Role of Conserved Histidines in Catalytic Activity and Inhibitor Binding of Human Recombinant Phosphodiesterase 4A

SUSANNE JACOBITZ, M. DOMINIC RYAN, MEGAN M. McLAUGHLIN, GEORGE P. LIVI, WALTER E. DeWOLF, JR., and THEODORE J. TORPHY

Dpartments of Pulmonary Pharmacology (S.J., T.J.T.), Immunopharmacology (T.J.T.), Physical and Structural Chemistry (M.D.R.), Molecular Recognition (W.E.D.), and Gene Expression Sciences (M.M.M.), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

SUMMARY

To identify critical amino acids within the central conserved region of recombinant human cAMP-specific phosphodiesterase 4 subtype A (rhPDE4A), we engineered the expression of point mutants in a fully active rhPDE4A/Met201–886. When histidine residues at positions 433, 437, 473, and 477, which are highly conserved among all PDE families, were changed independently to serine residues, cAMP hydrolyzing activities were substantially reduced or abolished. The ability of these mutants to bind prototypical PDE4 inhibitors [^{3}H]-(*R*)-rolipram or [^{3}H]RP 73401 was also decreased in parallel with the loss of catalytic activity. The parallel loss of catalytic activity and inhibitor binding suggests that these changes resulted from nonlocalized perturbations in the structure of the enzyme. More interesting results were obtained when histidine residues at positions 505 and 506 were changed independently to aspar

agines. The K_m value for cAMP increased 3-fold in H505N (K_m = 11 ± 3 μM) and 11-fold in H506N (K_m = 44 ± 6 μM) compared with the wild-type protein (K_m = 4 ± 1 μM). These mutant proteins bound [^{3}H]-(*R*)-rolipram and [^{3}H]RP 73401 with K_d values of 1.8 ± 0.4 and 0.3 ± 0.1 nM, respectively, for H505N, and 3.9 ± 0.9 and 0.5 ± 0.1 nM, respectively, for H506N. These values are nearly identical to those obtained with the wild-type rhPDE4A/Met201–886. In contrast, the IC$_{50}$ values for cAMP competition with either [^{3}H]-(*R*)-rolipram or [^{3}H]RP 73401 binding increased ~2-fold in H505N and ~13-fold in H506N compared with the wild type protein. These increases are virtually identical to the changes in the K_m value for cAMP in these mutants. We conclude that His506 and, perhaps, His505 are involved in binding of cAMP to PDE4A/Met201–886 but not in binding of PDE4-selective inhibitors.

The hydrolysis of the second messengers cAMP or cGMP is catalyzed by the cyclic nucleotide PDE superfamily of enzymes. Seven members of this family have been described. These PDEs differ with respect to substrate specificity, sensitivity to specific inhibitors, and protein sequence (1–3). One member of the superfamily is the cAMP-specific, low-K_m PDE, termed PDE4. The structure of PDE4 consists of a central conserved domain, which contains the catalytic region, and two upstream conserved regions that have regulatory functions (4, 5). Four genetically distinct subtypes of the PDE4 family (PDE4A–D) have been identified (6–13). Differences within the protein sequences of these subtypes occur mainly at their NH$_2$- and COOH-terminal ends (5). They share 80–84% sequence identity within the central conserved region, which spans ~340 amino acids. Catalytic activities of all PDE4 subtypes are selectively inhibited by compounds such as rolip-

ram, BRL 61063, and RP 73401, presumably through direct competition with substrate (14–19).[1]

The PDE4A subtype is the largest protein among the PDE4s, consisting of 886 amino acids with a native molecular mass of 110 kDa (5). A notable feature of PDEs is their ability to bind the archetypal PDE4-selective inhibitor (*R*)-rolipram with both a high affinity (K_d = 1 nM) and a low affinity (K_d = 50–100 nM) (19-21). This unique characteristic is thought to reflect the ability of hPDE4A to exist in two distinct and enzymatically active conformational states: one with a catalytic site that binds rolipram with a high affinity

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[1] W. E. DeWolf, M. M. McLaughlin, D. W. Green. S. M. Fisher, P. Hensley, E. O. Doughty, G. P. Livi, and T. J. Torphy. Kinetic analysis of purified human cAMP phosphodiesterase 4A: evidence for negative cooperativity in full-length and N-terminally truncated form, submitted for publication.

ABBREVIATIONS: PDE, phosphodiesterase; PDE4, cAMP-specific phosphodiesterase; rhPDE4, recombinant human cAMP-specific phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; HPDE4, high affinity rolipram-binding conformer of cAMP-specific phosphodiesterase; LPDE4, low affinity rolipram-binding conformer of cAMP-specific phosphodiesterase.

(termed HPDE4) and a second with a catalytic site that binds rolipram with a low affinity (termed LPDE4) (19).

A more recently synthesized compound, RP 73401, selectively inhibits PDE4 with very high potency ($IC_{50} \sim 0.5$ nM) (17, 19). This compound does not distinguish between HPDE4 and LPDE4 but instead binds with equal affinity ($K_d = 0.4$ nM) to both forms of the enzyme (19). Additional studies suggest that cAMP, rolipram, and RP 73401 all bind to a central conserved region of hPDE4A, spanning amino acids 332–722 (19).

