SH (2003) [3H]sildenafil binding to phosphodiesterase-5 is specific, kinetically heterogeneous, and stimulated by cGMP. *Mol Pharmacol* **63**(6):1364-1372.

Corbin JD and Francis SH (1999) Cyclic GMP phosphodiesterase-5: target of sildenafil. *J Biol Chem* **274**(20):13729-13732.

Fersht AR (1988) Relationships between apparent binding energies measured in site-directed mutagenesis experiments and energetics of binding and catalysis. *Biochemistry* **27**(5):1577-1580.

Fink TL, Francis SH, Beasley A, Grimes KA and Corbin JD (1999) Expression of an active, monomeric catalytic domain of the cGMP-binding cGMP-specific phosphodiesterase (PDE5). *J Biol Chem* **274**(49):34613-34620.

Francis SH, Bessay EP, Kotera J, Grimes KA, Liu L, Thompson WJ and Corbin JD (2002) Phosphorylation of isolated human phosphodiesterase-5 regulatory domain induces an apparent conformational change and increases cGMP binding affinity. *J Biol Chem* **277**(49):47581-47587.

Francis SH, Turko IV and Corbin JD (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog Nucleic Acid Res Mol Biol* **65**:1-52.

Galie N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M and Simonneau G (2005) Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* **353**(20):2148-2157.

Gerstner JA, Bell JA and Cramer SM (1994) Gibbs free energy of adsorption for biomolecules in ion-exchange systems. *Biophys Chem* **52**(2):97-106.

Gross-Langenhoff M, Hofbauer K, Weber J, Schultz A and Schultz JE (2006) cAMP is a ligand for the tandem GAF domain of human phosphodiesterase 10 and cGMP for the tandem GAF domain of phosphodiesterase 11. *J Biol Chem* **281**(5):2841-2846.

Hanson KA, Ziegler JW, Rybalkin SD, Miller JW, Abman SH and Clarke WR (1998) Chronic pulmonary hypertension increases fetal lung cGMP phosphodiesterase activity. *Am J Physiol* **275**(5 Pt 1):L931-941.

Huai Q, Liu Y, Francis SH, Corbin JD and Ke H (2004) Crystal structures of phosphodiesterases 4 and 5 in complex with inhibitor 3-isobutyl-1-methylxanthine suggest a conformation determinant of inhibitor selectivity. *J Biol Chem* **279**(13):13095-13101.

Jeremy JY, Ballard SA, Naylor AM, Miller MA and Angelini GD (1997) Effects of sildenafil, a type-5 cGMP phosphodiesterase inhibitor, and papaverine on cyclic GMP and cyclic AMP levels in the rabbit corpus cavernosum in vitro. *Br J Urol* **79**(6):958-963.

Kang KK, Ahn GJ, Ahn BO, Yoo M and Kim WB (2003) DA-8159, a new PDE5 Iihibitor, induces penile erection in conscious and acute spinal cord injured rabbits. *Eur Urol* **43**(6):689-695.

Kendirci M, Bivalacqua TJ and Hellstrom WJ (2004) Vardenafil: a novel type 5 phosphodiesterase inhibitor for the treatment of erectile dysfunction. *Expert Opin Pharmacother* **5**(4):923-932.

Martinez SE, Beavo JA and Hol WG (2002) GAF Domains: Two-Billion-Year-Old Molecular Switches that Bind Cyclic Nucleotides. *Mol Interv* **2**(5):317-323.

Martinez SE, Bruder S, Schultz A, Zheng N, Schultz JE, Beavo JA and Linder JU (2005) Crystal structure of the tandem GAF domains from a cyanobacterial adenylyl cyclase: modes of ligand binding and dimerization. *Proc Natl Acad Sci U S A* **102**(8):3082-3087.

McAllister-Lucas LM, Haik TL, Colbran JL, Sonnenburg WK, Seger D, Turko IV, Beavo JA, Francis SH and Corbin JD (1995) An essential aspartic acid at each of two allosteric cGMP-binding sites of a cGMP-specific phosphodiesterase. *J Biol Chem* **270**(51):30671-30679.

Mullershausen F, Koesling D and Friebe A (2005) NO-sensitive guanylyl cyclase and NO-induced feedback inhibition in cGMP signaling. *Front Biosci* **10**:1269-1278.

