A New Photoaffinity Analog of cGMP: Potent Inhibitor of Cyclic Nucleotide Phosphodiesterase†

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Received September 16, 1993®

A purine nucleotide analog, 2′-*O*-(4-benzoylbenzoyl)guanosine cyclic 3′,5′-monophosphate (BBcGMP), was synthesized from 4-benzoylbenzoic acid and cGMP. BBcGMP was found to inhibit several types of cyclic nucleotide phosphodiesterases (PDEs) at low concentrations. Besides its activity as a PDE inhibitor, the benzophenone moiety of BBcGMP provides it with UV light-induced photoreactivity toward organic compounds. This photoreactivity of BBcGMP was exploited to radiolabel specifically the light-stimulated cGMP PDE of the rod outer segment with [α-^{32}P]BBcGMP. These results suggest that benzoylbenzoic derivatives of cyclic nucleotides can be of general use in the inhibition of cyclic nucleotide PDEs. The results further suggest that radiolabeled cyclic nucleotide derivatives may be useful in the identification and active site determination of cyclic nucleotide-binding proteins and enzymes.

Numerous analogs of cGMP have been found to supply useful information concerning the identity, mechanism, and structure of various phosphodiesterase molecules and other molecules that bind cyclic nucleotides.[1–4] Certain of these analogs possess the capability to fluoresce in response to irradiation with short wavelength light,[4,5] and others have been employed as photoaffinity analogs of cGMP. Of the photoaffinity analogs, 8-N_3cGMP has been used to identify cGMP-dependent protein kinases[3] and the α-subunit of retinal rod cGMP phosphodiesterase (PDE);[6] 8-[(*p*-azidophenacyl)thio]-cGMP has been used to label and activate a cGMP-gated conductance in rod outer segment plasma membranes.[7] While the labeling of cGMP PDE with 8-N_3cGMP proved valuable as a rough determinant of the cyclic nucleotide-binding proteins in bovine retina, the low binding affinity of many proteins for 8-N_3cGMP (competition using 2 mM cGMP) leaves several questions as to the specificity of this label. For example, because of the low apparent affinity of PDE for this compound, it is questionable whether binding of 8-N_3-cGMP by PDE was at the catalytic site for PDE. Certain PDEs have a higher affinity for O^2-substituted cGMP analogs (*i.e.*, the rod outer segment PDE).[8] This suggested to us that a photoaffinity analog of this type should be a more specific substrate/ligand for these PDEs. It has been reported that 3′-*O*-benzoylbenzoyl derivatives of ATP and GTP appear to bind to ATP- and GTP-binding proteins, respectively, with high affinity.[9] We felt that a ribose ring-substituted benzoylbenzoyl derivative of cGMP would be a high affinity, specific photoaffinity ligand for certain PDE molecules. We report here the detailed methods of synthesis and characterization of a 2′-*O*-4-benzoylbenzoyl derivative of cGMP (Clack, J. W.; Stein, P. J. U.S. Patent No. 5,039,797; Aug. 13, 1991).

The biological studies presented here show that this compound serves as a potent inhibitor of several cyclic nucleotide phosphodiesterases. They also show that 2′-*O*-(4-benzoylbenzoyl)guanosine cyclic 3′,5′-monophosphate (BBcGMP) functions as a photoaffinity probe for a representative phosphodiesterase, the cGMP PDE derived from rod outer segments.

Results

Chemical. The synthesis of BBcGMP is essentially a one-step process. Following carbonyldiimidazole-mediated reaction with 4-benzoylbenzoic acid, the reaction products are dissolved in 10% MeOH/90% water and extracted twice with *n*-butanol. The butanol extracts over 90% of the dissolved carbonyldiimidazole and some unreacted benzoylbenzoic acid. Aqueous reactants and products are then chromatographed on a C_{18} reverse-phase column. Figure 1 shows the elution profile of the reaction mixture during a typical 10–100% MeOH gradient elution of the C_{18} column. The benzoylbenzoyl analog elutes well after the unreacted cGMP and before the benzoylbenzoic acid. Varying the volume of the methanol gradient used to elute the product and reactants can increase or decrease the degree of overlap between peaks. Yields are between 30% and 50%. BBBcGMP is stable at neutral pH and can be stored for several months at −20 °C without significant degradation.

