Phosphodiesterase-5 Gln\textsuperscript{817} Is Critical for cGMP, Vardenafil, or Sildenafil Affinity

ITS ORIENTATION IMPACTS cGMP BUT NOT cAMP AFFINITY\textsuperscript{a,b}

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The side group of an invariant Gln in cGMP- and cAMP-specific phosphodiesterases (PDE) is held in different orientations by bonds with other amino acids and purportedly discriminates between guanine and adenine in cGMP and cAMP. In cGMP-specific PDE5, Gln\textsuperscript{775} constrains the orientation of the invariant Gln\textsuperscript{817} side chain, which forms bidentate bonds with 5'-GMP, vardenafil, sildenafil, and 3-isobutyl-1-methylxanthine (IBMX) (Sung, B. J., Hwang, K. Y., Jeon, Y. H., Lee, J. I., Heo, Y. S., Kim, J. H., Moon, J., Yoon, J. M., Hyun, Y. L., Kim, E., Eum, S. J., Park, S. Y., Lee, J. O., Lee, T. G., Ro, S., and Cho, J. M. (2003) Nature 425, 98–102; Huai, Q., Liu, Y., Francis, S. H., Corbin, J. D., and Ke, H. (2004) J. Biol. Chem. 279, 13095–13101; Zhang, K. Y., Card, G. L., Suzuki, Y., Artis, D. R., Fong, D., Gillette, S., Hsieh, D., Neiman, J., West, B. L., Zhang, C., Milburn, M. V., Kim, S. H., Schlessinger, J., and Bollag, G. (2004) Mol. Cell 15, 279–286). PDE5\textsubscript{Q817A} and PDE5\textsubscript{Q775A} were generated to test the hypotheses that Gln\textsuperscript{817} is critical for cyclic nucleotide inhibitor affinity and that Gln\textsuperscript{775} immobilizes the Gln\textsuperscript{817} side chain to provide cGMP/cAMP selectivity. Allosteric cGMP binding and the molecular mass of the mutant proteins were unchanged compared with PDE5\textsubscript{WT}. For PDE5\textsubscript{Q817A}, \( K_m \) for cGMP or cAMP was weakened 60–80-fold, respectively. For PDE5\textsubscript{Q775A}, \( K_m \) for cGMP was weakened ~20-fold but was unchanged for cAMP. For PDE5\textsubscript{Q817A}, vardenafil, sildenafil, and IBMX inhibitory potencies were weakened 60–80, and 60-fold, respectively, indicating that Gln\textsuperscript{817} is a major determinant of potency, especially for vardenafil, and that binding of vardenafil and sildenafil differs substantially. Sildenafil and vardenafil affinity were not significantly affected in PDE5\textsubscript{Q775A}. It is concluded that Gln\textsuperscript{817} is a positive determinant for cGMP affinity and several inhibitors; Gln\textsuperscript{775}, which perhaps restricts rotation of Gln\textsuperscript{817} side chain, is critical for cGMP affinity but has no measurable effect on affinity for cAMP, sildenafil, or vardenafil.

Cyclic nucleotide (cN)\textsuperscript{2} phosphodiesterases (PDE) cleave the 3',5'-cyclic phosphate bond of cNs. Among cGMP- or cAMP-specific PDEs, affinities for the cNs differ by 100-fold or more (1–4). Co-crystals of PDE catalytic domains (C domain) with the products provide insight into mechanisms contributing to cAMP and cGMP binding and selectivity (5–11). In addition, inhibitors and 5'-GMP form some of the same contacts in PDE5 (12–14).

Information from the crystal structure of the PDE4B led Xu et al. (15) to propose that an invariant glutamine might provide for cN selectivity (Fig. 1A); they noted that the side chain of the invariant glutamine (Gln\textsuperscript{455}) is held in place by hydrogen bond (H-bond) with the Tyr\textsuperscript{403} hydroxyl and in this position forms a bidentate H-bond with two positions in adenine of cAMP (Fig. 1A). Xu et al. (15) suggested that the side chain of the invariant glutamine could rotate ~180° so that the positions of the Oe and Ne of the side chain would be reversed and could form a bidentate H-bond complex with the guanine of cGMP, which has different H-bonding potential.

