A 46-Amino Acid Segment in Phosphodiesterase-5 GAF-B Domain Provides for High Vardenafil Potency over Sildenafil and Tadalafil and Is Involved in Phosphodiesterase-5 Dimerization

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

Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee (M.A.B., R.Z., E.P.B., J.D.C., S.H.F.); and Department of Biochemistry and Biophysics and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina (H.K.)

Received July 7, 2006; accepted August 22, 2006

ABSTRACT

Phosphodiesterase-5 (PDE5) contains a catalytic domain (C domain) that hydrolyzes cGMP and a regulatory domain (R domain) that contains two mammalian cGMP-binding phosphodiesterase, Anabaena adenylyl cyclases, Escherichia coli FhlAs (GAFs) (A and B) and a phosphorylation site for cyclic nucleotide-dependent protein kinases (cNPKs). 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: PDE5/H90041-321 contained GAF-B domain, C domain, and the sequence between GAF-A and -B; PDE5/H90041-419 contained GAF-B and C domain; PDE5/H90041-465 contained the C domain and the C-terminal portion of GAF-B; and PDE5/H90041-534 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_m values 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 with 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.

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; Rybalkin et al., 2003; Mullershausen et al., 2005). 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; Wyatt et al., 1998; Rybalkin et al., 2003; Sopory et al., 2004). PDE5 is the target of erectile dysfunction medications [e.g., sildenafil (Viagra), tadalafil (Cialis), vardenafil (Levitra), and udenafil (Zydena)]; sildenafil (Revatio) has recently been approved for treatment of pulmonary arterial hypertension (Jeremy et al., 1997; Ballard et al., 1998; Corbin and Francis, 1999; Turko et al., 1999; Kang et al., 2003; Rotella, 2003; Sebkhi et al., 2003; Kendirci et al., 2004; Galie et al., 2005). 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 (Thomas et al., 1992; McAllister-Lucas et al., 1995; Corbin and Francis, 1999; 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 phosphodiesterase, *Anabaena* adenyl cyclases, *Escherichia coli* FHLAs (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 (Yamazaki et al., 1982; Francis et al., 2002; Martinez et al., 2005; Gross-Langenhoff et al., 2006). 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 (Turko et al., 1998; Rybalkin et al., 2003; Martinez et al., 2002; Zoraghi et al., 2004). 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 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 (Sung et al., 2003; Blount et al., 2004; Huai et al., 2004). 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 cocrystallographic images derived from the isolated C domain may not contain or reveal structural nuances that affect 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 (Turko et al., 1999; Blount et al., 2004). 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 features within the R domain influence the affinity of the PDE5 catalytic site for certain classes of inhibitors. In addition, we considered 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 played 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 accounted for this effect, and that the same 46 amino acids were required for GAF-B-mediated dimerization of PDE5.

Materials and Methods

Materials. \[^3H\]cGMP and DEAE-Sephacel were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). IBMX, histone type II-AS, *Crotalus atrox* snake venom, 5'-GMP, and cGMP were obtained from Sigma-Aldrich (St. Louis, MO). Full-length recombinant bovine PDE5 was isolated from infected Sf9 cells using Ni/NTA agarose (QIAGEN, Valencia, CA) 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 GE Healthcare for radiolabeling with tritium. Tadalafil (Fig. 1) was synthesized according to Daugan (2000). After confirming the compound structure by mass spectrometry, tadalafil was submitted to GE Healthcare for radiolabeling with tritium. High-performance liquid chromatography results from GE Healthcare indicated that \[^3H\]sildenafil was >98% pure, whereas the \[^3H\]tadalafil preparation was >99% pure. Vardenafil, \[^3H\]vardenafil, demethyl-vardenafil, and BAY 51-1871 (Fig. 1) were kindly provided by Bayer AG (Wuppertal, Germany). UK-122764 (Fig. 1) was generously provided by Pfizer Central Research (Sandwich, UK). All three \(^3\)H-labeled inhibitors that had been stored for more than a year were subjected to Sephadex G-25 chromatography, which adsors these PDE inhibitors and provides purification with high resolution (Corbin et al., 2003). All three \(^3\)H-inhibitors were resolved in single peaks and...
coeluted with purified unlabeled inhibitors, suggesting that the 3H-labeled inhibitors were not altered after storage.

