

# Analysis of a Mutation in Phosphodiesterase Type 4 that Alters Both Inhibitor Activity and Nucleotide Selectivity

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## ABSTRACT

Cyclic nucleotide phosphodiesterase type 4 (PDE4) is a cAMP-specific phosphodiesterase that is found as four distinct genes in the mammalian genome (PDE4A, 4B, 4C, and 4D). Mutation analysis was done to identify the amino acids involved in activity and inhibitor selectivity. Mutations at Asp333 were made in HSPDE4D3 based on mutations that affect rolipram sensitivity in RNPDE4B1. The PDE4D3 Asp-Asn mutant was resistant to inhibition by rolipram as well as several other PDE4 inhibitors tested. These results suggest that this residue is near the inhibitor binding pocket in PDE4D3. Sequence comparison of PDE4 with cGMP-specific PDE proteins shows a conserved aspartic acid at position 333 in PDE4D3 and a conserved asparagine at this position in PDE enzymes that hydrolyze

cGMP. Therefore, cGMP hydrolysis by PDE4D3 Asp-Asn was measured. PDE4D3 Asp-Asn hydrolyzes cGMP with kinetic constants similar to those observed for this protein with cAMP ( $K_m \sim 20 \mu\text{M}$ ,  $V_{\max} \sim 2 \mu\text{mol AMP/min/mg}$  recombinant protein). Under identical conditions, the  $K_m$  value for cAMP hydrolysis by wild-type PDE4D3 is  $3 \mu\text{M}$  and the  $V_{\max}$  value is  $1 \mu\text{mol AMP/min/mg}$  recombinant protein. In addition, the PDE4D3 Asp-Ala mutant protein could hydrolyze cGMP. Finally, the analogous mutation in HSPDE4B1 (Asp413Asn) also allows hydrolysis of cGMP. These results show that this aspartic acid residue is important in inhibitor binding and nucleotide discrimination and suggest this residue is in the active site of PDE4.

cAMP and cGMP are important second messengers involved in the regulation of multiple intracellular signaling pathways. The cyclic nucleotide phosphodiesterases (PDEs) are a family of enzymes that hydrolyze cAMP and cGMP. These enzymes, therefore, regulate intracellular cyclic nucleotide levels that influence many signaling pathways. Ten PDE families, each containing multiple genes, have been described (Beavo, 1995; Fisher et al., 1998a,b; Soderling et al., 1998a,b, 1999; Fujishige et al., 1999). Each family is defined primarily by sequence homology and shares similar substrate specificity, regulatory properties, and inhibitor profiles. Multiple proteins can be derived from individual genes by alternative splicing and the use of alternative transcriptional start sites. In addition to unique biochemical and regulatory properties, many of these proteins are differentially expressed in tissues and cell types and have distinct intracellular localization patterns.

The PDE4 family is characterized by specificity for cAMP hydrolysis and inhibition by rolipram. As many as 13 human and rodent PDE4 variants are translated from each of four distinct genes (Houslay et al., 1998; Conti and Jin, 1999). This PDE family is an important therapeutic target for the treatment of disease, most notably for the treatment of inflammation and asthma (Teixeira et al., 1997; Spina et al.,

1998; Torphy, 1998). The identification of the residues critical for inhibitor and substrate binding is an important aspect in finding specific and effective new drugs.

All PDE families share significant sequence homology. A conserved region of approximately 270 to 390 amino acid residues contains the catalytic domain of the PDE enzymes (Charbonneau et al., 1986; Houslay et al., 1998). In this region, the different PDE4 subtypes show greater than 85% identity. The members of a PDE family share greater sequence homology between each other than between members of a different family. This similarity is likely to account for similar substrate specificity and inhibitor profiles of enzymes within the same family. Conversely, the nonconserved amino acids are likely to confer specificity between families or subtypes. In the absence of structural information, mutagenesis techniques and other methods have been used to determine the amino acid residues important for substrate and inhibitor binding. PDE4 inhibitors appear to interact within the catalytic domain region (Pillai et al., 1993; Wilson et al., 1994; Jacobitz et al., 1996), although other domains do have an effect on the sensitivity of inhibitor binding (Saldou et al., 1998b; McPhee et al., 1999; Yarwood et al., 1999).

Extensive mutagenesis has been done on the catalytic domain of the cGMP-specific PDE5 to identify residues impor-

tant for catalysis. Twenty-three amino acid residues conserved in all PDEs were mutated, and several were found to allow this cGMP-specific PDE to hydrolyze cAMP, therefore altering the substrate specificity of this protein (Turko et al., 1998a). In addition, they found several residues that affect the rate of catalysis, suggesting these residues might be part of the catalytic center. Two other residues were shown to affect  $K_m$  values, suggesting that these residues may be involved in cGMP binding. Interestingly, the key residues involved in zaprinast binding, a PDE5 inhibitor, differ significantly from those that affect binding of cGMP (Turko et al., 1998b).

A different approach for probing important amino acid residues uses a yeast strain deficient in endogenous PDEs to select for inhibitor-resistant mutations after performing random mutagenesis (Atienza and Colicelli, 1998). Several residues in the PDE4B conserved domain were found to be required for rolipram sensitivity (Pillai et al., 1993). Using this same method, chimeric proteins consisting of portions of HSPDE3A and RNPDE4B1 conserved domains showed that the inhibitor specificity domain lies within a short sequence at the carboxyl end of the catalytic domain (Atienza et al., 1999). The carboxyl end was also found to be important in substrate recognition by using cAMP analogs to probe the catalytic site (Omburo et al., 1997).

By sequence alignment of all PDE families, it became clear that one residue at the amino-terminal end of the catalytic domain was conserved in all cAMP-specific PDEs but different in cGMP-specific PDEs. This residue is identical with one described as being important in rolipram sensitivity of PDE4B (Pillai et al., 1993). We mutated this residue in HSPDE4D3 and HSPDE4B1 to determine whether this residue is important for substrate selectivity. We also examined the inhibitor profile of the mutated PDE4D3 with inhibitors specific for each PDE family. We found that changing this residue allowed PDE4 proteins to hydrolyze cGMP and altered inhibitor sensitivity, including an increased sensitivity to zaprinast.