The proposed structure of BBcGMP is shown in Figure 2. Evidence that the benzoylbenzoyl moiety is attached to the 2′-oxygen of the ribose was obtained by infrared spectroscopy. The infrared spectrum of BB-cGMP was devoid of a sharp peak at a wavenumber of 3400 cm^{-1} (data not shown). This peak was present in spectra of the parent compound cGMP. Peaks found at or near this wavenumber are associated with stretching vibrations of free or hydrogen-bonded alcohol groups.[10,11] Loss of this alcohol peak was interpreted to be due to conjugation of the benzophenone moiety at the 2′-oxygen. Loss of the H-2′ was confirmed by ^{1}H NMR spectroscopy. A proton shift downfield from 4.42 ppm (presumably the H-2′ of cGMP) to 4.99 ppm (BBcGMP) was observed (data not shown). This proposed structure is consistent with observations from investigators who synthesized benzoylbenzoyl derivatives of nucleoside

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† Contribution from Connecticut Seed Ventures, Avon, CT, and a grant from the Indiana Lions Eye Bank.
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® Abstract published in *Advance ACS Abstracts,* June 15, 1994.

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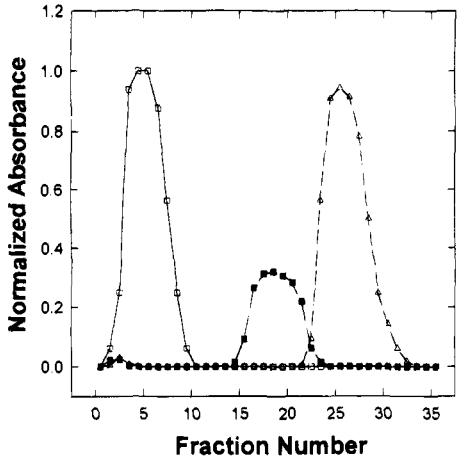

Figure 1. Elution pattern of reactants and product from C_{18}-derivatized silica column. Typically, 10 mL of dried reaction mixture was dissolved in 10% MeOH and applied to the column. Reactants and product were eluted with a 10–100% MeOH gradient. Column flow rate was approximately 2 mL/min; 4 mL fractions were collected; 1 μL of each fraction was applied to a lane of a silica gel TLC plate. Plates containing all lanes were developed, and spots of reactants and product were analyzed by image capture and analysis using Jandel Java image analysis software. Quantified spot densities are plotted according to spot R_f values. cGMP (R_f = 0.08; □—□) eluted as a broad peak first followed by a small peak of BBcGMP (R_f = 0.71; ■—■). Unreacted benzoylbenzoic acid (R_f = 0.91; △—△) eluted after almost all of the BBcGMP had eluted from the column.

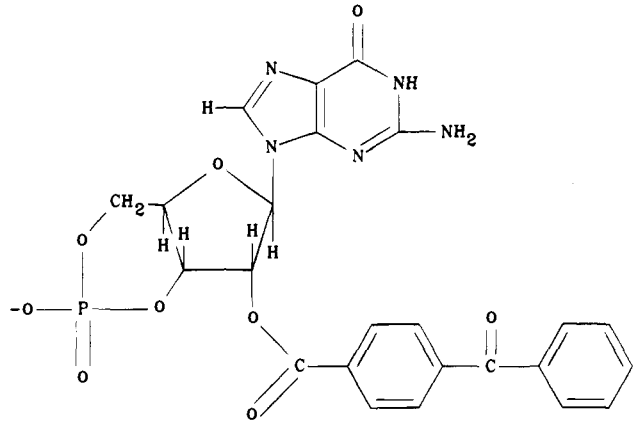

Figure 2. Structure of 2′-*O*-(4-benzoylbenzoyl)guanosine cyclic 3′,5′-monophosphate.

phosphates with unconstrained 3′-hydroxy groups.[9,12] (When noncyclic guanosine phosphate molecules are derivatized with 4-benzoylbenzoic acid, the initial site of covalent attachment is *via* an ester linkage through the 2′-oxygen; however, the covalent attachment migrates to the 3′-oxygen because of thermodynamic reasons. It is unlikely that this would occur in a molecule which already possesses an ester linkage at the 3′-position. Furthermore, this would create 3′-*O*-(4-benzoylbenzoyl)guanosine 5′-monophosphate, which migrates differently on our TLC system.)

