Identification of Competitive Antagonists of the Rod Photoreceptor cGMP-Gated Cation Channel: β-Phenyl-1,N^2-etheno-Substituted cGMP Analogues as Probes of the cGMP-Binding Site†

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ABSTRACT: cGMP is the natural activator of the cyclic nucleotide-gated channel originally isolated from rod photoreceptors but now known to be expressed in a wide variety of neural and non-neural cells. To identify antagonists of cGMP action and to better understand the interaction between cGMP and the channel protein, experimental studies were undertaken using four synthetic cGMP analogues, PET-cGMP, 8-Br-PET-cGMP, Rp-8-Br-PET-cGMPS, and Sp-8-Br-PET-cGMPS. With excised patches from either *Xenopus* oocytes expressing a cloned rat rod channel α-subunit or from native *Xenopus* rod photoreceptors, Rp-8-Br-PET-cGMPS competitively suppressed the cGMP-induced current with an IC_{50} of 25 μM and Sp-8-Br-PET-cGMPS inhibited this current with an IC_{50} of 105 μM. On the expressed rat rod channel, 8-Br-PET-cGMP behaved as a very weak partial agonist at high concentrations and an antagonist (IC_{50} = 64 μM) at lower concentrations when coapplied with cGMP. PET-cGMP did not activate channel currents alone but showed synergism when coapplied with subsaturating concentrations of cGMP. Because Sp-8-Br-PET-cGMPS is a potent activator of type I cGMP-dependent protein kinase, but a competitive antagonist of channel activation, it will be a useful reagent for discriminating between those effects of cGMP that are mediated by a protein kinase and those mediated by channel activation. Because the PET derivatives all contain a phenyl-substituted 5-membered ring system fused to the amino group in position 2 and the nitrogen in position 1 of the guanine ring, the results support the idea that N1 and N2 are important for channel activation. They also suggest a minor role for the cyclic phosphate group in binding or activation.

Cyclic nucleotide guanosine-3′,5′-cyclic monophosphate (cGMP)[1] exerts its physiological effect primarily through two classes of molecules, cGMP-dependent protein kinases (PKGs) and cyclic nucleotide-gated nonselective cation channels (CNGCs) (Schulman, 1995; Barnstable, 1993). CNGCs were originally isolated from rod and cone photoreceptors and olfactory receptor cells. Molecular studies have established that the three subtypes are encoded by distinct members of a gene family (Kaupp et al., 1989; Dhallan et al., 1990; Bonigk et al., 1993; Barnstable & Wei, 1995). Each appears to be a hetero-oligomer, probably a tetramer (Liu et al., 1996; Varnum & Zagotta, 1996), composed of α-subunits that can form active channels when expressed alone and β-subunits that do not form active channels when expressed alone but can modulate the channel properties of the α-subunits (Chen et al., 1993, 1994; Liman & Buck, 1994; Bradley et al., 1994). The exact stoichiometry of the subunits has yet to be elucidated. CNGCs of the rod and cone type are activated only by physiological concentrations of cGMP, while the olfactory type of channel is also activated by cAMP (Fesenko et al., 1985; Nakamura & Gold, 1987).

Recently, it has been shown that CNGCs are expressed in many other types of mammalian CNS neurons (Nawy & Jahr, 1990; Ahmad et al., 1994; Leinders-Zufall et al., 1995b; Kingston et al., 1996) and other tissues and cells such as heart, kidney, lymphocytes, and sperm (Ahmad et al., 1990, 1992; DiFrancesco & Tortora, 1991; Biel et al., 1994; Weyand et al., 1994; Yau, 1994; Finn et al., 1996). These channels may be important mediators of the response to diffusible messengers such as nitric oxide and carbon monoxide and may represent an important pathway for the entry of extracellular calcium into cells (Shiells & Falk, 1992; Ahmad et al., 1994; Leinders-Zufall et al., 1995a). CNGCs have been implicated in the modulation of synaptic efficacy during normal neural function as well as in pathophysiological processes following ischemia and other insults (Rieke & Schwartz, 1994; Zufall, 1996).

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[1] Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; 8-Br-cGMP, 8-bromo-cGMP; PET-cGMP, β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphate; 8-Br-PET-cGMP, β-phenyl-1,N^2-etheno-8-bromoguanosine 3′,5′-cyclic monophosphate; Sp- or Rp-8-Br-PET-cGMPS, β-phenyl-1,N^2-etheno-8-bromoguanosine 3′,5′-cyclic monophosphorothioate (*S*p or *R*p isomer, respectively); PKG, cGMP-dependent protein kinase; CNGC, cyclic nucleotide-gated nonselective cation channel.

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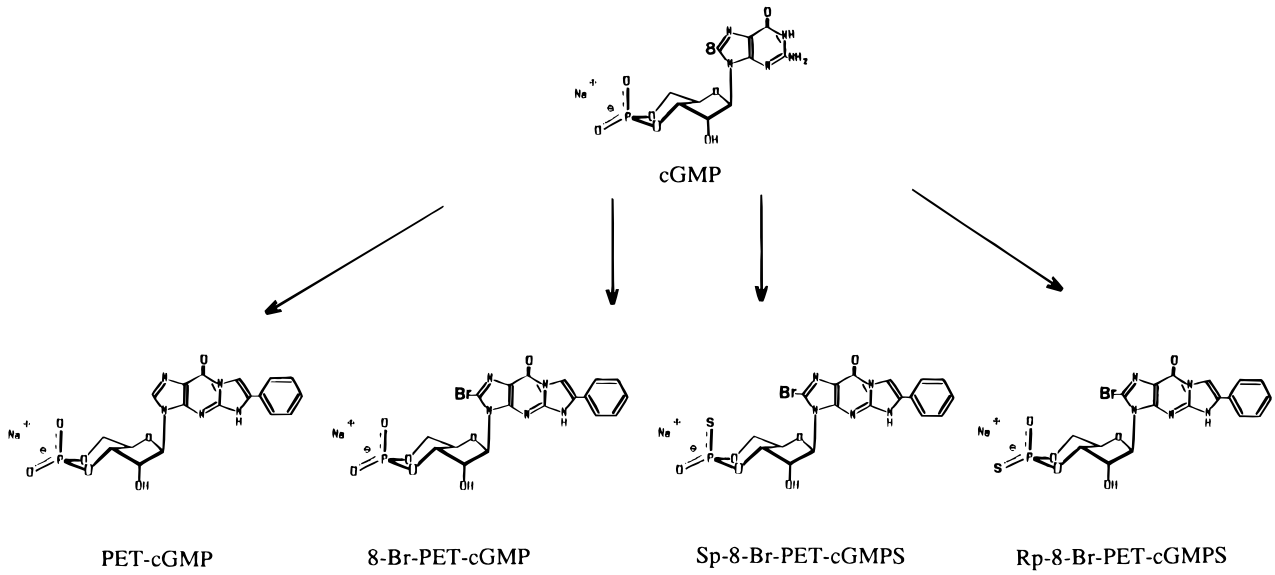

FIGURE 1: Chemical structures of cGMP and its derivatives PET-cGMP, 8-Br-PET-cGMP, Sp-8-Br-PET-cGMPS, and Rp-8-Br-PET-cGMPS.

