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Characterization of Cyclic Nucleotide Phosphodiesterases with Cyclic GMP Analogs: Topology of the Catalytic Domains

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SUMMARY

To help define essential interactions of cGMP with the catalytic site, we tested a series of cGMP analogs as competitive inhibitors of each cyclic nucleotide phosphodiesterase (PDE) family known to hydrolyze cGMP (PDE1, PDE2, PDE3, PDE5, and PDE6). IC_{50} values, relative to cGMP, were used to predict which functional groups of cGMP contribute to binding by the catalytic sites of each isozyme. The results indicate that the N1-nitrogen of cGMP contributes to binding at the catalytic site of all PDEs, probably as a hydrogen donor. All PDEs tested, with the exception of PDE2, also use the 6-oxo group, probably as a hydrogen acceptor. In contrast to other cGMP-binding enzymes, the 2-amino and 2′-hydroxyl groups of cGMP are not major requirements for binding to any PDE. The 8-bromo- and 8-*p*-chlorophenylthio-substituted analogs inhibit PDE1, PDE2, and PDE6 activity with high relative affinities, suggesting that these PDEs are not sterically hindered with bulky 8-position substitutions and that they do not preferentially bind the *anti*-conformation of cGMP. PDE3 and PDE5 have reduced apparent affinity for these analogs and therefore either are sterically hindered with these substitutions or bind cGMP in the *anti*-conformation. Overall, the data show substantial differences in structural requirements for cGMP binding to the catalytic sites of the different PDE families. Comparisons with published data show different structural requirements for binding to the catalytic, compared with noncatalytic, binding domains of PDEs. Even larger differences are seen between the requirements for binding to PDE catalytic sites and those for the cGMP-dependent protein kinase and the cGMP-gated cation channel.

Cyclic nucleotide PDEs constitute a complex family of enzymes that are differentially expressed in mammalian cells and vary widely in their substrate affinities and modes of regulation. Recent sequence analysis suggests that at least seven different families containing >20 distinct isozymes exist (1, 2). These include CaM-PDE (PDE1), cGS-PDE (PDE2), cGI-PDE (PDE3), cAMP-specific PDE (PDE4), cGB-PDE (PDE5), photoreceptor PDE (PDE6), and the recently identified cAMP-specific HCP1 (PDE7) families. Most families contain multiple members. All but the cAMP-specific PDEs are able to efficiently catalyze the hydrolysis of cGMP at micromolar substrate concentrations.

A substantial amount of primary sequence information for each of the PDE families has been reported. Alignment of these sequences reveals a region of approximately 270 amino acids near the carboxyl terminus that is conserved among all mammalian PDEs and contains the catalytic domain (3, 4). This catalytic domain is highly conserved within each PDE family. Greater than 60% sequence identity is observed among isozymes of the same family. In contrast, <35% sequence identity is observed between isozymes of different families. It seems likely that many of the conserved amino acids play a role in binding or catalysis, whereas the nonhomologous sequences serve isozyme-specific functions. The conserved domain serves as a distinguishing feature of the PDEs, because no significant homology is found between this region and any other protein in the Protein Identification Resource sequence database (3). The specific amino acids involved in catalysis and the precise location of the individual catalytic domains remain to be determined.

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ABBREVIATIONS: PDE, phosphodiesterase; 8-Br-cGMP, 8-bromo-cGMP; 2′-butyryl-cGMP, 2′-monobutyryl-cGMP; CaM-PDE, calcium/calmodulin-dependent phosphodiesterase; cGB-PDE, cGMP-binding phosphodiesterase; cGI-PDE, cGMP-inhibited phosphodiesterase; cGS-PDE, cGMP-stimulated phosphodiesterase; 1-CH_3-cGMP, 1-methyl-cGMP; cPuMP, purine riboside 3′,5′-cyclic monophosphate; DCCD, dicyclohexylcarbodiimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; N^2-butyryl-cGMP, N^2-monobutyryl-cGMP; 3′-NH-cGMP, 3′-amino-cGMP; 2-NH_2-cPuMP, 2-amino-purine riboside 3′,5′-cyclic monophosphate; 8-OH-cGMP, 8-hydroxyl-cGMP; pCPT, *para*-chlorophenylthio; ROS-PDE, rod outer segment phosphodiesterase; 6-SH-cGMP, 6-thio-cGMP.

The diversity of the PDEs is emphasized by the wide range of affinities for cAMP and cGMP found for the various PDE families, the observation that nonspecific PDE inhibitors, such as the alkylxanthines, are not equipotent for all PDE isozymes, and the fact that specific inhibitors for most of the isozymes have been identified (5). However, no structural data accounting for the general or isozyme-specific features of the PDE catalytic domain have been reported. A few modeling studies, based largely on inhibitor affinities, provide some information about possible structural features of the catalytic site (6, 7). Early evidence that PDE isozymes interact with the cyclic nucleotide substrate in an isozyme-specific fashion was suggested by data generated using cyclic nucleotide analogs (8, 9) Although only a few isozymes were tested in those early studies, the data suggested that cyclic nucleotides interact with PDE catalytic domains through isozyme-specific binding interactions.

Cyclic nucleotide analogs have proven to be a useful tool for determining which functional groups on the cyclic nucleotide molecule interact with the various cyclic nucleotide receptors (10). This information in turn helps to define the structure of the binding sites on the enzyme. In addition, several of the analogs also have proven to be effective agents in mimicking the effects of cGMP in intact cells. However, to interpret these types of studies, the specificity for binding to each of the possible "receptors" for cGMP in the cell must first be established. Nearly all of the points of interaction between cGMP and the cGMP-dependent protein kinases have been established by using an analog approach (11). Several studies on the noncatalytic, high affinity binding site of the cGS-PDE and cGB-PDE have yielded similar information (9, 12). Those studies that have attempted to address the binding interactions at the catalytic site have largely focused on contrasting the catalytic sites of cGB-PDE and cGS-PDE with their respective noncatalytic sites (9, 12). The few studies that have attempted to contrast different PDE catalytic sites have been hampered by the fact that impure PDE preparations were used or that only cAMP hydrolysis was examined (8, 13). Direct comparative data aimed at identifying general catalytic site binding interactions and isozyme-specific interactions have been lacking before this study.

This study includes a representative isozyme from each of the five families known to readily hydrolyze cGMP. Specifically, the isozymes tested include the 59-kDa CaM-PDE from bovine heart and lung (PDE1A), the soluble cGS-PDE from bovine adrenal gland (PDE2A), the soluble cGI-PDE from bovine heart (PDE3A), the soluble cGB-PDE from bovine lung (cGB-PDE or PDE5A), and the soluble ROS-PDE from bovine retina (PDE6A/B). The nomenclature used here follows the recently standardized format (2). This is the first study to test the same derivatives with representatives of all relevant isozyme families in a systematic way, using comparable methods of assay and analysis. The data indicate that each isozyme family displays a unique profile of interactions. In addition, PDE catalytic sites interact with cGMP in a way that is unique, compared with other cyclic nucleotide-binding molecules.

