Identification of overlapping but distinct cAMP and cGMP interaction sites with cyclic nucleotide phosphodiesterase 3A by site-directed mutagenesis and molecular modeling based on crystalline PDE4B

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Abstract

Cyclic nucleotide phosphodiesterase 3A (PDE3A) hydrolyzes cAMP to AMP, but is competitively inhibited by cGMP due to a low k_{cat} despite a tight K_m . Cyclic AMP elevation is known to inhibit all pathways of platelet activation, and thus regulation of PDE3 activity is significant. Although cGMP elevation will inhibit platelet function, the major action of cGMP in platelets is to elevate cAMP by inhibiting PDE3A. To investigate the molecular details of how cGMP, a similar but not identical molecule to cAMP, behaves as an inhibitor of PDE3A, we constructed a molecular model of the catalytic domain of PDE3A based on homology to the recently determined X-ray crystal structure of PDE4B. Based on the excellent fit of this model structure, we mutated nine amino acids in the putative catalytic cleft of PDE3A to alanine using site-directed mutagenesis. Six of the nine mutants (Y751A, H840A, D950A, F972A, Q975A, and F1004A) significantly decreased catalytic efficiency, and had k_{cat}/K_m less than 10% of the wild-type PDE3A using cAMP as substrate. Mutants N845A, F972A, and F1004A showed a 3- to 12-fold increase of K_m for cAMP. Four mutants (Y751A, H840A, D950A, and F1004A) had a 9- to 200-fold increase of K_i for cGMP in comparison to the wild-type PDE3A. Studies of these mutants and our previous study identified two groups of amino acids: E866 and F1004 contribute commonly to both cAMP and cGMP interactions while N845, E971, and F972 residues are unique for cAMP and the residues Y751, H836, H840, and D950 interact with cGMP. Therefore, our results provide biochemical evidence that cGMP interacts with the active site residues differently from cAMP.

Keywords: cAMP; cGMP; phosphodiesterase; site-directed mutagenesis; molecular modeling

Unlike most tissues, but similar to other blood cells, an increased intracellular level of cAMP is associated with the

inhibition of all platelet responses to agonists including such responses as shape change, aggregation, adhesion, and granule release (Salzman and Weisenberger 1972). Cyclic AMP is synthesized by adenylate cyclase under control of PGI₂ and PGE₁, PGD₂ and adenosine acting on separate receptors coupled to Gs protein. cAMP acting through protein kinase A inhibits agonist binding and phosphoinositide hydrolysis to diacylglycerol and IP₃. cAMP phosphodies-

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terases catalyze the degradation of cAMP. In contrast, agonists such as thrombin, ADP, and epinephrine antagonize the stimulation of adenylate cyclase by acting on Gi.

The catalytic regions of the 11 known families of cyclic nucleotide phosphodiesterases are well conserved (Soderling and Beavo 2000). In contrast, the regulatory regions comprising sites for phosphorylation, cGMP binding, and membrane insertion vary widely. Metallohydrolases, especially those binding catalytic zinc, require three ligands directly coordinating the metal ion as well as a fourth ligand, usually H₂O. The amino acid residues commonly found to act as zinc ligands are histidine and glutamate, and, more rarely, aspartate or cysteine. The three-ligand arrangement on a polypeptide chain also shows a highly predictable pattern. In all 11 families of cyclic nucleotide phosphodiesterases (Soderling and Beavo 2000), there is absolute conservation of two motifs, HNXXH(X)₂₄₋₂₆E and HDXXH(X)₂₄₋₂₆E. In the case of PDE3, a unique 44 amino acid insert lies between the second histidine and the glutamate of the first motif (Meacci et al. 1992).

Platelets contain two cAMP phosphodiesterases: the high K_m cyclic GMP-stimulated PDE2A and the low K_m cGMP-inhibited PDE3A (Hidaka and Asano 1976). Platelets also contain the specific cGMP phosphodiesterase, PDE5A. We have focused on the major cAMP phosphodiesterase in platelets, PDE3A, which we purified to homogeneity (Grant and Colman 1984). We cloned the enzyme from HEL cells, which are known to express megakaryocyte/platelet proteins, since mRNA levels are very low in platelets and libraries are hard to construct (Cheung et al. 1996). The resulting cDNA sequence was identical to a cloned enzyme from human myocardium (Meacci et al. 1992). To begin to define the region of the active site, we made four deletion mutants of HEL cell PDE3A. Two had full activity, but the two smaller ones did not, indicating that the active site lay between amino acids 679 and 1141 (Cheung et al. 1996).

