Critical Amino Acids in Phosphodiesterase-5 Catalytic Site That Provide for High-Affinity Interaction with Cyclic Guanosine Monophosphate and Inhibitors[†]

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ABSTRACT: The molecular bases for phosphodiesterase 5 (PDE5) catalytic-site affinity for cyclic guanosine monophosphate (cGMP) and potency of inhibitors are poorly understood. Cocrystal structures of PDE5 catalytic (C) domain with inhibitors reveal a hydrogen bond and hydrophobic interactions with Tyr-612, hydrogen bonds with Gln-817, a hydrophobic clamp formed by Phe-820 and Val-782, and contacts with His-613, Leu-765, and Phe-786 [Sung et al. (2003) *Nature 425*, 98−102; Huai et al. (2004) *J. Biol. Chem. 279*, 13095−13101]. Present results of point mutations of full-length PDE5 showed that maximum catalysis was decreased 2650-fold in H613A and 55-fold in F820A. Catalytic-site affinities for cGMP, vardenafil, sildenafil, tadalafil, or 3-isobutyl-1-methylxanthine (IBMX) were respectively weakened 14-, 123-, 30-, 51-, and 43-fold for Y612A; 63-, 511-, 43-, 95- and 61-fold for Q817A; and 59-, 448-, 71-, 137-, and 93-fold for F820A. The data indicate that these three amino acids are major determinants of affinity for cGMP and potency of selective and nonselective inhibitors, and that higher vardenafil potency over sildenafil and tadalafil results from stronger contacts with Tyr-612, Gln-817, and Phe-820. Affinity of V782A for cGMP, vardenafil, sildenafil, tadalafil, or IBMX was reduced 5.5-, 23-, 10-, 3-, and 12-fold, respectively. Change in affinity for cGMP, vardenafil, sildenafil, or IBMX in Y612F, H613A, L765A, or F786A was less, but affinity of H613A or F786A for tadalafil was weakened 37- and 17-fold, respectively. The results quantify the role of PDE5 catalytic-site residues for cGMP and inhibitors, indicate that Tyr-612, Gln-817, and Phe-820 are the most important cGMP or inhibitor contacts studied, and identify residues that contribute to selectivity among different classes of inhibitors.

Cyclic nucleotide (cN)[1] phosphodiesterases (PDEs) are key regulators of signaling by cyclic adenosine and guanosine monophosphate (cAMP and cGMP), and inhibition of PDEs by drugs can lead to elevation of intracellular cAMP/cGMP. The mammalian PDE superfamily is composed of 11 families of enzymes (*3*). Each PDE monomer contains a conserved carboxyl-terminal catalytic (C) domain composed of ∼270 amino acids (Figure 1). Nineteen C-domain residues are invariant among mammalian PDEs. The X-ray crystal structures of the isolated C domain of mammalian PDEs reveal strong similarity in the general topography of the catalytic site (*1*, *4*−*8*).

The similarity of PDE catalytic sites has complicated development of inhibitors that are highly selective for a particular PDE family or subfamily (*9*). Many nonspecific PDE inhibitors such as caffeine, theophylline, and 3-isobutyl-1-methylxanthine (IBMX) contain a heterocyclic ring that mimics the adenine or guanine in cNs; consequently, these can block interaction of cN with the catalytic site of many PDEs. Despite the similarity in the PDE catalytic sites, compounds have been identified in recent years that preferentially inhibit particular PDE families; examples include cilostazol (Pletal), a PDE3-selective inhibitor; rolipram, a PDE4-selective inhibitor; and sildenafil (Viagra), tadalafil (Cialis), vardenafil (Levitra), and udenafil (Zydena), PDE5-selective inhibitors (*3*). However, despite the pressing need to develop selective PDE inhibitors as therapeutic drugs, the molecular mechanisms by which conserved C domains of the various PDE families simultaneously recognize substrates and inhibitors as well as distinguish among selective inhibitors are poorly understood.

The recent availability of X-ray crystal structures of a number of PDE isolated C domains in complex with various inhibitors provides the promise of a more rational approach to the design of new inhibitors and further optimization of their potencies and selectivities (*1*, *7*, *8*, *10*−*15*). Even so, the information imparted by X-ray crystal structures is primarily descriptive, and in most instances the relative energy contributed by each contact to the potency and selectivity for binding of a particular inhibitor is unknown. To date, PDEs have been crystallized as isolated C domains without attached regulatory (R) domains. It is now clear that contacts with some inhibitors differ between the isolated C domain and its corresponding holoenzyme; this emphasizes

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[1] Abbreviations: PDE, cyclic nucleotide phosphodiesterase; cN, cyclic nucleotides; IBMX, 3-isobutyl-1-methylxanthine; C domain, catalytic domain; Ni-NTA, nickel−nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; KP(M) buffer, 10 mM potassium phosphate, pH 6.8 (plus 25 mM β-mercaptoethanol); ΔG, Gibbs free energy of binding; $\Delta\Delta G$, binding energy in enzyme−transition-state complexes.

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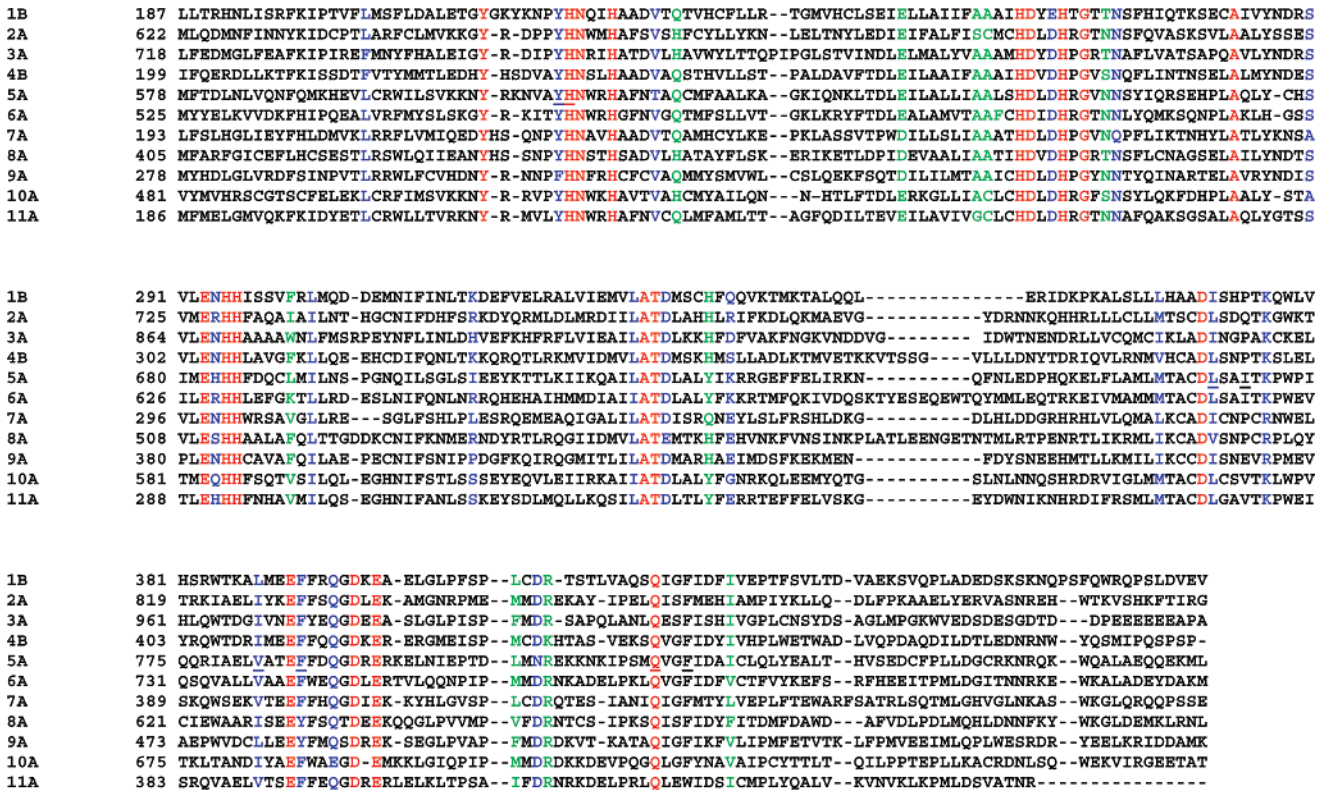