CNGCs are thought to undergo simple transitions between open and closed states, with no evidence for inactive or desensitized states (Yau & Nakatani, 1985; Baylor & Nunn, 1985; Matthews & Watanabe, 1987; Hodgkin & Nunn, 1988; Karpen et al., 1988; Goulding et al., 1994). Binding of cGMP stabilizes the open state, although the cooperative nature of the dose−response curve suggests interaction between binding sites and the need for multiple cGMP molecules to support channel opening (Zimmerman & Baylor, 1986). It is possible to analyze the detailed interactions of cGMP with its binding site because there is a good structural model for this domain of CNGCs. Cyclic nucleotide binding sites of both CNGCs and cGMP-dependent protein kinases show substantial sequence homology with a bacterial cAMP binding protein whose structure has been determined by X-ray crystallography (Weber & Steitz, 1987; Weber et al., 1989; Kumar and Weber, 1992). The binding site consists of a pocket formed by eight β-strands with its bottom formed by α-helices. A number of previous studies of the nucleotide−protein interactions in CNGCs have utilized site-modified cGMP ligands. Zimmerman et al. (1985) reported that 8-Br-cGMP, which introduced bromine at C8 position of the guanine ring, was more effective than cGMP. They also found that Sp-cGMPS and Rp-cGMPS, which are modified at the oxygen atom at the cyclic phosphate, were less effective than cGMP at activating the rod CNGC. Two cAMP derivatives, Rp-cAMPS and Rp-8-pCPT-cAMPS, antagonize cGMP activation of rod CNGCs with low potency and were not able to totally block activation by cGMP (Kramer & Tibbs, 1996). While these results have shown that modification of different positions on the cGMP molecule can alter the overall efficiency of channel activation, there has not been a systematic investigation of the effects of the different substituents. In an alternative approach, in vitro mutagenesis showed that substitution of an uncharged residue for an aspartate in the binding site α-helix decreased the open probability of the channel, suggesting that interaction between this residue and cGMP stabilized the open state (Varnum et al., 1995).

To investigate the role of different sites on the cGMP molecule, we have measured the effects of a related series of substituted cGMP molecules on the activity of a cloned rat rod CNGC α-subunit. Different substitutions were found to affect the ability of the cGMP derivative to bind to the channel and to stabilize the open state. Both Rp-8-Br-PET-cGMPS and Sp-8-Br-PET-cGMPS were found to be antagonists of channel activation. Since Sp-8-Br-PET-cGMPS is a potent activator of type I cGMP-dependent protein kinase, it can thus distinguish between actions of cGMP on these two classes of effector molecules.

MATERIALS AND METHODS

Specific Site Modified cGMP Analogues. cGMP and 8-Br-cGMP were obtained from Sigma. All other analogues of cGMP were synthesized by BIOLOG Life Science Institute (Bremen, Germany) using methods described elsewhere (Sekhar et al., 1992; Butt et al., 1995). The structures of the cGMP analogues PET-cGMP, 8-Br-PET-cGMP, Sp-8-Br-PET-cGMPS, and Rp-8-Br-PET-cGMPS are shown in Figure 1. PET-cGMP has been modified by a phenyl-substituted five-membered ring system fused to the amino group in position 2 and the nitrogen in position 1 of the purine ring. 8-Br-PET-cGMP also has a hydrogen in position 8 of the nucleobase substituted by bromine. Finally, the equatorial or axial exocyclic oxygen atom of the cyclic phosphate moiety was replaced by sulfur to give Rp-8-Br-PET-cGMPS or Sp-8-Br-PET-cGMPS. These compounds were stored as aliquots at −80 °C. Experimental solutions were prepared freshly from the stocks.

RNA Expression and Patch-Clamp Recording from Cloned Rat Rod cGMP-Gated Channel in Xenopus Oocytes. The cloned rat rod CNGC has been described elsewhere (Barnstable & Wei, 1995). The full length sequence was subcloned into a high-expression vector, PGEM-HE, that contains the 5′- and 3′- untranslated regions of the *Xenopus* β-globin gene flanking the polylinker (Liman & Buck, 1994). Plasmids were linearized with restriction endonuclease *Nhe*I (New England Biolabs, Beverly, MA) and then RNA transcribed *in vitro* by T7 RNA polymerase in the presence of 2 mM m7G(5′)ppp(5′)G (Ambion Inc., Austin, TX). RNA was precipitated and resuspended in diethyl pyrocarbonate-treated water. Oocytes were surgically removed from

Xenopus (*Xenopus* I, Ann Arbor, MI) after anaesthesia using 1.5 g/L tricaine (Sigma), and then incubated in 2 mg/mL type IA collagenase (Sigma) for 2 h in OR_3 medium (50% v/v L-15 medium, 30 μM HEPES, pH 7.5, 0.2 mg/mL gentamycin, and 50 U/mL nystatin) followed by extensive washing with OR_3. Fifty nanoliters of a 1 μg/μL solution of RNA was injected into each *Xenopus* oocyte using a Drummond microdispenser. Injected cells were cultured in OR_3 for 3−5 days. The follicular cell layer and vitelline membrane were removed from the oocytes prior to patch-clamp recording by incubating for 5 min in hyperosmotic stripping solution (200 mM KAsp, 20 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.4, and osomolarity 475 mos).

Patch-clamp experiments were performed in the standard inside-out configuration. Membrane patches were excised from oocytes, using 6−8 MΩ borosilicate patch electrodes, and recorded in the inside-out configuration at room temperature (22 °C). The internal and external solutions both contained 130 mM NaCl, 3 mM HEPES (pH 7.2), and 0.2 mM EDTA. A Ag−AgCl wire connected the bath solution to ground. Test solutions were locally applied to excised patches using a 12-channel gravity-fed microperfusion system combined with simultaneous bath perfusion. Inhibition curves were obtained by coapplication of saturating levels of cGMP with an increasing concentration of analogues. Wash times between each application were 1−2 min. Macroscopic patch currents were recorded in the mixed mode using a Dagan 3900A integrating patch-clamp amplifer (Dagan Corp., Minneapolis, MN), low-pass Bessel filtered at 2 kHz, and stored at 18.5 kHz using a digital tape recorder−VCR unit (Instrutech model VR10B, Great Neck, NY). The current record was also continuously monitored using a Gould model 220 chart recorder (Gould Corp., Cleveland, OH). Voltage ramps were generated using a Labmaster TL-1 DMA interface running pCLAMP6 software (Axon Instruments, Foster City, CA) on a 486DX2 computer. Voltage ramps in the presence of cGMP and its analogues were delivered from a standard holding potential of −70 mV and were leak-subtracted with drug-free controls.