Experimental Procedures

Materials. [8-^{3}H]cGMP (54 Ci/mmol) was obtained from New England Nuclear. cGMP, cAMP, cCMP, cTMP, cUMP, cIMP, N^2-butyryl-cGMP, 2′-deoxy-cGMP, 2′-butyryl-cGMP, 8-Br-cGMP (Fig. 1), DEAE-Sephadex A-25, trypsin, soybean trypsin inhibitor, DCCD, and *Crotalus atrox* snake venom were purchased from Sigma Chemical Co. Rabbit anti-mouse immunoglobulin was purchased from Boehringer Mannheim. DE-52 cellulose was purchased from Whatman. Heat-inactivated, formalin-fixed cells of the Cowan I strain of *Staphylococcus aureus* were used for immunoprecipitation experiments. Ultima Gold scintillation fluid was purchased from Packard. Tritiated samples were counted in a Packard Tricarb scintillation counter. 8-pCPT-cGMP was obtained from Biolog Life Science Institute (Bremen, Germany). 6-SH-cGMP, cPuMP, 2-NH_2-cPuMP, 8-OH-cGMP, and 3′-NH-cGMP were synthesized in the laboratory of Dr. Bernd Jastorff (University of Bremen, Bremen, Germany). 1-CH_3-cGMP was a generous gift from the laboratory of Dr. Jackie Corbin (Vanderbilt University). All nucleotide analogs were examined by reverse phase high performance liquid chromatography before assay, to assess purity. All chromatograms showed single peaks. Examination of each analog on a more sensitive scale revealed <0.1% contamination by cGMP (the limit of detection).

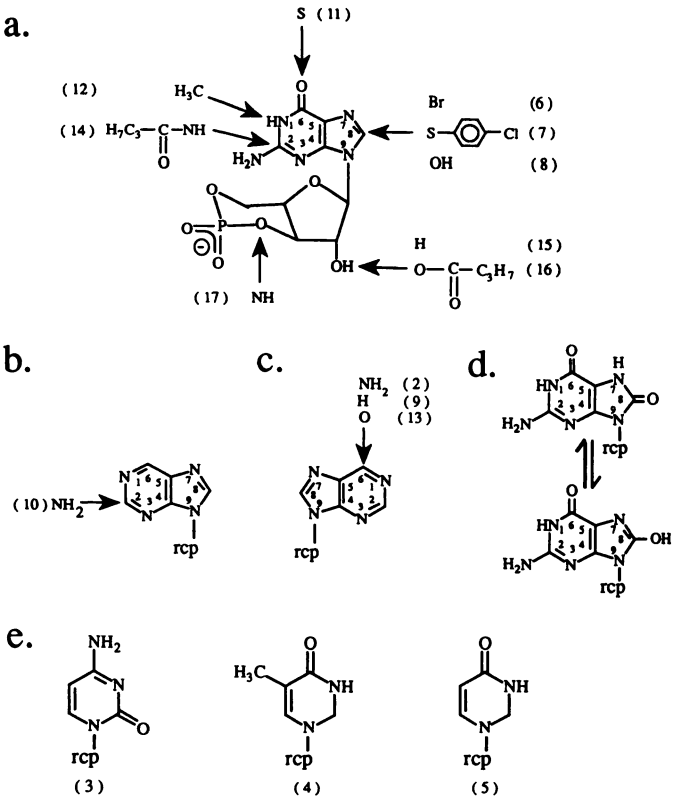

Fig. 1. Cyclic nucleotide structures. *rcp*, ribose cyclic phosphate. In each case the group that is substituted becomes the functional group on the carbon indicated (*arrows*). a, The reference compound is cGMP, shown in its *syn*-conformation. Compound **6** is 8-Br-cGMP. Compound **7** is 8-pCPT-cGMP. Compound **8** is 8-OH-cGMP. As shown in d, it exists in two tautomeric forms. Compound **11** is 6-SH-cGMP. Compound **12** is 1-CH_3-cGMP. Compound **14** is N^2-butyryl-cGMP. Compound **15** is 2′-deoxy-cGMP. Compound **16** is 2′-butyryl-cGMP. Compound **17** is 3′-NH-cGMP. b, Compound **10** is 2-NH_2-cPuMP, shown in the preferred *syn*-conformation. c, Compound **2** is cAMP. Compound **9** is cPuMP. Compound **13** is cIMP. d, Tautomeric forms of 8-OH-cGMP (compound **8**) are shown. In the predominant tautomeric form, the N7-nitrogen can act as a hydrogen donor. e, Compounds **3**, **4**, and **5** are cCMP, cTMP, and cUMP, respectively.

Preparation of PDEs. cGS-PDE and cGI-PDE were purified by immunoprecipitation. Solid-phase antibody reagents were prepared using the anti-cGS-PDE monoclonal antibody cGS-2 (14) and the anti-cGI-PDE monoclonal antibody cGI-5 (15), as described previously. Hypotonic extracts prepared from fresh bovine tissues were used di-

rectly or were chromatographed over DE-52 cellulose to yield a pool enriched in PDE activity. cGS-PDE was immunoprecipitated from bovine adrenal extract. The cGI-PDE was immunoprecipitated from a bovine cardiac PDE-enriched DEAE pool using the solid-phase antibody. These samples were stored in 20 mM Tris, pH 7.5/50% glycerol at −20° with no loss in catalytic activity over a period of several months. As described in more detail in the accompanying paper, a recombinant 59-kDa CaM-PDE was purified (16) from extracts of *Spodoptera frugiperda* insect cells that had been infected with a baculovirus construct containing a full length 59-kDa cDNA isolated from a bovine lung library.[2] The expressed protein was found to be >90% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. cGB-PDE was isolated from bovine lung through the blue dextran step according to a previously reported method (17). ROS-PDE, prepared according to the method of Gillespie *et al.* (18), was provided by Dr. Rabi Prusti (University of Washington, Seattle, Washington). In initial control experiments, IC_{50} curves generated for cGMP using immunoprecipitated enzyme were found to be identical to those obtained using highly purified enzyme, for CaM-PDE, cGS-PDE, and cGI-PDE (data not shown).

Each of the PDEs used in this study was assayed in such a way as to minimize known influences from noncatalytic sites. The cGB- and ROS-PDEs each have a high affinity, noncatalytic, cGMP binding site. Although cGMP binding to the noncatalytic site has been shown to increase the state of phosphorylation of the cGB-PDE, no changes in catalysis have been observed as a result of cGMP binding to the noncatalytic site of either PDE isozyme (19). The cGI-PDEs are competitively inhibited by cGMP. From sequence analysis, it was determined that no recognizable cyclic nucleotide binding domains other than the catalytic domain exist in the cGI-PDE sequence (20). Therefore, the cGB- and cGI-PDEs were assayed directly without further modification, on the assumption that all analog effects would be due to binding at the catalytic site. Highly purified ROS-PDE was activated before the assay by incubation with trypsin. The trypsin-activated ROS-PDE was stable on ice for several hours. The CaM-PDE was assayed in its fully activated state by the addition of calcium and calmodulin to the assay buffer.

The cGS-PDE presented a special problem for assay and interpretation. This isozyme contains a noncatalytic site that causes activation of the catalytic site when occupied by cGMP. Theoretically, a cGMP analog could cause inhibition either by binding to the catalytic site or by acting as an antagonist at the allosteric site. Therefore, this isozyme was assayed after prior activation by covalent modification with DCCD. This method was adapted from that of Stroop and Beavo (4). The results are depicted in Fig. 2 and the method is discussed in detail in the legend to Fig. 2.

