The Journal of Neuroscience, June 15, 1999, *19*(12):4786–4795

Activity-Dependent Modulation of Rod Photoreceptor Cyclic Nucleotide-Gated Channels Mediated by Phosphorylation of a Specific Tyrosine Residue

Elena Molokanova, Floyd Maddox, Charles W. Luetje, and Richard H. Kramer

Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

Cyclic nucleotide-gated (CNG) channels are crucial for phototransduction in vertebrate rod photoreceptors. The cGMP sensitivity of these channels is modulated by diffusible intracellular messengers, including Ca^{2+}/calmodulin, contributing to negative feedback during sensory adaptation. Membrane-associated protein tyrosine kinases and phosphatases also modulate rod CNG channels, but whether this results from direct changes in the phosphorylation state of the channel protein has been unclear. Here, we show that bovine rod CNG channel α-subunits (bRET) contain a tyrosine phosphorylation site crucial for modulation. bRET channels expressed in *Xenopus* oocytes exhibit modulation, whereas rat olfactory CNG channels (rOLF) do not. Chimeric channels reveal that differences in the C terminus, containing the cyclic nucleotide-binding domain, account for this difference. One specific tyrosine in bRET (Y498) appears to be crucial; replacement of this tyrosine in bRET curtails modulation, whereas installation into rOLF confers modulability. As the channel becomes dephosphorylated, there is an increase in the rate of spontaneous openings in the absence of ligand, indicating that changes in the phosphorylation state affect the allosteric gating equilibrium. Moreover, we find that dephosphorylation, which favors channel opening, requires open channels, whereas phosphorylation, which promotes channel closing, requires closed channels. Hence, modulation by changes in tyrosine phosphorylation is activity-dependent and may constitute a positive feedback mechanism, contrasting with negative feedback systems underlying adaptation.

Key words: rod photoreceptor; protein kinase; cyclic GMP; phototransduction; tyrosine kinase; phosphorylation

Cyclic nucleotide-gated (CNG) channels in vertebrate photoreceptors and olfactory receptor neurons generate changes in membrane potential and intracellular Ca^{2+} concentration in response to sensory stimuli. The change in internal Ca^{2+}, in conjunction with calmodulin and other Ca^{2+}-binding proteins, feeds back on CNG channels to lower their sensitivity to cyclic nucleotides during sensory adaptation (Kramer and Siegelbaum, 1992; Hsu and Molday, 1993; Chen and Yau, 1994; Nakatani et al., 1995; Kurahashi and Menini, 1997). Other diffusible intracellular messengers, such as diacylglycerol (Gordon et al., 1995) and nitric oxide (Broillet and Firestein, 1996) can also directly affect CNG channels and may play additional roles in sensory transduction and/or adaptation.

CNG channels are also modulated by changes in phosphorylation state catalyzed by Ser/Thr kinases (Muller et al., 1998) and phosphatases (Gordon et al., 1992) and by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Molokanova et al., 1997). Rod CNG channels expressed in *Xenopus* oocytes exhibit an increase in cGMP sensitivity that occurs spontaneously after excision of membrane patches containing the channels. This increase in cGMP sensitivity is unaffected by Ser/Thr phosphatase inhibitors but is suppressed by a PTP inhibitor, suggesting that it results from tyrosine dephosphorylation (Molokanova et al., 1997). In addition, oocyte patches contain active PTKs that reverse the effect, decreasing cGMP sensitivity after ATP application. This is prevented by specific inhibitors of PTKs but not by inhibitors of Ser/Thr kinases. These results indicate that rod CNG channels expressed in oocytes are associated with active PTPs and PTKs that catalyze changes in tyrosine phosphorylation state, thereby altering cGMP sensitivity. Additional studies (Molokanova et al., 1997) suggest that rod outer segments also contain PTPs and PTKs that modulate native CNG channels, but these enzymes are lost or inactive in excised patches from rods.

It has been unclear whether the CNG channel protein, or a distinct protein associated with the channels, is the substrate for PTPs and PTKs. Native rod CNG channels are heteromeric, composed of α- and β-subunits (Chen et al., 1993; Koerschen et al., 1995), but functional homomeric CNG channels with similar properties can be formed in *Xenopus* oocytes by expression of the α-subunit alone (Kaupp et al., 1989). Here, we show that the α-subunit of the bovine rod channel (bRET) contains crucial phosphorylation sites responsible for altering cGMP sensitivity. First, we show that modulation is dependent on the activation state of the channel, such that cGMP profoundly alters the ability of PTPs and PTKs to modulate the channel. This activity-dependence of phosphorylation and dephosphorylation may be physiologically important for regulating the extent of modulation in rods. Second, we exploit our observation that the rat olfactory CNG channel expressed in oocytes (rOLF) does not exhibit modulation by tyrosine phosphorylation. By studying chimeric bRET–rOLF channels, we have identified a specific tyrosine residue (Y498) within the cyclic nucleotide binding site of the rod channel that is crucial for modulation. Thus, cGMP sensitivity is

Received Jan. 22, 1999; revised March 31, 1999; accepted April 7, 1999.

This work was supported by grants from the National Institutes of Health (EY11877 to R.H.K. and DA08102 to C.W.L.) and the American Heart Association, Florida Affiliate (9502002 to R.H.K.). We thank Michael Vendiola for technical assistance, Alexei Savchenko for help with figures, and William Zagotta for providing chimeric channel constructs.

Correspondence should be addressed to Dr. Richard H. Kramer, P.O. Box 016189, University of Miami School of Medicine, Miami, FL 33101.

dynamically regulated by changes in the phosphorylation state of a specific tyrosine in the rod CNG channel protein.

MATERIALS AND METHODS

Expression and recording from oocyte CNG channels. A cDNA clone encoding the bovine rod photoreceptor CNG channel α-subunit (Kaupp et al., 1989) was used for *in vitro* transcription of mRNA, as described previously (Goulding et al., 1992), which was injected into *Xenopus* oocytes (50 nl per oocyte at 1 ng/nl). After 2–7 d, the vitelline membrane was removed from injected oocytes, which were then placed in a chamber for patch-clamp recording at 21–24°C. Glass patch pipettes (2–3 MΩ) were filled with a solution containing (in mM): 115 NaCl, 5 EGTA, and 10 HEPES, pH 7.5. This also served as the standard bath solution and cGMP perfusion solution, unless noted otherwise. After formation of a gigaohm seal, inside-out patches were excised, and the patch pipette was quickly (<30 sec) placed in the outlet of a 1 mm diameter tube for cGMP application. We used a perfusion manifold containing up to 15 different solutions that is capable of solution changes within 50 msec. A series of four to five cGMP concentrations (10–2000 μM cGMP) was applied to the patch. Application of the series required 20–30 sec and was repeated at 1 min intervals. In many experiments, 200 μM ATP (Mg salt) was applied transiently for 3 min starting 10.5 min after patch excision. cGMP, ATP, and sodium orthovanadate were obtained from Sigma (St. Louis, MO).

Current responses through CNG channels were obtained with an Axopatch 200A patch clamp (Axon Instruments, Foster City, CA), digitized, stored, and later analyzed on a 486 personal computer using pClamp 6.0 software. Membrane potential was held at −75 mV. Current responses were normalized to the maximal CNG current (I_{max}), elicited by saturating (2 mM) cGMP. Normalized dose–response curves were fit to the Hill equation: $I/I_{max} = 1/(1 + (K_{1/2}/[A])^n)$, where A is the cGMP concentration and n is the Hill coefficient, using a nonlinear least squares fitting routine (Origin; Microcal Software, Inc., Northampton, MA). Variability among measurements is expressed as mean ± SEM.