**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 polymerase chain reaction fragment was inserted into a pAcHLT-A vector (BD Biosciences Pharmingen, San Diego, CA) 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 lacking 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 (Thr322-Gly539) (Fig. 2). The full-length isolated R domain (PDE5Δ540-875) and isolated GAF-B mutants were prepared and characterized as described previously (Zoraghi et al., 2005).

All constructs were confirmed by DNA sequencing before cotransfection into Sf9 cells with BaculoGold linear baculovirus DNA (BD Biosciences Pharmingen) and isolated C domain (human Glu535-Gln860) (Fig. 2) used in these studies was kindly provided by Dr. Hengming Ke (University of North Carolina, Chapel Hill, NC). The plasmid was transformed into *E. coli* strain BL21 (CodonPlus) for overexpression. *Escherichia coli* containing the vector was grown in enriched LB medium (10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl per liter with 0.4% glucose, and 100 mg of AMP added after autoclaving) at 37°C to *A*°cw = 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 liters of *E. coli* cells was dispersed with 4 ml of extraction buffer (20 mM Tris, pH 8, 50 mM NaCl, 15 mM imidazole, and 1 mM *β*-mercaptoethanol) and 1 EDTA-free Complete protease inhibitor cocktail tablet per 50 ml of solution; Roche Diagnostics, 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 four times. After centrifugation at 9000 rpm in a Beckman JA-20 rotor for 20 min, the supernatant was applied to a Ni-NTA-free Complete protease inhibitor cocktail tablet per 50 ml of solution; Roche Diagnostics, 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 four times. After centrifugation at 9000 rpm in a Beckman JA-20 rotor for 20 min, the supernatant was applied to a Ni-NTA column (QIAGEN) equilibrated with buffer containing 200 mM Tris, pH 8, 0.3 M NaCl, 15 mM imidazole, and 1 mM *β*-mercaptoethanol. 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 Glu535-Gln860) (Fig. 2) used in these studies was kindly provided by Dr. Hengming Ke (University of North Carolina, Chapel Hill, NC). The plasmid was transformed into *E. coli* strain BL21 (CodonPlus) for overexpression. *Escherichia coli* containing the vector was grown in enriched LB medium (10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl per liter with 0.4% glucose, and 100 mg of AMP added after autoclaving) at 37°C to *A*°cw = 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 liters of *E. coli* cells was dispersed with 4 ml of extraction buffer (20 mM Tris, pH 8, 50 mM NaCl, 15 mM imidazole, and 1 mM *β*-mercaptoethanol) and 1 EDTA-free Complete protease inhibitor cocktail tablet per 50 ml of solution; Roche Diagnostics, 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 four times. After centrifugation at 9000 rpm in a Beckman JA-20 rotor for 20 min, the supernatant was applied to a Ni-NTA column (QIAGEN) equilibrated with buffer containing 200 mM Tris, pH 8, 0.3 M NaCl, 15 mM imidazole, and 1 mM *β*-mercaptoethanol. 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.

**Full length PDE5**

**PDE5Δ1-321**

**PDE5Δ1-419**

**PDE5Δ1-465**

**Isolated PDE5 C domain (Δ1-534)**

**Isolated PDE5 R domain**

**Isolated PDE5 GAF-B**

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

**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-Aldrich), [3H]cGMP (Amersham) (80,000–150,000 cpm/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-Aldrich), 10 mM GMP, and 10 mM cAMP in 100 mM Tris, pH 7.5. Snake venom 5′-nucleotidase (200 μg, Sigma-Aldrich) 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 [3H]guanosine was measured in a scintillation counter. To determine IC50 values for various inhibitors, the [3H]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 Km for each construct was determined using a range of cGMP concentrations. Unless indicated otherwise, PDE activity is expressed as picomoles per minute per milliliter of enzyme in the reaction tube.

