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Characterization of Cyclic Nucleotide Phosphodiesterases with Cyclic AMP Analogs: Topology of the Catalytic Sites and Comparison with Other Cyclic AMP-Binding Proteins

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SUMMARY

To define essential interactions of cAMP with the catalytic sites of cyclic nucleotide phosphodiesterases (PDEs) and to begin to map the topology of the sites, we have tested a series of cAMP analogs as competitive inhibitors of the PDEs that hydrolyze cAMP with high efficiency (PDE1, PDE2, PDE3, and PDE4). Comparisons of IC_{50} values, relative to cAMP, were used to predict which functional groups on cAMP interact with each isozyme. Common to all PDEs tested, except for the calcium/calmodulin-dependent PDE (CaM-PDE, PDE1), is an interaction at the N1-position of cAMP and a distinct lack of binding to the 2′-hydroxyl group of the ribose moiety. Only the cGMP-stimulated (PDE2) and cAMP-specific (PDE4) PDEs appear to interact strongly at the N7-position. The cGMP-inhibited PDE (cGI-PDE, PDE3) may interact less strongly with this nitrogen. The PDE4 and PDE3 both interact with cAMP through the 6-amino group, which most likely serves as a hydrogen bond donor. PDE4 and PDE3 appear to be able to bind to the *anti*-conformer of cAMP, whereas the PDE1 and PDE2 bind the *syn*-conformer. The CaM-PDE exhibits no appreciable specificity for any of the analogs tested, showing little or no interaction with the 6-amino group or with any of the ring nitrogens. Large differences exist in the nucleotide-binding requirements for the PDE catalytic sites, compared with the regulatory sites of cAMP-dependent protein kinase and the catabolite activator protein.

The hydrolysis of cAMP and cGMP is catalyzed by multiple cyclic nucleotide PDEs (1, 2). Recent reports reveal the existence of at least seven different PDE gene families and >20 different isozymes (1). These include the CaM-PDE (PDE1), the cGS-PDE (PDE2), the cGI-PDE (PDE3), the cAMP-specific PDE (PDE4), the cGMP-specific PDE (PDE5), the cGMP-specific photoreceptor PDE (PDE6), and a recenty discovered high affinity cAMP-specific PDE (PDE7) (3). The nomenclature used in this manuscript includes both the recently approved revisions and the more traditional descriptive names (1). Five isozyme families are known to efficiently hydrolyze cAMP at physiological substrate concentrations, namely the CaM-PDEs, the cGS-PDEs, the cGI-PDEs, and the cAMP-specific PDEs. Kinetically, the cGS-PDEs and most CaM-PDEs exhibit a higher affinity for cGMP than for cAMP, whereas the cGI-PDEs display a high affinity for both cGMP and cAMP (4–6). The cAMP-specific PDEs are the most specific of any of these PDEs for cAMP but also hydrolyze cGMP, albeit with very low affinity (7).

The primary sequence for each of the PDEs tested in this study has been determined (8–12). Sequence alignments reveal that all PDEs possess a conserved region of approximately 270 amino acids near the carboxyl terminus, which is thought to contain the catalytic domain (13). It is likely that the conserved residues account for common functional properties of PDEs, whereas the remaining residues confer isozyme specificity. A comparison of PDE sequences with other cyclic nucleotide binding domains, including those of cAdPK, cGMP-dependent protein kinase, *Escherichia coli* CAP, and the cGMP-gated ion channel, reveals no significant extended structural relationships (13, 14).

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ABBREVIATIONS: PDE, phosphodiesterase; 8-Br-cAMP, 8-bromo-cAMP; 2′-butyryl-cAMP, 2′-monobutyryl-cAMP; cAdPK, cAMP-dependent protein kinase; CaM-PDE, calcium/calmodulin-dependent phosphodiesterase; CAP, catabolite activator protein; 6-Cl-cPuMP, 6-chloro-purine riboside 3′,5′-cyclic monophosphate; cGI-PDE, cGMP-inhibited phosphodiesterase; cGS-PDE, cGMP-stimulated phosphodiesterase; cPuMP, purine riboside 3′,5′-cyclic monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 1-CH_3O-cAMP, 1-methoxy-cAMP; MOPS, 3-(*N*-morpholino)propanesulfonic acid; N^6-butyryl-cAMP, N^6-monobutyryl-cAMP; 2-NH_2-cPuMP, 2-amino-purine riboside 3′,5′-cyclic monophosphate; pCPT, *para*-chlorophenylthio; Sf9, *Spodoptera frugiperda*.

Several studies have attempted to predict the binding interactions of cyclic nucleotides with their respective cyclic nucleotide binding sites (15–19). However, to date few studies have addressed the molecular interactions between cAMP and the various PDE catalytic domains (17, 20, 21). In addition, inconsistent assay conditions and methods of analysis have made it difficult to compare the data from different studies.

In the accompanying paper, we characterized the interaction of cGMP with five representative PDE catalytic sites by using cGMP analogs (22). The present study is the first to define and compare the essential interactions of cAMP with the residues of the catalytic sites of representative members of the four major isozyme families currently reported to hydrolyze cAMP. The enzymes tested in this study include a 59-kDa CaM-PDE from bovine heart and lung, a soluble cGS-PDE from bovine adrenal gland, a cGI-PDE from bovine heart, and a cAMP-specific PDE from rat sertoli cells [RNPDE4D1A (1)]. Unfortunately, to date the PDE7 isozyme has not been isolated or expressed in sufficient quantity to allow large-scale analog studies to be conducted. Data showing how PDE catalytic sites bind cAMP in a way that is unique with respect to other cyclic nucleotide-binding molecules are presented. In addition, findings are presented demonstrating that each isozyme family displays a unique profile of binding interactions and that both cAMP and cGMP interact with the PDE in specific and unique fashions. Knowledge of the binding interactions and the structures of these PDE catalytic sites is pertinent to the development of selective inhibitors for manipulating intracellular levels of cyclic nucleotides experimentally, as well as for possible therapeutic applications.

