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Mapping the Functional Domains of Human Recombinant Phosphodiesterase 4A: Structural Requirements for Catalytic Activity and Rolipram Binding

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SUMMARY

To identify functional domains of the 886-amino acid human recombinant cAMP-specific phosphodiesterase (PDE) subtype A (rhPDE4A), we engineered the expression of seven mutant proteins containing both NH_2- and COOH-terminal truncations. The level of rhPDE4A protein expression in yeast was monitored by immunoblotting using enzyme-specific antisera. Biochemical profiles of the mutant proteins were compared with those of the full-length protein or a fully active truncated form of the enzyme (rhPDE4A Met265-886), lacking the first 264 amino acids. The smallest catalytically active fragment generated was Met332-722, which at 45 kDa is less than half the mass of the full-length enzyme (~110 kDa) but spans the most highly conserved region of the PDE superfamily. Two prototypical PDE4 inhibitors, rolipram and RP 73401, inhibited cAMP hydrolyzing activity of all truncated forms of the enzyme, with IC_{50} values of 70–2000 nM and 0.2–0.6 nM, respectively. [^{3}H](*R*)-Rolipram bound to two sites on Met265-886, a high affinity site (K_{d_1} = 0.7 ± 0.3 nM) and a low affinity site (K_{d_2} = 34 ± 10 nM). Interestingly, [^{3}H](*R*)-rolipram failed to bind to Met332-886 with high affinity, indicating that high affinity binding is not required for inhibition of enzyme activity. Low affinity rolipram binding was still present in Met332-886 (K_d = 101 ± 7 nM). In contrast to [^{3}H](*R*)-rolipram, [^{3}H]RP 73401 bound to a single class of high affinity sites on Met265-886 (K_d = 0.4 ± 0.1 nM). Further truncation of the enzyme to Met332-886 had no effect on [^{3}H]RP 73401 binding (K_d = 0.2 ± 0.03 nM). We conclude that the catalytic center of rhPDE4A lies between amino acids 332 and 722. Furthermore, amino acids 265–332 may form a high affinity binding site for rolipram that is outside of the catalytic domain. As a more likely alternative, these amino acids may not form a distinct binding site but instead may be required for the recombinant enzyme to assume a conformation that binds rolipram at the catalytic domain with a high affinity.

cAMP and cGMP have major roles in signal transduction processes. The hydrolysis of these second messengers is catalyzed by a family of enzymes called PDEs. Seven members of this family have been distinguished thus far, based on substrate specificity, kinetic properties, sensitivity to specific inhibitors, tissue distribution, and, recently, sequence-derived information (1–3). One member of the PDE family is the low K_m cAMP-specific PDE, also known as PDE4 (for nomenclature, see Ref. 3). This enzyme class is highly selective for cAMP (K_m = 3–18 μM) versus cGMP (K_m = >1 mM) and is selectively inhibited by two prototypical compounds, rolipram and Ro 20–1724, as well as by RP 73401 (4), a novel compound with an exceptionally high potency. Recently, considerable interest has been generated in PDE4 as a molecular target for novel anti-inflammatory and antiasthmatic drugs (5).

The mammalian PDE4 family is composed of four subtypes (PDE4A through PDE4D) that are encoded by different genes (6–15; for reviews, see Refs. 1 and 3). Sequence differences among the subtypes occur mainly at their NH_2- and COOH-terminal ends (12). Additional NH_2-terminal variation occurs within individual subtypes through the expression of alternative mRNA splice variants (10–14).

Certain tissues (e.g., rat brain) that contain large amounts of PDE4 activity also bind rolipram with a high affinity (K_d = 1–2 nM; Ref. 16). Cloning and expression of rhPDE4A and rhPDE4B demonstrated that catalytic activity and high affinity rolipram binding are indeed properties of the same protein (10, 17). Historically, it has been assumed that the high affinity rolipram-binding site is the catalytic site on PDE4 and that rolipram and similar compounds inhibit catalytic activity competitively. However, the following discrepancies bring into question these assumptions. 1) Rolipram inhibits PDE4 catalytic activity (K_i = ~1 μM) at concentra-

ABBREVIATIONS: PDE, phosphodiesterase; PDE4, cAMP-specific phosphodiesterase; hPDE4A, human cAMP-specific phosphodiesterase subtype A; hPDE-1, human cyclic AMP-specific phosphodiesterase subtype A (original clone); rhPDE4A, human recombinant cAMP-specific phosphodiesterase subtype A; PCR, polymerase chain reaction; bp, base-pair(s); ORF, open reading frame.

tions 500-fold above those needed to compete for high affinity binding (17). 2) Although both binding and catalytic activity are inhibited by structurally diverse PDE inhibitors, the relative potencies of these compounds for the two functions differ (17). 3) Rolipram does not inhibit PDE4A activity in a purely competitive manner (17, 18). Thus, although the high affinity rolipram-binding site is contained within PDE4, the relationship between this site and the catalytic site is not clear. One proposal is that the high affinity rolipram-binding site is an allosteric site (17, 18). Alternatively, it may represent the catalytic site on a fraction of the expressed enzyme that exists in a unique tertiary or quaternary conformation (17). In essence, the latter proposal holds that rhPDE4A exists in two conformational states, one of which binds rolipram at the catalytic site with a K_d value of ~1 nM, and a second that binds rolipram at the catalytic site with a K_d value of ~1 μM.

To obtain more information on the nature of the high affinity rolipram-binding site and its relationship to the catalytic site, we attempted to map the functional domains of rhPDE4A by assessing the activity of truncated forms of this enzyme. These studies were designed to answer three questions. First, what is the smallest catalytically active fragment of rhPDE4A? Second, can the high affinity rolipram-binding activity be separated from catalytic activity? Third, do truncations that abolish high affinity rolipram-binding also abolish the ability of rolipram to inhibit catalytic activity?

Materials and Methods

Inhibitors. Unlabeled and tritiated rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidone) and RP 73401 [*N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide] were synthesized by Dr. Siegfreid Christensen, Department of Medicinal Chemistry, and Dr. Richard Heys, Department of Synthetic Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, PA.

Yeast and bacterial strains. The PDE-deficient yeast strain *Saccharomyces cerevisiae* GL62 is isogenic to GL61 (*MAT*α *leu2 ura3*-52 *ade1 his3 trp1 lys2*-208 *pde1::LEU2 pde2::URA3 pep4::HIS3*), which is described elsewhere (10, 19). *Escherichia coli* strains JM109 (*end*A1 *rec*A1 *gyr*A96 *thi hsd* R17(r_k^-, m_k^+) *rel*A1 *sup*E44 λ^- Δ(*lac-pro*AB) [F′ *tra*D36 *pro*A^+B^+ *lac*$I^qZ\Delta M15$]) and BMH 71–18 mutS (*thi sup*E Δ(*lac-pro*AB) [*mut*S::Tn10] [F′ *pro*A^+B^+ *lac*$I^qZ\Delta M15$]) were purchased from Promega (Madison, WI).

DNA and protein sequence analysis. The first hPDE4A-encoding cDNA engineered for expression in this study (hPDE-1) has been described previously (8). It lacks ~600 bp of 5′ ORF sequence and predicts a truncated protein lacking ~200 amino acids at the amino terminus. Comparison of this sequence with the full-length hPDE4A-encoding cDNA sequence subsequently reported by Bolger *et al.* (12), which predicts an 886-amino acid protein, indicated that our hPDE4A clone is, in fact, truncated. Furthermore, the sequence we originally reported contained a sequencing error near the 5′ end that masked a small portion of the ORF. Resequencing of the entire hPDE-1 clone also revealed a few single-base-pair errors in the published sequence that alter individual amino acids; the revised sequence is now in agreement with that of Bolger *et al.* (12), with the following changes in our previously reported sequence: Y520D, R723M, R727P, E789A, and N798S. There remains one difference between our amino acid sequence and that of Bolger *et al.* (12): although Bolger *et al.* (12) predicts an alanine at position 736, our clone and that reported by Sullivan *et al.* (20) predicts a glutamate. We updated the version of our sequence in GenBank (accession no. M37744) to reflect these changes. Our original hPDE4A-encoding cDNA was engineered for expression using PCR (8, 19). The recombinant protein, which we designate Met201-886, contains nine non-native amino acids (MCPFPVTTV) at the NH_2 terminus fused to Pro210. We have shown previously that this truncated rhPDE4A protein has kinetic characteristics that are identical to those of the native enzyme (17, 21).