**[3H]cGMP Binding Assays.** To measure allosteric cGMP binding, Millipore filter binding assays were conducted in a total volume of 50 μl of reaction mixture that contained 10 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 0.5 mg/ml histone type II-A, 30 mM dithiothreitol, 0.2 mM sodium fluoride, and either 3 μM (for binding stoichiometry determination) or 0.05 to 4 μM (for binding affinity determination) of [3H]cGMP. The binding reaction was initiated by addition of enzyme and incubated on ice for 60 min. Then, 1 ml of ice-cold KP buffer (10 mM potassium phosphate, pH 6.8) was added to each sample, and samples were filtered immediately onto premoistened Millipore HAWP filters (pore size 0.45 μm). Filters were then washed with 2 ml of ice-cold KP buffer two times, dried, and counted using nonaqueous Ready Safe scintillation cocktail (Beckman Coulter, Inc.). Counts bound to PDE5 were corrected by subtraction of nonspecific binding (counter contribution of buffer that consisted of 0.2 mg/ml histone II-A, 30 mM theophylline (Sigma-Aldrich), 10 mM cGMP, and 10 mM MgCl2). Nonspecific binding was determined in PDE assay containing no PDE5 were run for each [3H]cGMP concentration. The bound activity was determined 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% to 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 at 4°C, and fractions (0.5 ml) were collected from the bottom of the tubes. 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 10% SDS-PAGE analysis and stained with Coomassie Blue. The intensity of the band was quantified using MetaMorph software (Molecular Devices, Sunnyvale, CA). The proteins of known S0.2 were—cytochrome c (1.86 S), protein kinase A-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 Compute pl/Mw (http://www.expasy.org/tools/pi_tool.html) based on the amino acid composition. This was compared with the experimentally determined relative molecular mass of the proteins to establish whether 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 eq. 3 (Gerstner et al., 1994):

$$\Delta G = -RT \ln K$$  \hspace{1cm} (3)

where R is the ideal gas constant (1.98 × 10-5 kcal/degree/mol), T is the temperature at which the assay was done (303 K), and K is the association constant for the interaction. ΔG values were calculated from the experimentally determined IC50 values for the inhibitors using the equation (eq. 4) of Cheng and Prusoff (1973):

$$K_i = \frac{IC_{50}}{1 + [S]/K_m}$$  \hspace{1cm} (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 eq. 5 (Fersht, 1988):

\[
\Delta \Delta G = \Delta G_{WT} - \Delta G_{mut}
\]

ΔΔG 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 ± S.E.M. as determined by Prism software (GraphPad Software Inc., San Diego, CA). The software uses the following equation: S.E.M. = S.D./√n

Where S.D. is determined as \[\sum(y_i - \bar{y})^2/(n-1)\] 1/2. All S.E.M.s reported fit within a 95% confidence interval, which quantifies the precision of the mean.

**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 under 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 assay 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.** IC₅₀ 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 by using 0.4 μM [³H]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 imidazotriazinone 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 IC₅₀ values of IBMX, a weak, nonspecific PDE inhibitor; for full-length PDE5, PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465; and for 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 IC₅₀ values of IBMX, a weak, nonspecific PDE inhibitor; for full-length PDE5, PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, and isolated C domain (Δ1-534) were also similar (Table 1). IC₅₀ 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 affect the potency of inhibition by IBMX, tadalafil, or sildenafil-based compounds.