FIGURE 1: Sequence alignment of catalytic domains of mammalian PDEs: 1B, hPDE1B (NP_000915); 2A, hPDE2A (AAC51320); 3A, hPDE3A (NP_000912); 4B, hPDE4B (NP_002591); 5A, hPDE5 (Np-001074); 6A, hPDE6A (NP_000431); 7A, hPDE7A (NP_002594); 8A, hPDE8A (NP_002596); 9A, hPDE9A (AAC39778); 10A, hPDE10A (NP_006652); 11A, hPDE11A (BAB16371). Nineteen invariant amino acids in C domains of mammalian PDEs are in red, highly conserved residues are in blue, and less conserved residues are in green. The PDE5 amino acids underlined are those that have been shown in the crystal structure of human PDE5 to be in contact with the fused double ring of the bound inhibitors as shown in Figure 2A and 2B. Except for Ile-768, all of these were mutated in the present study.

the importance of examining the impact of particular amino acids on affinity for ligands in the context of the holoenzyme (*16*, *17*). Cocrystal structures of PDE5 isolated C domain with 5′-GMP and several inhibitors reveal numerous contacts between C domain residues and either the product or inhibitors (Figure 2) (*1*, *2*, *9*, *11*, *15*); up to now, the structure of an active PDE C domain containing substrate has not been observed since the cyclic phosphate ring is hydrolyzed when the molecule is infused into the crystals (*12*) (H. Ke, personal communication).

Three PDE5-selective inhibitors [sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis)] are highly successful pharmacological agents for treatment of erectile dysfunction, and more recently, sildenafil (Revatio) has been approved for treatment of pulmonary arterial hypertension. Udenafil (Zydena), another PDE5-selective inhibitor, has recently been approved for marketing in Korea. While these inhibitors share some structural similarity with the PDE5 substrate, cGMP, they are 1000−40 000-fold more potent than cGMP or IBMX, a nonselective PDE inhibitor (*18*). The potency of PDE5-selective inhibitors is due to contacts that simulate interactions formed with cGMP as well as novel contacts that cGMP does not make.

Cocrystals of PDE5 with 5′-GMP, sildenafil, vardenafil, tadalafil, and IBMX have indicated that each ligand exploits some of the same contacts. In previous reports, we have quantified the contribution of Tyr-612 to affinity of PDE5 for cGMP and potency of vardenafil and sildenafil (*19*) and of Gln-817 to affinity for cGMP, vardenafil, sildenafil, and IBMX (*20*). Quantification of the role of PDE5 catalytic-site residues for interaction of PDE5 with tadalafil has not been reported. Herein, we provide a comprehensive quantification of the contributions of His-613, Tyr-612, Leu-765, Val-782, Phe-786, Gln-817, and Phe-820 to affinity for cGMP, vardenafil, sildenafil, tadalafil, and IBMX. On the basis of X-ray crystal structures of PDE5 in complex with these inhibitors (Figure 2) or the product, 5′-GMP (*1*, *2*, *11*, *15*), and previous mutagenesis studies (*19*−*22*), we hypothesize herein that Gln-817, Phe-820, and Tyr-612 are critical contacts for high-affinity interaction with cGMP, vardenafil, sildenafil, tadalafil, or IBMX. We also hypothesize that contributions involving these residues, as well as Phe-786, Val-782, and His-613, would significantly differ for the potencies of the inhibitors. To address these hypotheses, point mutations have been created to determine the importance of these amino acids in providing for potency of interaction of PDE5 with cGMP or inhibitors. Combined insights from X-ray crystallographic structures previously reported and the studies herein provide a more accurate profile of catalytic-site function and will optimize efforts to design drugs with improved affinities and selectivities.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]cGMP was purchased from Amersham Biosciences Inc. (Piscataway, NJ). IBMX, *Crotalus atrox* snake venom, cGMP, and histone type II-AS were obtained from Sigma Chemical Co. (St. Louis, MO). Sildenafil was purified from Viagra tablets by the method described in our earlier report (*23*). Tadalafil was synthesized according to

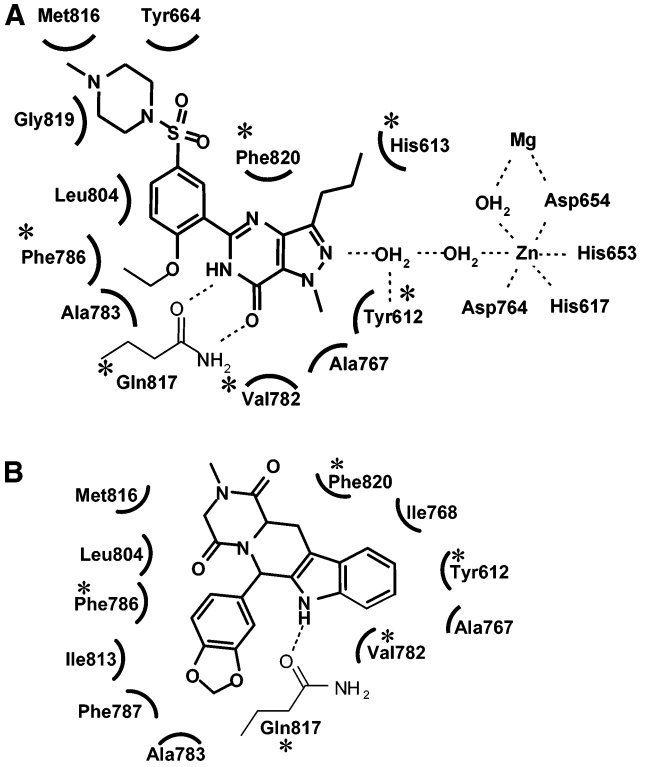

FIGURE 2: Schematic representation of interactions made by (A) sildenafil and (B) tadalafil with PDE5. Residues subjected to site-directed mutagenesis are indicated with asterisks. The hydrogen-bonding interactions are shown as dashed lines. Reproduced with permission from Sung et al. (2003) *Nature 425*, 98−102. Copyright 2003 Nature Publishing Group.

ref *24*. Vardenafil was kindly provided by Bayer AG (Wuppertal, Germany).