Spectral Properties. The absorption spectrum of BBcGMP at pH 7.4 is shown in Figure 3. The spectrum of BBcGMP exhibits two maxima at 259 nm (ϵ = 38 500 M^{-1} cm^{-1}) and approximately 198 nm. No guanine nucleotide 280 nm shoulder on the 259 nm peak was observed in this compound,[4] presumably because of the high extinction coefficient of the 259 nm peak of BBcGMP. We attempted to decrease the 259 nm peak

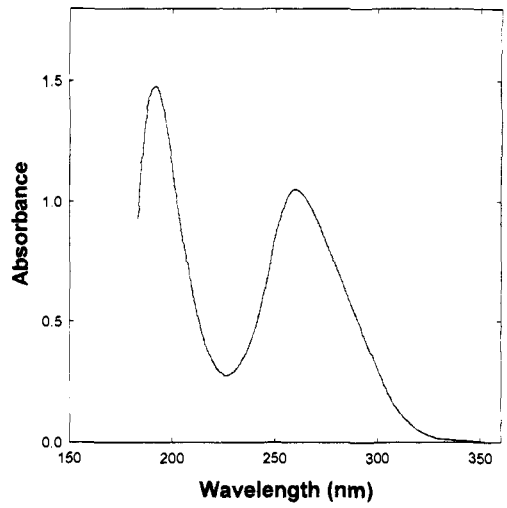

Figure 3. Absorbance spectrum BBcGMP. Sample and reference cuvettes were 10 × 10 mm^2 matched quartz cuvettes. The sample cuvette contained 27 μM BBcGMP dissolved in 4 mL of 10 mM HEPES, ph 7.4; the reference cuvette contained only the 10 mM HEPES buffer solution. Absorbance was determined using a Shimadzu UV-3000 dual beam, dual wavelength spectrophotometer in dual beam mode.

of BBcGMP by irradiating a 4 mL quartz cuvette containing 7 μM BBcGMP in 10 mM HEPES buffer (pH 7.4) with 254 nm light at a distance of 1 cm as demonstrated for 8-N_3cGMP by Gaehlen *et al.*,[3] but we were unable to do so. In fact, we observed a shift of the peak to about 252 nm accompanied by a slight (≤5%) increase in the extinction coefficient (data not shown). We cannot explain this phenomenon but presume that BBcGMP does not undergo a decrease in the peak thought to be responsible for photoreactivity because of the relatively low affinity that the benzoylbenzoyl moiety has for aqueous molecules such as H_2O.[13,14] Synthesis and application of benzoquinone-substituted ATP, GTP, 8-N_3ATP, and 8-N_3GTP have recently been reported by Rajagopalan *et al.*[15] Only derivatives containing N_3 were demonstrated to undero spectrophotometric changes correlated with UV-induced photodecomposition in water.

Phosphodiesterase Inhibitory Activity. BBcGMP was examined for its ability to act as substrate and/or to inhibit several cGMP and cAMP PDE molecules. The inhibitory activity of BBcGMP on the soluble rod outer segment (type III) PDE was examined using a variant of the technique of Yee and Liebman.[23] BBcGMP was a poor substrate for rod PDE, having a V_{max} of 1.1 mol of BBcGMP (mol of PDE)$^{-1}$ s^{-1}. By contrast, rod PDE has a robust V_{max} of (2.1–4.0) × 10^3 mol (mol of PDE)$^{-1}$ s^{-1} for the parent compound cGMP. The benzoylbenzoyl derivative proved to be a potent inhibitor of rod PDE. Figure 4 compares the effect of the apparent K_m and V_{max} of rod PDE for cGMP by 2 and 20 μM BBcGMP with that of 25 μM 8-Br-cGMP on a Lineweaver–Burke plot. Apparent K_m values were calculated from the x intercepts of the lines fitted to the data. The K_i value calculated (see figure legend for calculation) for 8-Br-cGMP was 5.9 μM. The averaged K_i for BBcGMP under these conditions was 0.3 μM. Both of the cGMP derivatives appeared to act as competitive inhibitors of rod cGMP PDE. The extremely low K_i of BBcGMP suggests that it can be bound by rod PDE (probably at the catalytic site) but is poorly hydrolyzed.