Expression plasmid construction. A yeast expression plasmid expressing rhPDE4A Met201-886 was described previously (19) and designated p138NB-hPDE1; we now refer to it as p138NB-Met201-886. A rhPDE4A Met265-886 expression plasmid was constructed as follows. A 1.3-kb *Nco*I/*Spe*I 3′ fragment of the original cDNA clone (hPDE-1; Ref. 8) was subcloned into the unique *Nco*I and *Sal*I polylinker sites of the yeast expression plasmid p138NB (10, 19) to create p138NB-hPDE1-3′. The overhangs created by *Spe*I and *Sal*I were filled using the Klenow fragment of DNA polymerase. The 5′ end of the cDNA was generated by PCR (22). The 5′ primer added an *Eco*RV site just 5′ to the ATG initiation codon for Met265-886, and the 3′ primer included the internal *Nco*I site of hPDE-1 (8). The 883-bp fragment was then cut with *Eco*RV and *Nco*I and subcloned into *Xho*I (Klenow-filled) and *Nco*I sites of p138NB-hPDE1-3′ to create p138NB-Met265-886.

Other constructs expressing NH_2- and COOH-terminal truncations of hPDE4A were generated from hPDE-1 using the Altered Sites mutagenesis system (Promega), as described below. cDNAs encoding NH_2-terminal truncations were constructed by introducing *Nde*I sites just 5′ to the following codons: Met305 (using the mutagenic oligonucleotide 5′-ATCCCATCACCCCATATGAAGGAACGAGAA-3′) or Met332 (using 5′-GATTTGGGACATATGCTGTAAGTGTGG-3′). The original construct contained an inherent *Nde*I site upstream of these sites (at the codon for amino acid residue 201). *Nde*I fragments of 312 and 393 bp were removed to obtain the desired deletion constructs. *Sal*I/*Xba*I fragments of 2.0 or 1.9 kb of the respective constructs were then subcloned into the unique *Xho*I and *Nhe*I polylinker sites of p138NB, creating p138NB-Met305-886 and p138NB-Met332-886. An internal deletion of hPDE4A coding sequence was constructed starting with p138-Met201-886 by taking advantage of two naturally occurring *Sac*I sites. Deletion of a 282-bp *Sac*I fragment resulted in an in-frame deletion of sequences encoding 94 amino acids between residues 271 and 365. The resulting plasmid is designated p138NB-Met201-886(Δ271-365).

For COOH-terminal truncations, translational stop codons were engineered into p138NB-Met201-886 at the sites encoding Trp678 (using mutagenic oligonucleotide 5′-CGCTGTAGTATCAGTCCCGGT-3′) or Ser722 (using 5′-TCTGGGCCCTTCATATTTCTTC-3′), resulting in deletion of sequences for 209 or 165 COOH-terminal amino acids and creating plasmids p138NB-Met201-678 and p138NB-Met201-722. An NH_2- and COOH-terminal double-truncation expression construct was made as follows. A 1.2-kb fragment of hPDE4A coding sequence was amplified by PCR (using hPDE-1 as the template) to contain an *Nhe*I site before the codon for Met332 and a stop codon and an *Asp*718I site at Ser722 (using the oligonucleotides 5′-GCCCCCGCTAGCACACTTACAGCC-3′ and 3′-TTCTTTATACTTCCATGGTCTAT-5′). The *Nhe*I/*Asp*718I PCR fragment was then subcloned into the unique *Nhe*I and *Kpn*I sites of p138NB, creating p138NB-Met322-722.

To use the Altered Site mutagenesis system, a 2.4-kb *Sal*I/*Xba*I fragment of hPDE-1 sequence (8) encoding Met201-886 was subcloned into the mutagenesis vector phagemid pALTER-1 (Promega). Propagation of single-stranded DNA and mutagenesis reactions were performed according to the manufacturer's instructions. Mutations were confirmed by DNA sequencing as described below.

To express the full-length rhPDE4A protein (Met1-886), a 2.8-kb *Sal*I/*Xba*I fragment containing the original cDNA clone (hPDE-1; Ref. 8) was subcloned into the same sites of pALTER (Promega), creating pA-hPDE1. The 1.2-kb 5′ *Sal*I/*Xho*I fragment from this plasmid was then subcloned into pBluescript (Stratagene, La Jolla, CA), creating pBS-hPDE1-5′; this resulted in unique sites available for adding the missing 5′ end of the ORF. The 0.2-kb *Sal*I/*Bst*EII

fragment of pBS-hPDE1-5′ was then removed and replaced with a gene assembly fragment that had been constructed in two pieces (a 0.29-kb *SalI/KpnI* piece and a 0.22-kb *KpnI/BstEII* piece), based on the sequence published by Bolger *et al.* (12). This manipulation involved PCR, added a *SalI* site just upstream of the initiating methionine, and included preferred codons for yeast expression. The resulting *SalI/XhoI* fragment from this plasmid (now 1.5 kb) was ligated with the 3′ end of the ORF contained in pA-hPDE1. The construct was confirmed by DNA sequencing, and the deduced translation product is identical to that of Bolger *et al.* (12) except for the alanine at position 736, as indicated above. The entire coding sequence carried on a *SalI/XbaI* fragment was then ligated into p138NB digested with *XhoI* and *NheI*, creating the yeast expression plasmid p138NB-Met1-886.

PCR amplification. PCRs (22) were performed according to the instructions of the supplier of *Taq* polymerase and the DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Briefly, reactions were performed in the presence of 10 mM Tris·HCl, pH 8.0, 50 mM KCl, 15 mM $MgCl_2$, 200 mM concentration of each of the four dNTPs, 4–8 ng of each primer, 5–20 ng of template DNA, and 2.5 units of *Taq* polymerase in a 100-μl final volume. Reaction mixtures were overlaid with 50 μl of mineral oil and subjected to one cycle of 8 min at 72°, followed by 25 cycles of denaturation (1 min at 94°), annealing (2 min at 55°), and extension (3 min at 72°). The products were subjected to a 1% agarose gel to confirm amplifications of the correct sizes. PCR products were digested with *NheI* and *Asp*718I and subcloned in the expression plasmid p138NB. 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) as described by the supplier.

Yeast expression system. The yeast expression plasmid p138NB contains the *TRP1* selectable marker and a partial 2-μ 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 (19). Expression plasmids for various hPDE4A proteins were transformed into *S. cerevisiae* strain GL62 using the lithium acetate method (23). Strain GL62 contains a deletion of both genes encoding the yeast cAMP PDEs (24–26) and is therefore 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 (19).

Quantification of protein expression. In studies describing the kinetic characteristics of the various mutants and Scatchard analyses for determining B_{max}, protein expression was quantified by immunoblotting with an antibody raised against a GalK-hPDE4A fusion protein (19) and ^{125}I-labeled protein A (specific activity, >30 μCi/μg; ICN Biomedicals, Cleveland, OH). Different amounts of protein lysates (100 ng to 10 μg) were analyzed in a single gel for each of the truncated proteins to ensure the linear range of detection. Blots were exposed to the phosphor screen of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for 2 hr and analyzed using ImageQuant software (Molecular Dynamics). To compare and normalize different expression levels, various amounts (10, 20, 50, 100, 200, 500, and 1000 ng) of partially purified hPDE4A Met265-886 (21) were run in parallel with the lysates to generate a standard curve. Because this preparation of Met265-886 was not homogeneous, data generated can be regarded only as relative V_{max} or B_{max} values (see Figs. 3 and 4 and Tables 2–4).