Fitting dose–response data to the allosteric Monod–Wyman–Changeux model. To fit dose–response curves to the Monod–Wyman–Changeux (1965) (MWC) model, we first needed to estimate spontaneous open probability (P_{sp}) and maximal open probability (P_{max}). To estimate P_{sp}, spontaneous openings were recorded with patch pipettes coated with Sylgard (Dow Corning Co., Midland, MI) at a holding potential of −80 mV and pipette and test solutions containing (in mM): 67 KCl, 30 NaCl, 10 EGTA, 1 EDTA, and 10 HEPES, pH 7.2. Mean spontaneous currents (I_{sp}) resulting from unliganded channel openings (filtered at 4 kHz, digitized at 20 kHz) were determined from the integral of the difference between the channel current and zero current baseline. P_{sp} was calculated from the equation: $P_{sp} = (I_{sp}/I_{max})\, P_{max}$, where I_{max} is the current elicited by saturating cGMP.

Spontaneous currents were measured at 1 min after excision in the absence of cGMP, and 2 mM cGMP was applied for ~8 min, followed by 1 min of extensive and continuous washing with cGMP-free solution, which flowed 2 ml/min. The patch pipette was inserted directly into the perfusion tube, and from the initial rate at which the macroscopic current deactivates after washout (<50 msec), we estimate that by 2 min later cGMP is completely removed.

To estimate P_{max}, we used noise analysis. Membrane currents, filtered at 10 kHz, were recorded over 60 sec in the absence and presence of 25–2000 μM cGMP and analyzed using Strathclyde Electrophysiological Software (Dr. John Dempster, University of Strathclyde, Glasgow, Scotland). Current variance values were fit according to the relation: $\sigma^2 = Ii - I^2/N$, where N is the number of CNG channels, and i is the unitary current, estimated from $i = V_m\, \gamma$, where V_m is the driving force (−80 mV) and γ is the single channel conductance (57 pS) (Liu et al., 1998). P_{max} was calculated as follows: $P_{max} = I_{max}/(Ni)$.

Currents through CNG channels at various concentrations of cGMP were expressed as open probability (P_o) according to $P_o = (I_A/I_{max})\,(P_{max} - P_{sp}) + P_{sp}$, where I_A is the current at a given ligand concentration. Open probabilities as a function of the ligand concentration were fit by the MWC model according to $P_o = (1 + [A]/K_o)^n/(L_o\,(1 + [A]/K_c)^n + (1 + [A]/K_o)^n)$, where K_o and K_c are dissociation constants of ligand binding to the channel in the open and close states, respectively, and $[A]$ is ligand concentration. Parameters of the MWC model were either directly determined (L_o, P_{max}) or obtained from the fits of the model (K_o/K_c). The equation $L_o = 1/P_{sp} - 1$ provides a value for the allosteric equilibrium constant (L_o) between the unliganded closed and open states of the channel.

Mutagenesis and construction of chimeric receptors. Mutant CNG channel subunits were constructed using PCR (Higuchi, 1990) and Pfu DNA polymerase. Our notation for mutant subunits is to list the naturally occurring residue, followed by the position of that residue, followed by the change that has been made. For example, the mutant bRET-Y498F is bRET in which tyrosine 498 has been changed to a phenylalanine. To minimize the amount of PCR product in the final construct that would have to be sequenced, a small cassette containing the mutation was cut from the PCR product and inserted into the wild-type construct using existing restriction sites. All PCR-derived portions of final constructs were confirmed by sequencing using Sequenase 2.0 from Amersham Life Sciences (Cleveland, OH). Pfu DNA polymerase was from Stratagene (La Jolla, CA).

RESULTS

Activity-dependence of modulation by tyrosine dephosphorylation

bRET channels expressed in *Xenopus* oocytes exhibit a gradual increase in their sensitivity to cGMP (a decrease in $K_{1/2}$) when membrane patches containing the channels are excised from the cell. Our previous studies (Molokanova et al., 1997) suggest that this decrease in $K_{1/2}$ results from tyrosine dephosphorylation by PTPs that are constitutively active in excised patches. Briefly exposing patches to ATP reverses this process, resulting in a decrease in the sensitivity to cGMP (an increase in $K_{1/2}$). Our evidence also suggests that this increase in $K_{1/2}$ is attributable to tyrosine phosphorylation by constitutively active PTKs in excised patches. To better understand whether bRET channels are modulated directly by acting as substrates for these PTK(s) and PTP(s), we examined how modulation is affected by the presence of ligands that selectively bind to CNG channels (i.e., cyclic nucleotides).

We first investigated whether the decrease in $K_{1/2}$ after patch excision, which results from tyrosine dephosphorylation, is influenced by the presence of cGMP. Changes in the $K_{1/2}$ after patch excision were monitored by repeatedly applying various concentrations of cGMP onto the patch (Fig. 1*A*). When these cGMP concentrations were applied at 1 min intervals, the change in $K_{1/2}$ occurred quickly (τ of 2.4 min), stabilizing at 52 ± 5% ($n = 55$) of its initial value within 10 min after excision. However, when the cGMP concentrations were applied less frequently, at 3 min intervals, the $K_{1/2}$ declined more slowly (τ of 6.1 min), decreasing to 66 ± 4% ($n = 7$) of its initial value after 13 min. At 2 min intervals, the $K_{1/2}$ declined at a rate that was intermediate to that elicited by 1 or 3 min application intervals. These observations suggest that the decline in $K_{1/2}$ associated with tyrosine dephosphorylation occurs more quickly when cGMP is present, accounting for the faster rate of decline when cGMP is applied more frequently.

To further examine the effect of ligands, we used a different paradigm, illustrated in Figure 1*B*. The initial $K_{1/2}$ was measured 1 min after patch excision, ligand was applied continuously during a "treatment" period for the subsequent 9 min, and then $K_{1/2}$ values were again monitored at 1 min intervals. When saturating cGMP (2 mM) was present during the treatment period, the $K_{1/2}$ declined dramatically during the first 10 min after excision. In contrast, when ligands were absent during the treatment period, the $K_{1/2}$ exhibited little change during the first 10 min, but subsequent exposure to cGMP could elicit a decline. With no ligand present during the treatment period, the $K_{1/2}$ declined by 12 ± 4% ($n = 12$), whereas with cGMP present, the $K_{1/2}$ declined by 52 ± 3% ($n = 14$).

Activation of CNG channels involves two processes: binding of ligands and opening of the channel. Whereas binding of cGMP to