Single-channel recordings were 4 pole Bessel filtered at 1 kHz and sampled at 20 kHz. Single-channel conductances were estimated from the Gaussian least-square fits to an all-points amplitude histogram using a Levenberg−Marquardt algorithm.

Photoreceptor Outer Segment Dissociation and Patch-Clamp Recording from Xenopus Retinal Rods. The native photoreceptor cGMP-gated channel was studied in acutely dissociated rods from *Xenopus.* After dark adaptation for more than 1 h, *Xenopus* were killed by decapitation and pithed. Eyes were dissected into frog Ringer's solution consisting of (in millimolar) 112 NaCl, 2.5 KCl, 1.6 $MgCl_2$, 1.0 $CaCl_2$, 10 glucose, and 10 HEPES at pH = 7.4, and retinas were removed carefully. Several 12 mm glass coverslips in a dish were precoated with poly(L-lysine) (1 mg/mL in water) and rinsed with Ringer's solution. Isolated retinas were teased apart with fine needles or chopped with a razor blade in the dishes and incubated for 20−30 min to allow intact rod outer segments to settle on the coverslips. Rods were continuously perfused with Ringer's solution before recording. cGMP-activated currents were measured with the following solution in the patch pipette and experimental chamber: 115 mM NaCl, 5 mM HEPES (pH 7.6), and 0.2 mM EDTA. The other experimental procedures were as above.

Data Analysis. IC_{50} curves were normalized to saturating doses of cGMP (100 μM in rat rod α-subunit expressed in *Xenopus* oocytes) and fitted to a power logistic function using ORIGIN software (Microcal, Northhampton, MA). Agonist dose−response curves were fitted to the Hill logistic equation. Points represent mean ± SD.

RESULTS

We have previously shown that inside-out patches isolated from *Xenopus* oocytes injected with RNA transcribed from a full length rat rod CNGC clone developed a large conductance increase when cGMP was applied and that these responses were not observed in uninjected oocytes (Barnstable & Wei, 1995). The EC_{50} and saturating concentrations for cGMP in these experiments were approximately 42 and 100 μM, respectively. The response properties were sustained and did not desensitize or run down over the time course of the experiments.

cGMP-Induced Currents of Rat Rod CNGC α-Subunits Expressed in Xenopus Oocytes Can Be Totally Blocked by Rp- and Sp-8-Br-PET-cGMPS. To identify possible antagonists of the rod CNGC, we first examined actions of componds Rp-8-Br-PET-cGMPS and Sp-8-Br-PET-cGMPS. Neither compound alone at 1−2 mM induced detectable currents. To test the antagonist action of these compounds, we applied them to isolated patches from *Xenopus* oocytes held at −70 mV in which inward sustained currents had been previously induced by bath application of a nearly saturating concentration of cGMP (100 μM). This current was rapidly and completely blocked by coapplication of 300 μM Rp-8-Br-PET-cGMPS (Figure 2A), and the effect was fully reversible upon removal of the antagonist. When cGMP was also removed from the bath, the current gradually disappeared. We next examined whether the stereoisomer, Sp-8-Br-PET-cGMPS, could also act as an antagonist. Figure 2B shows that 500 μM Sp-8-Br-PET-cGMPS totally suppressed the responses induced by 100 μM cGMP.

The dose dependence of the antagonism by Rp- and Sp-8-Br-PET-cGMPS was quantified by fitting a power logistic equation to the percentage of current remaining at different concentrations of Rp- and Sp-8-Br-PET-cGMPS. The data and fitted smooth curves are shown in Figure 2C. The curve for Rp-8-Br-PET-cGMPS yielded an IC_{50} of 25 μM at saturating concentrations of cGMP (100 μM), whereas the curve for Sp-8-Br-PET-cGMPS yielded an IC_{50} of 105 μM at saturating cGMP.

Rp-8-Br-PET-cGMPS Is a Competitive Inhibitor of cGMP at the cGMP-Binding Site of the Rod CNGC α-Subunit. To determine whether the antagonism of Rp-8-Br-PET-cGMPS was competitive with cGMP, we carried out a similar experiment but with a supersaturating concentration of cGMP (500 μM). Under these conditions, 100 μM Rp-8-Br-PET-cGMPS, which had caused approximately 90% inhibition of the response to 100 μM cGMP, caused only 5% inhibition (n = 3) (Figure 3A). This indicates that cGMP and Rp-8-Br-PET-cGMPS were acting competitively, most probably at the cGMP-binding sites of the CNGC. To confirm that Rp-8-Br-PET-cGMPS is a binding site antagonist rather than an open channel blocker, we first induced currents using 100 μM cGMP to confirm the presence of active channels. After