PDE assays. cGMP hydrolysis in the presence of analogs was measured at 30° using the standard radioligand assay (21). 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 [^{3}H]cGMP and cyclic nucleotide analog indicated. For CaM-PDE, the assay tubes contained 20 mM Tris, pH 7.5, 20 mM imidizole, 3 mM $MgCl_2$, 15 mM magnesium acetate, 200 μM $CaCl_2$, 4 μg/ml calmodulin, and the concentrations of [^{3}H]cGMP and cyclic nucleotide analog indicated. Assays were terminated by boiling for 1 min and were then treated with *C. atrox* snake venom for 5 min. Samples were chromatographed using DEAE-Sephadex A-25, and the nonabsorbed nucleoside was collected. All assays points are the average of duplicate values. At least three full dose-response curves with five or more concentrations, using at least two different PDE preparations, were used to generate each IC_{50} value.

For each isozyme tested, the substrate concentration 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. The CaM-PDE was assayed with 3 μM [^{3}H]cGMP, and cGI-PDE was assayed with 0.03 μM [^{3}H]-cGMP. To allow initial rates to be estimated, all assays were carried out at times and dilutions that allowed <30% maximal hydrolysis of the tritiated substrate. Theoretically, an artifactually high IC_{50} value could be obtained for an analog if it was hydrolyzed much more rapidly than the substrate. This seemed particularly possible for some analogs with cGI-PDE when cGMP was used as substrate, because the V_{max} for cGMP is much lower than that of cAMP. Therefore, in a few cases where the analog was found to be a better substrate than cGMP, maximal hydrolysis was limited to <10%. For example, with each analog and enzyme where hydrolysis was suspected, an aliquot of the reaction mixture containing 100–200 μM analog was analyzed by thin layer chromatography. This allowed the degree of analog hydrolysis during the assay to be compared with that of the labeled substrate. Finally, in the few cases (e.g., cGI-PDE with cAMP, cPuMP, 2-NH_2-cPuMP, or cIMP) where appreciable hydrolysis of analog was found at these higher analog concentrations, full time courses for cGMP PDE

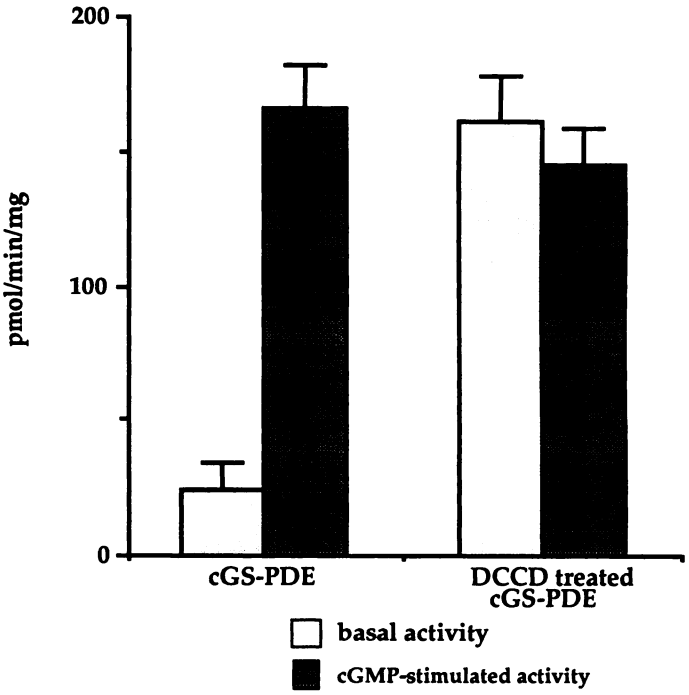

Fig. 2. Activation of cGS-PDE by DCCD. Hydrolysis by cGS-PDE is stimulated when cGMP is bound to high affinity, noncatalytic sites on the enzyme. Theoretically, cGMP analogs could also act as either agonists or antagonists at the allosteric site, thereby altering catalytic activity. Possible effects due to the binding of the cGMP analogs at the allosteric domain were eliminated by covalent modification of the enzyme before the assay. The following method of activation was adapted from the method of Stroop and Beavo (4) to be used with antibody-precipitated cGS-PDE, instead of highly purified enzyme as originally described. Five hundred microliters of the cGS-PDE-antibody-*S. aureus* suspension (~100–200 units of basal activity assayed at 1 μM cAMP) were pelleted and then resuspended in 200 μl of 20 mM MES, pH 6.5, 20 mM sodium phosphate, 100 mM NaCl, 0.02 mM EDTA, followed by the addition of cGMP to a final concentration of 0.7 μM cGMP and DCCD (dissolved in ethanol) to a final concentration of 400 μM. The reaction was carried out at 30° for 60 min and stopped by dilution (1/1) with 50 mM MOPS, pH 7.5, 2 mM EGTA, 37.5 mM EDTA, 25 mM β-mercaptoethanol. After an additional 20-min incubation to allow for hydrolysis of residual cGMP, the cGS-PDE was pelleted, rinsed twice with low-salt solution, and finally resuspended in 20 mM Tris, pH 7.5. Samples were assayed with 1 μM [^{3}H]cAMP, with or without 7 μM cGMP. cGMP and covalent modification using DCCD activated the cGS-PDE to the same extent. The covalently modified cGS-PDE could not be further activated by more cGMP. The DCCD-activated cGS-PDE was stable at 0° over a period of at least several days.

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

activity, using lower analog concentrations near the IC_{50} value, were determined. In no cases were increases in catalytic rates observed with time, as would be expected if rapid analog hydrolysis were occurring. Therefore, it was concluded that none of the reported IC_{50} values are likely to be artifactually high, for this reason.

Rationale for selection of specific analogs. To map the essential molecular interactions between cGMP and the catalytic sites of several different PDE families, a series of 12 systematically modified cGMP analogs were selected. In addition, cGMP, cAMP, and three cyclic pyrimidines were tested. 1-CH_3-cGMP was chosen to investigate the possibility of a hydrogen bond or steric hindrance at the N1-position. 8-Br-cGMP and 8-pCPT-cGMP were chosen to investigate possible steric hindrance due to substitution at the 8-position and also to help determine whether the enzymes prefer to bind cGMP in the *syn*- or *anti*-conformation. The ability of these two nucleotides to inhibit the different PDEs was also of interest because of their widespread use as activators of cGMP-dependent protein kinase and cGMP-gated ion channels. The introduction of an hydroxyl group at the C8-position is known to have large effects on analog binding to several cyclic nucleotide binding sites (11, 19). In particular, it indirectly addresses potential interactions at the 7-position. Therefore, it was of interest to test the effects of 8-OH-cGMP. 6-SH-cGMP, cIMP, cPuMP, and 2-NH_2-cPuMP were selected to provide information about the possibility of interactions at the 6-position. In addition, cIMP, 2-NH_2-cPuMP, and N^2-butyryl-cGMP provide information about interactions at the 2-position and, in the case of 2-NH_2-cPuMP, also preference for the *syn*- versus *anti*-conformation. The pyrimidine cyclic monophosphates were included to test the specificity for the purine ring. 2′-Deoxy-, 2′-butyryl-, and 3′-NH-cGMP were included to look for evidence of interactions between the enzyme and the 2′- and 3′-hydroxyl groups of the ribose moiety.

Hydrogen-bonding interactions. Many of the modifications made to the analogs used in this study eliminate or greatly decrease the potential for a hydrogen bond to form with the PDE. Hydrogen bonds commonly fall into the range of 1–3 kcal/mol, which predicts a change in IC_{50} of 10-fold or more due to loss of a hydrogen bond. Therefore, we have assumed that, if a modification removes an essential hydrogen bond, it would cause an increase in the IC_{50} of 10-fold or more.[3] Although smaller shifts might indicate a weaker hydrogen bond, they are more difficult to interpret due to the variability intrinsic to IC_{50} determinations. In the hydrogen bond pair, the term hydrogen bond donor is used in this manuscript to denote the atom to which the hydrogen is most tightly linked. The other atom of the pair is the hydrogen acceptor. Given the low pK_a of the 6-amino group on cAMP and the precedent for this group to act as a donor at other nucleotide binding sites (22), it is assumed that in the PDEs where it is used as part of the binding motif it also acts as a hydrogen bond donor. A similar assumption was made for the 2-amino group of cGMP. All chemical structures are depicted in Fig. 1.

