Movement of Gating Machinery during the Activation of Rod Cyclic Nucleotide-Gated Channels

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ABSTRACT In the visual and olfactory systems, cyclic nucleotide-gated (CNG) ion channels convert stimulus-induced changes in the internal concentrations of cGMP and cAMP into changes in membrane potential. Although it is known that significant activation of these channels requires the binding of three or more molecules of ligand, the detailed molecular mechanism remains obscure. We have probed the structural changes that occur during channel activation by using sulfhydryl-reactive methanethiosulfonate (MTS) reagents and *N*-ethylmaleimide (NEM). When expressed in *Xenopus* oocytes, the α-subunit of the bovine retinal channel forms homomultimeric channels that are activated by cGMP with a $K_{1/2}$ of ~100 μM. Cyclic AMP, on the other hand, is a very poor activator; a saturating concentration elicits only 1% of the maximum current produced by cGMP. Treatment of excised patches with MTS-ethyltrimethylamine (MTSET) or NEM dramatically potentiated the channel's response to both cyclic nucleotides. After MTSET treatment, the dose-response relation for cGMP was shifted by over two orders of magnitude to lower concentrations. The effect on channel activation by cAMP was even more striking. After modification, the channels were fully activated by cAMP with a $K_{1/2}$ of ~60 μM. This potentiation was abolished by conversion of Cys^{481} to a nonreactive alanine residue. Potentiation occurred more rapidly in the presence of saturating cGMP, indicating that this region of the channel is more accessible when the channel is open. Cys^{481} is located in a linker region between the transmembrane and cGMP-binding domains of the channel. These results suggest that this region of the channel undergoes significant movement during the activation process and is critical for coupling ligand binding to pore opening. Potentiation, however, is not mediated by the recently reported interaction between the amino- and carboxy-terminal regions of the α-subunit. Deletion of the entire amino-terminal domain had little effect on potentiation by MTSET.

INTRODUCTION

Ion channels directly gated by cyclic nucleotides are emerging as a key component of signaling systems in a variety of cell types throughout the body (Yau, 1994; Finn et al., 1996). This type of channel was first discovered in retinal rods and olfactory cilia, where they generate changes in membrane potential in response to external stimuli (Zimmerman, 1995). In retinal rods, for example, these channels close in response to the light-triggered hydrolysis of cGMP (Stryer, 1991). The resulting membrane hyperpolarization decreases neurotransmitter release at the synapse. Cyclic nucleotide-gated (CNG) ion channels have now been discovered in a number of other tissues, including the brain, heart, liver, and kidneys (Biel et al., 1994; Ruiz et al., 1996; Feng et al., 1996; Kingston et al., 1996; Leinders-Zufall et al., 1995). Although their exact role in these tissues remains speculative, some evidence suggests that they may provide an alternative pathway for the regulated entry of extracellular calcium (Kaupp, 1995; Frings et al., 1995) or act as downstream effectors in nitric oxide signaling systems (Rieke and Schwartz, 1994; Zufall et al., 1997; Savchenko et al., 1997).

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The cGMP-activated channel of retinal rods is a tetramer composed of homologous 63-kDa α- and 240-kDa β-subunits (Cook et al., 1987; Kaupp et al., 1989; Chen et al., 1993; Korschen et al., 1995; Liu et al., 1996). Each subunit contains a transmembrane domain and a cytoplasmic region that binds cGMP (Henn et al., 1995; Brown et al., 1995). The transmembrane domains of four subunits come together to form a cation-specific pore (Liu et al., 1996), which remains closed in the absence of cGMP. When expressed in *Xenopus* oocytes, the α-subunit of the bovine retinal channel forms a homomultimeric channel that responds to cGMP with a $K_{1/2}$ of ~100 μM (Kaupp et al., 1989). Cyclic AMP, on the other hand, is a very weak activator of this channel; saturating concentrations elicit only 1% of the maximum current evoked by cGMP (Goulding et al., 1994; Gordon and Zagotta, 1995a). Although it is known that significant activation of the rod CNG channel requires the binding of three or more molecules of cGMP (Haynes et al., 1986; Zimmerman and Baylor, 1986; Ruiz and Karpen, 1997), details of the molecular rearrangement that leads to pore opening remain obscure.

A central question in the study of ligand-gated ion channels is how the binding of ligand is allosterically coupled to opening of the ion-conducting pathway. In the absence of definitive methods, information on ion channel structure has been inferred indirectly from functional measurements after site-specific mutation and/or modification. The recently developed family of sulfhydryl-specific methanethiosulfonate (MTS) reagents has revolutionized the study of the pore structure, membrane topology, and dynamic gating move-

ments of ion channels. Advantages of MTS reagents over previously developed sulfhydryl reagents include exceptional specificity for cysteine residues and rapid kinetics, which allows complete modification in minutes with low concentrations of reagent. Akabas and colleagues originally used these reagents in combination with cysteine-substitution mutagenesis to probe the pore structure of nicotinic acetylcholine channels and $GABA_A$ receptors (Akabas et al., 1994; Akabas and Karlin, 1995; Xu and Akabas, 1996). More recently, this technique has been used to demonstrate voltage-dependent movement of the S4 helix, a putative voltage sensor, during activation of both voltage-gated sodium and potassium channels (Yang and Horn, 1995; Larsson et al., 1996). In previous studies on CNG channels, Sun et al. (1996) used this technique to demonstrate that many pore residues are accessible to MTS reagents applied from either side of the membrane, suggesting that the selectivity filter is a thin iris in a larger diameter pore.

In the current study, we demonstrate that modification of Cys^{481} in the bovine retinal CNG channel with NEM or MTS-ethyltrimethylamine (MTSET) greatly potentiates the channel's response to cyclic nucleotides. This modification occurs much more rapidly when the channel is open and can increase the efficacy of channel activation by cAMP by 100-fold. This residue is located in the linker region between the transmembrane and cGMP-binding domains of the channel. These results suggest that this linker region moves significantly during the activation process and may be an important cog in the channel-gating machinery.

MATERIALS AND METHODS

Methanethiosulfonate reagents were purchased from Toronto Research Chemicals (North York, ON, Canada) and were used within 10 min of dilution into buffer. Cyclic nucleotides were purchased from Sigma (St. Louis, MO). All other reagents were of the highest purity available. Curve-fitting was done with SigmaPlot software (Jandel Scientific, San Rafael, CA). Fits of the Hill equation were typically made using logarithmic values to emphasize points low on the dose-response curve that are more sensitive to changes in the limiting slope.