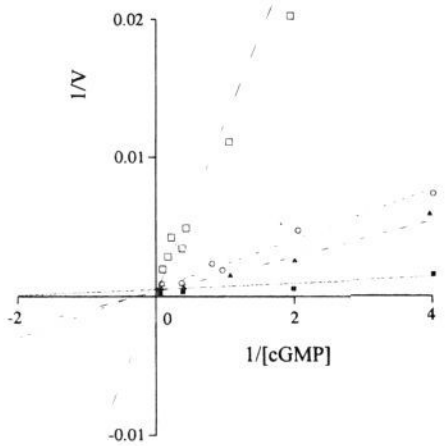

Figure 4. Lineweaver–Burke analysis of rod cGMP PDE inhibition by 8-Br-cGMP and BBcGMP. Inverse enzyme velocity ($1/V$, mol of cGMP hydrolyzed (mol of PDE)$^{-1}$ s^{-1}) was plotted as a function of inverse concentration (1/[cGMP] (1×10) M^{-1}). Inverse velocities were determined for several concentrations of cGMP in the absence (■–■) and presence of 25 μM 8-Br-cGMP (▲– –▲), 2 μM BBcGMP (○– –○), and 20 μM BBcGMP (□–□). The lines associated with each set of symbols were fit to the data by linear least-squares regression analysis.

Table 1. Concentrations for Half-Maximal Inhibition of Various PDE Preparations[a]

phosphodiesterase type	IC_{50} (μM)
rat bladder dome cAMP	15.8
rat bladder dome cGMP	0.3
human platelet cAMP	3.4

[a] Rat bladder PDE was assayed per Wells *et al.*[24] Human platelet PDE activity was assayed as described by Thompson *et al.*[11]

The effect of BBcGMP on other PDE types was tested. Table 1 shows the effectiveness of BBcGMP as an inhibitor in three different PDE systems. BBcGMP effectively inhibited both cAMP and cGMP PDEs. The K_i or IC_{50} of the derivative is less than 1 μM for cGMP PDE preparations. While BBcGMP inhibited cAMP PDE systems, the K_i or IC_{50} for BBcGMP in such enzyme systems was consistently 10–50 times higher than that for cGMP PDE systems.

Photoaffinity Labeling of Rod PDE by [α-^{32}P]-BBcGMP. The benzophenone moiety of BBBcGMP provides it with UV light-induced photoreactivity by formation of a highly reactive diradical triplet state at the ketone group separating the phenyl groups (see Figure 2). The benzoylbenzoyl moiety exhibits photoreactivity preferentially toward organic molecules rather than toward aqueous molecules. By contrast, azido derivatives, when photoactivated, can react with aqueous molecules such as H_2O, lowering the efficiency with which the dehydroazepine intermediate covalently links with organic molecules.[3,14] This makes benzoylbenzoyl nucleotide derivatives extremely valuable photoaffinity agents.[3,12]

Given the high affinity for BBcGMP demonstrated by rod light-activated PDE, we attempted to photolabel this enzyme. In the experiment shown in Figure 5, specific labeling of rod cGMP PDE from toad rod outer segments by [α-^{32}P]BBcGMP is shown in lane B.[16–18] Addition of 50 μM unlabeled cGMP decreased the [α-^{32}P]BBcGMP labeling of PDE to approximately 8% of that observed in lane B (lane C), indicating that the labeling of PDE

