Irreversible Activation of Cyclic Nucleotide-Gated Ion Channels by Sulfhydryl-Reactive Derivatives of Cyclic GMP†

R. Lane Brown,* Tammie L. Haley, and Sheila D. Snow

Neurological Sciences Institute, Oregon Health Sciences University, 1120 NW 20th Avenue, Portland, Oregon 97209

Received September 3, 1999; Revised Manuscript Received October 27, 1999

ABSTRACT: First discovered in the sensory epithelium of the visual and olfactory systems, cyclic nucleotide-gated (CNG) ion channels have now been found in tissues throughout the body. Native rod CNG channels are tetramers composed of homologous, but distinct, α- and *β*-subunits. The goal of this study was to develop a novel method for targeting covalent attachment of cGMP to individual subunit types. Toward this goal, we have found that treatment of membrane patches expressing rod α-subunit channels with sulfhydryl-reactive derivatives of cGMP resulted in irreversible activation. The persistent currents were sensitive to block by both Mg^{2+} and tetracaine. Pretreatment of the patch with the sulfhydryl-blocking reagents *N*-ethylmaleimide (NEM) and bis-dithionitrobenzoic acid (DTNB) prevented covalent activation; the effect of DTNB was reversed by reduction with DTT. Furthermore, the process of covalent activation was dramatically slowed by the presence of an excess of 8-Br-cGMP. These results suggested that covalent activation resulted from the tethering of cGMP near the channel's ligand-binding sites by reaction with an endogenous cysteine. The α-subunit of the rod channel contains seven cysteine residues, and we set out to determine the site of attachment by site-directed mutagenesis. Surprisingly, irreversible activation was not abolished by elimination of all seven cysteine residues. This result suggests that the site of attachment is on a tightly associated protein, rather than on the channel protein itself. To further investigate these results, we treated patches containing irreversibly activated channels with 100 *μ*g/mL trypsin and discovered two modes of covalent activation. One type developed rapidly and was removed by trypsin treatment, and the second developed slowly and was resistant to trypsin treatment. Both types of covalent activation were present in all mutants tested and were also present when CNG channels were expressed in HEK-293 cells. These results suggest that CNG channel subunits may associate with endogenous proteins when they are expressed in heterologous systems.

The cyclic nucleotide-gated (CNG) ion channel of retinal photoreceptors generates the cell's electrical response by tracking light-induced changes in the intracellular level of cGMP (*1*, *2*). The rod CNG channel is a tetramer composed of 63 kDa α- and 240 kDa *β*-subunits (*3*−*7*). Both subunit types contain a transmembrane domain and pore region in the amino-terminal portion and a cyclic nucleotide-binding site at the carboxy-terminus (*8*−*10*). While the basic structure of both subunits is similar, each subunit type possesses a number of distinct functional characteristics. For instance, the α-subunit contains a histidine residue in the linker region between the transmembrane and cyclic nucleotide-binding domains that is required for potentiation of the channel's response by transition metal divalent cations (*11*, *12*). The *β*-subunit, on the other hand, binds the calcium−calmodulin complex, thereby lowering the channel's apparent affinity for cGMP (*13*−*15*). In addition, the *β*-subunit contains a glutamic acid-rich domain of unknown function at its amino-terminus (*7*).

Activation of CNG ion channels is a multistep process in which a series of ligand-binding steps is followed by an allosteric transition that leads to channel opening (*16*−*19*). Because each binding step is a dynamic equilibrium with ligands binding and dissociating on a millisecond time scale, it has been difficult to assess the contributions of individual binding steps to overall channel activation. Recently, these obstacles have been surmounted by covalently tethering a photoaffinity analogue of cGMP (APT-cGMP) to the channel's binding sites (*10*, *20*). The ability to study the activation of channels with a fixed complement of attached ligands allowed us to identify a functional heterogeneity in the binding sites of native channels (*21*). Ruiz and Karpen have extended this analysis to the single channel level (*22*). By analyzing the behavior of channel species with zero, one, two, three, or four ligands covalently attached, they have developed a model for channel activation that exhibits "dynamic flexibility". All channel species can open to any one of three conductance states, but binding of more ligands favors longer openings with increased conductance (*23*).

We are trying currently to develop a method to target specific subunit types for the covalent attachment of ligand. This approach will allow us to address similar questions using macroscopic currents and to more accurately assess the functional properties of each subunit type in the heteromeric

† This work was supported by grants from the National Eye Institute (EY11397), the Medical Research Foundation of Oregon, and the Oregon Lions Sight and Hearing Foundation.

* To whom correspondence should be addressed. Phone: (503) 413-7438. Fax: (503) 413-7229. E-mail: brownla@ohsu.edu.

10.1021/bi9920735 CCC: $19.00
Published on Web 12/11/1999

channel. The photoaffinity probe is not well suited for this purpose because photolysis generates a highly reactive and rather nonselective nitrene species. Although the labeling was primarily confined to the cGMP-binding sites, APT-cGMP did not seem to distinguish significantly between the α- and β-subunits in the native channel (*10*). One strategy to limit attachment to a single subunit type is to use sulfhydryl-reactive derivatives of cGMP, which attach to the channel via reaction with endogenous cysteine residues. After the site of attachment has been identified by site-specific mutagenesis, mutant subunits could be created that were resistant to covalent activation. Here we report the production of sulfhydryl-reactive derivatives of cGMP that irreversibly activate current through CNG channels when applied to the cytoplasmic face of excised membrane patches. Surprisingly, however, removal of all seven cysteine residues from the rod CNG channel α-subunit did not hinder the development of irreversible activation. These results suggest that irreversible activation may result from attachment of cGMP moieties to a number of cysteine residues in close proximity to the cGMP-binding site; some of these sites may reside on a protein that is tightly associated with the channel, rather than the channel itself.

MATERIALS AND METHODS

Cyclic nucleotides, TPCK-treated trypsin, collagenase (type IA), NEM, and DTNB were purchased from Sigma (St. Louis, MO). The heterobifunctional cross-linkers sulfosuccinimidyl[4-iodoacetyl]aminobenzoate (sulfo-SIAB) and *N*-succinimidyl 3-[2-pyridyldithio]propionate (SPDP) were from Pierce (Rockford, IL), and divinyl sulfone was from Aldrich (Milwaukee, WI). Methanethiosulfonate reagents were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). All buffers and reagents were of the highest purity available. *Xenopus laevis* were purchased from Xenopus One (Ann Arbor, MI). *Rana pipiens* were purchased from Charles Sullivan (Nashville, TN). Electrospray mass spectrometric analysis of the cyclic nucleotide derivatives was performed at the W. Alton Jones Protein Science Center (Lake Placid, NY).

Synthesis of Sulfhydryl-Reactive Derivatives of cGMP. All sulfhydryl-reactive derivatives of cGMP were synthesized from 8-thio-cGMP, which was prepared as described previously (*24*). Reaction progress was monitored by thin-layer chromatography on silica.

Vinylsulfonoethylthio-cGMP (VSET-cG). Fifty milligrams (0.13 mmol) of purified 8-thio-cGMP (ammonium salt) was dissolved in 10 mL of 50 mM sodium borate, pH 8.5. A 20-fold excess of divinyl sulfone (0.3 g; 0.25 mL) was added, and the reaction mix was incubated for 24−48 h at room temperature. Excess divinyl sulfone and solvent were removed by repeated drying in a Speed-Vac (Savant, Holbrook, NY). The product, VSET-cG, was dissolved in water and purified by reversed-phase HPLC on a 250 mm × 22 mm Econosphere C18 column (Alltech, Deerfield, IL) using a water to methanol gradient (1%/min) with 5 mM ammonium acetate (pH 3.9) throughout. The flow rate was 5 mL/min, and the column eluate was monitored for absorbance at 280 nm using a diode-array detector (HPLC System 1100; Hewlett-Packard, Palo Alto, CA).