Channel mutagenesis and expression

Complementary DNA encoding the bovine retinal CNG channel and several mutants were kindly provided by S. Gordon and W. Zagotta. The cDNA had been modified to include unique restriction sites and was inserted into the pGEMHE vector including 5′ and 3′ sequences from the β-globin gene to improve expression (Gordon and Zagotta, 1995b; Liman et al., 1992). Other mutations were made with the Quick Change kit (Stratagene, La Jolla, CA). The appropriate restriction fragment containing the mutation was then subcloned into a vector of known sequence and sequenced in full with the ^{33}P-Terminator kit (Amersham, Cleveland, OH) to guard against second-site mutations. For in vitro transcription, the vector was linearized with *Pst*I; complementary RNA was transcribed from the T7 promoter and capped using the mMessage mMachine kit (Ambion, Austin, TX). RNA was precipitated with lithium chloride and quantified by its absorbance at 260 nm.

Xenopus oocytes were prepared and injected with cRNA (~50 ng) by standard methods (Stuhmer, 1992). After 3–5 days of incubation at 18°C, the vitelline membrane was removed and channel function was assayed by patch-clamp recording in the inside-out configuration (Hamill et al., 1981). Electrode resistance was typically between 0.5 and 1.0 MΩ, and recordings were made at room temperature (20–22°C). Control solution contained 130 mM NaCl, 3 mM HEPES, and 0.2 mM EDTA, pH 7.4. Cyclic nucleotides and/or sulfhydryl reagents were added to the bath solution as indicated. Currents were elicited by 25-ms voltage pulses to ±50 mV and were recorded with an Axopatch 200A amplifier with a Digidata 1200 interface (Axon Instruments, Foster City, CA). The data were sampled at 10 kHz, filtered at 1 kHz, and analyzed with Pclamp 6.0 software (Axon Instruments). Solutions were applied to the cytoplasmic face of the patch via the "sewer pipe" method, using the RSC-100 perfusion system (Molecular Kinetics, Pullman, WA). Currents induced by the application of cyclic nucleotides were determined by subtracting a control trace obtained in their absence. Currents were not corrected for series resistance errors; the maximum error in any patch was estimated at <10%. All dose-response relations were recorded at +50 mV. Large currents were measured within 5 ms of the voltage steps to eliminate artifacts due to hindered diffusion (Zimmerman et al., 1988). In some cases the dose-response relations for patches were normalized by their individual $K_{1/2}$ values, so that results from patches with differing cGMP affinities could easily be compared (Brown et al., 1993).

RESULTS

Inside-out membrane patches were excised from *Xenopus* oocytes expressing wild-type or mutant CNG α-subunit channels derived from bovine retinal cDNA. Most versions of the channel were activated robustly by the bath application of cGMP. The dose-response relations were fit using the Hill equation (see legend Fig. 1) to extract parameters of

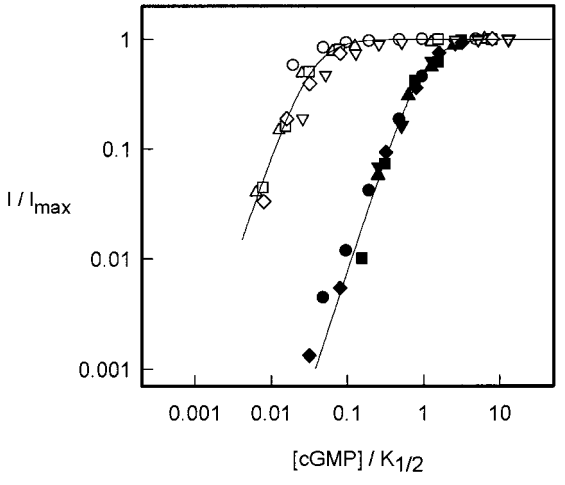

FIGURE 1 Treatment of retinal CNG channels with NEM potentiates their response to low concentrations of cGMP. Shown are normalized cGMP dose-response relations for five patches containing expressed α-subunit channels before (*filled symbols*) and after (*open symbols*) a 5-min exposure to 1 mM NEM in the presence of 2 mM cGMP. Each symbol type represents data from a different patch. The data from each patch were fit using the Hill equation ($I/I_{max} = [cG]^n/([cG]^n + K^n_{1/2})$) to determine the $K_{1/2}$, the concentration that elicits a half-maximum response for that cyclic nucleotide, and the Hill coefficient, n, which serves as an index of cooperativity. The best-fit parameters for this set of patches were $K_{1/2} = 70 \pm 24$ μM, $n = 2.1 \pm 0.2$ before NEM exposure and $K_{1/2} = 2.3 \pm 0.8$ μM, $n = 2.0 \pm 0.1$ after. The average maximum current in these five patches was 5821 pA. To combine the data from patches with variable $K_{1/2}$ values, the data from each patch were subsequently expressed relative to its respective $K_{1/2}$ value, using the following equation: $I/I_{max} = ([cG]/K_{1/2})^n/1 + ([cG]/K_{1/2})^n$.

$K_{1/2}$, the concentration of cGMP required to stimulate half-maximum channel activity, and the Hill coefficient, n, which is a rough measure of the number of ligands required for channel activation. The original dose-response relations for the wild-type channels were fit with a $K_{1/2} = 70 \pm 24$ μM and $n = 2.1 \pm 0.2$ ($N = 5$). Brief treatment of the patch with 1 mM NEM in the presence of saturating cGMP (2 mM) dramatically enhanced the channel's response to cGMP. As shown in Fig. 1, the dose-response relation for cGMP was shifted to lower concentrations by more than a factor of 10. The $K_{1/2}$ for cGMP decreased from 70 μM to 2.3 ± 0.8 μM ($N = 5$) with little change in the shape of the curve; the Hill coefficient remained near 2.1. Gordon et al. (1997) and Finn et al. (1995) have reported similar results. In the hands of Finn and colleagues, however, the dose-response relation became much shallower after NEM treatment. Serre et al. (1995) and Balakrishnan et al. (1990) reached a similar conclusion by measuring ion flux through vesicles containing native rod outer segment channels.

Modification with MTSET, an MTS reagent that donates a positive charge to cysteine residues, had an even more dramatic effect (Fig. 2). After only 1 min of exposure to 500 μM MTSET, the channel responded vigorously to submicromolar concentrations of cGMP. Before treatment, the current elicited by this concentration of cGMP was undetectable. After treatment with MTSET, the channel's $K_{1/2}$ for cGMP plummeted from 103 to 0.5 μM, whereas the Hill coefficient remained near 2. Most patches (8 of 11) also showed a 10–20% increase in maximum current after treatment with MTSET. In two patches, however, the maximum current decreased; in these cases the typical increase may have been obscured by an unexplained loss of channel activity in the patch. The effect on channel activation by cAMP was even more striking. Before modification, wild-type channels were poorly activated by cAMP. Application of a saturating concentration of cAMP (10 mM) elicited only 0.80 ± 0.48% of the maximum current induced by cGMP. The apparent affinity was also significantly lower; the concentration of cAMP that elicited a half-saturating response was ~650 μM. This value is slightly lower than those previously reported by Gordon and Zagotta (1995a) and Goulding et al. (1994). After exposure to MTSET, however, the channels responded fully to cAMP, with a $K_{1/2} = 54$ μM and $n = 1.7$.