As a first step to elucidate the catalytic mechanism of PDE3A, we performed and previously published chemical modification studies on the histidines present in the catalytic domain of the enzyme, isolated from human platelets with diethyl pyrocarbonate (DEP) (Ghazaleh et al. 1996). This reaction resulted in a time- and concentration-dependent inactivation and hydroxylamine promptly reactivated PDE3A consistent with modification of histidines. The inactivation was accompanied by an increase in a difference spectrum at 240 nm consistent with N-carbethoxylation of histidine residues. The extrapolated value for complete inactivation shows that 2.0 mols of histidine per mol of the enzyme are responsible for most of the loss in PDE3A activity. AMP protects two histidine groups against ¹⁴C-DEP incorporation, and cGMP also protects two histidine groups. However, when both nucleotides are included, four groups are protected, indicating that cAMP and cGMP each protects two different groups. These results suggest but do not

prove that there are different cAMP and cGMP sites. However, the fact that cGMP is a competitive inhibitor led to the hypothesis that the sites may overlap.

In a second study, we therefore produced six-point mutations of conserved histidines and glutamic acid residues in the catalytic domain of PDE3A (Fig. 1) (Zhang and Colman 2000). Despite adequate expression, H752 and H756 had activities of less than 0.1% of the wild type due to effects on catalysis and/or metal binding. Two of the mutants showed significantly different K_m for cAMP. E866A exhibited a 10-fold increase and E971A a 5-fold increase. Thus, these two amino acids lie in the cAMP binding site. When cGMP was tested as an inhibitor, the K_i for the first histidine of the second motif, H836A, showed a 178-fold increase in K_i and the related glutamate residue, E866A, showed a 27-fold increase in the K_i of cGMP. The data indicates that these two amino acids display defective interactions with cGMP. Thus, E866 lies in both the cGMP and cAMP sites, H836 only in the cGMP site, and E971 in the cAMP site. These observations confirmed our hypothesis that the sites overlap and account for the behavior of cGMP as a competitive inhibitor.

To better interpret these results and to rationally design new mutants, we have now created a molecular homology model of PDE3A based on the three-dimensional structure of the catalytic region (residues 152–528) of PDE4B2B (Xu et al. 2000), the only three-dimensional structure of PDE currently determined. In addition, the sequence homology of the catalytic region of PDE4B2B is over 50%. We chose five nonconserved residues which constituted the putative boundaries of a deep pocket thought to be the catalytic cleft in the molecule. We also examined four additional conserved residues which lay close to identified residues in the cAMP and/or cGMP binding sites previously described (Zhang and Colman 2000). The description of the molecular model and the characterization of these amino acids by site-directed mutagenesis to alanine form the basis of this report.

Results

Modeling of cAMP/cGMP binding

The binding of cAMP to PDE4B active site was built on the basis of the electron density for the structure of PDE4B (Xu et al. 2000) and the structure of PDE4B complexed to cAMP (Ke et al., unpubl.). The model of cAMP binding to PDE3A was generated from the superposition between PDE3A and PDE4B (Fig. 2). In this model, the phosphate portion of cAMP is located next to Zn and forms hydrogen bonds with His752 as well as the Zn binding residues of Asp837 and Asp950 (Fig. 3). The sugar group of cAMP forms van der Waals interactions with Tyr751, Leu910, and Ile968 while the adenine ring of cAMP interacts with hy-

Fig. 1. Alignment of the catalytic domain PDE3A with PDE4B. Positions where sequences of all 11 mammalian family members are identical are noted with asterisks. Locations of mutants generated in this study are indicated in black boxes. The mutants made in the previous experiments (Zhang and Colman 2000) are displayed in gray boxes.