As predicted, in crystal structures of cGMP-specific PDE5, the invariant glutamine (Gln\textsuperscript{817}) side chain is immobilized by H-bond to Gln\textsuperscript{775} (12, 13). In a co-crystal structure of PDE5 C domain with the 5'-GMP product, the Gln\textsuperscript{817} side chain forms a bidentate H-bond with the N-1 proton and C-6 carbonyl oxygen of guanine (Fig. 1A) (14). In x-ray crystal structures of dual specificity PDEs, the side chain of the invariant glutamine can adopt both configurations shown in Fig. 1A, allowing for interaction with either cN (9, 14). Based on these observations, orientation of the side chain of the invariant glutamine was referred to as the "glutamine switch" (14).

Crystal structures of PDE5 C domain bound with the inhibitors sildenafil or vardenafil revealed that atoms in these inhibitors that are analogous to substituents at N-1 and C-6 in cGMP form a bidentate H-bond with the Gln\textsuperscript{817} side chain (Fig. 1B) (12). 3-Isobutyl-1-methylxanthine (IBMX), a weak inhibitor, also forms a bidentate H-bond with Gln\textsuperscript{817}, but its orientation and interactions differ (Fig. 1B) (13). X-ray crystal structures of isolated C domains of PDEs co-crystallized with ligands provide invaluable insights into contacts and topography of the catalytic sites. However, the importance of these contacts must be experimentally quantified in holoenzymes to assess the role of these interactions in the enzyme-ligand complex and to provide appropriate direction for improved drug design. The combined approaches provide a more accurate profile of catalytic site function.

Based on the prediction of the glutamine switch as the determinant of cN selectivity in PDEs and supporting x-ray crystallographic evidence, we hypothesized that Gln\textsuperscript{817} is a critical contact for interaction with cGMP, vardenafil, sildenafil, or IBMX and that H-bonding of the Gln\textsuperscript{817} side chain with Gln\textsuperscript{775} optimizes the configuration for contact with cGMP, vardenafil, sildenafil, and IBMX but not with cAMP. Removal of the H-bonding potential provided by Gln\textsuperscript{775} was predicted to free the rotational constraint of Gln\textsuperscript{817}, weaken affinity for cGMP, and improve affinity for cAMP substantially. To address these questions, point mutations of Gln\textsuperscript{817} and Gln\textsuperscript{775} were created to determine (a) the importance of Gln\textsuperscript{817} in providing for potency of interaction of PDE5 with cGMP,
cAMP, or inhibitors and (b) the role of Gln775 in providing for cGMP versus cAMP specificity in PDE5 and its impact on sildenafil or vardenafil potency.

EXPERIMENTAL PROCEDURES

Materials—[3H]cGMP was purchased from Amersham Biosciences. IBMX, Crotalus atrox snake venom, cGMP, and histone type II-AS were obtained from Sigma. Sildenafil was purified from Viagra tablets as described in our earlier report (16).

Generation of Wild-type and Mutant (Q817A and Q775A) hPDE5A1—Human cDNA coding for full-length PDE5A1 (courtesy of Dr. K. Omori, Tanabe-Seiyaku Pharmaceutical Co. Ltd., Saitama, Japan) was used as the template to generate full-length PDE5A1 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 EcoRI and NotI unique sites of the baculovirus expression vector pAcHLT-A (Pharmingen). pAcHLT-A vector contains a His6 sequence that precedes the coding region. This step resulted in plasmid pAcA-PDE5 (Met1–Asn875), which generated N-terminally His-tagged recombinant hPDE5A1. The QuikChange site-directed mutagenesis kit (Stratagene) was used to make point mutations (Gln817 to Ala (Q817A) and Gln775 to Ala (Q775A)) in the pAcA-PDE5 expression vector according to the manufacturer’s protocol using the following pairs of mutagenic oligonucleotides (altered bases are underlined): 1) for Q817A, 5'-ATCCCAAGTATGGCAGTTGGGTTCATAG-3' and 5'-CTATGAACCCAACTGCCATACTTGGGAT-3'; 2) for Q775A, 5'-AACCCCTGGCCCTATTGCA-
Expression of Wild-type and Mutant hPDESA1—Sf9 cells (BD Pharmering) were cotransfected with BaculoGold linear baculovirus DNA (BD Pharmering) and one of the hPDEA1 constructs (PDE5wt
(Met1–Asn875), PDE5Q817A (Met1–Asn875/Q817A) and PDE5Q775A
(Met1–Asn875/Q775A) in the pAcHL-T baculovirus expression vector by the calcium phosphate method according to the protocol from BD Pharmering. At 5 days post-infection, the cotransfection supernatant was collected, amplified three times in Sf9 cells, and used directly as viral stock for expression 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 harvested 92 h post-infection.