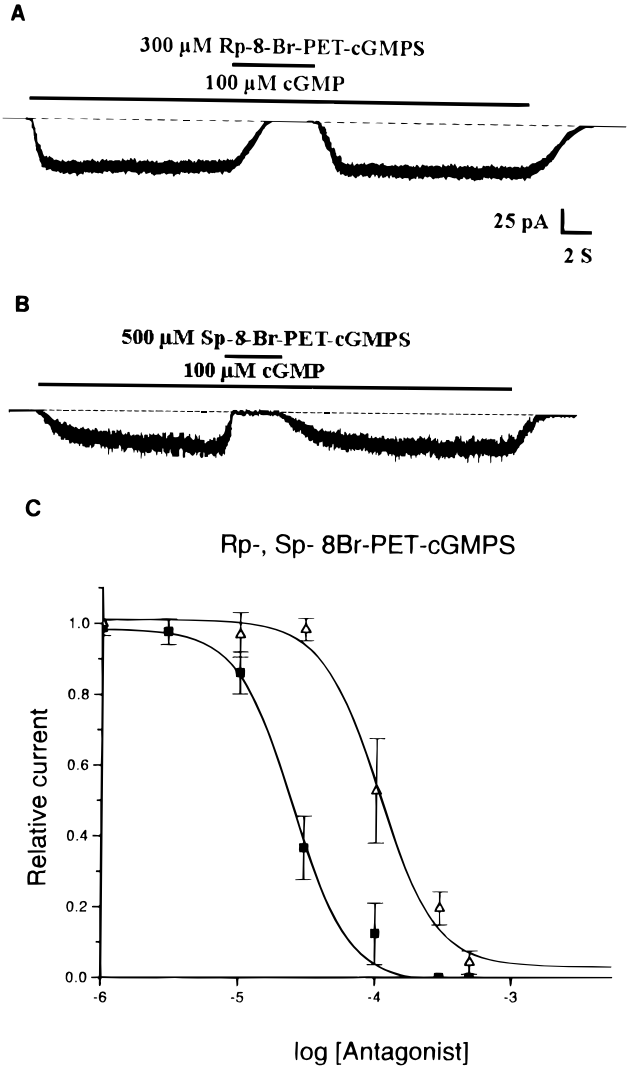

FIGURE 2: Rp- and Sp-8-Br-PET-cGMPS inhibit the activity of rat rod cGMP-gated channel α-subunits expressed in *Xenopus* oocytes. (A) Rp-8-Br-PET-cGMPS blocks the cGMP-induced current from excised patches. When 100 μM cGMP was applied to an inside-out patch, a large inward current was observed at −70 mV. In the presence of cGMP, the inward current could be rapidly blocked by coperfusion of 300 μM Rp-8-Br-PET-cGMPS. Upon washout of Rp-8-Br-PET-cGMPS, the cGMP induced inward current fully recovered. (B) Inhibition of cGMP responses in excised patches by Sp-8-Br-PET-cGMPS. Currents induced by 100 μM cGMP were completely blocked by 500 μM Sp-8-Br-PET-cGMPS. Holding potential, −70 mV. Upon washout of Sp-8-Br-PET-cGMPS, the cGMP-induced inward current fully recovered. (C) Dose−response curves of inside-out patch currents of 100 μM cGMP coapplied with increasing concentrations of Rp-8-Br-PET-cGMPS (solid squares) and Sp-8-Br-PET-cGMPS (open triangles). The cGMP-induced inward currents were reduced by half (IC_{50}) at a concentration of 25 μM Rp-8-Br-PET-cGMPS ($n = 5$) and 105 μM Sp-8-Br-PET-cGMPS ($n = 5$).

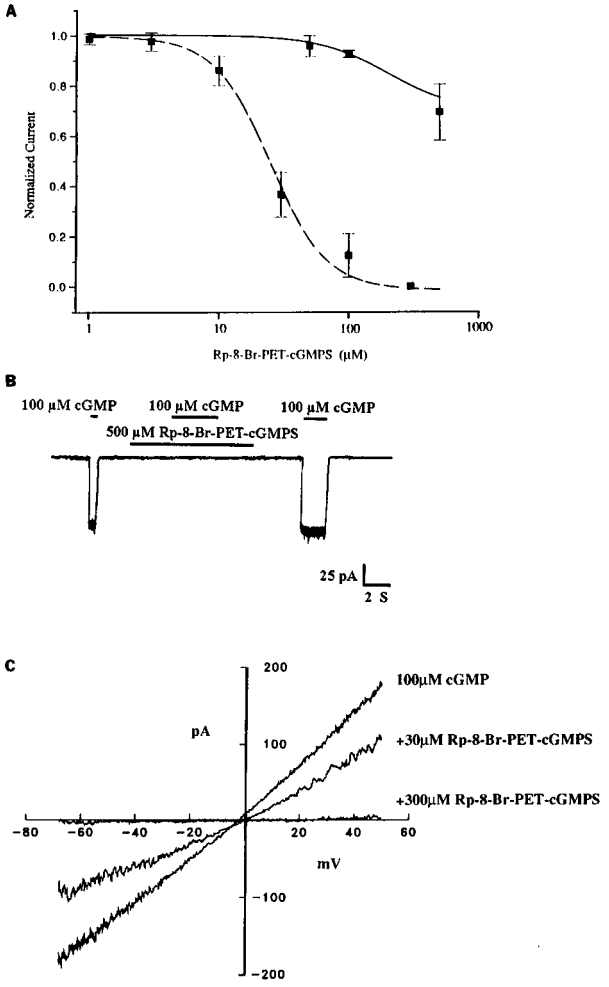

FIGURE 3: Rp-8-Br-PET-cGMPS is a competitive antagonist of cGMP for the rat rod CNGC α-subunit. (A) Comparison of dose−response relations for 100 μM cGMP (dashed line) and 500 μM cGMP (solid line) coapplied with increasing concentrations of Rp-8-Br-PET-cGMPS. When supersaturating doses of cGMP (500 μM) were used, the inhibitory effects were reduced ($n = 3$). (B) Applications of 100 μM cGMP induced a sustained inward current at −70 mV. After washout, 500 μM Rp-8-Br-PET-cGMPS was applied but no inward current was observed. During this period, a pulse of 100 μM cGMP in 500 μM Rp-8-Br-PET-cGMPS was applied. No transient current was induced by this procedure. Upon washout, the response to 100 μM cGMP fully recovered. (C) Current−voltage relation of the current induced by 100 μM cGMP in the absence and in the presence of 30 and 300 μM Rp-8-Br-PET-cGMPS. In the control condition, the cGMP-induced current reversed near 0 mV. This current was partially and completely blocked in the presence of 30 and 300 μM Rp-8-Br-PET-cGMPS and showed voltage-independent inhibitions. Patch currents have been leak-subtracted.

washout, the patch was bathed in 500 μM Rp-8-Br-PET-cGMPS, and during this time, a pulse of 100 μM cGMP in 500 μM Rp-8-Br-PET-cGMPS was applied. Within the resolution of the experimental method, no transiently decaying currents were induced by this procedure as might be expected if Rp-8-Br-PET-cGMPS required channel opening prior to its blocking action. After Rp-8-Br-PET-cGMPS washout, the currents could again be induced by 100 μM cGMP, indicating that all the effects were reversible, as shown in Figure 3B. A further indication that Rp-8-Br-PET-cGMPS was not acting as a channel blocker is that the block showed no voltage dependence, as shown in Figure 3C. The block by Sp-8-Br-PET-cGMPS showed similar competitive behavior and lack of voltage dependence (data not shown).