Aminoethylthio-cGMP (AET-cG). Aminoethylthio-cGMP was a key intermediate in the synthesis of the two sulfhydryl-reactive derivatives described below. Purified 8-thio-cGMP (100 mg; 0.25 mmol) was dissolved in 20 mL of 50 mM sodium borate, pH 8.5, and a 10-fold excess of 2-bromoethylamine hydrobromide (0.5 g) was added. The reaction mix was incubated at room temperature for 24−48 h. The product, aminoethylthio-cGMP (AET-cG), was purified by reversed-phase HPLC as described above, and then dried in a Speed-Vac to remove the methanol and ammonium acetate.

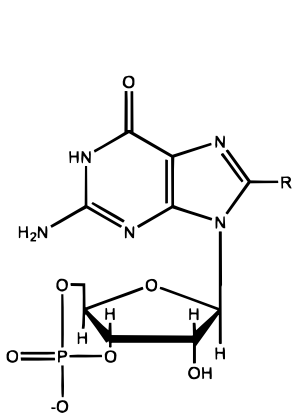
Iodoacetamidobenzoylamidoethylthio-cGMP (IABET-cG). AET-cG (50 mg; ∼0.10 mmol) was dissolved in 10 mL of 50 mM sodium borate, pH 8.5. A 2-fold excess of sulfo-SIAB (100 mg) was added, and the reaction was incubated at room temperature in the dark for 6−12 h. The product, IABET-cG, was purified by reversed-phase HPLC as described and dried in a Speed-Vac.

2-Pyridyldithiopropylaminoethylthio-cGMP (PDT-cG). AET-cG (50 mg; ∼0.1 mmol) was dissolved in 10 mL of 50 mM sodium borate, pH 8.5. A 2-fold excess of SPDP (60 mg) was first dissolved in a small volume of acetonitrile, and then added to the solution of AET-cG. The reaction mixture was incubated for 6−12 h at room temperature. The product, PDT-cG, was purified by reversed-phase HPLC and dried in a Speed-Vac.

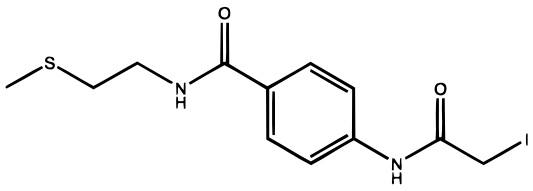
Determination of Cyclic Nucleotide Concentrations. Concentrations of cyclic nucleotide derivatives were determined by UV absorbance. An extinction coefficient of 12 950 M^{-1} cm^{-1} was used for cGMP. The extinction coefficient of VSET-cG was assumed to be the same as AET-cG [17 700 M^{-1} cm^{-1} (*24*)]. To determine the extinction coefficient for IABET-cGMP, a highly purified sample was dried repeatedly until it reached a constant weight in a Speed-Vac. This assured that all of the ammonium acetate from the HPLC purification step had been removed. The absorbance of this sample was then measured after it had been diluted in buffer, and the extinction coefficient was calculated assuming that the product was an anhydrous acetate salt. The extinction coefficient of PDT-cGMP was determined by two different methods. The first method was a gravimetric method similar to that described for IABET-cGMP. In the second method, the concentration of PDT-cGMP was estimated by the increase in A_{343} generated by the release of pyridine-2-thione when PDT-cGMP is reduced by β-mercaptoethanol (The molar extinction coefficient of pyridine-2-thione is 8.08×10^3 M^{-1} cm^{-1}). The concentration determined in this manner was then compared to the absorbance prior to reduction. The extinction coefficients that were determined for PDT-cGMP using these two methods agreed within 10%. Molecular weights, spectral data, and apparent affinities for IABET-, PDT-, and VSET-cGMP are given in Table 1.

Mutagenesis and Expression of the Rod CNG Channel. Complementary DNAs encoding the α-subunit of the wild-type bovine rod CNG channel (bROD), as well as the ΔN- and ΔC-bROD, and the cysteine-free mutants were gifts from the laboratory of Dr. W. N. Zagotta (University of Washington, Seattle, WA). The cDNA had been modified using silent mutations to create a user-friendly gene with a number of unique restriction sites. Other cysteine point mutations were created using the Quick-Change kit (Stratagene, La Jolla, CA). Complementary RNA was transcribed using the mMessage mMachine kit (Ambion, Austin, TX), and *Xenopus* oocytes were selected and injected as previously

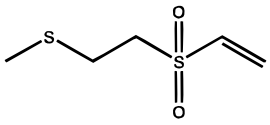
Table 1: Structures and Physical Properties of Sulfhydryl-Reactive Derivatives of Cyclic GMP



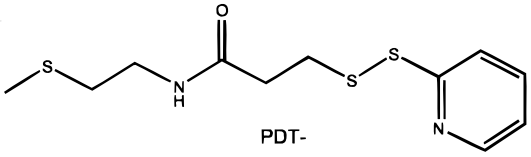
Cyclic GMP



IABET-



VSET-



PDT-

derivative	calcd mol wt (NH_4^+ salt)	*m/e*	(mM^{-1} cm^{-1})	λ_{max} (nm)	$K_{1/2}$ (μM)
IABET-cG	725	706.3	26.8	272	1.7
VSET-cG	513	494.3	17.7	276	1.7
PDT-cG	635	616.0	19.5	282	1.2

described (*25*). All procedures involving animals were carried out as prescribed by institutional guidelines.

For some experiments, CNG channels were expressed in HEK-293 cells. These cells were transfected with a pcDNA vector (Invitrogen, Carlsbad, CA) containing the gene encoding the wild-type rod channel α-subunit (a gift from Dr. Robert Molday, University of British Columbia) by treatment with pFX-6 (Invitrogen, Carlsbad, CA) using the procedure of Biel et al. (*26*). Cells were cotransfected with a plasmid encoding green fluorescent protein (Clontech, Palo Alto, CA). Two days after transfection, cells expressing exogenous protein were identified by fluorescence, and CNG channel activity was assayed by patch-clamp recording from excised inside-out patches.

Preparation of Rod Photoreceptors. Rod photoreceptors were prepared as described previously (*21*). Briefly, light-adapted *R. pipiens* were sacrificed by rapid decapitation followed by bilateral pithing of the brain and spinal cord. After removal and hemisection of the eye cups, the retinas were gently peeled from the pigment epithelium and placed in a modified Ringer solution containing 111 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 6.0 mM $MgCl_2$, 10 mM D-glucose, 0.02 mM EDTA, and 3.0 mM Hepes, pH 7.6. The bottom of the experimental chamber was made tacky by brief treatment with 1 mg/mL poly-L-lysine hydrobromide (30000−70000 mol wt; Sigma, St. Louis, MO) or 0.1 mg/mL concanavalin A (Sigma) followed by a thorough rinse with Ringer solution. Small pieces of retina were placed in the chamber and teased apart with fine forceps to release individual rod outer segments.