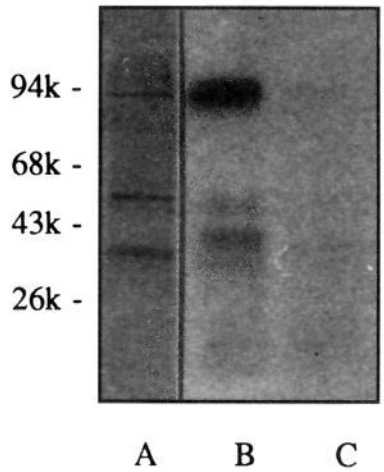

Figure 5. Photoaffinity labeling of rod disk membrane cGMP PDE. [α-^{32}P]BBcGMP was incubated with 100 μg (total protein; other soluble nucleotide-binding proteins included transducin, cGMP-dependent protein kinase, α- and β-tubulin, and myosin) of partially purified rod PDE. Coomassie blue stained and autoradiograph lanes were analyzed by image capture and analysis using Jandel Java image analysis software. Peaks were digitized, and integrated densities were calculated to determine relative protein amounts and [α-^{32}P]-BBcGMP labeling. Analysis of PAGE gels of the partially purified PDE determined that PDE constituted approximately 3.5% of the total protein. Reaction conditions and subsequent workup are described in the Experimental Section. Lane A: Coomassie blue stained PAGE of low-ionic strength purified PDE. The protein with molecular weight of approximately 92 kDa corresponds to the α-subunit of PDE. Lane B: autoradiograph of PDE treated with [α-^{32}P]BBcGMP. Lane C: autoradiograph of PDE treated with [α-^{32}P]BBcGMP in the presence of 50 μM cGMP.

was specific to a domain that binds cGMP. Much higher concentrations of GMP (1 mM), GDP (1 mM), or cAMP (500 μM) were required to significantly decrease labeling by [α-^{32}P]BBcGMP (data not shown). Figure 6 shows the degree of UV-induced fixation of [α-^{32}P]BBcGMP to rod outer segment (ROS) proteins as a function of irradiation time. Fixation of [α-^{32}P]BBcGMP increased linearly with irradiation time and saturated at approximately 300–400 s. The apparent maximal ratio of ligand to protein fixed as determined by regression analysis was 2.56 mmol/mol of protein. This ratio is somewhat low when compared to fixation ratios of other nucleotide photoaffinity analogs.[13] However, the major protein constituent of ROS membranes, rhodopsin, constitutes approximately 80–90% of the total protein found in ROS. Rod cGMP PDE is ≤1% of the total protein in the outer segment. Thus, it is possible that the ratio of ligand to actual substrate enzyme(s) is much higher.

Discussion

BBcGMP was initially developed as a photoaffinity probe for the rod light-sensitive cation channel. However, the affinity of the channel appears to be higher for 8-substituted and lower for 2′-substituted cGMP derivatives.[5,7,8,19] In patch clamp experiments, the concentration of BBcGMP that elicits a half-maximum increase in the chord conductance of the toad outer segment cGMP-sensitive cation channel is approximately 150 μM, almost 10-fold higher than the K_m of 17 μM for cGMP (unpublished results). Therefore, one

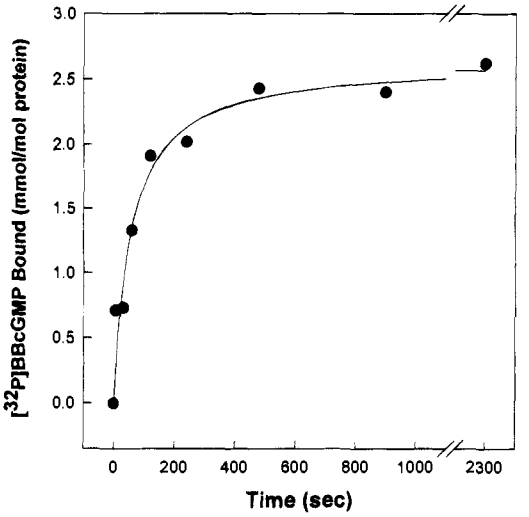

Figure 6. Dependence of [α-^{32}P]BBcGMP fixation to ROS membranes on UV irradiation time. [α-^{32}P]BBcGMP was incubated with ROS membranes for 5 min and irradiated for the indicated times. Reaction conditions and subsequent workup are described in the Experimental Section. Fixation amount was calculated by subtracting control (unirradiated) sample radioactivity from irradiated sample radioactivity and comparing sample radioactivity with that of known amounts of similarly quenched [α-^{32}P]BBcGMP. Each data point is the average of duplicate samples. The data were well fit by a single exponential function with τ = 56 s and a maximum ligand fixation ratio of 2.56 mmol/mol of protein.