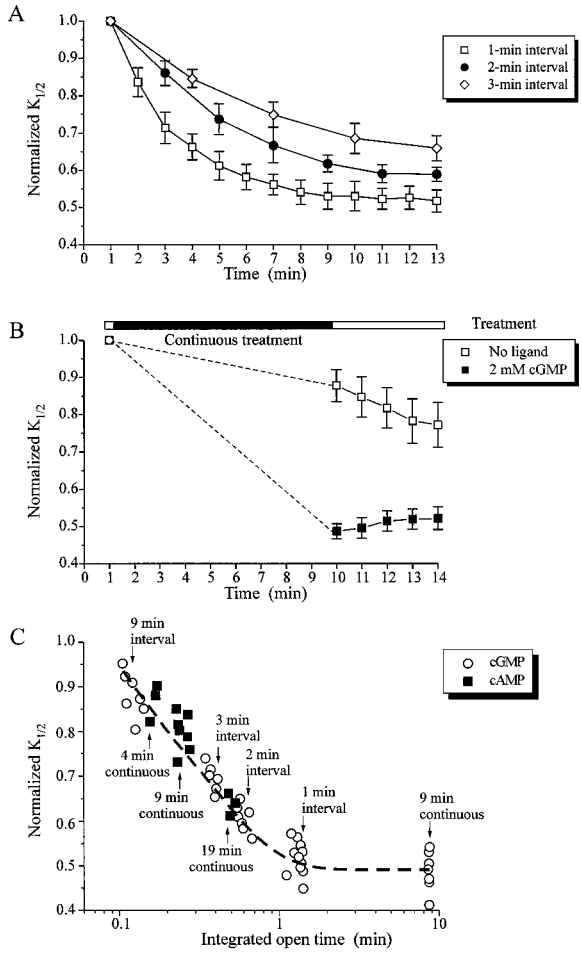

Figure 1. Activity-dependence of modulation by tyrosine dephosphorylation of bRET channels. *A*, Decline of $K_{1/2}$ for activation by cGMP (increase in sensitivity) with time after excision of membrane patches. To determine $K_{1/2}$ values, four concentrations of cGMP (50, 100, 250, and 2000 μM) were repeatedly applied on different patches at 1 ($n = 55$), 2 ($n = 8$), or 3 ($n = 7$) min intervals, starting 1 min after patch excision. In this and other figures, data are normalized to the initial $K_{1/2}$ value obtained 1 min after excision, unless indicated otherwise. Note that $K_{1/2}$ declines more quickly with more frequent cGMP application. *B*, Application of ligand (2 mM cGMP) during treatment period (9 min) promotes the decline of $K_{1/2}$ for cGMP, whereas with no ligand, $K_{1/2}$ changes little ($n = 10$–14 each). *C*, cGMP and cAMP treatment promote similar changes in the $K_{1/2}$ for cGMP, depending only on the integrated open time of CNG channels. Integrated open time is defined as the total current activated by ligand during the treatment period, divided by the maximal integrated current activated by saturating (2 mM) cGMP. Each point represents an individual patch with the final $K_{1/2}$ normalized to the initial $K_{1/2}$ value obtained at 1 min after patch excision. Patches were examined over a 10 min period, except for those treated with cAMP for 19 min. Patches were repeatedly exposed to various concentrations of ligand (interval paradigm, as in *A*) or continuously exposed to saturating ligand (continuous treatment, as in *B*). The *dashed line* is a single exponential fit to the data with a time constant of 0.4 min.

rod CNG channels is tightly coupled to opening, cAMP is less effective in triggering opening, such that saturating cAMP elicits only ~2% of the current activated by saturating cGMP (Kaupp et al., 1989; Goulding et al., 1992). To distinguish whether the acceleration of dephosphorylation results from ligand binding or channel opening, we compared the effects of cGMP and cAMP. We observed that saturating cAMP was much less effective than saturating cGMP in promoting a decline in $K_{1/2}$ as a result of dephosphorylation, suggesting that simple occupancy of the binding sites is insufficient to account for effect of ligand. In Figure 1*C*, we take into account the different efficacies of cAMP versus cGMP and the different application paradigms (e.g., continuous vs interval application) by determining the integrated open time for each experiment. The integrated open time represents the average time spent by each channel in the open state and was estimated by calculating the total current activated by ligand during the treatment period (usually 10 min), divided by the maximal integrated current activated by saturating cGMP. Figure 1*C* shows that the decrease in $K_{1/2}$ is promoted equally well by cAMP and cGMP once the difference in efficacy of these ligands is accounted for by calculating integrated open time. The decrease in $K_{1/2}$ depends only on the integrated open time with either ligand and follows the same trajectory, with a time constant of ~0.4 min. At integrated open times greater than ~1.5 min, additional exposure to ligand results in no further change in $K_{1/2}$, suggesting that dephosphorylation is complete. These experiments strongly suggest that channel opening rather than ligand binding is the crucial factor that promotes dephosphorylation.

Activity-dependence of modulation by tyrosine phosphorylation

We next examined whether the effect of ATP exposure on $K_{1/2}$ is influenced by the presence of cGMP. In previous work (Molokanova et al., 1997), we showed that treatment of patches with ATP could suppress or reverse the decrease in $K_{1/2}$ that occurs after patch excision and that the effect of ATP is blocked by specific inhibitors of PTKs. Figure 2*A* shows that treatment with ATP increases the $K_{1/2}$ of RET channels for cGMP, partially reversing the decline in $K_{1/2}$ that occurs during the first 10 min after patch excision. However, if a saturating concentration of cGMP (2 mM) is included along with ATP during the treatment period, no change in $K_{1/2}$ occurs. Thus, cGMP can completely suppress the increase in $K_{1/2}$ attributed to tyrosine phosphorylation.

How does addition of cGMP prevent the effect of ATP on the channels? Because tyrosine dephosphorylation is promoted by opening the channels (Fig. 1), it is possible that cGMP inhibits the ATP effect not by inhibiting PTK, but rather by promoting PTP activity. According to this scenario, even if the channels are tyrosine phosphorylated in the presence of ATP plus cGMP, they are more rapidly dephosphorylated by the enhanced PTP activity. If this were true, addition of vanadate, which inhibits PTPs (Swarup et al., 1982) and suppresses the apparent dephosphorylation of the rod CNG channel (Molokanova et al., 1997), should restore the effect of ATP. However, Figure 2*B* shows that there was no significant change in $K_{1/2}$ when ATP and cGMP were coapplied, even when 200 μM vanadate was added ($p < 0.05$). Hence, it is likely that inhibition of the ATP effect by saturating cGMP results from suppression of PTK activity rather than promotion of PTP activity.

Further studies (Fig. 2*B*) show that saturating cAMP (20 mM) also prevented the increase in $K_{1/2}$ during ATP treatment. The similarity of the actions of cAMP and cGMP suggest that simple occupancy of the ligand binding site could account for the prevention of modulation by tyrosine phosphorylation. Our observation that dephosphorylation is accelerated by ligands rules out the possibility that ligands simply indiscriminately occlude access to the phosphorylation site by both PTK and PTP enzymes. However, we cannot rule out the possibility that bound ligand selectively prevents access by PTKs. An additional possibility is that cAMP prevents tyrosine phosphorylation by inducing conforma-

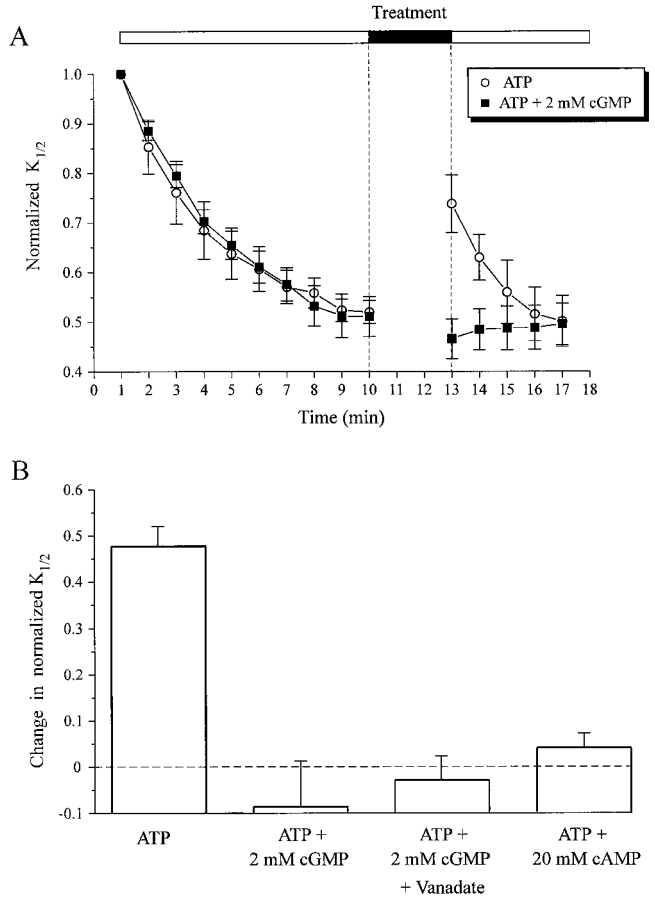

Figure 2. Activity-dependence of modulation by tyrosine phosphorylation of bRET channels. *A*, Increase in $K_{1/2}$ for activation by cGMP (decrease in sensitivity) after exposure to 200 μM ATP. Note that the presence of 2 mM cGMP during the treatment period (3 min) prevents the change in $K_{1/2}$ in response to ATP (n = 12 each). *B*, Summary data for experimental protocol illustrated in *A*. Change in normalized $K_{1/2}$ during the treatment period (10–13 min after excision) with the different treatments indicated. n = 38 for ATP alone; n = 8–12 for other treatments.