The suppression of rod CNGC currents by Rp-8-Br-PET-cGMPS could involve two possible mechanisms: a decrease in the open probability of a single CNGC or a decrease in single-channel conductance. To distinguish these mechanisms, we examined single-channel currents with and without a low concentration of Rp-8-Br-PET-cGMPS sufficient to suppress approximately half of the patch current induced by 100 μM cGMP. Figure 4A shows a recording from an oocyte patch containing only one rod CNGC. Coapplication of 40 μM Rp-8-Br-PET-cGMPS and 100 μM cGMP increased the interval between channel openings and decreased

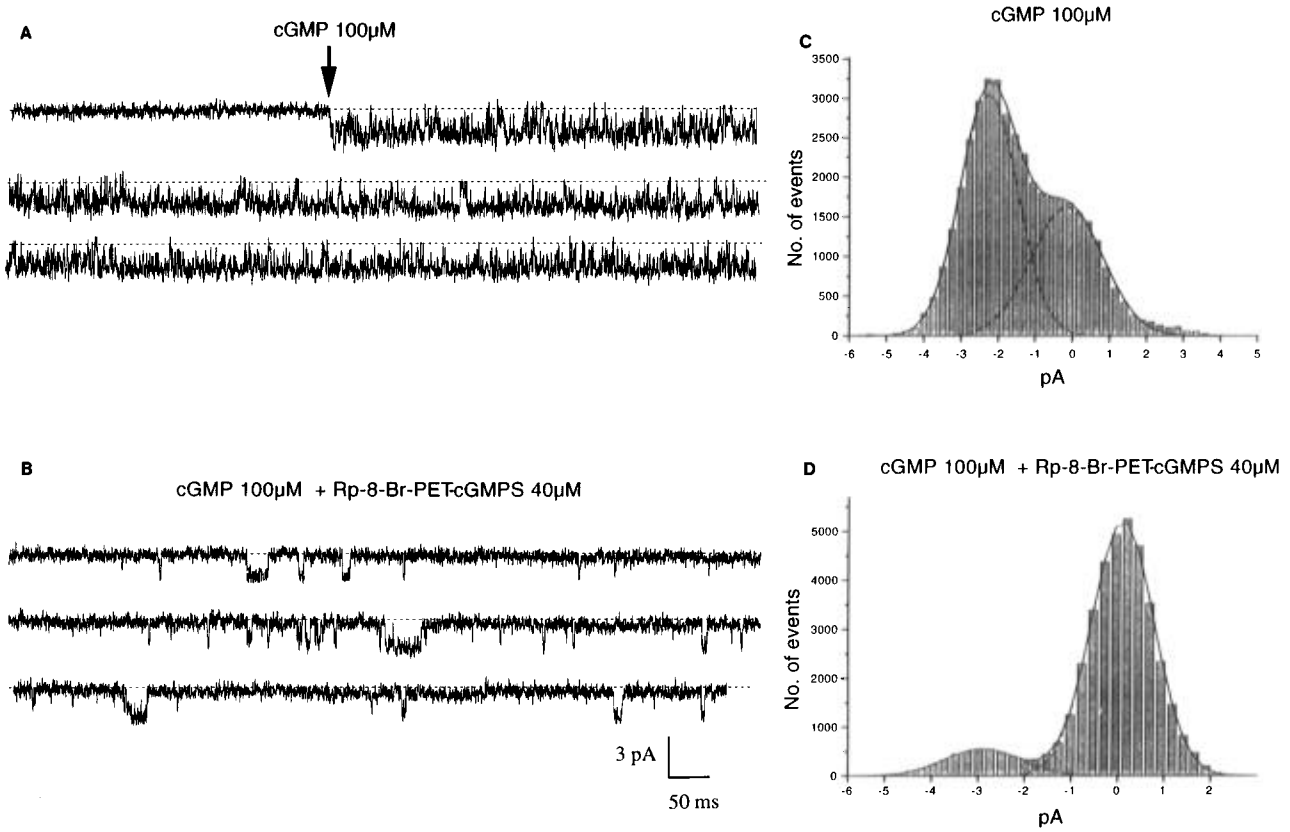

FIGURE 4: Single-channel analysis of Rp-8-Br-PET-cGMPS antagonism on rat rod CNGC α-subunit. The holding potential of the recordings was −70 mV. (A) Representative openings of a single cyclic nucleotide-gated channel. Application of cGMP (arrow) induced a rapidly flickering current in the open state. (B) Same channel in the presence of the antagonist Rp-8-Br-PET-cGMPS and cGMP. The fast cGMP-induced channel openings were reduced, revealing a series of openings of with a longer duration. The single-channel conductance remained the same. (C and D) All-points amplitude histograms of the channel in the presence of 100 μM cGMP and in the presence of the antagonist, respectively. Events shorter than 1 ms were excluded.

the duration of these openings (Figure 4B). Amplitude histograms of each condition showed two peaks corresponding to the open and closed states (Figure 4C,D). Rp-8-Br-PET-cGMPS caused a decrease in open probability with only a minimal change in the single-channel conductance (32.1 without and 39.1 pS with Rp-8-Br-PET-cGMPS).

Antagonism of Rod CNGC Activity by Other PET-Substituted cGMP Molecules. Rp- and Sp-8-Br-PET-cGMPS contain three separate modifications of the cGMP structure. To determine the relative contributions of each of these substituents, we examined the properties of other related compounds. Figure 5A shows the effect of PET-cGMP on the cGMP-gated channel. In the control condition, 1 mM cGMP induced a large current, confirming the presence of channels. After washout, application of 2.5 mM PET-cGMP alone did not lead to detectable channel current in the same patch. After PET-cGMP was removed from the chamber, a current of the same amplitude could again be induced by 1 mM cGMP, indicating that all the effects were reversible. Thus, PET-cGMP was incapable of inducing any channel activity by itself. On the other hand, when different concentrations of PET-cGMP were coapplied with cGMP (50 μM), the currents induced were larger than with 50 μM cGMP alone and approached the magnitude seen with 1 mM cGMP, shown in Figure 5B. The magnitude of the effect of PET-cGMP was dependent on the cGMP concentration. Low concentrations of cGMP (10 μM) that induced only very small currents showed little change even when 1 mM PET-cGMP was coapplied (data not shown). These results suggest that PET-cGMP cannot initiate channel opening, but

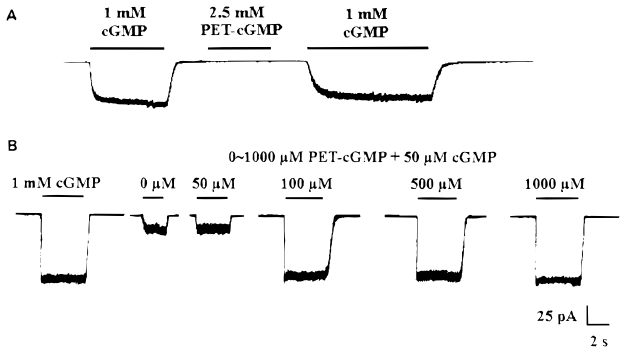

FIGURE 5: Effects of PET-cGMP on rat rod CNGC α-subunits. (A) cGMP (1 mM) induced a 96 pA current. After washout, 2.5 mM PET-cGMP alone did not lead to detectable channel current in the same patch. After PET-cGMP was removed from the chamber, the same amplitude current could again be induced by 1 mM cGMP. (B) The first record shows the maximum current induced in a patch by 1 mM cGMP. Subsequent records show the responses of the same patch to 50 μM cGMP coapplied with 50, 100, 500, and 1000 μM PET-cGMP. Concentrations of PET-cGMP above 50 μM induced maximal responses.

when one or more cGMP binding sites are occupied by cGMP, it can bind and complete the transition to the fully open state.

