Hydropathic Analysis and Mutagenesis of the Catalytic Domain of the cGMP-Binding cGMP-Specific Phosphodiesterase (PDE5). cGMP versus cAMP Substrate Selectivity†

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ABSTRACT: The mechanism of discrimination between cGMP and cAMP in the catalytic site of the cGMP-binding cGMP-specific phosphodiesterase (BTPDE5A1 or PDE5) has been investigated. A hydropathy analysis of the catalytic domains of different families of PDEs suggests that substrate selectivity of PDEs could result from the pattern of hydrophobic/hydrophilic residues in a short segment surrounding a conserved Glu that has been shown to be critical for cGMP binding in the catalytic domain of PDE5. This implies that the substrate selectivity of PDE5 could be altered by replacing the residues within this segment that are conserved in cGMP-specific PDEs with the conserved residues in the corresponding positions of cAMP-specific PDEs. The A769T/L771R, W762L/Q765Y, and W762L/Q765Y/A769T/L771R mutant PDE5s were expressed in High Five cells, and their substrate selectivities were compared with that of wild-type PDE5. The results indicate that the substrate-binding site of PDE5 contains positive elements for accommodating cGMP, as well as negative elements that discriminate against binding of cAMP, and that the cGMP/cAMP selectivity of PDE5 can be shifted 106-fold by substituting four residues of PDE5 with the residues in the corresponding positions of PDE4.

The number of cell processes that are regulated by cAMP or cGMP is legion. Recent direct measurements reveal that cAMP (*1*), and presumably cGMP as well, can diffuse in the cytosol as freely as in a simple electrolyte solution, and the estimated range of cyclic nucleotide action during its lifetime is about 100−200 μm. cAMP and cGMP are chemically similar compounds that in some instances interact with the same intracellular protein targets through a process known as "cross-activation". However, in most cases cells possess several means to discriminate cAMP and cGMP signaling.

One means of cellular "discrimination" between cAMP and cGMP is the selectivity exhibited by members of the 3′:5′- cyclic nucleotide phosphodiesterase (PDE)[1] superfamily, which catalyzes the hydrolysis of 3′:5′-cyclic nucleotides to the corresponding nucleoside 5′-monophosphates (*2*). While these enzymes have the same function, i.e., degradation of cyclic nucleotides, significant differences in cyclic nucleotide specificity of their catalytic sites have been noted (*3*). Comparison of the different PDE sequences reveals a region of 270 amino acids located in the carboxyl-terminal portion of the proteins, which is remarkably conserved among all members of the superfamily (*4*). Several lines of evidence indicate that this region comprises the catalytic domain (*5*−*11*). Even though this sequence is highly conserved, the catalytic function varies in different PDEs from those that are highly specific for cGMP, to those with mixed cGMP/cAMP-specificity, to those that are exclusively cAMP-specific. Recently, we replaced 23 conserved residues in the catalytic domain of the BTPDE5A1. The most dramatic decrease in substrate binding was seen with Y602A and E775A mutants (*12*). It was concluded that these two residues are important for cGMP binding in the catalytic site of PDE5, perhaps by making direct contacts with the cGMP molecule. These data created the initial basis for structural and functional mapping of the PDE5 catalytic site.

In an attempt to decipher elements that contribute to the cyclic nucleotide selectivity in the substrate-binding site of PDE5, hydropathy plots of the catalytic domains of different PDEs have been generated and certain residues that are conserved in cGMP-specific PDEs have been replaced with the residues conserved in cAMP-specific PDEs in the corresponding positions.

EXPERIMENTAL PROCEDURES

Materials. [8-^{3}H]cGMP and [5,8-^{3}H]cAMP were purchased from Amersham Corp. cGMP, cAMP, histone VIII-S, *C. atrox* snake venom, 3-isobutyl-1-methylxanthine (IBMX), and zaprinast were obtained from Sigma. Hydroxyapatite was from Bio-Rad.

Site-Directed Mutagenesis. cGB-8/14 clone encodes a full-length bovine lung BTPDE5A1 (*13*). The QuikChange site-directed mutagenesis kit (Stratagene) has been used to make point mutations in the cGB-8/14 clone in the pBac-

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[1] Abbreviations: PDEs, 3′:5′-cyclic nucleotide phosphodiesterases; PDE5, cGMP-binding cGMP-specific phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine.