The critical amino acids within the catalytic core of hPDE4A that are responsible for substrate binding and catalysis have not been identified. However, Jin et al. (8) demonstrated that the substitution of Thr349 with either alanine or proline in rat PDE4D (corresponding to Thr544 in hPDE4A) abolished catalytic activity. In contrast, the replacement of Thr349 with a serine, a more conservative change, yields an enzyme that retains a degree of catalytic activity. This implies an important role for Thr349 as a H-bond donor in PDE4. Other studies on the rolipram sensitivity of spontaneous single-site mutations of rat rPDE4B have yielded interesting information on inhibitor binding (22). Three catalytically active mutants were identified as being rolipram resistant: D239N (position 440 in hPDE4A), T405A (position 606), and Val449A (position 650). In addition to being insensitive to rolipram, the T405A mutant is insensitive to IBMX.

In an attempt to improve our understanding of the molecular basis for substrate binding, substrate turnover, and inhibitor binding in PDE4, we evaluated the functional consequences of changing single amino acids within the central conserved region of hrPDE4A. The objectives were to (i) identify specific amino acids that are critical for catalytic activity and (ii) determine whether the amino acids that are important for substrate binding or turnover are also important for inhibitor binding.

Experimental Procedures

Inhibitors. Unlabeled and tritiated rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidone), RP 73401 [N-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide], and BRL 61063 (1,3-dicyclopropylmethyl-8-aminoxanthine) were synthesized by Dr. Siegfried Christensen (Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, PA) and Dr. Richard Heys (Department of Synthetic Chemistry, SmithKline Beecham Pharmaceuticals). Tritiated (R)-rolipram and RP 73401 had specific activities of 5.7×10^4 and 5.15×10^4 dpm/pmol, respectively.

Expression plasmid construction. The PDE-deficient Saccharomyces cerevisiae strain GL62 is isogenic to GL61 (MATα leu2 ura3-52 ade1 his3 trp1 lys2-208 pde1::LEU2 pde2::URA3 pep4::HIS3) and has been previously described (9, 23). A yeast expression plasmid expressing rhPDE4A was previously described as p138NB/Met201–886 (19). It lacks ~600 base pairs of 5′ open reading frame sequence and predicts a truncated protein lacking ~200 amino acids at the NH₂ terminus (4, 5). The recombinant protein, which we designate Met201–886, was engineered to contain nine non-native amino acids (MCPF-PVTTV) at the NH₂ terminus fused to Pro210. Thus, this construct encodes a fusion protein consisting of a nonapeptide coupled to Pro210 on hPDE4A. Our original hPDE4A-encoding cDNA was designated hPDE-1 and engineered for expression using polymerase chain reaction (4, 23, 24). The amino acid sequence of this protein is identical to that of h6.1 as described by Sullivan et al. (25). This truncated rhPDE4A

protein has kinetic characteristics that are identical to those of the native enzyme (21, 25).

Site-directed mutagenesis. A 2.4-kb SalI/XbaI fragment of hPDE-1 sequence (4) encoding Met201–886 was subcloned into the mutagenesis vector phagemid pALTER-1 (Promega, Madison, WI). The oligonucleotides used for mutating amino acids were 5′-TGCGTGCAGGCTGTTGGAGTAGGCCA-3′ for H433S, 5′-GTCA-GCTGCGGACAGGCTGTTATGGTAG-3′ for H437S, 5′-GGGTGATC-CACATCGGAGATGGCAGCCGCG-3′ for H473S, 5′-ACCCCA-GGGGAATCCACATCGTGGATG-3′ for H477S, 5′-CTCATCGTTGA-ACATGAGCGCC-3′ for Y496T, 5′-CTCATCGTTGAACATGAGCG-CC-3′ for Y496F, 5′-GGCCAGGTGGTTATTCTCGAG-3′ for H505N, 5′-CACGGCCAGGTTGTGATTCTC-3′ for H506N, 5′-GAAGATGTCGG-AGTTGTACTC-3′ for C519S, and 5′-CATGAGCGCCAGCTCCGA-GCTGCTGTTGATGAGGAACTGG-3′ for the double mutant T489S/N490S. Oligonucleotides were synthesized by Dr. Ganesh Sathe (Department of Gene Expression Sciences, SmithKline Beecham Pharmaceuticals). Propagation of single-stranded DNA and mutagenesis reactions were performed according to the supplier's directions (Promega). Briefly, 1.25 pmol of phosphorylated mutant oligonucleotide and 0.25 pmol of ampicillin repair oligonucleotide were annealed to 0.05 pmol of single-stranded template DNA. The annealing reaction mixture was maintained at 70° for 10 min and allowed to cool slowly to room temperature for 30 min before it was placed on ice. Second-strand synthesis was at 37° for 90 min in the presence of all four nucleotides, 10 units of T4 DNA polymerase, and 2 units of T4 DNA ligase.

The entire synthesis reaction mix was transformed into Escherichia coli BMH 71–18 mut S, and cells were grown for 24 hr at 37° in the presence of 125 μg of ampicillin/ml in liquid LB. Plasmid DNA from these cultures was prepared by alkaline lysis (26), and 0.1 μg of plasmid DNA was transformed into E. coli JM 109. Single colonies of the resulting transformants were picked, and plasmid DNA was prepared. To confirm that the desired base changes had occurred, several clones were sequenced by dideoxy sequencing with Sequenase 2.0 (United States Biochemical, Cleveland, OH) according to the manufacturer's instructions.

Expression of single-site mutants. The yeast expression plasmid p138NB contains the TRP1-selectable marker and a partial 2-micron sequence for maintenance at high copy number. Expression is driven by the copper-inducible CUP1 promoter with a polylinker site just downstream, followed by the yeast CYC1 transcriptional terminator (23). Expression plasmids containing various single-site mutants of rhPDE4A/Met201–886 (Fig. 1) were transformed into S. cerevisiae strain GL62 according to the lithium acetate method (27). Expression of PDE4 was induced by 150 μM $CuSO_4$ and allowed to proceed for 6 hr. Cells were harvested, washed, resuspended, and lysed as previously described (23). Strain GL62 contains deletions of both genes encoding the yeast cAMP PDEs and therefore is devoid of endogenous enzymatic activity. It also contains a deletion of the PEP4 gene, which encodes an aspartyl protease; stable rhPDE4A expression in yeast is dependent on the absence of this protease (23).