Based on the model of cAMP, the binding of cGMP was manually built into the active site of PDE3A (Fig. 4). Since the difference between cAMP and cGMP occurs on the base rings of the nucleotides, we feel that cGMP and cAMP must have similar molecular conformation and the phosphate portion of cGMP and cAMP must have similar binding. However, the orientation of the guanine ring is uncertain because the volume of the active site (440 Å³) (Xu et al. 2000) is significantly larger than that of cGMP (estimated 250 Å³). In addition, unlike PDE3A, PDE4B does not bind cGMP in its active site and thus no structure with cGMP is available. There are two ways that cGMP can be modeled. The first model is that the guanine of cGMP occupies the similar location as the adenine of cAMP. In this model, the residues interacting with cGMP will be essentially identical to cAMP. However, the extra atom N2 of guanine might form a hydrogen bond with a backbone oxygen of the Phe989 loop. The results suggest that cGMP or its hydrolysis product GMP will be sequestered in the active site to inhibit the PDE3A activity. However, the hydrolysis of cGMP pro-

In the second model which is preferred and displayed (Fig. 4), we have placed the phosphate portion of cGMP into a similar position to that of cAMP while the guanine ring orients to a different hydrophobic pocket that is comprised of His913, Phe914, Phe1004, and Ile1008. In this model, the phosphate group of cGMP contacts His752 and Asp950 in a similar way as cAMP, and the sugar group of cGMP remains to interact with Tyr751 and Leu910 but no longer with Ile968. The dramatic change is that the guanine of cGMP is located in a different hydrophobic pocket and forms van der Waals interactions with Phe1004 and Phe1008 and a hydrogen bond with His913. The presence of the hydrogen bond between His913 and the guanine ring will lock cGMP at the active site to inhibit PDE3A.

Fig. 2. Homology model of PDE3A. Superposition of C α atoms of PDE4B catalytic domain (yellow) over a PDE3A model (green). The zinc position is marked with a ball, while the red stick represents a model of PDE3A inhibitor clostamide.

Mutagenesis

Eight new mutants were made and one, H840, previously made in yeast (Cheung et al. 1996) was reconstructed in SF9 insect cells for more careful analysis. In each case, the chosen amino acid was mutated to alanine. Five mutants (T844, N845, L910, F972, and Q975) were chosen by reference to the homology model to represent the boundaries of the putative substrate binding site. Three (Y751, D950, and F1004) were highly conserved (Fig. 1) and found to lie in the putative active site (Fig. 3). The mutants were analyzed by SDS electrophoreses and each was a single band of 60 kD (Fig. 5). Similarly, on a Western blot, a single band was detected with anti-PDE3A antibody for each mutation (Fig. 6).

Table 1 presents the kinetic data. The k_{cat} of the mutants ranged from 0.35% to 77% of recombinant PDE3A, and a similar range was noted for the catalytic efficiency k_{cat}/K_m . We defined a third amino acid which lies in the cAMP site with a K_m elevated 13-fold (F1004); a fourth amino acid, N845, with a K_m elevated 4-fold; and a fifth, F972, with a K_m elevated 3-fold. We also have located four additional amino acids in the cGMP site that markedly increased the K_i : Y751A, 8.8-fold increase; D950A, 53-fold increase; H840A, 69-fold increase; and F1004A, over 130-fold. Fig. 7A illustrates representative determinations of the K_m for

cAMP for the mutants N845A and F1004A. Figure 7B presents a sample determination of the K_i for cGMP for the mutant Y751A. The value is nine times the K_i for recombinant PDE3A.