Generation of Wild-Type and Mutant hPDE5A1 Constructs. Human cDNA coding for full-length PDE5A1 (courtesy of Dr. K. Omori, Tanabe-Seiyaku Pharmaceutical Co. Ltd., Saitama, Japan) was used as a template to generate the full-length enzyme by introduction of start and stop codons at appropriate loci. The resulting PCR fragment was cloned into pCR 2.1-TOPO vector (Invitrogen), verified by sequencing, and then ligated into the *Eco*RI and *Not*I unique sites of the baculovirus expression vector pAcHLT-A (Pharmingen). pAcHLT-A vector contains a His_6 sequence in front of the coding region. This step resulted in plasmid pAcA-PDE5 (M_1-N_{875}), which generated N-terminally His-tagged recombinant hPDE5A1. The quick change site-directed mutagenesis kit (Stratagene) was used to make point mutations (Tyr-612 to Ala, His-613 to Ala, Leu-765 to Ala, Val-782 to Ala, Phe-786 to Ala, Gln-817 to Ala, and Phe-820 to Ala) in the pAcA-PDE5 expression vector according to the manufacturer's protocol by use of the following pairs of mutagenic oligonucleotides (altered bases underlined): (1) for H613A, 5′-GAATGTTGCCTATGCTAATTGGAGACATG-3′ and 5′-CATGTCTCCAATTAGCATAGGCAACATTC-3′; (2) for L765A, 5′- GATGACAGCTTGTGATGCTTCTGCAATTAC-3′ and 5′-GTAATTGCAGAAGCATCACAAGCTGTCATC-3′; (3) for V782A, 5′-GATAGCAGAACTTGCAGCAACTGAATT-3′ and 5′-AATTCAGTTGCTGCAAGTTCTGCTATC-3′; (4) for F786A, 5′-CTTGTAGCAACTGAAGCTTTTGATCAAGGAG-3′ and 5′-CTCCTTGATCAAAAGCTTCAGTTGCTACAAG-3′; and (5) for F820A, 5′-GTATGCAAGTTGGGGCCATAGATGCCATCT-3′ and 5′-AGATGGCATCTATGGCCCCAACTTGCATAC. The primer pairs for generating Y612F, Y612A, and Q817A mutants have been described previously (*19*, *20*). The presence of the desired mutation was verified by sequencing the entire DNA segment.

Expression of hPDE5A1 Proteins. Sf9 cells (BD Pharmingen) were cotransfected with BaculoGold linear baculovirus DNA (BD Pharmingen) and one of the hPDE5A1 constructs in the pAcHLT-A baculovirus expression vector by the calcium phosphate method according to the protocol from BD Pharmingen. At 5 days postinfection, the cotransfection supernatant was collected, amplified three times in Sf9 cells, and then used directly as viral stock without additional purification of recombinant viruses. 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 typically infected with 100 μL of viral stock/flask and then harvested at 92 h postinfection.

Purification of hPDE5A1 Proteins. The Sf9 cell pellet from each T-175 flask ($\sim 2 \times 10^7$ cells) was resuspended in 3 mL of ice-cold buffer (20 mM Tris-HCl, pH 8, and 100 mM NaCl) containing protease inhibitor mixture lacking EDTA (Complete; Roche Molecular Biochemicals) as recommended by the manufacturer. Cell suspension was homogenized in 10−20-mL aliquots on ice by 2 × 6-s bursts in an Ultra-Turrex microhomogenizer (Tekmar) with a 20-s recovery between bursts. Cell homogenate was centrifuged (10 000 rpm in a Beckman JA-20 rotor) for 20 min at 4 °C, and the supernatant was applied to a Ni-NTA agarose (Qiagen) column (1 × 2 cm) equilibrated with buffer containing 20mM Tris-HCl, pH 8, and 100 mM NaCl. The column was washed with 100 mL of the equilibration buffer followed by washes with the same buffer containing a stepwise gradient of imidazole (0.8−20 mM). The same buffer containing 100 mM imidazole was then soaked into the resin, and the column was incubated for 2 h at 4 °C before 10 1-mL fractions were collected. Fractions containing PDE5 protein were pooled and dialyzed versus 2000 volumes of ice-cold 10 mM potassium phosphate, pH 6.8, and 25 mM β-mercaptoethanol (KPM buffer) containing 150 mM NaCl to remove imidazole. All purification steps were done at 4 °C; enzyme was flash-frozen in KPM buffer containing 150 mM NaCl and 10% sucrose and stored at −70 °C. Activity in frozen samples was stable for at least 10 months.

SDS−PAGE. Purity and physical integrity of proteins were assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Protein samples were boiled for 4 min in the presence of 10% SDS, 2 M β-mercaptoethanol, and 0.1% bromphenol blue and subjected to SDS−12% PAGE before visualization by Coomassie Brilliant Blue staining.

cGMP Binding. 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-AS, 30 mM DL-dithiothreitol, 0.2 mM sildenafil, and either 3 μM (for stoichiometry determination) or 0.05−4 μM (for binding affinity determination) of [^{3}H]-cGMP (*25*). The binding reaction was initiated by addition of enzyme and incubated on ice for 60 min. One milliliter of cold buffer containing 10 mM potassium phosphate, pH 6.8 (KP buffer), was added to each sample, and samples were

filtered immediately onto premoistened Millipore HAWP filters (pore size 0.45 μm). Filters were washed with 2 mL of cold KP buffer two times, dried, and counted in nonaqueous 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 included for each [^{3}H]cGMP concentration.

PDE Catalytic Activity and Inhibitor Selectivity of hPDE5A1 Proteins. PDE activity was determined by a modification of the assay described previously (*20*). Reaction mixture contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 0−750 μM cGMP and [^{3}H]cGMP (60 000−150 000 cpm/assay tube) as substrate, and one of the PDE5 proteins in a total volume of 50−100 μL. Assay incubation time was 10−20 min at 30 °C. The apparent K_m and V_{max} or k_{cat} values were determined by nonlinear regression analysis of data with Prism GraphPad Software. In all studies, less than 10% of total [^{3}H]cGMP was hydrolyzed during the reaction. To determine IC_{50} values for sildenafil, vardenafil, tadalafil or IBMX, PDE catalytic activity was assayed in triplicate in three experiments in the presence of a wide range of inhibitor concentrations (from 1 to 10^9 pM) and 0.5 μM cGMP as substrate.