## Materials and Methods

**Sequence Alignment of PDE Families.** A multiple sequence alignment was constructed of all available cyclic nucleotide PDEs. Sequences included were those identified as PDEs (<http://weber.u.washington.edu/~pde/Nomenclature.html>) and any other sequence with recognizable sequence similarity to these sequences using the PsiBlast algorithm (Altschul et al., 1997). For this analysis, the recently discovered proteins of the PDE8, PDE9, and PDE10 classes were not included. For pairs of sequences sharing more than 99% sequence identity, only the longer sequence was retained. Sequences were clustered on the basis of their sequence similarity. At a cutoff of 30% sequence identity, two primary clusters are identified: one containing the cAMP-preferring enzymes (PDE1, PDE4, PDE3, and PDE7 for a total of 33 sequences), and one containing the cGMP-preferring enzymes (PDE2, PDE5, and PDE6, for a total of 18 sequences).

A consensus sequence was constructed for each cluster by selecting at each position the amino acid with the highest BLOSUM62 score (Henikoff and Henikoff, 1993) versus all the residues observed at that position. A position is regarded as being "conserved" if the consensus residue matches 90% of the residues at that position with a nonnegative BLOSUM62 score.

**Plasmids, Cloning, and Expression.** HSPDE4D3 (Nemoy et al., 1996) and HSPDE4B1 (Bolger et al., 1993) were gifts from Dr. Marco

Conti (Stanford University Medical Center). The full-length inserts were subcloned into the baculovirus transfer vector pFastbac1 (Life Technologies, Gaithersburg, MD). Recombinant viral DNA as a bacmid in *Escherichia coli* was obtained using the Bac-to-Bac Baculovirus Expression Kit (Life Technologies), and 10  $\mu$ l of the bacmid prep was used for transfection. The viral stock from the transfection was amplified, and protein was produced by infecting a T-150 containing S/21 cells at 50% confluency with 100  $\mu$ l of amplified virus. Cells were collected after 72 h and used to prepare the lysate.

**Preparation of S/21 Cell Lysate Containing PDE4B1 and PDE4D3.** S/21 cells were collected by centrifugation, washed twice in cold PBS, and resuspended at a concentration of  $1 \times 10^8$  cells/4 ml in a buffer containing 20 mM bis-Tris, pH 6.5, 2.5 mM dithiothreitol, 10 mM EDTA, 2 mM benzamidine, 100 mg/ml bacitracin, and 50  $\mu$ M phenylmethylsulfonyl fluoride. Cells were lysed by sonication and centrifuged at 100,000g for 1 h. Supernatant was collected and assayed for PDE4 activity.

**Western Blot Analysis.** PDE4 carboxyl-terminal glutathione S-transferase (GST) fusion plasmids and antibodies specific for PDE4B (K118) and PDE4D (M3S1) were gifts from Dr. Marco Conti. PDE4 carboxyl-terminal GST fusion proteins were used to estimate the quantity of PDE4B and PDE4D wild-type and mutant proteins expressed in S/21 lysates (Iona et al., 1998). Because the antibodies are selective for an extreme carboxyl-terminal epitope, mutation of a single amino acid in the catalytic domain is not expected to affect antibody recognition. Varying concentrations of the PDE4 carboxyl-terminal GST proteins and the experimental samples were loaded so that the signal from the experimental samples fell within the range of signals achieved with the PDE4 carboxyl-terminal GST proteins. The PDE4-specific antibodies and secondary antibodies directly conjugated to fluorescein were used to visualize the PDE4 proteins (ECF Western Blotting Kit; Amersham, Piscataway, NJ). The signal was measured using a Fluorimager SI (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant (Molecular Dynamics).

**Mutagenesis of PDE4B1 and PDE4D3.** Mutagenesis was done on HSPDE4D3 and HSPDE4B1 in pFastbac1 construct using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used to introduce the Asp333 mutation in PDE4D3 were 5'-CCATGCTGCAAATGTTGTCCAGTCTAC-3' and 5'-GTAGACTGGACAACATTTCAGCATGG-3'. The primers used to introduce this mutation in HSPDE4B1 were 5'-CTGCACGCTGCTAATGTAGCCAGTCG-3' and 5'-CGACTGGGCTACATTAGCAGCGTGCAG-3'.

**PDE4 Activity Assay.** PDE inhibitors were obtained from BIOMOL (Plymouth Meeting, PA). To determine  $IC_{50}$  values for these inhibitors, the compound was diluted in DMSO at 50 $\times$  the desired concentration, and 2  $\mu$ l was spotted onto a 96-well plate (Costar, Cambridge, MA). The  $IC_{50}$  value for cGMP was obtained by diluting cGMP in H<sub>2</sub>O at 50 $\times$  the desired concentration after adjusting the pH of the solution to 7.5. These samples were assayed in 100  $\mu$ l of reaction mixture containing 40 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 45.6 mM  $\beta$ -mercaptoethanol, 0.2  $\mu$ M cAMP, and 0.04  $\mu$ Ci of [<sup>3</sup>H]cAMP (Amersham). PDE4 lysate was diluted between 100- and 10,000-fold, depending on the protein prep, into the final assay mixture. Mock infected S/21 cell lysates show no endogenous PDE activity for cAMP or cGMP at these dilutions. Incubations were performed at 37°C for 30 to 60 min, and reactions were terminated by the addition of 50  $\mu$ l of 0.5 $\times$  scintillation proximity assay (SPA) beads containing zinc sulfate (Amersham). Reactions were incubated with beads for 25 min at room temperature then counted on a Trilux 96-well plate scintillation counter (Wallac, Turku, Finland). The experiments were performed with 10 dilutions of compound in duplicate. The data are plotted as a percentage of the inhibition obtained with 500  $\mu$ M 3-isobutyl-1-methylxanthine (IBMX). Standard inhibitor concentrations and specificities for the PDE inhibitors in Table 2 are taken from BIOMOL.

Kinetic measurements were done in reactions containing 40 mM Tris-HCl, pH 8, 10  $\mu$ M MgCl<sub>2</sub>, 45.6 mM  $\beta$ -mercaptoethanol, 2%

DMSO with or without 500  $\mu$ M IBMX, and Sf21 lysate containing an equal amount of recombinant mutant and wild-type protein as determined by Western blot analysis. Substrate concentration was varied at a constant ratio of  $^3$ H-labeled cyclic nucleotide to unlabeled cyclic nucleotide (1:50). Reactions were terminated, and radiolabeled nucleotide was quantified as described earlier. Product formation was measured at 5, 10, 15, and 20 min, and the rate of hydrolysis was determined by the slope of the linear regression of micromoles of product formed per minute. The background was measured in reactions containing 500  $\mu$ M IBMX to control for any endogenous PDEs, and these counts were subtracted from the total counts at each concentration of substrate. A linear standard curve of CPM per micromole nucleotide was determined using dilutions of standard [ $^3$ H]AMP stock.