Quantification of protein expression. Expression of mutant and wild-type proteins was monitored by immunoblotting as previously described (19, 23). Some of the mutant proteins described in this study expressed to lesser amounts compared with the nonmutated hrPDE4A/Met201–886 (Fig. 2). In studies in which Scatchard analyses for determining B_{max} and kinetic analyses were described, protein expression of the various mutants was quantified in more detail by immunoblotting with an antibody raised against a GalK-hPDE4A fusion protein (23) and ^{125}l-labeled protein A (specific activity, >30 μCi/μg, ICN Biomedicals, Irvine, CA). Different amounts of protein lysates (100 ng to 10 μg) were analyzed in a single gel for each of the mutated proteins to ensure a linear range of detection. Blots were exposed to the phosphor screen of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for 2 hr and analyzed using the software ImageQuant. To compare and normalize different expression levels, various amounts of lysates containing hPDE4A/
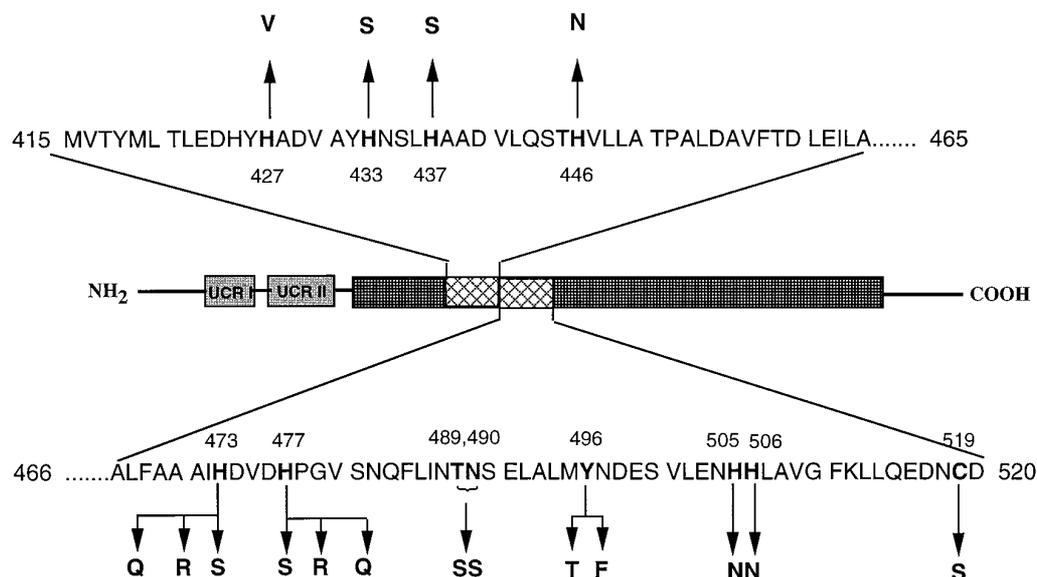
Fig. 1. Functional domains of hrPDE4A and amino acid sequence between positions 415 and 520. The proposed sequence was obtained from Livi *et al.* (4) and Bolger *et al.* (5). *Highlighted*, amino acids that were changed in the current study. *Numbers*, positions within rhPDE4A; *arrows*, newly introduced amino acids; *UCR I and II*, upstream conserved regions I and II (5); *hatched regions*, catalytic domain contained within residues 332–722 (19).
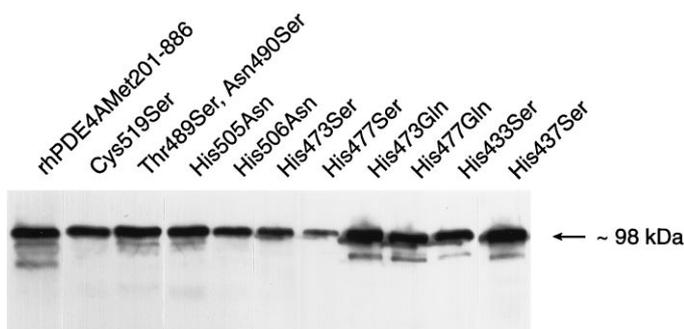
Fig. 2. Immunoblot analysis of the expression of single-site mutants of rhPDE4A in *S. cerevisiae*. Supernatant fractions of lysates of strain GL62 containing single-site mutants of rhPDE4A were prepared as described in the text. Proteins were separated, transferred, probed, and analyzed as previously described (19, 23). *Lane 1*, 5 μl of rhPDE4A/Met201–886; *lane 2*, 10 μl of C519S; *lane 3*, 15 μl of T489S/N490S; *lane 4*, 10 μl of H505N; *lane 5*, 10 μl of H506N; *lane 6*, 20 μl of H473S; *lane 7*, 20 μl of H477S; *lane 8*, 20 μl of H473Q; *lane 9*, 20 μl of H477Q; *lane 10*, 20 μl of H433S; *lane 11*, 20 μl of H437S.

Met201–886 (100 ng to 10 μg) were run side by side with the mutant lysates.