Richter W and Conti M (2004) The oligomerization state determines regulatory properties and inhibitor sensitivity of type 4 cAMP-specific phosphodiesterases. *J Biol Chem* **279**(29):30338-30348.

Rotella DP (2003) Tadalafil Lilly ICOS. *Curr Opin Investig Drugs* **4**(1):60-65.

Rybalkin SD, Yan C, Bornfeldt KE and Beavo JA (2003) Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ Res* **93**(4):280-291.

Sebkhi A, Strange JW, Phillips SC, Wharton J and Wilkins MR (2003) Phosphodiesterase type 5 as a target for the treatment of hypoxia-induced pulmonary hypertension. *Circulation* **107**(25):3230-3235.

Siegel LM and Monty KJ (1966) Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases. *Biochim Biophys Acta* **112**(2):346-362.

Sopory S, Kaur T and Visweswariah SS (2004) The cGMP-binding, cGMP-specific phosphodiesterase (PDE5): intestinal cell expression, regulation and role in fluid secretion. *Cell Signal* **16**(6):681-692.

Sung BJ, Hwang KY, Jeon YH, Lee JI, Heo YS, Kim JH, Moon J, Yoon JM, Hyun YL, Kim E, Eum SJ, Park SY, Lee JO, Lee TG, Ro S and Cho JM (2003) Structure of the catalytic domain of human phosphodiesterase 5 with bound drug molecules. *Nature* **425**(6953):98-102.

Thomas MK, Francis SH, Beebe SJ, Gettys TW and Corbin JD (1992) Partial mapping of cyclic nucleotide sites and studies of regulatory mechanisms of phosphodiesterases using cyclic nucleotide analogues. *Adv Second Messenger Phosphoprotein Res* **25**:45-53.

Thomas MK, Francis SH and Corbin JD (1990) Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase. *J Biol Chem* **265**(25):14964-14970.

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**(1):124-130.

Turko IV, Francis SH and Corbin JD (1998) Binding of cGMP to both allosteric sites of cGMP-binding cGMP-specific phosphodiesterase (PDE5) is required for its phosphorylation. *Biochem J* **329 (Pt 3)**:505-510.

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**(2 Pt 2):H448-455.

Yamazaki A, Bartucca F, Ting A and Bitensky MW (1982) Reciprocal effects of an inhibitory factor on catalytic activity and noncatalytic cGMP binding sites of rod phosphodiesterase. *Proc Natl Acad Sci U S A* **79**(12):3702-3706.

Zoraghi R, Bessay EP, Corbin JD and Francis SH (2005) Structural and functional features in human PDE5A1 regulatory domain that provide for allosteric cGMP binding, dimerization, and regulation. *J Biol Chem* **280**(12):12051-12063.

Zoraghi R, Corbin JD and Francis SH (2004) Properties and functions of GAF domains in cyclic nucleotide phosphodiesterases and other proteins. *Mol Pharmacol* **65**(2):267-278.

Zoraghi R, Corbin JD and Francis SH (2006) Phosphodiesterase-5 Gln817 Is Critical for cGMP, Vardenafil, or Sildenafil Affinity: ITS ORIENTATION IMPACTS cGMP BUT NOT cAMP AFFINITY. *J Biol Chem* **281**(9):5553-5558.

Footnotes:

Work was supported by NIH DK40299, NIH DK58277, and American Heart Association Postdoctoral Fellowship 032525B.

For reprint requests, contact: Sharron H. Francis, 702 Light Hall, Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615. Tel: 615 322 4383; Fax: 615 343 3794; E-mail: sharron.francis@vanderbilt.edu

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 1. Chemical structures of cGMP and inhibitors.

Fig. 2. Schematic of full-length PDE5 and PDE5 truncation constructs.
All constructs were prepared in the Sf9 expression system as outlined in *Materials and Methods* with the exception of isolated C domain which was purified from both E. *coli* and infected Sf9 cells as also outlined in *Materials and Methods*.

Fig. 3. Vardenafil potency of inhibition for full-length PDE5 and isolated C domain.
Full-length PDE5 (PDE5) or isolated PDE5 C domain (C domain) (10 µl; 0.1 nM final concentration in assay) was added to the PDE assay reaction mixture containing increasing concentrations of vardenafil. PDE activity was determined in a 15-min incubation as described in *Materials and Methods* using 0.4 µM (final concentration) [^{3}H]cGMP as substrate. Data represent experiments performed in triplicate where *n=3*.