Results

IC_{50} determinators. IC_{50} values for 17 cyclic nucleotides with five different PDE isozymes were determined at a subsaturating substrate level, which in all cases was one third of the K_m or lower. Because the assays were performed at low substrate levels, the IC_{50} values approach the K_i, the inhibition constant of the analog. The IC_{50} values are reported in Table 1 as the mean of two to 10 determinations. To facilitate comparisons between PDE isozymes, relative IC_{50} values (IC_{50}') were calculated as the $IC_{50(analog)}$ divided by the $IC_{50(cGMP)}$. Relative IC_{50}

[3] In general, the free energy change due to a particular substitution can be estimated as $\Delta G = -RT\ln[K_{d(analog)}/K_{d(cGMP)}]$, in which ΔG is the change in free energy. 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 (40). According to this equation, an 10-fold increase in the IC_{50} value yields a ΔG of approximately 1.4 kcal/mol.

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 three to 10 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 in Experimental Procedures, 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 matches the compound number in Fig. 1. Values are mean ± standard deviation.

Analog	CaM-PDE		ROS-PDE		cGB-PDE		cGS-PDE		cGI-PDE	
	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*
	μM		μM		μM		μM		μM	
No substitution										
1 cGMP	7.3 ± 1.2	3	28 ± 9.3	6	5.6 ± 1.3	8	49 ± 11	8	0.4 ± 0.1	10
2 cAMP	150 ± 15	3	> 500	4	> 500	3	27 ± 20	3	1.3 ± 0.5	6
3 cCMP	>5000	3	>1500	5	> 500	3	>1500	2	>500	3
4 cTMP	1300 ± 290	3	>1000	5	~ 500 ± 0.0	2	>2500	2	630 ± 230	3
5 cUMP	6300 ± 580	3	>1000	5	> 500	3	>5000	2	>500	3
Imidizole ring substitutions										
6 8-Br-cGMP	27 ± 21	3	63 ± 23	3	180 ± 25	3	220 ± 130	3	28 ± 9.6	4
7 8-pCPT-cGMP	60 ± 26	3	43 ± 14	3	29 ± 4.8	4	62 ± 10	3	210 ± 55	5
8 8-OH-cGMP	ND[a]		325 ± 130	4	800 ± 560	2	3200 ± 1000	2	>100	2
Pyrimidine ring substitutions										
9 cPuMP	190 ± 54	4	> 500	3	3700 ± 350	3	62 ± 26	4	10 ± 5.5	10
10 2-NH_2-cPuMP	240 ± 40	3	1700 ± 420	5	2200 ± 350	2	43 ± 12	5	36 ± 13	7
11 6-SH-cGMP	ND	3	70 ± 10	3	25 ± 5.0	3	85 ± 50	4	0.5 ± 0.1	4
12 1-CH_3-cGMP	230 ± 26	3	440 ± 120	4	250 ± 87	3	250 ± 87	3	21 ± 6.4	5
13 cIMP	7.3 ± 1.2	3	32 ± 10	3	10 ± 4.4	3	63 ± 7.6	3	2.2 ± 1.7	9
14 N^2-Butyryl-cGMP	57 ± 15	3	233 ± 76	3	120 ± 30	4	250 ± 100	5	54 ± 21	7
Ribose subsitutions										
15 2′-Deoxy-cGMP	18 ± 11	3	23 ± 2.9	3	7.2 ± 2.4	3	55 ± 15	3	0.6 ± 0.3	4
16 2′-Butyryl-cGMP	8.3 ± 2.1	3	39 ± 11	5	7.1 ± 2.9	5	10 ± 4.4	5	0.9 ± 0.5	5
17 3′-NH-cGMP	ND		80 ± 17	3	31 ± 3.6	3	43 ± 10	5	1.8 ± 0.8	3

[a] ND, not determined.

values allow comparison of the affinity of a particular analog for each PDE isozyme with the affinity of cGMP for that isozyme. The relative IC_{50} values are presented in Table 2. A brief description of the results obtained from modifications at each position is given below and is discussed in more detail in Discussion.

6-Position [2-NH_2-cPuMP (10), cIMP (13), and 6-SH-cGMP (11)]. Comparison of cGMP and 2-NH_2-cPuMP should be a rather direct test for the importance of the 6-oxo group for binding. For each PDE except cGS-PDE, removal of this group greatly increases the IC_{50} value. Similarly, 6-SH-cGMP has IC_{50} values similar to those of cGMP for each of these isozymes. Both of these sets of data suggest that the 6-oxygen (or sulfur) functions as a hydrogen bond acceptor in the binding pocket. The complete lack of effect of any substitution at this position seen for cGS-PDE suggests that this enzyme does not use this position for binding.

6-Position [cAMP (2) and cPuMP (9)]. Comparison of the relative IC_{50} values for cAMP and cPuMP also provides an indirect assessment of the function of the 6-NH_2 group for binding. For the CaM-, cGS-, ROS-, and cGB-PDEs, the relative affinity is decreased to about the same extent for both nucleotides. This suggests that for these PDEs the 6-NH_2 group does not participate in binding as a hydrogen bond acceptor and does not substitute for oxygen. It may even have a negative effect. The case with cGI-PDE is more complex, in that cAMP has an equal or perhaps even higher affinity, compared with cPuMP. As discussed in more detail below, this may mean that cGI-PDE can bind the *anti*-conformation of the cyclic nucleotides and the 6-NH_2 group on cAMP functions as a hydrogen bond donor. However, as mentioned previously, the relative differences between the IC_{50} values for cPuMP and cAMP are not large and therefore are difficult to interpret with confidence.

1-Position [1-CH_3-cGMP (12)]. As shown in Table 2, the 1-methyl substitution causes a large increase (5–49-fold) in the relative IC_{50} values for the different PDEs. Such an increase is consistent either with a highly constrained steric requirement that is sensitive to the relatively small methyl group or with a hydrogen bond at this position.

2-Position [cIMP (13), cPuMP (9), 2-NH_2-cPuMP (10), and N^2-butyryl-cGMP (14)]. Removal of the 2-NH_2 group from cGMP to produce cIMP does not decrease the IC_{50} for any PDE, with the possible exception of cGI-PDE. Similarly, the addition of a 2-NH_2 group to cPuMP to form 2-NH_2-cPuMP does not increase the apparent affinity of the derivative for any PDE. All of these data taken together suggest, therefore, that the 2-NH_2 group does not participate in the binding of cGMP to most of the PDEs. The fact that a large bulky substitution of a butyryl group at this position causes an increased IC_{50} for all PDEs suggests that the area is sterically constrained for most PDEs.

7-Position [8-OH-cGMP (8)]. As mentioned in Rationale for selection of specific analogs, modification of the 8-position by an hydroxyl group serves in part as an indirect measurement of possible interactions at the 7-position. The data show that the different PDE isozymes interact with 8-OH-cGMP with greatly varying relative affinities. The relative IC_{50} values range from >100-fold for the cGB- and cGI-PDEs to only 11-fold for the ROS-PDE. Several possible interpretations of these results are explored in more detail in Discussion. The simplest is that the 7-nitrogen may act as a hydrogen bond acceptor for all of the PDEs.