The results reported above suggest that modification of endogenous cysteine residues can have profound effects on the function of CNG channels. Identification of the residue(s) involved may provide clues to the location of the channel's gating machinery and dynamic changes that occur during activation. The α-subunit of the rod CNG channel has a total of seven cysteine residues. Only four of these, however, are predicted to lie on the cytoplasmic face of the channel. Cys^{35} is found near the amino terminus of the protein in a region of the channel that is thought to be proteolytically removed in the native tissue (Molday et al., 1991). Cys^{481} lies in a linker region between the channel's transmembrane and cGMP-binding domains. The two remaining cysteine residues, Cys^{505} and Cys^{573}, reside within the cGMP-binding domain itself. To identify the site of modification that is responsible for the dramatic functional effects, each cysteine residue was mutated individually to a nonreactive alanine or threonine residue. The mutant channels were expressed and challenged with the MTSET reagent. The results shown in Fig. 3 indicate that modification of Cys^{481} is necessary for the potentiation of the channel's response to both cyclic nucleotides. Mutation of Cys^{35}, Cys^{505}, or Cys^{573} left potentiation by MTSET largely unaffected, whereas mutation of Cys^{481} virtually eliminated the effect of MTSET on channel function. Only a slight (perhaps twofold) increase in cGMP sensitivity occurs in the C481A mutant after MTSET treatment. Furthermore, experiments with the triple mutant C35A/C505T/C573A

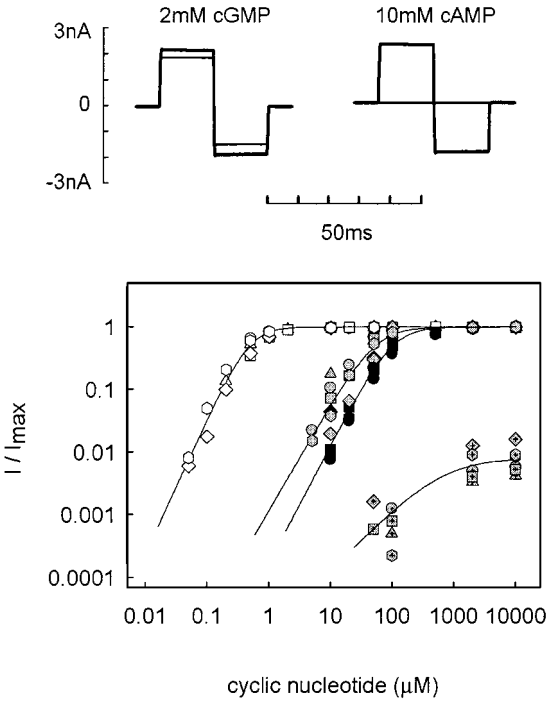

FIGURE 2 Exposure of retinal CNG channels to MTSET potentiates their response to cGMP and dramatically increases the efficacy of cAMP. The top panel of the figure shows currents evoked by saturating concentrations of cGMP and cAMP before (*thin lines*) and after (*thick lines*) treatment with MTSET. From a holding potential of 0 mV, the patch was stepped to +50 mV and then to −50 mV before it was returned to 0 mV. The bottom panel shows dose-response curves for patches before and after 2 min of treatment with MTSET in the presence of 2 mM cGMP. Each symbol shape represents data from a different patch. Black symbols and white symbols represent, respectively, the dose-response relation for cGMP before and after treatment with MTSET. The gray symbols with and without a + represent, respectively, the relation for cAMP before and after treatment. Solid lines show fits to the Hill equation using the parameters listed below. The fit parameters for cGMP were $K_{1/2} = 103$ μM and $n = 1.9$ before and $K_{1/2} = 0.5$ μM and $n = 2.15$ after potentiation. For cAMP these parameters were $K_{1/2} = 647$ μM and $n = 1.0$ before and $K_{1/2} = 54$ μM and $n = 1.7$ after potentiation. When compared to the maximum cGMP-induced current measured after potentiation, the current elicited by saturating cAMP increased from 0.8% before potentiation to virtually 100% thereafter. The average maximum current in these five patches was 7184 ± 4839 pA.