**Statistics and Data Analysis.** Data from two or more experiments were pooled to afford the opportunity to assess model aptness and provided efficient parameter estimation.  $IC_{50}$  values (and associated standard errors) were estimated with a two-parameter nonlinear model regressing response on the common logarithm of dose with the statistical software package JMP (SAS Institute, Cary, NC). Then  $\log_{10} IC_{50}$  values were compared with each other in a pairwise fashion via a Z-statistic (Zhou et al., 1997). Final estimates of  $IC_{50}$  values on the original (nonlogarithm) scale were determined via an antilogarithm transformation and a  $\delta$ -method estimate of the corresponding S.E. values (Billingsley, 1986).  $K_m$  and  $V_{max}$  values (and associated S.E. values) were also estimated in SAS JMP via a standard Michaelis-Menton model.  $K_m$  and  $V_{max}$  values were subsequently compared with other  $K_m$  and  $V_{max}$  estimates, respectively, via a standard Z-statistic.

Each of the three parameters,  $IC_{50}$ ,  $K_m$ , and  $V_{max}$ , formed a group or class of statistical comparisons. Members of each class were not statistically compared with members of another (i.e.,  $IC_{50}$  estimates from one set of experimental conditions were compared only other  $IC_{50}$  values from others and not with  $K_m$  and  $V_{max}$  values). Thus, the level of statistical significance for all comparisons was chosen to be  $P = .05/3 = .0167$  to adjust for multiplicity of statistical testing within a class of parameters.

## Results

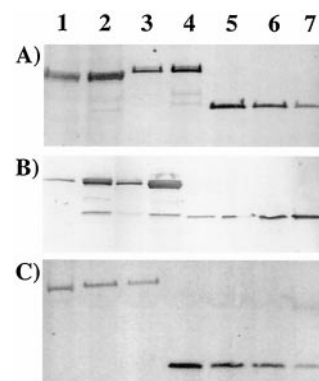
**Sequence Comparisons of PDE Families.** A comparison of the consensus sequence for the catalytic domain of cAMP-specific PDEs with that of cGMP-specific PDEs shows that there are sequence differences between the cAMP-specific and cGMP-specific families. One of these differences is residue 333 in the amino terminus of the PDE4D3 catalytic domain (Fig. 1). The cAMP-preferring enzymes conserve an aspartic acid at this position, whereas the cGMP-preferring enzymes conserve an asparagine. It is known that this position is in the inhibitor binding site because it affects rolipram binding (Pillai et al., 1993). Based on these data, we hypoth-

PDE4D	V A Y H N N I H A A D V V Q S T H V L L S T P A
all cAMP-specific	n p Y H N s i H A a D V t q S t h y l l s t p a
all cGMP-specific	v t Y H N W r H g F N v g q t m F t l l m t g k

**Fig. 1.** Alignment of PDE4 with consensus sequence for cAMP-specific PDEs and consensus sequence for cGMP-specific PDEs. The three sequences listed include a consensus sequence created from all human and rat PDE4D subtypes and cAMP-specific and cGMP-specific consensus sequences constructed as described in *Materials and Methods*. Capital letters indicate conserved positions. The first two putative metal-binding histidines are boxed in dark gray. The amino acid mutated in PDE4D3 and PDE4B1 is boxed in light gray. The aspartic acid in the PDE4D sequence corresponds to residue number 333 of PDE4D3, 413 of PDE4B1, and 440 of PDE4A4B (GenBank accession numbers are U50159, L20966, and L20965, respectively).

esized that the difference between Asp and Asn could contribute to the substrate specificity of these two main classes of PDEs. To test this hypothesis, we made mutations at this residue, as described by Pillai et al. (1993), and tested the biochemical properties of the mutant proteins.

**Expression of PDE4 Wild-Type and Mutant Proteins.** Mutant forms of the PDE4D3 protein were made, and the mutant and wild-type proteins were expressed using a baculovirus expression system. To generate reliable kinetic data, activity assays must be done at relatively similar PDE protein concentrations. To determine the PDE4 protein concentration in each of the lysate samples, Western blot analysis was done on the lysate from the infected cells (Fig. 2). The expressed proteins are recognized by a PDE4D monoclonal antibody and are the predicted size for PDE4D3 (93 kDa), as expected because the antibodies recognize the PDE4D-specific carboxyl-terminal region of this protein. The concentration of recombinant protein in each lysate was estimated by comparison with known concentrations of purified PDE4D carboxyl-terminal GST fusion protein. The lysate containing PDE4D3 Asp333Asn had recombinant protein at a concentration of approximately 27 ng/ml, and the lysate containing PDE4D3 wild-type had recombinant protein at a concentration of approximately 4  $\mu$ g/ml. Because the amount of protein and the activity of the protein can vary among baculovirus infections, we compare only proteins made at the same time and under the same conditions. The mutant PDE4D3 Asp333Ala (and wild-type) was produced in a separate infection, and the amount of PDE4D3 wild-type and Asp333Ala in the lysate was determined in an analogous fashion (Fig. 2B). The lysate containing PDE4D3 Asp333Ala had recombinant protein at a concentration of approximately 225 ng/ml, and the lysate containing PDE4D3 wild type had recombinant protein at a concentration of approximately 600 ng/ml. The slight difference in mobility between wild-type and PDE4D3(Asp333Asn) mutant proteins in Fig. 2A (lanes 1, 3,