8-Position [8-Br-cGMP (6) and 8-pCPT-cGMP (7)]. The 8-bromo and 8-pCPT substitutions have little effect on the relative affinities of cGMP for the CaM-, ROS-, and cGS-PDEs. Because these substitutions tend to force most of the analog into the *syn*-conformation, these results strongly suggest

TABLE 2

Relative IC_{50} values

For each isozyme the IC_{50} values are normalized to the IC_{50} for cGMP for that particular isozyme, to facilitate comparison between the various isozymes. The relative IC_{50} value (IC_{50}') is $IC_{50(analog)}/IC_{50(cGMP)}$ 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	ROS-PDE	cGB-PDE	cGS-PDE	cGI-PDE
No substitution					
1 cGMP	1.0	1.0	1.0	1.0	1.0
2 cAMP	20	>18	>90	0.6	3.0
3 cCMP	>680	>55	>90	>30	>1200
4 cTMP	180	>36	89	>50	1500
5 cUMP	860	>36	>90	>100	>1200
Imidizole ring substitutions					
6 8-Br-cGMP	3.7	2.3	32	4.5	65
7 8-pCPT-cGMP	8.2	1.5	5.2	1.3	490
8 8-OH-cGMP	ND[a]	12	140	66	>230
Pyrimidine ring substitutions					
9 cPuMP	26	>18	660	1.3	23
10 2-NH_2-cPuMP	33	62	390	0.9	84
11 6-SH-cGMP	ND	2	4	1.7	1.2
12 1-CH_3-cGMP	32	16	45	5.1	49
13 cIMP	1.0	1.0	1.8	1.3	5.1
14 N^2-Butyryl-cGMP	8.0	10	21	5.1	130
Ribose substitutions					
15 2′-Deoxy-cGMP	2.4	0.8	1.3	1.1	1.4
16 2′-Butyryl-cGMP	1.1	1.4	1.3	0.2	2.1
17 3′-NH-cGMP	ND	2.9	5.5	0.9	4.2

[a] ND, not determined.

that these three enzymes do not preferentially bind the *anti*-conformation of cGMP. They also suggest that these PDEs have a relatively open binding site in this region for this conformation. In contrast, both 8-substitutions cause a large increase in the relative IC_{50} for the cGI-PDE and a more modest increase for the cGB-PDE. This suggests that inhibition of activity for both the cGI- and cGB-PDEs occurs by preferential binding of the *anti*-conformer of cGMP and/or that these two isozymes are more sterically constrained in this region.

Pyrimidine ring [cCMP (3), cUMP (5), and cTMP (4)]. These derivatives were included to test for the specificity of the various PDE isozymes for the purine ring. Although several cyclic pyrimidine monophosphate PDE activities have been reported (23, 24), there is little information in the literature about whether highly purified PDEs from each of the families represented in this study hydrolyze these nucleotides. The extremely high IC_{50} values suggest that none of these PDEs is responsible for cyclic pyrimidine monophosphate hydrolysis in mammalian tissues.

Ribose moiety [2′-deoxy-cGMP (15), 2′-butyryl-cGMP (16), and 3′-NH-cGMP (17)]. Deletion of the 2′-hydroxyl group has effects of ≤3-fold for all isozymes. Addition of a large butyryl group at this position also causes little if any decrease in affinity and, in fact, with the cGS-PDE a higher apparent affinity was consistently observed for 2′-butyryl-cGMP. The 3′-amino substitution also causes very small effects. In all cases the relative increase in IC_{50} is ≤5-fold. These data indicate that, in contrast to other cGMP-binding proteins, the 2′-hydroxyl group on the ribose ring appears unimportant for binding to the PDE catalytic sites. The relatively small increases due to substitution of the 3′-hydroxyl group suggest that it also is not involved in binding.

Discussion

Common interactions. Mapping the topology of the catalytic site for representative members of several different PDE isozyme families clearly demonstrates that each isozyme family binds cGMP through a unique set of molecular interactions (Fig. 3). These unique interactions in turn lead to the distinct kinetic properties of the different PDEs. Surprisingly, the cGMP-specific isozymes, the ROS- and cGB-PDEs, appear not to interact with cGMP via its 2-NH_2 group, as has been observed for the cGMP-dependent protein kinases and the cGMP-gated ion channel. Instead, the cGMP specificity is determined mainly by the 6-position oxygen. In addition, this study indicates that modification of the N1-position of cGMP decreases the affinity for all PDE catalytic sites, suggesting that this nitrogen is important for binding. This interaction is common to all PDEs tested. Finally, alterations of the ribose 2′- and 3′-positions do not increase the IC_{50} for any of the PDEs, suggesting that the 2′- and 3′-hydroxyl groups are not involved in cGMP binding to the catalytic sites of the PDEs.

For most of the PDEs, removal of the 6-oxo group or substitution for it by an amino group increases the IC_{50} for the analog, compared with its unmodified control. This suggests that a hydrogen bond in which the 6-oxygen acts as the hydrogen acceptor is common to all PDEs except the cGS-PDE. Because many substitutions do not alter the affinity for the cGS-PDE, it apparently interacts with cGMP through the smallest number of interactions. Conversely, the cGI-PDE, which exhibits the highest affinity for cGMP, interacts with cGMP through the greatest number of interactions, as evidenced by its sensitivity to nearly any modification of the guanine ring. Finally, a pyrimidine ring is not sufficient for high affinity binding. The rationale for each of these conclusions is discussed in more detail in the following paragraphs. The proposed binding motifs based on these interpretations are depicted in Fig. 3.