Purification of Wild-type and Mutant hPDESA1—Purification steps were done at 4 °C. The Sf9 cell pellet for each T-175 flask (2–10 x 107 cells) was resuspended in 3 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 0.3 M NaCl) containing Complete™ protease inhibitor (Roche Molecular Biochemicals) as recommended by the manufacturer. Cell suspension was homogenized in 10–20 ml aliquots on ice by two 6-s bursts in an Ultra Turrex microhomogenizer (Tekmar) with a 20-s recovery between bursts. Cell homogenate was centrifuged (20 min, 10,000 rpm, in a Beckman JA-20 rotor). The supernatant was applied to a nickel-nitriotriacetic acid-agarose (Qiagen) column (1 x 2 cm) equilibrated with lysis buffer. The column was sequentially washed with 100 ml of lysis buffer and a stepwise gradient of imidazole (0.8 to 20 mM) in lysis buffer. Lysis buffer containing 100 mM imidazole was soaked into the resin, and after 2 h, ten 1-ml fractions were collected. Elutions containing PDE5 protein were dialyzed versus 2000 volumes of 10 mM potassium phosphate, pH 6.8, 25 mM β-mercaptoethanol, and 150 mM NaCl, flash-frozen in the same buffer containing 10% sucrose, and stored at −70 °C. Activity in frozen samples was stable for at least 10 months.

SDS-PAGE of hPDESA1 Constructs—The purity and integrity of proteins were assessed using SDS-PAGE. Protein samples were boiled for 4 min in the presence of 10% SDS, 2 M β-mercaptoethanol, and 0.1% bromophenol blue and subjected to 12% SDS-polyacrylamide gel electrophoresis before visualization by Coomassie Brilliant Blue staining.

cGMP Binding—To measure allosteric cGMP-binding, Millipore HAWP filters (pore size 0.45 μm) were premoistened Millipore HAWP filters (pore size 0.45 μm). Filters were washed twice with 2 ml of cold KP buffer, dried, and counted using non-aqueous Ready Safe scintillation mixture (Beckman). Counts bound to PDE5 were corrected by subtraction of nonspecific binding (+1 mM unlabeled cGMP). Blanks containing no PDE5 were run for each [3H]cGMP concentration.

Catalytic Activity—PDE activity was determined as described (17). Reaction mixture contained 50 mM Tris HCl, pH 7.5, 10 mM MgCl2, 0.3 mg/ml bovine serum albumin, and either cGMP (0–750 μM and [3H]cGMP (60,000–150,000 cpm/assay tube)) or cAMP (0–1500 μM and [3H]cAMP (60,000–150,000 cpm/assay tube)) as substrate and one of the PDE5 proteins in a total volume of 50–100 μl. Incubation time was 10–20 min at 30 °C. Apparent Km and Vmax were determined by non-linear regression analysis of data using Prism Graphpad software. In all studies, <10% of total [3H]cNMP was hydrolyzed. To determine IC50 for sildenafil, vardenafil, or IBMX, PDE catalytic activity was assayed in triplicate in the presence of a range of inhibitor concentrations (1–1,000,000,000 pM) with 0.5 μM cGMP as substrate. Ki values were calculated using the equation Ki = IC50/1 + [S]/Km.

