Activation of Retinal Rod cGMP-Gated Channels: What Makes for an Effective 8-Substituted Derivative of cGMP?†

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ABSTRACT: Analogs of cGMP bearing diverse substituents at the C_8 position of the guanine ring system have been shown to activate the cGMP-activated channel of retinal rods at concentrations lower than cGMP itself. In an effort to understand this behavior, we synthesized eight novel C_8-substituted derivatives and tested their ability to activate channels in excised patches from salamander rod outer segments. We express the effectiveness of each analog as a ratio (in brackets) of the concentration required to open half of the channels in a patch to that required of 8-Br-cGMP, previously shown to be about 10 times more effective than cGMP. Five of the derivatives contained a thio substitution at C_8: *n*-propylthio-cGMP [0.61], sulfoethylthio-cGMP [0.90], carboxyethylthio-cGMP [0.97], aminoethylthio-cGMP [2.8], and (trimethylamino)ethylthio-cGMP [8.5]. Three of the derivatives contained an amino substitution at C_8: carboxyethylamino-cGMP [22], *n*-propylamino-cGMP [25], and aminoethylamino-cGMP [230]. The results indicate that thio-substitution at C_8 produces more effective analogs than does amino-substitution, regardless of the chemical nature of the terminal functional group. Derivatives containing neutral and apolar tails opened channels at much lower concentrations than their positively-charged counterparts with the same C_8 substituent. Analogs having negatively-charged tails were also more effective than those with positive charge but not quite as effective as those with neutral tails. ^{1}H NMR measurements of the H2′ chemical shift, previously shown to be sensitive to conformation about the *N*-glycosidic bond, revealed no significant differences in the *syn–anti* equilibrium between pairs of amino- or thio-substituted derivatives that opened channels at widely differing concentrations. The disparities in analog effectiveness most likely reflect differences in the chemical interactions between the C_8-substitutions or neighboring atoms affected by the substitutions and the channel's binding sites.

Ion channels directly gated by cyclic GMP (cGMP) are now known to generate the electrical response to light in retinal rods (Fesenko et al., 1985; Yau & Baylor, 1989; Kaupp, 1991). In the dark, cGMP holds channels open, allowing Na^+ and Ca^{2+} to enter and partially depolarize the rod. A light-activated enzymatic cascade lowers the cGMP concentration, thereby hyperpolarizing the rod [reviewed in McNaughton (1990), Stryer (1991), Lagnado & Baylor (1992)]. In addition to retinal rods and cones, cyclic nucleotide-gated channels have also been identified recently in olfactory cilia (Nakamura & Gold, 1987), cochlear hair cells (Kolesnikov et al., 1991), pinealocytes (Dryer & Henderson, 1991), and renal epithelial cells (Marunaka et al., 1991).

Patch-clamp and ion-flux studies of rod channel activation by cyclic nucleotides and their analogs have provided some information on the specificity of the channel's binding sites for cGMP. Modifications to the cyclic phosphate, ribose, and most positions on the guanine ring system generally produced compounds that were less effective than cGMP itself (Fesenko et al., 1985; Zimmerman et al., 1985; Karpen et al., 1988; Tanaka et al., 1989). In contrast, the introduction of a variety of substituents at the C_8 position of the guanine ring system produced analogs that were more effective than cGMP. For example, 8-Br-cGMP and 8-fluoresceinylcarbamoylmethylthio-cGMP were reported to open channels at 10-fold and 30–200-fold lower concentrations than cGMP (Zimmerman et al., 1985; Koch & Kaupp, 1985; Caretta et al., 1985; Tanaka et al., 1989). It has been suggested that bulky substituents at C_8 enhance analog potency by shifting the conformational equilibrium about the *N*-glycosidic bond to the *syn* form.

The present study was inspired by the finding that a several hundred-fold higher concentration of 8-aminoethylamino-cGMP (AEA-cGMP)[1] was required to open channels in excised membrane patches from salamander rods than was required of 8-Br-cGMP. This substitution was one of several made in attempts to provide a convenient handle for attachment of the cGMP moiety to an affinity resin. The ineffectiveness of AEA-cGMP may have been due to the amine-substitution at C_8, the primary amine tail which bears a positive charge at physiological pH, or a combination of both. To distinguish among these alternatives, we systematically varied each position and tested the resulting analogs in patch-clamp experiments.

MATERIALS AND METHODS

Cyclic GMP (sodium salt) and ethylene diamine were purchased from Sigma. Bromine, 1-bromopropane, and

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[1] Abbreviations: AEA-cGMP, 8-aminoethylamino-cGMP; AET-cGMP, 8-aminoethylthio-cGMP; CEA-cGMP, 8-carboxyethylamino-cGMP; CET-cGMP, 8-carboxyethylthio-cGMP; *n*PA-cGMP, 8-*n*-propylamino-cGMP; *n*PT-cGMP, 8-*n*-propylthio-cGMP; SET-cGMP, 8-sulfoethylthio-cGMP; TMAET-cGMP, 8-(trimethylamino)ethylthio-cGMP.