Experimental Procedures

Materials. [8-^{3}H]cAMP was obtained from NEN. cGMP, cAMP, cIMP, N^6-butyryl-cAMP, 2′-deoxy-cAMP, 2′-butyryl-cAMP, 8-Br-cAMP, 8-pCPT-cAMP, DEAE-Sephadex A-25, trypsin, soybean trypsin inhibitor, dicyclohexylcarbodiimide, and *Crotalus atrox* snake venom were purchased from Sigma. Rabbit anti-mouse immunoglobulin was obtained from Boehringer Mannheim. DE-52 cellulose was purchased from Whatman. Solid-phase immunoabsorbant was prepared from heat-inactivated, formalin-fixed cells of the Cowan I strain of *Staphylococcus aureus* as described in the companion paper (22). Ultima Gold scintillation fluid was purchased from Packard. Tritiated samples were counted in a Packard Tricarb scintillation counter. 1-CH_3O-cAMP, 6-Cl-cPuMP, and 7-deaza-cAMP were synthesized in the laboratory of Dr. B. Jastorff, according to the method of Genieser *et al.* (23). All nucleotide analogs were examined by reverse phase high performance liquid chromatography before assay, to assess purity. All chromatograms showed single peaks. Examination on a more sensitive scale revealed <0.1% contamination by cAMP (the limit of detection). The expression vector (pCMVPDE3) for a cAMP-specific PDE (PDE4D1A) (1) was obtained from Dr. M. Conti (Stanford University) (24, 25).

Preparation of PDEs. cGI-PDE and cGS-PDE were purified by immunoprecipitation, as described in the accompanying study (22). Three different types of CaM-PDE preparations were used. In initial studies, CaM-PDE isolated from bovine heart extracts as a *S. aureus*/antibody immunoprecipitate (28) was used. In most studies a recombinant CaM-PDE was purified (26) from extracts of Sf9 insect cells that had been infected with a baculovirus construct containing a full length cDNA for the 59-kDa bovine lung enzyme.[2] The expressed protein was >90% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Initial studies comparing the immunoprecipitated heart preparation with the recombinant CaM-PDEs gave substantially different IC_{50} values for cGMP (see Fig. 2). These results suggested that there might be two or more different kinetic forms of PDE present in the heart preparation, one of which had higher affinity for cGMP. Therefore, for nearly all of the studies, except those in Fig. 2, the recombinant 59-kDa CaM-PDE (PDE1A1) was used. In a few studies, a 61-kDa CaM-PDE (PDE1A2) was tested to determine whether amino acids outside of the catalytic domain might effect specificity. As expected, both recombinant isozymes gave essentially identical results with all analogs. This was expected, because these two splice variants have identical catalytic domains. The recombinant cAMP-specific PDE (PDE4D1A) used in these studies was transiently expressed in COS-7 cells. COS-7 cells were maintained at 37° in Dulbecco's modified Eagle's medium supplemented with 10% Nuserum, in a water-saturated 7% CO_2 atmosphere. Transfection of plasmid DNA into COS-7 cells was performed by the method of electroporation (27). COS-7 cells expressing rat PDE3.1 (pCMVPDE3) were harvested on day 2 after transfection. Medium was removed from each plate of transfected cells by aspiration. The cells were rinsed twice with phosphate-buffered saline and resuspended in buffer containing 40 mM Tris, pH 7.5, 5 mM EDTA, 15 mM benzamidine, 15 mM β-mercaptoethanol, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin (1 ml of buffer/100-mm plate). The cells were lysed with 30 strokes in a Dounce homogenizer. The whole-cell extract was used in the PDE assay.

Each of the PDEs used in this study was assayed in such a way as to minimize influences from noncatalytic sites. The cGI-PDE is competitively inhibited by cGMP (28). From sequence analysis, it was determined that no recognizable noncatalytic cyclic nucleotide binding domains exist in the cGI-PDE sequence (12). Therefore, the cGI-PDE was assayed directly, without modification. Several PDEs exist in active and inactive states. The CaM-PDE was assayed in its fully activated state by the addition of calcium and calmodulin to the assay buffer. The cGS-PDE was activated by covalent modification with dicyclohexylcarbodiimide (22, 29).

PDE assays. cAMP hydrolysis in the presence of analogs was measured at 30° using the standard radioligand assay (4). Assay tubes contained 40 mM MOPS, pH 7.5, 0.8 mM EGTA, 15 mM magnesium acetate, 0.2 mg/ml bovine serum albumin, and the concentrations of cAMP and cyclic nucleotide analog indicated. For CaM-PDE, the assay tubes contained 20 mM Tris, pH 7.5, 20 mM imidazole, 3 mM $MgCl_2$, 15 mM magnesium acetate, 200 μM $CaCl_2$, 4 μg/ml calmodulin, and the concentrations of cAMP and cyclic nucleotide derivative indicated. Assays were terminated by boiling for 1 min and were then treated with *C. atrox* snake venom for 5 min at 30°. Nonhydrolyzed cAMP was removed by chromatography using DEAE-Sephadex A-25 eluted with 20 mM Tris·HCl, pH 7.5. All assay points are the averages of duplicate values. For each isozyme tested, the substrate level in the assay was one third of the K_m or less. By using low substrate concentrations, the IC_{50} values determined approach the K_i. CaM-PDE was assayed with 1 μM cAMP, cGI-PDE was assayed with 0.1 μM cAMP, cGS-PDE was assayed with 10 μM cAMP, and cAMP-specific PDE was assayed with 0.5 μM cAMP. Less than 30% of the cAMP was hydrolyzed during the assays. Because the assays were linear with time, it was assumed that little hydrolysis of the analogs occurred during the assay and therefore that IC_{50} values determined were not artifactually overestimated. In addition, with most of the key compounds, i.e., cPuMP, 2-NH_2-cPuMP, cIMP, cGMP, and 8-Br-cAMP, thin layer chromatography (see accompanying paper for details) was carried out on reaction mixtures to confirm that these compounds were not better substrates than cAMP itself.