Previous studies suggested that modifications in position 8 of the guanine nucleobase enhanced the channel activator properties of cGMP (Zimmerman et al., 1985; Koch & Kaupp, 1985; Tanaka et al., 1989; Brown et al., 1993). We,

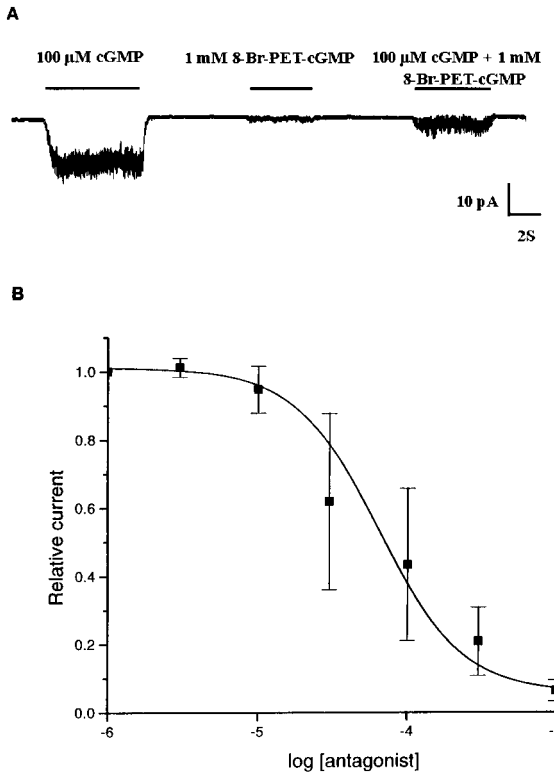

FIGURE 6: Effects of 8-Br-PET-cGMP on rat rod CNGC α-subunits. (A) 8-Br-PET-cGMP (1 mM) increased the membrane noise without giving a clear current, as compared with the 22 pA currents induced by 100 μM cGMP at −70 mV. Coapplication of both compounds reduced the current to 25% of the control value. (B) Dose−response curve generated by coapplying different concentrations of 8-Br-PET-cGMP with 100 μM cGMP. From this curve, 8-Br-PET-cGMP exhibits an IC_{50} of 64 μM.

therefore, compared the properties of 8-Br-PET-cGMP with those of PET-cGMP. 8-Br-PET-cGMP, at concentrations up to 1 mM, just occasionally induced channel openings that resulted in currents of less than 2 pA, as compared with 22 pA currents induced by 100 μM cGMP (shown in Figure 6A). To test whether 8-Br-PET-cGMP could act as a cGMP antagonist, different concentrations of 8-Br-PET-cGMP were coapplied with 100 μM cGMP as shown in Figure 6B. 8-Br-PET-cGMP reduced the cGMP-induced currents with an IC_{50} of 64 μM, suggesting that the two compounds were competing for the same binding sites.

Inhibition of Native CNGCs in Rod Photoreceptors by Sp-8-Br-PET-cGMPS and Rp-8-Br-PET-cGMPS. To determine whether Sp-8-Br-PET-cGMPS and Rp-8-Br-PET-cGMPS acted as antagonists for native channels of α- and β-subunits as well as for the cloned rat rod CNGC α-subunit, we studied their effects on channels in patches of membrane from freshly isolated *Xenopus* rod photoreceptors. To test whether Sp- or Rp-8-Br-PET-cGMPS had any agonist effect on the channels, patches were tested using saturating doses of cGMP (100 μM) followed by high concentrations of either Sp- or Rp-8-Br-PET-cGMPS. As shown in Figure 7, neither compound induced any current in patches where cGMP could induce a current of about 500 pA.

We next examined both the mechanisms and the voltage dependence of antagonism by the Rp and Sp compounds. Preliminary experiments using a fixed concentration of cGMP and increasing concentrations of Sp-8-Br-PET-cGMPS or Rp-8-Br-PET-cGMPS showed that both compounds acted as antagonists and that the IC_{50}s were similar to those

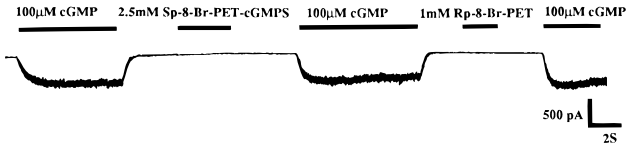

FIGURE 7: Sp-8-Br-PET-cGMPS and Rp-8-Br-PET-cGMPS do not induce currents in native rod CNGCs. Saturating concentrations of cGMP induced a 500 pA current in excised patches from a native rod. After washout, high concentrations of Sp-8-Br-PET-cGMPS or Rp-8-Br-PET-cGMPS alone did not lead to detectable channel current in the same patch. After these compounds were removed from the chamber, the same amplitude current could again be induced by 100 μM cGMP.

derived for the cloned rat rod α-subunit (data not shown). More extensive dose−response curves using fixed concentrations of Sp-8-Br-PET-cGMPS or Rp-8-Br-PET-cGMPS in the presence of increasing concentrations of cGMP revealed the mechanism of antagonism to be competitive in nature (Figure 8A,C). Higher concentrations of antagonists displaced the curves to higher concentrations of cGMP but did not change either the curve shape or saturation. Schild plot analysis revealed a curve with a slope of 1, suggesting that a single antagonist molecule is sufficient to block rod CNGC activation. For Sp-8-Br-PET-cGMPS, the curve gave a slope of 1.4, suggesting that more than one molecule of this antagonist must bind to block the channel.

In control conditions of cGMP alone, the current−voltage relationship showed the native rod CNGC to be outwardly rectifying, as reported previously (Yau & Baylor, 1989). When a series of doses of antagonist, up to those giving complete suppression of the cGMP-induced current, were coapplied with cGMP, both Sp-8-Br-PET-cGMPS and Rp-8-Br-PET-cGMPS produced a voltage-independent block of the current in excised patches of native rod channels (Figure 8B,D).