PDE4 assay. Reaction mixtures contained (in final concentrations) 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 50 μM 5′-[^{14}C]AMP, 1 μM [^{3}H]cAMP, 50 μM 5′-AMP, 1 μM cAMP, and 0.05% bovine serum albumin. Reactions were initiated by the addition of 50 μl of yeast supernatant of several different dilutions, depending on the activity of the mutant proteins, to a 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 described previously (27). For kinetic measurements, [^{3}H]cAMP was kept constant at 0.035 μM, whereas the cAMP concentration varied between 0.05 and 1000 μM, and reaction time varied between 1 and 60 min, so that assays were conducted in the linear range of reaction with ≤20% substrate hydrolysis. For inhibitor studies, yeast supernatants were incubated for 10 min in the presence of various concentrations of inhibitor before initiation of the reaction. Specific activities indicated are corrected against different expression levels of the constructs unless otherwise specified. Kinetic data and inhibitor studies were analyzed using the KaleidaGraph computer program (Synergy Software, Reading, PA).

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 described previously (16, 17). Reactions were started by adding 20–50 μl of PDE4A extracts to the reaction mixtures.

For saturation binding experiments, the concentration of [^{3}H](*R*)-rolipram (5.7×10^4 dpm/pmol) ranged from 0.25 to 400 nM, and the concentration of [^{3}H]RP 73401 (5.15×10^4 dpm/pmol) ranged from 0.2 to 10 nM. Nonspecific binding was estimated in the presence of 100-fold excess of unlabeled ligand. For competition binding experiments, 1 nM [^{3}H]RP 73401 and various concentrations of competitor were used. For competition experiments with cAMP, reaction volumes were increased to 5 ml. In addition, the reaction was conducted at 4° for 30 min rather than at 30° for 1 hr. These modifications were made to limit hydrolysis of the competitor cAMP during the incubation period.

[^{3}H](*R*)-Rolipram binds to rhPDE4A with two affinities: K_{d_1} = 1–2 nM and K_{d_2} = 40–500 nM (21). To distinguish between high affinity and low affinity rolipram binding, two different filtration assays were used. For high affinity rolipram binding, reactions were stopped by placing the reaction mixtures on ice before subjecting them to rapid filtration (Brandel Cell Harvester, Gaithersburg, MD) through Whatman 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 is described in greater detail elsewhere (16, 17) and is called the standard filtration assay.

To detect both low affinity and high affinity rolipram binding, an equilibrium filtration technique was used. This technique maintains equilibrium conditions of the ligand/receptor complex by eliminating the filter washing protocol normally used to separate bound and free ligand and to reduce nonspecific binding. Because the ligand/receptor complex is not diluted during the separation procedure, ligand bound to low affinity sites does not dissociate. In this assay, the reaction mixture was filtered on a 96-well plate with polyvinylidene difluoride filter (Millipore, Bedford, MA), previously equilibrated with 0.3% polyethylenimine and 10 μM unlabeled (*R*)-rolipram. Plates were dried immediately and counted via liquid scintillation counting. Nonspecific binding was 30–60% of the specific binding. Analysis of saturation and competition binding experiments were carried out using the Acufit (Beckman Instruments, Palo Alto, CA) or the GraphPAD Inplot 3.0 (San Diego, CA) computer program. Extremely high nonspecific binding of [^{3}H]RP 73401 precluded the use of this ligand in the equilibrium filtration assay.

Results

Catalytic activities of truncated forms of rhPDE4A. A cDNA clone encoding a nearly full-length PDE4A (lacking ~600 bp of 5′ ORF sequence) was previously isolated from a human monocyte cDNA library (8). The full-length sequence was subsequently reported by Bolger *et al.* (12) and found to predict a protein of 886 amino acids. We and others have shown that most known PDEs contain a highly conserved internal region consisting of ~340 amino acids, leading to the speculation that this region encodes the catalytic domain of

the enzyme. Based on a comparison of published protein sequences of cloned human PDE4s (i.e., PDE4A, PDE4B, PDE4C, and PDE4D; Refs. 8, 10–12, 15, 28), rat PDE4s (6, 7, 9, 14, 29), and the *Drosophila dunce* gene product (30), there is a core conserved region that spans amino acids ~343 to ~714 (using hPDE4A coordinates; see also Fig. 1), which is predicted to correspond to the catalytic domain (for reviews, see Refs. 21 and 31). Deletion mutagenesis studies of rat PDE4B and PDE4D support this prediction (9, 32). In rat PDE4B, an active enzyme of minimum length was determined to be from residues 72–498 (427 of 562 amino acids) (32), whereas the smallest active rat PDE4D was determined to be from residues 146–485 (340 of 584 amino acids) (9). These regions include but are not limited to the domain that is highly conserved in most PDEs.

We wanted to extend this type of analysis to hPDE4A by first determining the smallest catalytically active fragment of the enzyme. Such an analysis also served as a starting point to answer the following questions. Is the high affinity rolipram-binding activity of rhPDE4A (17) separable from catalytic activity? If so, are catalytically active fragments of the enzyme that lack the high affinity binding site still sensitive to inhibition by rolipram?

We began by engineering the expression of a series of truncated hPDE4A proteins in yeast (Fig. 1). Protein expression was carried out using a yeast strain in which both genes encoding the yeast cAMP PDEs were deleted (19) and therefore lacks endogenous enzymatic activity. [^{3}H](*R*)-Rolipram binding is not detectable in extracts from yeast without prior induction of rhPDE4A activity (17). The original recombinant protein expressed in this study (Met201-886) lacks 200 NH_2-terminal amino acids. Three other mutant proteins containing NH_2-terminal truncations were also expressed (Met265-886, Met305-886, and Met332-886), as well as one mutant protein containing an internal 94-amino acid deletion from residues 271–365 [Met201-886(Δ271-365)]. In addition, two mutant proteins, starting at Met201 and possessing COOH-terminal deletions, were expressed (Met201-722 and Met201-678). Finally, one protein lacking a significant portion of both the NH_2- and COOH-termini was expressed (Met332-722). Protein expression was monitored by immunoblotting using anti-rhPDE4A antisera (8).

Met1-886, Met201-886, and Met265-886 were expressed to similar levels (Fig. 2) and had similar catalytic activities and ligand-binding properties (Table 1). The apparent molecular masses of these proteins are slightly larger than those deduced from the cDNA sequence, which may reflect some degree of post-translational modification (17, 19). Met305-886 was expressed at a significantly lower level compared with the other NH_2-terminally truncated enzymes (Fig. 2) and was virtually devoid of cAMP-hydrolyzing activity and [^{3}H](*R*)-rolipram-binding activity (Table 1).

Met332-886 was expressed as an ~80-kDa immunoreactive band (Fig. 2). This protein had remarkable cAMP hydrolyzing activity with a normalized V_{max} value of 6.3 ± 0.3 nmol/min/μg of protein and a K_m value of 3 ± 1 μM (Table 2). This corresponded to a 5-fold increase in catalytic efficiency (V_{max}/K_m) compared with the kinetic data for Met265-886 (V_{max} = 1.3 nmol/min/μg of protein, K_m = 3 μM; Table 2) and a 7-fold increase compared with Met1-886 (V_{max} = 2.9 ± 0.6 nmol/min/μg of protein, K_m = 10.0 ± 1.4 μM). The internal deletion protein [Met201-886(Δ271-365)], although expressed at significant levels (Fig. 2), did not hydrolyze cAMP.