Calculation of Free Energy of Binding. All values determined represent three measurements, each in triplicate. The value for the Gibbs free energy change, ΔG, that occurs by association of either cGMP, vardenafil, sildenafil, tadalafil, or IBMX with PDE5 is related to the equilibrium association constant for the interaction and was calculated from

$$\Delta G = -RT \ln K \quad (1)$$

where $K = K_m$ or K_i, R is the ideal gas constant (equal to 1.98×10^{-3} kcal deg^{-1} mol^{-1}), and T is the temperature at which the assay was done (303 K). K_i values were calculated from the experimentally determined IC_{50} values for the inhibitors:

$$K_i = IC_{50}/1 + [S]/K_m \quad (2)$$

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

$$\Delta\Delta G = \Delta G_{WT} - \Delta G_{mut} \quad (3)$$

$\Delta\Delta G$ is the change in free energy of binding in enzyme−transition-state complexes attributable to the substituted group.

RESULTS

Expression and Purification of hPDE5A1 Proteins. Proteins were His-tagged constructs of full-length hPDE5A1; they included PDE5A1 containing wild-type sequence, $PDE5_{WT}$ (M1−N875), and mutants containing the following substitutions: Tyr612Phe ($PDE5_{Y612F}$), Tyr612Ala ($PDE5_{Y612A}$), His613Ala ($PDE5_{H613A}$), Leu765Ala ($PDE5_{L765A}$), Val782Ala ($PDE5_{V782A}$), Phe786Ala ($PDE5_{F786A}$), Gln817Ala ($PDE5_{Q817A}$), and Phe820Ala ($PDE5_{F820A}$). All proteins were expressed at high levels (~2−3 mg/L of culture) in a baculovirus/Sf9 expression system, and detected on SDS−PAGE followed by western blot analysis with a polyclonal PDE5-specific antibody (results not shown). Following purification as described under Experimental Procedures, each exhibited a high degree of purity (>98%) as determined by SDS−PAGE [12% (w/v) gel] stained with Coomassie Brilliant Blue (Figure 3) and migrated with essentially the same mobility as that of $PDE5_{WT}$ (a 99-kDa band). This migration correlated well with the predicted molecular weight based on amino acid composition of hPDE5. This indicated that all mutated PDE5s were expressed as full-length proteins. PDE5 proteins were characterized with respect to their allosteric cGMP-binding properties, kinetics of catalytic activity, and potency of inhibition by vardenafil, sildenafil, tadalafil, or IBMX.

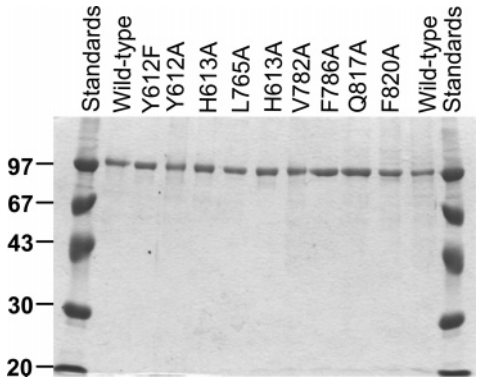

FIGURE 3: SDS−PAGE of purified wild-type hPDE5A1 and mutants. A 5 μg aliquot of wild-type or mutant hPDE5A1 protein that had been expressed in Sf9 cells and purified to apparent homogeneity through the Ni-NTA chromatography step as described under Experimental Procedures was applied to each lane. Low molecular weight standards (Pharmacia Biotech) were applied as size markers. Proteins were visualized by staining the gel with Coomassie Brilliant Blue dye. The two lanes of H613A represent two preparations that were separately expressed and purified.

Structural Integrity of PDE5 Mutants. To address overall structural integrity of the mutant PDE5 constructs, stoichiometry and affinity of [^{3}H]cGMP binding to the allosteric cGMP-binding sites in the R domain of PDE5 proteins were determined as described under Experimental Procedures. All PDE5A mutants bound cGMP with affinities (K_D ~ 190−210 nM) comparable to that of $PDE5_{WT}$ (K_D ~ 200 nM). The stoichiometry of cGMP binding for each mutant protein was 0.5−0.55 mol of [^{3}H]cGMP per PDE5A1 monomer, which was similar to that of $PDE5_{WT}$ (0.6 mol of [^{3}H]cGMP per monomer; data not shown). These values approximated those reported for native and non-His-tagged recombinant bovine PDE5 (*22*, *26*). The data indicated that overall structures of the proteins were preserved and that differences in their kinetic parameters and inhibitor affinities were not due to nonspecific conformational effects induced by mutations.

Kinetic Analyses of PDE5 Proteins. The kinetic characteristics of catalytic function (k_{cat} and K_m for cGMP) of each purified PDE5 construct were determined as described under Experimental Procedures (Table 1, Figure 4). The impact of each of the mutated amino acids on enzyme function fell into three categories: (1) those defective mainly in k_{cat}, (2) those defective mainly in K_m, and (3) those defective in both parameters. $PDE5_{WT}$ had a K_m for cGMP of 2.9 ± 0.8 μM and V_{max} of 1.3 ± 0.3 μmol min^{-1} mg^{-1} (k_{cat} value of 2.2 ± 0.4 s^{-1}). Only $PDE5_{H613A}$ and $PDE5_{F820A}$ had markedly reduced k_{cat} values, less than 0.04% and 1.8% that for $PDE5_{WT}$, respectively (Table 1). $PDE5_{Y612A}$, $PDE5_{L765A}$,

Table 1: Kinetic Parameters of Wild Type and PDE5A1 Mutants[a]

hPDE5A1	K_m, μM	K_m (*x*-fold effect)	k_{cat}, s^{-1}	k_{cat}/K_m ($\times 10^6$), M^{-1} s^{-1}	$\Delta\Delta G$ ($\Delta G_{WT} - \Delta G_{mut}$), kcal/mol
WT	2.9 ± 0.8	1	2.2 ± 0.4	0.758	
Y612F	6.1 ± 0.8	2	1.7 ± 0.2	0.272	0.45
Y612A	41.6 ± 2.1	14	0.8 ± 0.05	0.019	1.6
H613A	7.1 ± 2.4	2.4	0.00083 ± 0.00005	0.000012	0.5
L765A	27.7 ± 1.5	9.6	1.3 ± 0.1	0.047	1.4
V782A	15.9 ± 2.3	5.5	2.5 ± 0.2	0.157	1.00
F786A	29.7 ± 2.7	10	3.7 ± 0.2	0.124	1.4
Q817A	182 ± 5.2	63	3.8 ± 0.2	0.021	2.5
F820A	172 ± 5.5	59	0.04 ± 0.005	0.00023	2.5

[a] Values represent the average of three experiments of triplicate determinations ± standard deviations. *x*-fold effect for K_m was determined by dividing the K_m value for mutant by that for wild type for each protein. Change in the free energy of binding in enzyme−transition-state complexes attributable to the substituted group ($\Delta\Delta G$) was calculated by subtracting the ΔG value for mutant from that for wild type for each protein.