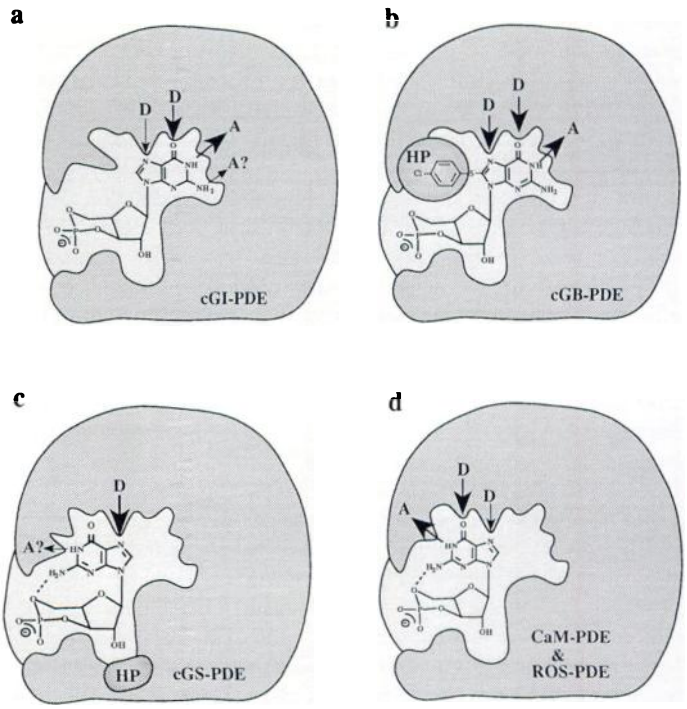

Fig. 3. Interactions of cGMP with the catalytic site of PDEs. Models of the binding of cGMP to the various catalytic domains are proposed based on the data described in Tables 1 and 2. *Large arrows*, stronger interactions; *small arrows*, weaker interactions. *Direction of the arrows*, probable arrangement of the hydrogen bond, with the arrow pointing towards the hydrogen acceptor. *A*, hydrogen acceptor; *D*, hydrogen donor; *HP*, hydrophobic pocket. a, cGMP is proposed to bind to cGI-PDE in its *anti*-conformation. The purine ring of cGMP appears to interact with residues of the cGI-PDE catalytic domain through three distinct interactions, at the N1-, N7-, and 6-oxo positions, as well as possibly a weaker interaction with the 2-amino group. This interaction is shown as being with a hydrogen acceptor only by analogy to similar interactions of purine amino groups with binding sites on other enzymes. b, cGMP is proposed to bind to cGB-PDE in its *anti*-conformation. The purine ring of cGMP appears to interact with residues of the cGI-PDE catalytic domain through three distinct interactions, at the N1-, N7-, and 6-oxo positions. *Darker patch*, probable hydrophobic region that may interact in a positive manner with the pCPT group of 8-pCPT-cGMP or 8-pCPT-cAMP. The 8-pCPT group is shown interacting with a hydrophobic pocket. c, cGMP is proposed to bind to cGS-PDE in its *syn*-conformation. The purine ring appears to interact with residues of the cGS-PDE catalytic domain through two distinct interactions, at the N1- and N7-positions. The site is drawn as being relatively open and able to accommodate large substitutions at most positions on the base or ribose. *Darker patch*, possible hydrophobic region that binds the 2′-butyryl group of 2′-butyryl-cGMP. d, cGMP is proposed to bind to both the CaM- and ROS-PDEs in its *syn*-conformation. The purine ring appears to interact with residues of the CaM- and ROS-PDE catalytic domains through two distinct interactions, at the N1- and 6-oxo-positions, as well as perhaps at the N7-position, at least for the ROS-PDE. The site is drawn as being relatively open and able to accommodate large substitutions at most positions on the base or ribose.

6-Position modifications. The complete absence of a functional group at the 6-position (cPuMP and 2-NH_2-cPuMP)

causes a large increase in the IC_{50} in all cases except for the cGS-PDE. In addition, the 6-thio substitution causes little if any increase in the IC_{50} (4-fold or less) for all of the enzymes. This is not unexpected, because the sulfur can also act as a hydrogen acceptor, albeit a weaker one. The likelihood of an important hydrogen bond at the 6-position for the other PDEs is also supported by the relatively high affinity of cIMP for the PDE catalytic site. The CaM-, ROS-, and cGB-PDEs are all more potently inhibited by cIMP than by cAMP, suggesting that a good hydrogen acceptor is required at the 6-position for efficient binding to these enzymes. Taken together, all of the data are consistent with the 6-oxygen of cGMP acting as a hydrogen acceptor for the ROS-, cGB-, and CaM-PDEs. Neither the absence of a 6-oxo group nor the presence of any of the other 6-position substitutions decreases the apparent affinity for the cGS-PDE, suggesting that this position is not involved in cGMP binding as a substrate for this isozyme.

For the cGS- and cGI-PDEs, the relative IC_{50} values for cGMP, cAMP, and 6-SH-cGMP are nearly equal. Both the cGS- and cGI-PDEs, which bind cGMP and cAMP with approximately equal affinities, do not discriminate between a 6-amino group and a 6-oxo group; in addition, the relative IC_{50} for cIMP with the cGI-PDE is only modestly increased (5-fold). However, in contrast to the cGS-PDE, the cGI-PDE does require some substituent in this position, as evidenced by the high IC_{50} for cPuMP and 2-NH_2-cPuMP. Therefore, it appears that, whereas the cGS-PDE does not utilize an interaction with the 6-position, the cGI-PDE makes use of either an oxygen or amino group at this position. In this latter case, it may be that residues of the cGI-PDE catalytic domain are arranged in such a way that they can act as either hydrogen bond donors or acceptors, depending on the substrate.

1-Position modifications. A methyl substitution at the 1-position has a negative effect of 5–49-fold on the affinity of cGMP for each of the PDEs tested. The addition of a methyl group prevents the N1-nitrogen from being a hydrogen donor. It also may cause steric hindrance if the binding site is highly constrained in this region. A likely interpretation of these data is that the increase in IC_{50} reflects participation of the hydrogen on the N1-nitrogen in a hydrogen bond with some acceptor group on each of the PDE catalytic sites. Although the methyl group is relatively small, it is of course possible that all of the PDEs have sites that are extremely constrained in this region and that the decreased affinities caused by the methyl substitution are due to steric hindrance at this point. Further work using substitutions with larger groups is necessary to completely dismiss this possibility. It should also be pointed out that the 6-oxo group greatly influences the ability of the N1-nitrogen to act as a hydrogen donor. In fact, in all cases where cGMP and cIMP are better inhibitors than cAMP, a major effect of the 6-oxygen may be to allow a hydrogen bond involving the N1-position to form and in this manner indirectly allow the N1-nitrogen to be a major determinant of substrate specificity.

2-Amino-position modifications. The data obtained with 2-NH_2-cPuMP and cIMP suggest that none of the PDEs, with the possible exception of the cGI-PDE, interacts with the 2-position amino group. The cGMP-dependent protein kinases use a hydrogen-bonding interaction at the 2-NH_2 group as part of their high affinity binding motif (25). It was expected that, if this were also the case with the PDE catalytic sites, removal of the 2-NH_2 group (as in cIMP) would greatly decrease the IC_{50}, compared with cGMP. As can be seen in Tables 1 and 2, with the possible exception of cGI-PDE, cIMP has essentially the same IC_{50} as cGMP. Similarly, if the 2-amino group were important, one might expect the IC_{50} for cPuMP to be greater than that for 2-NH_2-cPuMP. As shown in Table 2, 2-NH_2-cPuMP does not have a higher affinity than cPuMP for any of the PDEs. However, the addition of a bulky group on the 2-NH_2 group has a modest 5–10-fold effect on the CaM-, ROS-, and cGS-PDEs, perhaps due to steric effects. In contrast, the cGI-PDE shows sensitivity to bulky additions at the 2-NH_2 position. Lack of a 2-amino group, as seen with cIMP, increases the relative IC_{50} with this isozyme about 5-fold, suggesting a possible weak hydrogen-bonding interaction of cGI-PDE with cGMP through the 2-amino group. However, because the magnitude of the increase is not large, compared with experimental variation, some question about this conclusion remains. In addition, 2-NH_2-cPuMP does not have higher affinity than cPuMP.