Fig. 4. IC_{50} of vardenafil for full-length PDE5, isolated C domain, isolated C domain plus isolated R domain, and isolated C domain plus isolated GAF-B.
Full-length PDE5 (PDE5) or isolated PDE5 C domain (C domain) (10 µl; 0.1 nM final concentration in assay) was added to the PDE assay reaction mixture containing increasing concentrations of vardenafil. Isolated C domain (10 µl; 0.1 nM) was incubated with either isolated R domain (R domain) or isolated GAF-B module (GAF-B). PDE activity was determined in a 15-min incubation as described in *Materials and Methods using* 0.4 µM (final concentration) [^{3}H]cGMP as substrate. Data represent experiments performed in triplicate where *n=3*.

Fig 5. Sephacryl S-200 gel filtration of PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, and isolated C domain (Δ1-534).
50 µg of either PDE5Δ1-321 (A), PDE5Δ1-419 (B), PDE5Δ1-465 (C), or isolated C domain (Δ1-534) (D) was applied to a Sephacryl S-200 column equilibrated in 20 mM Tris (pH 8) and 100 mM NaCl at 4°C. The internal standards apoferritin (2 mg/ml) and cytochrome *c* (5 mg/ml) were included with each construct. Elution of internal standards was determined by absorbance at 400 nm. The elution position of PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, or isolated C domain (Δ1-534) was determined by PDE assays that were performed as outlined in *Materials and Methods*. Each graph is a representative of one of the three experiments performed for each construct.

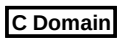
Fig 6. Sucrose density gradient centrifugation of PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, and isolated C domain (Δ1-534).
20 µg of either PDE5Δ1-321 (A), PDE5Δ1-419 (B), PDE5Δ1-465 (C), or isolated C domain (Δ1-534) (D) were subjected to sucrose density gradient centrifugation (5-20%) as described in *Materials and Methods*. Internal standards included in each sample are 2 mg/ml of aldolase and 5 mg/ml cytochrome *c*. Following centrifugation, 0.5 ml fractions were collected. To determine position of the internal standard aldolase, 20 µl of fraction was loaded on a 10% SDS-PAGE gel and stained with Coomassie blue. The resulting protein bands were quantified as described in *Materials and Methods*. Absorbance at 400 nm was used to determine the elution positions of cytochrome *c*. To determine the location of the N-terminal construct, PDE assays were performed according to the

protocol described in *Materials and Methods.* Each graph is representative of one of three experiments performed for each construct.

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Comparison of Potency of PDE5 Inhibitors on Catalytic Activity of Full-Length PDE5 and PDE5 N-Terminal Truncation Constructs.

Either full-length PDE5, PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, or isolated C domain (Δ1-534) (10 µl; 0.1 nM final concentration in assay) was added to the PDE assay reaction mixture containing increasing concentrations of inhibitors. PDE activity was determined in a 15-min incubation as described in *Materials and Methods* using 0.4 µM (final concentration) [^{3}H]cGMP as substrate. Data represent experiments performed in triplicate where *n=3*.

	IC_{50}						
	µM	nM					
			Sildenafil-based		Vardenafil-based		
	IBMX	Tadalafil	Sildenafil	UK-122764	Vardenafil	Demethyl-Vardenafil	BAY- 51187
PDE5 	4.6 ± 0.7	2.9 ± 0.3	2.3 ± 0.4	5.4 ± 0.5	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.3
PDE5Δ1- 321 	4.5 ± 0.8	3.6 ± 0.4	2.5 ± 0.4	5.5 ± 1.5	0.2 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
PDE5Δ1- 419 	4.4 ± 0.6	3.8 ± 1.1	2.6 ± 0.4	5.1 ± 1.9	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2
PDE5Δ1- 465 	4.8 ± 2.4	3.6 ± 1.3	3.0 ± 0.5	5.5 ± 0.8	2.7 ± 0.5	5.6 ± 1.2	3.1 ± 0.8
C domain (Δ1-534) 	4.3 ± 1.2	3.6 ± 0.5	2.8 ± 0.1	5.1 ± 0.8	2.9 ± 0.3	3.2 ± 0.5	2.9 ± 0.4