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PAK9 expression vector (Clontech) according to the protocol from Stratagene. The following pairs of mutagenic oligonucleotides were used: (1) A769T/L771R: 5′-CAA CAA CGG ATA ACA GAA CGT GTT GCC ACT G-3′ and 5′-C AGT GGC AAC ACG TTC TGT TAT CCG TTG TTG-3′; (2) W762L/Q765Y, 5′-GCA ATT ACA AAA CCC TTG CCT ATT TAC CAA CGG-3′ and 5′-CCG TTG GTA AAT AGG CAA GGG TTT TGT AAT TGC-3′. The altered bases are underlined. To make a W762L/Q765Y/A769T/L771R mutant, the A769T/L771R mutant DNA was used as a template in the mutagenesis reaction with the W762L/Q765Y pair of mutagenic oligonucleotides. To avoid theoretically possible random mutations, the 1073 bp fragments containing the desired mutations were excised from cGB-8/14 by using *Kpn* I/*Bst*1107I digestion and resubcloned in a wild-type pBacPAK9 vector using the same restriction sites.

E. coli XL1-blue cells were used for all transformations. DNA fragments were purified by the freeze−squeeze method from agarose slices by using SPIN-X centrifuge filter units (Costar). DNA was purified from large-scale vector preparations by using a QIAGEN Plasmid Maxi kit according to the manufacturer's protocol (QIAGEN Inc.). All DNA segments subjected to mutagenesis and subcloning reactions were sequenced in their entirety to ensure the presence of the desired mutation and proper in-frame subcloning.

Expression of Wild-Type and Mutant PDE5s. Sf9 cells were cotransfected with *Bsu*36 I-digested BacPAK6 viral DNA (Clontech) and one of the mutated cGB-8/14 clones in the pBacPAK9 expression vector by the lipofection method according to the protocol from Clontech. At 3 days postinfection, the cotransfection supernatant was collected, amplified twice in Sf9 cells, and then used as virus stock directly for expression without additional purification of recombinant viruses. High Five cells (Invitrogen) grown at 27 °C in complete Grace's insect medium (Invitrogen) with 10% fetal bovine serum (Intergen) and 10 mg/mL gentamicin (Life Technologies, Inc.) in T-185 flasks were infected by 5 mL of virus stock *per* flask. The culture medium was harvested at 96 h postinfection. The level of recombinant enzyme production was normalized to purified wild-type PDE5 activity (2.5 μmol/mg/min) according to the amount of total protein in the impure sample and the PDE5 activity of the sample adjusted to that of wild type.

Purification of Wild-Type and Mutant PDE5s. The culture medium, 240−260 mL, was fractionated by sequential ammonium sulfate precipitation at 4 °C. The fraction precipitated by 25−40% saturation was resuspended in 30 mL of 10 mM sodium phosphate buffer, pH 7.2, and centrifuged at 48000*g* for 30 min at 4 °C. The supernatant was loaded onto an hydroxyapatite (Bio-Rad) column (1.5 × 15 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.2. The column was washed with 100 mL of 70 mM sodium phosphate buffer, pH 7.2, and then eluted with 120 mM sodium phosphate buffer, pH 7.2, at a flow rate of 5 mL/h. The pool containing BTPDE5A1 activity was diluted with 6 volumes of ice-cold deionized water and concentrated to approximately 1 mL by using an Amicon filtration cell equipped with a PM-30 membrane. All purification steps were performed at 4 °C. The final preparation was stored in 20% glycerol at −70 °C.