PDE assay. Reaction mixtures contained (final concentrations) 50 mM Tris·HCl, pH 7.5, 5 mM MgCl$_2$, 50 μM 5′-[^{14}C]AMP (400 dpm/nmol; New England Nuclear Research Products, Boston, MA), 1 μM [^{3}H]cAMP (2000 dpm/pmol; New England Nuclear), and 0.05% bovine serum albumin. Reactions were run at 30° initiated by the addition of 50 μl of yeast supernatant of several different dilutions, depending on the activity of the mutant proteins, to 100 μl final volume and terminated after 30 min by placing the reaction mixtures in a heating block at 100° for 1 min. Separation and estimation of cyclic nucleotides and 5′-nucleotides were conducted as previously described (28). For kinetic measurements, [^{3}H]cAMP was kept constant at 0.035 μM and supplemented with nonlabeled cAMP to give final concentrations of 0.045–100 μM. Reaction times were varied between 1 and 60 min, so that ≤20% of the substrate was hydrolyzed during the assay. For inhibitor studies, the enzyme was preincubated at 30° for 10 min before the start of the reaction through the addition of the appropriate mixture of substrate and inhibitor.

For the determination of K_i values, assays were conducted using 0.5, 1, 2, and 5 μM cAMP for Met201–886; 2, 5, 10, and 20 μM cAMP for H505N; and 10, 20, 40, and 60 μM cAMP for H506N. Specific

activities were calculated by first determining the different expression levels of the constructs as described above.

Inhibitor binding assay. Binding assays were performed at 30° for 1 hr in 0.5 ml of PDE buffer (50 mM Tris·HCl, pH 7.5, 5 mM MgCl$_2$) containing 0.05% bovine serum albumin as previously described (20, 21). Reactions were started by the addition of 20–50 μl of PDE4A to the reaction mixtures.

For saturation binding experiments, the concentration of [^{3}H]-(*R*)-rolipram (5.7 × 10^4 dpm/pmol) was 0.2–52 nM, and the concentration of [^{3}H]RP 73401 (5.15 × 10^4 dpm/pmol) was 0.2–10 nM. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled ligand. For cAMP competition binding experiments, 1 nM [^{3}H]RP 73401 was used as the radiolabel, and the concentration of cAMP was 0.3–1000 μM. Assay volumes were increased to 5 ml, and equilibration was carried out at 4° for 30 min. These modifications to the assay were made to limit hydrolysis of the competitor cAMP during the incubation period. Preliminary experiments were conducted to validate these modifications. Under standard experimental conditions for the radioligand binding assay, 83 ± 12% and 41 ± 6% of total cAMP was hydrolyzed at substrate concentrations of 3 and 30 μM, respectively. In contrast, substrate degradation was negligible under the modified assay conditions, so that <2% of the total cAMP content was lost using either substrate concentration.

Binding reactions were stopped by placement of the mixtures on ice before subjecting them to rapid filtration (Brandel Cell Harvester, Montreal, Quebec, Canada) through Whatman (Clifton, NJ) GF/B filters that had been equilibrated with 0.3% polyethylenimine. The filters were washed three times with 2.5 ml of ice-cold PDE buffer each time, dried, and counted via liquid scintillation counting. Nonspecific binding was 1–10% of the specific binding. This assay has been previously described in greater detail (20, 21). Under these conditions, [^{3}H]RP 73401 detects both conformational states of PDE4A (i.e., LPDE4 plus HPDE4), whereas [^{3}H]-(*R*)-rolipram binds only to HPDE4 (18). Analyses of saturation and competition binding experiments were carried out using the Acufit computer program (Beckman Instruments, Columbia, MD).

Results and Discussion

In the current study, single amino acids within the conserved region of rhPDE4A were changed to examine the involvement of functional groups in substrate catalysis and inhibitor binding. Fig. 1 shows the amino acid sequence of rhPDE4A between positions 415 and 520 within the central conserved region and the residue changes therein. These

TABLE 1

Catalytic and binding activities of hrPDE4A/Met201-886 and various point mutants

Catalytic activities were determined using a single substrate concentration of 1 μM cAMP, and rolipram binding was determined using 2 nM [³H]-(R)-rolipram to detect HPDE4 (high affinity binding) as described in the text. All data are normalized for different expression levels by immunoblot analysis using rhPDE4A/Met201-886 as a standard. Data indicate mean $\pm$ standard error with the number of experiments indicated in parentheses. Values without standard error represent single determinants.
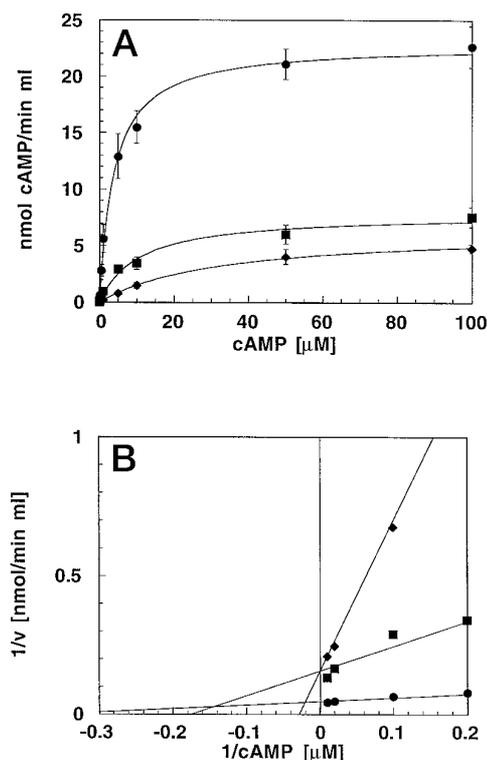
Mutant strain	Catalytic activity	Rolipram binding
	pmol of cAMP/min/μl	*pmol/ml*
Met201-886	31 $\pm$ 5 (4)	59 $\pm$ 10 (3)
H427V	41 $\pm$ 3 (3)	48
H446N	25 $\pm$ 3 (3)	35
H433S	<0.5 (3)	<1 (3)
H437S	<0.5 (3)	<1 (3)
H473S	7 $\pm$ 1 (3)	4
H473R	7 $\pm$ 3 (3)	3
H473Q	<0.5 (3)	<1
H477S	12 $\pm$ 5 (3)	3
H477R	0.6 $\pm$ 0.4 (3)	2
H477Q	<0.5 (3)	<1
H505N	5.5 $\pm$ 0.7 (6)	45 $\pm$ 13 (3)
H506N	3 $\pm$ 1 (6)	47 $\pm$ 5 (3)
Y496T	23 $\pm$ 0.5 (3)	48
Y496F	5 $\pm$ 1 (3)	15
T489S/N490S	38 $\pm$ 0.1 (3)	45
C519S	12 $\pm$ 0.02 (3)	55