Removal of 164 amino acids from the COOH terminus did not decrease catalytic activity (Met201-722; Table 1), whereas removal of 208 amino acids resulted in a protein devoid of both catalytic and [^{3}H] (*R*)-rolipram binding activities (Met201-678; Table 1). Although these data suggest that

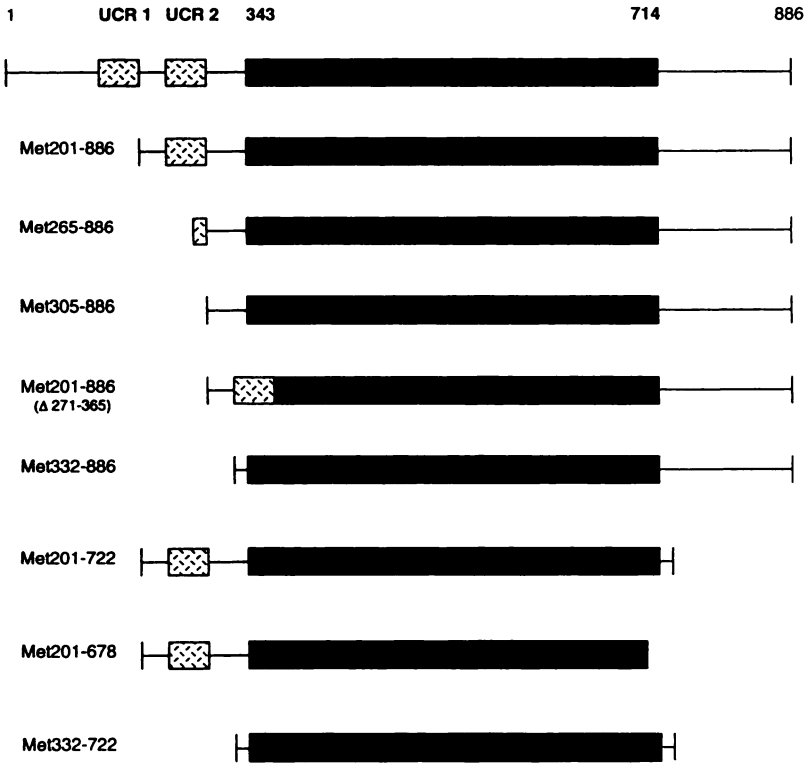

Fig. 1. Schematic diagram of NH_2- and COOH-terminal deletion mutant rhPDE4A proteins showing proposed functional domains. Data were obtained from Livi *et al.* (8) and Bolger *et al.* (12). *Dark areas*, putative conserved central domain of the PDE4 family; *light areas* (*UCR 1* and *UCR 2*), upstream conserved regions 1 and 2 as described in Bolger *et al.* (12).

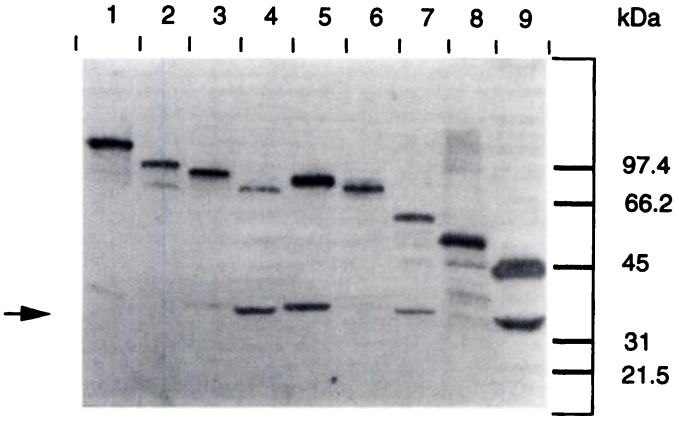

Fig. 2. Expression of truncated forms of rhPDE4A in *S. cerevisiae*. Supernatant fractions of lysates of strain GL62 containing truncated forms of rhPDE4A were prepared as described in Materials and Methods. Proteins were separated on a 10% acrylamide gel containing 1% sodium dodecyl sulfate and transferred by semidry electroblotting onto polyvinylidene difluoride membrane (Millipore), as described (19). Blots were probed with antisera raised against a GalK-PDE4A fusion protein expressed in *E. coli* (8). *Lane 1*, 0.04 μl of Met1-886; *lane 2*, 0.04 μl of Met201-886; *lane 3*, 0.04 μl of Met265-886; *lane 4*, 0.4 μl of Met201-886(Δ271-365); *lane 5*, 20 μl of Met305-886; *lane 6*, 0.04 μl of Met332-886; *lane 7*, 10 μl of Met201-722; *lane 8*, 10 μl of Met201-678; *lane 9*, 20 μl of Met332-722. The antisera cross-reacted with a ~34-kDa yeast protein (*arrow*), which was also present in nontransformed yeast extracts.

TABLE 1

Kinetic characteristics and ligand binding of truncated forms of rhPDE4A

cAMP hydrolysis and ligand binding were determined in cell lysates at a single substrate concentration of 1 μM cAMP for V_0 and 2 nM [^{3}H](R)-rolipram or 1 nM [^{3}H]RP 73401 for ligand binding using the standard filtration assay (see Materials and Methods). All data are normalized for different expression levels of PDE4A protein by immunoblot analysis using a partially purified preparation of Met265-886 as a standard. Data represent mean ± standard error. The number of experiments is given in parentheses.

Protein	cAMP hydrolysis	Ligand binding	
	V_0[a]	Rolipram	RP 73401
	pmol/min/μg	*pmol/μg*	
Met1-886	160 ± 3 (4)	1.0, 1.2 (2)	2.5 ± 0.6 (3)
Met201-886	170 ± 5 (4)	1.2 ± 0.2 (3)	3.7 ± 0.4 (3)
Met265-886	130 ± 3 (4)	1.0 ± 0.1 (6)	3.7 ± 0.4 (5)
Met305-886	10 ± 0.2 (3)	0	NT[b]
Met332-886	450 ± 8 (4)	0	3.8 ± 0.2 (8)
Met201-722	190 (1)	0.7, 1.0 (2)	2.6 ± 0.3 (3)
Met201-678	6 ± 0.7 (4)	0	0
Met332-722	210 ± 20 (4)	0	2.4 ± 0.6 (3)

[a] Initial velocity.
[b] NT, not tested.

amino acids 678–722 are critical for both catalytic activity and high affinity rolipram-binding activity, it cannot be ruled out that loss of activity is due to improper protein folding. The smallest protein expressed contained only the conserved central domain (Met332-722). This protein appeared as a 45-kDa immunoreactive band (Fig. 2), less than half the size of the full-length protein, yet it was still catalytically active, with a V_{max} value of 2.8 ± 0.3 nmol/min/μg of protein and a K_m value of 6.0 ± 0.7 μM cAMP (Table 2). This activity was inhibitable by rolipram and RP 73401 (Table 2).

Saturation analysis of [^{3}H](R)-rolipram and [^{3}H]RP 73401 binding. Using the newly developed equilibrium filtration assay, two [^{3}H](R)-rolipram binding sites were detected in Met265-886, one with a K_{d_1} value of 0.7 ± 0.3 nM and one with a K_{d_2} value of 34 ± 10 nM (four experiments; Table 3). A Scatchard analysis from a representative experiment is shown in Fig. 3. These results are similar to those reported previously using independent techniques to detect low and high affinity binding sites for Met265-886 (21).

Additional studies were conducted in which a side-by-side comparison of the binding characteristics of [^{3}H](R)-rolipram and [^{3}H]RP 73401 was carried out using Met265-886 and Met332-886 (Fig. 4A). In contrast to the two classes of [^{3}H](R)-rolipram binding sites present on Met265-886 (Figs. 3 and 4A and Table 3), only a single, low affinity site (K_d = 101 ± 7 nM; four experiments) was present in Met332-886 (Fig. 4A and Table 3). The standard filtration assay detected only a single, high affinity rolipram-binding site in Met265-886 (K_d = 0.3 nM; Fig. 4B), as well as in the full-length protein (K_d = 0.5 nM; data not shown). Low affinity [^{3}H](R)-rolipram binding was not detected using the standard filtration assay (Fig. 4B).

[^{3}H]RP 73401 bound to a single site (n_H = 0.8 ± 0.1) in the Met265-886 protein with a K_d value of 0.4 ± 0.1 nM (Fig. 4C and Table 4). In contrast to the results with [^{3}H](R)-rolipram, truncation of the protein further (i.e., Met332-886) did not result in a loss of high affinity [^{3}H]RP 73401 binding. On the contrary, the affinity of [^{3}H]RP 73401 for Met332-886 was virtually identical to its affinity for Met265-886 (Fig. 4C and Table 4). Similarly, the K_d value of [^{3}H]RP 73401 for Met1-886, the full-length protein, was virtually identical to its K_d value of the truncated forms (Table 4).