Catalytic Activity of BTPDE5A1. PDE catalytic activity was measured by using a modification of the assay procedure described previously (*14*). Incubation mixtures contained 40 mM MOPS, pH 7.5, 0.5 mM EGTA, 15 mM magnesium acetate, 0.15 mg/mL bovine serum albumin, cGMP and [8-^{3}H]cGMP (150 000 cpm/assay) or cAMP and [5,8-^{3}H]-cAMP (150 000 cpm/assay), and one of the BTPDE5A1 samples, in a total volume of 250 μL. Under these conditions the reaction rate using 1 μM cGMP or 20 μM cAMP was approximately linear for more than 10 min for wild-type and mutant proteins that were tested, and an incubation time of 10 min at 30 °C was selected for all kinetic measurements. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. After cooling, 20 μL of 10 mg/mL *C. atrox* snake venom was added followed by a 20-min incubation at 30 °C. Nucleoside products were separated from unreacted nucleotides by using DEAE Sephadex A-25 equilibrated with 20 mM Tris-HCl buffer, pH 7.5, and counted. In all studies, less than 15% of the total substrate was hydrolyzed during the reaction. The apparent K_m values for cGMP or cAMP were determined from Lineweaver−Burk plots after assaying PDE activity in duplicate at 1−250 μM cGMP or 20−1000 μM cAMP, respectively. To determine IC_{50} values for zaprinast or rolipram, the PDE activity was assayed in duplicate in the presence of 0.05−30 μM zaprinast or 20−1500 μM rolipram, respectively. For each mutant or wild-type enzyme tested, the substrate (cGMP) level in the assay was one-third of the K_m. All values determined represent at least three measurements using at least two different PDE preparations.

cGMP-Saturation Binding Assay. The cGMP-saturation binding assay was conducted in a total volume of 60 μL containing 10 mM sodium phosphate buffer, pH 6.8, 1 mM EDTA, 0.2 mM IBMX, 0.5 mg/mL histone VIII-S, and 0.5−25 μM [8-^{3}H]cGMP. The reaction was initiated by addition of enzyme. Following a 60-min incubation on ice, assay mixtures were filtered onto premoistened Millipore HAWP filters (pore size 0.45 μm), which were then rinsed 4 times with a total of 4 mL of cold 10 mM sodium phosphate buffer, pH 6.8, with 1 mM EDTA, and then dried and counted. The data were corrected by subtraction of nonspecific binding, which was defined as either the [8-^{3}H]cGMP bound in the absence of BTPDE5A1 or the [8-^{3}H]cGMP bound in the presence of a 100-fold excess of unlabeled cGMP. A similar 2−4% of nonspecific binding was obtained with each method. The data were subjected to nonlinear least-squares analysis, using the program MINSQ II (Micromath Scientific Software, Salt Lake City, UT) to obtain the dissociation constant (K_d).

Other Methods. SDS-electrophoresis in 10% polyacrylamide gels and Western blot analysis were done as previously described (*14*). Total protein concentrations were determined by the method of Bradford (*15*), using bovine serum albumin as the standard.

RESULTS

Hydropathy Analysis. Globular proteins fold into structures that maximize burial of hydrophobic side chains while simultaneously exposing hydrophilic side chains to solvent. It is believed that not all amino acids in a sequence contribute equally to the formation of a native structure, since some features are far more important than others. Kauzmann reviewed the forces that maintain the structure of globular