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β-alanine were from Fluka. Sodium methoxide, 2-bromoethylamine hydrobromide, 3-bromopropionic acid, 2-bromoethanesulfonic acid (sodium salt), (2-bromoethyl)trimethylammonium bromide, and *n*-propylamine were purchased from Aldrich. Thiourea and ammonium acetate (HPLC grade) were from Fischer. All solvents were HPLC grade from either Fischer or Mallinkrodt. All other chemicals were reagent grade.

Thin-Layer Chromatography Methods. Two TLC methods were used to monitor reaction progress and to characterize cGMP derivatives. The most commonly used TLC system was silica gel (kieselgel 60 F_{254}; 0.25 mm; EM Science), developed with 1-butanol/acetic acid/water (5:3:2). A second system was poly(ethyleneimine) cellulose (PEI–cellulose; 0.1 mm; Brinkmann), developed with 200 mM lithium chloride and 50 mM triethanolamine, pH 7.9. Both matrices contained a fluorescent dye excited at 254 nm, and products were visualized by ultraviolet shadowing using short-wavelength UV excitation from a mineral light (Model UVGL-25; UVP, San Gabriel, CA). Products containing a primary amino group were also identified on the silica system using a ninhydrin spray (0.2% ninhydrin in 1-butanol/acetic acid (99.5:0.5)) followed by heating to visualize the pink-red adduct.

Synthesis of 8-Br-cGMP. Bromination of cGMP was performed according to a modification of a previously published method (Geahlen, et al., 1979). A 1:100 bromine/water solution (5.46 mL) was added to a 27.4-mL solution of 500 mM ammonium acetate, pH 3.9 with acetic acid, containing 300 mg of cGMP. This was allowed to react for 2 h at room temperature. To ensure complete conversion of cGMP to 8-Br-cGMP, a second 1.37-mL aliquot of the bromine/water stock was added to the reaction mixture. Formation of 8-Br-cGMP was monitored by silica TLC. The reaction mixture was then dried to constant weight in a Speed-Vac (Savant) to remove residual bromine and ammonium acetate.

Synthesis of 8-Thio-cGMP. 8-Br-cGMP (ammonium salt) (150 mg) was divided among four screw-cap Eppendorf tubes. Redistilled DMSO (600 μL) and 75 mg of thiourea were added to each tube, and the solutions were heated on a Temp-Block at 115 °C for 17 h. PEI–cellulose TLC indicated that the conversion of 8-Br-cGMP to an isothiuronium salt intermediate was complete, and the reaction mixes were pooled and diluted to 25 mL with methanol. This intermediate was decomposed to 8-thio-cGMP by the addition of 200 mg of sodium methoxide. The crude product was dried in a Speed-Vac, resuspended in water, adjusted to neutral pH with acetic acid, and partially purified by anion-exchange chromatography on a Whatman DE-52 matrix in the acetate form. The crude 8-thio-cGMP was loaded at 1 mL/min onto a 1.7- × 25-cm column, equilibrated with water. The column was then washed with five volumes of water, and the 8-thio-cGMP was eluted with 1 N ammonium acetate, pH 7.0. The column effluent was monitored for absorbance at 274 nm. The fractions containing the 8-thio-cGMP were pooled and dried repeatedly in a Speed-Vac to remove ammonium acetate.

Synthesis of 8-Aminoethylthio-cGMP (AET-cGMP), 8-(Trimethylamino)ethylthio-cGMP (TMAET-cGMP), 8-Carboxyethylthio-cGMP (CET-cGMP), 8-Sulfoethylthio-cGMP (SET-cGMP), and 8-n-Propylthio-cGMP (nPT-cGMP). Partially purified 8-thio-cGMP (50 mg) was dissolved in 25 mL of methanol for the synthesis of *n*PT-cGMP or 25 mL of water for the syntheses of the other thio-substituted analogs. A 10-fold molar excess of 2-bromoethylamine hydrobromide, (2-bromoethyl)trimethylammonium bromide, 3-bromopropionic acid, 2-bromoethanesulfonic acid (sodium salt), or 1-bromopropane was added for the respective syntheses of AET-, TMAET-, CET-, SET-, and *n*PT-cGMP, and the solutions were brought to pH 9.0 by the addition of either acetic acid or ammonium hydroxide. The solutions were allowed react at room temperature, and product formation was monitored by silica TLC. The reaction mixtures were once again dried in a Speed-Vac.

Synthesis of 8-Aminoethylamino-cGMP (AEA-cGMP). 8-Br-cGMP (ammonium salt) (50 mg) and 550 μL of ethylene diamine were dissolved in 5 mL of DMSO. This solution was distributed among four screw-cap Eppendorf tubes and incubated at 120 °C on a Temp-Block. The reaction was monitored by silica TLC and was halted after 17 h. The reaction mixture was repeatedly diluted with 100 mL of water and dried in a Speed-Vac to remove DMSO and ethylene diamine. The AEA-cGMP was dissolved in 100 mL of water and partially purified by anion-exchange chromatography on Whatman DE-52 as described above.