Rationale for selection of cAMP derivatives. To map the essential molecular interactions between cAMP and the catalytic site of several different PDE families, a series of 11 systematically modified cAMP analogs were selected. Purine cyclic monophosphates are found to have two conformational minima, *syn* and *anti*. cGMP has a lower energy in the *syn*-conformation and therefore the population is skewed

[2] W. T. Sonnenburg, D. Seger, and J. A. Beavo, unpublished observations.

toward the *syn*-conformer, whereas cAMP, cIMP, and cPuMP prefer the *anti*-conformation (30). In general, the introduction of bulky substituents in the 8-position shifts the population toward the *syn*-conformation, due to steric hindrance between the purine and ribose moieties (31–33). 8-Br-cAMP and 8-pCPT-cAMP were chosen to investigate steric and bulky group influences at the 8-position, as well as conformational preference. Although cAMP itself has no functional group at the 2-position, the analog pair of cPuMP and 2-NH_2-cPuMP were used in combination with the 8-position analogs to assess the preference of the various PDEs for the *syn*- or *anti*-conformer. cIMP, 6-Cl-cPuMP, N^6-butyryl-cAMP, and cPuMP were selected to provide information about the presence of interactions at the 6-position. 7-Deaza-cAMP was chosen to determine whether a binding interaction occurs at the N7-position. Limited amounts of analog were available, so only three PDEs were tested with this derivative. 1-CH_3O-cAMP was selected to investigate possible interactions at the N1-position. 2′-Deoxy-cAMP and 2′-butyryl-cAMP were included to test for interactions between the enzyme and the 2′-hydroxyl group of the ribose. All chemical structures are shown in Fig. 1.

Results

IC_{50} determinations. Dose-response curves over a wide range of concentrations were obtained for all of the isozymes, with at least two different preparations of enzyme. In all cases except one, reproducible Michaelis-Menten kinetics were observed. However, with ACC-1 antibody-purified CaM-PDE obtained from bovine heart, great variation was seen between preparations. Moreover, the IC_{50} curve for cGMP obtained with the immunoprecipitated heart CaM-PDE was always much lower than the curve obtained with recombinant 59-kDa CaM-PDE (Fig. 2). This suggested that different bovine heart CaM-PDE preparations might contain varying amounts of two or more CaM-PDEs having different affinities for cGMP. Therefore, nearly all of the analogs were tested using recombinant CaM-PDE (PDE1A) expressed in baculovirus-infected Sf9 cells. With this enzyme, IC_{50} curves typical of a single, kinetically pure enzyme were obtained (Fig. 2). Unfortunately, not enough of four analogs, i.e., 7-deaza-cAMP, 1-CH_3O-cAMP, 2-NH_2-cAMP, and 6-Cl-cPuMP, was available to obtain full curves for the recombinant enzyme. A representative experiment with two of the more important analogs, 7-deaza- and 1-CH_3O-cAMP, is shown in Fig. 2a. Neither analog gave substantially different curves, compared with cAMP itself.

IC_{50} values based on full dose-response curves for 13 cyclic nucleotides with four different PDE isozymes were determined at a substrate level of one third the K_m or lower. Therefore, the IC_{50} values approach the inhibition constant, K_i, and the dissociation constant, K_d. The IC_{50} values are reported in Table 1 as the mean of three to nine determinations. To facilitate comparisons between PDE isozymes, relative IC_{50} values (IC_{50}') were calculated. The relative IC_{50} is the $IC_{50(analog)}/IC_{50(cAMP)}$. The use of relative IC_{50} values allows comparison of the affinity of a particular analog with the affinity of cAMP for each PDE isozyme. The relative IC_{50} values are presented in Table 2.

7-Position [7-deaza-cAMP (11)]. This substitution has only a small effect on the cGI-PDE. However, a 76-fold increase in IC_{50} was observed for the cAMP-specific PDE and a some-

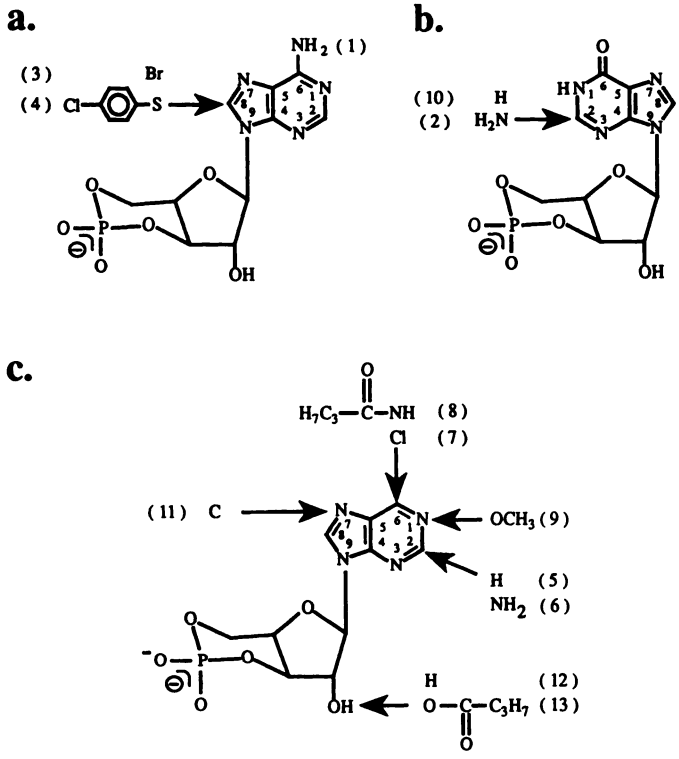

Fig. 1. Cyclic nucleotide structures. a, The reference compound is cAMP (**1**), shown here in its *anti*-conformation. Compound **3** is 8-Br-cAMP. Compound **4** is 8-pCPT-cAMP. b, The reference structure is cGMP (compound **2**). Compound **10** is cIMP. c, The reference structure is cPuMP (**5**). Compound **6** is 2-NH_2-cPuMP. Compound **7** is 6-Cl-cPuMP. Compound **8** is N^6-butyryl-cAMP. Compound **9** is 1-CH_3O-cAMP. Compound **11** is 7-deaza-cAMP (the carbon replaces the nitrogen). Compound **12** is 2′-deoxy-cAMP. Compound **13** is 2′-butyryl-cAMP. In each case the group that is substituted to create the analog replaces the entire functional group indicated (*arrows*).