Fig. 3. Kinetic analysis of cAMP-hydrolyzing activity of rhPDE4A/Met201–886, H505N, and H506N. Total cAMP concentration was varied from 0.045 to 100 μM, as described in the text. A, Data analysis was performed by a nonlinear fit of the data to the hyperbolic equation $v = (V_{max} \times [S])/(K_m + [S])$ using the KaleidaGraph computer program. B, Double-reciprocal plot of substrate hydrolysis at 5–100 μM cAMP. Values are mean $\pm$ standard error of three to six separate experiments and are normalized for different expression levels using Met201–886 as a standard. ●, Met201–886. ■, H505N. ◆, H506N. The determined kinetic parameters are given in Table 2.

changes were performed using the NH₂-terminally truncated form rhPDE4A/Met201–886. This protein retains the previously described high affinity rolipram-binding activity and possesses the same kinetic properties as the full-length protein Met1–886 (19, 21).

Rationale for mutating histidine residues. A major focus of our effort was on the role of histidine residues in the catalytic domain. The rationale for targeting histidine residues was based on previous observations suggesting an important role of this amino acid in the function of PDEs. First, hydrolysis of cAMP by PDEs is thought to occur by nucleophilic substitution reactions that involve base/acid-catalyzed hydrolysis of the phosphoester bond (30). Second, studies using substrate analogues suggest that cAMP binds to the enzyme via hydrogen bonding between the protein and N6 and N7 of the adenine ring (31, 32). Thus, because histidine can function as a H-bond donor as well as a H-bond acceptor at physiological pH, this amino acid may be involved in both substrate binding and hydrolysis. Finally, divalent cations such as Mg²⁺ are required for cAMP hydrolysis and (R)-rolipram binding (20, 21).[1] These divalent cations bind directly to the enzyme,[1] and histidine, along with serine, may be required for metal binding. Alteration of these residues could thus yield an inactive enzyme. Consistent with a critical role for histidine, the histidine-modifying reagent diazopyrocarbonate abolishes catalytic activity of rhPDE4A (33).

Because of the potential importance of histidine residues in the expression of PDE catalytic activity, our initial targets for mutagenesis were His427, His433, His437, His446, His473, His477, His505, and His506. These are conserved among all mammalian PDEs, perhaps reflecting a critical structural or functional role.

Modification of His427 and His446. His427 and His446 of rhPDE4A are not present in the *Drosophila* dunce gene product (34). Instead, valine and asparagine are present at these positions. The *Drosophila* dunce gene product is a

member of the PDE4 family that is insensitive to inhibition by Ro 20–1724 and other archetypical PDE4 inhibitors (35). We reasoned that the lack of a histidine residue at either position could be responsible for insensitivity to PDE4 inhibitors. However, contrary to our expectations, H427V and H446N in rhPDE4A were still catalytically active and bound (R)-rolipram with high affinity (Table 1). Moreover, these mutants were sensitive to inhibition of catalytic activity by (R)-rolipram (not shown). Therefore, His427 and His446 are not required for the activity of the prototypical PDE4 inhibitor rolipram in rhPDE4A.

Modification of His433, His437, His473, and His477. Catalytic activity was abolished when His433 or His437 was changed to serine and was reduced by 60–98% when His473 or His477 was changed to serine or arginine (Table 1). Similarly, (R)-rolipram binding was either not detectable or reduced by >90% in the corresponding mutants (Table 1). These changes were considered to be fairly conservative because serine and arginine as well as glutamine (see Table 1) can function as H-bond donors at physiological pH. The results indicate that these histidine residues are required for catalytic activity and (R)-rolipram binding under standard assay conditions. One possibility is that they are involved directly with inhibitor and substrate binding. His473 and His477 are the integral histidine residues in the PDE consensus sequence HDX₂HX₄N (36). Alternatively, His433 and

TABLE 2

Kinetic characteristics and inhibitor sensitivities of H505N and H506N of hrPDE4A/Met201-886

For kinetic measurements, substrate concentration was varied from 0.045 to 100 μM cAMP. Values for V_{max} and K_m were determined as described in the text using the computer program KaleidaGraph. For inhibitor studies, cell lysates were incubated at room temperature for 10 min with various concentrations of inhibitor: 0.03–300 μM (R)-rolipram and 0.03–300 nM RP 73401. The reaction was initiated by the addition of 1 μM cAMP. V_{max} values were normalized for different expression levels using rhPDE4A/Met201-886 as a standard, as described in Experimental Procedures. Data indicate the mean $\pm$ standard error of three separate experiments.