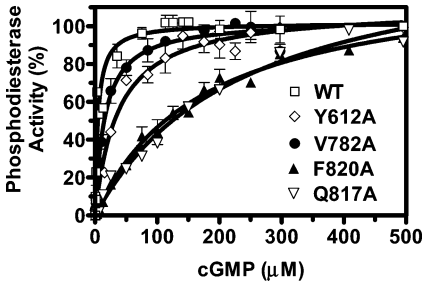

FIGURE 4: Comparison of cGMP hydrolysis by $PDE5_{WT}$, $PDE5_{Y612A}$, $PDE5_{V782A}$, $PDE5_{Q817A}$, and $PDE5_{F820A}$. Phosphodiesterase assays were conducted as described under Experimental Procedures. The data are derived from one of three experiments performed in triplicate and analyzed by use of Prism GraphPad Software (single-site model). Error bars indicate range of values within the single experiment shown. Experiments were conducted at a substrate concentration of 0−750 μM cGMP as described under Experimental Procedures.

$PDE5_{F786A}$, $PDE5_{Q817A}$, and $PDE5_{F820A}$ exhibited the most significant reductions in affinity for substrate compared to $PDE5_{WT}$ (14-, 10-, 10-, 63-, and 59-fold, respectively).

Effect of Mutation of His-613 on PDE5 Catalytic Function and Affinity for Inhibitors. PDE_{H613A} profoundly lowered catalytic activity (k_{cat} = 0.00083 ± 0.00005), but affinity for cGMP was decreased only 2.5-fold (K_m = 7.1 ± 2.4 μM) (Table 1); this result was verified for two separate preparations of purified H613A (Figure 3). Potency of $PDE5_{H613A}$ inhibition by vardenafil, sildenafil, tadalafil, or IBMX (IC_{50} 0.78, 13.2, 81, or 42 000 nM, respectively) was weaker than that for $PDE5_{WT}$ (IC_{50} 0.12, 3.3, 2.2, or 3500 nM, respectively) (Table 2). This mutation produced a particularly significant decline in tadalafil affinity (37-fold), compared to a more modest decline in affinity for vardenafil, sildenafil or IBMX (7-, 4-, or 12-fold, respectively). These results are in good agreement with our previous report (*22*) that substitution of the corresponding residue in bovine PDE5A (His-603) with Ala produced a marked decrease in k_{cat} (39-fold) and a modest decline in affinity for the cGMP substrate (3-fold) and the inhibitor zaprinast (6-fold), suggesting that His-613 is likely to be important for efficient catalysis.

Effect of Mutation of Tyr-612 on PDE5 Catalytic Function and Affinity for Inhibitors. We previously showed that $PDE5_{Y612A}$ had a 15-fold decrease in affinity for cGMP, whereas k_{cat} decreased only 2.5-fold (*19*). $PDE5_{Y612A}$ had a ∼123-fold loss in affinity for vardenafil which was much higher loss than that for sildenafil, tadalafil, or IBMX [30-, −51-, and 43-fold, respectively (Table 2)]. Substitution of phenylalanine for Tyr-612 only slightly reduced affinity for cGMP (2-fold) or tadalafil (∼3-fold), but affinity for sildenafil, vardenafil, or IBMX improved ∼2-fold. The k_{cat} of $PDE5_{Y612F}$ was unchanged compared to that of $PDE5_{WT}$. Our previous work (*22*) showed that the corresponding mutant ($PDE5_{Y602A}$) in bovine PDE5 exhibited dramatic increases in K_m for cGMP (32-fold), with a modest decrease in k_{cat} and affinity for zaprinast (4- and 7-fold, respectively), suggesting that this residue is particularly important for cGMP binding in the catalytic site. Zaprinast is a potent competitive inhibitor of PDE5, but the key residues for its binding differ significantly from those for cGMP, vardenafil, sildenafil, and tadalafil.

Effect of Mutation of Gln-817 on PDE5 Catalytic Function and Affinity for Inhibitors. Our previous results showed that substitution of Gln-817 by alanine ($PDE5_{Q817A}$) significantly altered affinity of the enzyme for cGMP, sildenafil, vardenafil, or IBMX, but maximum catalytic activity was not compromised (*20*). In the present studies the effect of this mutation on tadalafil potency was compared to the effect on affinity for cGMP and the other three inhibitors. $PDE5_{Q817A}$ again exhibited a 63-fold decrease in affinity for cGMP (K_m = 182 ± 5.2 μM) (Figure 4, Table 1) and potency for inhibitors was also decreased: vardenafil (511-fold), sildenafil (43-fold), tadalafil (95-fold), and IBMX (61-fold) (Figure 5, Table 2).

Effects of Mutation of Amino Acids Involved in Hydrophobic Clamp on PDE5 Catalytic Function and Affinity for Inhibitors. The cocrystal structures of PDE5 in complex with inhibitors revealed a "hydrophobic clamp" that is formed by Phe-820 and Val-782 in the inhibitor-binding pocket. The fused double rings of 5′-GMP, sildenafil, vardenafil, tadalafil, and IBMX are sandwiched (Figure 2) between the hydrophobic side chains of these residues. To examine the impact of the interactions of Phe-820 and Val-782 with substrate and inhibitors, these residues were individually replaced by alanine ($PDE5_{F820A}$ and $PDE5_{V782A}$, respectively).

$PDE5_{F820A}$ had a significant reduction in affinity for cGMP and catalytic activity. K_m for cGMP was weakened 59-fold (172 ± 5.5 μM) compared to that for $PDE5_{WT}$ (2.9 ± 0.8 μM) (Figure 4, Table 1), and catalytic activity (k_{cat} = 0.04 ± 0.005) was reduced 55-fold compared to $PDE5_{WT}$ (Figure 4, Table 1). Potencies of vardenafil, sildenafil, tadalafil, and IBMX were also significantly decreased (448-, 71-, 137-, and 93-fold, respectively) (Figure 5, Table 2) compared to $PDE5_{WT}$. This mutation produced a particularly dramatic decline in vardenafil affinity compared to that for sildenafil, tadalafil, or IBMX. The potency of vardenafil over

Table 2: Comparison of Effect of PDE5 Mutations on Potency of Inhibition by Vardenafil, Sildenafil, Tadalafil, or IBMX[a]

	IC_{50}, nM				rel IC_{50} (inhibitor potency, *x*-fold effect)			
hPDE5A1	vardenafil	sildenafil	tadalafil	IBMX	vardenafil	sildenafil	tadalafil	IBMX
wild type	0.12 ± 0.02	3.3 ± 0.5	2.2 ± 0.4	3.5 ± 0.3	1	1	1	1
Y612F	0.06 ± 0.01	1.3 ± 0.3	5.8 ± 1.2	1.9 ± 0.2	0.5	0.4	2.6	0.5
Y612A	14.7 ± 1.6	98.3 ± 4.7	112 ± 4.2	152 ± 11.2	123	30	51	43
H613A	0.78 ± 0.1	13.2 ± 1.8	81 ± 5.1	42.3 ± 1.1	7	4	37	12
L765A	0.49 ± 0.07	10.1 ± 0.8	9.1 ± 0.4	3.0 ± 0.1	4	3	4	0.9
V782A	2.75 ± 0.1	31.9 ± 1.5	6.7 ± 0.9	42.5 ± 2.1	23	10	3	12
F786A	0.83 ± 0.1	20.6 ± 2.2	37.5 ± 2.5	43.3 ± 2.5	7	6	17	12
Q817A	61.3 ± 3.1	141 ± 3.5	210 ± 6.5	214 ± 4.3	511	43	95	61
F820A	53.7 ± 3.5	235 ± 12	301 ± 13	327 ± 17	448	71	137	93

[a] All values are mean ± SEM of three experiments of triplicate determinations. To facilitate comparison, the IC_{50} values for vardenafil, sildenafil, tadalafil and IBMX for $PDE5_{WT}$ were taken as 1.0. The corresponding values for the PDE5 mutants were divided by the $PDE5_{WT}$ value to calculate the *x*-fold change.