Electrophysiological Measurements. Currents through CNG channels from all three types of preparation were recorded using a Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Data were acquired using a Digidata 1200 controlled by pCLAMP6.0 software (Axon Instruments). The electrodes used to record from oocytes had resistances between 0.5 and 1.5 MΩ; those used to record from HEK cells and photoreceptors had resistances of 2−3 MΩ. The latter electrodes were bent slightly about 1 mm from the tip using a miniature oxygen−acetylene torch to facilitate a more vertical approach to the cells. Gigaohm seals on photoreceptors and HEK cells were initially obtained in Ringer solution. Following patch excision, currents were measured in control buffer containing 130 mM NaCl, 0.2 mM EDTA, and 3 mM Hepes, pH 7.4. Seals on oocytes were routinely obtained in control solution.

Cyclic nucleotide-stimulated currents were determined from inside-out patches by subtracting currents in a nucleotide-free control solution from those obtained when various concentrations of cyclic nucleotide were added. Solutions were applied to the intracellular face of patches using an array of tubes controlled by a Biologic RSC-100 (Molecular Kinetics, Pullman, WA). Following permanent activation, the "leak" current was estimated following application of either 10 mM $MgCl_2$ or 500 μM tetracaine (*27*). These concentrations of channel blockers would inhibit greater than 95% of the channel current. Although magnesium is a somewhat less specific blocker than tetracaine, it was used in most cases because it was more readily reversible. Patches that showed a significant decrease in the seal resistance (determined by comparing the original leak current to the current present in 10 mM $MgCl_2$ after covalent activation) were discontinued. Results were analyzed using SigmaPlot (Jandel Scientific, San Rafael, CA). When results were plotted as histograms, the error bars indicate the standard deviation among the values from different patches.

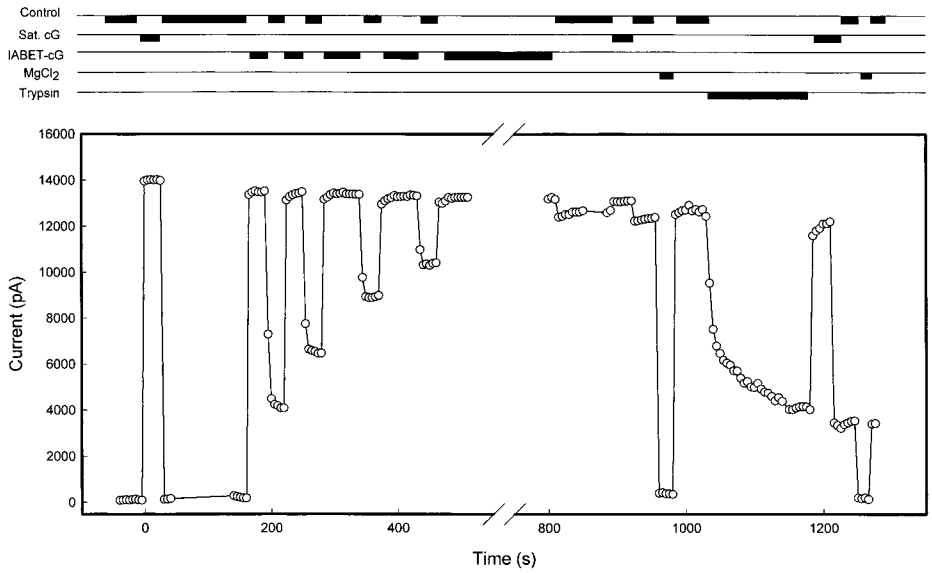


FIGURE 1: Covalent activation of rod α-subunit CNG channels by IABET-cGMP. The open circles indicate the current elicited by +50 mV pulses given every 5 s. The intracellular face of the patch was exposed to solutions indicated by the thicker lines at the top of the figure. Currents were not leak-subtracted. Application of a saturating concentration of cGMP evoked a 14 nA current that returned to baseline within 5 s of cGMP removal. A brief application of IABET-cGMP also elicited a near maximal current, but its effect was not fully reversed upon removal. Instead, a persistent current developed with continued exposure that reached 95% of the maximum current at 800 s. This persistent current was strongly blocked by 10 mM $MgCl_2$. These results strongly suggest that the persistent current was due to covalent activation of the CNG channels by tethering of cGMP near its binding site. Application of 100 μg/mL trypsin, however, rapidly removed 80% of the covalent activation in this patch with little effect on the maximum current.

RESULTS

Irreversible Activation of CNG Channels by Sulfhydryl-Reactive Derivatives of cGMP. Three new sulfhydryl-reactive derivatives of cGMP were synthesized and characterized in this study. Their structures and physical properties, including absorbance maximums, extinction coefficients, and molecular weights, are given in Table 1. All three derivatives activated the CNG channel when applied at low micromolar concentrations to inside-out patches excised from oocytes expressing the rod α-subunit. Concentrations above ~20 μM elicited saturated responses from the channel, similar to that obtained in 1 mM cGMP. Approximate $K_{1/2}$ values for these derivatives are also given in Table 1. These values were determined by measuring a dose−response relation following modification of the channel with sulfonatoethylmethanethiosulfonate (MTSES). We have previously shown that this MTS compound efficiently blocks sulfhydryl groups on the α-subunit of the rod CNG channel without altering the dose−response relation of the channel (*25*). It was not possible to measure an accurate dose−response relation using unmodified channels because application of these derivatives caused irreversible channel activation. During this process, the dose−response relation shifts to lower concentrations and becomes significantly shallower.

Exposure of rod α-subunit CNG channels to IABET-cGMP caused irreversible activation (Throughout this paper the terms "irreversible", "permanent", and "covalent" are used interchangeably to describe activation that cannot be removed by extended perfusion of the patch with nucleotide-free control solution.). As shown in Figure 1, the application of 50 μM IABET-cGMP to an excised patch elicited a maximal current response. Upon removal of IABET-cGMP, however, the patch current did not return to the initial control level. Instead, the patch current remained at ~30% of maximum even after extended perfusion with nucleotide-free control. Repeated exposure to IABET-cGMP for a total of 5−7 min resulted in a persistent current that was >95% of the maximum current elicited by the application of a saturating concentration of cGMP. This persistent current was virtually abolished by the addition of 10 mM $MgCl_2$ (see Figure 1) or 500 μM tetracaine (data not shown) to the bath solution. These data suggest that the persistent current was caused by irreversible activation of channels due to the covalent attachment of IABET-cGMP near the channel's ligand-binding sites. Most of this covalent activation could be removed by treatment with 100 μg/mL trypsin, a result that will be discussed below in greater detail. Treatment of patches with PDT-cG or VSET-cG resulted in similar irreversible activation. The time course of permanent activation by VSET-cG was similar, but that of PDT-cGMP was remarkably rapid (see Figure 7). In fact, application of 25 μM PDT-cGMP led to nearly complete irreversible activation within 10 s.