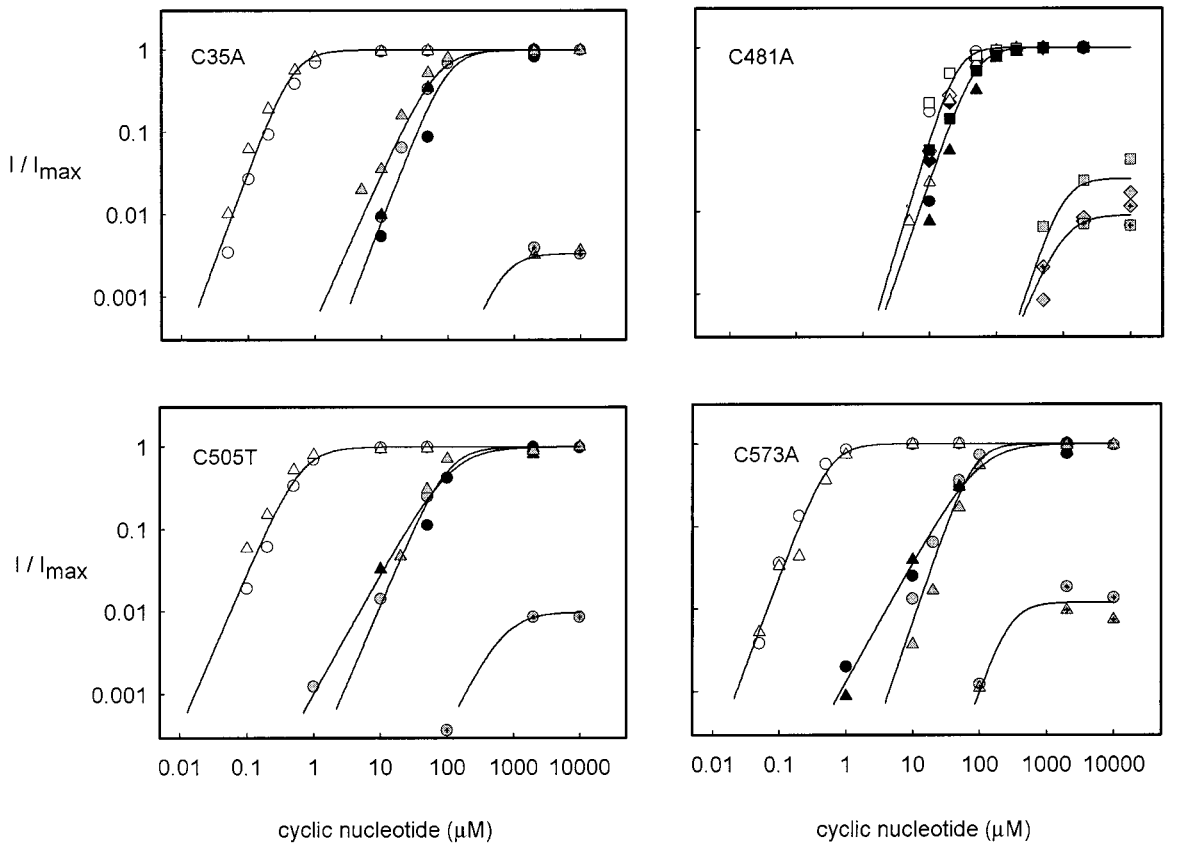

FIGURE 3 Modification of Cys^{481} is necessary and sufficient for potentiation of the retinal CNG channel. Patches containing mutant channels in which single cysteine residues at positions 35, 481, 505, or 573 were replaced with a nonreactive alanine or threonine were challenged with a 2-min application of 500 μM MTSET in the presence of 2 mM cGMP. Shown are dose-response relations for cGMP before (*black symbols*) and after (*open symbols*) treatment and cAMP before (*gray symbols with +*) and after (*gray symbols without +*). Once again, each symbol type in a panel represents data from a different patch. Maximum currents in the patches were as follows. C35A: ●, 6148 pA; ▲, 2458 pA. C481A: ●, 306 pA; ▲, 9265 pA; ■, 920 pA; ◆, 9335 pA; C505T: ●, 1614 pA; ▲, 425 pA; C573A: ●, 6538 pA; ▲, 4570 pA. The lines are derived from composite fits of the Hill equation, using the following parameters. C35A: cGMP before, $K_{1/2}$ = 99 μM, n = 2.0; cGMP after, $K_{1/2}$ = 0.47 μM, n = 2.2; cAMP before, $K_{1/2}$ = 650 μM, n = 2.0, fractional activation at saturation = 0.004; cAMP after, $K_{1/2}$ = 69 μM, n = 1.8. C481A: cGMP before, $K_{1/2}$ = 49 μM, n = 2.4; cGMP after, $K_{1/2}$ = 26 μM, n = 2.7; cAMP before, $K_{1/2}$ = 1000 μM, n = 2.0, fractional activation = 0.008; cAMP after, $K_{1/2}$ = 1000 μM, n = 2.5, fractional activation = 0.025. C505T: cGMP before, $K_{1/2}$ = 116 μM, n = 1.45; cGMP after, $K_{1/2}$ = 0.65 μM, n = 1.89; cAMP before, $K_{1/2}$ = 697 μM, n = 1.67, fractional activation = 0.008; cAMP after, $K_{1/2}$ = 101 μM, n = 1.91. C573A: cGMP before, $K_{1/2}$ = 98 μM, n = 1.45; cGMP after, $K_{1/2}$ = 0.56 μM, n = 2.16; cAMP before, $K_{1/2}$ = 276 μM, n = 2.0, fractional activation = 0.015; cAMP after, $K_{1/2}$ = 81 μM, n = 2.4.

indicated that the modification of Cys^{481} alone was sufficient for potentiation by MTSET. The $K_{1/2}$ for cGMP decreased from an initial value of ~28 μM to ~0.3 μM after MTSET treatment, and the fractional activation by cAMP increased from ~0.015 to virtually 1.0. Unfortunately, this triple mutant could not be characterized fully because of poor expression (<200 pA maximum current at +100 mV in saturating cGMP after MTSET treatment).

We next set out to determine if the rate of Cys^{481} modification depended upon the activation state of the channel. Fig. 4 compares the dose-response relations for wild-type channels after a 5-min treatment with NEM in the presence or absence of saturating cGMP. In the presence of 2 mM cGMP, the dose-response relation had reached its maximum left shift during this period of time. For this set of patches, the average $K_{1/2}$ shifted from 70 ± 24 μM before NEM treatment to 2.3 ± 0.8 μM afterward. In the absence of cGMP, however, the left shift was much less pronounced; the $K_{1/2}$ shifted from a value of 80 ± 8 μM before treatment to 55 ± 6 μM afterward. The most obvious sign of modification is a slight decrease in the Hill coefficient from 2.1 to 1.7. This decrease in slope would be expected for a heterogeneous population of channels in which a small subset had been modified. These data suggested that Cys^{481} is significantly more accessible to small solutes when the channel is open. We tested this hypothesis further by using MTSET. In this case, we were surprised to find that the channel's dose-response relation was already significantly shifted after a 5-min treatment with 500 μM MTSET in the absence of cGMP (data not shown). This result suggests that MTS reagents may have limited access to Cys^{481} when the channel is closed.

To measure the state-dependent difference in modification rates for MTSET, we lowered the concentration to 100

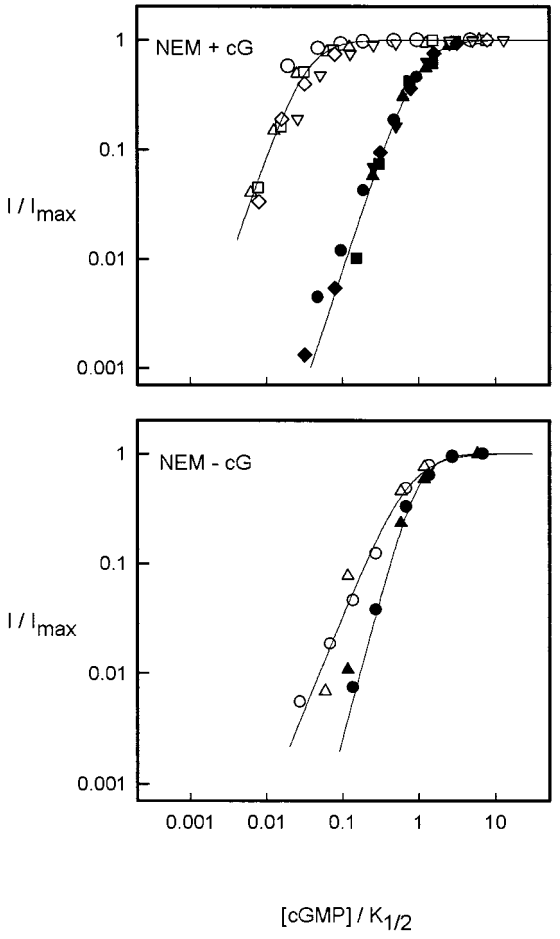

FIGURE 4 Modification of Cys^{481} by NEM occurs much faster in the presence of cGMP. Shown are normalized dose-response curves for cGMP before and after a 5-min treatment of patches with 1 mM NEM. The top panel presents data obtained when 2 mM cGMP was present during NEM treatment; the bottom panel presents data from patches treated with NEM in the absence of cGMP. The fit parameters for the top panel are given in Fig. 1. The fit parameters for the bottom panel were as follows: before NEM treatment, $K_{1/2} = 80 \pm 5$ μM, $n = 2.2 \pm 0.2$; after NEM treatment, $K_{1/2} = 55 \pm 3$ μM; $n = 1.7 \pm 0.2$.