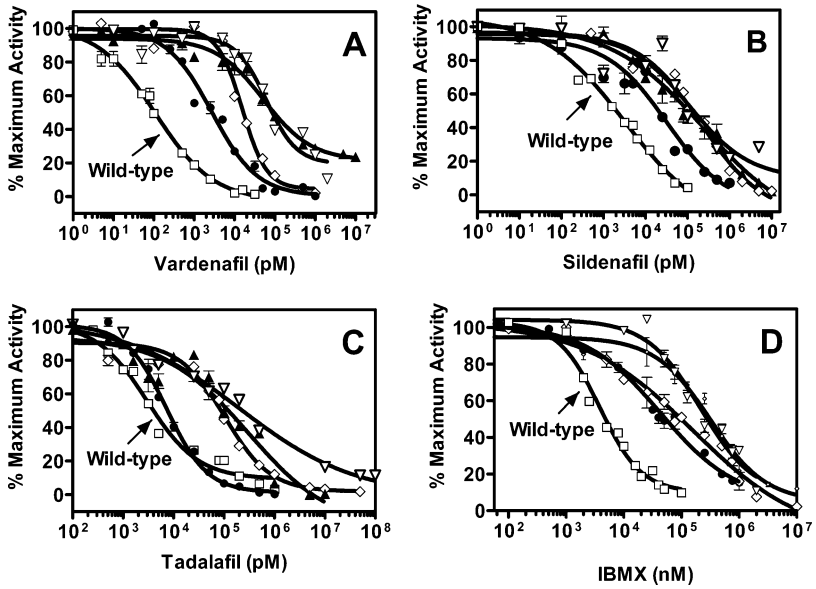

FIGURE 5: Potency of inhibition of catalytic activity of $PDE5_{WT}$, $PDE5_{Y612A}$, $PDE5_{V782A}$, $PDE5_{Q817A}$, and $PDE5_{F820A}$ by (A) vardenafil, (B) sildenafil, (C) tadalafil, and (D) IBMX. Potency of inhibition of (□) $PDE5_{WT}$, (◇) $PDE5_{Y612A}$, (●)$PDE5_{V782A}$, (▽) $PDE5_{Q817A}$, or (▲) $PDE5_{F820A}$ was determined as described under Experimental Procedures with 0.5 μM cGMP substrate. The data are derived from one of three experiments performed in triplicate and analyzed with Prism GraphPad Software (single-site model). Error bars indicate range of values within the single experiment shown. The final concentration of PDE5 proteins used in these studies was 0.45−0.70 nM.

sildenafil declined sharply from 28-fold in $PDE5_{WT}$ to 4-fold in $PDE5_{F820A}$.

Changes in affinity of $PDE5_{V782A}$ for cGMP and catalytic function were much less than that of $PDE5_{F820A}$; $PDE5_{V782A}$ exhibited a ~6-fold loss in affinity for cGMP as substrate ($K_m = 15.9 \pm 2.3\ \mu M$) (Figure 4, Table 1) and no significant change in k_{cat}. The deleterious effects of the alanine substitution of Val-782 on potency of vardenafil, sildenafil, tadalafil, and IBMX were substantially less (23-, 10-, 3-, and 12-fold, respectively) (Figure 5, Table 2) than the effects of substitution of Phe-820. The relative influence of Phe-820 in the hydrophobic clamp compared to that of Val-782 (*x*-fold effect of F820A vs V782A) on affinity for cGMP, vardenaflil, sildenafil, tadalafil, and IBMX varied somewhat (10.7-, 19.4-, 7.1-, 45.7-, and 7.8-fold, respectively), but the influence of Phe-820 was always substantially greater for all five ligands. These results revealed the particular importance of Phe-820 in the hydrophobic clamp to provide effective hydrophobic interaction for PDE5 inhibitors.

Influence from Other Catalytic-Site Residues on PDE5 Catalytic Activity and Inhibitor Affinity. The importance of other hydrophobic contacts (Phe-786 and Leu-765) previously identified in the substrate and inhibitor binding pocket of PDE5 were also quantified. $PDE5_{F786A}$ showed a 10-fold decrease in affinity for cGMP ($K_m = 29.7 \pm 2.7\ \mu M$) (Table 1) with no decline in catalytic activity ($k_{cat} = 3.7 \pm 0.2$); this revealed the involvement of hydrophobic interactions of Phe-786 in substrate binding. Potency of inhibition of $PDE5_{F786A}$ by vardenafil, sildenafil, tadalafil, or IBMX was significantly weaker than that for $PDE5_{WT}$ (7-, 6-, 17-, and 12-fold, respectively) (Table 2). Mutation of Leu-765 ($PDE5_{L765A}$), which is in close proximity with inhibitors in the X-ray cocrystal structures of PDE5 C domain (Figure 2), weakened affinity for cGMP by ~10-fold but had a modest effect on k_{cat} (Table 1). Change in affinity of $PDE5_{L765A}$ for vardenafil, sildenafil, tadalafil, or IBMX was modest (4-, 3-, 4-, and 0.9-fold, respectively).

Contribution of Catalytic-Site Amino Acids to Free Energy of Binding of cGMP or Inhibitors. The energy involved in interactions of cGMP, vardenafil, sildenafil, tadalafil, or IBMX with $PDE5_{WT}$ were quantified from the K_m for cGMP or the K_i for the respective inhibitors by calculating the Gibbs free energy of binding (ΔG) of 7.7, 13.8. 11.0, 12.1, and 7.5 kcal mol^{-1}, respectively, as described under Experimental Procedures. These values reflected the varying affinities with which the respective ligands bind to the PDE5 catalytic site.