Calculation of Free Energy of Binding—The Gibbs free energy change, ΔG, which occurs by association of a ligand with PDE5, is related to the equilibrium association constant for the interaction and was calculated using Equation 1,

$$
\Delta G = -RT \ln K
$$

(Eq. 1)

where K = Km or Ki calculated from IC50 values for the inhibitors as described above R is the ideal gas constant (equal to 1.98 x 10^{-3} kcal/degree/mol), and T is the temperature at which the assay was done (303 K). Values reported represent three measurements, each in triplicate. High-affinity interaction is indicated when ΔG is a large negative value. The contribution of a substituted amino acid side chain to the Gibbs free energy of binding in enzyme-transition state complexes was calculated from the biochemical potency (Km or Ki values calculated from the IC50 values for the inhibitors) using Equation 2,

$$
\Delta \Delta G = \Delta G_{WT} - \Delta G_{mut}
$$

(2)

where ΔΔG is the change in free energy of binding in enzyme-transition state complexes attributable to the substituted group (18, 19).

RESULTS AND DISCUSSION

Enzymes in these studies were His-tagged constructs that had been expressed in Sf9 cells, purified, and characterized as described under “Experimental Procedures.” Structural integrity of each was verified by pattern of migration on SDS-PAGE and allosteric cGMP-binding properties (see supplemental material).

Effect of Gln817 Substitution on PDE5 Catalytic Function—Kinetic characteristics of catalytic function of purified PDE5 constructs were determined as described under “Experimental Procedures” (Fig. 2A, Table 1). PDE5WT had a Km for cGMP of 2.9 ± 0.8 μM and a kcat of 2.2 ± 0.4 s⁻¹. Gln817 was replaced by alanine (PDE5Q817A), which lacks the Oe and Ne groups that in the PDE5 crystal structure were shown to form a bidentate H-bond with 5′-GMP, sildenafil, or vardenafil; these contacts were inferred to also occur with cGMP. PDE5Q817A site affinity for cGMP was decreased 60-fold (Km = 180 ± 52 μM) (Fig. 2A, Table 1), indicating that the Gln817 side chain is extremely important for high-affinity interaction with cGMP; kcat (3.8 ± 0.2 s⁻¹) was slightly improved.

PDE5WT hydrolyzes cAMP with ~100-fold lower affinity (Km = 290 ± 80 μM) than for cGMP; kcat for cAMP hydrolysis was 1.6 ± 0.2 s⁻¹. PDE5Q817A affinity for cAMP (Km = 630 ± 42 μM) was slightly weaker (~2-fold) than that of PDE5WT; kcat (3.0 ± 0.3 s⁻¹) was slightly improved (Fig. 2B, Table 1). Catalytic efficiency (kcat/Km) for hydrolysis of cGMP declined 36-fold in PDE5Q817A (Table 1) but was unchanged for cAMP. The selective impact of removal of the Gln817 side chain on affinity and catalytic efficiency for cGMP versus cAMP as substrate was consistent with the importance of this contact with PDE5 and 5′-GMP. The lack of a deleterious effect of the mutation on kcat suggested that the mutation did not introduce a globally deleterious effect on the catalytic site.

CAACCGATAGCAG-3’ and 5’-CTGCTATCCGTGTGCAATAGG-GACGGGTT-3’. The presence of the desired mutation was verified by sequencing the entire DNA segment.
PDE5 Gln<sup>817</sup> Role in Affinity for cGMP, cAMP, and Inhibitors

Effect of Gln<sup>775</sup> Substitution on PDE5 Catalytic Function—The $k_{cat}$ (4.6 ± 0.2 s<sup>−1</sup>) for hydrolysis of cGMP and $k_{cat}$ (3.4 ± 0.2) for hydrolysis of cAMP by PDE5<sub>Q775A</sub> were slightly improved compared with PDE5<sub>WT</sub> (Table 1). $K_m$ for cGMP was weakened 20-fold (60 ± 3.3 μM) compared with that for PDE5<sub>WT</sub> (Fig. 2A, Table 1), but $K_m$ for cAMP was essentially unchanged ($K_m$ = 247 ± 18 μM) (Fig. 2B, Table 1). Catalytic efficiency ($k_{cat}/K_m$) of PDE5<sub>Q775A</sub> for cGMP was decreased 10-fold and improved ~3-fold for cAMP. Selectivity for cGMP over cAMP declined from ~100-fold in PDE5<sub>WT</sub> to ~4-fold in both mutants, but this was due to the loss in affinity for cGMP.