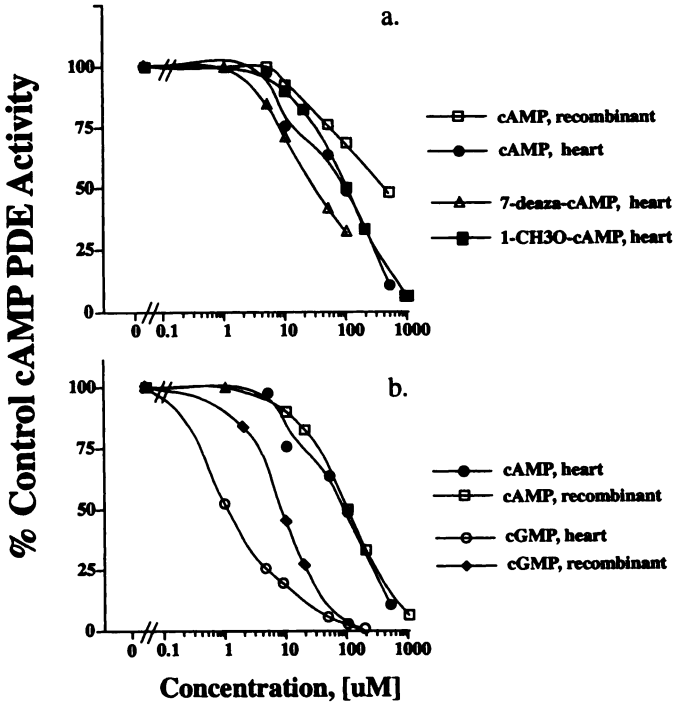

Fig. 2. Inhibition of different CaM-PDEs by cAMP, cGMP, 7-deaza-cAMP, and 1-CH_3O-cAMP. a, Effects of unlabeled cAMP analogs on a mixture of CaM-PDEs isolated from bovine heart by antibody precipitation or on a single recombinant 59-kDa CaM-PDE (PDE1A). b, Effects of unlabeled cAMP and cGMP on a mixture of CaM-PDEs isolated from bovine heart by antibody precipitation or on a single kinetic form of CaM-PDE isolated from Sf9 cells infected with a recombinant 59-kDa CaM-PDE.

TABLE 1

IC_{50} values

All IC_{50} curves consisted of six assay points (in duplicate), one with no analog present and five with increasing concentrations of analog. The IC_{50} values are the mean of two to nine determinations. *n*, The exact number of determinations made using a particular analog >, The highest concentration used in the assay is shown and little or no inhibition was observed at that concentration of analog. ~, In some cases, even though 50% inhibition was not reached, an estimate could be made. Under the assay conditions described, the IC_{50} approaches the K_i because the substrate concentration is significantly less than the K_m. In each case the compound number noted matched the compound number in Fig. 1. Values are mean ± standard deviation for $n > 3$, or mean ± range for $n = 2$.

Analog	CaM-PDE		cGS-PDE		cGI-PDE		cAMP-specific PDE	
	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*
	μM		μM		μM		μM	
No substitution								
1 cAMP	95 ± 40	4	36 ± 2.9	6	0.56 ± 0.19	9	1.9 ± 0.4	7
2 cGMP	8.3 ± 3.5	3	46 ± 11	4	0.13 ± 0.03	4	>200	3
Imidazole ring substitutions								
3 8-Br-cAMP	120 ± 16	3	150 ± 41	4	2.5 ± 1.0	4	25 ± 8.7	3
4 8-pCPT-cAMP	48 ± 7.6	3	98 ± 24	4	6.7 ± 1.2	3	16 ± 4.8	4
Pyrimidine ring substitutions								
5 cPuMP	120 ± 23	3	65 ± 8.7	3	4.9 ± 1.8	3	>200	3
6 2-NH_2-cPuMP	ND[a]		60 ± 23	5	20 ± 2.9	5	>200	3
7 6-Cl-cPuMP	ND		45 ± 8.7	3	3.7 ± 0.76	3	140 ± 41	3
8 N^6-Butyryl-cAMP	530 ± 58	3	550 ± 200	3	22 ± 13	4	44 ± 14	4
9 1-CH_3O-cAMP	ND		>500	2	3.8 ± 1.3	3	23 ± 2.9	3
10 cIMP	7.0 ± 3.0 ND	3	35 ± 13	3	1.0 ± 0.19	4	>200	3
11 7-Deaza-cAMP			400 ± 150	3	2.6 ± 1.3	4	140 ± 8.7	3
2′-Ribose subsitutions								
12 2′-Deoxy-cAMP	200 ± 50	3	57 ± 5.8	3	1.0 ± 0.46	5	5.7 ± 0.6	3
13 2′-Butyryl-cAMP	120 ± 30	3	9.3 ± 1.2	3	0.45 ± 0.14	4	3.0 ± 0	3

[a] ND, not determined.

TABLE 2

Relative IC_{50} values

For each isozyme the IC_{50} values are normalized to the IC_{50} for cAMP for that particular isozyme to facilitate comparison between the various isozymes. The relative IC_{50} value (IC_{50}') is $IC_{50\,(analog)}/IC_{50\,(cAMP)}$ for each isozyme. The data used to determine the relative IC_{50} values are reported in Table 1. In each case the compound number noted matches the compound number in Fig. 1.