Correlations of mutagenesis results with the model

The K_i values for the mutants proposed to alter cGMP interactions are 10- to over 100-fold elevated compared to the recombinant PDE3A consistent with the hypothesis that H-bonds are involved. Although the cGMP binding model needs to be confirmed by crystal structures, our site-directed mutagenesis supports the model of different binding of cGMP from cAMP and agrees with the previous differential protection of the nucleotides against histidine modification (Ghazaleh et al. 1996). Among the nine amino acids that have been mutated to alanine, Thr844, Asn845, and Gln975 have no interactions with the current models of cAMP and cGMP, consistent with the mutagenesis that the mutants showed no substantial impact on the catalytic activity and binding affinity of the nucleotides. Phe1004 interacts with both bases of cAMP and cGMP and its mutation to alanine resulted in about 300-fold decrease of k_{cat}/K_m of cAMP and over 100-fold increase of K_i of cGMP (Table 1), indicating the critical role of Phe1004 for cGMP binding and catalytic activity. An interesting residue is Asp950 that interacts with zinc and also forms hydrogen bond with phosphate group of cAMP/cGMP (Figs. 3 and 4). Mutation of Asp950 to alanine resulted in a net loss of 100-fold in catalytic activity and 50-fold in cGMP binding. Mutation of the residues Tyr751, Leu910, and Phe972 that interact with either cAMP or cGMP showed significant impact on the catalytic activity and nucleotide binding.

Discussion

Two previous studies from our laboratory employing site-directed mutagenesis (Cheung et al. 1998; Zhang and Coleman 2000) have focused on the 26 perfectly conserved amino acids in PDE2, PDE3A, PDE4B, and PDE5A (Turko et al. 1998). A total of 12 conserved amino acids were mutated in the two studies with particular emphasis on the two canonical metal binding motifs, HNXXH and HDXXH, with the associated "downstream" acidic residue in this case glutamate. Although preliminary conclusions about some of the amino acids involved in catalysis and cAMP and cGMP interaction sites were suggested, without a molecular model the spatial proximity of these residues could not be visualized.

The three-dimensional atomic structure of the catalytic domain of PDE4B2B (Xu et al. 2000) provided considerable insight into the mechanism of catalysis and specificity of PDE4. Ultimate understanding of the catalytic properties and the binding of metals, substrates, and inhibitors of

Fig. 3. Model of cAMP binding site. The nine residues mutated in this study and the two residues, E866 and E971, previously identified as involved in cAMP binding (Zhang and Colman 2000) are shown as is the position of the Zn^{++} atom against the alpha carbon backbone ribbon of the PDE3A homology model. The positioning of cAMP is described in Materials and Methods.

PDE3A will await the crystallization of the enzyme. In the interim, we prepared a molecular model of PDE3A which could be used to select new amino acids for mutation and to integrate previous observations. We also have used the information about the metal binding residues and cAMP binding residues of PDE4B to help interpret our data. The unaltered immunoreactivity of the mutations and their mutation to alanine minimizes but does not rule out conformational changes due to different patterns of folding.

The structure of the catalytic domain residues 152–528 of PDE4B2B is made up of 17 alpha helices which fold into three subdomains. At the junction of these domains is a deep pocket which accommodates the substrate cAMP and two metal ions. Twenty-one conserved amino acids lie within or near this pocket (Xu et al. 2000) and both metal ions are located near the bottom. One of the metals, Zn^{++} , is bound with high affinity and assumed to be structural, and its role in catalysis is uncertain. Previously, the mutations H752A and H756A exhibited less than one-thousandth the catalytic efficiency of the truncated wild-type enzyme without appreciable change in the K_m for cAMP or the K_i of cGMP. We assumed that Zn^{++} at the bottom of the active site in PDE3A is in the homologous position to that in PDE4 crystal and interacts with H756 of PDE3A. Both amino acid residues H752 and H756 are probably involved in the catalysis or Zn^{++} binding (Xu et al. 2000). It is possible that

these two histidines are the ones protected by cAMP against the inactivation by DEP (Ghazaleh et al. 1996). H752 in PDE3A are fully prepared to protonate the O3' linkage and is stabilized by hydrophobic ring stacking including Y751 and H840.