In contrast, the IC₅₀ values of the three vardenafil-based compounds for the proteins of different lengths differed markedly. The IC₅₀ 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 IC₅₀ values for vardenafil that were very similar to that for full-length PDE5 (0.2 ± 0.1 and 0.4 ± 0.1 nM, respectively) (Table 1). However, the shorter constructs that lacked a complete group of sildenafil (Fig. 1). Previous studies established that the IC₅₀ values of vardenafil for full-length PDE5 compared with that for the isolated C domain is also illustrated in Fig. 3. IC₅₀ values for two other vardenafil-based compounds were also determined in head-to-head experiments. The first compound, demethyl-vardenafil, contained the [5,1-]/[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); IC₅₀ values

### Table 1

Comparison of potency of PDE5 inhibitors on catalytic activity of full-length and N-terminal truncation constructs of PDE5

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PDE5</th>
<th>PDE5Δ1-321</th>
<th>PDE5Δ1-419</th>
<th>PDE5Δ1-465</th>
<th>C Domain (Δ1-534)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBMX</td>
<td>4.6 ± 0.7</td>
<td>4.5 ± 0.8</td>
<td>4.4 ± 0.6</td>
<td>4.8 ± 2.4</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>Tadalafil</td>
<td>2.3 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>2.6 ± 0.4</td>
<td>3.0 ± 0.5</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>Sildenafil-Based</td>
<td>5.4 ± 0.5</td>
<td>5.5 ± 1.5</td>
<td>5.1 ± 1.9</td>
<td>5.5 ± 0.8</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>Vardenafil</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Demethyl-Vardenafil</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>BAY-51-1871</td>
<td>4.0 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>3.1 ± 0.8</td>
<td>2.9 ± 0.4</td>
</tr>
</tbody>
</table>
(0.3 ± 0.1 nM) determined here agreed with those previous findings. Demethyl-vardenafil had a similar IC₅₀ (0.6 ± 0.2 nM) for PDE5α1-321 and PDE5α1-419 (0.4 ± 0.1 nM). However, PDE5α1-465 and the isolated C domain (Δ1-534), both of which lacked an intact GAF-B, had ~10-fold higher IC₅₀ values (5.6 ± 1.2 and 3.2 ± 0.5 nM, respectively).

BAY 51-1871 also contains the [5,1]-[1,2]triazine ring of vardenafil, but a carbonyl group replaces the methyl group (Fig. 1). The IC₅₀ values determined for full-length PDE5, PDE5α1-321, and PDE5α1-419 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 IC₅₀ values for PDE5α1-465 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 [³H]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 [³H]vardenafil and the membrane filtration binding assay described under Materials and Methods. Maximum [³H]vardenafil binding to both proteins was ~0.5 mol/mol PDE5 monomer. The specificity of [³H]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, and tadafin) as well as other heterocyclic inhibitors that are selective for other PDEs [rolipram (PDE4), milrinone (PDE3), erythroid-9-(2-hydroxy-3-nonyl)adenine (PDE2), or vinpocetine (PDE1)]. High concentrations of rolipram, milrinone, erythroid-9-(2-hydroxy-3-nonyl)adenine, or vinpocetine did not affect [³H]vardenafil binding (3 nM), whereas unlabeled vardenafil, milrinone, or tadafin blocked more than 90% of the binding (data not shown). A 100,000-fold excess of either cAMP, 5'-GMP, or IBMX had no effect on [³H]vardenafil binding to either isolated C domain or full-length PDE5 (data not shown).

The Kᵦ of [³H]vardenafil was determined for isolated C domain and full-length PDE5 by performing a binding isotherm with increasing concentrations of [³H]vardenafil in the absence of cGMP in a head-to-head manner. The Kᵦ obtained for full-length PDE5 was 1.1 ± 0.5 nM (n = 3), a value similar to that previously determined in this laboratory (Blount et al., 2004). The Kᵦ of vardenafil for isolated C domain was 7.3 ± 3.4 nM (n = 3). This ~7-fold increase in Kᵦ of isolated C domain for vardenafil was similar to the ~10-fold increase in the IC₅₀ of vardenafil described above.