Analog	IC_{50}'			
	CaM-PDE	cGS-PDE	cGI-PDE	cAMP-specific PDE
No subsitution				
1 cAMP	1.0	1.0	1.0	1.0
2 cGMP	0.09	1.3	0.23	>100
Imidazole ring substitutions				
3 8-Br-cAMP	1.2	4.2	4.4	13
4 8-pCPT-cAMP	0.51	2.7	12	8.2
Pyrimidine ring substitutions				
5 cPuMP	1.2	1.8	8.8	>100
6 2-NH_2-cPuMP	ND[a]	1.7	36	>100
7 6-Cl-cPuMP	ND	1.2	6.6	71
8 N^6-Butyryl-cAMP	5.6	15	40	23
9 1-CH_3 O-cAMP	ND	>14	6.8	12
10 cIMP	0.07	0.97	1.8	>100
11 7-Deaza-cAMP	ND	11	6.5	76
2′ ribose subsitutions				
12 2′-Deoxy-cAMP	2.1	1.6	1.8	3.0
13 2′-Butyryl-cAMP	1.3	0.25	0.80	1.6

[a] ND, not determined.

what smaller (11-fold) increase in IC_{50} was seen for the cGS-PDE. As mentioned above, in initial experiments (Fig. 2a) using a mixture of CaM-PDEs isolated from bovine heart by ACC-1 immunoprecipitation (26), the IC_{50} curve obtained for 7-deaza-cAMP was not shifted substantially to the right at any analog concentrations and, if anything, was shifted toward lower values. This suggests that the CaM-PDEs present in this preparation do not interact with the 7-position. Unfortunately, not enough derivative was available to allow very high concentrations to be used or to allow additional experiments to be carried out with the kinetically pure recombinant CaM-PDE used in the rest of the studies.

6-Position [cPuMP (5), 6-Cl-cPuMP (7), cIMP (10), and N^6-butyryl-cAMP (8)]. Removal of the 2-NH_2 group to form cPuMP does not cause an increase in the IC_{50} for either the CaM-PDE or the cGS-PDE. However, disruption of this position by substitution of a 6-chloro group or addition of a 6-butyryl group results in a dramatically increased IC_{50} for the cAMP-specific PDE, suggesting the presence of a hydrogen bond between the 6-NH_2 group of cAMP and the cAMP-specific isozyme. For cPuMP and 6-Cl-cPuMP, a relatively smaller effect is observed with the cGI-PDE, indicating a possible weaker interaction of the 6-NH_2 group of cAMP with this isozyme. cIMP has an affinity similar to that of cAMP for the

cGS- and cGI-PDEs. It has an even higher apparent affinity, compared with cAMP, for the CaM-PDE, much more like that of cGMP. This might be expected, because this PDE is able to hydrolyze cGMP and utilizes the 6-oxo group as part of its binding motif but does not utilize the 6-amino group. Substitution of a carbonyl oxygen for the amino group, however, cannot be accommodated by the cAMP-specific PDE.

8-Position [8-Br-cAMP (3) and 8-pCPT-cAMP (4)]. Neither 8-bromo nor 8-pCPT substitutions increase the IC_{50} for the CaM-PDE; however, both cause an 8–13-fold increase in the IC_{50} for the cAMP-specific PDE. Little or no increase is seen for the cGS- and cGI-PDEs with the 8-bromo substitution. However, a 12-fold increase is seen for the 8-pCPT substitution with the cGI-PDE.

1-Position [1-CH_3O-cAMP (9)]. The increase in the IC_{50} values for the cGS-PDE and cAMP-specific PDE suggest an interaction with cAMP at this position for each isozyme. These may be hydrogen bonds; however, additional effects due to steric hindrance cannot be excluded. Unfortunately, not enough analog was available to obtain complete dose-response curves for the recombinant CaM-PDE. However, as can be seen in Fig. 2a, using an immunoprecipitated CaM-PDE preparation from bovine heart this curve is not shifted substantially to the right, suggesting little or no interaction at the 1-position for either of the PDEs present in this preparation.

2-Position [2-NH_2-cPuMP (6) and cPuMP (5)]. Large increases in IC_{50} values were observed with cPuMP and 2-NH_2-cPuMP, compared with cAMP, for the cGI-PDE and cAMP-specific-PDE. No effect of these changes was seen with the CaM- and cGS-PDEs.

2′-Position [2′-deoxy-cAMP (12) and 2′-butyryl-cAMP (13)]. Changes at the 2′-position that either eliminate a hydrogen-bonding possibility or introduce steric hindrance have only negligible effects on the IC_{50} values of all PDEs tested. In the case of cGS-PDE, the butyryl group decreases the IC_{50}, perhaps due to a hydrophobic interaction with a hydrophobic region near, but not contained within, the catalytic site.

Discussion

Rationale. The differences in IC_{50} values among the cAMP analogs with the four PDE isozymes clearly demonstrate that the binding motifs of the isozymes are not identical to each other. They are also not identical for cAMP and cGMP. For three of the four PDEs, cAMP is bound to the catalytic site by several distinct interactions with the purine moiety. The isozyme-specific interactions presumably contribute to the differing specificities and kinetic properties observed for the various PDE isozymes. Based on the study presented in the accompanying manuscript, using cGMP analogs (22), it was expected that cAMP would interact with residues of the catalytic site, at least in part, through hydrogen bonds. Therefore, several of the analogs used in this study were chosen because they could act to disrupt a hydrogen-bonding possibility. Because a hydrogen bond generally provides 1–3 kcal/mol, an increase in the IC_{50} of 10-fold or more is considered to be consistent with the occurrence of a hydrogen bond at that position.[3] Smaller changes in the IC_{50} values are more difficult to interpret but may indicate weaker hydrogen bonds. Increases in IC_{50}' values of 100-fold or more seem likely to be due to a combination of several effects.