Hydrophobic and other interactions. It is clear that some of the chemical modifications to the guanine ring alter not only the hydrogen-bonding potential but also the overall dipole moment, the general and regioselective polarity, the pK_a, and the highest occupied molecular orbital/lowest unoccupied molecular orbital values (26, 27). Because these factors may influence binding between a heterocyclic base and a protein, interpretation of the effects of these substitutions is more difficult. This is especially true for the 8-hydroxyl substitution, which would be expected to have a number of effects on the guanine ring. In addition to increasing the *syn* population, this substitution allows a tautomeric rearrangement to occur, as shown in Fig. 1. In cGMP, the N7-nitrogen is basic and can therefore act as a hydrogen acceptor (28). However, with 8-OH-cGMP the lactam tautomer is less likely to act as a hydrogen acceptor at the N7-position. It could, however, serve as a hydrogen donor. The 8-hydroxyl substitution would also be expected to alter the dipole moment of the purine base. This, in turn, might have effects on binding affinity, in addition to altering the hydrogen-bonding potential at other positions on the guanine ring. For example, addition of an hydroxyl group at the C6-position is thought to alter the intramolecular hydrogen bond strength of the 2-NH_2 group of cGMP (29). Therefore, effects of this 8-hydroxyl substitution on the affinity of the analog for the PDE are difficult to interpret. However, particularly for the cGB-, cGS-, and cGI-PDEs, where very large increases in the IC_{50} are seen for this analog, it seems likely that at least part of the increase in the IC_{50} could be due to loss of a hydrogen bond at the N7-position. The fact that the disruption of a hydrogen bond at the N7-position is not the only reason for the increased IC_{50} values observed with 8-OH-cGMP is suggested by the data in the accompanying manuscript (30), where a 7-deaza substitution of cAMP increases the IC_{50} less dramatically. Based in part on the observation that 7-deaza-cAMP does not greatly alter the IC_{50} for the cGI-PDE, a weak interaction with the N7-position is proposed. In contrast, the disruption of binding due to the 7-deaza-cAMP substitution is much greater than that observed here for 8-OH-cGMP with the cGS-PDE. A previous study with this enzyme demonstrated an increase in the K_i for 7-deaza-cGMP, compared with cGMP (8). These data, taken together, support a hydrogen-bonding interaction at the N7-position with the cGS-PDE. In addition, the data suggest that the increase in IC_{50} due to an 8-hydroxyl substitution of cGMP is caused by more than just a change in

hydrogen bonding at the N-7-position for most of the PDEs. Regardless of the mechanism, because this substitution does not cause the same increase in IC_{50} for all PDEs it does show that different modes of binding are used by the different PDE isozymes. Even two isozymes as similar as the ROS- and cGB-PDEs are differentially affected by the 8-hydroxyl substitution.

A role for hydrophobic interactions due to π-electron stacking in cAMP binding to partially purified preparations of the cGS- and CaM-PDEs was proposed in two early derivative studies (9, 13). This proposal was made in part because little effect of substitutions expected to disrupt hydrogen bonds to the purine ring was seen. It should be noted, however, that in the first study with an enzyme preparation enriched in a cGS-PDE no analogs likely to have decreased hydrogen-bonding possibilities at the N1-position were tested. In fact, in the present study this isozyme was also the least sensitive to substitution on the guanine ring. In the second study, an enzyme preparation enriched in a CaM-PDE displayed a substantially decreased apparent affinity for the 1-NO-cAMP and benzimidizole derivatives. Because cAMP was used as the substrate 1-CH_3-cGMP was not tested, and because the authors were most likely working with a mixture of PDEs perhaps some potential hydrogen-bonding interactions proposed for cGMP in this study were not detected. It should be noted that, in the present study, none of the data rule out a possible role for hydrophobic interactions. However, it is clear that several specific hydrogen bonds with substituents of the guanine ring are likely to play a critical role in nucleotide binding to the catalytic sites of these PDEs.

Steric effects. Several of the modifications add rather large, bulky substituents to the guanine ring. In the cases of the CaM-, ROS-, and cGS-PDEs, where no change in IC_{50} is caused by the bulky substitutions, it is clear that these enzymes must have a rather open architecture, i.e., must be able to accommodate a large group without steric hindrance. Specifically, the IC_{50} values for these three isozymes are not greatly increased by the bulky bromo and pCPT substitutions at the 8-position, although the 8-fold effect of the pCPT group on the IC_{50} for the cGS-PDE may suggest some steric effect for this enzyme. In addition, these three isozymes show 8–20-fold decreased affinity due to the large 2-NH_2-butyryl substitution, suggesting some steric interaction at this site. The cGI- and cGB-PDEs appear to be the most constrained at their catalytic domains. These two isozymes are less able to tolerate bulky substitutions at the 2-NH_2- and 8-positions than are the other PDEs. This observation is particularly striking in the case of cGI-PDE, where the bulky 2-NH_2-butyryl substitution increases the IC_{50} >100-fold, compared with the parent compound, cGMP; this may be due to a combined effect of disruption of a required hydrogen-bonding interaction and steric hindrance. The possibility of steric effects due to 1-position substitutions was discussed previously.

***Syn*- versus *anti*-conformational effects.** It is intrinsically difficult to determine whether a substitution causes a change in IC_{50} because of its effect on the *syn*- versus *anti*-conformation of the analog or because of steric hindrance with residues on the enzyme. In the present study we assume that, if the PDE binding site exhibits a strict preference for the *anti*-conformation, then the IC_{50} is increased by approximately the same amount as the analog population equilibrium is shifted towards the *syn*-conformer by the substitution. For example, substitution of a bromo group at the 8-position, which would be expected to substantially decrease the percentage of the nucleotide in the *anti*-conformation, also would be expected to substantially increase the amount of derivative required for 50% inhibition if it bound only in the *anti*-conformation. Some direct evidence for this has been obtained for 8-Br-cAMP, which binds less tightly to the *Escherichia coli* catabolite activator protein than does cAMP itself (31). The X-ray crystal structure of the catabolite activator protein shows cAMP to be bound in the *anti*-conformation (32). Conversely, and perhaps more importantly, if the IC_{50} is not changed by this type of substitution then one can conclude that the PDE does not preferentially bind the *anti*-conformer.

Although cGMP exists as both *syn*- and *anti*-conformers, in contrast to cAMP it has a lower energy minimum in the *syn*-conformation; therefore, the population equilibrium somewhat favors this conformation. The source of the conformational preference has been attributed to an intramolecular hydrogen bond, in the *syn*-conformation, between a hydrogen atom on the guanine 2-amino group and the axial phosphate oxygen atom. Estimates of the magnitude of this effect vary; however, modeling studies suggest a difference of at least 2 kcal/mol and perhaps as much as 5 kcal/mol (29). cAMP and cPuMP derivatives substituted with a 2-NH_2 group also show increased preference for the *syn*-conformation; moreover, the strength of this bond is substantially increased by the presence of the 6-oxo group of cGMP (29). The dynamic equilibrium of the *syn*- and *anti*-conformers of 8-Br-cGMP in solution, as determined by NMR spectrometry, is shifted largely toward the *syn*-conformation, such that <5% exists in the *anti*-conformation (33). Those authors did not specifically test 8-pCPT-cGMP; however, they did show that, in general, the larger the substitution at the 8-position the larger the population in the *syn*-conformation. To our knowledge there is no published measurement for the conformational preference of 8-pCPT-cGMP. Because the sulfur is slightly smaller than the bromine, 8-pCPT-cGMP may show less preference for the *syn*-conformation than does 8-Br-cGMP. In any case, we assume that either substitution would increase the IC_{50} for any PDE preferring the *anti*-conformer. The 8-bromo and 8-pCPT substitutions, which shift the equilibrium of the analog towards the *syn*-conformation, have little effect on the affinity of cGMP for the CaM-, ROS-, and cGS-PDEs. This lack of substituent effect strongly suggests that the CaM-, ROS-, and cGS-PDEs do not prefer the *anti*-conformation and either can accommodate both the *syn*- and *anti*-conformations or, more likely, prefer the *syn*-conformation.