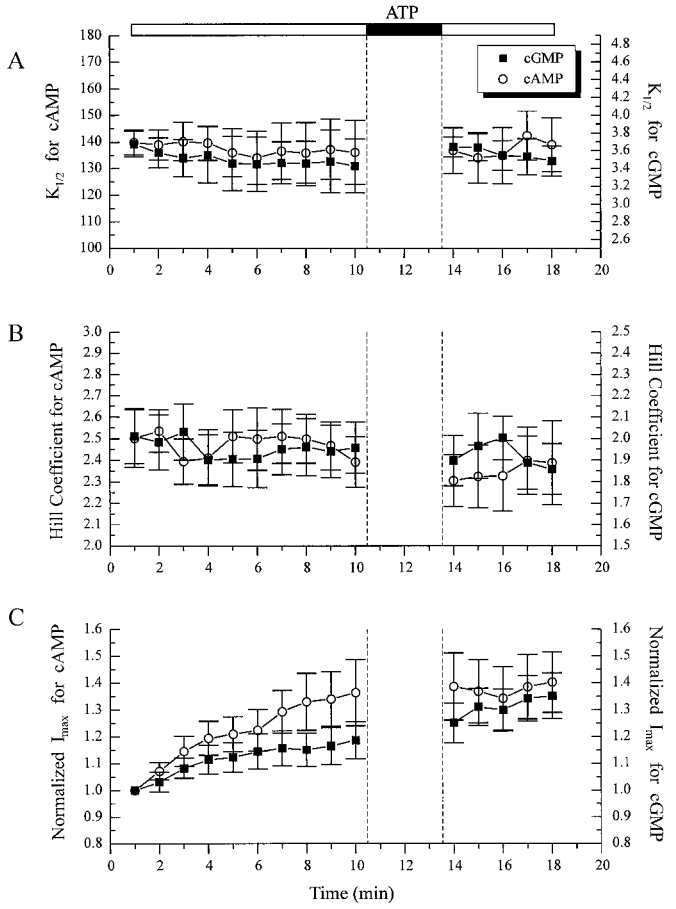

Figure 3. The rOLF channel does not exhibit modulation by tyrosine phosphorylation or dephosphorylation. *A*, $K_{1/2}$ values for activation by cGMP and cAMP measured at 1 min intervals after patch excision. Note that there is no significant change after either excision or ATP application. *B*, There is also no significant change in Hill coefficient values after excision and ATP application. *C*, Maximal current (I_{max}) elicited by saturating cGMP (2 mM) or cAMP (20 mM) increase with time after excision. I_{max} values were normalized to the initial I_{max}, measured at 1 min after patch excision. n = 14 for cGMP; n = 9 for cAMP.

tional changes that are not associated with full activation of the channels. The observation that the rates of both dephosphorylation and phosphorylation are profoundly altered by cyclic nucleotides strongly suggests that the CNG channel proteins themselves are substrates for PTPs and PTKs.

Identification of a protein region containing the tyrosine phosphorylation site

To investigate the molecular basis of modulation, we investigated the behavior of the related CNG channel rOLF and exploited the observation that this channel does not exhibit modulation by tyrosine phosphorylation. Figure 3 shows the behavior of rOLF channels expressed in *Xenopus* oocytes after patch excision. Various concentrations of cGMP or cAMP were applied at 1 min intervals, and resulting dose–response curves were fit with the Hill equation, and the three free parameters, $K_{1/2}$, Hill coefficient, and maximal current, were analyzed. Unlike bRET channels, the $K_{1/2}$ values of activation were stable after patch excision and showed no significant changes after application of ATP, either with cGMP or cAMP as the ligand (Fig. 3*A*). Likewise, the Hill coefficient exhibited no significant change over time (Fig. 3*B*). The maximal current elicited by saturating concentrations of cGMP or cAMP (I_{max}) did change, increasing progressively with time after excision (Fig. 3*C*). However, this increase was unaffected by vanadate, a PTP inhibitor (data not shown), or by ATP, indicating that the increase in I_{max} is unrelated to tyrosine phosphorylation or dephosphorylation. Thus, rOLF channels expressed in oocytes do not appear to be modulated by tyrosine phosphorylation. One of several possible explanations is that they may be lacking one or more critical tyrosine residues that constitute the phosphorylation site.

To test this possibility, we used chimeric CNG channels containing segments of the rOLF sequence substituted into the corresponding positions of bRET and segments of bRET substituted into the corresponding positions of rOLF (Gordon and Zagotta, 1995b). We found that substitution of the cytoplasmic C-terminal segment, which contains the conserved cyclic nucleotide binding site, was capable of conferring sensitivity to tyrosine phosphorylation. Thus, bRET channels containing the C terminus of rOLF behaved like rOLF channels, exhibiting no changes in $K_{1/2}$ after patch excision or ATP application (Fig. 4*A*). In contrast, rOLF channels containing the C terminus of bRET exhibited striking changes in $K_{1/2}$ after excision and ATP application (Fig. 4*B*). Hence, chimeric channels containing the C

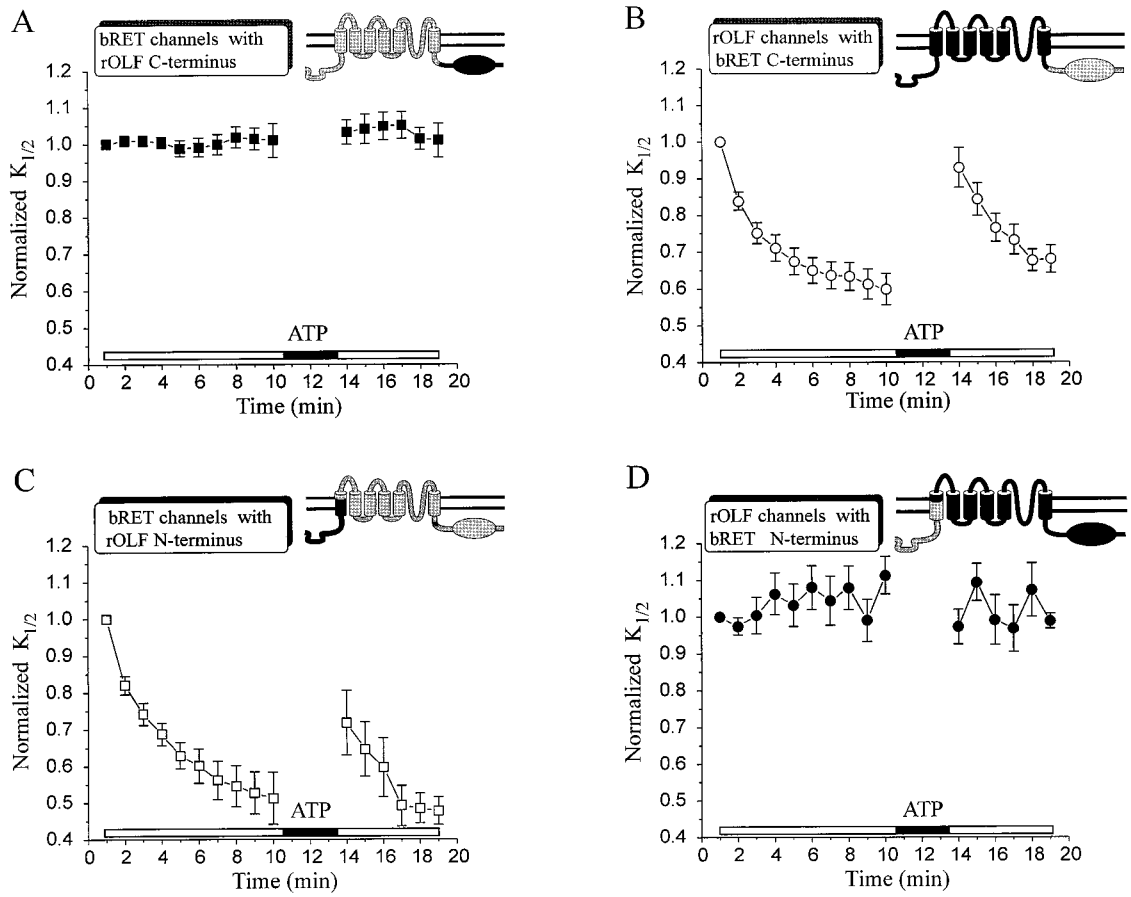

Figure 4. The difference in modulability of bRET and rOLF channels results from differences in the cytoplasmic C-terminal domain. *A–D*, Changes in $K_{1/2}$ for cGMP with time after patch excision and after application of 200 μM ATP. The *icons* illustrate each chimeric construct, with *gray* representing portions of the bRET channel and *black* indicating portions of the rOLF channel. The chimeras used in parts *A–D* correspond to CHM1, CHM18, CHM15, and CHM16 of Gordon and Zagotta (1995). n = 8–10 patches for each panel.