	Met201-886	H505N	H506N
V_{max} (nmol/min/ml)	23 $\pm$ 3	7.9 $\pm$ 2.3	6.4 $\pm$ 1.1
K_m (μM)	4 $\pm$ 1	11 $\pm$ 3	44 $\pm$ 6
IC$_{50}$ (nM)			
(R)-Rolipram	100 $\pm$ 20	140 $\pm$ 11	280 $\pm$ 20
RP 73401	0.3 $\pm$ 0.2	0.8 $\pm$ 0.1	0.7 $\pm$ 0.3

His437 or His473 and His477 may be involved in the binding of metal to the enzyme. As pointed out by Francis *et al.* (37), the corresponding histidine tandems in PDE5 represent divalent cation binding motifs composed of two histidines, separated by three amino acids, in conjunction with a glutamic acid located 25–35 residues downstream [HXXXH(X)$_{25-35}$E]. PDE5 seems to be a zinc hydrolase, and these histidine tandems are critical for chelation of this metal (37). The presence of these histidine tandems, along with their requirement for catalytic activity, are consistent with the suggestion that PDE4s are also zinc hydrolases. Finally, of course, we cannot eliminate the possibility that these histidines are important in maintaining the structural integrity of the enzyme.

Differential effects of H505N and H506N. Changing either His505 or His506 to asparagine reduced the cAMP-hydrolyzing activities of the mutants by 80–90% (Table 1). These results are consistent with those of Jin *et al.*, who reported a complete loss of catalytic activity when His311 in rat PDE4D (which corresponds to His506 in hPDE4A) was changed to asparagine, aspartic acid, or tyrosine (8). However, despite the substantial decrease in catalytic activities of the His505 and His506 mutants, [³H]-(R)-rolipram binding was virtually unaffected (Table 1). This is in contrast to the loss of both catalytic and binding activity observed in the His433, His437, His473, and His477 mutants.

Superficially, the results with the His505 and His506 mutants suggested that these residues are important for the binding or catalysis of substrate but not the inhibitor. This prompted a more detailed examination of cAMP hydrolysis and inhibitor binding. Michaelis-Menten analysis of cAMP hydrolysis revealed a 3-fold increase in the K_m value and a 65% reduction in the V_{max} value for H505N and a 11-fold increase in the K_m value and a 72% reduction in the V_{max} value for H506N (Fig. 3, Table 2). The catalytic activities for the mutant enzymes remained sensitive to (R)-rolipram and RP 73401, with IC$_{50}$ values similar to those of hPDE4A/Met201–886 (Table 2). These results indicate that alteration of His505 and His506 reduces both the affinity and turnover of cAMP but has little effect on inhibitor binding, suggesting a difference in the mode of substrate binding versus inhibitor binding.

The effect of altering His505 and His506 on the potency of several inhibitors was explored in greater depth by examin-
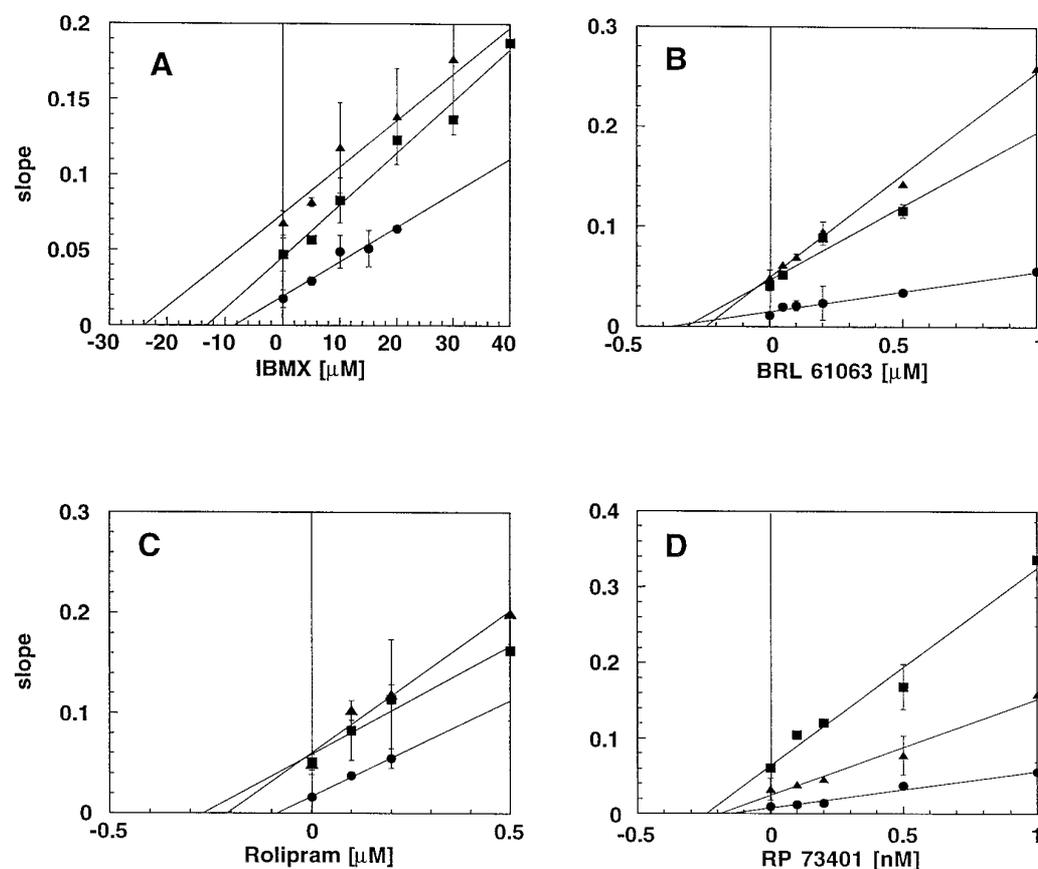

Fig. 4. Inhibition of Met201–886, H505N, and H506N by IBMX, BRL 61063, (R)-rolipram, and RP 73401. Substrate concentrations were 0.5–5 μM cAMP for Met201–886, 2–20 μM cAMP for H505N, and 10–60 μM cAMP for H506N. For determination of K_i values, the slopes of individual double-reciprocal plots, determined as described in Table 3 (36), versus the inhibitor concentrations were replotted and fit to a straight line using KaleidaGraph. The point of intersection of each line with the horizontal axis gives $-K_i$ values, assuming that each inhibitor is competitive with substrate. K_i values are summarized in Table 3. Values are mean $\pm$ standard error of three experiments. ●, Met201–886. ■, H505N. ▲, H506N.