**Fig. 2.** Western blot analysis to determine concentration of PDE4 wild-type and mutant proteins in Sf21 lysates. PDE4-GST proteins were used as a standard to determine protein concentrations. A, determination of PDE4D3 wild-type and PDE4D3 Asp-Asn proteins. Lane 1, 15  $\mu$ l of PDE4D3 Asp-Asn lysate; lane 2, 15  $\mu$ l of PDE4D3 Asp-Asn lysate plus 0.1  $\mu$ l of wild-type PDE4D3 lysate; lanes 3 and 4, 0.2 and 0.4  $\mu$ l of wild-type PDE4D3 lysate, respectively; and lanes 5–7, 400, 200, and 100 ng of PDE4D-GST, respectively. B, determination of PDE4D3 wild-type and PDE4D3 Asp-Ala proteins. Lanes 1 and 2, 0.1 and 1  $\mu$ l of wild-type PDE4D3 lysate, respectively; and lanes 3 and 4, 1 and 10  $\mu$ l of PDE4D3 Asp-Ala lysate, respectively; and lanes 5–7, 50, 100, 200, and 400 ng of PDE4D-GST protein, respectively. C, determination of PDE4B wild-type and mutant proteins. Lane 1, 20  $\mu$ l of PDE4B1 Asp-Asn lysate; lanes 2 and 3, 0.4 and 0.2  $\mu$ l of wild-type PDE4B1 lysate, respectively; and lanes 4–7, 40, 20, 10, and 4 ng of PDE4B-GST protein, respectively.



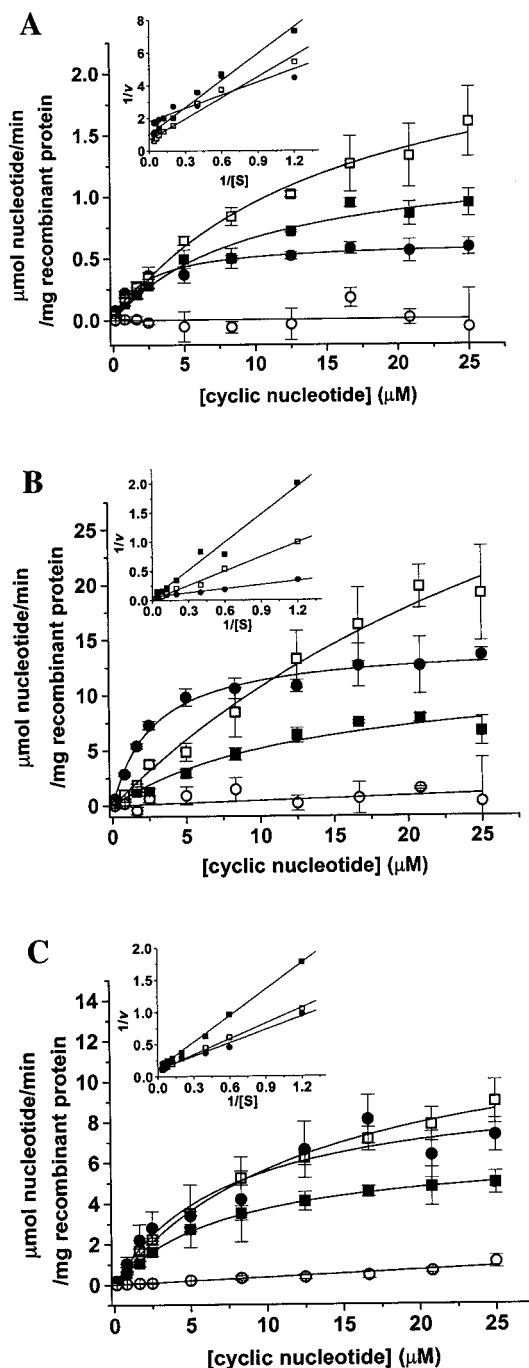
and 4) is due to differences in sample volume applied to the gel, as mixing wild-type and mutant protein shows a single band on the gel (Fig. 2A, lane 2).

The same mutation was made in PDE4B1, and the wild-type and mutant proteins were expressed. Western blot analysis of the recombinant proteins shows a single band that is recognized by a PDE4B-specific polyclonal antibody in both the lysate containing the PDE4B1 wild-type protein and the lysate containing the PDE4B1 Asp413Asn protein (Fig. 2C). These bands are the predicted size for PDE4B1 (93 kDa). The concentration of recombinant protein in each lysate was also calculated by comparison with known concentrations of purified PDE4B carboxyl-terminal GST. The lysate containing PDE4B1 Asp413Asn had recombinant protein at a concentration of approximately 0.37 ng/ml, and the lysate containing PDE4B1 had recombinant protein at a concentration of approximately 15 ng/ml.

**Kinetic Analysis of PDE4 Wild-Type and Mutant Proteins.** PDE4D3 wild-type, PDE4D3 Asp333Asn, and PDE4D3 Asp333Ala proteins were able to hydrolyze cAMP. This agrees with previous data showing that PDE4B with the analogous Asp-Asn mutation could hydrolyze cAMP (Pillai et al., 1993). A more complete characterization of the cAMP hydrolysis was done by determining the  $K_m$  and  $V_{max}$  values for cAMP with the wild-type and the mutant protein (Fig. 3). The  $V_{max}$  for cAMP is 0.76  $\mu\text{mol AMP/min/mg}$  recombinant wild-type protein. This value is similar to the published values (Salanova et al., 1998; Saldou et al., 1998a). PDE4D3 Asp333Asn has a  $V_{max}$  value for cAMP that is approximately 2-fold of the  $V_{max}$  value for cAMP with PDE4D3 wild-type (Table 1). The  $K_m$  value for cAMP of the mutant protein is 16  $\mu\text{M}$ , 3-fold greater than that observed for the wild-type protein (Table 1). This minor difference in kinetic parameters suggests that the Asp333Asn mutation has not altered the ability of the protein to convert cAMP to AMP. In agreement with Pillai et al. (1993), no high-affinity binding of rolipram could be observed in the PDE4D3 Asp333Asn mutant extract, whereas the wild-type extract shows normal high-affinity binding (unpublished observations). This is consistent with the decrease in rolipram sensitivity expected for this mutant.