would not expect BBcGMP to serve as a useful photoaffinity probe of the rod outer segment cGMP-gated cation channel.

Fortunately, BBcGMP has proven to be an extremely potent inhibitor of the light-activated rod PDE with a K_i at least 10-fold lower than those of other known inhibitors. The inhibitory capability of this compound is not limited to this enzyme. PDE molecules belonging to other than the type III family are also inhibited by low concentrations of BBcGMP. BBcGMP has also proved useful as a high-affinity cGMP photoaffinity analog. Given these two complementary uses, BBcGMP (or other BB-derivatized cyclic nucleotides) could serve as a powerful tool to probe the identity, structure, and mechanism of action of a variety of cyclic nucleotide phosphodiesterases.

It may also be possible to utilize this compound or class of compounds *in vivo*, as either a diagnostic or curative agent. For example, it has long been known that PDE inhibitors such as theophylline are useful in the treatment of asthma and related conditions. A recent study has shown that 8-Br-cGMP, when applied topically to the eye of the rabbit, can cause significant decreases in intraocular pressure.[20] Thus, it is possible that PDE inhibitors such as BBcGMP may be useful in the reduction of intraocular pressure in glaucoma. Indeed, preliminary studies using rabbits with artificially induced ocular hypertension as a model for glaucoma[21] indicate that topical application of BBcGMP can substantially lower intraocular pressure for substantial lengths of time.[22] It is likely that further studies of this class of compounds will reveal additional uses for this type of PDE inhibitor.

Experimental Section

Materials. cGMP was purchased from Sigma. 1,1'-Carbonyldiimidazole and 4-benzoylbenzoic acid were purchased from Aldrich. Octadecyl (C_{18})-derivatized silica gel for reverse-phase flash chromatography was obtained from Sigma. Kieselgel 60 F_{254} silica TLC plates were obtained from EM Science (Cherry Hill, NJ). All other reagents were of reagent or biochemical research grade.

TLC. All TLC was performed with 1-butanol:CH_3COOH:water (5:2:3, v/v/v) on silica gel. Compounds were detected on chromatograms using a Mineralight Model UVG-11 short wavelength UV lamp. Spots on TLC were subjected to video image capture and analysis using an Imaging Technology frame capture board and Jandel Scientific Java image analysis software (object analysis and integrated intensity mode) to determine elution of reactants/product from the reverse-phase column and purity of the analog.

Chemical Synthesis of 2'-*O*-(4-benzoylbenzoyl)guanosine Cyclic 3',5'-Monophosphate. cGMP (Na salt, 1 g) was dissolved in 20 mL of water at 25 °C. 1,1'-Carbonyldiimidazole (2 g) and 4-benzoylbenzoic acid (1 g) were dissolved in 10 mL of dimethylformamide (DMF). The DMF solution was poured slowly into the cGMP solution while continuously stirring. The mixture was stirred overnight in darkness. Products were then aliquotted into shell vials and vacuum-centrifuged for 4–6 h. The resulting oil was dissolved in 20 mL of water, this solution was extracted three times with an equal amount of 1-butanol to remove the carbonyldiimidazole. A 10% aqueous methanol solution was added to the aqueous phase, and this solution was applied to a C_{18} silica reverse-phase column (5 × 15 cm^2) equilibrated with 10% methanol. The column was eluted with a 10–50% methanol gradient at a flow rate of 60 mL/h. Fractions were taken every 4 min (4 mL fractions). Aliquots of each fraction (0.2–0.5 μL) were chromatographed on silica gel. The order of elution was cGMP, BBcGMP, 4-benzoylbenzoic acid. Peak fractions containing BBcGMP were pooled and vacuum-centrifuged to dryness. Yield expressed as a percentage of cGMP was typically 25–35%. The pooled, dried BBcGMP was checked for purity by TLC; R_f values on silica gel were cGMP, 0.08; BBcGMP, 0.71; benzoylbenozic acid, 0.95. In addition, [α-^{32}P]BBcGMP was synthesized from [α-^{32}P]cGMP (see below), purified in the same manner, and chromatographed by TLC on silica gel. Regions of the plate corresponding to the R_f values of the reactants and product were scraped into vials containing scintillation cocktail (Amersham) and counted. Purity of the radiolabeled analog was determined to be better than 99% by this method. The molecular formula of BBcGMP is $C_{24}H_{19}N_5O_9P$, and its formula weight is 553.