TABLE 3
K_i values of inhibitors against Met201-886, H505N, and H506N

cAMP concentrations were 0.5–5 μM for Met201-886, 2–20 μM for H505N, and 10–60 μM for H506N, as described in the text. Inhibitor concentrations were 0.1, 0.2, and 0.5 μM (R)-rolipram; 0.5, 1, 2, and 5 nM RP 73401; 5, 10, 15, 20, 30, and 40 μM IBMX; and 0.1, 0.2, 0.5, and 1 μM BRL 61063. Values indicate the mean $\pm$ standard error (n = 3) or the values from two separate experiments. K_i values were determined by replotting the slope of each double-reciprocal plot versus the corresponding inhibitor concentration (39) using the KaleidaGraph computer program and assuming a competitive mode of inhibition.

Inhibitor	Met201-886	H505N	H506N
(R)-Rolipram (μM)	0.15, 0.2	0.2, 0.3	0.2, 0.3
RP 73401 (μM)	0.0002, 0.0003	0.0004, 0.0002	0.0004, 0.0003
BRL 61063 (μM)	0.35 $\pm$ 0.05	0.3 $\pm$ 0.1	0.25 $\pm$ 0.1
IBMX (μM)	5.0 $\pm$ 1.5	8.5 $\pm$ 2.5	23 $\pm$ 3

ing enzyme activity in the presence of a broad range of cAMP and inhibitor concentrations (Fig. 4, Table 3). In addition to RP 73401 and rolipram, the PDE4-selective inhibitor BRL 61063 and the nonselective inhibitor IBMX were examined. The latter two compounds have structural similarities to cyclic nucleotides and, like RP 73401 and rolipram, are believed to bind to the catalytic site of PDEs. Interestingly, the K_i values for IBMX varied in a fashion similar to those of cAMP; the K_i value increased 2-fold for H505N and 5-fold for H506N (Table 3). In contrast, the K_i values for BRL 61063, RP 73401, and (R)-rolipram were unaltered in these histidine mutants (Table 3). These data suggest that cAMP and the nonselective inhibitor IBMX bind to hPDE4A in a manner distinct from that of the PDE4-selective inhibitors.

To evaluate the effect of mutation of His505 and His506 on inhibitor and substrate binding, radioligand binding studies were conducted. Mutation of hPDE4A/Met201–886 to H505N or H506N had virtually no effect on the binding affinities of [³H]-(R)-rolipram and [³H]RP 73401 (Table 4). K_d values for [³H]RP 73401 ranged from 0.2 to 0.5 nM and were 6–8-fold lower than those for [³H]-(R)-rolipram. The B_{max} value for [³H]RP 73401 was 2–3-fold greater than that for [³H]-(R)-rolipram, which reflects the fact that the former ligand binds with the same high affinity to two unique structural conformers of hPDE4A, whereas rolipram binds with high affinity to only one form (19). The B_{max} value for the radioligands was essentially unchanged in H505N but was reduced by 60–70% in H506N. The decreased B_{max} value of H506N suggests that

TABLE 4
Ligand binding characteristics of H505N, H506N, and hrPDE4A/Met201-886

Saturation binding experiments were conducted using ligand concentrations of 0.2–52 nM [³H]-(R)-rolipram or 0.2–10 nM [³H]RP 73401 in the filtration assay as described in the text. Scatchard analyses were performed using the Acufit computer program. Ligand competition binding studies were 1 nM [³H]RP 73401 and 2 nM [³H]-(R)-Rolipram. IC$_{50}$ values were calculated using the Cheng-Prusoff equation (40): IC$_{50}$ = IC$_{50}$/[ligand]/(K_d + 1). Data were normalized for different expression levels of PDE4A protein by immunoblot analysis using rhPDE4A/Met201-886 as a standard. Data represent the mean $\pm$ standard error of three to six separate experiments.