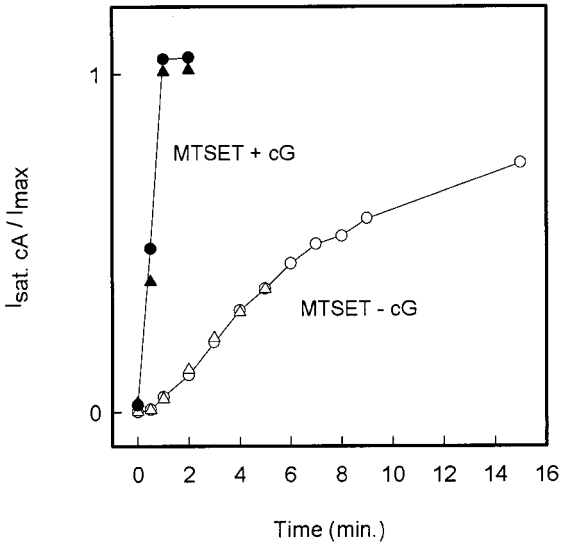

FIGURE 5 Modification of Cys^{481} by MTSET occurs much faster when the channel is open. The current elicited by 10 mM cAMP is compared to that elicited by 2 mM cGMP as a function of the time of exposure to 100 μM MTSET. Each symbol type represents the time course from a different patch. Open symbols represent time courses obtained in the absence of cGMP, and filled symbols represent those obtained in the presence of cGMP.

μM and measured the time course of the increase in cAMP efficacy (Fig. 5). The patch was treated with MTSET for the indicated time (in either the presence or absence of 2 mM cGMP) and then washed extensively with control solution. It was then challenged with 10 mM cAMP, and the current was compared to the maximum elicited by cGMP. In the presence of 2 mM cGMP, MTSET modification was complete within 1 min. In the absence of cGMP, the increase in cAMP efficacy had reached only 60% after 16 min of treatment. This indicates that the reaction is slowed by at least 30-fold when the channel is in the closed state.

Noting that potentiation of channel function by MTSET was more robust than potentiation by NEM, we were curious to see if the chemical nature of the modifying group would have a substantial impact on the degree of potentiation. To investigate this question, we treated patches containing the expressed channel with MTSES, a reagent that donates a negatively charged sulfonic acid group. As shown in Fig. 6, this treatment produced no shift in the cGMP dose-response relation. This reagent, however, did react with Cys^{481} because this treatment blocked potentiation by subsequent application of MTSET (data not shown). These results suggest that the degree of channel potentiation depends upon the chemical nature of the modifying group. Reagents that donate a positive charge cause the largest degree of potentiation, followed by neutral or hydrophobic reagents. Reagents that impart a negative charge have little, if any, effect on the dose-response relations. These conclusions are supported by limited experiments with other sulfhydryl reagents such as DTNB and iodoacetamide derivatives of biotin (data not shown). Surprisingly, a 10-min treatment of patches with 10 mM iodoacetic acid did not appear to modify Cys^{481}.

Gordon et al. recently reported a state-dependent interaction between the N-terminal and C-terminal regions of the rod CNG channel (1997). To determine if this interaction plays a role in channel potentiation by MTSET, we tested an N-terminal deletion mutant of the channel. This mutant lacks virtually the entire rod channel N-terminus. It is a chimera containing amino acids 131–138 of the olfactory channel fused to position 165 of the rod sequence. This position is located three residues before the first putative transmembrane helix. This deletion mutant (ΔN-BRET) expressed well, yielding 1–2-nA currents at +50 mV in saturating cGMP. The cGMP dose-response relation for ΔN-BRET was fit to the Hill equation with a $K_{1/2} = 82 \pm 16$ μM and $n = 2.15 \pm 0.45$. This deletion had no effect on channel potentiation by MTSET (Fig. 7). After treatment

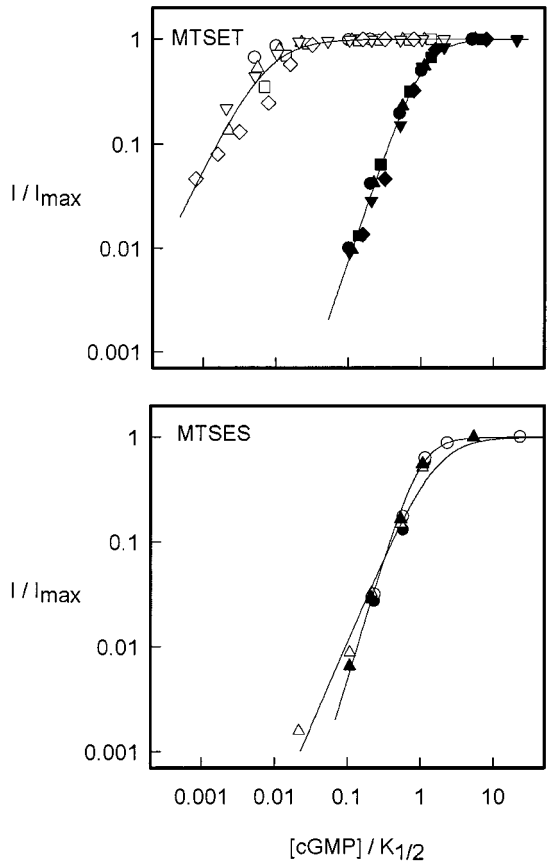

FIGURE 6 Modification of the retinal CNG channel with MTSES does not lead to potentiation. Cyclic GMP dose-response relations for excised patches containing wild-type BRET were measured before (*filled symbols*) and after (*open symbols*) a 5-min exposure to either 500 μM MTSET or MTSES in the presence of 2 mM cGMP. Each symbol shape represents data from a different patch. Solid lines show normalized fits of the combined data to the Hill equation. Before normalization the best-fit parameters were as follows: cGMP before MTSET, $K_{1/2} = 84 \pm 16$ μM, $n = 2.0 \pm 0.2$; cGMP after MTSET, $K_{1/2} = 0.61 \pm 0.11$ μM, $n = 1.5 \pm 0.2$; cGMP before MTSES, 89 ± 5 μM, $n = 2.3 \pm 0.2$; cGMP after MTSES, 142 ± 8 μM, $n = 1.7 \pm 0.3$.

with MTSET, the $K_{1/2}$ was shifted to 0.75 ± 0.35 μM with little change in the Hill coefficient. The response of ΔN-BRET to cAMP before and after MTSET treatment was also similar to that of the wild-type channel. Before treatment with MTSET, exposure to saturating cAMP (10 mM) evoked only 1–2% of the maximum current elicited by cGMP, and the $K_{1/2}$ was 1–2 mM. After treatment, cAMP activated the channel fully with a $K_{1/2}$ of 118 ± 33 μM. These data suggest that the interaction between the N- and C-termini is not essential for basic channel function and is not involved in the potentiation of channel activity by MTSET modification.