DISCUSSION

We have used both a cloned α-subunit of the rat rod CNGC, which forms homomeric tetramers, and the native rod CNGC from *Xenopus*, which consists of α- and β-subunits in a heteromeric tetramer, to investigate the interactions of different sites on the cGMP molecule with its binding site and to identify a potent class of channel antagonists. Previous studies of the rod CNGC have shown that the presence of the β-subunit can alter some detailed channel properties such as rapid fluctuations from the open state (flickering), sensitivity to calcium calmodulin, and sensitivity to channel blockers such as 1-*cis*-diltiezam (Chen et al., 1993), although basic properties such as ion selectivity, conductance, and cyclic nucleotide selectivity appear to be determined by the α-subunit (Kaupp et al., 1989). For the olfactory channel, however, the β-subunit plays a more substantial role in determining properties such as cyclic nucleotide selectivity (Liman & Buck, 1994; Bradley et al., 1994). In agreement with previous studies, we found that the native rod channels showed an increased sensitivity to cGMP. Rp-8-Br-PET-cGMPS and Sp-8-Br-PET-cGMPS acted as competitive antagonists with both types of channel. Since the potency was similar with the homomeric or heteromeric channels, the results suggest either that binding to the α-subunit alone is sufficient to give inhibition or that the compounds bind to the α- and β-subunits with equal affinity. Schild plot analysis of the inhibition by Rp-8-Br-

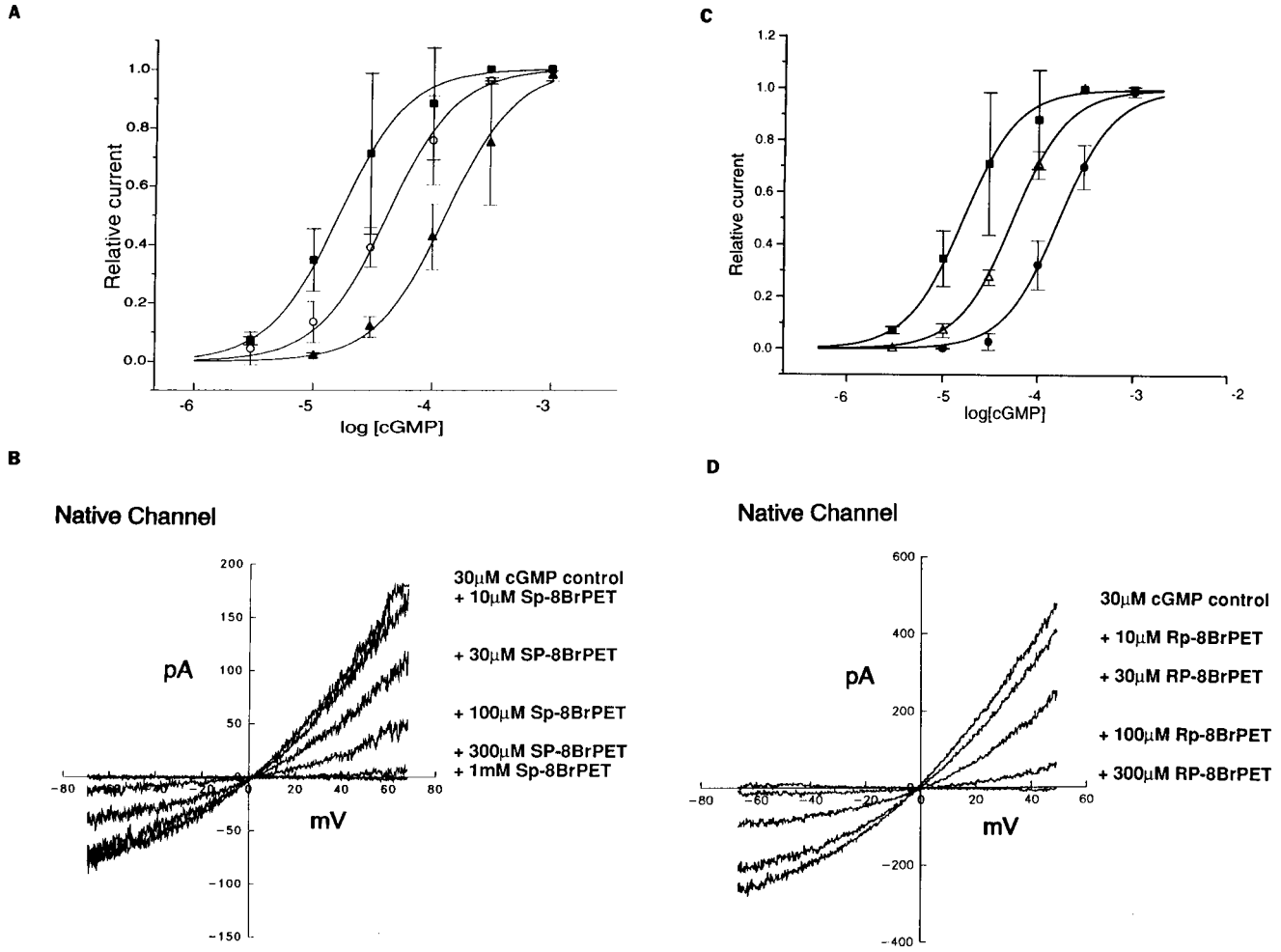

FIGURE 8: Sp-8-Br-PET-cGMPS and Rp-8-Br-PET-cGMPS are competitive antagonists of cGMP on native rod CNGCs. (A) Three dose−response relationships show the currents induced by different concentrations of cGMP alone (solid squares) and coapplied with 30 μM Sp-8-Br-PET-cGMPS (open circles) or 120 μM Sp-8-Br-PET-cGMPS (solid triangles). The fitted curves shifted in parallel to the right with increasing concentrations of Sp-8-Br-PET-cGMPS, indicating that inhibition by this compound is competitive. Cyclic GMP dose−response relationships were fitted to the Hill equation. Best fits for the exponent ranged from 1.4 to 2.0. A value of 1.5 is shown in all curves. The holding potential of the recordings was −70 mV. (B) Current−voltage relations of the current induced by 30 μM cGMP in the absence and in the presence of different concentrations of Sp-8-Br-PET-cGMPS. The responses decreased with increasing concentrations of Sp-8-Br-PET-cGMPS. The inhibition showed no voltage dependence. (C) Three dose−response relationships show the currents induced by different concentrations of cGMP alone (solid squares) or coapplied with 10 μM Rp-8-Br-PET-cGMPS (open triangles) or 80 μM Rp-8-Br-PET-cGMPS (solid circles). The fitted curves shifted in parallel to the right with increasing concentrations of Rp-8-Br-PET-cGMPS. Cyclic GMP dose−response relationships were fitted to the Hill equation. Best fits for the exponent ranged from 1.4 to 2.0. A value of 1.5 is shown in all curves. The holding potential of the recordings was −70 mV. (D) Current−voltage relations of the current induced by 30 μM cGMP in the absence and in the presence of different concentrations of Rp-8-Br-PET-cGMPS. The inhibition showed no voltage dependence.