Initial experiments showed that the mutant protein, unlike the wild-type, could hydrolyze cGMP. To characterize cGMP hydrolysis in the mutant protein, the  $K_m$  and  $V_{max}$  values for cGMP were determined (Fig. 3). The  $K_m$  and  $V_{max}$  values for cGMP with PDE4D3 wild-type could not be determined because the levels of cGMP hydrolysis were so low that a rate could not be calculated. PDE4D3 Asp333Asn has a  $V_{max}$  value for cGMP that is 3.5  $\mu\text{mol GMP/min/mg}$  recombinant protein, which is similar to the  $V_{max}$  value for cAMP with this same protein (Table 1). This indicates that the Asp333Asn mutant protein can convert cGMP to GMP as efficiently as it can convert cAMP to AMP. Also, the data show that the rate of cGMP hydrolysis by the mutant protein is at least 10-fold greater than the rate of cGMP hydrolysis by the wild-type protein based on the minimum level of cGMP hydrolysis that can be detected in our assay at 30  $\mu\text{M}$  substrate concentration in a 20-min incubation. PDE4D3 Asp333Asn has a  $K_m$  value for cGMP that is less than 2-fold greater than the  $K_m$  value for cAMP with the same protein (Table 1). These results suggest that the Asp333Asn mutant protein binds cAMP and cGMP with a similar affinity.

A direct assessment of the change in nucleotide selectivity between the wild-type and mutant protein is hampered by the inability to measure cGMP hydrolysis by the wild-type protein. Therefore, we measured the ability of cGMP to in-



**Fig. 3.** Michaelis-Menten analysis of PDE4D3 and PDE4B1. Cyclic nucleotide hydrolysis by wild-type and mutant proteins of PDE4. ■, cAMP hydrolysis by mutant protein. ●, cAMP hydrolysis by wild-type protein. □, cGMP hydrolysis by mutant protein. ○, cGMP hydrolysis by wild-type protein. A, cyclic nucleotide hydrolysis by HSPDE4D3 wild-type and Asp333Asn. B, cyclic nucleotide hydrolysis by HSPDE4B1 wild-type and Asp413Asn. C, cyclic nucleotide hydrolysis by HSPDE4D3 wild-type and Asp333Ala. Insets to each figure show the Lineweaver-Burke transformation of the data set. These are representative data, and the error bars show the S.D. of duplicate points from one experiment. The experimental design and data analysis are described in *Materials and Methods*.

hibit cAMP hydrolysis by these proteins. PDE4D3 wild-type shows a 50% reduction in cAMP hydrolysis ( $IC_{50}$ ) at 360  $\mu M$ , whereas PDE4D3 Asp333Asn shows the same reduction at 24  $\mu M$ . Thus, cAMP hydrolysis by PDE4D3 Asp333Asn is inhibited at a significantly lower concentration of cGMP than cAMP hydrolysis by PDE4D3 wild type (Fig. 4). Comparison of cGMP  $IC_{50}$  with cAMP  $K_m$  for these proteins can give an indication of selectivity between cGMP and cAMP. For the wild-type protein, the ratio of cGMP  $IC_{50}$  to the  $K_m$  for cAMP is 88 (360:4.1). This same ratio is 1.7 for the mutant protein (24:14). The difference in ratios between the wild-type and mutant protein represents an apparent 50-fold change in nucleotide selectivity. These results show that the Asp333Asn mutation has increased the affinity of the protein for cGMP.

To determine whether alteration in nucleotide selectivity with the Asp333Asn mutation in PDE4D3 was a subtype-specific effect, we measured the  $K_m$  and  $V_{max}$  values for cAMP and cGMP with both PDE4B1 wild-type and Asp413Asn mutant proteins (Fig. 3). The wild-type protein has a  $V_{max}$  value for cAMP of 14  $\mu mol$  AMP/min/mg recombinant protein. This value is within the range of the published values (Rocque et al., 1997). The wild-type and the mutant proteins had a very similar  $V_{max}$  value for cAMP. This suggests that the PDE4B1 mutant protein, like the PDE4D3 Asp333Asn mutant, can hydrolyze cAMP as efficiently as wild-type (Table 1). PDE4B1 Asp413Asn has a  $K_m$  value for cAMP that is approximately 5-fold greater than the  $K_m$  value for cAMP with PDE4B1 wild type, indicating that the mutant protein binds cAMP with a lower affinity than the wild-type protein (Table 1). Thus, the data with PDE4B1 agree with the results we obtained from comparing the cAMP hydrolysis by wild-type and Asp333Asn mutant of PDE4D3. PDE4B1 Asp-Asn has a  $K_m$  value for cGMP that is approximately 2-fold higher than the  $K_m$  value for cAMP. The  $V_{max}$  value for cGMP is approximately 4-fold greater than the  $V_{max}$  value for cAMP (Table 1). These results suggest that the mutant protein has a similar affinity for cAMP and cGMP and can efficiently convert cGMP to GMP.

Pillai et al. (1993) have shown that an alanine at this residue also affects rolipram binding. To determine whether the change in nucleotide selectivity was dependent on an asparagine at this residue, we altered the aspartic acid at residue 333 to an alanine in PDE4D3. The analysis was done on wild-type and mutant protein lysates that had been in-

fectured and isolated at the same time and under the same conditions to minimize possible differences due to protein production. Similar to the Asp-Asn mutation, PDE4D3 Asp-Ala could hydrolyze cAMP as well as wild-type and was also capable of hydrolyzing cGMP (Table 1). Also, the rates and  $K_m$  values for cAMP hydrolysis and cGMP hydrolysis by PDE4D3 Asp333Ala were similar. In addition,  $K_m$  value for cGMP hydrolysis by PDE4D3 Asp333Ala is similar to the  $K_m$  value for cGMP by PDE4D3 Asp333Asn (Table 1). This demonstrates that the change in nucleotide selectivity is due to the removal of the aspartic acid at residue 333.

**Pharmacological Characterization of PDE4D3 Wild-Type and Mutant Proteins.** Altering the analogous aspartic acid residue to an asparagine in a truncated form of PDE4B makes the protein less sensitive to inhibition by rolipram (Pillai et al., 1993). This mutation also makes the cAMP hydrolytic activity of PDE4D3 less sensitive to inhibition by rolipram (Table 2). To determine whether this mutation alters the sensitivity of PDE4D3 to PDE inhibitors other than rolipram, the inhibitor concentration at 50% PDE activity ( $IC_{50}$ ) was calculated for 14 PDE inhibitors with PDE4D3 Asp333Asn and wild-type (Table 2). Almost all of the inhibitors tested showed some statistically significant change in  $IC_{50}$ , between wild-type, cAMP hydrolysis by the mutant protein, and cGMP hydrolysis by the mutant protein. The exceptions include the nonspecific inhibitor IBMX and two compounds that do not inhibit, erythro-9-(2-hydroxy-3-nonyl)adenine HCl (EHNA) and quazinson. However, milrinone and dipyrindamole show changes that are not likely to be significant biologically. Based on the results from these experiments, the inhibitors were divided into three groups: compounds that are less potent, compounds that show the same potency, or compounds that are more potent for mutant protein activity compared with wild-type protein activity.