Synthesis of 8-n-Propylamino-cGMP (nPA-cGMP). 8-Br-cGMP (ammonium salt) (50 mg) and 800 μL of *n*-propylamine were suspended in 2 mL of methanol and heated at 120 °C in an acid digestion bomb. After 20 h an additional 400 μL of *n*-propylamine was added, and the incubation continued for 16 h. Because *n*PA-cGMP was not well resolved from 8-Br-cGMP on the silica TLC systems used, reaction progress was monitored by reversed-phase HPLC (see below). When complete, the reaction mixture was dried in a Speed-Vac. The *n*PA-cGMP was partially purified by anion-exchange chromatography on Whatman DE-52 as described above.

Synthesis of 8-Carboxyethylamino-cGMP (CEA-cGMP). 8-Br-cGMP (ammonium salt) (100 mg) was dissolved in 3.5 mL of water containing 1.5 g of β-alanine. This mixture was incubated at 100 °C in an acid digestion bomb for 4 days while the reaction progress was monitored by silica TLC. After 90 h the reaction progress seemed to have halted, with approximately 50% of the 8-Br-cGMP consumed. The crude CEA-cGMP was diluted to 50 mL with water and chromatographed on Whatman DE-52 as described above.

HPLC Purification of 8-Substituted cGMP Analogs. All analogs were purified to homogeneity using anion-exchange followed by reversed-phase chromatography on a Gilson HPLC system. Anion-exchange chromatography was performed on an Alltech Adsorbosphere-SAX semipreparative column using a gradient from 5 to 250 mM ammonium acetate, pH 5 with acetic acid. Reversed-phase chromatography was performed on a Vydac semipreparative C18 column using a 0–45% gradient of methanol in water (5 mM ammonium acetate, pH 5 with acetic acid throughout). Both columns were developed with a flow rate of 2.5 mL/min. Column effluents were monitored at 260 or 274 nm. Overall yields after purification were typically 20–25%. Reversed-phase chromatography of purified analogs indicated a purity of >99% based on UV absorbance.

Electrophysiology. The effectiveness of each analog in opening cGMP-activated channels was tested on excised inside-out membrane patches from rod outer segments of the larval tiger salamander, *Ambystoma tigrinum* [maintained as described in Baylor & Nunn (1986)]. Experiments were performed in visible light at 22 °C. Following rapid decapitation, the brain and spinal cord were pithed, both eyes were removed and hemisected, and the retinas were isolated into a Ringer solution containing 111 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 0.02 mM EDTA, and 3 mM HEPES, pH 7.6. A small piece of retina

was transferred to the experimental chamber (fluid volume ~ 100 μL) and teased with fine needles to release isolated whole rods and rod outer segments. The floor of the chamber had been pretreated with 1 mg/mL poly(L-lysine hydrobromide) (30 000–70 000 mol wt; Sigma), followed by thorough rinsing. This caused the cells to stick and prevented them from moving when the patch electrode approached. Patch electrodes were fabricated from borosilicate glass and had orifices ~1 μm in diameter and resistances near 3 MΩ. Using a miniature O_2/acetylene torch, a right-angle bend was made ~1 mm from the tip. An outer segment was then easily approached from the direction perpendicular to the chamber floor. After a seal (1–10 GΩ) was formed, often requiring light suction, the electrode was rapidly lifted from the cell, excising a patch of membrane. Cyclic nucleotide-activated currents were measured with the following solution in the patch pipet and experimental chamber: 130 mM NaCl, 2 mM HEPES, pH 7.6, 0.02 mM EDTA. The seal resistance normally decreased somewhat in the low-divalent solution. The chamber solution was changed within ~20 s by a gravity-driven perfusion system. Currents were recorded with an Axopatch 1D amplifier and low-pass filtered (eight-pole Bessel). The records were sampled, stored, and analyzed using a Gateway 386 computer and pCLAMP 5.5.1 software (Axon Instruments, Foster City, CA). Cyclic nucleotide-activated currents were obtained from the difference in currents with and without the nucleotide. The difference currents were corrected for the voltage drop across the pipet series resistance using the following relation: $I = I_r V/(V - I_r R_p)$, where I is the corrected current, I_r is the raw difference current, V is the command potential, and R_p is the measured pipet resistance. Strictly, this correction applies only when the current–voltage relation is linear. The deviation from linearity is small between 0 and +50 mV, however, and at worst the linear correction should have underestimated the current by only about 6%.

At the beginning of each patch, and up to several more times for patches that lasted, a brief dose–response relation was determined for 8-Br-cGMP (usually at three concentrations). A fit to the data was obtained with the Hill equation:

$$I/I_{max} = [\text{8-Br-cGMP}]^n/(K_{1/2}{}^n + [\text{8-Br-cGMP}]^n) \quad (1)$$

where I_{max} is the current obtained at a saturating concentration of cyclic nucleotide, $K_{1/2}$ is the concentration that causes a half-maximal current, and n is an index of cooperativity. Analog concentrations were then expressed relative to the most recent $K_{1/2}$ for 8-Br-cGMP measured on the same patch. A fit to combined data from different patches was obtained using a modified form of the Hill equation, in which both the numerator and the denominator of the equation describing activation by an 8-substituted derivative (8-R-cGMP) were divided by the factor $K_{1/2}(\text{8-Br-cGMP})^n$:

$$I/I_{max} = \{[\text{8-R-cGMP}]/K_{1/2}(\text{8-Br-cGMP})\}^n/ \{\{K_{1/2}/K_{1/2}(\text{8-Br-cGMP})\}^n + \{[\text{8-R-cGMP}]/K_{1/2}(\text{8-Br-cGMP})\}^n\} \quad (2)$$