The results of this study were combined with our previous investigation (Zhang and Colman 2000). Taken together, we identified five amino acids (N845, E866, E971, F972, and F1004) which showed an increase of the K_m for cAMP ranging from 3- to 13-fold when they are mutated to alanine. The model for cAMP has a high probability of being accurate since the position of cAMP is very similar to that of cAMP in the structure of the crystal of PDE4B. To decide whether there is significant interaction between the amino acid residues and the nucleotides, we have taken 3.5 to 4 Å representing the hydrophobic interactions. The best examples of this are F972, F989, and F1004 (Fig. 3), which form the boundaries of the deep concave hydrophobic pocket for accommodation of adenine moiety of cAMP, and correspond to hydrophobic pocket residues F414, M431, and F446 in PDE4B (Xu et al. 2000). The residues N845 and E866, while not close enough to cAMP to be considered in the binding site, are gatekeepers which guard the opening to the active site cleft and thus prevent cAMP from entering the pocket. The final amino acid we have identified as interacting with cAMP, E971, corresponds to E413 in PDE4B

Fig. 4. Model of cGMP binding site. The nine residues mutated in this study and the two residues, E866 and H836, previously identified as involved in cGMP binding (Zhang and Colman 2000) are shown in a similar way as in Figure 3.

which stabilizes H234 (H752 in PDE3A), a known participant in catalysis probably through the second metal required for enzymatic activity. The binding of this residue to a metal may cause a conformational change bringing it closer to cAMP which is not represented by the model. The model supports the lack of interaction of T844, L910, and Q975

mutations, which does not affect the kinetic behavior of the enzyme with cAMP or cGMP while F1004 is involved in both cGMP and cAMP binding.

We have also identified six amino acids that increase the K_i for cGMP from 9- to 200-fold (Fig. 4). Two, F1004 and E866, are common to both cyclic nucleotides and have been

Fig. 5. Analysis of purified proteins of PDE3A by SDS-PAGE gel. Recombinant PDE3A and mutant proteins were separated in 12% SDS-PAGE gel and proteins were detected by Coomassie blue stain.

Fig. 6. Analysis of the purified mutant proteins using Western blotting. Recombinant PDE3A and mutant proteins were resolved using 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membrane was probed with anti-platelet PDE3A antibody.

Table 1. Kinetic parameters of PDE3A mutants on cAMP

	K_m (μM)	k_{cat} (s^{-1}) $\times 100$	k_{cat}/K_m ($\mu\text{M}/\text{s}^{-1}$)	K_i cGMP (μM)
Recombinant				
PDE3A	0.21 ± 0.023	189.0 ± 23.6	8.58	0.76 ± 0.11
Y751A	0.53 ± 0.03	10.6 ± 1.56	0.20	6.71 ± 0.60
H840A	0.46 ± 0.03	0.68 ± 0.03	0.015	52.2 ± 8.6
T844A	0.35 ± 0.02	45.20 ± 1.58	1.29	0.46 ± 0.10
N845A	0.92 ± 0.26	146.0 ± 24.19	1.58	0.88 ± 0.062
L910A	0.50 ± 0.07	29.6 ± 2.65	0.593	1.72 ± 0.15
D950A	0.23 ± 0.01	1.81 ± 0.10	0.076	40.1 ± 2.26
F972A	0.65 ± 0.10	36.5 ± 3.15	0.562	1.67 ± 0.07
Q975A	0.24 ± 0.05	21.1 ± 2.20	0.878	0.24 ± 0.02
F1004A	272 ± 0.61	8.68 ± 4.26	0.031	>100

All determinations represent the mean $\pm$ SD of at least three separate experiments. The bold values of K_m and K_i are at least 3-fold elevated compared to recombinant PDE3A and the differences are statistically significant ($P < 0.001$) using Student's *t*-test.

discussed above. D950 is predicted to form at least one hydrogen bond with cGMP and thus helps to fix its position in the molecule. Y751 is close enough to participate in van der Waals-type interactions. The corresponding amino acid in PDE5A, Y603, is considered one of the main contributors to the binding of cGMP, the substrate of PDE5A (Turko et al. 1998). Although H836 and H840 are not within 4 Å of cGMP in its current orientation, a previous study identified two histidines protected by cGMP against interaction by DEP (Ghazaleh et al. 1996). By analogy to PDE4B, H836 is a ligand for Zn^{++} and thus may in PDE3A be closer to cGMP than currently predicted by this model. H840 is perfectly conserved in the 11 PDE families and may be involved in binding of the additional metal ion such as Mn^{++} necessary for catalysis but whose exact location is still unclear. It should be noted that these two histidines are among the most quantitatively perturbed by mutation as judged by the 176-fold and 69-fold increase in K_i for cGMP.