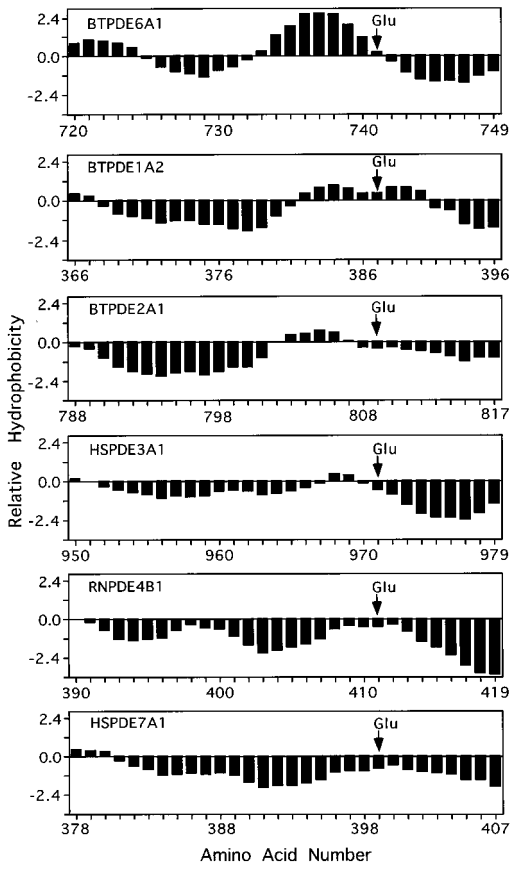

FIGURE 1: Hydropathy analysis of selected sequences in the catalytic domain of different PDE families. Hydropathy plots were generated by using the program SCIPROTEIN (SciVision, Lexington, MA) based on the algorithm of Kyte and Doolittle (*17*). A window size of seven residues and an amphiphilicity window size of 11 have been used. *Abscissa* values represent amino acid numbers, while *ordinate* values indicate the relative hydrophobicity. Values above the axis denote hydrophobic regions; values below the axis indicate the hydrophilic regions. Sequences used were from BTPDE6A1 (*18*), BTPDE1A2 (*19*), BTPDE2A1 (*20*), HSPDE3A1 (*21*), RNPDE4B1 (*22*), and HSPDE7A1 (*23*). The first two letters in the PDE's abbreviations represent species (HS for *Homo sapiens*, BT for *Bos taurus*, and RN for *Rattus norvegicus*). The invariant Glu residue, which is involved in cGMP binding in the BTPDE5A1 (*12*) is marked.

proteins (*16*) and pointed out that hydrophobic interactions are largely entropic in nature, in contrast to other bonds, which are mostly enthalpic. It is generally accepted that hydrophobic interactions are the major forces that drive a protein molecule toward collapse and folding. Computer programs give a remarkable correspondence between hydrophobic and hydrophilic components of proteins and known interior and exterior portions, respectively, of these proteins as determined by crystallography (*17*). In the present work the program SCIPROTEIN (SciProtein, Lexington, MA) was used to generate the hydropathy plots of catalytic domains of different PDEs.

To date, at least 15 individual PDE genes have been described (*3*). Most PDE genes have more than one alternatively spliced mRNA transcribed from them, which increases considerably the number of mammalian PDE gene products. Fifty-two different sequences (8 for PDE1, 2 for PDE2, 2 for PDE3, 28 for PDE4, 1 for PDE5, 9 for PDE6, and 2 for PDE7) have been analyzed in this report. The substrate selectivity of these proteins varies widely from

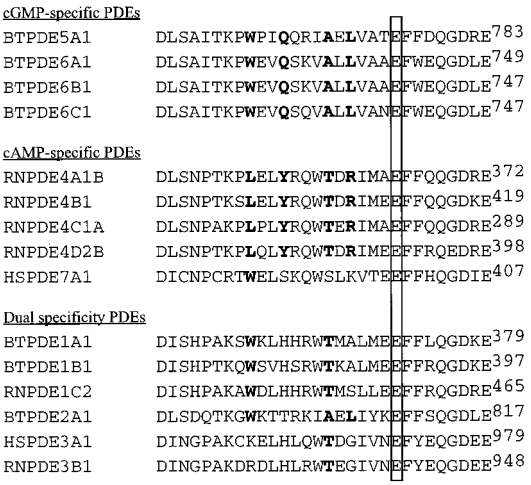

FIGURE 2: Alignment of selected sequences from the catalytic domain of different PDEs. The sequences around the invariant Glu residue (shown in the open box), which is involved in cGMP binding in the BTPDE5A1 (*12*), were aligned. Bold designates the residues which are identical in all known cGMP- or cAMP-specific PDEs only. All sequences are arranged in three blocks: cGMP-specific PDEs (PDE5 and PDE6 families), cAMP-specific PDEs (PDE4 and PDE7 families), and PDEs with mixed specificity (PDE1, PDE2, and PDE3 families). The first two letters in the PDE's abbreviations represent species (HS for *Homo sapiens*, BT for *Bos taurus*, and RN for *Rattus norvegicus*). Sequences used were from BTPDE5A1 (*26*), BTPDE6A1 (*18*), BTPDE6B1 (*27*), BTPDE6C1 (*28*), RNPDE4A1B (*29*), RNPDE4B1 (*22*), RNPDE4C1A (*30*), RNPDE4D2B (*29*), HSPDE7A1 (*23*), BTPDE1A2 (*19*), BTPDE1B1 (*31*), RNPDE1C2 (*32*), BTPDE2A1 (*20*), HSPDE3A1 (*21*), and RNPDE3B1 (*33*).