The double-truncated protein (Met332-722) had the same properties as Met332-886; i.e., high affinity rolipram binding was absent, whereas RP 73401 binding was still observed (Table 1).

To determine whether there is a domain at the NH_2 terminus for high affinity binding of rolipram (and possibly RP 73401) that is distinct from the catalytic core of the enzyme, proteins that had COOH-terminal truncations but still contained the region between amino acids 201 and 331 were examined. As mentioned above, Met201-722 was catalytically active, whereas Met201-678 was inactive. Similarly, high affinity rolipram binding was present in Met201-722 but not in Met201-678 (Table 1). These results do not support the existence of a rolipram-binding site that is distinct from the catalytic site (i.e., a distinct site formed by amino acids 201–331).

Inhibition by rolipram and RP 73401 of rhPDE4A Met265-886 and Met332-886 catalytic activity. The catalytic activity of both Met265-886 and Met332-886 was inhibited by (R)-rolipram and RP 73401 (Fig. 5). (R)-Rolipram inhibited catalytic activity of Met265-886 with an IC_{50} value of 0.07 ± 0.02 μM and that of Met332-886 with an IC_{50} value of 2.05 ± 0.05 μM (Table 2). Interestingly, the concentrations of (R)-rolipram required to inhibit enzyme activity of Met265-886 were much greater than those required to interact with the high affinity binding site (K_d = 0.7 ± 0.3 nM) but similar to those required to interact with the low affinity binding site (K_d = 34 ± 10 nM). In contrast to the 30-fold difference in the potency of (R)-rolipram against catalytic activity of the two proteins, RP 73401 inhibited cAMP-hydrolyzing activity of both Met265-886 and Met332-886 with IC_{50} values of 0.3 ± 0.1 and 0.23 ± 0.03 nM (Table 2). These IC_{50} values were

TABLE 2

Comparison of enzymatic properties and inhibitor sensitivities of NH_2- and COOH-terminal truncated forms of rhPDE4A

For kinetic measurements of cAMP hydrolysis, substrate concentrations were varied between 0.05 and 100 μM (see Materials and Methods). Kinetic determinants V_{max} and K_m were estimated using the KaleidaGraph computer program. For inhibitor studies, concentrations were varied between 0.03 and 300 nM (RP 73401) or 0.03 and 300 μM [(*R*)-rolipram] at a substrate concentration of 1 μM cAMP. Enzyme activities were followed after preincubation of cell lysates with inhibitor for 10 min at room temperature. V_{max} data are normalized for different expression levels of PDE4A protein using a partially purified preparation of Met265-886 as a standard. Data represent mean ± standard error of three experiments, except for Met201-722 (one experiment).

	Met1-886	Met265-886	Met332-886	Met201-722	Met332-722
V_{max} (nmol/min/μg)	2.9 ± 0.6	1.30 ± 0.02	6.3 ± 0.3	1.5	2.8 ± 0.3
K_m (μM)	10.0 ± 1.4	3.0 ± 0.4	3.0 ± 1.1	14.2	6.0 ± 0.7
IC_{50} (nM)					
RP 73401	0.6 ± 0.1	0.3 ± 0.1	0.23 ± 0.03	0.2	0.4 ± 0.1
(*R*)-Rolipram	325 ± 18	70 ± 20	2050 ± 50	120	1800 ± 140

TABLE 3

[^{3}H](*R*)-Rolipram binding in rhPDE4A Met265-886 and Met332-886

Saturation binding data were determined using a ligand concentration of 2-400 nM [^{3}H](*R*)-rolipram in the equilibrium filtration assay (see Materials and Methods). Scatchard analysis was carried out using the Acufit computer program. Data are normalized for different expression levels of PDE4A protein using a partially purified preparation of Met265-886 as a standard. Hill coefficients (n_H) were obtained through replotting of saturation binding data as log[$B/(B_{max} - B)$] versus log[free ligand]. Data represent the mean ± standard error of four experiments.

	Met265-886	Met332-886
K_{d_1} (nM)	0.7 ± 0.3	
K_{d_2} (nM)	34 ± 10	101 ± 7
n_H	0.7 ± 0.1	0.9 ± 0.1
B_{max1} (pmol/μg)	0.04 ± 0.01	
B_{max2} (pmol/μg)	0.3 ± 0.1	0.31 ± 0.08

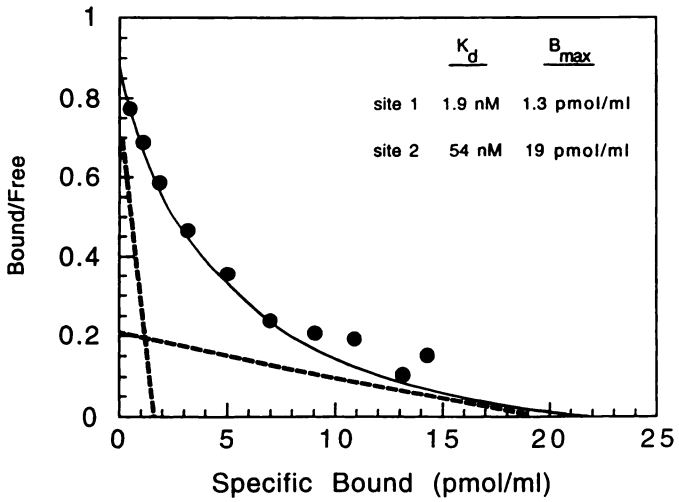

Fig. 3. Scatchard analysis for high and low affinity binding of [^{3}H](*R*)-rolipram to rhPDE4A Met265-886. The supernatant fraction (100,00 × *g*) of yeast cell lysates containing rhPDE4A Met265-886 was prepared, and specific binding of 0.5–250 nM [^{3}H](*R*)-rolipram using the equilibrium filtration assay was conducted as described in Materials and Methods. The amount of specific binding was not normalized for the expression level of PDE4A protein.

identical to the K_d values of RP 73401 for both of these truncated forms of rhPDE4A (Table 4).

Neither the PDE3 inhibitor siguazodan (30 μM) nor the PDE5 inhibitor zaprinast (30 μM) inhibited the catalytic activity of Met265-886, Met332-886, Met201-722, or Met332-722 (data not shown). These results indicate that none of the NH_2-, COOH-, or (NH_2 + COOH)-terminal truncations resulted in loss of the typical sensitivity of PDE4A to pharmacological agents. Thus, the determinants for inhibitor specificity for PDE subtypes seem to lie within the catalytic domain rather than the NH_2 or COOH terminus.

Competition binding assays. The ability of (*R*)-rolipram and cAMP to compete for [^{3}H]RP 73401 (1 nM) binding on Met265-886 and Met332-886 is shown in Fig. 6. Consistent with the presence of two (*R*)-rolipram binding sites on Met265-886, the competition curve for (*R*)-rolipram was biphasic. Analysis using the Inplot computer program revealed an improvement in a two-site fit over a one-site fit. IC_{50} values of 0.2 ± 0.03 nM and 57 ± 6 nM (four experiments) were calculated, with the high affinity binding sites representing 23% of the total. Interestingly, the data obtained with Met332-886 also fit a two-site model (IC_{50} = 1.1 ± 0.2 and 4.1 ± 0.9 μM, four experiments), although the percentage of high affinity sites (13%) was less than that of Met265-886. Regardless, just as (*R*)-rolipram was considerably less potent as an inhibitor of the catalytic activity of Met332-886 versus Met265-886 (Table 2 and Fig. 5), it is also less potent as a competitor of [^{3}H]RP 73401 binding in Met332-886 (Fig. 6).

Also shown in Fig. 6 is the ability of cAMP to compete for [^{3}H]RP 73401 binding. The competition curves were monophasic using either Met265-886 or Met332-886, with IC_{50} values of 53 ± 15 and 55 ± 5 μM, respectively.