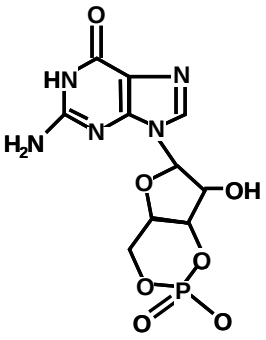
Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Determination of molecular mass for N-terminal constructs.
Stokes radius was determined for PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, and isolated C domain (Δ1-534) by gel filtration chromatography while the sedimentation coefficient was calculated from sucrose density gradient centrifugation results as described under *Materials and Methods*. The values are given as the mean ± SEM where $n=3$. Molecular mass (M_r) was calculated according to the Siegel and Monty equation (Eq. 2 in *Materials and Methods*). The calculated M_r was compared to the predicted M_r to determine the quaternary structure of the N-terminal construct.

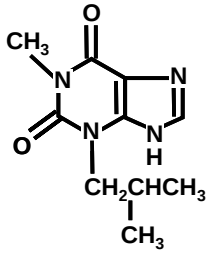
		Stokes Radius Å	Sedimentation Coefficient S	Calculated M_r kDa	Predicted M_r kDa	Assigned Quaternary Structure
PDE5Δ1-321	B – C Domain	46.9 ± 0.46	5.83 ± 0.15	113	63.7	dimer
PDE5Δ1-419	B – C Domain	44.7 ± 1.45	5.13 ± 0.09	94.8	52.4	dimer
PDE5Δ1-465	B – C Domain	34.8 ± 2.03	3.03 ± 0.17	43.6	47.2	monomer
C domain (Δ1-534)	C Domain	24.5 ± 0.51	2.65 ± 0.15	26.8	37.7	monomer