cGMP-specific to cAMP-specific with many enzyme species hydrolyzing both cyclic nucleotides (*3*). Our primary focus was to determine if the substrate selectivities of these PDEs might result from a difference in the patterns of hydrophobicity/hydrophilicity in the catalytic domain of these enzymes. This analysis identified such a correlation in a short sequence located near the invariant Glu residue that is thought to interact directly with cGMP in a cGMP-specific PDE (PDE5). The hydropathy profiles around this Glu residue for selected PDEs with varying cGMP/cAMP substrate selectivity are shown in Figure 1. BTPDE6A1 (*18*) belongs to the PDE6 family, which comprises several different gene products. All of these PDEs are highly specific for cGMP and exhibit very similar hydropathy profiles in the region shown in Figure 1. The hydropathy profile of PDE5 is very similar to that for PDE6 and is shown in Figure 4. BTPDE1A2 (*19*) is a relatively cGMP-specific PDE that belongs to the PDE1 family, which includes three calmodulin-stimulated PDEs with very different substrate selectivity (*3*). BTPDE2A1 (*20*) belongs to the PDE2 family, of which only one gene has been identified to date. PDE2 hydrolyzes both cAMP and cGMP, and V_{max} values are very similar for both substrates. HSPDE3A1 (*21*) belongs to the PDE3 family, and two different gene products have been identified for this family. Both of these proteins have a high affinity for cAMP and cGMP, but the V_{max} for cGMP is lower than that for cAMP. RNPDE4B1 (*22*) belongs to the PDE4 family, which comprises four different gene products. All of these proteins are cAMP-specific PDEs and exhibit very similar hydropathy profiles in the region shown in Figure 1. HSPDE7A1 is the only member of PDE7 family (*23*). This protein has a high affinity and selectivity for cAMP.

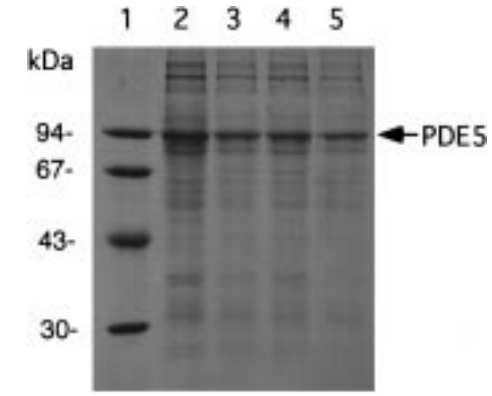

FIGURE 3: SDS−polyacrylamide gel electrophoresis of partially purified wild-type and mutant BTPDE5A1s. Lane 1, molecular mass standards; lane 2, wild-type enzyme; lane 3, W762L/Q765Y mutant; lane 4, A769T/L771R mutant; lane 5, W762L/Q765Y/A769T/L771R mutant. Wild-type and mutant enzymes, 10 μg, that had been purified through the hydroxyapatite step described in MATERIALS AND METHODS were applied to each lane. Proteins were visualized by staining the gel with Coomassie Brilliant Blue dye. The molecular masses in kilodaltons of protein standards are indicated on the left.