Table 3: Comparison of Effect of PDE5 Mutations on Affinity for Vardenafil, Sildenafil, Tadalafil, or IBMX[a]

	ΔG_{mut}, kcal/mol				$\Delta\Delta G$ ($\Delta G_{WT} - \Delta G_{mut}$), kcal/mol			
hPDE5A1	vardenafil	sildenafil	tadalafil	IBMX	vardenafil	sildenafil	tadalafil	IBMX
wild type	13.8	11.0	12.1	7.5				
Y612F	14.2	12.4	11.5	8.0	−0.4	−1.4	0.6	−0.5
Y612A	10.9	9.8	9.7	5.4	2.9	2.0	2.4	2.3
H613A	12.7	11.0	9.9	6.1	1.1	0.8	2.2	1.5
L765A	13.0	11.1	11.2	7.7	0.8	0.7	0.9	0.0
V782A	11.9	10.5	11.4	6.1	1.9	1.4	0.7	1.5
F786A	12.6	10.7	10.4	6.1	1.2	1.1	1.7	1.5
Q817A	10.0	9.6	9.3	5.2	3.7	2.3	2.7	2.5
F820A	10.1	9.3	9.1	4.9	3.7	2.6	3.0	2.7

[a] All values are average of three experiments of triplicate determinations. Change in the free energy of binding in enzyme−transition-state complexes attributable to the substituted group ($\Delta\Delta G$) was calculated by subtracting the ΔG value for mutant from that for wild type for each protein for each inhibitor.

In mutant proteins, the magnitude of the contribution of the substituted amino acid side chains to the binding energy in enzyme−transition-state complexes ($\Delta\Delta G$) was quantified by calculating the Gibbs free energy of binding (ΔG) of cGMP, vardenafil, sildenafil, tadalafil, and IBMX in PDE5 mutants compared to the free energy of binding of these ligands in $PDE5_{WT}$ (Tables 1 and 3).

The change in the free energy of binding ($\Delta\Delta G$) of $PDE5_{Y612A}$ for cGMP, vardenafil, sildenafil, tadalafil, or IBMX was equivalent to 1.6, 2.9, 2.0, 2.4, and 2.3 kcal mol^{-1}, respectively (Table 3). The free energy of binding ($\Delta\Delta G$) of $PDE5_{Q817A}$ with cGMP, vardenafil, sildenafil, tadalafil, or IBMX was substantially weakened compared to $PDE5_{WT}$ as reflected in $\Delta\Delta G$ values that were decreased by 2.5, 3.7, 2.3, 2.7, and 2.5 kcal mol^{-1}, respectively (Table 3). The magnitude of the change in free energy of binding of $PDE5_{Y612A}$ for vardenafil and tadalafil as well as the change in $PDE5_{Q817A}$ for cGMP and inhibitors was consistent with loss of at least one hydrogen bond (*27*, *28*). The loss of free energy of binding ($\Delta\Delta G$) in the $PDE5_{F820A}$ mutant for cGMP, vardenafil, sildenafil, tadalafil, and IBMX was equivalent to 2.5, 3.7, 2.6, 3.0, and 2.7 kcal mol^{-1}, respectively (Table 3), suggesting that stacking interactions might have a role in higher binding affinity of PDE5 toward vardenafil in comparison with cGMP and other inhibitors.

DISCUSSION

PDE5 provides a major portion of the cGMP-hydrolyzing activity in many tissues; it is the target of sildenafil (Viagra), tadalafil (Cialis), vardenafil (Levitra), and udenafil (Zydena), all of which are competitive inhibitors of PDE5 and used for treatment of male erectile dysfunction and other maladies associated with vascular diseases. However, the molecular bases for interaction of either substrate, cGMP, or inibitors with the PDE5 catalytic site are still poorly understood. In the present study, Tyr-612, His-613, Leu-765, Val-782, Phe-786, Gln-817, and Phe-820, which in X-ray structures of cocrystals of PDE5 with either 5′-GMP or inhibitor were shown to be in close proximity to these ligands, have been individually mutated to alanine. The importance of these residues to catalysis and interaction with either substrate or inhibitors varies substantially. All mutations diminish catalytic efficiency, in most cases due to effects on K_m for the substrate, cGMP. Tyr-612, Gln-817, and Phe-820 play prominent roles in interaction of PDE5 with catalytic-site ligands irrespective of their absolute potencies; Phe-820 and His-613 also importantly impact catalytic rate. The k_{cat} values of $PDE5_{Y612A}$, $PDE5_{V782A}$, $PDE5_{F786A}$, and $PDE5_{Q817A}$ are either equal to or exceed that for $PDE5_{WT}$, and k_{cat} is modestly lowered in $PDE5_{Y612A}$ and $PDE5_{L765A}$. Allosteric cGMP-binding function in all mutants is similar to that of $PDE5_{WT}$. In combination, these characteristics make it unlikely that the effects of these mutations are the result of general impairment of the protein, although, as with any study using site-directed mutagenesis, this possibility cannot be ruled out. It is unlikely that the catalytic-site mutations alter quaternary structure of PDE5 since the high-affinity (K_D < 20 pM) dimerization contacts within the enzyme structure are located in the regulatory domain (*25*).

The importance of contact(s) provided by invariant Tyr-612 for binding four inhibitors of differing potencies and structures exceeds that for substrate (cGMP) affinity or catalysis. According to the X-ray cocrystal structures, Tyr-612 contributes both hydrophobic and hydrophilic interactions in the PDE5 catalytic site (*1*, *2*, *11*), and the $PDE5_{Y612A}$ mutant has a dramatic loss in affinity for cGMP and inhibitors. A nitrogen in the five-member ring of sildenafil or vardenafil forms a hydrogen bond with a water that is hydrogen-bonded to the Tyr-612 hydroxyl and another water that coordinates to the catalytic-site Zn^{2+} (Figure 2A) (*1*, *11*). Tadalafil does not make similar contacts (Figure 2B) (*1*). Removal of the Tyr-612 hydroxyl ($PDE5_{Y612F}$) actually increases affinity for sildenafil, vardenafil, and IBMX. This is surprising since the hydrogen-bond bridge involving the hydroxyl (Figure 2A) would have been predicted to increase affinity (*1*, *11*). However, this hydroxyl may compromise interaction with the inhibitors by altering orientation of the phenyl ring or reducing volume in the catalytic pocket. Tadalafil or cGMP affinity is modestly reduced despite the fact that tadalafil lacks the hydrogen-bond contact (Figure 2B) (*1*). The results indicate that Tyr-612 contributes substantially to the potency difference between vardenafil and sildenafil or tadalafil and that the phenyl ring is critical for potency of interaction with all five catalytic-site ligands.

Gln-817 is invariant in mammalian PDEs; this glutamine has been suggested to be an important determinant of substrate affinity in the PDE superfamily, but evaluation of the effect of point mutation of this residue on substrate affinity and specificity has been tested only in PDE5 (*15*, *20*). The X-ray crystal structure of PDE5 isolated C domain in complex with the low-affinity product, 5′-GMP, implies that the Gln-817 side chain forms a bidentate hydrogen bond

with guanine (*15*). The X-ray cocrystal structure of PDE5 with sildenafil, vardenafil, or IBMX exhibits similar bidentate hydrogen bonding with Gln-817, whereas tadalafil forms a single hydrogen bond (*1*, *2*, *11*). $PDE5_{Q817A}$ exhibits major loss in affinity for cGMP, vardenafil, sildenafil, tadalafil, and IBMX but not for cAMP, a low-affinity substrate, which suggests that the low affinity of cAMP interaction may result in part from poor contact with Gln-817 (*20*). Loss in vardenafil potency in $PDE5_{Q817A}$ is 5−12 times greater than that for the other ligands. $PDE5_{Q817A}$ selectivity for vardenafil over sildenafil or tadalafil, compared to $PDE5_{WT}$, declines from 28- to ∼2-fold and from 18- to ∼3-fold, respectively. $PDE5_{Q817A}$ k_{cat} for cGMP is greater than that for $PDE5_{WT}$ (1.7-fold); affinity for cAMP is only 2-fold weaker than that for $PDE5_{WT}$, and k_{cat} for cAMP, which is similar to that for cGMP, is unaffected (*20*). This indicates that the deleterious effect of this mutation on cGMP catalysis is not due to generalized impairment of the catalytic site.