The fit parameters were n and the ratio $K_{1/2}/K_{1/2}$(8-Br-cGMP), a measure of the effectivness of an analog compared with 8-Br-cGMP. The reasons for treating the data in this way are given in the Results. Least-squares curve fitting to collected results and preparation of figures was done using SigmaPlot 5.0 software (Jandel Scientific, San Rafael, CA).

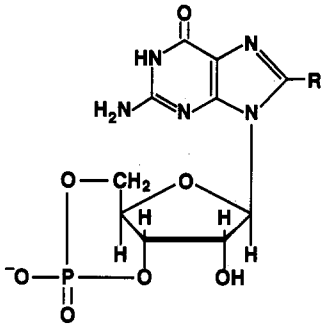
NMR Methods. Spectra were recorded at ambient temperature in D_2O on a Bruker AM500 NMR spectrometer, operating at 500 MHz for ^1H measurements and at 126 MHz for ^{13}C measurements. The ^1H NMR and ^{13}C NMR chemical shifts were determined with dioxane as an internal reference at 3.53 and 66.5 ppm, respectively. ^1H NMR measurements were carried out at two concentrations (1–2 and 10–20 mM), while ^{13}C NMR measurements were carried out only at the higher concentration range for reasons of sensitivity. The lower concentrations were used to minimize possible intermolecular stacking interactions. All ^1H chemical shifts varied by only 0.02 ppm or less between the two concentration levels, which shows that stacking interactions were, indeed, limited. The chemical shift of the H2′ resonance, the main spectral parameter of interest, varied by 0.01 ppm or less between the two concentrations. This chemical shift is reported at the lower concentration only. The assignment of the H2′ resonance was obtained from homonuclear decoupling experiments and by comparison with previously reported assignments and coupling constants for cyclic nucleotides in general (Schweizer & Robins, 1973; Kainosho & Ajisaka, 1975). The assignment of the C4 resonance was based on the presence of a vicinal carbon–hydrogen coupling constant ($J_{C4-H1'}$) and the chemical shift. For carbon–hydrogen coupling constant measurements, the sweep width was 20 kHz and the data size was 64K. The data were processed with zero-filling to 256K to give a digital resolution of 0.16 Hz/point.

RESULTS

Structural, Chromatographic, and Spectral Characteristics of 8-Substituted cGMP Derivatives. The structures of the C_8 substituents of all eight cGMP analogs are given in Table I. Two of the analogs (AET-cGMP and AEA-cGMP) had been previously synthesized using different methods (Dills et al., 1976). The identities of the derivatives were confirmed by fast atom bombardment mass spectrometry; m/e ratios of the molecular ions are given in Table I. For four of the analogs, the disappearance of the H8 resonance in the ^1H NMR spectra and the appearance of resonances expected for each substituent provided further confirmation of the structures. Also given in Table I are the R_f values characteristic of each purified analog on our silica TLC system and the wavelengths of peak absorbance. The λ_{max} for each thio-substituted analog (274 nm) agreed closely with the value of 273 nm reported previously for 8-methylthio-cGMP at pH 1 (Miller et al., 1973). Similarly, the two absorbance peaks (at 260 and 298 nm) observed for each amino-substituted analog were also seen previously by Miller et al. (1973) for 8-methylamino-cGMP at pH 7 (258 and 297 nm). The differences in λ_{max} values were within the resolution of our spectrophotometer (±2 nm). The ratios of the absorbances at the two peak wavelengths (A_{260}/A_{298} = 2.0) agreed with the previous value to within 2%. The derivatives synthesized in this study contained unconjugated substituents at C_8; we therefore assumed that the five thio-substituted analogs had the same extinction coefficient, 17 700 M^{-1} cm^{-1}, reported previously for 8-methylthio-cGMP. Similarly, we assumed that the three amino-substituted derivatives had the extinction coefficient of 15 300 M^{-1} cm^{-1} reported for 8-methylamino-cGMP. These values were used to estimate the concentrations of the derivatives in patch-clamp experiments.