These results indicated that restriction of the orientation of the Gln<sup>817</sup> side arm by Gln<sup>775</sup> is a positive determinant for cGMP affinity in the PDE5 catalytic site, but removal of that restriction does not improve cAMP affinity. This brings into question the importance of the orientation of the side chain of the invariant glutamine in determining cN selectivity in other PDEs as well. In the crystal structure of the PDE9 C domain, a cGMP-specific PDE, the side chain of the invariant glutamine is not tethered to restrict orientation (10). In combination, the results provided herein refute the assertion that an H-bond contribution of the glutamine side chain in cGMP-specific PDEs provides for discrimination against cAMP. In addition, the 20-fold change in affinity of PDE5<sub>Q775A</sub> for cGMP suggests that Gln<sup>775</sup> may contribute to cGMP binding affinity by processes not mediated through Gln<sup>817</sup>.

Effect of Gln<sup>817</sup> Substitution on Affinity of PDE5 Catalytic Site for Inhibitors—X-ray co-crystal structures of PDE5 with vardenafil, sildenafil, or IBMX revealed that, like 5'-GMP, all three inhibitors form contacts with the Gln<sup>817</sup> side chain (12, 13). To quantify the role of the Gln<sup>817</sup> side chain for inhibitor potencies, IC<sub>50</sub> values were determined as described under "Experimental Procedures." Substrate concentration (0.5 μM [<sup>3</sup>H]cGMP) was significantly lower than the $K_m$ for each construct, so that IC<sub>50</sub> should approach $K_i$.

Potency of inhibition of PDE5<sub>Q817A</sub>, by vardenafil, sildenafil, or IBMX (IC<sub>50</sub> values of 61, 141, or 214,000 nM, respectively) was substantially weaker than that for PDE5<sub>WT</sub> (IC<sub>50</sub> values of 0.1, 2.9, and 3500 nM, respectively) (Fig. 3, Table 2). The dramatic difference in effect of the Q817A mutation on affinity for vardenafil (610-fold) versus cGMP (60-fold) or sildenafil (48-fold) indicated that despite purportedly similar interactions between Gln<sup>817</sup> and these ligands, as indicated in the x-ray crystal structures, significant quantitative differences exist. The 13-fold disparity between the effect on potencies of vardenafil and sildenafil challenges the interpretation that the two inhibitors bind similarly at this position in PDE5. The relative selectivity for vardenafil over sildenafil in PDE5<sub>Q817A</sub> declined sharply from 29-fold in PDE5<sub>WT</sub> to 2.3-fold in PDE5<sub>Q817A</sub> (Table 2). This emphasizes the particular importance of Gln<sup>817</sup> as a determinant of vardenafil potency and selectivity between vardenafil and sildenafil. The similar change in potency for sildenafil and IBMX (48- and 60-fold, respectively) was surprising and suggests that despite the difference in potency (~1200-fold) of these inhibitors and the H-bonding pattern (Fig. 1B), the relative importance of Gln<sup>817</sup> for binding of each inhibitor is similar.

Effect of Gln<sup>775</sup> Substitution on PDE5 Affinity for Sildenafil or Vardenafil—The potency of sildenafil or vardenafil inhibition of PDE5<sub>Q775A</sub> catalytic activity, assessed as described under "Experimental Procedures," did not differ significantly from that of PDE5<sub>WT</sub> (Table 2). Thus, freeing the putative rotation potential of the Gln<sup>817</sup> side chain had little if any effect on the affinity for these inhibitors compared with a 20-fold effect on affinity for cGMP.