DISCUSSION

Activation of the rod CNG channel is initiated by the binding of three or four molecules of cGMP. These binding events are followed by a poorly understood allosteric rearrangement that leads to the opening of a transmembrane pore. In this study we have used sulfhydryl-modifying reagents to gain insights into the dynamic structural rearrangements that occur during channel gating. Modification of Cys^{481}, located in the linker region between the transmembrane and cGMP-binding domains, can have dramatic effects on channel function. Modification of this residue by the neutral, somewhat hydrophobic reagent NEM or by the positively charged MTSET caused a left shift in the cGMP dose-response relation and increased the ability of cAMP to promote channel opening. Finn et al. (1995), Gordon et al. (1997), Donner et al. (1990), Serre et al. (1995), and Balakrishnan et al. (1990) have previously described potentiation by modification of the channel with NEM. Surprisingly, modification with the negatively charged reagent MTSES had little effect. These results suggest that electrostatic interactions may play an important role in the potentiation process. It will be interesting to test this hypothesis further by site-specific mutagenesis. In this regard, Zong et al. (1998) have recently shown that the response of CNG3, the cone/kidney channel, to cAMP is dramatically enhanced by the neutralization of two acidic amino acids in this linker region. These results further strengthen the hypothesis that electrostatic interactions are important to the allosteric coupling between ligand binding and channel activation.

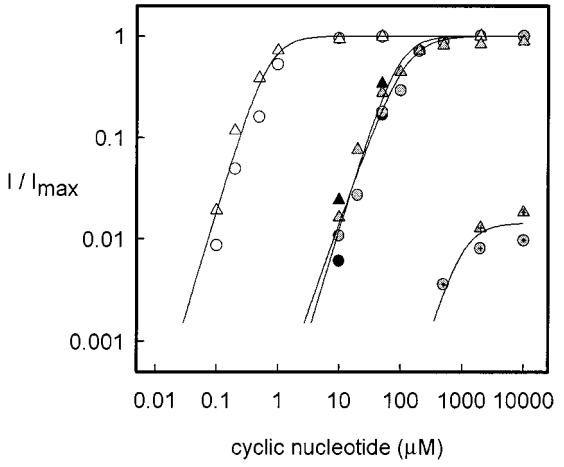

FIGURE 7 Potentiation does not require an interaction between the carboxy- and amino-termini of the channel. Shown are cGMP and cAMP dose-response relations for excised patches containing ΔN-BRET before and after treatment with MTSET. Symbols have the same meaning as in Fig. 2. Solid curves are fits to the Hill equation with the following parameters: cGMP before, $K_{1/2} = 89$ μM, $n = 2.0$; cGMP after, $K_{1/2} = 0.7$ μM, $n = 2.0$; cAMP before, $K_{1/2} = 1000$ μM, $n = 2.0$, fractional activation = 0.014; cAMP after, $K_{1/2} = 118$ μM, $n = 1.7$.

Modification of Cys^{481} with MTSET creates a version of the rod channel that is functionally similar to the olfactory CNG channel—the $K_{1/2}$ for cGMP is in the low micromolar range, and the channel can be fully activated by cAMP. Recent results from two laboratories have suggested that some of the functional differences between the olfactory and retinal channels are due to a stimulatory interaction between amino acids 60–90 of the olfactory channel amino

terminus and the carboxy-terminal linker and cyclic nucleotide-binding domains (Liu et al., 1994; Varnum and Zagotta, 1997). Deletion of this region shifts the dose-response relations for both nucleotides to higher concentrations and significantly reduces the efficacy of cAMP. A similar effect is achieved when this stimulatory interaction is blocked by the binding of the Ca^{2+}-calmodulin complex to the N-terminus. This interaction between the N- and C-termini is either weaker (Gordon et al., 1997) or nonexistent (Varnum and Zagotta, 1997) in the rod channel. It was interesting to speculate that the potentiation effect described in this manuscript arose from the stabilization of a favorable interaction between the N- and C-termini of the rod channel by the modification of Cys^{481}. This hypothesis was not supported, however, by subsequent experiments. Deletion of the entire N-terminus of the rod channel had little effect on potentiation by MTSET. It is interesting to note that the N-terminus of the channel is also not required for potentiation of the channel's response by nickel (Gordon and Zagotta, 1995b). These results suggest that other interactions within the channel can have profound effects on channel function.

To gain further insight into the molecular mechanism of channel potentiation by modification of Cys^{481}, we constructed a model of channel activation that could explain this behavior. Finding potentiation by MTSET reminiscent of that caused by micromolar concentrations of Ni^{2+} (Karpen et al., 1993; Gordon and Zagotta, 1995a), we adapted the model originally proposed by Gordon and Zagotta for Ni^{2+} potentiation. In this model (Fig. 8), two ligand-binding steps are followed by an allosteric closed-to-open transition. The original dose-response relation for cGMP is well fit by this model, using intrinsic binding constants of 100 μM (K_G) and an open-closed equilibrium constant that strongly favors the open state ($L_G = 0.15$). The original dose-response relation for cAMP was well fit, using a similar intrinsic binding constant ($K_A = 400$ μM) in conjunction with an unfavorable opening equilibrium constant ($L_A = 100$). These parameters are consistent with the low open probability (~1%) for the unmodified channel in the presence of saturating concentrations of cAMP and are similar to those proposed by Gordon and Zagotta. Like potentiation by Ni^{2+}, the potentiation by MTSET can be explained by assuming that modification simply makes the channel easier to open without changing the intrinsic binding affinities. In more precise terms, modification decreases L_G and L_A while leaving K_G and K_A unchanged. To account for the dramatic shifts in the dose-response relations, the open-closed equilibrium constants were decreased by a factor of 20,000. These results suggest that the peptide region surrounding Cys^{481} plays an important role in coupling the binding of ligand to opening of the channel pore. Potentiation of channel activation by Ni^{2+} has been explained by a similar mechanism (Gordon and Zagotta, 1995a). It is interesting to note that His^{420}, which is required for potentiation of the channel by Ni^{2+}, is also found in the linker region between the transmembrane and cyclic nucleotide-binding domains. Modification of cysteine residues in the native olfactory channel also has dramatic effects on the channel's open-closed equilibrium. Broillet and Firestein (1996) have shown that treatment of membrane patches excised from olfactory receptors with NEM can cause CNG channels to open spontaneously in the absence of cyclic nucleotides.