Covalent Activation Occurs via the Reaction with Endogenous Sulfhydryl Groups. Pretreatment of patches with the sulfhydryl-reagents *N*-ethylmaleimide (NEM) or dithio-bis-nitrobenzoic acid (DTNB) blocked permanent activation (Figure 2). Patches exposed to 25 μM IABET-cGMP for more than 7 min reached a limiting activation level of 98 ± 4% in control solution. Pretreatment of patches with 1 mM NEM for 5−10 min did not induce measurable spontaneous channel activity, and the maximum patch current in the presence of saturating concentrations of cGMP was also largely unchanged. This treatment, however, did block permanent activation when the patch was subsequently treated with IABET-cGMP. Instead of the ~98% permanent activation obtained with naïve patches, the extent of irreversible activation was limited to only 5 ± 2%. Similar results were obtained upon pretreatment with 1 mM DTNB for 5−10 min. Once again pretreatment had little effect on

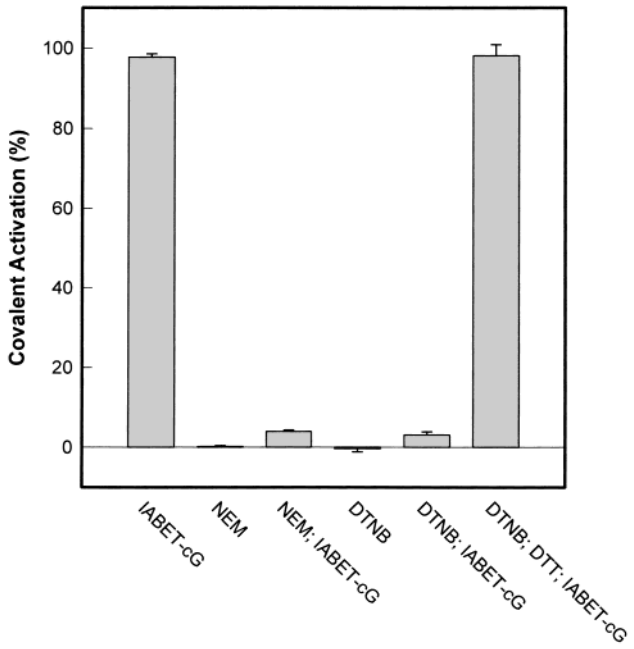


FIGURE 2: Treatment of excised patches of *Xenopus* oocyte membrane containing expressed bovine retinal α-subunit channels with IABET-cG resulted in irreversible activation via tethering to endogenous cysteine residues. Patches were treated with a saturating concentration of IABET-cGMP (25 μM) and then washed extensively in nucleotide-free control solution. The seal resistance and "leak" current were estimated by blocking the channel current with either 10 mM $MgCl_2$ or 500 μM tetracaine. The extent of covalent activation was then determined by comparing the difference current present in control solution with that elicited by a saturating concentration of cGMP. IABET-cGMP was applied repeatedly until the extent of covalent activation reached a limiting value (≥5 min total application time). This treatment typically resulted in covalent activation of $>98 \pm 2\%$ of the channels ($N = 5$). Pretreatment (5 min) of the patch with the sulfhydryl-specific reagents NEM or DTNB significantly blocked covalent activation (NEM, $5 \pm 0.8\%$; DTNB, $4 \pm 1.5\%$; $N = 4$ for each). Because DTNB reacts with sulfhydryls to form mixed disulfides, its blocking effect could subsequently be reversed by treatment with DTT which regenerates free sulfhydryl groups ($96 \pm 4\%$).

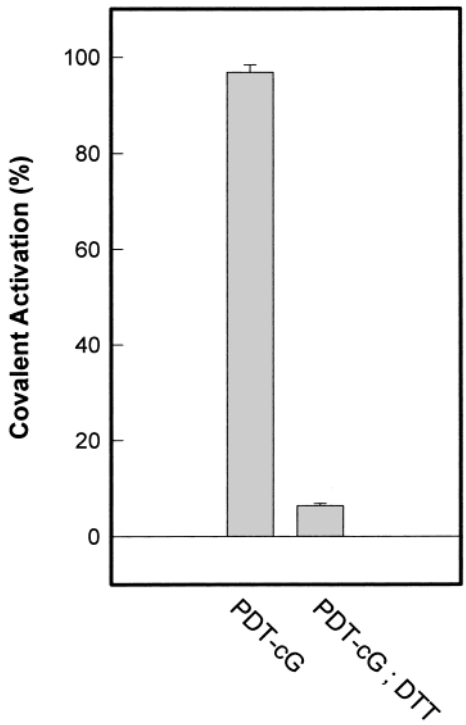


FIGURE 3: PDT-cGMP covalently activated the channel by forming a mixed disulfide that could be cleaved by DTT. To confirm that covalent activation was due to modification of a cysteine and not some other nucleophilic residue, we tested a derivative that forms reversible mixed disulfide linkages with sulfhydryls. Exposure of an excised patch to 25 μM PDT-cGMP for 1 min resulted in covalent activation of virtually all of the CNG channels. This covalent activation could be reversed by a subsequent 30 min treatment with 10 mM DTT, which reductively cleaves disulfides. This type of reversibility is specific to modification of sulfhydryl groups. The values shown are the means of results from three different patches.

spontaneous channel activity or maximum current, but it prevented covalent activation by IABET-cGMP. Because DTNB reacts with sulfhydryl groups to form mixed disulfides, this modification should be reversed by treatment with reducing agents such as β-mercaptoethanol and dithiothreitol (DTT). Treatment of the DTNB-modified patches with 20 mM DTT for 10 min restored the ability of the channels to be irreversibly activated by subsequent application of IABET-cGMP.

Similar experiments were also performed with PDT-cGMP using the sulfhydryl-reagent methanethiosulfonate-ethylsulfonic acid (MTSES). Pretreatment of patches with 500 μM MTSES for 5 min in control solution substantially blocked covalent activation by PDT-cGMP. After treatment with MTSES, application of 25 μM PDT-cGMP for 10 min caused less than 5% covalent activation. This compares with almost complete covalent activation of unmodified channels in just 10 s (data not shown).

To further test if our cGMP derivatives were, in fact, reacting with sulfhydryl groups, we performed several more control experiments. First, we checked to see if the permanent activation caused by PDT-cGMP was reversed by treatment with DTT. Unlike the iodoacetamide and vinyl sulfone derivatives, which are alkylating agents, dithiopyridine derivatives react with sulfhydryl groups via disulfide exchange. The resulting mixed disulfide linkage can be reductively cleaved by treatment with DTT. As shown in Figure 3, treatment with DTT did reverse the covalent activation achieved with PDT-cGMP. As a final control, we monitored reaction of both the IABET- and PDT-cGMP derivatives with free cysteine, lysine, and histidine in solution. In these experiments, 50 μM IABET- or PDT-cGMP was incubated in control patch-clamp solution containing the amino acid to be tested at a concentration of 1 mM. Reaction progress was monitored by reversed-phase HPLC using the same conditions described for the purification of the cGMP derivatives. While both derivatives reacted rapidly with cysteine, no reaction was detectable with either lysine or histidine even after 12−15 h at room temperature. In light of these results, it seems virtually certain that irreversible activation by our sulfhydryl-reactive derivatives occurs via reaction with sulfhydryl groups.