Previous studies using the standard filtration assay demonstrated the ability of cAMP to compete for rolipram binding at the high affinity rolipram-binding site in Met201-886 (17). In the current study, we attempted to examine competition with cAMP at the low affinity [^{3}H](*R*)-rolipram-binding site in Met265-886 as well as in Met332-886 by using the equilibrium filtration assay. Although competition for low affinity rolipram binding with cAMP was detected, suggesting that rolipram binds to the catalytic site, the inhibitor constants obtained were inconsistent (20–100 μM). The variability of the results was due to high nonspecific binding in the equilibrium filtration assay as well as the extensive hydrolysis of competitor (i.e., cAMP) that occurred under these assay conditions.

Discussion

In this study, we attempted to map key functional domains of hPDE4A by engineering the expression of a variety of truncated forms of the enzyme. Our objectives were 1) to identify the smallest catalytically active form of the enzyme, 2) to determine whether the structural domain responsible for catalytic activity is distinct from that for high affinity rolipram-binding activity, and 3) to determine whether truncations that abolish high affinity rolipram-binding activity also abolish the ability of rolipram to inhibit catalytic activity. The overall goal of these studies was to provide a foundation for understanding the relationships among protein structure, catalytic activity, and inhibitor interactions.

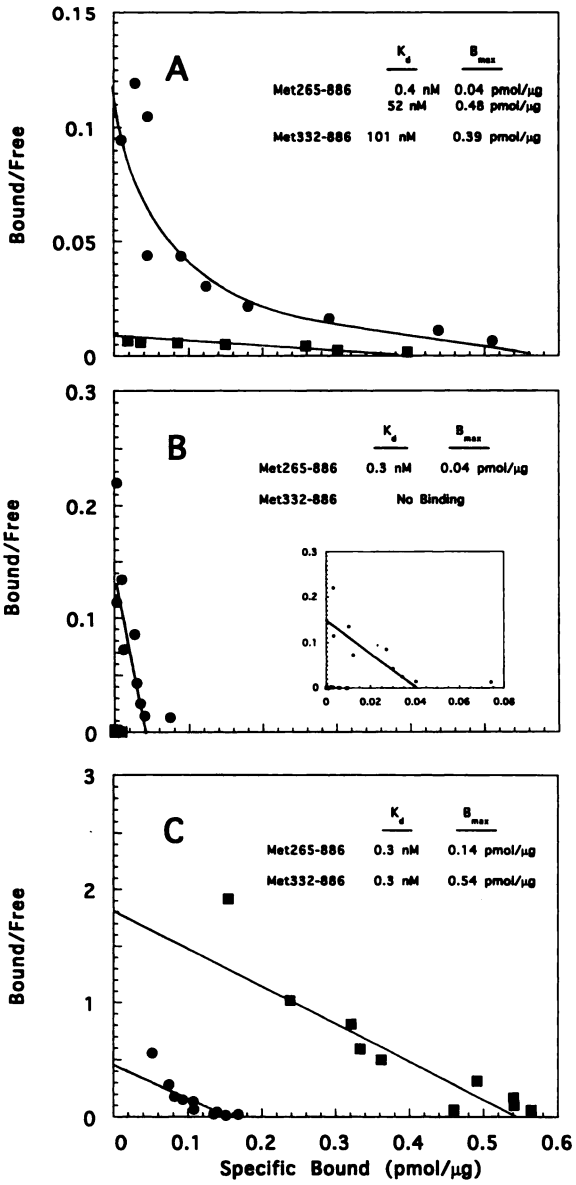

Fig. 4. Scatchard analyses of [^{3}H](*R*)-rolipram and [^{3}H]RP 73401 binding to truncated forms of rhPDE4A. Supernatant fractions (100,000 × *g*) of yeast cell lysates containing hPDE4A Met265-886 (●) or Met332-886 (■) were prepared as described in Materials and Methods. A, Specific binding of 0.5–400 nM [^{3}H](*R*)-rolipram was measured using the equilibrium filtration assay to detect both high and low affinity binding sites. B, Specific binding of 0.5–400 nM [^{3}H](*R*)-rolipram was measured using the standard filtration assay to detect high affinity binding sites (see Materials and Methods). *Inset*, expansion of *x* axis. C, Specific binding of 0.2–10 nM [^{3}H]RP 73401 was measured using the standard filtration assay. The amount of specific binding (B_{max}) was normalized for the expression levels of Met265-886 and Met332-886 protein. Data are representative of four independent experiments (Table 3).

TABLE 4

[^{3}H]RP 73401 binding in rhPDE4A Met1-886, Met265-886, and Met332-886

Saturation binding data were obtained using a ligand concentration of 0.2–10 nM [^{3}H]RP 73401 in the standard filtration assay (see Materials and Methods). Scatchard analysis was carried out using the Acufit computer program. Hill coefficients (n_H) were obtained through replotting of saturation binding data as log[$B/(B_{max} - B)$] versus log[free ligand]. B_{max} values are normalized for different expression levels of PDE4A protein using a partially purified preparation of Met265-886 as a standard. Data represent the mean ± standard error of three to six experiments as indicated in parentheses.

	Met1-886	Met265-886	Met332-886
K_d (nM)	0.4 ± 0.1 (3)	0.4 ± 0.1 (6)	0.20 ± 0.03 (4)
n_H	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
B_{max} (pmol/μg)	0.52 ± 0.04	0.14 ± 0.01	0.64 ± 0.04

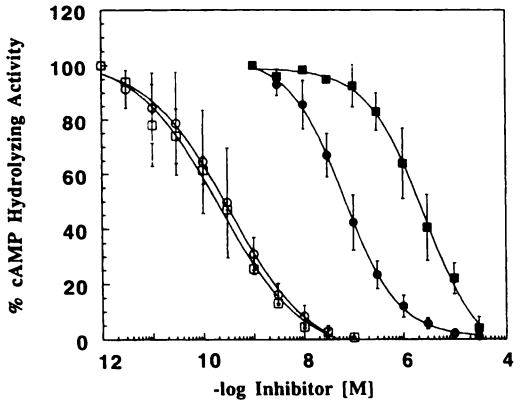

Fig. 5. Inhibition of catalytic activity in truncated forms of rhPDE4A. Assays were run using 100,000 × *g* supernatant fractions of yeast cell lysates as described in Materials and Methods. Inhibition of cAMP-hydrolyzing activity with (*R*)-rolipram (● and ■) or RP 73401 (○ and □) was monitored after preincubation of the supernatant fractions with different concentrations of inhibitor for 10 min at room temperature. Activity (normalized for expression levels) in the absence of inhibitor was 59 ± 4 pmol/min/μg for Met265-886 (○ and ●) or 97 ± 11 pmol/min/μg for Met332-886 (□ and ■). Data represent the mean ± standard error of three experiments.

Recent studies indicate that four major subtypes of human PDE4 exist (12). PDE4A is the largest of these subtypes, consisting of 886 amino acids and having an apparent molecular mass of 110 kDa. Sequence comparisons reveal three regions of high homology among the four subtypes (8, 12). These include a central region consisting of ~340 amino acids and two upstream conserved regions near the NH_2 terminus that contain ~40 amino acids each. There is strong sequence homology among the central regions of PDEs from all families, which suggests that this area contains the catalytic domain; this suggestion is supported by deletion mutagenesis studies of two rat PDE4s (9, 32).

In the current study, our assessment of enzyme activity in a variety of truncated human PDE4A proteins confirmed this proposal. Deletions of ≤331 amino acids from the NH_2 terminus failed to reduce enzyme activity, whereas an internal deletion between amino acids 271–365 abolished activity. These results suggest that the NH_2 terminus can be eliminated without destroying catalytic activity up to the region between amino acids 332–365. On the COOH terminus, 114 amino acids can be deleted without destroying catalytic activity. Indeed, the double-truncated protein, Met332-722, was fully active, suggesting that amino acids 332–722 represent the minimum or close to the minimum requirements for enzyme activity of hPDE4A.