terminus of bRET exhibited modulation, whereas channels that contained the C terminus of rOLF did not.

We also examined chimeras of the N-terminal cytoplasmic domain, which has also been implicated in the activation of CNG channels (Goulding et al., 1994; Gordon and Zagotta, 1995; Gordon et al., 1997). The N terminus of rOLF also contains a domain that binds calmodulin, which can modulate cyclic nucleotide sensitivity (Liu et al., 1994). We found that substitution of the N-terminal domain had no effect on modulation by tyrosine phosphorylation. Thus, bRET channels containing the N terminus of rOLF exhibited large changes in $K_{1/2}$ after excision and ATP application (Fig. 4*C*), whereas rOLF channels containing the N terminus of bRET did not exhibit changes in $K_{1/2}$ (Fig. 4*D*). Hence, whereas the specific sequence of the C terminus of bRET appears to be crucial for determining whether modulation by tyrosine phosphorylation can occur, the specific nature of the N terminus appears to be less important and may not contain relevant tyrosine phosphorylation sites.

Identification of a specific tyrosine residue involved in modulation

The cytoplasmic C-terminal domain of bRET contains 10 tyrosine residues. Three of these are not conserved with respect to the rOLF sequence. Because modulability by tyrosine phosphorylation maps to this region of the channel protein, we generated three new channel constructs in which each of these tyrosines in bRET was substituted with the amino acid in the complementary position of rOLF. One of the mutant constructs (bRET-Y541C) could not be functionally expressed in *Xenopus* oocytes and therefore could not be analyzed. Figure 5*A* shows a comparison of the behavior of the remaining two constructs with that of bRET and rOLF. The bRET-Y454N channel exhibits changes in $K_{1/2}$ after excision and ATP application that are indistinguishable from those observed in bRET. In contrast, the bRET-Y498F channel exhibited very little modulation, with only a minor decrease in $K_{1/2}$ during the first 10 min after excision and no significant change resulting from application of ATP. The modulability of bRET-Y498F is greatly reduced with respect to bRET, but comparison with rOLF shows that it is not completely eliminated. In other respects, the bRET-Y498F channels are similar to bRET channels. Thus, the initial $K_{1/2}$ after excision was 132 ± 8 μM (n = 12), slightly higher than the initial $K_{1/2}$ of bRET (117 ± 6 μM; n = 55). Moreover, the maximal open probability of bRET-Y498F channels at saturating cGMP was ~0.85, similar to that of bRET (Goulding et al., 1994), and the channels retained their selective activation by cGMP versus cAMP (data not shown). The simplest explanation for these observations is that Y498 is a phosphorylation site that contributes to modulation of the rod CNG channel.

To test whether this tyrosine is sufficient for inducing modulation, we constructed a mutant of the rOLF channel in which a

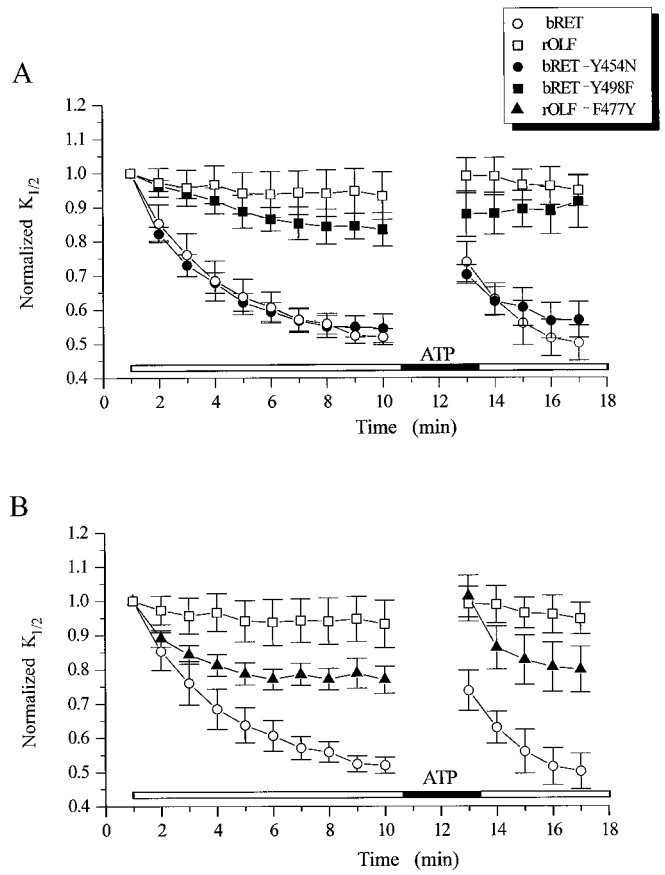

Figure 5. Identification as Y498 as a site crucial for modulation by phosphorylation. Decline in $K_{1/2}$ for cGMP with time after patch excision and increase in $K_{1/2}$ with 200 μM ATP application, indicative of tyrosine dephosphorylation and phosphorylation, respectively. Changes in $K_{1/2}$ resulting from both processes are shown for mutant bRET (*A*) and mutant rOLF (*B*) channels. Data from wild-type bRET (n = 55) and rOLF (n = 14) are shown in both *A* and *B* for comparison. n = 12 patches for each mutant.

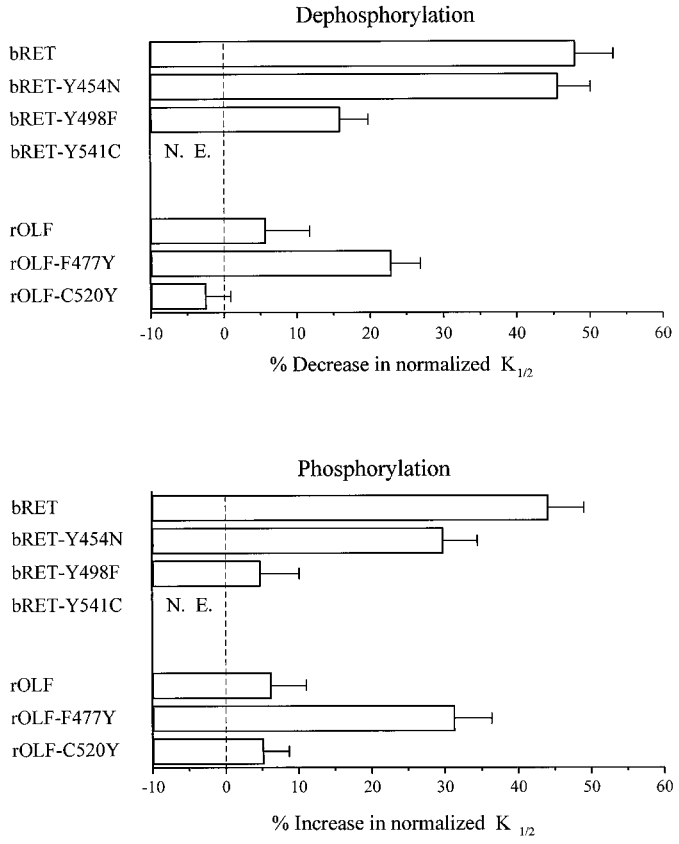

Figure 6. Modulation by dephosphorylation and phosphorylation of bRET and rOLF point mutants. *Top*, Dephosphorylation was estimated by monitoring the change in $K_{1/2}$ for cGMP during the first 10 min after patch excision, normalized to the initial $K_{1/2}$ (1 min after excision). *Bottom*, Phosphorylation was estimated by monitoring the change in $K_{1/2}$ for cGMP evoked by a 3 min application of 200 μM ATP, as shown in Figure 5. $K_{1/2}$ values after ATP were normalized to values immediately before ATP application. There was no expression (*N.E.*) of mutant bRET-Y541C. Numbers of experiments for each *bar* are the same as Figure 5, except rOLF-C520Y in which n = 8.