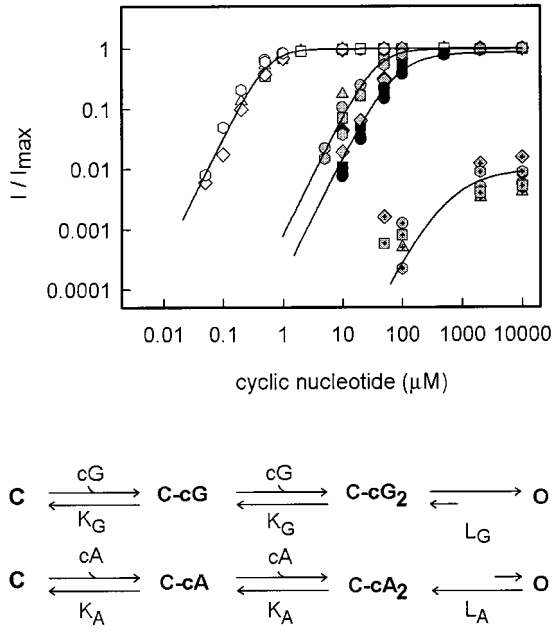

FIGURE 8 Potentiation arises from a decrease in the open-closed equilibrium constant rather than a change in the intrinsic binding affinity. The data and symbols are the same as those shown in Fig. 2. Solid lines show fits obtained from the simple model of channel activation shown above and were derived using the following equation: $I/I_{max} = [cN]^2/([cN]^2 + L * ([cN]^2 + 2 * [cN] * K + K^2)$. Each current is compared to the maximum patch current obtained in saturating cGMP after potentiation; under these conditions, the open probability of the channel is ~ 1. The parameters used for the fits shown were as follows. Before MTSET: $K_G = 200$ μM; $K_A = 500$ μM; $L_G = 0.15$; $L_A = 100$. After MTSET: $K_G = 200$ μM; $K_A = 500$ μM; $L_G = 0.0000075$; $L_A = 0.005$.

The rate of Cys^{481} modification by either NEM or MTSET was strongly dependent upon the activation state of the channel. The rate of modification was greatly enhanced by the presence of saturating cGMP, suggesting that the solvent exposure of the peptide region containing Cys^{481} was dramatically increased by the binding of ligand or by the allosteric transition leading to opening of the pore. These data suggest that the peptide region containing Cys^{481} may move significantly during the activation process. An alternative explanation is that other regions of the channel move to uncover Cys^{481}. Gordon et al. (1997) recently reported that both modification of Cys^{481} by NEM and formation of a disulfide bond between Cys^{481} and Cys^{35} were facilitated by channel activation. These results also indicate that Cys^{481} is more accessible when the channel is in the open conformation. The data presented here suggest that the linker region between the transmembrane and cyclic nucleotide-binding domains of the rod CNG channel moves

during activation and plays a critical role in the coupling of ligand binding to pore opening. In the future, identification of its interaction partners may identify other cogs in the gating machinery and shed light on the molecular rearrangements that occur during channel activation.

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REFERENCES

Akabas, M. H., and A. Karlin. 1995. Identification of acetylcholine receptor channel-lining residues in the M1 segment of the alpha-subunit. *Biochemistry.* 34:12496–12500.

Akabas, M. H., C. Kaufmann, P. Archdeacon, and A. Karlin. 1994. Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the alpha subunit. *Neuron.* 13:919–927.

Balakrishnan, K., J. Padgett, and R. A. Cone. 1990. Calcium flux in rod outer segment membranes: NEM potentiates the effects of cGMP. *Biophys. J.* 57:371A (Abstr.).

Biel, M., X. Zong, M. Distler, E. Bosse, N. Klugbauer, M. Murakami, V. Flockerzi, and F. Hofmann. 1994. Another member of the cyclic nucleotide-gated channel family, expressed in testis, kidney, and heart. *Proc. Natl. Acad. Sci. USA.* 91:3505–3509.

Broillet, M.-C., and S. Firestein. 1996. Direct activation of the olfactory cyclic nucleotide-gated channel through modification of sulfhydryl groups by NO compounds. *Neuron.* 16:377–385.

Brown, R. L., R. J. Bert, F. E. Evans, and J. W. Karpen. 1993. Activation of retinal rod cGMP-gated channels: what makes for an effective 8-substituted derivative of cGMP? *Biochemistry.* 32:10089–10095.

Brown, R. L., R. Gramling, R. J. Bert, and J. W. Karpen. 1995. Cyclic GMP contact points within the 63-kDa subunit and a 240-kDa associated protein of retinal rod cGMP-activated channels. *Biochemistry.* 34: 8365–8370.

Chen, T. Y., Y. W. Peng, R. S. Dhallan, B. Ahamed, R. R. Reed, and K. W. Yau. 1993. A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. *Nature.* 362:764–767.

Cook, N. J., W. Hanke, and U. B. Kaupp. 1987. Identification, purification, and functional reconstitution of the cyclic GMP-dependent channel from rod photoreceptors. *Proc. Natl. Acad. Sci. USA.* 84:585–589.

Donner, K., S. Hemila, G. Kalamkarov, A. Koskelainen, I. Pogozheva, and T. Rebrik. 1990. Sulfhydryl binding reagents increase the conductivity of the light-sensitive channel and inhibit phototransduction in retinal rods. *Exp. Eye Res.* 51:97–105.

Feng, L., I. Subbaraya, N. Yamamoto, W. Baehr, and N. Kraus-Friedmann. 1996. Expression of photoreceptor cyclic nucleotide-gated cation channel alpha subunit (CNGCalpha) in the liver and skeletal muscle. *FEBS Lett.* 395:77–81.

Finn, J. T., M. E. Grunwald, and K. W. Yau. 1996. Cyclic nucleotide-gated ion channels: an extended family with diverse functions. *Annu. Rev. Physiol.* 58:395–426.

Finn, J. T., J. Li, and K.-W. Yau. 1995. C-terminus involvement in the gating of cyclic nucleotide-activated channels as revealed by Ni^{2+} and NEM. *Biophy. J.* 68:A253 (Abstr.).

Frings, S., R. Seifert, M. Godde, and U. B. Kaupp. 1995. Profoundly different calcium permeation and blockage determine the specific function of distinct cyclic nucleotide-gated channels. *Neuron.* 15:169–179.

Gordon, S. E., M. D. Varnum, and W. N. Zagotta. 1997. Direct interaction between amino- and carboxyl-terminal domains of cyclic nucleotide-gated channels. *Neuron.* 19:431–441.