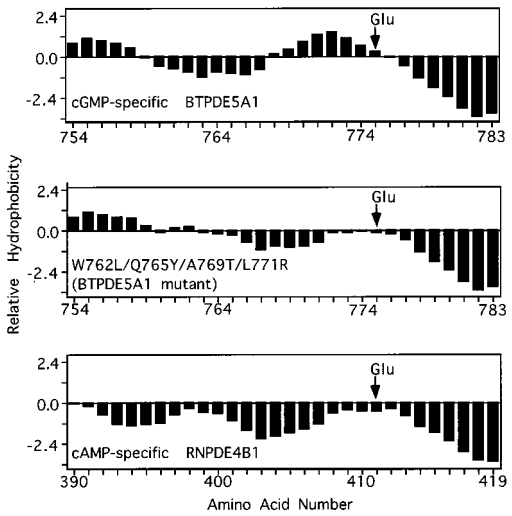

FIGURE 4: Comparison of hydropathy plots of the wild-type BTPDE5A1 and its W762L/Q765Y/A769T/L771R mutant. Hydropathy plots were generated by using the program SCIPROTEIN (SciVision, Lexington, MA) based on the algorithm of Kyte and Doolittle (*17*). A window size of seven residues and an amphiphilicity window size of 11 have been used. *Abscissa* values represent amino acid numbers, while *ordinate* values indicate the relative hydrophobicity. Values above the axis denote hydrophobic regions; values below the axis indicate the hydrophilic regions. The first two letters in the PDE's abbreviations represent species (BT for *Bos taurus* and RN for *Rattus norvegicus*). The invariant Glu residue, which is involved in cGMP binding in the BTPDE5A1 (*12*), is marked.

Comparison of the hydropathy profiles in Figure 1 reveals that the gradual transition of substrate selectivity from essentially pure cGMP-specific to essentially pure cAMP-specific is accompanied by gradual changes in relative hydrophobicity of the segments of sequences surrounding the invariant Glu residue in these PDEs. This increase in cAMP selectivity is associated with the predicted appearance of nine amino acid residues including the invariant Glu at the exterior of the protein molecule. Using the generalization that turns in protein structure occur at those sites in the polypeptide chain where the hydrophobicity is at a local minimum (*24*), it is assumed that a structural element with strong hydrophobicity located between two turns in a cGMP-binding site is not present in a cAMP-binding site.

Mutagenesis Strategy. The common functions of protein superfamilies are preserved by evolutionary conservation of essential amino acids for folding and/or function. The tolerance of proteins for a variety of amino acid substitutions (*25*) demonstrates that properties of short sequences do not dictate the overall structure of a protein. Local sequence changes over long periods of evolution, which maintain the stability of a protein and do not negatively affect its common function, may lead to creation of a specific new property of the protein. The common function of PDEs is to catalyze the hydrolysis of 3′:5′-cyclic nucleotides to the corresponding nucleoside 5′-monophosphates, and the specific function of a particular PDE is to hydrolyze cGMP, cAMP, or both. Figure 2 shows the amino acid sequences in the putative region responsible for at least part of the substrate selectivity of PDEs. This region is highly conserved in all PDEs. In addition to several residues that are invariant among all PDEs, some residues are conserved among either cGMP- or cAMP-specific PDEs. Trp, Gln, Ala, and Leu residues (shown in bold in Figure 2) are invariant among all cGMP-specific PDEs (PDE5 and PDE6 families), while the corresponding positions are occupied by Leu, Tyr, Thr, and Arg residues (shown in bold in Figure 2) in the cAMP-specific PDEs (PDE4 family). PDE7, which is cAMP-specific, does not exhibit this feature. Despite this fact, the hydropathy profile of PDE7 in this region (Figure 1) is very similar to those of the other cAMP-specific PDEs. PDEs with mixed specificity (PDE1, PDE2, and PDE3) have either a combination of residues found in the cGMP- and cAMP-specific PDEs or do not have homology at all in the corresponding positions (Figure 2). Taking all of these observations into account, three mutants that modify this sequence in PDE5 have been generated in order to examine the effects of these changes on cGMP/cAMP substrate selectivity.

Expression and Purification of PDE5 Mutants. The residues conserved in the cGMP-specific PDEs (Trp, Gln, Ala, and Leu) have been replaced in PDE5 with the conserved residues in the corresponding positions of the cAMP-specific PDE4s (Leu, Tyr, Thr, and Arg, respectively). Two double mutants (A769T/L771R and W762L/Q765Y) and one multiple mutant (W762L/Q765Y/A769T/L771R) were expressed in High Five cells as described under Experimental Procedures. The levels of expression of these mutants (1−2 mg/100 mL of culture) were comparable to that of the wild-type enzyme (6 mg/100 mL of culture). The wild-type and mutant PDE5s were purified from culture medium by using ammonium sulfate precipitation and hydroxyapatite chromatography as described under "Materials and Methods". There was no noticeable difference in binding to and subsequent elution of these proteins from the hydroxyapatite column compared to that for the wild-type enzyme. Figure 3 shows a Coomassie Blue-stained SDS−polyacrylamide gel of partially purified mutants obtained following the hydroxyapatite column step. It can be seen that the PDE5 protein band at approximately 93 kDa was the main protein in the preparation. All mutated proteins migrated on the gel with essentially the same mobility as that of wild-type PDE5. The identity of the recombinant