tyrosine residue was introduced into the appropriate position, substituting for a phenylalanine (rOLF-F477Y). We find that this "reverse mutant" channel does exhibit changes in $K_{1/2}$ after patch excision and ATP application that are significantly larger than those seen in rOLF, although the modulation is not as profound as that exhibited by bRET (Fig. 5*B*). To determine whether the changes in $K_{1/2}$ in rOLF-F477Y indeed result from introduction of a tyrosine phosphorylation site, we tested the effects of vanadate, a PTP inhibitor, and erbstatin, a PTK inhibitor. In the presence of 100 μM vanadate, the $K_{1/2}$ for cGMP decreased only by 8 ± 4% (n = 4) during the first 10 min after excision compared with 23 ± 7% (n = 8) without vanadate present. Moreover, in four experiments, application of 50 μM erbstatin completely blocked the effect of ATP on the $K_{1/2}$ for cGMP, consistent with introduction of a tyrosine phosphorylation site. These results strongly support the hypothesis that Y498 of the bRET channel is a tyrosine phosphorylation site that regulates cGMP sensitivity.

Figure 6 quantifies the results of our mutagenesis experiments. Modulation resulting from dephosphorylation and phosphorylation were both large in bRET channels but insignificant in rOLF channels. Mutant bRET-Y541C did not functionally express. Modulation of mutant bRET-Y454N (n = 12) by both dephosphorylation and phosphorylation were similar to bRET. In contrast, modulation of bRET-Y498F was greatly reduced compared with bRET ($p < 0.05$). After patch excision, the $K_{1/2}$ of rOLF and bRET channels decreased by 6 ± 7 (n = 14) and 48 ± 3% (n = 55), respectively. The decrease in $K_{1/2}$ of the bRET-Y498F channel was only 16 ± 5% (n = 12), accounting for at least 75% of the difference between bRET and rOLF.

Introduction of the tyrosine into rOLF (rOLF-F477Y) had the opposite effect, installing modulability by both phosphorylation and dephosphorylation. The decrease in $K_{1/2}$ evoked by dephosphorylation of this channel was ~23 ± 7% (n = 8), approximately half the decrease observed in bRET channels. The rOLF-F477Y channel exhibited a 31 ± 7% increase in $K_{1/2}$ resulting from phosphorylation, ~75% as large as the increase observed with bRET. Hence, this data strongly implicates residue Y498 of bRET as a phosphorylation site that modulates cGMP sensitivity.

The failure of bRET-Y541C to express functionally prevented us from obtaining information about a potential role for Y541. As an additional attempt to gain information about this site, we constructed rOLF-C520Y, inserting a tyrosine into rOLF at a position equivalent to Y541 of bRET. This construct was capable of functional expression but did not show any modulation. Hence, this tyrosine residue cannot confer modulability on rOLF, suggesting that it is not involved in modulation of bRET.

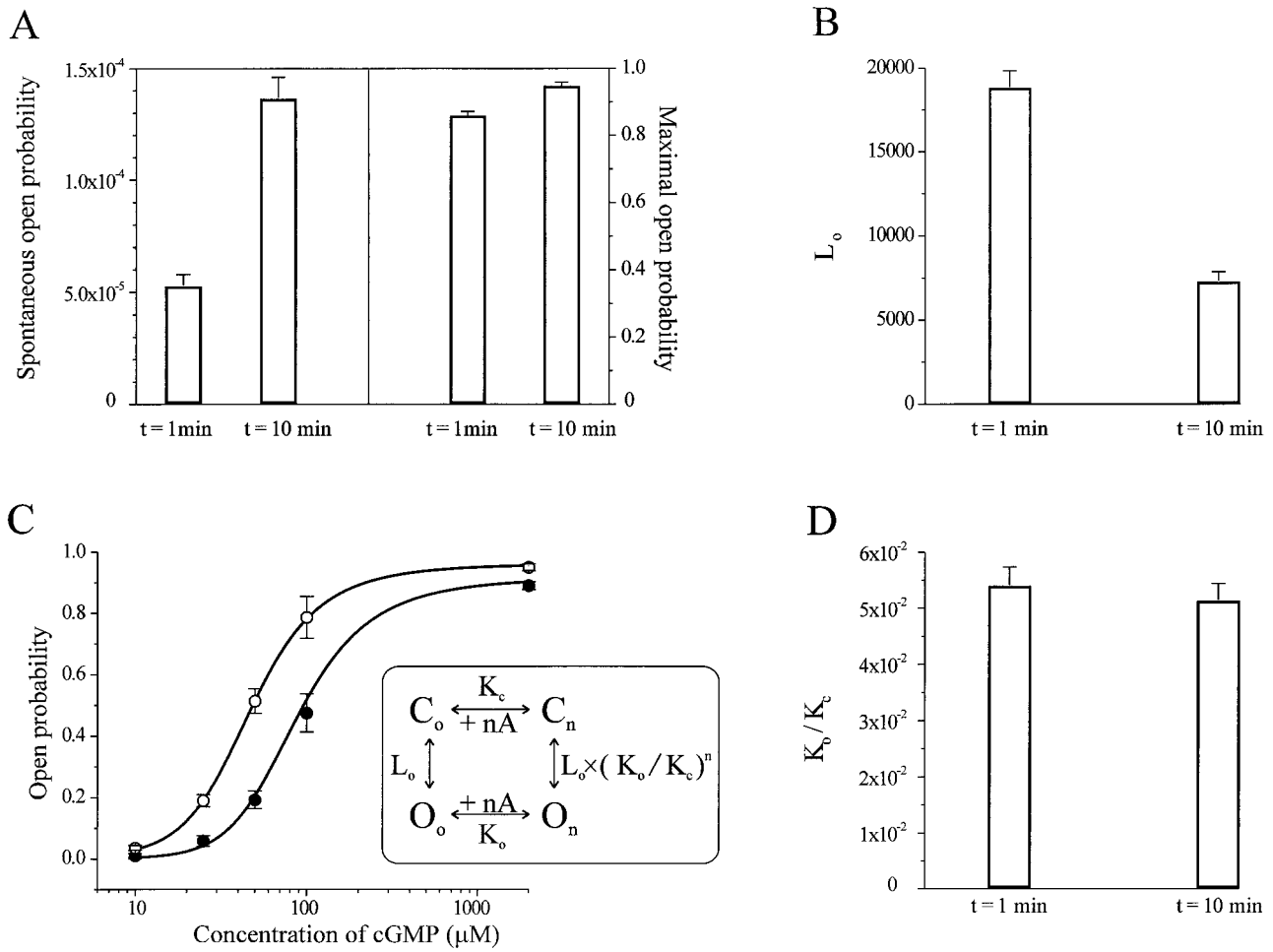

Figure 7. The MWC model of CNG channel activation suggests that modulation of cGMP-sensitivity results from a change in the equilibrium of the allosteric gating transition. *A*, Open probability of RO133 channels in the complete absence of ligand (spontaneous open probability, *left panel*) and in the presence of 2 mM cGMP (maximal open probability, *right panel*), determined at 1 and 10 min after patch excision. See Materials and Methods for how these parameters are calculated. *B*, Equilibrium gating constant (L_o) at 1 and 10 min after patch excision, calculated from spontaneous open probability. *C*, Fits of the MWC model (*solid lines*) to data representing activation of RO133 channels obtained 1 min (*closed circles*) and 10 min (*open circles*) after patch excision. *Inset* represents MWC model with *C* and *O* representing closed and open channels, with zero to *n* ligands bound, *A* representing agonist, and K_o and K_c representing dissociation constants for binding to the open and closed states. *D*, Ratio of dissociation constants (K_o/K_c) as a result of fits to the model. $n = 7$ patches for 1 and 10 min for each panel.