PET-cGMPS suggested that binding of a single molecule was sufficient to cause inhibition. A similar analysis for Sp-8-Br-PET-cGMPS gave a slope value of 1.4, providing a further indication that it is a less effective antagonist and may require more than one molecule to bind to the channel to cause inhibition.

Rp-8-Br-PET-cGMPS was originally described as an antagonist of type I cGMP-dependent protein kinase with an apparent K_I of 0.03 μM (Butt et al., 1995). Thus, this compound cannot be used to distinguish actions of cGMP involving CNGCs from those involving protein kinase. Sp-8-Br-PET-cGMPS, on the other hand, is an antagonist of the rod CNGC but an agonist of type I cGMP-dependent protein kinase. Since both Rp-8-Br-PET-cGMPS and Sp-8-Br-PET-cGMPS are membrane-permeant and resistant to hydrolysis by phosphodiesterases, they will be useful for elucidating physiological roles for CNGCs and cGMP-dependent protein kinases in intact cells.

The results of this study also provide useful information about the interactions between different parts of the cGMP molecule and the channel protein. Such interactions can be discussed with some precision because a structural model of the cyclic nucleotide binding site of CNGC has been proposed on the basis of the homology with the bacterial CAP protein. This protein contains a cAMP binding site, and its structure has been defined by X-ray crystallography (Weber & Steitz, 1987). Several models have been proposed for the way in which cGMP fits into the CNGC binding site, and these differ in the configuration of the cyclic nucleotide that binds [reviewed in Zagotta and Siegelbaum (1996)]. The actions of cGMP can be divided into a binding interaction that facilitates the transition between open and closed states and a gating interaction that stabilizes the open state.

The analogues we have studied have modifications at three different parts of the cGMP molecule that are thought to interact with the channel protein (see Figure 1). These are

the presence or absence of a bromine atom at the 8 position of the guanine ring, the presence or absence of a thiophosphate group in the cyclic phosphate moiety, and a PET substitution of the guanine ring. The net effect of each analogue on channel activity, either alone or in combination with cGMP, is the sum of the interactions at each of these three sites.

Hydrophobic substitutions at the 8 position of cGMP generally increase the activity of the compounds by a mechanism thought to involve increasing the affinity of the interaction with the cGMP binding domain (Brown et al., 1993; Scott & Tanaka, 1995). For example, the EC_{50} for channel activation by cGMP is 17 μM but for 8-Br-cGMP is 1.6 μM (Zimmerman et al., 1985). A similar increase in binding affinity may explain the different properties of PET-cGMP and 8-Br-PET-cGMP. High concentrations of 8-Br-PET-cGMP caused an increase in membrane noise, whereas PET-cGMP applied alone did not induce any current, suggesting that increased affinity for the channel caused by the 8-Br group allowed the binding energy of 8-Br-PET-cGMP to promote rapid transitions between the open and closed states. When coapplied with cGMP, however, PET-cGMP increased the response to maximal levels. It is possible that PET-cGMP binds so weakly to the closed state of the channel that it cannot increase the channel openings or act as an effective competitor of cGMP. There is evidence that binding of one or more cGMP molecules to the channel tetramer causes alterations in the affinity of the other binding sites (Karpen & Brown, 1996). If PET-cGMP were to bind effectively to one of these altered binding sites, or to the fully open state, this could explain the synergistic action. Such binding would tend to increase the channel open time and thus the observed current.

*R*p diastereoisomers of cGMP have been shown to act as inhibitors of type I PKG (Vaandrager & De Jonge, 1994), and *S*p diastereoisomers act as activators (Butt et al., 1990). This suggests that the interaction of PKG with this phosphate group is critical for enzyme activation and that the isomeric position of the thiophosphate S atom (*R*p vs *S*p) is sufficient to define antagonist or agonist. Unlike 8-Br-PET-cGMP, neither Rp-8-Br-PET-cGMPS nor Sp-8-Br-PET-cGMPS induced currents in membrane patches when applied alone (figure 7A). This suggests that introduction of the sulfur atom in either isomeric position causes a small decrease in either the binding or gating interactions of the compounds.

On the other hand, all three compounds were found to be antagonists of the rod CNGC when coapplied with cGMP. Comparison of the IC_{50}s for Rp-8-Br-PET-cGMPS, 8-Br-PET-cGMP, and Sp-8-Br-PET-cGMPS (25, 64, and 105 μM, respectively) suggests that the interactions of the cyclic phosphate group with the channel protein do have a small effect, with the *R*p form serving as a more effective antagonist, but are probably less important than in activation of cGMP-dependent protein kinase.

The conformational change that accompanies channel opening is thought to allow the formation of hydrogen bonds between the aspartate side chain at position 597 (corresponding to position 604 in the bovine channel) and the N1 and N2 groups of the guanine ring. This model is supported by the results of a mutation analysis of this aspartate residue on the ligand selectivity of the bovine rod CNGC α-subunit in which neutralization (D604Q) or replacement with a nonpolar amino acid (D604M) produced dramatic changes in relative agonist (cAMP, cGMP, and cIMP) efficacy (Varnum et al., 1995). Both Rp-8-Br-PET-cGMPS and Sp-8-Br-PET-cGMPS act competitively at the cGMP-binding site of the channel, and their actions are completely reversible. In both compounds, N1 and N2 of the guanine ring are substituted by a phenyl ring which will prevent hydrogen bond formation with the aspartate residue of the channel. Thus, while both compounds can bind efficiently, they either do not allow the conformational change necessary for channel activation or cannot stabilize it; hence, they are effective antagonists.

Together, our results indicate that site-modified cGMP analogues can be powerful tools for probing the structure of the cGMP binding site of rod type CNGCs. Further experiments will be necessary to determine whether these compounds can also serve as antagonists of olfactory and cone types of CNGCs. Comparisons of the sequences of bovine CNGCs show that, while the rod and cone/testis channels have an aspartate residue that can interact with the guanine ring N1 and N2 groups, the olfactory channel has a glutamate residue. This suggests that the PET derivatives will also act as antagonists of the cone/testis channel. The effects on the olfactory channel are harder to predict. Because this CNGC can be activated by both cAMP and cGMP, it is thought that the critical interactions for channel activation reside in groups such as the cyclic phosphate ester rather than in the purine ring. For example, Sp-cGMPS has been shown to be an agonist for the olfactory channel, whereas Rp-cGMPS is an antagonist (Kramer & Tibbs, 1996).

There is increasing evidence that CNGCs play a role in mediating the responses to a number of agents that change cGMP concentrations, including diffusible messengers such as NO and pathways through certain G protein-coupled receptors. Analogues of cyclic nucleotides, such as those described in this report, can selectively activate or inhibit these channels and will be valuable reagents for the study of CNGC function in many regions of the CNS.

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