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

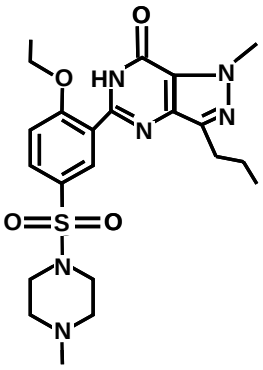
cGMP



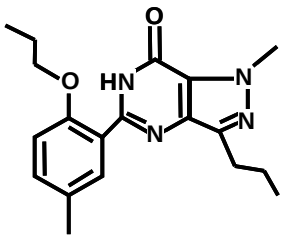
IBMX



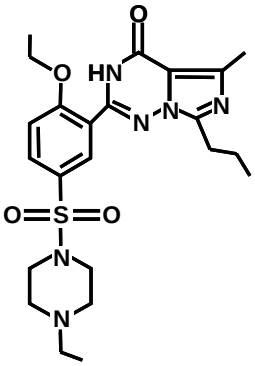
Sildenafil



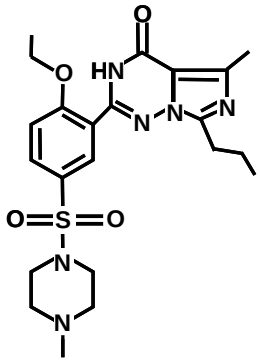
UK-122764



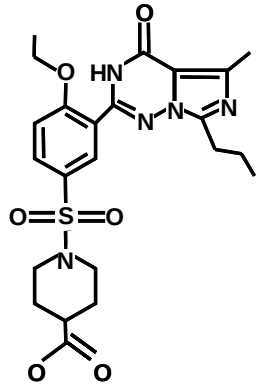
Vardenafil



Demethyl-Vardenafil



BAY 51-1871



Tadalafil

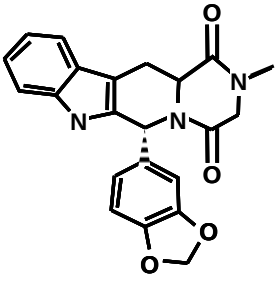


Fig. 1

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

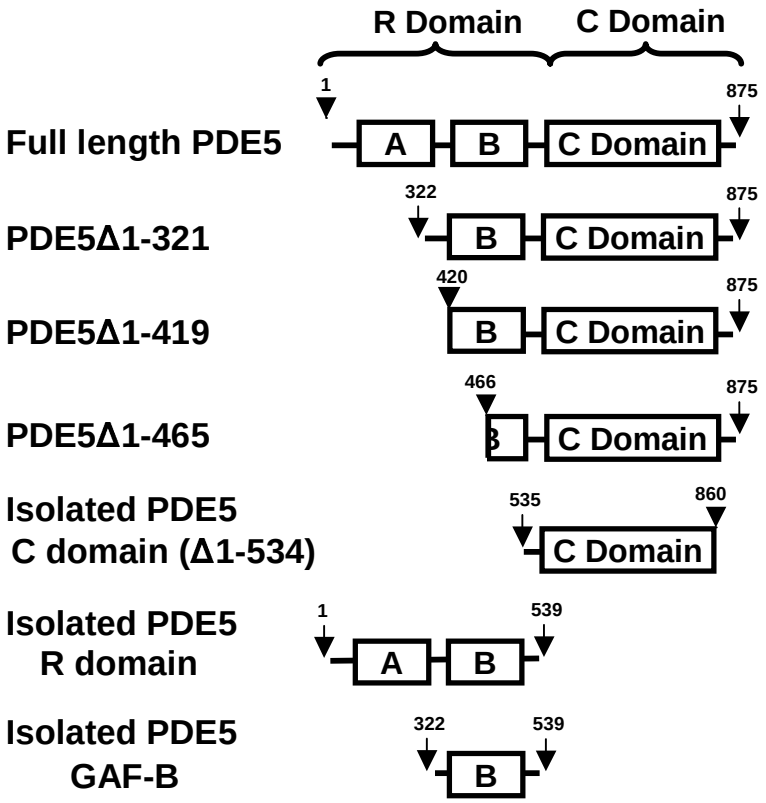


Fig. 2