Modulation by tyrosine phosphorylation results from changes in the allosteric channel opening transition

To understand how tyrosine phosphorylation affects the activation of CNG channels, we used the classic allosteric model of Monod et al. (1965), which was adapted for CNG channels (Stryer, 1987; Goulding et al., 1994). According to this model, all four subunits of a CNG channel undergo a concerted allosteric transition between open (O) and closed (C) states. In the absence of ligand, the equilibrium gating constant of this transition is termed L_o. Ligand can bind to both closed and open channels, but the dissociation constant of binding to the open channel (K_o) is lower than that for the closed channel (K_c). Sequential binding of ligand to each of the four subunits causes a progressive shift in the equilibrium gating constant by the factor $(K_o/K_c)^n$, where n is the number of ligand molecules bound to the channel. According to this model, changes in the apparent affinity of ligand as a result of phosphorylation could be caused by altering the allosteric opening equilibrium (change in L_o) and/or by altering the ratio of dissociation constants of binding to open versus closed states (change in K_o/K_c).

To determine whether the value of L_o changes during dephosphorylation, we examined spontaneous opening of CNG channels (Tibbs et al., 1997) in the complete absence of ligand at 1 and 10 min after patch excision. Because these openings do not involve ligand binding, they can be used as a unambiguous indicator of channel gating. The small single channel conductance of bRET channels makes it very difficult to measure current through spontaneous open channels. Therefore, we used a chimeric channel (RO133; Goulding et al., 1993) composed of the bRET channel substituted with the pore-forming region of the catfish olfactory channel, which has a threefold larger single channel conductance that bRET. We find that RO133 exhibits behaviors characteristic of tyrosine dephosphorylation and phosphorylation that are qualitatively and quantitatively similar to those observed in bRET, namely a decrease in $K_{1/2}$ after patch excision and an increase in $K_{1/2}$ after ATP application (data not shown).

Figure 7*A* shows how open probability of RO133 channels changes during the first 10 min after patch excision. The spontaneous open probability of these channels increased from 5.3×10^{-5} to 1.37×10^{-4} (160% increase). As a control, we performed

the same analysis on patches from oocytes expressing rOLF channels, which are much more sensitive to cGMP but do not exhibit modulation by tyrosine phosphorylation. For rOLF channels, spontaneous open probability was $2.31 \times 10^{-3} \pm 0.91 \times 10^{-3}$ at 1 min and $2.19 \times 10^{-3} \pm 0.88 \times 10^{-3}$ at 10 min after patch excision ($n = 4$). Thus, using the same procedure, we find no increase in spontaneous openings between 1 and 10 min after excision. Our observation that the increase in spontaneous openings is specific for bRET also indicates that channels native to oocytes are unlikely to contribute, because they should be present in both RO133 and rOLF patches.

The maximal open probability of RO133 channels, determined from noise analysis of current in the presence of saturating ligand (2 mM cGMP), increased from 0.86 to 0.95 (10% increase) during the first 10 min after patch excision. We observed a similar increase in maximal current in bRET channels of 13 ± 5%; ($n = 55$), although we previously presented data from a single patch that showed no significant increase (Molokanova et al., 1997). With cAMP as the agonist, which has a much lower efficacy that cGMP, we observed a much larger increase in maximal current of 122 ± 17% ($n = 10$) over the first 10 min.

Our measurement of spontaneous open probability allows the direct determination of L_o. Figure 7*B* shows that L_o decreases 2.6-fold after channel dephosphorylation. Using the calculated values for L_o and the directly determined values of maximal open probability, dose–response curves of RO133 channel activation at 1 and 10 min after excision were fitted with the MWC model (Fig. 7*C*). The ratio of dissociation constants for binding to open versus closed states (K_o/K_c) was a free parameter, and this value was obtained from the best fit of the model to our data. Figure 7*D* shows that the values of K_o/K_c required for the best fit were not significantly different at 1 versus 10 min after patch excision ($p < 0.01$). Therefore, the decrease in $K_{1/2}$ resulting from tyrosine dephosphorylation can be fully accounted for by changes in L_o without requiring a change in K_o/K_c. Hence, the increase in apparent affinity for cGMP caused by tyrosine dephosphorylation results, at least in part, from an energetically more favorable opening reaction.

DISCUSSION

Identification of the tyrosine phosphorylation site responsible for modulation

Our results indicate that modulation of bRET channels expressed in oocytes results from direct phosphorylation and dephosphorylation of tyrosine residues located in the bRET polypeptide. One particular tyrosine, Y498, appears to have a major importance for modulation. When this tyrosine is removed from bRET (bRET-Y498F), modulation is reduced by 75%. When a tyrosine is introduced into the corresponding position of rOLF (rOLF-F477Y), modulation appears, although the changes in cGMP sensitivity are only ~50% as great as those observed in bRET. The simplest interpretation of our results is that Y498 is a phosphorylation site that alters cGMP sensitivity in bRET. The residual modulation observed when this site is removed from bRET suggests that phosphorylation of additional tyrosines also contributes. The partial introduction of modulability to rOLF after installation of the tyrosine is also consistent with this idea. Additional tyrosines that may contribute to modulation are likely to reside in the C terminus because the chimeric bRET channel containing the rOLF C terminus exhibits a complete loss of modulation, and the chimeric rOLF channel containing the bRET C terminus exhibits a complete gain of modulation.

Many cyclic nucleotide-binding proteins, including CNG channels, cAMP and cGMP-dependent protein kinases (PKA and PKG, respectively), and the catabolite gene activator protein (CAP), a bacterial transcription factor, contain a conserved cyclic nucleotide binding site (Shabb and Corbin, 1992). In each of these proteins, the amino acid position corresponding to Y498 is highly conserved, containing either a tyrosine or a phenylalanine. Type I PKG and CAP both contain a tyrosine, but it is not known whether the activities of these proteins are modulated by tyrosine phosphorylation. Whereas the rat olfactory CNG channel (rOLF) contains a phenylalanine (Dhallan et al., 1990), the catfish olfactory channel contains a tyrosine (Goulding et al., 1992), and studies have shown that this channel expressed in *Xenopus* oocytes does exhibit changes in $K_{1/2}$ after patch excision and ATP application (R. Kramer, unpublished observations), suggesting that it too is susceptible to modulation by tyrosine phosphorylation.

The crystal structure of CAP shows that cyclic nucleotides bind in a pocket between an eight-stranded β-roll and a long α-helix (the C-helix), with two additional α-helices supporting the structure (Weber and Steitz, 1987). The amino acid corresponding to Y498 (Y23 in CAP) is located in the β1 strand, with the side group on the periphery of the β-roll, accessible to the aqueous environment. The predicted position of Y498 in CNG channels is removed from contact sites with cyclic nucleotides, consistent with our finding that phosphorylation affects allosteric gating transitions but not necessarily binding of ligands. The β1 strand is located close to the N-terminal end of the cyclic nucleotide-binding domain. Hence, by occurring between the ligand binding site and the rest of the channel protein, the tyrosine phosphorylation site is located in a strategic position for influencing coupling of ligand binding to channel gating.