	Met201-886	H505N	H506N
K_D (nM)			
(R)-Rolipram	1.3 $\pm$ 0.5	1.8 $\pm$ 0.4	3.9 $\pm$ 0.9
RP 73401	0.2 $\pm$ 0.04	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1
B_{max} (pmol/ml)			
(R)-Rolipram	1.0 $\pm$ 0.1	0.8 $\pm$ 0.2	0.3 $\pm$ 0.05
RP 73401	2.4 $\pm$ 0.3	2.1 $\pm$ 0.1	1.0 $\pm$ 0.1
IC$_{50}$ cAMP (μM)			
[³H]-(R)-Rolipram	49 $\pm$ 8	68 $\pm$ 23	626 $\pm$ 196
[³H]RP 73401	42 $\pm$ 12	78 $\pm$ 12	552 $\pm$ 129
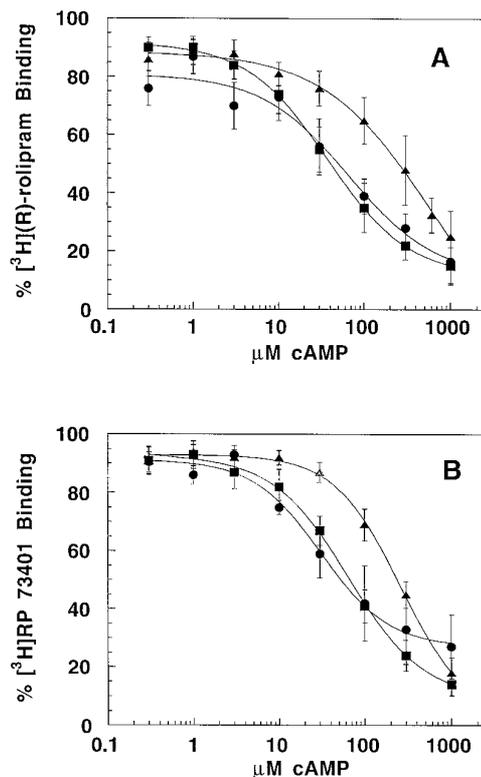
Fig. 5. Competition for [³H]-(R)-rolipram and [³H]RP 73401 binding by cAMP in rhPDE4A/Met201–886, H505N, and H506N mutants. Competition for [³H]-(R)-rolipram (2 nM, A) and [³H]RP 73401 (1 nM, B) binding was assayed in 5 ml at 4° for 30 min as described in the text. Values are mean $\pm$ standard error of five separate experiments. ●, Met201–886. ■, H505N. ▲, H506N. IC$_{50}$ values are indicated in Table 4.

the reduced cAMP V_{max} value of this mutant reflects a decrease in the proportion of expressed enzyme molecules that are catalytically active rather than a true change in substrate turnover (i.e., k_{cat}).

Rolipram and RP 73401 compete with cAMP for binding at the catalytic site (19).[1] Consequently, we evaluated the ability of cAMP to compete for 2 nM [³H]-(R)-rolipram and 1 nM [³H]RP 73401 binding in hPDE4A/Met201–886 as well as in the H505N and H506N mutants. In line with the modest increase in the cAMP K_m values of H505N, the cAMP IC$_{50}$ value increased only slightly in this mutant (Table 4, Fig. 5). Likewise, the 11-fold increase in the cAMP K_m value for H506N was mirrored by a 13-fold increase in the IC$_{50}$ value for cAMP in this mutant. Interestingly, sequence comparison of hPDE3A, hPDE4A, and hPDE7A reveals the presence of a conserved peptide SerValLeuGluAsnHisHis, in which the two histidines are analogous to His505 and His506 in PDE4A (results not shown). This peptide also seems to be conserved among all other PDEs sequenced thus far, again suggesting an important involvement of this motif in the structure or function of PDEs.

Mutational analysis of conserved, nonhistidine amino acids. Other functional amino acids that might be involved with substrate binding and catalysis include those containing a hydroxyl group such as serine, threonine, or tyrosine. These residues could serve as electron donors for nucleophilic attack. In addition, a seven-amino acid stretch, GluLeuAlaLeuMetTyrAsn (position 491–497 in hrPDE4A), is conserved among most but not all PDEs and is also contained

within the regulatory subunit RII$_a$ of cAMP-dependent protein kinase (38). Tyr496 within this sequence is conserved among all PDEs sequenced thus far. The Y496T change in hrPDE4A resulted in only a modest reduction in catalytic activity and [^{3}H]-(R)-rolipram binding, whereas the Y496F change markedly reduced these activities (Table 1). This implies that a hydroxyl group at this position is required for optimal catalytic activity and inhibitor binding.

The *Drosophila* dunce gene product also contains the above-mentioned seven-amino acid stretch. Directly preceding this peptide, however, is a sequence of three consecutive serine residues that differs from the sequence found in human PDE4A (see Fig. 1). To study the potential influence of this serine stretch toward rolipram sensitivity, we changed T489S and N490S simultaneously in hrPDE4A. No changes in catalytic activity or (R)-rolipram binding were noted in this mutant (Table 1).

Finally, replacement of Cys519 with serine reduced catalytic activity by ~60% but had no effect on [^{3}H]-(R)-rolipram binding (Table 1). Although the biochemical characteristics of C519S were not examined in detail, this result hints that, similar to His506, Cys519 may influence catalytic activity but not inhibitor binding.

Conclusions. Taken collectively, these data suggest that His506 of hPDE4A is a key residue involved in the binding of cAMP- but not PDE4-selective inhibitors. On the other hand, His505 has a lesser role in cAMP binding but seems to influence substrate turnover. None of the nondestructive amino acid changes made in this study had an impact on the binding of PDE4-selective inhibitors. Previous studies by Pillai *et al.* (22), however, demonstrated that alteration of Asp239 in rat PDE4B (position 440 in hPDE4A) to an asparagine decreased the inhibitory potency of rolipram by 400-fold. Interestingly, the same change had virtually no effect on the inhibitory potency of IBMX (22). Conversely, results from the current study indicate that changing His506 to asparagine increased the K_i value of IBMX by 5-fold but had no effect on the affinity of rolipram, RP 73401, or BRL 61063. An implication of these results is that substrate analogues such as IBMX bind to PDE4A in a manner that is different from structurally diverse PDE4-selective inhibitors. Although further mutational analyses may yield useful information, clear insights into the roles of specific amino acids in cAMP hydrolysis and inhibitor binding await more definitive structural approaches.

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Send reprint requests to: Theodore J. Torphy, Ph.D., Department of Pharmacology, UW2532, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406. E-mail: theodore_j_torphy@sbphrd.com