Gordon, S. E., and W. N. Zagotta. 1995a. A histidine residue associated with the gate of the cyclic nucleotide-activated channels in rod photoreceptors. *Neuron.* 14:1–20.

Gordon, S. E., and W. N. Zagotta. 1995b. Localization of regions affecting an allosteric transition in cyclic nucleotide-activated channels. *Neuron.* 14:857–864.

Goulding, E. H., G. R. Tibbs, and S. A. Siegelbaum. 1994. Molecular mechanism of cyclic-nucleotide-gated channel activation. *Nature.* 372: 369–374.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100.

Haynes, L. W., A. R. Kay, and K. W. Yau. 1986. Single cyclic GMP-activated channel activity in excised patches of rod outer segment membrane. *Nature.* 321:66–70.

Henn, D. K., A. Baumann, and U. B. Kaupp. 1995. Probing the transmembrane topology of cyclic nucleotide-gated ion channels with a gene fusion approach. *Proc. Natl. Acad. Sci. USA.* 92:7425–7429.

Karpen, J. W., R. L. Brown, L. Stryer, and D. A. Baylor. 1993. Interactions between divalent cations and the gating machinery of cyclic GMP-activated channels in salamander retinal rods. *J. Gen. Physiol.* 101:1–25.

Kaupp, U. B. 1995. Family of cyclic nucleotide gated ion channels. *Curr. Opin. Neurobiol.* 5:434–442.

Kaupp, U. B., T. Niidome, T. Tanabe, S. Terada, W. Bönigk, W. Stühmer, N. J. Cook, K. Kangawa, H. Matsuo, T. Hirose, T. Miyata, and S. Nuna. 1989. Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. *Nature.* 342:762–766.

Kingston, P. A., F. Zufall, and C. J. Barnstable. 1996. Rat hippocampal neurons express genes for both rod retinal and olfactory cyclic nucleotide-gated channels: novel targets for cAMP/cGMP function. *Proc. Natl. Acad. Sci. USA.* 93:10440–10445.

Körschen, H. G., M. Illing, R. Seifert, F. Sesti, A. Williams, S. Gotzes, C. Colville, F. Müller, A. Dosé, M. Godde, L. Molday, U. B. Kaupp, and R. S. Molday. 1995. A 240 kDa protein represents the complete beta subunit of the cyclic nucleotide-gated channel from rod photoreceptor. *Neuron.* 15:627–636.

Larsson, H. P., O. S. Baker, D. S. Dhillon, and E. Y. Isacoff. 1996. Transmembrane movement of the Shaker K^+ channel S4. *Neuron.* 16:387–397.

Leinders-Zufall, T., H. Rosenboom, C. J. Barnstable, G. M. Shepherd, and F. Zufall. 1995. A calcium-permeable cGMP-activated cation conductance in hippocampal neurons. *Neuroreport.* 6:1761–1765.

Liman, E. R., J. Tytgat, and P. Hess. 1992. Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron.* 9:861–871.

Liu, D. T., G. R. Tibbs, and S. A. Siegelbaum. 1996. Subunit stoichiometry of cyclic nucleotide-gated channels and effects of subunit order on channel function. *Neuron.* 16:983–990.

Liu, M., T. Y. Chen, B. Ahamed, J. Li, and K. W. Yau. 1994. Calcium-calmodulin modulation of the olfactory cyclic nucleotide-gated cation channel. *Science.* 266:1348–1354 (erratum: *Science.* 266:1933).

Molday, R. S., L. L. Molday, A. Dosé, I. Clark-Lewis, M. Illing, N. J. Cook, E. Eismann, and U. B. Kaupp. 1991. The cGMP-gated channel of the rod photoreceptor cell characterization and orientation of the amino terminus. *J. Biol. Chem.* 266:21917–21922.

Rieke, F., and E. A. Schwartz. 1994. A cGMP-gated current can control exocytosis at cone synapses. *Neuron.* 13:863–873.

Ruiz, M., and J. W. Karpen. 1997. Single cyclic nucleotide-gated channels locked in different ligand-bound states. *Nature.* 389:389–392.

Ruiz, M. L., B. London, and B. Nadal-Ginard. 1996. Cloning and characterization of an olfactory cyclic nucleotide-gated channel expressed in mouse heart. *J. Mol. Cell. Cardiol.* 28:1453–1461.

Savchenko, A., S. Barnes, and R. H. Kramer. 1997. Cyclic-nucleotide-gated channels mediate synaptic feedback by nitric oxide. *Nature.* 390: 694–698.

Serre, V., M. Ildefonse, and N. Bennett. 1995. Effects of cysteine modification on the activity of the cGMP-gated channel from retinal rods. *J. Membr. Biol.* 146:145–162.

Stryer, L. 1991. Visual excitation and recovery. *J. Biol. Chem.* 266: 10711–10714.

Stühmer, W. 1992. Electrophysiological recording from *Xenopus* oocytes. *Methods Enzymol.* 207:319–339.

Sun, Z. P., M. H. Akabas, E. H. Goulding, A. Karlin, and S. A. Siegelbaum. 1996. Exposure of residues in the cyclic nucleotide-gated channel pore: P region structure and function in gating. *Neuron.* 16:141–149.

Varnum, M. D., and W. N. Zagotta. 1997. Interdomain interactions underlying activation of cyclic nucleotide-gated channels. *Science.* 278: 110–113.

Xu, M., and M. H. Akabas. 1996. Identification of channel-lining residues in the M2 membrane-spanning segment of the GABA(A) receptor alpha1 subunit. *J. Gen. Physiol.* 107:195–205.

Yang, N., and R. Horn. 1995. Evidence for voltage-dependent S4 movement in sodium channels. *Neuron.* 15:213–218.

Yau, K. W. 1994. Cyclic nucleotide-gated channels: an expanding new family of ion channels [comment]. *Proc. Natl. Acad. Sci. USA.* 91: 3481–3483.

Zimmerman, A. L. 1995. Cyclic nucleotide gated channels. *Curr. Opin. Neurobiol.* 5:296–303.

Zimmerman, A. L., and D. A. Baylor. 1986. Cyclic GMP-sensitive conductance of retinal rods consists of aqueous pores. *Nature.* 321:70–72.

Zimmerman, A. L., J. W. Karpen, and D. A. Baylor. 1988. Hindered diffusion in excised membrane patches from retinal rod outer segments. *Biophys. J.* 54:351–355.

Zong, X., H. Zucker, F. Hofmann, and M. Biel. 1998. Three amino acids in the C-linker are major determinants of gating in cyclic nucleotide-gated channels. *EMBO J.* 17:353–362.

Zufall, F., G. M. Shepherd, and C. J. Barnstable. 1997. Cyclic nucleotide gated channels as regulators of CNS development and plasticity. *Curr. Opin. Neurobiol.* 7:404–412.