Excess 8-Br-cGMP Dramatically Slows Covalent Activation by IABET-cGMP. If irreversible activation is due to tethering of cGMP within the binding sites of the channel, the time course of covalent activation should be dramatically slowed by the presence of competing ligand. To investigate this possibility, we included 2 mM 8-Br-cGMP during the treatment of the patch with 50 μM IABET-cGMP. As shown in Figure 4, the rate of covalent activation was significantly

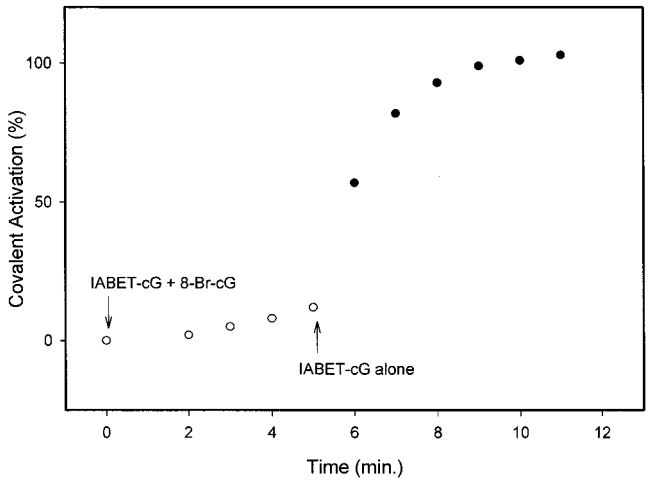


FIGURE 4: Excess 8-Br-cGMP slows covalent activation by IABET-cGMP. A patch from an oocyte expressing the rod CNG channel α-subunit was exposed to 25 μM IABET-cGMP in the presence or absence of 2 mM 8-Br-cGMP. Plot depicts the time course of covalent activation. The 8-Br-cGMP was present in during the first 5 min of exposure (○) but was absent during minutes 6−12 (●). Similar results were seen in two other patches.

reduced. After the excess cGMP was removed, covalent activation of the channels in this patch proceeded along a typical time course. These results indicate that efficient covalent activation requires concentration of the IABET-cGMP in the channel's binding sites and suggest that the cysteine involved in covalent activation is in close proximity to the binding site. The predicted length of the linker between the guanine ring of cGMP and the sulfhydryl-reactive group is ∼15 Å for IABET-cGMP, ∼10 Å for PDT-cGMP, and ∼7 Å for VSET-cGMP.

Mutation of Cysteine Residues in the Channel Does Not Eliminate Covalent Activation. In the next series of experiments, we set out to determine the site of attachment that produces irreversible channel activation. There are seven cysteine residues scattered throughout the α-subunit of the channel (Figure 5). Two of these residues, cysteines 169 and 314, are located in regions of the channel predicted by hydropathy plots to lie within the plasma membrane, and cysteine 184 is predicted to be in an exterior loop. The remaining four cysteine residues are expected to lie on the cytoplasmic face of the channel. Cysteine 35 is located in the amino-terminal region of the channel, prior to the first transmembrane domain. Cysteine 481 lies in a linker region between the channel's transmembrane and cyclic nucleotide-binding domain. The final two cysteine residues, at positions 505 and 573, are found within the cyclic nucleotide-binding domain itself. Each of these residues was individually mutated to either alanine or threonine, depending on which mutation resulted in better expression and/or less perturbation of channel properties (i.e., the dose−response relation for cGMP; see Table 2). These mutant forms of the CNG channel were challenged with a saturating concentration of IABET-cGMP (25−50 μM) to determine if any were resistant to irreversible activation. As shown in Figure 6, all single mutants were still irreversibly activated by this treatment. Furthermore, none exhibited a significant change in the kinetics of permanent activation (data not shown).

Because covalent activation persisted in several double and triple mutants, we obtained a functional cysteine-free version

Table 2: Summary of Expression and Activation Properties of CNG Channel Mutants[a]

channel type	$K_{1/2}$ (mM)	n	expression level	cov. act. with IABET-cG
bRod	79 ± 20	2.3 ± 0.3	++++	yes
C35A	86 ± 17	2.4 ± 0.3	++	yes
C169T	84 ± 15	2.3 ± 0.3	++++	yes
C186T	156	2.3	+++	yes
C314T	133	2.0	+	yes
C481A	39 ± 6	2.4 ± 0.8	+++	yes
C505T	67 ± 22	2.3 ± 0.3	+++	yes
C573A	98 ± 17	1.45 ± 0.6	++++	yes
C573T	280 ± 12	1.9 ± 0.2	++	yes
C573S	233 ± 24	1.8 ± 0.1	++++	yes
ΔN-bROD	89 ± 24	2.01 ± 0.3	++++	yes
ΔC-bROD	118 ± 21	1.97	++++	yes
Cys-free	8.6 ± 0.44[b]	2.1[b]	+++	yes

[a] This table compares the activation parameters and expression levels of a series of mutants in which one or more cysteine residues was removed. Typical expression levels are encoded as follows: (++++) maximum currents > 2000 pA; (+++) 500−1999 pA; (++) 200−499 pA; (+) 10−199 pA; and (NE) no detectable expression in >3 batches of oocytes. All values are ± SD except for the Cys-free mutant which is ± SEM. All patches for which a SD is listed have N values ranging from 4 to 20. The values for the C186T and C314T are from single patches. [b] Values taken from ref *28*.

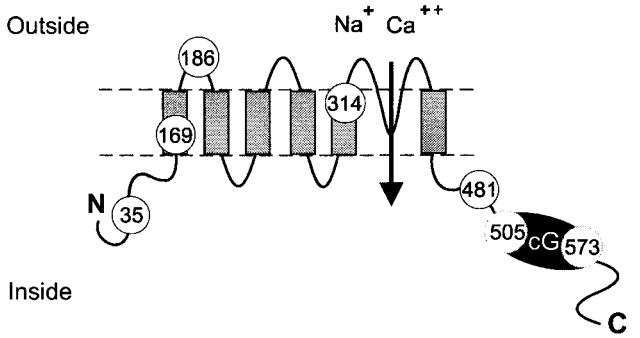


FIGURE 5: Location of cysteine residues within the α subunit of the bovine retinal CNG channel. The proposed membrane topology of the retinal CNG channel includes six transmembrane helices, a pore loop, and a carboxy-terminal cGMP-binding domain. Seven cysteine residues are present in the α-subunit. One, C186, is predicted to lie on the external face of the channel, and two others, C169 and C314, are predicted to be buried within the membrane. The remaining four are predicted to lie on the cytoplasmic face of the channel. The first, C35, lies near the amino-terminus. The second, C481, lies in the loop connecting the transmembrane domain to the cGMP-binding site. The final two, C505 and C573, are found within the cGMP-binding site itself. C505 is predicted to be within 12 Å of the bound cGMP (*42*).

of the channel (C35A/C169S/C186S/C386S/C481F/C505V/C573V) as a gift from W. N. Zagotta, G. Flynn, and K. Matulef (University of Washington). The functional behavior of this channel was similar to wild-type, although the apparent affinity was slightly higher [$K_{1/2}$ = 8.6 μM (*28*)]. This decrease in $K_{1/2}$ is most likely due in part to the C481F mutation (*25*, *28*). When the Cys-free mutant was challenged with PDT-cGMP, irreversible activation was undiminished (Figure 6). Similar results were obtained with IABET-cGMP.