Contribution of Gln<sup>817</sup> to Free Energy of Binding of cGMP and PDE Inhibitors—The interactions of cGMP, vardenafil, sildenafil, or IBMX with PDE5<sub>WT</sub> were quantified by calculating the Gibbs free energy of binding (ΔG) of 7.7, 13.8, 11.9, and 7.6 kcal mol<sup>−1</sup>, respectively, as described under "Experimental Procedures" (18, 19), and using the $K_m$ for cGMP or the $K_i$ calculated from the respective IC<sub>50</sub> values for the inhibitors. IC<sub>50</sub> was determined under conditions in which IC<sub>50</sub> should not differ substantially from the $K_i$. The change in free energy of binding (ΔΔG) of PDE5<sub>Q817A</sub> for cGMP, vardenafil, sildenafil, or IBMX (2.5, 3.8, 2.4, and 2.5 kcal mol<sup>−1</sup>) was consistent with loss of one H-bond because an H-bond contribution to the binding energy of a ligand for its receptor

### Table 1

Effect of PDE5 Q817A and Q775A mutations on affinity for cGMP and cAMP as substrates

| hPDE5A1 proteins | cGMP | cGMP | cGMP | cAMP | cAMP | cAMP | cAMP | cAMP | cAMP | cAMP |
|------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                  | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> | K<sub>m</sub> |
| WT               | 2.9 ± 0.8 | 1 | 2.2 ± 0.4 | 0.758 | 290 ± 8.0 | 1 | 1.6 ± 0.2 | 0.005 | 100 |
| Q817A            | 180 ± 5.2 | 60 | 3.8 ± 0.2 | 0.021 | 630 ± 42 | 2 | 3.0 ± 0.3 | 0.005 | 3.5 |
| Q775A            | 60 ± 3.3 | 20 | 4.6 ± 0.2 | 0.076 | 247 ± 18 | 1 | 3.4 ± 0.2 | 0.014 | 4.1 |

*IC<sub>50</sub> values for each inhibitor are given in the text.*

*Values are mean ± S.E. of three experiments of triplicate determinations.*

*Fold selectivity for cAMP was determined by dividing K<sub>m</sub> value for cAMP by that for cGMP for each of the proteins. WT, wild-type.*
Thus, contrary to accepted dogma, features in the PDE5 catalytic site predominantly for cGMP binding affinity but does not affect cAMP affinity.

The final concentration of PDE5 proteins used in these studies was 0.045–0.065 nM.

Results were analyzed using Prism Graphpad software. The IC50 values for vardenafil, sildenafil, and IBMX for PDE5WT were taken as 1.0. The corresponding values for the PDE5Q817A or PDE5Q775A constructs were divided by the IC50 values.

Comparison of the effect of PDE5 Q817A mutation on potency of inhibition by vardenafil, sildenafil, or IBMX

**FIGURE 3.** Inhibition of catalytic activity of PDE5WT and PDE5Q817A by vardenafil (A), sildenafil (B), and IBMX (C). Assays were performed as described under “Experimental Procedures” using 0.5 μM [3H]cGMP as substrate and a wide range of inhibitor concentrations. Data shown are representative of three experiments and values are mean ± S.E. of triplicate determinations. Results were analyzed using Prism Graphpad software. The final concentration of PDE5 proteins used in these studies was 0.045–0.065 nM.