Channel Activation by 8-Substituted cGMP Derivatives. To determine why AEA-cGMP was ineffective in activating channels, we synthesized seven other derivatives (Table I), in which either an amino or thio group was directly attached to

Table I: Structures, Chemical Properties, and Channel Activation Parameters of 8-Substituted cGMP Derivatives



derivative	C_8 substitution (R)	m/e^a	$R_f{}^b$	λ_{max} (nm)	$K_{1/2}$/ $K_{1/2}$(8-Br-cGMP)	I_{max}/ I_{max}(8-Br-cGMP)
*n*PT-cGMP	$-SCH_2CH_2CH_3$	420 (+)	0.48	274	0.61	1.0
SET-cGMP	$-SCH_2CH_2SO_3^-$	484 (–)	0.22	274	0.90	0.99
CET-cGMP	$-SCH_2CH_2CO_2^-$	448 (–)	0.39	274	0.97	1.0
AET-cGMP	$-SCH_2CH_2NH_3^+$	421 (+)	0.23	274	2.8	0.79
TMAET-cGMP	$-SCH_2CH_2N(CH_3)_3^+$	463 (+)	0.066	274	8.5	0.69
CEA-cGMP	$-NHCH_2CH_2CO_2^-$	433 (+)	0.28	260, 298	22	1.06
*n*PA-cGMP	$-NHCH_2CH_2CH_3$	403 (+)	0.41	260, 298	25	0.83
AEA-cGMP	$-NHCH_2CH_2NH_3^+$	404 (+)	0.13	260, 298	230	0.62

[a] Mass/charge ratio of the molecular ion. (+) or (–) indicates whether the identified molecular ion was positive or negative. [b] Ratio in silica TLC of the migration of the derivative to the migration of the solvent front.

C_8 and in which the terminal functional group (following an ethylene spacer) was positive, negative, or neutral at physiological pH. Figure 1 shows pairwise comparisons of dose–response relations for amino- versus thio-substituted derivatives having the same terminal functional groups. Steady-state current amplitudes at +50 mV were expressed relative to the value at a saturating concentration of the same analog. Concentrations were expressed relative to the concentration of 8-Br-cGMP that gave a half-maximal current on the same patch, $K_{1/2}$(8-Br-cGMP). The reasons for expressing analog concentrations in this way are as follows: the concentration of cGMP that gives a half-maximal current can vary widely between patches (Zimmerman & Baylor, 1986; Gordon et al., 1992). The same holds true for all of the cGMP analogs reported here. We observed for each analog tested, however, that the *ratio* of the concentration that gave a half-maximal current to the corresponding concentration of 8-Br-cGMP was nearly constant across patches. Gordon et al. (1992) had made a similar observation for cGMP versus 8-Br-cGMP. Because patches last for a finite period of time, normalizing analog concentrations by the effective concentration of 8-Br-cGMP allows us to combine data on the same analog from different patches and to compare analogs that were not tested on the same patch. A fit to the combined data for an analog was obtained using eq 2 (the parameter $K_{1/2}/K_{1/2}$(8-Br-cGMP) is given for each analog in Table I).

The results in Figure 1 indicate that thio-substituted derivatives were much more effective than amino-substituted derivatives, regardless of the nature of the terminal functional group, which also had a large impact on the absolute effectiveness (see below). *n*PT-cGMP opened channels at a 41-fold lower concentration than *n*PA-cGMP, AET-cGMP opened channels at an 82-fold lower concentration than AEA-cGMP, and CET-cGMP was 23-fold more effective than CEA-cGMP.

Figure 2 shows a comparison of dose–response relations for *n*PT-cGMP and TMAET-cGMP. *n*PT-cGMP opened channels at a 14-fold lower concentration than TMAET-cGMP. In general, analogs containing neutral and hydrophobic terminal groups were much more effective than those containing a positive charge, regardless of the nature of the substituent at the guanine ring system. *n*PA-cGMP opened channels at a 9.2-fold lower concentration than AEA-cGMP, and *n*PT-cGMP was 4.6-fold more effective than AET-cGMP (Table I). The difference in effectiveness between TMAET-cGMP and AET-cGMP suggests that permanent positive charge may be more deleterious than positive charge that can be affected by the local chemical environment. The bulkier terminal functional group of TMAET-cGMP is probably not the relevant difference here. TMAET-cGMP is considerably less bulky than 8-fluoresceinylcarbamoylmethylthio-cGMP (Caretta et al., 1985; Tanaka et al., 1989) and 8-*p*-azidophenacylthio-cGMP (Brown et al., 1993), both more effective channel activators.

Figure 3 shows a comparison of dose–response relations for SET-cGMP and TMAET-cGMP. SET-cGMP opened channels at a 9.4–fold lower concentration than TMAET-cGMP. Analogs containing negative terminal groups were consistently more effective than those containing a positive charge. CEA-cGMP opened channels at a 10-fold lower concentration than AEA-cGMP, and CET-cGMP was 2.9-fold more effective than AET-cGMP (Table I). As noted above, the positive charge on AET-cGMP may be modulated by the local environment of the binding site. Conversely, there is no evidence that the negative charge on CET-cGMP is modulated; SET-cGMP, which contains a strongly acidic terminal group, opened channels at about the same concentration as CET-cGMP (Table I).

*n*PT-cGMP was more effective than either CET-cGMP or SET-cGMP (Table I), suggesting that thio-substituted analogs containing a neutral and hydrophobic terminal functional group may be somewhat more effective than those containing a negatively-charged one. *n*PA-cGMP and CEA-cGMP were nearly equally effective (Table I), suggesting that for amino-substituted analogs it makes little or no difference whether the terminal group is neutral or negative for this spacer length.