Brief Treatment with Trypsin Removes the Initial Type of Covalent Activation. We then examined one possible explanation for our perplexing results. Irreversible activation might occur if the sulfhydryl-reactive cGMP derivatives were attaching to a protein that was intimately associated with

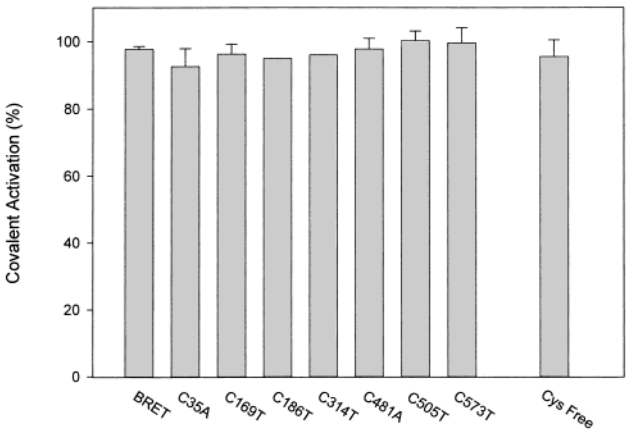


FIGURE 6: Mutation of individual cysteine residues in the channel does not eliminate covalent attachment by IABET-cGMP. As shown at right, even elimination of all cysteine residues did not abolish covalent activation. Values shown are the mean from at least four patches, except for the C186T, which was from two patches.

the channel, rather than the channel itself. If this associated protein were highly sensitive to digestion by trypsin, it might be possible to remove covalent activation, while leaving the CNG channels functionally intact. This type of experiment is shown in the latter half of Figure 1. CNG channels in a patch from an oocyte expressing the bovine rod α-subunit were covalently activated by application of IABET-cGMP. Following irreversible activation, the patch was treated with 100 μg/mL trypsin in control buffer. As the trypsin solution was applied, the patch current declined precipitously, eventually reaching a level about 20% of the original after 1.5 min. This decline was not, however, due to the degradation of channel activity. Application of 2 mM cGMP restored the patch current to >90% of the original maximum. When IABET-cGMP was subsequently reapplied to these trypsin-treated patches, covalent activation was still possible, but the kinetics were slowed by more than an order of magnitude. This slow form of covalent activation, however, was insensitive to trypsin treatment. When these patches were treated with trypsin for a second time, there was only a slow decline in covalent activation that paralleled the loss of the maximum current obtained in the presence of 2 mM cGMP (data not shown).

To further investigate these two modes of covalent activation, we turned to the PDT-cGMP derivative because of its more rapid kinetics. As shown in Figure 7, a five second treatment was sufficient to irreversibly activate >95% of the channels in a patch. When these patches were exposed to trypsin, we found that the type of covalent activation resulting from this brief application of PDT-cGMP was readily removed without significant loss of maximum current. Other patches were exposed to PDT-cGMP for 1 min. This resulted in covalent activation of virtually all of the channels. Subsequent treatment of these patches with trypsin revealed a second type of covalent activation that occured on a longer time scale and was resistant to trypsin.

The question of how trypsin reverses the rapid type of permanent activation is intriguing. Trypsin treatment could release an associated protein either by clipping off the segment of the channel to which it is bound or by degrading the channel-associated protein itself. A quick examination of the channel's primary structure reveals several lysine-

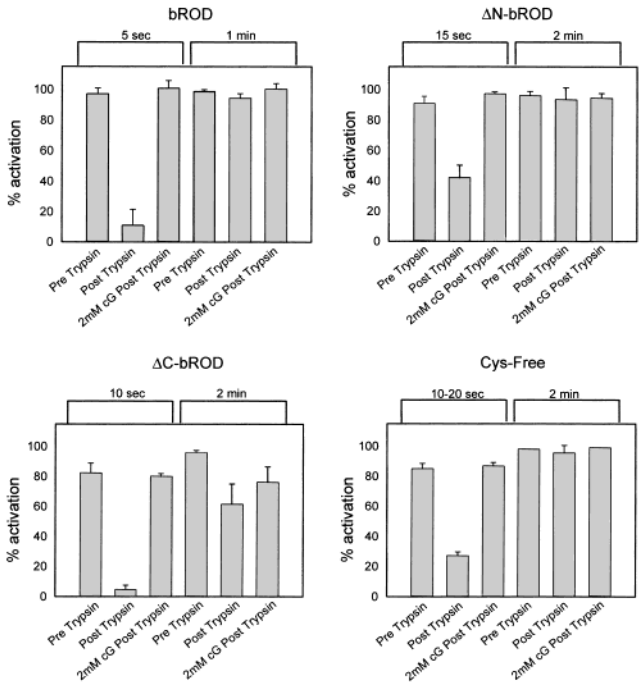


FIGURE 7: Two types of covalent activation are revealed by trypsin treatment. Wild-type α-subunit channels (bROD) and amino- and carboxy-terminal deletion mutants (ΔN- and ΔC-bROD) were exposed to 25 μM PDT-cGMP for times indicated at the top of each panel. Because of its lower $K_{1/2}$, the Cys-free mutant was treated with 5 μM PDT-cGMP. After determining the extent of irreversible activation (Pre trypsin), patches were exposed to 100 μg/mL trypsin for 1.5 min. Subsequently, the patch current was measured in control solution (post-trypsin) and in the presence of 2 mM cGMP (2 mM cGMP post-trypsin). All comparisons are made with the original patch current measured in 2 mM cGMP. All values shown are the mean (± SD) from at least three patches.

rich regions in the amino-terminal region, which might be the initial target for trypsin cleavage. This notion is supported by the results of Cook et al., who reported that mild trypsinization of native membranes removed approximately 4 kDa from the amino-terminus of the channel (*29*). In light of the fact that covalent activation persisted after mutation of the only cysteine in the amino-terminal region (C35), it seemed unlikely that the PDT-cG was attaching directly to this region of the α-subunit. To test if this region was the binding site for an associated protein, we expressed a deletion mutant (ΔN-bROD) lacking virtually the entire amino-terminus up to the first transmembrane helix (*25*). We have also expressed a carboxy-terminal deletion mutant that is truncated at position 608 (ΔC-bROD), just a few residues beyond the cGMP-binding site. These mutants, as well as the cysteine-free mutant, were challenged with PDT-cG and tested for the presence of both forms of covalent activation. Not surprisingly, there was some minor variability (<3-fold) among the mutant channels in the time required for the development of trypsin-sensitive and/or -insensitive covalent activation. For instance, in the ΔN-bROD channel, the rapid phase of covalent activation was slightly slowed so that it required a 15 s exposure to achieve 90% covalent activation. This longer exposure to PDT-cG, however, also resulted in a greater degree of trypsin-resistant covalent activation. This kinetic variability among the mutants caused slight differences in the fraction of covalent activation generated by a brief exposure to PDT-cG that was trypsin sensitive. As shown in Figure 7, however, both the N- and C-terminal