Interestingly, the catalytic efficiency of Met332-886 PDE4A was 7-fold greater than that of the full-length enzyme and 5-fold greater than that of the Met265-886 protein. Although the molecular basis for this observation is not clear, it is consistent with the proposal of Conti *et al.* (33) that the NH_2 terminus of PDE4 contains an inhibitory domain.

In addition to binding and hydrolyzing its substrate cAMP, PDE4s bind rolipram and certain other PDE4-selective inhibitors with high affinity (10, 16, 17). In the current study,

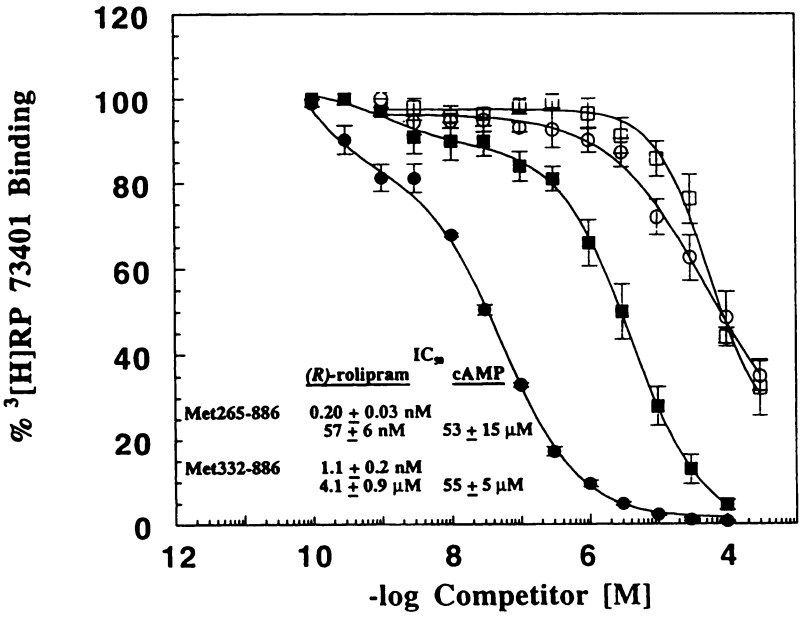

Fig. 6. Competition by (*R*)-rolipram and cAMP for [^{3}H]RP73401 binding in Met 265-886 (*circles*) and Met 332-886 (*squares*) of rhPDE4A. Competition for [^{3}H]RP 73401 (1 nM) binding with (*R*)-rolipram, ● and ■) was assayed in 0.5 ml at 30° for 30 min, whereas competition with cAMP (○ and □) was assayed in 5 ml at 4° for 30 min. Curve fitting and calculated apparent IC_{50} values were obtained using the Inplot computer program, which favors a two-site curve fitting over a one-site fit for competition with (*R*)-rolipram. Data represent the mean ± standard error of four experiments.

two classes of rolipram-binding sites were detected on rhPDE4A Met265-886 using an equilibrium filtration assay. In contrast to the two sites detected on Met265-886, only a single low affinity site was detected on Met332-886. Despite the loss of the high affinity rolipram-binding site in Met332-886, this mutant protein retained full catalytic activity. Furthermore, (*R*)-rolipram still inhibited the catalytic activity of Met332-886 albeit with a 30-fold loss in potency. Thus, the loss of the high affinity rolipram-binding site in hPDE4A mutant proteins was not associated with a loss of catalytic activity, nor was it associated with a loss in the ability of rolipram to inhibit catalytic activity.

In contrast to the results with (*R*)-rolipram, RP 73401 bound with high affinity (K_d = ~0.3 nM) to a single class of sites on both Met265-886 and Met332-886. That this binding site is the catalytic site is supported by the fact that cAMP competed for [^{3}H]RP 73401 binding on both proteins. Also consistent with this proposal is that RP 73401 inhibited catalytic activity of both proteins with an IC_{50} value (~0.3 nM) that is identical to its dissociation constant for binding. If the RP 73401 binding site is indeed the catalytic site, then it follows that the low affinity rolipram-binding site is also the catalytic site. This is the case because (*R*)-rolipram competed with [^{3}H]RP 73401 for binding in Met332-886.

A major question that remains is the identity and function of the high affinity rolipram-binding site. One possibility is that this site is an allosteric site (17, 18). Regarding this proposal, it is noteworthy that the current results indicate that rolipram inhibits PDE4A catalytic activity of Met332-886, a truncated form of the enzyme in which the high affinity rolipram-binding activity has been engineered out. Thus, even if the high affinity rolipram-binding site is an allosteric site, rolipram also seems to be capable of interacting at the catalytic site. In fact, several pieces of information argue against the high affinity rolipram-binding site being an allosteric site. First, the relative stoichiometry of binding is inconsistent with the proposal of an allosteric site. Assuming that there is only one allosteric site and one catalytic site per molecule of PDE4, then the relative stoichiometry of high affinity binding sites to low affinity binding sites should be 1:1. Although somewhat variable, the relative stoichiometry of rolipram binding to high affinity versus low affinity sites was typically 1:10 and never exceeded 1:4. Second, high affinity rolipram binding was not detected in a COOH-terminal truncated protein (Met201-678) that was devoid of catalytic activity yet contained the NH_2-terminal region between amino acids 265–332 that seemed to be required for high affinity binding. Although it cannot be ruled out that loss of catalytic activity and rolipram binding in this protein was the result of improper folding, this result does not support the existence of a distinct rolipram-binding site outside of the catalytic domain. Third, RP 73401 competes for high affinity [^{3}H](*R*)-rolipram binding with an IC_{50} value of ~0.5 nM,[1] and as shown in the current study, RP73401 seems to interact exclusively at the catalytic site. Fourth, the loss of high affinity rolipram-binding activity in rhPDE4A Met332-886 had no effect on either the K_d or B_{max} value of the low affinity site (i.e., catalytic site), suggesting the absence of cooperative interactions between multiple sites. Finally, results from a previous study indicate that a low concentration of (*R*)-rolipram has no effect on the IC_{50} values of other PDE4 inhibitors with respect to their ability to inhibit cAMP catalysis (17). Taken collectively, these results suggest that rolipram does not act through a distinct, high affinity allosteric site to regulate catalytic activity.

An alternative explanation for the high affinity rolipram-binding site is that it represents the catalytic site on a proportion of the expressed PDE4 molecules that exist in a distinct conformational state (17). For example, PDE4 may fold in two ways, one of which yields a protein that binds rolipram at the catalytic site with high affinity (K_d = ~1 nM) and a second that binds rolipram at the catalytic site with a lower affinity (K_d = ~50 nM). According to this model, the role of amino acids 265–332 is not to form a distinct binding site but rather to increase the probability that a portion of the enzyme will assume a conformation that allows (*R*)-rolipram to bind to the catalytic site with high affinity. Consistent with this proposal are the observations that little (com-

[1] T. J. Torphy and M. Burman, unpublished observations.

petition studies with [^{3}H]RP 73401) or no (saturation studies with [^{3}H](*R*)-rolipram) high affinity rolipram binding sites were detected in Met332-886. If PDE4A can exist in two conformations, then why does RP 73401 seem to interact with only a single binding site? One explanation may be that unlike rolipram, RP 73401 has little or no selectivity for the catalytic site of either conformation. If PDE4A can indeed assume two distinct catalytically active conformations with different affinities for rolipram, then one would predict atypical (i.e., shallow) curves for the inhibitory effect of rolipram on cAMP hydrolysis. Indeed, we and others typically observe such a pattern (9, 10, 17, 18). Curiously, grossly atypical rolipram inhibition curves were not detected in the current study with the use of Met265-886. The failure to observe this behavior may simply reflect the fact that the enzyme preparations used for this study generally contained only a small fraction (≤10%) of the high affinity rolipram-binding form.