### Vardenafil Affinity of PDE5α1-465 or Isolated PDE5 C Domain Is Not Affected by Addition of Either Excess Isolated R Domain or Isolated GAF-B.

Addition of either isolated GAF-B module (IC₅₀ = 9.1 ± 1.0 nM) or isolated R domain (IC₅₀ = 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 full-length PDE5 or PDE5 C domain (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 under Materials and Methods using the experimentally determined Stokes radius and sedimentation coefficient for each. When PDE5α1-321 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 it had a calculated Stokes radius of 46.9 ± 0.46 Å (Table 2). Upon sucrose density gradient centrifugation, PDE5α1-321 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 mo-
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 mass (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 ± 0.17 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.

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 relative molecular mass 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.

### 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. It is surprising that a portion of the high-affinity inhibition of PDE5Δ1-321, PDE5Δ1-419, PDE5Δ1-465, and isolated C domain (Δ1-534) by gel filtration chromatography, whereas the sedimentation coefficient was calculated from sucrose density gradient centrifugation results as described under Materials and Methods. The values are given as mean ± S.E.M. where n = 3. Relative molecular mass (Mr) was calculated according to the Siegel and Monty equation (eq. 2 under Materials and Methods). The calculated Mr was compared with the predicted Mr to determine quaternary structure of the N-terminal construct.

**Table 2** Determination of molecular mass for N-terminal constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Stokes Radius</th>
<th>Sedimentation Coefficient</th>
<th>Mr, kDa</th>
<th>Assigned Quaternary Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE5Δ1-321</td>
<td>46.9 ± 0.46</td>
<td>5.83 ± 0.15</td>
<td>113</td>
<td>63.7 Dimer</td>
</tr>
<tr>
<td>PDE5Δ1-419</td>
<td>44.7 ± 1.45</td>
<td>5.13 ± 0.09</td>
<td>94.8</td>
<td>52.4 Dimer</td>
</tr>
<tr>
<td>PDE5Δ1-465</td>
<td>34.8 ± 2.03</td>
<td>3.03 ± 0.17</td>
<td>43.6</td>
<td>47.2 Monomer</td>
</tr>
<tr>
<td>C domain (Δ1-534)</td>
<td>24.5 ± 0.51</td>
<td>2.65 ± 0.15</td>
<td>26.8</td>
<td>37.7 Monomer</td>
</tr>
</tbody>
</table>
of PDE5 by the three vardenafil-based compounds requires the R domain. Direct determination of the $K_D$ for $[^3H]$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 whether the effect of GAF-B applies to high-affinity inhibitors per se or whether 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 upstream conserved region 2 domain in high-affinity riplipram 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 affect 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 cocystal structures of the isolated C domain of PDEs and their inhibitors (Corbin et al., 2005; Zoraghi et al., 2006). The cocystal 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 seem 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- 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 affect the catalytic sites of many PDEs. However, there is no known mechanism by which this 46-amino acid segment in PDE5 affects 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 mechanisms. 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 cyclic nucleotide-dependent protein kinases (Zoraghi et al., 2004). Some evidence suggests that GAFB 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 article 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 version 2.0; http://www.cbs.dtu.dk/services/TMHMM/) 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 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. Because 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.

Acknowledgments

We especially thank Drs. Kenji Omori and Jun Kotera of TanabeSeiyaku 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 AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827. We thank Bayer AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827. We thank Bayer AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827. We thank Bayer AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827. We thank Bayer AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827. We thank Bayer AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827. We thank Bayer AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827. We thank Bayer AG for providing vardenafil, [3H]vardenafil, demethyl-vardenafil, and BAY 51-2827.


Turko IV, Ballard SA, Francis SH, and Corbin JD (1999) Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. Mol Pharmacol 56:124–130.


Address correspondence to: Dr. Sharron H. Francis, Department of Molecular Physiology and Biophysics, 702 Light Hall, Vanderbilt University School of Medicine, Nashville, TN 37232-0615. E-mail: sharron.francis@vanderbilt.edu