The cGMP derivatives differed from each other not only in their effective concentrations but also in the maximum current obtained at saturating concentrations. The ratios of the maximum current at +50 mV induced by each analog to the maximum current induced by 8-Br-cGMP are given in Table I. Four of the analogs gave essentially the same

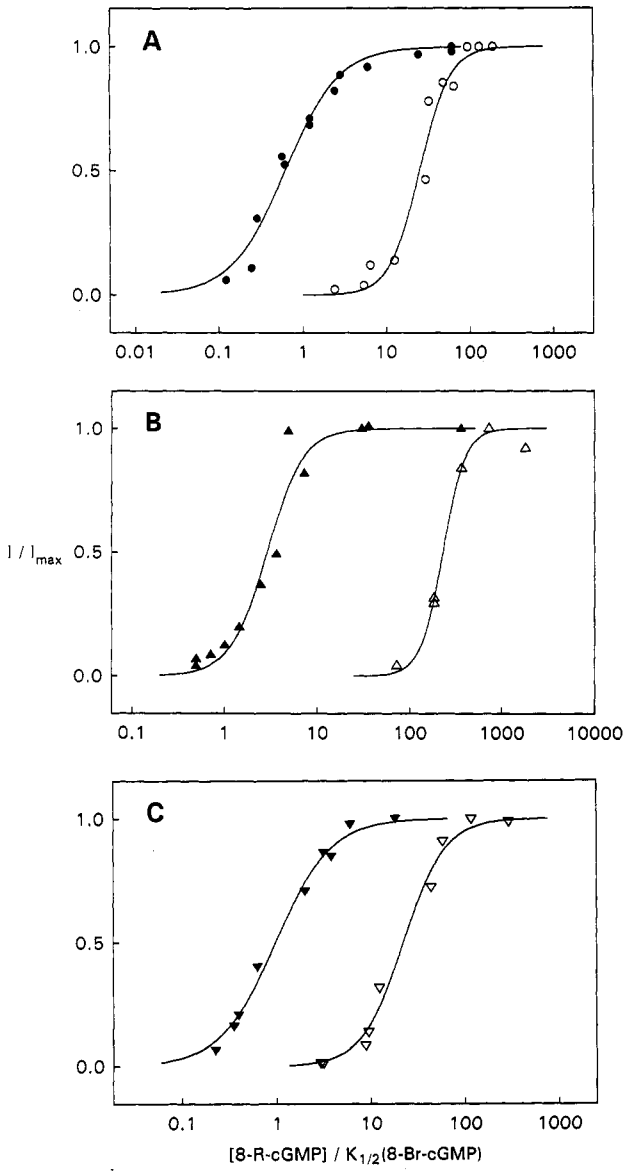


FIGURE 1: Comparison of dose–response relations for 8-amino- versus 8-thio-substituted derivatives containing the same terminal functional group. Steady-state cyclic nucleotide-activated currents measured at +50 mV were normalized by the saturating value (I_{max}). Concentrations were normalized by the effective 8-Br-cGMP concentration as described in the text and plotted on a logarithmic scale. (A) *n*PT-cGMP data (●) were obtained on two patches with fit $K_{1/2}$(8-Br-cGMP) values of 0.82 and 1.8 μM. The smooth curve is a nonlinear least-squares fit of eq 2 to the *n*PT-cGMP data with n = 1.4 and $K_{1/2}/K_{1/2}$(8-Br-cGMP) = 0.61. *n*PA-cGMP data (○) were from three patches with $K_{1/2}$(8-Br-cGMP) values of 1.5, 1.7–2.0, and 2.7 μM. For the second patch, the $K_{1/2}$(8-Br-cGMP) value was determined several times and found to decrease during the patch (Gordon et al., 1992). Smooth curve: n = 2.4 and $K_{1/2}/K_{12}$(8-Br-cGMP) = 25. (B) AET-cGMP data (▲) were obtained on two patches with $K_{1/2}$(8-Br-cGMP) values of 1.4 and 1.7–2.0 μM. Smooth curve: n = 2.2 and $K_{1/2}/K_{1/2}$(8-Br-cGMP) = 2.8. AEA-cGMP data (△) were from one patch with a $K_{1/2}$(8-Br-cGMP) value of 2.8 μM. Consistent data were obtained on another patch in which the maximum AEA-cGMP-induced current was not determined but in which comparisons were made with 8-Br-cGMP-induced currents. Smooth curve: n = 3.6 and $K_{1/2}/K_{1/2}$(8-Br-cGMP) = 230. (c) CET-cGMP data (▼) were obtained on two patches with $K_{1/2}$(8-Br-cGMP) values of 1.4 and 2.2 μM. Smooth curve: n = 1.5 and $K_{1/2}/K_{1/2}$(8-Br-cGMP) = 0.97. CEA-cGMP data (▽) were from two patches with $K_{1/2}$(8-Br-cGMP) values of 1.1–1.7 and 2.1 μM. Smooth curve: n = 2.0 and $K_1/_2/K_{1/2}$(8-Br-cGMP) = 22.

maximum current as 8-Br-cGMP: *n*PT-cGMP, SET-cGMP, CET-cGMP, and CEA-cGMP. 8-Br-cGMP had been previously shown to cause the same maximum current as cGMP.