Despite absolute differences in PDE5 affinity for these inhibitors, and disparate hydrogen-bonding patterns reported or predicted from X-ray crystallographic structures, the loss in free energy for each is similar. In particular, the loss in free energy of binding in $PDE5_{Q817A}$ for sildenafil and IBMX, both of which form bidentate bonds with Gln-817 in the X-ray crystal structures, is the same as the loss in free energy for tadalafil binding, which forms only one hydrogen bond with Gln-817 in the cocrystal X-ray structure (*1*, *2*, *11*). Since the absolute energy that is involved in a hydrogen bond between a ligand and its protein receptor varies considerably (*27*−*29*), it cannot be stated with certainty that the hydrogen-bonding patterns for ligands suggested from the present studies differ from those detected in the X-ray cocrystal structures with isolated C domain. However, as was concluded many years ago, the results of studies demonstrating the high affinity of PDE5 for cGMP analogues containing a substituent at the N-1 position [1-CH_3-cGMP, 1, N^2-phenyletheno-cGMP, β-(2-napthyl)-1,N^2-etheno-cGMP, and 1,N^2-(4-methoxyphenyletheno)-cGMP] indicate that hydrogen bonding via the N-1 hydrogen of the pyrimidine of cGMP is not a key component of affinity for cGMP or cGMP analogues (*30*−*32*). Rather, the combined results of analogue studies, mutagenesis studies, and calculation of the loss in free energy support the interpretation that there is functionally a single hydrogen bond contact between Gln-817 in the catalytic site of PDE5 and the C-6 carbonyl oxygen (or the equivalent thereof) in cGMP, vardenafil, sildenafil, or IBMX.

We have earlier reported another difference between predictions from X-ray crystal structure of PDE5 isolated C domain and effects of mutations in the holoenzyme. In that study, mutation of Gln-775 ($PDE5_{Q775A}$), which in the X-ray crystal structure forms a hydrogen bond with Gln-817, thereby orienting the Gln-817 side chain for purportedly optimal bidentate hydrogen bonding with substrate, has no effect on affinity for vardenafil or sildenafil (*20*); in X-ray crystal structures, vardenafil and sildenafil are predicted to require the same fixed orientation of the Gln-817 side chain for forming these bonds. The differences between the predicted importance of bonds in the X-ray crystal structures and the impact of removal of these bonds by point mutation or amino-teminal truncation mutants on interaction in solution with several ligands (cGMP, vardenafil, sildenafil, IBMX, and cAMP) emphasize the importance of assessing functional significance of amino acids in the context of the holoenzyme (*16*).

A hydrophobic clamp anchors substrate or inhibitors in the active site of mammalian PDEs (*1*, *2*, *5*, *8*, *10*−*12*). The phenylalanine that forms one side of the clamp (Phe-820 in PDE5A) is conserved in almost all mammalian PDEs, and another conserved hydrophobic residue (Val-782 in PDE5) forms the other side. In X-ray cocrystal structures, all four inhibitors interact with the clamp (and based on modeling, cGMP does also). The five-membered rings in vardenafil, sildenafil, and IBMX form a face-to-face $\pi-\pi$ electron stacking interaction with the Phe-820 phenyl ring (Figure 2A).

The dramatic difference in effect of the F820A mutation on vardenafil versus cGMP or sildenafil affinity indicates that, despite purportedly similar interactions between Phe-820 and vardenafil, sildenafil, or 5′-GMP, respectively, as indicated in the X-ray cocrystal structures, significant differences exist. The results also emphasize the importance of Phe-820 along with Gln-817 as a determinant of vardenafil potency and selectivity over sildenafil or tadalafil. The similar magnitude of change in affinity for sildenafil and IBMX in $PDE5_{F820A}$ indicates that, despite the ∼1200-fold difference in potencies, the relative importance of Phe-820 for binding of each is similar.

Substitution of Val-782 impacts affinity of the inhibitors far less than that for substitution of Phe-820. $PDE5_{V782A}$ exhibits a 3-fold decline in tadalafil potency compared to the 137-fold decline in $PDE5_{F820A}$. This suggests that tadalafil primarily contacts Phe-820 in the clamp. In X-ray cocrystal structures Leu-765 is closely approximated to the inhibitors, but $PDE5_{L765A}$ has modest effects on inhibitor potency with slightly greater impact on cGMP affinity. $PDE5_{F786A}$ exhibits modest effects on potency of vardenafil, sildenafil, IBMX, or cGMP, whereas tadalafil inhibitory potency is reduced 17-fold. This suggests that targeting differences in the impact of amino acids in the hydrophobic clamp region on inhibitor potency within and among PDE families may be useful in designing selective PDE inhibitors for particular PDEs.

His-613, which is invariant in all class I PDEs, is closely juxtaposed to the heterocyclic ring of several inhibitors in the X-ray cocrystal structures but lacks direct contact. $PDE5_{H613A}$ affinity for cGMP, vardenafil, sildenafil, or IBMX is modestly impaired, but tadalafil potency is affected more (37-fold). This suggests that tadalafil positioning in the catalytic site directly or indirectly exploits structural features provided by His-613. As reported previously, $PDE5_{H613A}$ has a major loss in catalytic function (*22*). Taken together, the data indicate that His-613 provides for optimal substrate hydrolysis in PDE5 and high affinity for certain classes of inhibitors.

To date, X-ray crystal structures of PDE catalytic sites share many conserved physical features that provide contacts for binding substrate as well as selective and nonselective inhibitors (*5*, *7*−*9*, *11*, *12*, *33*). Structural variation within and external to the catalytic sites provides for major differences in interaction with inhibitors, but the differences are not fully defined. We recently reported selective impact of PDE5 R domain on potency of vardenafil over sildenafil or tadalafil (*16*), and similar observations have been made regarding PDE4 affinity for rolipram (*17*, *34*). Combined insights derived from X-ray crystallographic structures of

isolated C domains and enzymatic studies using mutated PDE holoenzymes allow for better quantification of the impact of highly conserved structural features on potency of inhibitors of varying structures. These insights will aid significantly in the design of more potent and selective inhibitors toward different PDE families or even within a PDE family. This is the first comprehensive study to address the quantitative contribution of amino acids in the catalytic site of any PDE using the X-ray crystal structure for direction.

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