The first group of inhibitors is less potent against PDE4D3 Asp333Asn than PDE4D3 wild type. Rolipram and all of the PDE4-specific inhibitors tested fall into this group. This group also includes vinpocetine, trequinsin, and MY-5445. The compounds in this group have inhibitory activity against cAMP hydrolysis by PDE4D3 wild-type that is greater than their inhibitory activity against cAMP hydrolysis by PDE4D3 Asp333Asn. Interestingly, the  $IC_{50}$  value for cGMP hydrolysis is similar to that for cAMP hydrolysis by the mutant

TABLE 1

Hydrolysis of cyclic nucleotide by the wild-type, Asp-Asn, and Asp-Ala mutant proteins of PDE4D3 and PDE4B1

$K_m$  and  $V_{max}$  values for hydrolysis of cAMP by wild-type PDE4D3 and PDE4B1 and hydrolysis of cAMP and cGMP by two mutants of PDE4D3 and one mutant of PDE4B1 are shown. Experimental procedures and biostatistical analysis are described in *Materials and Methods*.

	PDE4D3				PDE4B1			
	$K_m$	S.E.	$V_{max}$	S.E.	$K_m$	S.E.	$V_{max}$	S.E.
	$\mu M$		$\mu mol/min/mg$ recombinant protein		$\mu M$		$\mu mol/min/mg$ recombinant protein	
Wild-type cAMP hydrolysis	2.7***	$\pm 0.79$	0.76***	$\pm 0.06$	3.2*	$\pm 0.52$	14	$\pm 0.62$
Asp-Asn cAMP hydrolysis	16	$\pm 3.7$	1.6***	$\pm 0.19$	20	$\pm 11$	12	$\pm 3.7$
Asp-Asn cGMP hydrolysis	23	$\pm 8.1$	3.5	$\pm 0.71$	40	$\pm 18$	49	$\pm 15$
Wild-type cAMP hydrolysis	5.8	$\pm 1.35$	9.7	$\pm 0.76$				
Asp-Ala cAMP hydrolysis	7.1	$\pm 1.2$	7.9	$\pm 1.2$				
Asp-Ala cGMP hydrolysis	12	$\pm 6.2$	9.7	$\pm 2.3$				

\*  $P < .0167$ , for cAMP hydrolysis by PDE4D3 (PDE4B1) wild-type and cAMP hydrolysis by PDE4D3 Asp-Asn.

\*\*  $P < .0167$ , for cAMP hydrolysis by PDE4D3 wild-type and cGMP hydrolysis by PDE4D3 Asp-Asn.

\*\*\*  $P < .0167$ , for cAMP hydrolysis by PDE4D3 Asp-Asn and cGMP hydrolysis by PDE4D3 Asp-Asn.

protein. These results suggest that the Asp333Asn mutation decreases the affinity of PDE4D3 for these compounds.

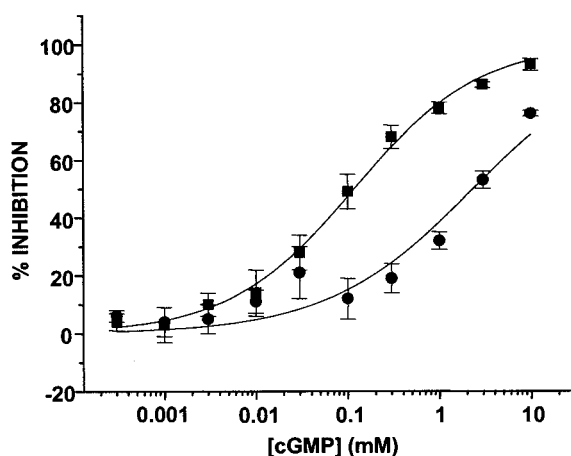
The inhibitory activity of the second group of compounds is not altered by the Asp-Asn mutation. EHNA, milrinone, quazinone, dipyridamole, and IBMX are in this group. These compounds have approximately the same  $IC_{50}$  value for cAMP hydrolysis by PDE4D3 wild-type and hydrolysis of both nucleotides by PDE4D3 Asp333Asn. These data demonstrate that the interaction of these compounds with PDE4D3 is not altered by the Asp333Asn mutation.

The third group of inhibitors has greater activity against PDE4D3 Asp333Asn than PDE4D3 wild-type. This group of inhibitors includes 8-methoxy-IBMX, cilostamide, and zaprinast. These compounds inhibit cAMP and cGMP hydrolysis by PDE4D3 Asp333Asn at a lower concentration than cAMP hydrolysis by PDE4D3 wild-type. These data suggest that

the mutant protein has a higher affinity for these compounds than that observed for the wild-type protein. Zaprinast is unique in this group because it is 5- to 12-fold more potent against cGMP hydrolysis by PDE4D3 Asp333Asn than against cAMP hydrolysis by either the mutant protein or the wild-type protein. Cilostamide shows a similar trend as zaprinast. These results show that zaprinast selectively inhibits cGMP hydrolysis by PDE4D3 Asp333Asn.

## Discussion

Mutation of a single amino acid in PDE4D3 and PDE4B1 alters the substrate selectivity and inhibitor profiles of these enzymes. The aspartic acid, at residue 333 in PDE4D3 or residue 413 in PDE4B1, is conserved in PDEs that favor cAMP hydrolysis and has previously been shown to be important in the mechanism of inhibition by rolipram, the PDE4-specific inhibitor. An asparagine is conserved at the same position in PDE families that prefer cGMP hydrolysis. Mutation of the aspartic acid to an asparagine converts the cAMP-specific PDE4s to PDEs that can also hydrolyze cGMP. Similar changes in the enzymatic characteristics of both PDE4D3 and PDE4B1 were observed. The modified proteins are still able to hydrolyze cAMP. In addition, PDE4B1 Asp413Asn, PDE4D3 Asp333Asn, and PDE4D3 Asp333Ala bind and hydrolyze cGMP almost as well as cAMP. The rates of hydrolysis ( $V_{max}$ ) of cAMP by mutated and wild-type proteins are similar, and the rates of hydrolysis of cGMP by the mutated proteins are approximately the same as the rate of cAMP hydrolysis by these proteins. Western blot analysis shows that full-length soluble proteins are expressed. These data suggest that this mutation has not severely compromised the protein structure or the ability of these PDEs to perform the normal function of cAMP hydrolysis. Thus, this report shows that a large fraction of the nucleotide selectivity in PDE4 proteins depends on a single residue and that this residue is involved in the binding of specific inhibitors for several PDE families (as well as PDE4).