7-Position interactions [7-deaza-cAMP (11)]. The substitution of a carbon atom for the 7-position nitrogen eliminates hydrogen-bonding possibilities at this point. This substitution resulted in large differential effects with one of the PDE isozymes surveyed, the cAMP-specific PDE. The high IC_{50}' values for this analog suggest that a hydrogen bond occurs with the cAMP-specific PDE at the N7-position, with the nitrogen acting as an acceptor. The lower IC_{50} values for cGS-PDE and perhaps also the cGI-PDE suggest a weaker interaction between the N7-nitrogen of cAMP and these isozymes. The data in Fig. 2a indicate that the curves for 7-deaza-cAMP are, if anything, shifted to the left, compared with those for cAMP. This suggests that neither of the CaM-PDEs present in this preparation used an interaction at the 7-position. It should be noted that a cDNA clone from human heart for a CaM-PDE reported to have a higher affinity for substrate has recently been described (34). It seems likely that this isozyme may be present in the ACC-1-precipitated CaM-PDE preparation used in the experiments reported in Fig. 2b and may be the cause of the relatively high affinity for cGMP.

6-Position interactions [cPuMP (5), 2-NH_2-cPuMP (6), 6-Cl-cPuMP (7), cIMP (10), and cGMP (2)]. In the accompanying manuscript, it was observed that the cGMP-hydrolyzing PDEs required a hydrogen bond acceptor functionality at the 6-position of cGMP. In the present study with cAMP, removal of the 6-NH_2 group to give cPuMP or replacement of the group with a chlorine atom to give 6-Cl-cPuMP eliminates the possibility of hydrogen bond formation at this position. The complete lack of effect of removal of the 6-amino group on the relative IC_{50} values for the CaM- and cGS-PDEs clearly indicates that these two isozymes do not have positive interactions with cAMP at the 6-position. Conversly, the large effect of substitution or removal of the 6-amino group on the relative IC_{50} value for the cAMP-specific PDE suggests that this enzyme does utilize this group as a major part of its binding specificity. Because the cAMP-specific PDE does not tolerate either cIMP or cGMP, the amino group is likely to serve as a hydrogen donor. This requirement for a 6-amino group is apparently the determining factor of cAMP specificity for this isozyme.

Conversly, the smaller but reproducible effect of alteration (e.g., 7-fold for 6-Cl-cPuMP) or removal (e.g., 9-fold for cPuMP) of the 6-amino group on the relative IC_{50} for cGI-PDE points to a weak interaction of this group with the cGI-PDE isozyme. This enzyme is unique, in that it binds, with high affinity, analogs having either a carbonyl (e.g., cGMP or cIMP) or amino (e.g., cAMP, 7-deaza-cAMP, or 2′-deoxy-cAMP) functionality at the 6-position. Several possibilities are consistent with these data. The most obvious is that the binding site is open in this region and that this position plays little direct role in the binding affinity for cAMP. However, the relatively high affinity of this isozyme for both cAMP and cGMP makes this less likely. A second possibility is that there may be a relatively weak hydrogen bond that can form with either the oxygen of cGMP as an acceptor or the nitrogen of cAMP as a donor. The relatively small effect of complete removal of this group (e.g., cPuMP) suggests that the quantitative importance

[3] The free energy change due to a particular substitution can be estimated using the following mathematical relationship, in which ΔG is the change in free energy: $\Delta G = -RT\ln[K_{d(\text{analog})}/K_{d(\text{cAMP})}]$. R, the ideal gas constant, is equal to 1.98×10^{-3} kcal/deg/mol and T, the temperature at which the assay took place, is equal to 303°K (39). Therfore, an increase in the IC_{50} value of 10-fold results in a ΔG of approximately 1.4 kcal/mol.

of any interaction at this site for cAMP binding to the cGI-PDE is relatively small.

An N^6-butyryl substitution significantly increases the relative IC_{50} for all of the PDEs, with the possible exception of the CaM-PDE. The very large IC_{50} value for N^6-butyryl-cAMP with cGI-PDE may be due to the additive effects of steric hindrance and loss of the hydrogen bond. The cAMP-specific PDE and the cGS-PDEs are also unable to accommodate the bulky butyryl substitution. Notably, the CaM-PDE, with its very open binding site, tolerates the N^6-butyryl substituent significantly better than do the other PDEs.

8-Position and conformational effects. As discussed in the companion paper, substitutions of bromo or pCPT groups at the 8-position of cGMP are known to cause most of the analog to exist preferentially in the *syn*-conformation. Therefore, it is clear that enzymes such as the CaM- and cGS-PDEs, for which the 8-substitutions cause no increase in the relative IC_{50}, are likely to be able to bind cAMP in the *syn*-conformation (22). Both 8-Br cAMP and 8-pCPT cAMP are thought to be shifted more toward the *syn*-conformation than is cAMP itself, which prefers the *anti*-conformation. However, the degree to which a bulky substitution at the 8-position affects the *syn*/*anti* equilibrium is less well defined for cAMP than for cGMP. Because there is no possibility for additional stabilization of the *syn*-conformation by hydrogen bonding of the 2-NH_2 group to the axial oxygen of the cyclic phosphate ring, the preference for the *syn*-conformation is likely to be less for 8-Br-cAMP than for 8-Br-cGMP. To our knowledge, the absolute degree of this preference has not been determined (31). No data for the *syn*/*anti* population equilibrium are available for 8-pCPT-cAMP. Although the data strongly suggest that the 8-position analogs can bind in the *syn*-conformation, this does not prove that unmodified cAMP might not also be able to bind in its preferred *anti*-conformation, particularly because the architecture of the site apears to be relatively open.

Based on the increased IC_{50}' values observed for both of the 8-position analogs, the cAMP-specific PDE either prefers the *anti*-conformer or is sterically constrained in this region. Unfortunately, cPuMP and 2-NH_2-cPuMP do not bind well to the cAMP-specific PDE. Therefore, even though 2-NH_2-cPuMP prefers the *syn*-conformation, the extremely large IC_{50}' values (both >100) of these two derivatives for the cAMP-specific PDE do not allow a meaningful comparison to be made. Additional work is needed to unequivocally establish whether cAMP binds in the *anti*-conformation to the cAMP-specific PDE catalytic site.