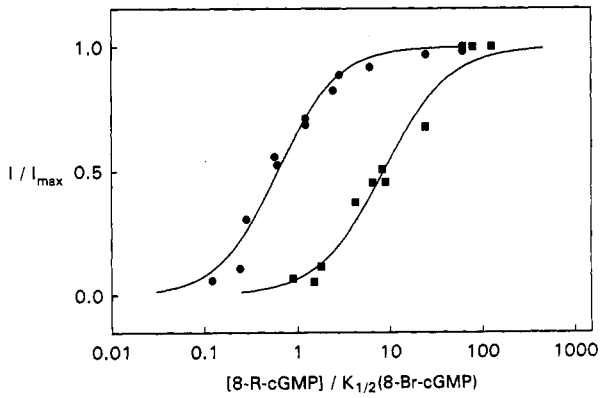


FIGURE 2: Comparison of dose–response relations for *n*PT-cGMP (●) and TMAET-cGMP (■). Cyclic nucleotide-activated currents and concentrations are expressed as described in Figure 1 and the text. *n*PT-cGMP data and fit are the same as in Figure 1. TMAET-cGMP data were obtained on three patches with fit $K_{1/2}$(8-Br-cGMP) values of 1.1, 1.4, and 2.0 μM. The smooth curve is a fit of eq 2 to the data with n = 1.2 and $K_{1/2}/K_{1/2}$(8-Br-cGMP) = 8.5.

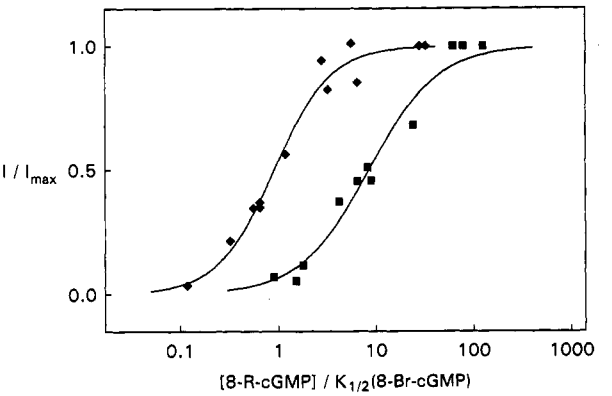


FIGURE 3: Comparison of dose–response relations for SET-cGMP (◆) and TMAET-cGMP (■). Cyclic nucleotide-activated currents and concentrations are expressed as described in Figure 1. TMAET-cGMP data and fit are the same as in Figure 2. SET-cGMP data were obtained on three patches with fit $K_{1/2}$(8-Br-cGMP) values of 1.6, 1.7, and 1.8 μM. The smooth curve is a fit of eq 2 to the data with n = 1.5 and $K_{1/2}/K_{1/2}$(8-Br-cGMP) = 0.90.

The other four analogs caused a smaller maximum current at both +50 and −50 mV, AEA-cGMP being the weakest activator. Three of the four "partial" activators contain a positive charge. *n*PA-cGMP, which does not, gave a somewhat higher maximum current than the other three. The relative variations in the maximum currents (38%) were small compared with the relative variations in the effective concentrations (approximately 400-fold). The reason for the lower maximum currents has not yet been determined.

On some patches two of the positively-charged derivatives, AET-cGMP and AEA-cGMP, appeared to block the current a high concentrations. The evidence for this is two-fold: (1) higher concentrations sometimes gave a smaller steady-state current at +50 mV than lower concentrations (see Figure 1B for AEA-cGMP) and (2) currents at the higher concentrations exhibited a time-dependent droop following a switch from −50 to +50 mV, and a time-dependent increase on returning to −50 mV (data not shown). This was not observed at lower concentrations. Voltage-dependent block may have occurred because the positively-charged groups attached to C_8 were driven into the pore at positive membrane potentials. Because the cGMP moiety was too large to pass through the pore, Na^+ currents were impeded while the pore was occupied by the amino group. Perhaps consistent with this interpretation, voltage-dependent block was not observed with TMAET-

Table II: NMR Measurements of H2′ Chemical Shifts and C4–H1′ Coupling Constants for cGMP and 8-Substituted Derivatives[a]

nucleotide	δH2′	$^3J_{C4-H1'}$	nucleotide	δH2′	$^3J_{C4-H1'}$
cGMP	4.55	4.6	AET-cGMP	4.70	ND[b]
8-Br-cGMP	4.76	5.2	*n*PA-cGMP	4.76	ND[b]
*n*PT-cGMP	4.68	5.1	AEA-cGMP	4.77	5.5

[a] All spectra were determined in D_2O. Chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) are in hertz. [b] Not determined.

cGMP; trimethylammonium ions have been shown to be impermeant, in contrast to ammonium ions which are more permeant than Na^+ (Picco & Menini, 1993). Block was most likely not the cause of the apparently incomplete activation by high concentrations of AET-cGMP and AEA-cGMP because (1) block was not observed on some patches in which the current clearly reached a saturating level and (2) on patches in which block was observed, the maximum current at −50 mV (where block was mostly relieved) was still significantly less than that induced by saturating 8-Br-cGMP.