Table 1: Kinetic Parameters of Wild-Type and Mutant Proteins[a]

protein	K_m for cGMP, μM	K_m for cAMP, μM	fold increase in cAMP/cGMP selectivity
wild-type	2 ± 0.4	330 ± 20	1
A769T/L771R	8 ± 1.5	84 ± 5	16
W762L/Q765Y	36 ± 3.2	77 ± 4	77
W762L/Q765Y/ A769T/L771R	43 ± 3.4	67 ± 3	106

[a] Apparent K_m values were determined from reciprocal plots of $1/v_0$ versus 1/[S]. The results represent the average of three determinations $\pm$ standard deviation.

Table 2: Comparison of Functional Parameters of Wild-Type and Mutant PDE5s[a]

protein	K_d for cGMP, μM	IC_{50} for zaprinast, μM	IC_{50} for rolipram, μM
wild-type	1.3 ± 0.2	0.3 ± 0.01	480 ± 20
A769T/L771R	1.3 ± 0.2	0.7 ± 0.02	480 ± 20
W762L/Q765Y	1.3 ± 0.2	10.0 ± 0.30	480 ± 20
W762L/Q765Y/ A769T/L771R	1.3 ± 0.2	10.0 ± 0.30	480 ± 20

[a] The results represent the average of three determinations $\pm$ standard deviation.

proteins was verified by Western blot analysis (data not shown).

Characterization of PDE5 Mutants. To analyze the mutant versus wild-type proteins with respect to their substrate selectivities, the apparent K_m values for cGMP and cAMP were determined from Lineweaver−Burk plots (Table 1). Relative to wild-type enzyme, the K_m values for cGMP for all of the mutant PDEs were increased, whereas the K_m values for cAMP were decreased by as much as 5-fold. The increased affinity for cAMP indicated that these mutations were not generally deleterious to the structure of this region of the PDE5. A change in the substrate selectivity caused by the mutations was calculated by using the equation shown below.

$$\text{increase in cAMP/cGMP selectivity} = [(K_m, \text{cGMP})_{\text{mutant}}/(K_m, \text{cGMP})_{\text{wild-type}}] \times [(K_m, \text{cAMP})_{\text{wild-type}}/(K_m, \text{cAMP})_{\text{mutant}}]$$

The reciprocal behavior of the two possible functions (cGMP or cAMP binding in the catalytic site) implied that alterations made in PDE5 shifted the substrate selectivity of this enzyme. Indeed, the W762L/Q765Y/A769T/L771R mutant had almost equal K_m values for cGMP and cAMP, whereas the wild-type PDE5 exhibits more than a 100-fold difference. Therefore, this protein could be qualified as an enzyme with mixed selectivity, in contrast to wild-type enzyme, which is highly selective for cGMP. The hydropathy profile of the W762L/Q765Y/A769T/L771R mutant (Figure 4) was very similar to that of members of the PDE4 family, whereas wild-type PDE5 (Figure 4) had a profile which was typical for cGMP-specific PDEs (Figure 1).

The PDE5 mutants were further characterized by assessing cGMP binding to the allosteric cGMP-binding sites (Table 2). PDE5 contains two allosteric cGMP-binding sites that are located in the amino terminal half of the protein molecule and which are distinct from the site of cGMP hydrolysis. The [^{3}H]cGMP filter-binding assay was used to examine the affinity of cGMP binding to the wild-type and mutant PDE5s. The [^{3}H]cGMP-binding concentration curves were indistinguishable (data not shown) for these proteins, and the data when subjected to nonlinear least-squares analysis showed no difference in dissociation constants (K_d). These results, taken together with the findings of a similar level of expression, unchanged chromatographic behavior, and a gain of function in the increased affinity for cAMP as substrate, supported the interpretation that the structural integrity of the mutant proteins was not changed and that the overall structure of the wild-type enzyme was preserved in the mutants.