The interpretation of the IC_{50} data for cGI-PDE is the most difficult. Although there is little effect of the 8-Br substitution, there is a 12-fold increase in the IC_{50}' value of 8-pCPT-cAMP for the cGI-PDE. Another potentially relevant piece of data presented here is the approximately 4-fold increase in the relative IC_{50} of 2-NH_2-cPuMP (compared with cPuMP) for the cGI-PDE isozyme. This analog also is thought to favor the *syn*-conformation, due to the intramolecular hydrogen bonding to the axial oxygen atom of the cyclic phosphate (30, 31). This increase in IC_{50}', like that seen for 8-pCPT-cAMP, would be consistent with a preference of cGI-PDE for the *anti*-conformation of cAMP, as appears to be the case with cGMP. However, the relative lack of effect of 8-bromo substitution of cAMP does not appear consistent with this interpretation. The increased IC_{50}' values seen for 8-pCPT-cAMP and 2-NH_2-cPuMP also could be due to steric considerations. Although we favor the interpretation of preference for the *anti*-conformer, as shown in Fig. 3, definitive evidence for both nucleotides with this isozyme must probably await the results of crystal structure determination. Finally, it should be mentioned that, because there are homologous internal repeat sequences (13) within the catalytic domain of the PDEs, it is not impossible to imagine that cAMP, cGMP, and different analogs might be able to bind in more than one conformation to some of the PDEs.

General steric effects. The insensitivity of the CaM- and cGS-PDEs to the 8-position substitutions suggests that these isozymes possess an open binding site, whereas the increase in relative IC_{50} values observed for the cGI-PDE and cAMP-specific PDE suggests that these isozymes have a more constrained catalytic site. The bulky butyryl substitution at the 6-position also serves to determine steric requirements. Here it was observed that the CaM-PDE also was best able to accommodate additional bulky groups at this position. Again, the cGI-PDE and cAMP-specific PDE appear to have the most constrained binding sites.

Sites on other cyclic nucleotide-binding proteins. In addition to acting as a substrate for PDEs, cAMP interacts at noncatalytic sites on several other proteins. The major intracellular receptor for cAMP in mammalian cells is the cAdPK. Analog studies with this enzyme reveal a binding motif distinct from that observed for PDEs (16, 35). cAMP is bound in the *syn*-conformation to both cAdPK binding sites, by stacking interactions between the adenine ring and aromatic amino acids in the protein. In contrast to the PDE catalytic site, no hydrogen bonds are formed between the base and the binding site. It was found that 8-substituted derivatives carrying a large hydrophobic group are well tolerated and that 7-deaza-cAMP is bound with affinity similar to that of cAMP. Positive binding interactions with the ribose moiety are observed. cAMP is bound by specific hydrogen bonds to the 5′- and 3′-oxygens and the 2′-hydroxyl group and by an ionic interaction with the exocyclic phosphate oxygens (36).

Another well characterized cAMP receptor protein, the CAP, stimulates transcription from several operons in *E. coli*. As in the case of cAdPK, cAMP has been found to be bound to CAP through interactions with the ribose moiety at the 2′-hydroxyl group, 3′-oxygen, 5′-oxygen, and charged phosphate group. In addition, CAP appears to interact with cAMP at the base moiety through a hydrogen-bonding interaction at the N6-position (37). An X-ray diffraction analysis of a CAP dimer with two molecules of cAMP bound reveals binding in the *anti*-conformation and distinct interactions with amino acid side chains (38), which is consistent with the molecular interactions predicted from the analog studies. Alignment of the CAP sequence with the four regulatory subunits of cAdPK reveals a striking homology (38). In fact, approximately 40% sequence identity is observed between the cAMP binding domain of CAP and those of the type I and type II regulatory subunits of cAdPK. Comparison of the PDE catalytic sites with the binding domains of cAdPK and CAP reveals no significant homology (13). This lack of similarity is consistent with the fact that large differences in the binding requirements are observed between either CAP or the cAdPKs and the PDE catalytic sites. Most striking is the fact that the cAMP regulatory sites in cAdPK and CAP tolerate no substitutions on the ribose moiety, whereas no essential binding interactions involve the