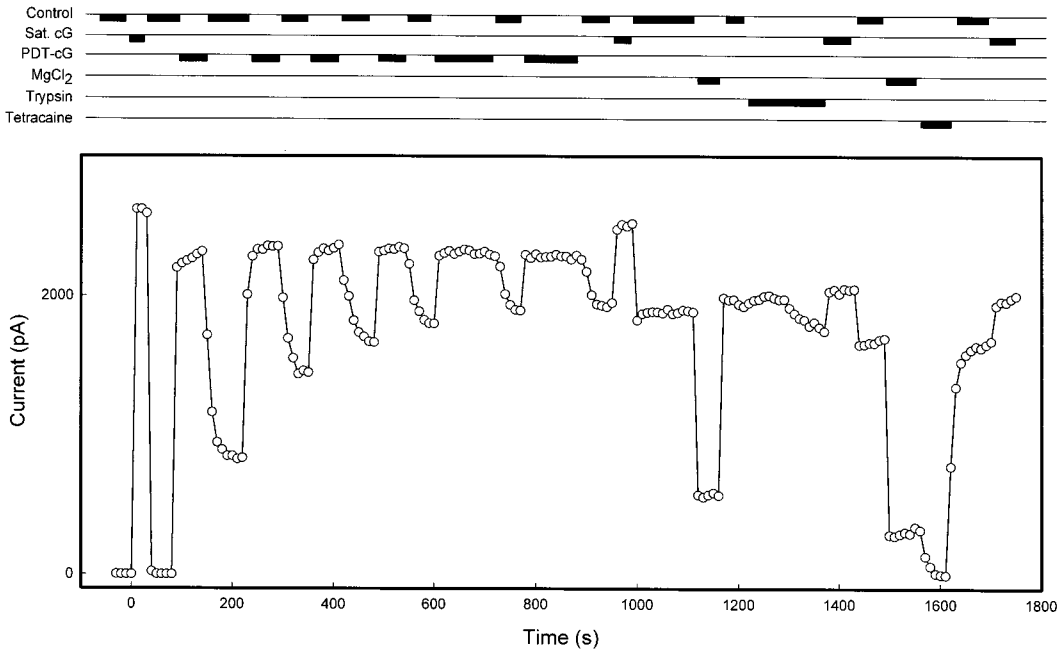


FIGURE 8: Treatment with PDT-cGMP causes covalent activation of the native rod CNG channel. The open circles indicate the current elicited by +50 mV pulses given every 10 s. The intracellular face of the patch was exposed to solutions indicated by the thicker lines at the top of the figure. Currents were not leak-subtracted. Similar results were seen on two other patches.

truncation mutants as well as the cysteine-free mutant displayed both trypsin-sensitive and trypsin-insensitive forms of covalent activation.

Are These Perplexing Results Confined to CNG Channels Expressed in Oocytes? Covalent Activation of CNG Channels Expressed in HEK-293 Cells and Native Photoreceptor Channels. To determine if both forms of covalent activation occurred only when the α-subunit was expressed in oocytes, we expressed the rod CNG channel α-subunit in HEK-293 cells. A number of ion channels have been shown to exhibit aberrant behavior when expressed in oocytes due to coassembly with endogenous proteins (see Discussion). Once again, the CNG channels expressed in HEK cells displayed both trypsin-sensitive and trypsin-insensitive forms of covalent activation (data not shown). If covalent activation is due to attachment of cGMP to an auxiliary protein, the protein is also present in HEK-293 cells.

We next tested our three sulfhydryl-reactive derivatives on native photoreceptor channels. For all analogues, covalent activation of native channels proceeded much more slowly than for its heterologously expressed counterparts. A time course of irreversible activation by treatment with PDT-cGMP is shown in Figure 8. While this analogue covalently activated expressed channels within a few seconds, it took several minutes to covalently activate native channels. When native channels were treated with IABET-cG or VSET-cG, covalent activation was even slower, taking 1−2 h for maximum effect. It was also difficult to achieve >80−90% covalent activation in native channels. These differences are not due to the presence of the β-subunit in the native channels. This possibility was tested by expression of heteromeric channels containing both α- and β-subunits in *Xenopus* oocytes (data not shown). With regard to covalent activation, the heteromeric channel was virtually indistinguishable from the α-subunit alone. The observed differences might be due to partial oxidation of the endogenous cysteine residues. Alternatively, the covalently attached ligand might be less effective at promoting the allosteric conformational change that leads to channel opening. As shown in Figure 8, the covalent activation of the native channels was insensitive to trypsin treatment. Thus, native photoreceptor channels seem to lack the fast, trypsin-sensitive type of covalent activation that occurs when CNG channels are expressed in oocytes or HEK cells.

Covalent Activation by the Photoaffinity Analogue APT-cGMP Was Resistant to Removal by Trypsin. We have previously reported covalent activation of native and expressed rod CNG channels by treatment with a photoaffinity analogue of cGMP, APT-cGMP, in the presence of UV light (*20*, *21*). In light of our results with the sulfhydryl-reactive derivatives of cGMP, we wanted to test another form of covalent activation for sensitivity to trypsin. When channels were covalently activated with APT-cGMP as previously described, the persistent current was immune to trypsin treatment (data not shown). We have previously shown that this photoaffinity probe attaches to hydrophobic residues in the β-roll structure of the binding site (*10*).

DISCUSSION

We have developed sulfhydryl-reactive derivatives of cGMP that irreversibly activate CNG channels by attaching to endogenous cysteine residues. We attempted to identify the site of attachment by site-specific mutagenesis of the α-subunit of the rod CNG channel expressed in *Xenopus* oocytes. Surprisingly, mutation of all seven cysteine residues in the α-subunit did not abolish irreversible activation. Truncation of the N- or C-terminal region of the channel subunit was likewise ineffective. A mild trypsin treatment, however, was able to remove a significant portion of a rapidly developing form of covalent activation. These data suggest that the cysteine residues responsible for covalent activation belong to a protein that is tightly associated with the channel, rather than the channel itself. Similar results were obtained

when the rod CNG channel was expressed in HEK-293 cells. Although the native channel can be irreversibly activated by treatment with sulfhydryl-reactive derivatives of cGMP, the covalent activation is resistant to trypsin. These results suggest that the expressed channel may associate with a protein present in both HEK-293 cells and oocytes. This accessory protein may not, however, associate with the channel in the native photoreceptor environment.

If IABET- and PDT-cGMP irreversibly activate the channel via attachment to an associated protein rather than the channel itself, the association must be very tight. The associated protein must extend to within 10−15 Å of the bound cGMP, and it must not dissociate from the channel, even with extended washing of the patch (1 h or more). Furthermore, this protein must bind to the "core" region of the channel, since it appears to associate with both N- and C-terminal deletion mutants. Thus far, few candidates have been found that can associate with the rod CNG channel. Results from several labs have shown that the complex of calmodulin (or a related calcium-binding protein) and calcium binds to the native rod photoreceptor channel and lowers its apparent affinity for cGMP (*13*). This association, however, is mediated by the β-subunit of the channel (*30*). Although the α-subunit of the olfactory CNG channel is known to bind calcium-calmodulin, no such association has been seen for the rod α-subunit (*31*, *32*). Recent results have also hinted at an association between the photoreceptor channel and the $Na^+/Ca^{2+},K^+$ exchanger (*33*, *34*). What proteins might associate with the CNG channel when it is expressed in heterologous systems? Molokanova, Trivedi, and Kramer have reported that the apparent affinity of the rod CNG channel is regulated by tyrosine phosphorylation when it is expressed in oocytes (*35*). The tyrosine kinase responsible for this regulation remains associated with excised membrane patches for at least several minutes, maybe longer. Furthermore, the same group has reported that genistein, a tyrosine kinase inhibitor that binds specifically to the ATP-binding site of the kinase, alters CNG channel function by a mechanism independent of phosphorylation (*36*). ATP, which competes for the genistein/ATP-binding site, and erbstatin, which prevents binding of substrates to many tyrosine kinases, antagonize the inhibitory action of genistein. The authors interpret these data to indicate that the kinase may associate directly with the channel. In the future, these putative interactions need to be confirmed using more definitive techniques.