**Fig. 4.** cGMP Inhibition of cAMP hydrolysis by PDE4D3 wild-type and the Asp333Asn mutant. Data were obtained using wild-type protein (●) or mutant protein (■). Each data point represents the average and S.D. of duplicates for a representative experiment. The average  $IC_{50}$  value for cGMP from two separate experiments is  $360 \pm 120 \mu M$  for wild-type protein and  $24 \pm 12 \mu M$  for mutant protein. The experimental design and data analysis are described in *Materials and Methods*.

**TABLE 2**

$IC_{50}$  values for PDE4D3 wild-type and PDE4D3 Asp-Asn proteins

Inhibitor concentrations at 50% activity for cAMP hydrolysis by PDE4D3 wild-type protein and cAMP and cGMP hydrolysis by PDE4D3 Asp440Asn protein. Cyclic nucleotide hydrolysis and biostatistical analysis were performed as described in *Materials and Methods*.

Compound	PDE Selectivity	$IC_{50}$	PDE4D3 Wild-Type		PDE4D3 Asp-Asn			
			cAMP		cAMP		cGMP	
			$IC_{50}$	S.E.	$IC_{50}$	S.E.	$IC_{50}$	S.E.
		$\mu M$	$\mu M$		$\mu M$		$\mu M$	
8-Methoxymethyl IBMX	1	4	90.9***	5.1	39.7	0.3	39.8	0.5
Vincocetine	1	20	47.1***	0.7	76.7	1.2	>100	
EHNA	2	0.8	>100		>100		>100	
Cilostamide	3	0.005	>100***		58.9***	0.5	35.7	0.4
Milrinone	3	0.3	23.0***	0.3	37.8	0.4	34.2	0.6
Quazinone	3	0.6	>100***		>100***		70.6	2.3
Trequinsin	3	0.0003	0.3***	0.005	2.2***	0.02	1.2	0.02
Rolipram	4	1	0.3***	0.01	34.4	0.9	47.5	1.4
Ro 20-1724	4	2	1.9***	0.1	63.4	1.9	63.9	1.0
Etazolate	4	2	0.6***	0.003	6.8***	0.04	4.7	0.04
Dipyridamole	5	0.9	4.0*	0.1	8.9***	0.1	3.2	0.04
Zaprinast	5	0.8	72.4***	1.1	27.7***	0.04	5.8	0.05
MY-5445	5	0.6	12.5***	0.2	>100		>100	
IBMX	None		11.9	0.4	14.0	0.5	11.9	0.4

\*  $P < .0167$ , for cAMP hydrolysis by PDE4D3 wild type and cAMP hydrolysis by PDE4D3 Asp440Asn.

\*\*  $P < .0167$ , for cAMP hydrolysis by PDE4D3 wild type and cGMP hydrolysis by PDE4D3 Asp440Asn.

\*\*\*  $P < .0167$ , for cAMP hydrolysis by PDE4D3 Asp440Asn and cGMP hydrolysis by PDE4D3 Asp440Asn.



The wild-type PDE4 protein does not measurably hydrolyze cGMP. We cannot measure a  $K_m$  value to directly compare the fold-change in cGMP to cAMP selectivity between the wild-type and mutated proteins, so we measured the  $IC_{50}$  for cGMP inhibition of cAMP hydrolysis by both proteins and compared these numbers with the  $K_m$  value for cAMP to determine the absolute change in selectivity. The ratio of cGMP  $IC_{50}$  to cAMP  $K_m$  for the wild-type protein is 87, and for the mutated protein, the ratio is 1.7, showing an apparent 50-fold change in selectivity for cGMP. The ratio of cGMP  $K_m$  and cAMP  $K_m$  values of the mutated protein is 1.9, which is very close to the  $IC_{50}:K_m$  ratio, suggesting that cAMP and cGMP compete for binding at the same catalytic site. These data suggest that the Asp-Asn mutation results in a protein with an increased affinity for cGMP. However, we cannot rule out the possibility that this mutation has an effect on the catalytic mechanism involved in the hydrolysis of cGMP. These data taken together suggest that the selectivity of the cAMP-specific PDE4 has not been switched to a cGMP-specific PDE but that the specificity requirements have been relaxed, allowing for the binding and hydrolysis of both cyclic nucleotides. This idea is depicted in the model described in Fig. 5.

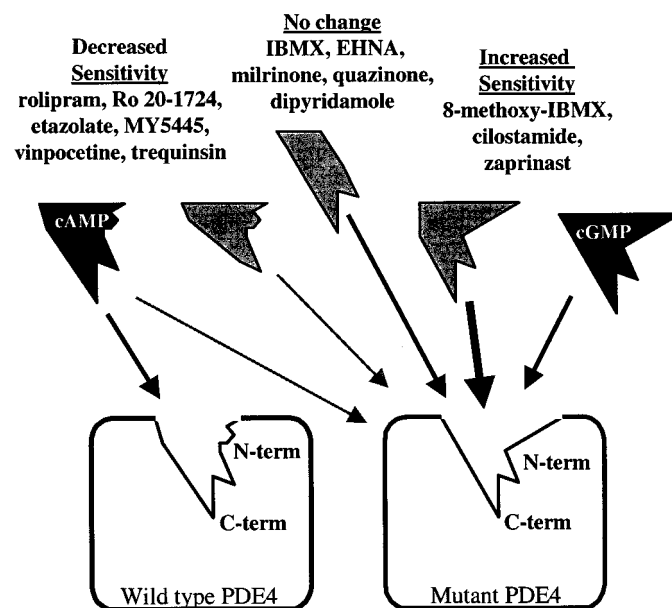
In addition to replacing the aspartic acid with an asparagine, we changed this residue to an alanine to determine the amino acid requirement at this position. This substitution was also shown to alter rolipram inhibition (Pillai et al., 1993). The results show that the kinetic parameters and substrate selectivity are very similar to those obtained for the asparagine mutation. This suggests that it is not the presence of an asparagine in this position that enhances cGMP binding but rather the presence of an aspartic acid that is important in excluding cGMP.