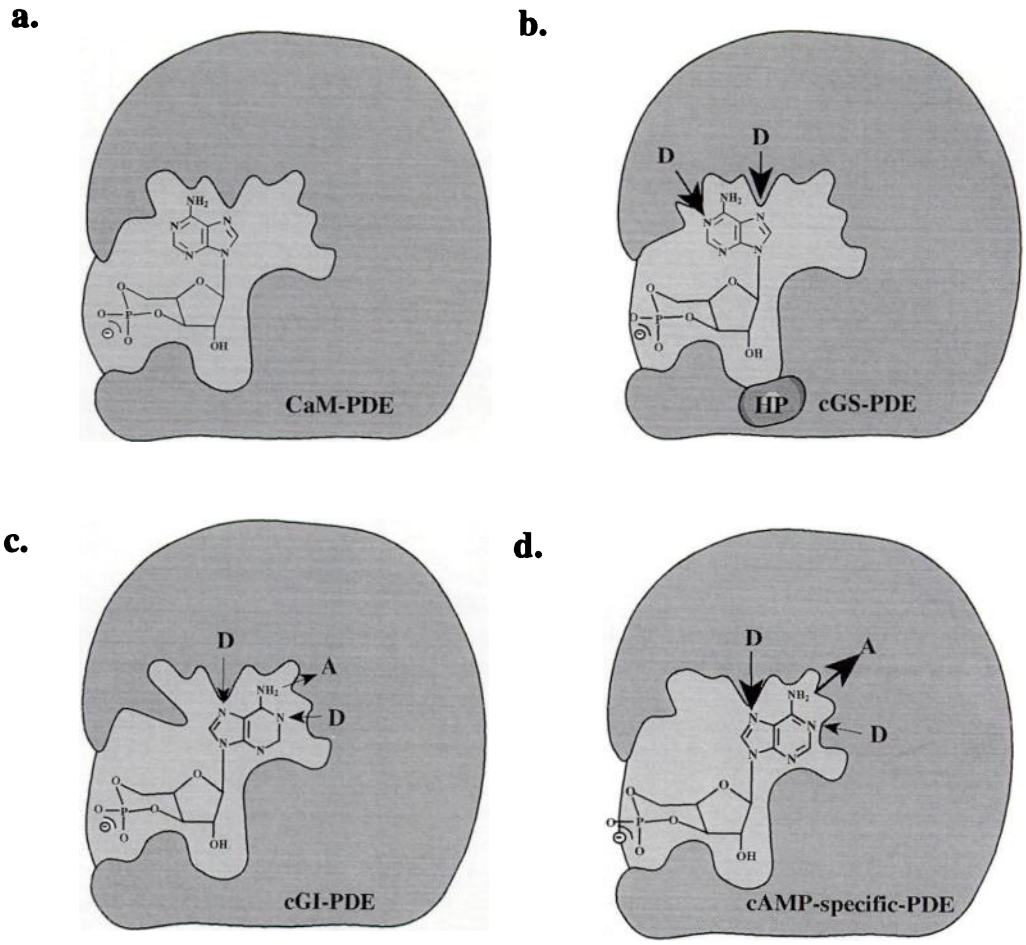

Fig. 3. Binding interactions of cAMP with the catalytic site of PDEs. Models of the binding of cAMP to the various catalytic domains are proposed based on the data described in Fig. 2 and Table 2. *Large arrows*, probable strong hydrogen-bonding interactions; *small arrows*, weaker interactions. *Direction of the arrows*, probable arrangement of the hydrogen bond, with the arrow pointing towards the hydrogen acceptor. *A*, hydrogen acceptor; *D*, hydrogen donor; *HP*, hydrophobic pocket. a, cAMP is proposed to bind to CaM-PDE in its *syn*-conformation, based largely on the fact that the 8-substituted analogs have IC_{50}' values near 1. Neither the 1- or 7-position nitrogens nor the 6-amino group of the adenine base of cAMP appears to have any appreciable interaction with the residues of the CaM-PDE, because all analogs have affinities similar to that of cAMP itself. The binding site is large enough to accomodate bulky 8- and 2′-position groups. The open architecture may also be able to accomodate the *anti*-conformer of cAMP. b, cAMP is proposed to bind to cGS-PDE in its *syn*-conformation. The adenine base of cAMP appears to interact with residues of the cGS-PDE catalytic domain through two distinct interactions, at the N1- and N7-positions. *Darker patch*, possible hydrophobic region that binds to the butyryl group of 2′-butyryl-cAMP. As with the CaM-PDE, the relatively open architecture may also be able to accomodate the *anti*-conformer of cAMP but may be a bit more constrained near the 6-NH_2 group. c, cAMP is proposed to bind to cGI-PDE in its *anti*-conformation. The adenine base of cAMP appears to interact with residues of the catalytic domain of the cGI-PDE through three distinct interactions, at the N1-, N7-, and 6-amino positions. The relatively high IC_{50}' value for 8-pCPT-cAMP suggests the presence of a negatively interacting steric or charged region near the 8-position. d, cAMP is proposed to bind to the cAMP-specific PDE in its *anti*-conformation. The adenine base of cAMP appears to interact with residues of the catalytic domain of the cAMP-specific PDE through three distinct interactions, at the N1-, N7-, and 6-amino positions. The major determinant of substrate specificity for this isozyme appears to be the 6-NH_2 group.

ribose moiety with the catalytic sites of PDEs. This may favor fast and efficient hydrolysis of the 3′-ester bond by the PDEs.

The binding motifs for cAMP proposed in Fig. 3 (and for cGMP in the accompanying manuscript) (22) are consistent with the hypothesis that there appears to be a core of general interactions common to all PDEs, in addition to multiple isozyme-specific interactions. The data presented in this study suggest that the binding of cAMP to the catalytic site of all PDEs except the CaM-PDE requires an interaction with the N1-position of the adenine ring. One important difference for all isozymes is the fact that, due to the presence of the 6-oxo group in cGMP, the N1-nitrogen probably acts as a hydrogen donor in the case of cGMP, whereas the 6-NH_2 group of cAMP makes it more likely that the N1-nitrogen is a hydrogen acceptor in the case of cAMP. If so, this in turn implies that cAMP and cGMP may not bind to the PDEs with their purine moieties in identical positions. At least this suggests that some of the PDEs may have different groups that can act as either hydrogen donors or acceptors in the region. It could also easily explain why cAMP and cGMP analogs with 1-substitutions behave differently with the CaM-PDE. Presumably only a hydrogen acceptor is present in this region for this PDE.

Additional isozyme-specific interactions include a hydrogen bond at the N7-position in the case of the cGS-PDE and cAMP-specific PDE and a hydrogen bond at the N6-position for the cGI-PDE and cAMP-specific-PDE, with a hydrogen donor functionality being required by the cAMP-specific PDE. A feature common to the CaM- and cGS-PDEs is that they appear

to have a relatively open catalytic site for both nucleotides, whereas the cGI-PDE appears to have a more constrained site. Also common to both the cAMP and cGMP binding motifs is an apparent conservation of the effect of bulky 8-position substitution on binding, i.e., the CaM- and cGS-PDEs effectively bind both cAMP and cGMP substituted at the 8-position, implying that these PDEs prefer the *syn*-conformation and that they are not sterically constrained in this region. Similarly, the cGI-PDE is greatly affected by this type of substitution, suggesting that it may bind both cAMP and cGMP in their *anti*-conformations or that it is highly constrained in this region.

All of the proposed binding motifs display a distinct lack of binding with the ribose moiety. A lack of binding with the ribose moiety has been noted previously for the cGS-PDE. These data serve to distinguish the PDE family from other cyclic nucleotide-binding molecules. Taken together, these data show that the binding interactions with cAMP are unique for the PDEs, compared with other cAMP-binding proteins, and that they are also different for different PDE family members.

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