All of these assignments in PDE3A structure are based on the kinetic properties of the mutants, but the spatial properties are based on the molecular model. Because PDE4B does not bind cGMP in its active site, the exact position of this nucleotide is less certain than cAMP. The hypotheses regarding the interactions of cGMP particularly remain to be confirmed or denied when a three-dimensional structure is available for the PDE3A catalytic domain.

Materials and methods

Materials

^3H -cAMP was purchased from Amersham Pharmacia Biotech. Oligonucleotides were synthesized by Life Technologies, Inc. cAMP, cGMP, and other reagents used in this study were purchased from Sigma unless stated otherwise.

A

B

Fig. 7. Kinetic analysis of PDE3A and mutants. Double-reciprocal Lineweaver-Burk plots derived from kinetic curves are shown. The assays were performed as described under "Experimental Procedures". A plot of a typical measurement out of three determinations is depicted for each of three mutants. Panel A shows the K_m values of recombinant PDE3A, N845A, and F1004A were determined with Lineweaver-Burk plots. Panel B shows the K_i of cGMP for mutant Y751A was calculated in presence of various concentrations of cGMP.

Expression of PDE3A mutants

Expression of PDE3A mutants in a baculovirus system and preparation of Sf9 lysates were as described (Zhang and Colman 2000). Briefly, the vector pBS3031 was digested by XhoI and SacI. The DNA fragment coding for PDE3A was subcloned into baculovirus vector pBlueBacHis2B (Invitrogen) which has a hexahistidine-tag to generate pBBH3031. Sf9 cells were cotransfected with pBBH3031 and linearized AcMNPV DNA (Invitrogen) to produce recombinant viruses using lipofection as previously described (Zhang and Colman 2000). After 96 h infection by the recombinant virus, the cells were collected and sonicated in a lysis buffer (50 mmole/L Tris-HCl, pH 7.8, 10 mmole/L MgCl_2 with 0.5 $\mu\text{g}/\text{mL}$ pepstatin, 0.5 $\mu\text{g}/\text{mL}$ leupeptin, 2 $\mu\text{mole}/\text{L}$ benzamidine, 10 $\mu\text{g}/\text{mL}$ soybean trypsin inhibitor and 50 $\mu\text{mole}/\text{L}$ tosyl phenylalanyl chloromethylketone). Cell debris was removed by centrifugation at 15,000g for 30 min at 4°C. The supernatant was either stored at -80°C or further purified.

Construction of PD3A mutants

All PDE3A mutants were constructed using a QuikChange Kit (Stratagene) as described previously (Zhang and Colman 2000). Pairs of complementary oligonucleotide primers that contain desired mutants were synthesized as follows:

(1) Y751A, 5'-GGGATATTCCTGCTCATAACAGAATCCATGCC-3' and 5'-GGCATGGATTCTGTTATGAGCAGGAATATCCC-3';

(2) H840A, 5'-CCATGCACGATTATGATGCTCCAGGAAAGGAC and 5'-GTCCTTCCTGGAGCATCATAATCGTGCATGG-3';

(3) T844A, 5'-CATCCAGGAAGGGCTAATGCTTTCCTGG-3' and 5'-CCAGGAAAGCATTAGCCCTTCCTGGATG;

(4) N845A, CCAGGAAGGACTGCTGCTTTCCTGGTTGC-3' and 5'-GCAACCAGGAAAGCAGCAGTCCTTCCTGG;

(5) L910A, 5'-GGCCACTGACGCGAAGAAACACTTTGAC-3' and 5'-GTCAAAGTGTTCCTTCGCGTCAGTGGCC-3';

(6) D950A, 5'-GTTGGCTGCTATCAATGG TCCAGC-3' and 5'-GCTGGACCATTGATAGCAGCCAAC-3';

(7) F972A, 5'-GGTATTGTCAATGAAGCTTATGAACAGGG-3' and 5'-CCCTGTTTCATAAGCTTCATTGACAATACC-3';

(8) Q975A, 5'-GAATTTTATGAAGCGGGTGATGAAGAGGCC-3' and 5'-GGCCTCTTCATCACCCGCTTCATAAAATTC-3';

(9) F1004A, CAGGAATCCGCCATCTCTCACATTG-3' and 5'-CAATGTGAGAGATGGCGGATTCCTG-3'.