Several previous reports have suggested that the carboxyl end of the conserved domain is at least in part responsible for conferring substrate-binding specificity (Omburo et al., 1997; Atienza and Colicelli, 1998). Our data suggest that at least one residue in the amino terminus of this domain is also very important. Therefore, it is likely that both ends of the catalytic domain interact to form the active site.

We tested several inhibitors, PDE4 specific as well as those specific for other PDE families, and found some interesting differences between the wild-type and the modified proteins. As previously shown for the PDE4 catalytic domain (Pillai et al., 1993), the mutated enzymes showed reduced sensitivity to PDE4-specific inhibitors. This reduction is probably due to a loss of specific interactions that are necessary for efficient competition of substrate hydrolysis by this class of inhibitors (Fig. 5). There is an approximately 10-fold difference in the  $IC_{50}$  values determined from those previously (Pillai et al., 1993). There are, however, several differences between these two studies and the proteins used to determine these values. These differences include the length of the protein, the origin of the sequence, and the expression systems used to make the proteins. The previous studies were performed with the conserved domain of rat PDE4B1 expressed in yeast, whereas the study described here was performed with full-length human PDE4D3 and PDE4B1 produced with a baculovirus expression system. Any one of these differences could account for the differences seen in inhibitor sensitivities. It is important to note, however, that the shift in PDE4 inhibitor sensitivity is in the same direction. Three other inhibitors, vin-

pocetine, trequinsin, and MY-5445, also have a decreased ability to inhibit the mutated form of the proteins even though they are not specific for PDE4. These data suggest that these inhibitors also make contact with this region in PDE1, PDE3, and PDE5, respectively.

It is interesting to note that mutation of this residue enhanced sensitivity of the protein to several inhibitors. In contrast to rolipram, zaprinast, cilostamide, and 8-methoxy-IBMX showed an increased potency for inhibition of the mutated protein for both cAMP and cGMP. Most interesting and unexpected was the effect of zaprinast, the cGMP-specific PDE5 inhibitor. Zaprinast is able to selectively inhibit cGMP hydrolysis by the mutated PDE4D3 (12-fold greater than wild-type cAMP hydrolysis), whereas cAMP hydrolysis is only moderately affected (2.6-fold greater than wild type). This suggests that the structural change caused by altering this residue allows access of both cGMP and zaprinast to areas of the protein that are not available in the wild-type protein. The inhibition of another PDE5 inhibitor, MY-5445, has lost demonstrable inhibitory activity for the mutated protein. These two compounds do not appear to share any obvious common structural characteristics, suggesting that



**Fig. 5.** Model for the interactions of inhibitors and cyclic nucleotides with wild-type and mutant proteins of PDE4. This is a model for inhibitors and cyclic nucleotides interacting with the wild-type and Asp-Asn mutant PDE4. For simplicity and because the observed kinetic parameters of hydrolysis do not change dramatically, we discuss this model in terms of changes in the active site, but the present data cannot rule out changes in overall secondary or tertiary structure of the protein that influence the results. The inhibitors are shaded gray, the nucleotides are black, and the proteins are white. This model shows the amino and carboxyl termini of the conserved region of the PDE4 protein in close proximity to each other. The gray lines represent a decrease in affinity, the black lines represent no change in affinity, and the bold lines show an increase in affinity. cAMP is able to bind the active site of both proteins, but the mutant protein has fewer interactions with cAMP. cGMP binding to the mutant PDE4 protein is enhanced by additional contacts. The inhibitors that are less potent against the Asp-Asn mutant protein make more contacts in wild-type PDE4 than in mutant PDE4. The inhibitors that are equally potent against both proteins make interactions that are conserved between the two proteins. The compounds that have greater inhibitory activity against PDE4 Asp-Asn have additional contacts compared with wild-type protein. In particular, zaprinast more closely mimics cGMP binding to the mutant protein.

these two inhibitors interact with different residues in the PDE active site. Cilostamide, a PDE3 inhibitor, follows the same pattern as zaprinast, although the differences between the inhibition of cGMP and cAMP hydrolysis are not as pronounced. Interestingly, PDE3 is characterized by cGMP inhibition of cAMP hydrolysis at the catalytic site. PDE3 is able to bind cGMP but has an approximately 10-fold lower  $V_{\max}$  value (Degerman et al., 1997). Like PDE4, PDE3 contains an aspartic acid residue at the analogous position. Although this mutation has not been made in PDE3, the data suggest that this residue may also be involved in the catalytic rate of hydrolysis in PDE3 and PDE4.

The change in specificity observed may be due to a direct interaction with the nucleotide base. The adenine and guanine bases differ markedly in their patterns of hydrogen bond donors and acceptors. In particular, at position 6, the substituent changes from an exclusively hydrogen bond-donating amino group in adenine to an exclusively hydrogen bond-accepting carbonyl group in guanine. In studies using synthetic nucleotide analogs of cAMP and cGMP, position 6 of the nucleotide base has been shown to be critical for base discrimination (Beltman et al., 1995; Butt et al., 1995). One explanation for our results is that PDE4D3 (Asp333) directly hydrogen bonds to the adenine amino group. This interaction would be unavailable on cGMP binding. Because the asparagine side chain can be both a hydrogen bond donor and an acceptor, the Asp333Asn mutant would be able to hydrogen bond equally well with either purine substrate. An alanine at this position, which has no hydrogen bonding potential, also showed reduced discrimination between cGMP and cAMP.

A change in nucleotide specificity on the change of an asparagine to an aspartic acid has been demonstrated in the enzyme thymidylate synthase (Hardy and Nalivaika, 1992; Liu and Santi, 1992, 1993). As seen by x-ray crystallography, in thymidylate synthase an asparagine forms two hydrogen bonds with the pyrimidine ring of the dUMP substrate. The change of that asparagine to aspartate created a dCMP-specific methylase.

Thus, our data confirm that this residue is in the active site and extend previous observations to include substrate selectivity as well as inhibitor selectivity. Taken together, these results imply that changing this residue affects the overall structure of the active site of PDE4.

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