Synthesis and Purification of ^{32}P-Labeled BBcGMP. [α-^{32}P]BBcGMP was prepared in the same manner but in smaller amounts. [α-^{32}P]cGMP (10 Ci/mmol; ICI Biomedicals) was dissolved in 1 mL of H_2O and added dropwise to 1 mL of DMF containing 0.1 g of 1,1-carbonyldiimidazole and 0.05 g of 4-benzoylbenzoic acid. All succeeding purification steps were performed in a manner similar to that described above but on a 10-fold smaller scale.

Enzyme Assay. Rod outer segment soluble phosphodiesterase activity in the presence of photoactivated rhodopsin and transducin was determined by measuring proton evolution resulting from hydrolysis of cGMP to GMP.[23] Enzyme velocities were determined by calibration of initial slopes with a known concentration of HCl. Guinea pig soluble and particulate cAMP PDE activities were assayed as per Wells *et al.*[24] Human platelet PDE activity was assayed as described by Thompson *et al.*[25]

Photoaffinity Labeling of Rod Outer Segment cGMP-Binding Proteins—Autoradiography. Partially purified rod phosphodiesterase was prepared by sequential high- and low-ionic strength washes of rod outer segment membranes as previously described.[26] Partially purified PDE was incubated with 750 nM [α-^{32}P]BBcGMP (20 Ci/mmol, 0.25 μCi) at 20 °C for 2 min in the presence or absence of 50 μM cGMP. The reaction mixture was irradiated at 4 °C for 60 s at a distance of 7 cm with short wavelength UV light (254 nm, 500 μW/cm^2; Model UVG-54; UVP, Inc., San Gabriel, CA). The reaction was quenched by addition of 5 mM dithiothreitol and

10 mM Tris, pH 7.4. Samples were run on SDS–PAGE gels as described by Laemmli.[27] The concentration of the resolving gel was 9.5% acrylamide. Gels were stained with Coomassie brilliant blue, dried, and autoradiographed with Kodak X-Omat film.

Photoaffinity Labeling of Rod Outer Segment cGMP-Binding Proteins—Filtration. Bovine rod outer segment vesicles were prepared as described previously.[28] Aliquots (100 μL) of an incubation buffer (10 mM Tris, 100 mM NaCl, pH 7.4) containing a ROS vesicle suspension ([rhodopsin] = 50 μM) were incubated in the presence of 750 nM [α-^{32}P]BBcGMP (20 Ci/mmol, 0.25 μCi) at 4 °C for 5 min. Samples were then irradiated with UV light under the conditions described above for 0–2300 s. Fixation was stopped by removing samples from irradiation and mixing with an excess of 10% trichloroacetic acid. Samples were then filtered on 25 mM Whatman GFA filters in a Millipore vacuum manifold and washed twice with 3 mL of the incubation buffer. The filters were then dried, added to scintillation cocktail, and counted in a Packard scintillation counter.

Acknowledgment. The authors thank Marsha Wheeler for determining the inhibitory effect of BB-cGMP on rat bladder dome PDE and Dr. Mark Rasenick for platelet PDE assays. We also thank Dr. William Miller for insightful comments and Dr. Marvin Sears for the supportive environment in which much of this study was performed.

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