The altered bases are underlined. PBlueBacHis3031 plasmid DNA was employed as a template of PCR with *Pfu* DNA polymerase. The PCR products were treated with *Dpn* I in order to digest the parent double strand DNA chains. The top 10 *E. coli* competent cells were used for all transformations. Plasmid DNA was purified using a QIAGEN Inc. Plasmid Miniprep kit according to the manufacturer's protocol (QIAGEN). The sequences of mutants were confirmed by automated DNA sequencing.

Purification of PDE3A mutants and Western blot analysis

The histidine-tagged PDE3A proteins were purified on a Ni⁺⁺-NTA column as previously described (Zhang and Colman 2000). Proteins in Sf9 cells lysates were separated by SDS-PAGE gel electrophoresis in 10% gel and transferred to nitrocellulose membrane. The membrane was blocked in TNA buffer containing 5% nonfat dry milk and 0.05% Tween 20 for 1.5 h at room temperature, and then incubated with anti-platelet PDE3A polyclonal antibody (1:1000 dilution) in TNA buffer for another 1 h. After washing the membrane three times, the immunoreactivity was detected with horseradish-conjugated anti-rabbit IgG. Bands were visualized with substrate system (Bio-Rad) according to the manufacturer's protocol.

PDE activity assay

Enzymatic activity was measured in a total volume of 0.1 mL containing 50 mmole/L Tris-HCl pH 7.8, 10 mmole/L MgCl₂, and ³H-cAMP (40,000 cpm/assay) at 24°C for 30 min. The reactions were terminated by addition of 0.2 mL of 0.2 mole/L ZnSO₄ and 0.2 mL of 0.2 mole/L Ba(OH)₂. The samples were vortexed and centrifuged at 10,000g for 3 min. The labeled product of the reaction ³H-5' AMP was precipitated with BaSO₄, and the unreacted

³H-cAMP remained in the supernatant. Radioactivity in the supernatant was determined by liquid scintillation counter. K_m and k_{cat} were calculated by Lineweaver-Burk plot with various concentrations of cAMP by Microsoft Excel Program. The K_m, K_i, and k_{cat} values were expressed as mean ± SD for three independent measurements. It would have been desirable to determine K_m for cGMP hydrolysis. However, cGMP is primarily a competitive inhibitor. Since the k_{cat} is more than 10-fold less than cAMP, it is difficult to accurately measure the K_m. Therefore, we used the K_i as a measure of the affinity of PDE3A for cGMP.

Protein assay

Protein concentrations were measured using bicinchoninic acid Protein Assay Reagent Kit (Pierce Chemical Company) with bovine serum albumin as a standard.

Molecular modeling

Members of the phosphodiesterase protein families display primary sequence homology and ternary structure conservation for their catalytic domains. The crystal structure of the catalytic domain of PDE4B (Xu et al. 2000) was used as a template to build the model of a highly related protein, human platelet cGMP inhibited cAMP phosphodiesterase (PDE3A). The model was built manually by the systematic side chain replacement (Baglia et al. 1991) according to the sequence alignment shown in Fig. 1. The small (1–5 amino acids) amino acid insertions/deletions between PDE4B and PDE3A were manually realigned to accommodate the three-dimensional structure. After loop grafting, explicit hydrogens were added and the final structure was subjected to four cycles of dynamics (200 steps)/minimization (50 steps) followed by minimization to energy convergence. Minimization was done by the conjugate gradient method using Kollman All-Atom force field and Gasteiger-Huckle charges at the dielectric constant 4 and non-bonded cut-off 12Å. The cAMP model was built into the active site of PDE4 according to the electron density in the complex structure of PDE4-cAMP (Ke et al. unpubl.). Based on the model of cAMP, the binding of cGMP was manually built into the active site of PDE3A by using program O (Jones 1982).

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