In summary, our results indicate that the catalytic domain of rhPDE4A resides between amino acids 332–722. In addition, elimination of amino acids 265–331 results in a loss of high affinity rolipram-binding activity without a loss in catalytic activity or the ability of rolipram to inhibit cAMP hydrolysis. Finally, the results suggest that the high affinity rolipram-binding site on PDE4A is not an allosteric site; rather, it is the catalytic site on a distinct enzyme conformer. However, additional studies are required before a definitive explanation of the nature and role of this site can be offered.

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References

1. Beavo, J. A., and D. H. Reifsnyder. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol. Sci.* **11:**150–155 (1990).
2. Bentley, J. K., and J. A. Beavo. Regulation and function of cyclic nucleotides. *Curr. Opin. Cell Biol.* **4:**233–240 (1992).
3. Beavo, J. A., M. Conti, and R. J. Heaslip. Multiple cyclic nucleotide phosphodiesterases. *Mol. Pharmacol.* **46:**399–405 (1994).
4. Ashton, M. J., D. C. Cook, G. Fenton, J.-A. Karlsson, M. N. Palfreyman, D. Raeburn, A. J. Ratcliffe, J. E. Souness, S. Thurairatnam, and N. Vicker. Selective type IV phosphodiesterase inhibitors as antiasthmatic agents: the syntheses and biological activities of 3-(cyclopentyloxy)-4-methoxybenzamides and analogues. *J. Med. Chem.* **37:**1696–1703 (1994)
5. Torphy, T. J., and B. J. Undem. Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax* **46:**512–524 (1991).
6. Swinnen, J. V., D. R. Josef, and M. Conti. Molecular cloning of rat homologues of the *Drosophila melanogaster* dunce cAMP phosphodiesterase: evidence for a family of genes. *Proc. Natl. Acad. Sci. USA* **86:**5325–5329 (1989).
7. Colicelli, J., C. Birchmeier, J. Michaeli, K. O'Neill, M. Riggs, and M. Wigler. Isolation and characterization of a mammalian gene encoding a high-affinity cAMP phosphodiesterase. *Proc. Natl. Acad. Sci. USA* **86:**3599–3603 (1989).
8. Livi, G. P., P. Kmetz, M. M. McHale, L. B. Cieslinski, G. M. Sathe, D. P. Taylor, R. L. Davis, T. J. Torphy, and J. M. Balcarek. Cloning and expression of cDNA for a human low-K_m, rolipram sensitive cyclic AMP phosphodiesterase. *Mol. Cell. Biol.* **10:**2678–2686 (1990).
9. Jin, S.-L. C., J. V. Swinnen, and M. Conti. Characterization of the structure of a low K_m, rolipram-sensitive cAMP phosphodiesterase. *J. Biol. Chem.* **267:**18929–18939 (1992).
10. McLaughlin, M. M., L. B. Cieslinski, M. Burman, T. J. Torphy, and G. P. Livi. A low-K_m, rolipram-sensitive, cAMP-specific phosphodiesterase from human brain. *J. Biol. Chem.* **268:**6470–6476 (1993).
11. Obernolte, R., S. Bhakta, R. Alvarez, C. Bach, P. Zuppan, M. Mulkins, K. Jarnagin, and E. R. Shelton. The cDNA of a human lymphocyte cyclic-AMP phosphodiesterase (PDE IV) reveals a multigene family. *Gene* **129:**239–247 (1993).
12. Bolger, G.,T. Michaeli, T. Martins, T. St. John, B. Steiner, L. Rodgers, M. Riggs, M. Wigler, and K. Ferguson. A family of human phosphodiesterases homologous to the *dunce* learning and memory gene product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol. Cell. Biol.* **13:**6558–6571 (1993).
13. Bolger, G. B., L. Rodgers, and M. Riggs. Differential CNS expression of alternative mRNA isoforms of the mammalian genes encoding cAMP-specific phosphodiesterases. *Gene* **149:**237–244 (1994).
14. Monaco, L., E. Vicini, and M. Conti. Structure of two rat genes coding for closely related rolipram-sensitive cAMP phosphodiesterases. *J. Biol. Chem.* **269:**347–357 (1994).
15. Baecker, P. A.,R. Obernolte, C. Bach, C. Yee, and E. R. Shelton. Isolation of cDNA encoding a human rolipram-sensitive cyclic AMP phosphodiesterase (PDE IV_D). *Gene* **138:**253–256 (1994).
16. Schneider, H. H., R. Schmiechen, M. Brezenski, and J. Seidler. Stereospecific binding of the antidepressant rolipram to brain protein structures. *Eur. J. Pharmacol.* **127:**105–115 (1986).
17. Torphy, T. J., J. M. Stadel, M. Burman, L. B. Cieslinski, M. M. McLaughlin, J. R. White, and G. P. Livi. Coexpression of human cAMP-specific phosphodiesterase activity and high affinity rolipram binding in yeast. *J. Biol. Chem.* **267:**1798–1804 (1992).
18. Souness, J. E., C. Maslen, and L. C. Scott. Effects of solubilization and vanadate/glutathion complex on inhibitor potencies against eosinophil cyclic AMP-specific phosphodiesterase. *FEBS Lett.* **302:**181–184 (1992).
19. McHale, M. M., L. B. Cieslinski, W.-K. Eng, R. K. Johnson, T. J. Torphy, and G. P. Livi. Expression of human recombinant cAMP phosphodiesterase isozyme IV reverses growth arrest phenotypes in phosphodiesterase-deficient yeast. *Mol. Pharmacol.* **39:**109–113 (1991).
20. Sullivan, M., M. Egerton, Y. Shakur, A. Marquardsen, and M. D. Houslay. Molecular cloning and expression, in both COS-1 cells and *S. cerevisiae*, of a human cytosolic type-IV_A, cyclic AMP specific phosphodiesterase (hPDE-$IVA_{-h6.1}$). *Cell. Signalling* **6:**793–812 (1994).
21. Torphy, T. J., W. E. DeWolf, Jr., D. W. Green, and G. P. Livi. Biochemical characteristics and cellular regulation of phosphodiesterase IV. *Agents Actions* **43:**51–71 (1993).
22. Mullis, K. B., and F. A. Faloona. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155:**335–350 (1987).
23. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153:**163–168 (1983).
24. Nikawa, J.-I., P. Sass, and M. Wigler. Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7:**3629–3636 (1987).
25. Sass, P., J. Field, J.-I. Nikawa, T. Toda, and M. Wigler. Cloning and characterization of the high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **83:**9303–9307 (1986).
26. Wilson, R. B., and K. Tatchell. *SRA5* encodes the low-K_m cyclic AMP phosphodiesterase of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8:**505–510 (1988).
27. Torphy, T. J., and L. B. Cieslinski. Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol. Pharmacol.* **37:**206–214 (1990).
28. Engels, P., M. Sullivan, T. Müller, and H. Lübbert. Molecular cloning and functional expression in yeast of a human cAMP-specific phosphodiesterase subtype (PDE IV-C). *FEBS Lett.* **358:**305–310 (1995).
29. Davis, R. L., H. Takaysasu, M. Eberwine, and J. Myers. Cloning and characterization of mammalian homologs of the *Drosophila* dunce$^+$ gene. *Proc. Natl. Acad. Sci. USA* **86:**3604–3608 (1989).
30. Chen, C.-N., S. Denome, and R. L. Davis. Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the *Drosophila* dunce$^+$ gene, structural gene for cAMP phosphodiesterase. *Proc. Natl. Acad. Sci. USA* **83:**9313–9317 (1986).
31. Torphy T. J., and G. P. Livi. Phosphodiesterase isozymes in airways, in *Pharmacology of the Respiratory Tract* (K. F. Chung and (P. J. Barnes, eds.). Marcel Dekker, New York, 177–222 (1993).
32. Pillai, R., K. Kytle, A. Reyes, and J. Colicelli. Use of a yeast expression system for the isolation and analysis of drug-resistant mutants of a mammalian phosphodiesterase. *Proc. Natl. Acad. Sci. USA* **90:**11970–11974 (1993).
33. Conti, M., G. Nemoz, C. Sette, and E. Vicini. Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocr. Rev.* **16:**370–389 (1995).

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