The cGI- and cGB-PDEs both are sensitive to substitution at the 8-position. For the cGI-PDE, 8-Br-cGMP and 8-pCPT-cGMP are each very poor inhibitors. These data, taken by themselves, do not allow one to distinguish between possible steric hindrance of the 8-position substituent by residues in the nucleotide binding pocket and effects of the substituent on the *syn*- versus *anti*-conformation of the analog, because the larger the group the greater would be the effect on both parameters. However, for the cGI-PDE these data can be combined with the increased IC_{50} observed for 2-NH_2-cPuMP, which favors the *syn*-conformation, compared with that for cPuMP, which is likely to be mostly in the *anti*-conformation, to indicate that cGI-PDE may preferentially bind cGMP in its *anti*-conformation. In addition, this enzyme has a comparable affinity for cAMP, which prefers the *anti*-conformation. Theoretically, the data are also consistent with the possibility that this isozyme

preferentially binds cGMP in the *syn*-conformation but cAMP (and possibly cPuMP and cIMP) in the *anti*-conformation. However, this would also require that the 8-bromo group be sterically hindered when bound in the *syn*-conformation even though the imidazole part of the purine ring is not sterically hindered when bound as the *anti*-conformer, which seems unlikely. Therefore, cGMP is shown in Fig. 3 as being bound in the *anti*-conformation.

For the cGB-PDE, the IC_{50} for 8-Br-cGMP is greatly increased, suggesting that this isozyme is sterically constrained or that it also prefers substrates in the *anti*-conformation. However, the IC_{50} value observed for 8-pCPT-cGMP is only slightly higher than that for cGMP, a result not predicted if cGB-PDE preferentially binds to the *anti*-conformation. This apparent discrepancy probably can be explained by the earlier observation that a structurally related compound, 8-pCPT-cAMP, is a potent inhibitor of the cGB-PDE even though both cAMP and 8-Br-cAMP are poor inhibitors of cGMP hydrolysis by this isozyme (34). This high affinity binding of 8-pCPT-cAMP has been attributed to positive interactions between the large pCPT group and residues located near the catalytic site (34). From the present data, it is clear that an 8-pCPT substitution on cGMP does not increase its affinity and in fact may decrease it 5-fold (Table 2). Therefore, if there is an interaction of the pCPT group with the enzyme, it seems likely that it must occur with the small fraction of the nucleotide population in the *anti*-conformation. Otherwise, it is difficult to see why 8-pCPT-cGMP, which has the same substitution as 8-pCPT-cAMP and should be predominately in the *syn*-conformation, is a poorer inhibitor than cGMP itself. The most likely explanation is that cGB-PDE binds both 8-pCPT-cAMP and 8-pCPT-cGMP in the *anti*-conformation. The reason why 8-pCPT-cGMP has a lower apparent affinity than 8-pCPT-cAMP is probably that 8-pCPT-cAMP more readily assumes the *anti*-conformation, because it does not have the *syn*-conformation stabilized by internal hydrogen bonding between the 2-NH_2 group and the exocyclic oxygen. Several other studies also have addressed the ability of 8-pCPT-cGMP to inhibit cGB-PDE. The present results are in accordance with two previously published studies (12, 19). In addition, there is one report suggesting an even higher IC_{50} for 8-pCPT-cGMP (34). All of these data are consistent with the idea that the cGB-PDE prefers the *anti*-conformer of cGMP, and it is drawn that way in Fig. 3. The 8-pCPT group and putative hydrophobic pocket are also depicted.

One piece of data that may not be consistent with this hypothesis is the observation that 2-NH_2-cPuMP and cPuMP appear to have approximately equal affinities for the cGB-PDE (i.e., IC_{50}' of ~400 versus ~670) (Table 2). One might expect 2-NH_2-cPuMP to exist in the *syn*-conformation to a greater extent than cPuMP, due to internal hydrogen bonding of the 2-amino group with the cyclic phosphate oxygen (29). Nevertheless, the data in Table 2 imply that it is not a poorer inhibitor. Perhaps cGMP can bind to cGB-PDE in either conformation but the hydrophobic interaction with the 8-pCPT group occurs only with the *anti*-conformer. Alternately, the IC_{50} values for both of these analogs are very high and therefore difficult to estimate precisely. It also becomes very difficult to rule out the possibility of trace contamination with some other cyclic nucleotide when such high levels are required for inhibition.

Cyclic pyrimidine monophosphates. For all PDEs tested, the cyclic pyrimidines have extremely high IC_{50} values. Some inhibition could be observed at very high concentrations for each enzyme. However, most values were so high that meaningful IC_{50} values could not be estimated. The very high values do suggest that none of these enzymes are responsible for hydrolyzing cCMP (23) or cUMP (24) *in vivo*.

Noncatalytic PDE binding sites. The noncatalytic cGMP binding sites of the cGS- and cGB-PDEs have been extensively studied by other researchers using cGMP analogs (9, 12, 35, 36). The noncatalytic site of ROS-PDE remains largely uncharacterized. Both cGS-PDE and cGB-PDE noncatalytic sites have been shown to be constrained around the 8-position or perhaps bind the *anti*-conformer. The cGB-PDE site is thought to form hydrogen bonds through the 6-oxo group and N1-nitrogen. Both binding sites interact with the 2-amino group of cGMP and also the 2′-hydroxyl group of the ribose ring. In both examples, cGMP is bound through more functional groups to the noncatalytic site than to the catalytic domain. In particular, the ribose ring plays a role in binding that was not detected at any PDE catalytic site. A general noncatalytic PDE binding motif for cGMP appears to include constraint about the 8-position (or an *anti*-conformational preference) and positive binding interactions at the 6-oxo, N1-nitrogen, and 2-amino moieties and with the 2′-hydroxyl group of the ribose cyclic phosphate.

Sites on other cyclic nucleotide-binding proteins. cGMP serves to regulate other cellular functions in addition to cyclic nucleotide metabolism. Two examples for which binding interactions with cGMP have been investigated are the cGMP-dependent protein kinase and the cGMP-gated cation channel. The cGMP-dependent protein kinase is a widely distributed cGMP receptor thought to play a role in smooth muscle relaxation as well as in platelet, kidney, and intestinal function. The cGMP-gated channel is highly localized in photoreceptor outer segments. cGMP is a direct agonist of the channel. The binding interactions of cGMP with the domains of the cGMP-dependent protein kinase and the cGMP-gated channel of the retina have been studied using an analog approach similar to that presented in the present study (11, 37–39). Common to the binding motifs of these two proteins are hydrogen bonds at the 6-oxo and 2-amino groups, as well as essential interactions with the ribose moiety. The kinase does not interact with cGMP at the N1-position. The N1-position has not been investigated with respect to the channel. The 8-position substitutions result in analogs that are good activators of both the kinase and the channel, whereas the 8-substituted analogs are relatively poor inhibitors of PDE activity of the cGB- and cGI-PDEs. A common binding motif for non-PDE allosteric sites, based on the two examples, includes hydrogen bonds with the 6-oxo and N2-amino groups, positive binding interactions about the ribose moiety, lack of constraint about the 8-position, and likely preference for the *syn*-conformer.

From this limited survey, it is evident that the PDE catalytic domains interact with cGMP in a unique fashion, compared with other cGMP-binding molecules. Only the PDE catalytic sites lack interactions with the 2′-ribose hydroxyl group. The general lack of a binding interaction at the 2-amino position also serves to distinguish the PDE catalytic domain from other guanosine nucleotide binding sites. All of the binding sites discussed, whether catalytic or allosteric, interact with the guanine ring at the 6-oxo position, with the single exception of the cGS-PDE, for which no interaction is observed. Finally,

the N1-position is important for binding to the catalytic and noncatalytic sites on all of the PDEs.

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