The effect of the mutations introduced into PDE5 on inhibitor sensitivity was evaluated by using two inhibitors: zaprinast and rolipram. Zaprinast is a potent inhibitor for the cGMP-specific PDEs (PDE5 and PDE6 families), whereas rolipram is a highly potent and selective inhibitor for one of the cAMP-specific PDEs (PDE4 family). The primary question was whether a change in substrate selectivity was accompanied by a similarly directed change in inhibitor selectivity. The potency of zaprinast to inhibit cGMP hydrolysis was decreased 33-fold in the W762L/Q765Y and W762L/Q765Y/A769T/L771R mutants and 2-fold in the A769T/L771R mutant, but neither of the three mutants exhibited a change in sensitivity to rolipram (Table 2). Interpretation of these data was complicated by the fact that the location of the zaprinast- or rolipram-binding sites is unknown. It is generally believed that zaprinast interacts with the catalytic site of PDE5, whereas rolipram interaction with PDE4 may be more complex (*3*, *22*, *30*, *34*) and involve amino acids which are outside the catalytic domain of PDE4 (*11*). Collectively, the data presented in Table 2 are consistent with currently accepted models for PDE inhibition by these inhibitors.

DISCUSSION

A cyclic nucleotide can be subdivided into two distinct segments: the phosphate-ribose and the purine base moieties. It is possible that the shape of the sugar or orientation of the nucleotide base with respect to the ribose (syn or anti conformations) may contribute to the cyclic nucleotide selectivity of a particular enzyme. There is no doubt that the chemical nature of the base plays an important role in substrate discrimination. Comparison in the base region shows that cGMP and cAMP have similar hydrogen bonding potential in the imidazole ring but quite different hydrogen bonding potential in the N-1, N-6, and C-2 positions. The amino group in cAMP at N-6 can act as a donor in hydrogen bonding, while the carbonyl group at the same position in cGMP is an acceptor. Another important difference between these nucleotides is that guanine has a higher dipole moment than does adenine and could therefore form stronger stacking interactions with polarizable amino acids such as tryptophan or phenylalanine. Cumulatively, PDEs may resemble other cyclic nucleotide-binding proteins that have key amino acid residues responsible for the recognition of the given nucleotide. For example, cGMP binds to and activates mammalian cAMP-dependent protein kinase very weakly, but a change of Ala^{334} to Thr in the cyclic nucleotide-binding site dramatically increases the affinity with which cGMP is bound to this enzyme (*35*). Similar effects have been published

(*36*−*38*) for proteins that bind nucleoside triphosphates, which are chemically related to cyclic nucleotides. In each instance, the single mutation of a conserved residue has led to the alteration of the nucleotide triphosphate selectivity. For at least one protein it has been shown that in addition to selective and favorable interactions with GTP in the GTP-binding site, there are also unfavorable interactions that discriminate against ATP (*39*).

The basis for substrate selectivity in the active sites of PDEs is unknown. The hydropathy analysis suggests that substrate selectivity of PDEs could be a function of the spatial structure of the substrate-binding site. The results of mutagenesis show that it is possible to alter the substrate selectivity of the PDE5 predictably by replacing the residues conserved in the cGMP-specific PDEs with the residues conserved in the corresponding positions of the cAMP-specific PDE4 family. These mutations might change the local structural arrangement in the catalytic site sufficiently to weaken positive interaction(s) for cGMP and negative interaction(s) for cAMP. These findings support the hypothesis of a dual control mechanism of PDE substrate-binding site occupancy. That is, cyclic nucleotide selectivity could be the result of multiple structural features: those that are favorable for a particular cyclic nucleotide provided by certain key residues that make direct contact with that molecule and those that are unfavorable for another cyclic nucleotide provided by steric hindrance.

The results of the present study provide the first specific information pertaining to structural features of PDEs that contribute to the affinity and selectivity with which the catalytic sites of these enzymes interact with cGMP and cAMP. It is evident from these studies that this region of PDEs provides a significant portion of the interaction of these enzymes with the cyclic nucleotides. Thus, changes in only a few residues in PDEs, and perhaps in other enzymes, could provide for marked differences in substrate selectivity without changing the general catalytic function.

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