Unexpected interactions between heterologously expressed ion channels and endogenous oocyte proteins have confounded the study of other types of channels, such as the minK and the G-protein regulated inwardly rectifying (GIRK) potassium channels. For example, injection of cRNA encoding the 130-amino acid minK peptide was sufficient to produce slowly activating voltage-dependent potassium currents in Xenopus oocytes. Furthermore, mutational analysis indicated that this peptide constituted part of the pore region of these channels. This ability to form channels was, however, confined to the oocyte system; attempts to express minK channel activity in many cell lines were mostly unsuccessful (*37*). Subsequent experiments revealed that functional expression of the minK channel required coassembly with the K_vLTQ protein present as part of the endogenous complement of oocyte proteins (*38*, *39*). In the case of GIRK channels, the GIRK1 isoform is unable to form functional homomultimeric channels when expressed in many cell lines. Functional expression requires coassembly with the cardiac inward rectifier (CIR). It seemed odd to obtain substantial currents following injection of Xenopus oocytes with cRNA encoding only GIRK1. Subsequent experiments have shown that this robust expression is due to the presence of the Xenopus version of the CIR protein (*40*). Finally, the NR1 subunit of the human NMDA receptor has been shown to coassemble with a Xenopus version of NR2 to form functional channels (*41*). Our current results advise some caution regarding the interpretation of experimental data for heterologously expressed versions of the CNG channel. If this channel does, in fact, associate with endogenous regulatory proteins, mutagenesis may alter channel function by altering interactions with associated proteins rather than interactions intrinsic to the channel itself.

ACKNOWLEDGMENT

The authors thank Drs. S. Gordon, G. Flynn, and W. N. Zagotta, and K. Matulef for the gift of the rod CNG α-subunit clone as well as several mutants. The authors would like to thank Drs. J. Karpen and M. Ruiz for critically reviewing the manuscript, and three Coleman-Wheeler Summer Fellows, Heather Berger, Amy Milburn, and Sungyon Bang, for technical assistance.

REFERENCES

1. Stryer, L. (1991) *J. Biol. Chem. 266*, 10711−10714.
2. Zimmerman, A. L. (1995) *Curr. Opin. Neurobiol. 5*, 296−303.
3. Cook, N. J., Hanke, W., and Kaupp, U. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 585−589.
4. Liu, D. T., Tibbs, G. R., and Siegelbaum, S. A. (1996) *Neuron 16*, 983−990.
5. Kaupp, U. B., Niidome, T., Tanabe, T., Terada, S., Bonigk, W., Stuhmer, W., Cook, N. J., Kangawa, K., Matsuo, H., and Hirose, T. (1989) *Nature 342*, 762−766.
6. Chen, T. Y., Peng, Y. W., Dhallan, R. S., Ahamed, B., Reed, R. R., and Yau, K. W. (1993) *Nature 362*, 764−767.
7. Korschen, H. G., Illing, M., Seifert, R., Sesti, F., Williams, A., Gotzes, S., Colville, C., Muller, F., Dose, A., and Godde, M. (1995) *Neuron 15*, 627−636.
8. Kaupp, U. B. (1995) *Curr. Opin. Neurobiol. 5*, 434−442.
9. Heginbotham, L., Abramson, T., and MacKinnon, R. (1992) *Science 258*, 1152−1155.
10. Brown, R. L., Gramling, R., Bert, R. J., and Karpen, J. W. (1995) *Biochemistry 34*, 8365−8370.
11. Karpen, J. W., Brown, R. L., Stryer, L., and Baylor, D. A. (1993) *J. Gen. Physiol. 101*, 1−25.
12. Gordon, S. E., and Zagotta, W. N. (1995) *Neuron 14*, 177−183.
13. Hsu, Y. T., and Molday, R. S. (1993) *Nature 361*, 76−79.
14. Grunwald, M. E., Yu, W. P., Yu, H. H., and Yau, K. W. (1998) *J. Biol. Chem. 273*, 9148−9157.
15. Weitz, D., Zoche, M., Muller, F., Beyermann, M., Korschen, H. G., Kaupp, U. B., and Koch, K. W. (1998) *EMBO J. 17*, 2273−2284.
16. Karpen, J. W., Zimmerman, A. L., Stryer, L., and Baylor, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1287−1291.
17. Sunderman, E. R., and Zagotta, W. N. (1999) *J. Gen. Physiol. 113*, 601−620.
18. Sunderman, E. R., and Zagotta, W. N. (1999) *J. Gen. Physiol. 113*, 621−640.
19. Li, J., Zagotta, W. N., and Lester, H. A. (1997) *Q. Rev. Biophys. 30*, 177−193.
20. Brown, R. L., Gerber, W. V., and Karpen, J. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5369−5373.

21. Karpen, J. W., and Brown, R. L. (1996) *J. Gen. Physiol. 107*, 169−181.
22. Ruiz, M., and Karpen, J. W. (1997) *Nature 389*, 389−392.
23. Ruiz, M., and Karpen, J. W. (1999) *J. Gen. Physiol. 113*, 873−895.
24. Brown, R. L., Bert, R. J., Evans, F. E., and Karpen, J. W. (1993) *Biochemistry 32*, 10089−10095.
25. Brown, R. L., Snow, S. D., and Haley, T. L. (1998) *Biophys. J. 75*, 825−833.
26. Biel, M., Altenhofen, W., Hullin, R., Ludwig, J., Freichel, M., Flockerzi, V., Dascal, N., Kaupp, U. B., and Hofmann, F. (1993) *FEBS Lett. 329*, 134−138.
27. Fodor, A. A., Gordon, S. E., and Zagotta, W. N. (1997) *J. Gen. Physiol. 109*, 3−14.
28. Matulef, K., Flynn, G. E., and Zagotta, W. N. (1999) *Neuron 24*, 1−20.
29. Cook, N. J., Molday, L. L., Reid, D., Kaupp, U. B., and Molday, R. S. (1989) *J. Biol. Chem. 264*, 6996−6999.
30. Chen, T. Y., Illing, M., Molday, L. L., Hsu, Y. T., Yau, K. W., and Molday, R. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11757−11761.
31. Liu, M., Chen, T. Y., Ahamed, B., Li, J., and Yau, K. W. (1994) *Science 266*, 1348−1354.
32. Varnum, M. D., and Zagotta, W. N. (1997) *Science 278*, 110−113.
33. Bauer, P. J., and Drechsler, M. (1992) *J. Physiol. (London) 451*, 109−131.
34. Molday, R. S., and Molday, L. L. (1998) *Vision Res. 38*, 1315−1323.
35. Molokanova, E., Trivedi, B., Savchenko, A., and Kramer, R. H. (1997) *J. Neurosci. 17*, 9068−9076.
36. Molokanova, E., Savchenko, A., and Kramer, R. H. (1999) *J. Gen. Physiol. 113*, 45−56.
37. Lesage, F., Attali, B., Lakey, J., Honore, E., Romey, G., Faurobert, E., Lazdunski, M., and Barhanin, J. (1993) *Recept. Channels 1*, 143−152.
38. Barhanin, J., Lesage, F., Guillemare, E., Fink, M., Lazdunski, M., and Romey, G. (1996) *Nature 384*, 78−80.
39. Sanguinetti, M. C., Curran, M. E., Zou, A., Shen, J., Spector, P. S., Atkinson, D. L., and Keating, M. T. (1996) *Nature 384*, 80−83.
40. Hedin, K. E., Lim, N. F., and Clapham, D. E. (1996) *Neuron 16*, 423−429.
41. Soloviev, M. M., and Barnard, E. A. (1997) *J. Mol. Biol. 273*, 14−18.
42. Kumar, V. D., and Weber, I. T. (1992) *Biochemistry 31*, 4643−4649.

BI9920735