The fit values of the Hill coefficient, *n*, varied among the different analogs, ranging from 1.2 for TMAET-cGMP to 3.6 for AEA-cGMP (see the legends to Figures 1–3). The data suggest a possible trend, in which lower affinity analogs activate channels with a higher degree of cooperativity. We hesitate to draw such a conclusion at this time, however, for two reasons. First, the value of the Hill coefficient depends somewhat on the range of concentrations tested, since the Hill equation does not fit entire dose–response relations measured over a wide range of concentrations (Zimmerman & Baylor, 1986; Haynes et al., 1986). The value obtained at low concentrations is generally a more reliable indicator of cooperativity in the activation mechanism. In the present study, however, we were primarily interested in determining the analog concentrations that produced a half-maximal current. Second, the values of *n* varied significantly from patch to patch even for the same analog. This was observed previously for other cyclic nucleotides [see Kaupp (1991)]. The basis for these variations are not yet understood.

NMR Estimation of Conformational Differences about the N-Glycosidic Bond. To determine if shifts in the *syn–anti* conformational equilibrium could account for the dramatic differences in analog effectiveness, we employed NMR measurements previously shown to be sensitive to conformation about the *N*-glycosidic bond. Cho and Evans (1991) previously reported that both the H2′ chemical shift (δH2′) and the magnitude of the C4–H1′ vicinal coupling constant ($^3J_{C4-H1'}$) reflect variations in the *syn*/*anti* ratio. For guanosine and its 8-substituted derivatives in DMSO, δH2′ ranged from 4.38 to 4.96 ppm, and $^3J_{C4-H1'}$ ranged from 2.5 to 5.4 Hz. In both cases, higher numbers were attributed to compounds that preferentially assume a *syn* conformation. Similar measurements are presented in Table II for cGMP and selected 8-substituted derivatives in D_2O. δH2′ values measured in both D_2O and DMSO varied over a much narrower range than those reported by Cho and Evans; a limited survey of $^3J_{C4-H1'}$ values indicated a similar trend. This may indicate that the measurements are less sensitive to conformation in cyclic nucleotides, due to the specific sugar conformation imposed by the cyclic phosphate. Alternatively, these compounds may simply exhibit less conformational variability. In either case, gross conformational differences should be apparent in the measurements. A striking finding was that the H2′ chemical shifts for *n*PA-cGMP and AEA-cGMP were nearly identical, despite a 9.2-fold difference in the concentrations required to open channels. This suggests that conformational differences do not account for the wide disparities in analog effectiveness. Similarly, conformational effects most likely do not account for the different potencies within 8-thio-substituted analogs or between 8-thio- and 8-amino-substituted analogs.

DISCUSSION

In an attempt to understand what chemical features enable certain 8-substituted derivatives to activate cGMP-gated channels at lower concentrations than cGMP itself, we have systematically varied both the atom attached to C_8 and the charge characteristics of the terminal functional group following an ethylene bridge. The results indicate that both positions dramatically affect analog potency. In general, 8-thio-substituted derivatives were much more effective than 8-amino-substituted derivatives, and derivatives containing neutral or negatively-charged tails were preferred over those with positively-charged tails.

Whereas it has been proposed that bromine and other substituents produce effective derivatives primarily by biasing the equilibrium about the *N*-glycosidic bond in favor of the *syn* conformation, the ^1H NMR data presented suggest that this effect is only part of the story. The effective concentrations of the analogs tested here varied over a nearly 400-fold range, while the H2′ chemical shifts showed little, if any, variation. The dominant factor in determining analog effectiveness appears to be chemical interactions between the C_8 substitutions or neighboring atoms affected by the substitutions and the channel's binding sites.

Amino-substitution at C_8 perturbs the electronic structure of the guanine ring system, as evidenced by the appearance of a second absorbance peak at longer wavelength (Table I). The nature of these structural changes and whether they are responsible for the ineffectiveness of amino-substituted derivatives remain to be determined. The potency of derivatives containing apolar tails may indicate the proximity of a hydrophobic pocket in the channel's binding sites. That negatively-charged tails were preferred over positive ones also indicates the importance of electrostatic interactions. These analogs, in combination with expression (Kaupp et al., 1989) and site-specific mutagenesis, should prove to be useful probes in future studies of the channel binding sites (Kumar & Weber, 1992).

The channel contains an 80 amino acid region that exhibits some sequence homology to the cGMP binding regions of cGMP-dependent protein kinase (Kaupp et al., 1989; Kaupp, 1991). At a functional level, 8-Br-cGMP and certain other 8-substituted derivatives have been shown to activate the kinase at low concentrations (Miller et al., 1973; Corbin et al., 1986; Sekhar et al., 1992). Like the channel, the kinase also prefers 8-thio-substituted to 8-amino-substituted derivatives of cGMP. It may be informative to use the cGMP analogs presented here to further investigate the structural and functional kinship between these two cGMP-binding proteins.

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