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

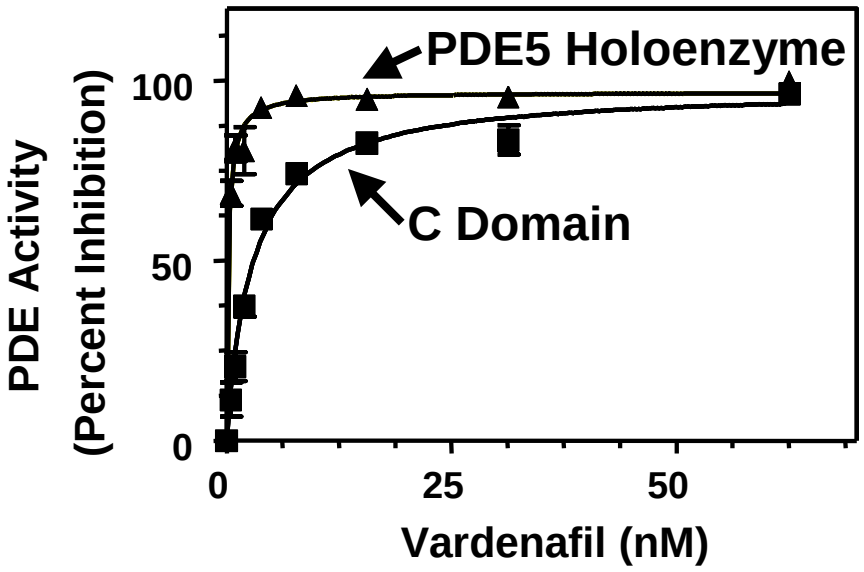


Fig. 3

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

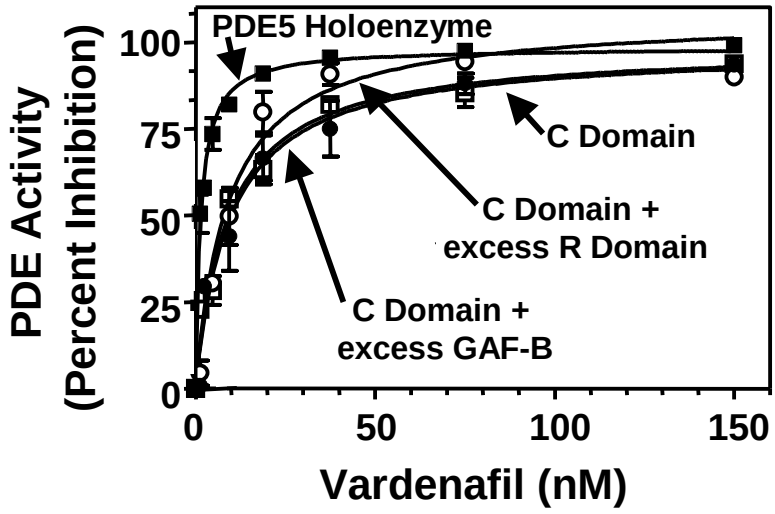


Fig. 4

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

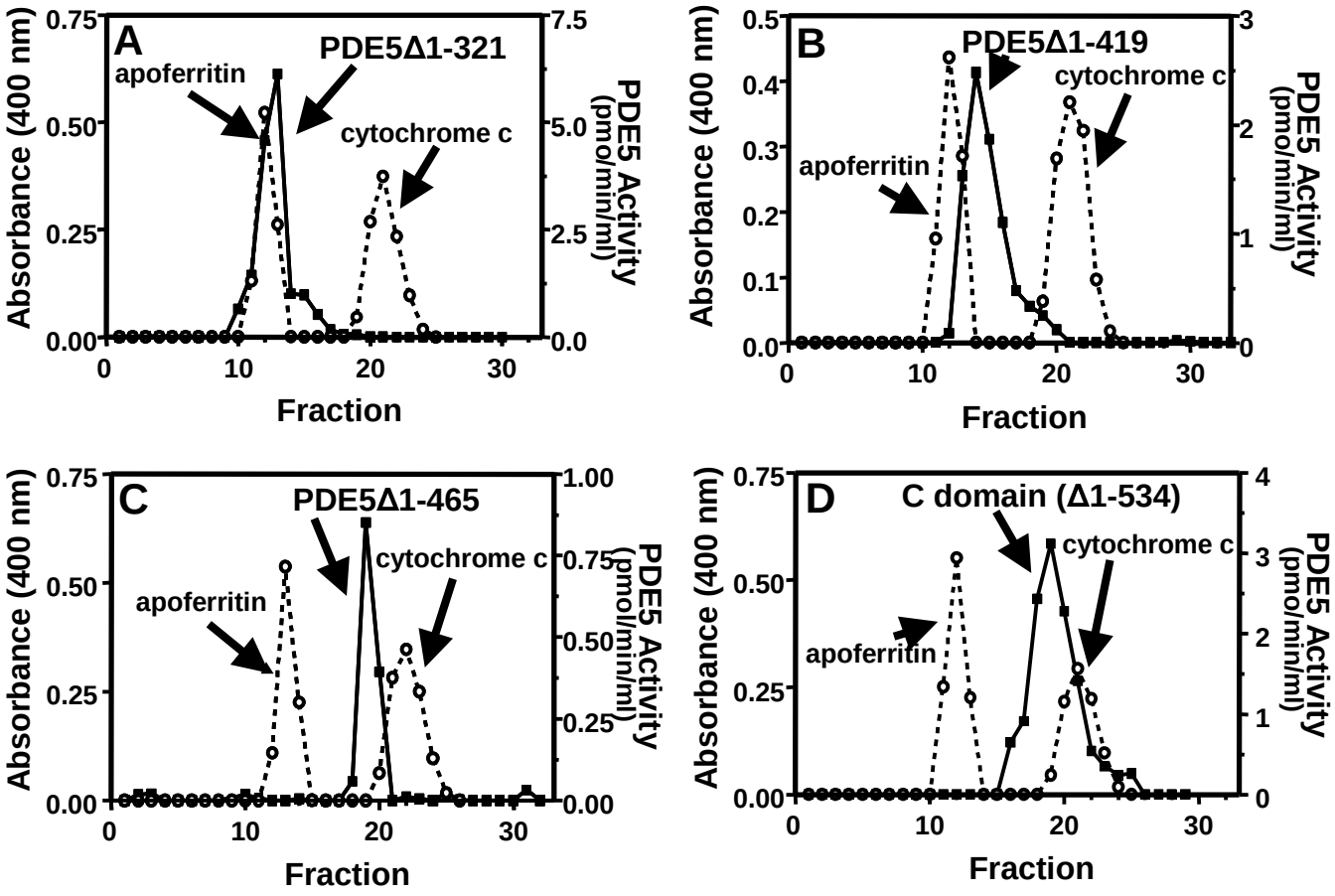


Fig. 5

Molecular Pharmacology Fast Forward. Published on August 22, 2006 as DOI: 10.1124/mol.106.028688
This article has not been copyedited and formatted. The final version may differ from this version.