**TABLE 2**

Comparison of the effect of PDE5 Q817A mutation on potency of inhibition by vardenafil, sildenafil, or IBMX

| hPDE5A1 | IC50 (nM) Vardenafil | IC50 (nM) Sildenafil | IC50 (nM) IBMX | IC50 (nM) Vardenafil | IC50 (nM) Sildenafil | IC50 (nM) IBMX | -fold |
|---------|---------------------|---------------------|----------------|---------------------|---------------------|---------------------|-------|
| Wild type | 0.1 ± 0.02          | 2.9 ± 0.4          | 3.5 ± 0.3      | 1                   | 1                   | 1                   | ND    |
| Q817A   | 61 ± 3.1            | 141 ± 3.5          | 214 ± 4.3      | 610                 | 48                  | 60                  | 2.3   |
| Q775A   | 0.08 ± 0.01         | 2.6 ± 0.4          | ND             | 0.8                 | 0.9                 | ND                  | 33    |

**PDE5 Gln817 Role in Affinity for cGMP, cAMP, and Inhibitors**

Concluding Remarks—Mutation of invariant Gln817 causes a ~60-fold decline in cGMP affinity but only a slight change in cAMP affinity; the decline in cGMP/cAMP selectivity from 100-fold to ~4-fold is due almost entirely to loss in cGMP binding affinity. As indicated by ablation of the H-bond provided by Gln817 to immobilize the Gln817 side chain, rotational constraint of the Gln817 side chain is a positive determinant for cGMP binding affinity but does not affect cAMP affinity. Thus, contrary to accepted dogma, features in the PDE5 catalytic site other than Gln817 and the orientation of its side group restrict PDE5 affinity for cAMP. This challenges the interpretation that rotational constraint of the glutamine switch is a major determinant of PDE cN selectivity.

Discrimination between the two cNs in the various PDE catalytic sites is likely to be mechanistically diverse and to result from subtle differences in the various sites. Evidence suggests that the invariant glutamine can in some instances provide a substantial portion of the affinity for interaction with cGMP or cAMP. However, cN selectivity of a site relies on multiple factors including access of the cN to the pocket and formation of multiple contacts within the site. In solution, cGMP and cAMP are in equilibrium between syn and anti conformers. It seems unlikely that conformational features of the cNs in solution influence PDE catalytic site selectivity, but different catalytic sites could impose conformational changes on the cNs, thereby influencing contacts that foster high affinity.

The 20-fold decline in affinity for cGMP upon removal of Gln775 (PDE5Q775A) could suggest that Gln775 directly contacts cGMP or stabilizes other important interactions. A co-crystal structure of PDE5 with cGMP will be required to resolve these possibilities. Notably, there was no change in affinity for sildenafil or vardenafil in PDE5Q775A, although the side chain of Gln817 would be predicted to be in an unfavorable orientation for binding the inhibitors part of the time. This reveals further differences in the binding of cGMP and sildenafil or vardenafil.

The contribution of Gln817 to PDE5 affinity for vardenafil is 10-fold greater than that for cGMP or sildenafil. This could be due to a substantially stronger bidentate H-bond between the Gln817 side chain and vardenafil than with the other ligands, but electron negativity of the pyrimidine N-1 does not differ significantly between vardenafil and sildenafil (data not shown). Alternatively, Gln817 could influence other contacts between vardenafil and PDE5 more than for other ligands. Gln817 is also critical for PDE5 selectivity for vardenafil over sildenafil.

The results presented herein and in our previous report (20) emphasize that interpretation of the importance of contacts detected in x-ray co-crystal structures of PDE C domains and ligands is limited. The energetic contribution of each amino acid must be experimentally quantified in holoenzymes to more accurately assess the role of interactions and to provide direction for improved drug design. A comparison of differences in the effect of alanine substitution for Gln817 (60-fold) or Tyr612 (15-fold) (20) on cGMP affinity or potency of inhibition by vardenafil, sildenafil, and IBMX reveals that Gln817 plays a central role in binding each of these. The unexpected differences in the role of Gln817 in the potency of vardenafil, sildenafil, and IBMX may be due in part to the fact that current studies used PDE5 holoenzyme, whereas the crystal structures utilized isolated C domain in complex with ligands. More consideration must be given to the impact of the regulatory domain of PDEs on ligand interaction at the catalytic site because holoenzymes are the pharmacological targets (21–24). Subtle changes in catalytic site
topography may manifest as marked changes in function and affinity for ligands. Results from both x-ray crystallography and mutational analyses have limitations, but the combined approaches provide critical direction in rational design of highly specific and potent inhibitors for PDEs.

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