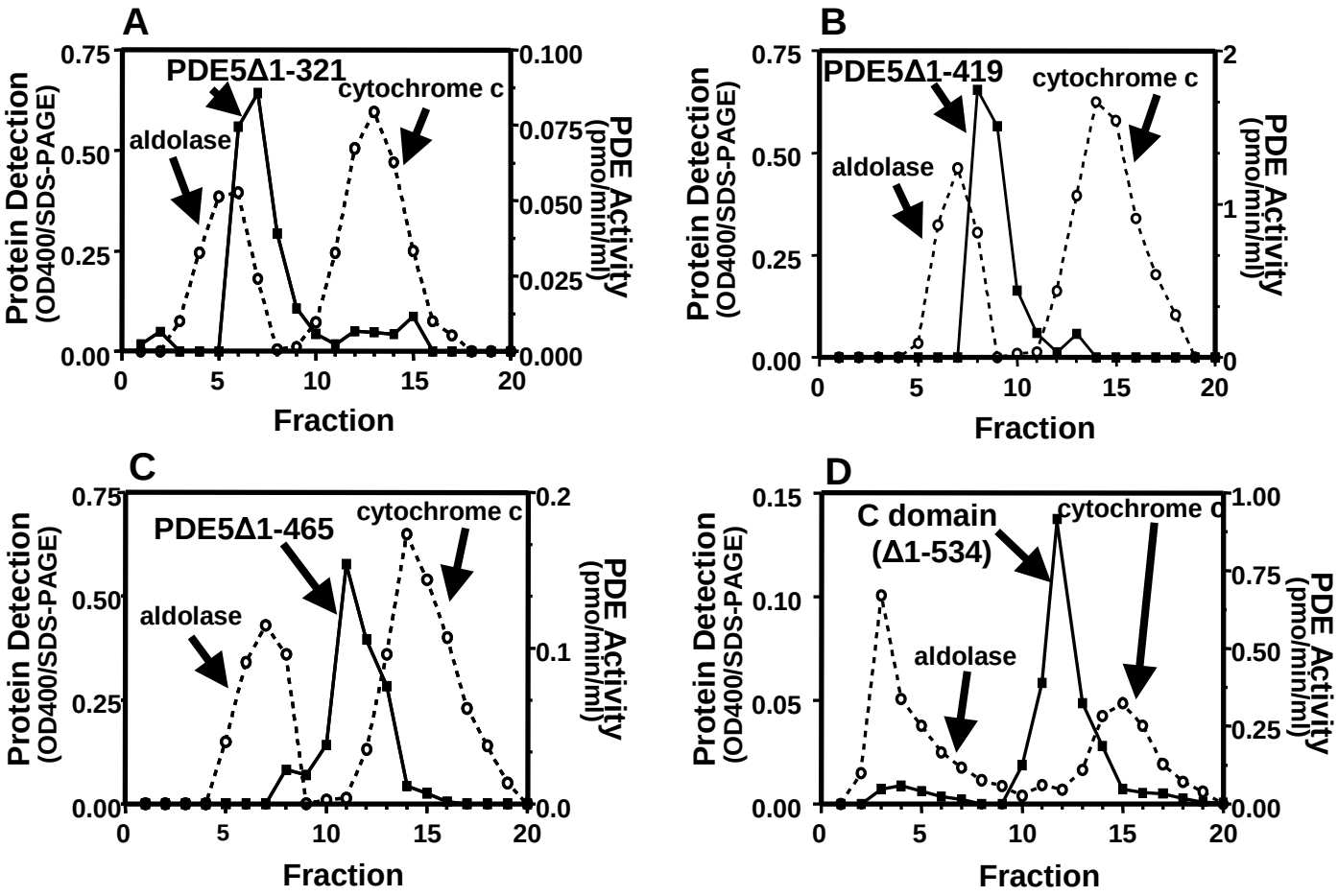


Fig. 6