Molecular basis for the difference in modulability of bRET and rOLF channels

The absence of a tyrosine in the position of rOLF (F477) corresponding to Y498 of bRET partly explains why rOLF does not exhibit modulation. We have considered several other structural and functional differences between bRET and rOLF that could also contribute to the difference in modulability. First, the binding of ligands appears to be coupled more tightly to the opening of rOLF channels than in bRET channels (Zagotta and Siegelbaum, 1996). It is conceivable that even if tyrosine phosphorylation occurred in rOLF as it does in bRET, it would have less of an impact in the context of such favorable gating. Our results suggests that this cannot explain the lack of modulation in rOLF. The free energy change of channel opening in rOLF channels with bound cAMP is much less than with bound cGMP, making the allosteric opening transition much less favorable and similar to that of the bRET channel with cGMP as the agonist (Goulding et al., 1994; Gordon and Zagotta, 1995). However, even with cAMP as the agonist, the rOLF channel still does not exhibit modulation.

Second, modulation may be impaired in rOLF because of a possible lack of recognition sites for binding PTKs and PTPs. Several types of ion channels, including nicotinic acetylcholine receptors (Swope and Huganir, 1994; Fuhrer and Hall, 1996) and certain voltage-gated K^+ channels (Holmes et al., 1996), contain specific domains that mediate "docking" to src homology domains 2 and 3 (SH2 and SH3) that are common in PTKs and PTPs (Fantl et al., 1993). The binding site for an SH2 domain is characterized by proline-rich regions, and examination of the predicted bRET and rOLF sequences fail to reveal such motifs in

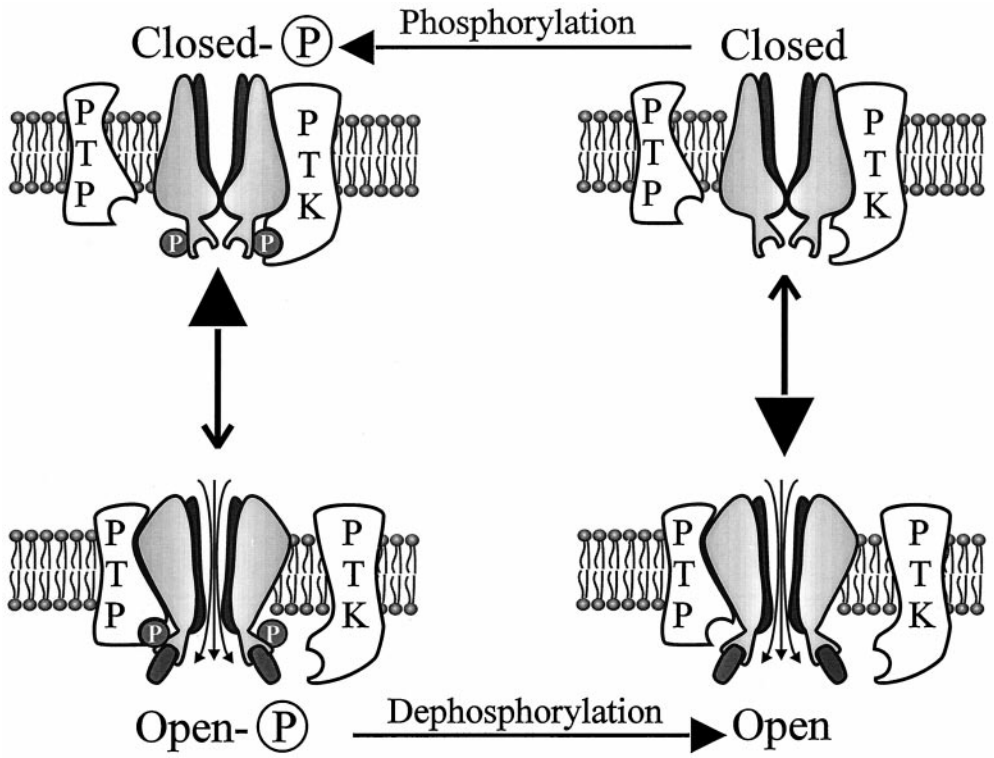

Figure 8. Schematic model illustrating positive feedback resulting from activity-dependence of modulation by tyrosine phosphorylation and dephosphorylation. The *top* and *bottom* represent closed and open channels, respectively, and the *left* and *right* represent phosphorylated and dephosphorylated channels, respectively. The *ovals* represent cGMP molecules. The relative *thickness* of *arrowheads* represents changes in the favorability of gating.

cytoplasmic portions of the proteins, suggesting that other uncharacterized binding domains mediate interaction with PTKs and PTPs. The observation that introduction of a tyrosine into rOLF can confer modulability implies that at least to some extent PTKs and PTPs can effectively bind and recognize the rOLF channel as a substrate.

Activity-dependence of tyrosine phosphorylation and dephosphorylation

Both phosphorylation and dephosphorylation of bRET are activity-dependent, such that cyclic nucleotides alter the ability of PTKs and PTPs to modulate the channel. Cyclic nucleotides prevent phosphorylation by PTKs, which could occur if bound ligand occluded the phosphorylation site, sterically preventing access by the enzymes. We cannot distinguish between this possibility and an additional one in which the binding of ligands produces conformational changes short of channel opening, which influence the ability of PTKs to catalyze phosphorylation by PTKs.

In contrast, PTP activity is accelerated by cyclic nucleotides. Our studies comparing cGMP and cAMP suggest that the activity-dependence of dephosphorylation results from channel activation rather than ligand binding per se. Hence, the extent to which a ligand promotes dephosphorylation is directly related to its ability to open CNG channels (Fig. 1*C*). The catalytic rates of PTPs appear to be dependent on the specific conformation of the channel (e.g., closed vs open), which can be altered much more effectively by cGMP than by cAMP.

The conformation of the channel might differentially alter the accessibility of a specific phosphorylation site to PTKs and PTPs that are already bound to the channel. An alternate, more simple possibility is that the conformation of the channel determines whether PTKs or PTPs can bind to the channel in the first place. Figure 8 illustrates a model in which the PTKs and PTPs can bind and unbind from the bRET channel, with the closed state allowing PTK binding and the open state allowing PTP binding. The activity-dependence reflects the exclusivity of PTK or PTP binding. The slow kinetics of phosphorylation and dephosphorylation could result from the slow kinetics of association of the enzymes with the channel, which may be rate-limiting.

One interesting feature of the model is that it is capable of exhibiting positive feedback. Thus, when the channel opens (e.g., in the presence of cGMP), binding of PTP, which dephosphorylates the channel, promotes channel opening, further accelerating dephosphorylation. In contrast, when the channel is closed (and/or when ligand is absent), binding of PTK leads to phosphorylation, promoting channel closing and further phosphorylation. The channel potentially has four tyrosine phosphorylation sites, so according to the above scheme, it is possible that dephosphorylation of one site increases the likelihood of dephosphorylation of additional sites. To test this possibility, it will be necessary to determine how many phosphorylation events are required to change the cGMP sensitivity of a single channel.

The positive feedback implied in this model is in sharp contrast to negative feedback systems, such as those mediated by intracellular Ca^{2+}, which contribute to relatively rapid (seconds to minutes) adaptation processes in photoreceptors (Koutalos and Yau, 1996). It is interesting to speculate about the functional role of modulation by tyrosine phosphorylation and what its positive feedback characteristics might play in rods. Our previous studies show that native CNG channels in rods can be modulated by native PTKs and PTPs in a manner similar to that observed in expressed bRET channels (Molokanova et al., 1997). Recent studies show that certain growth factors, especially insulin-like growth factor I (IGF-1), modulate cGMP-sensitivity of native rod channels, apparently by changing their tyrosine phosphorylation state (A. Savchenko and R. Kramer, unpublished observations). From these observations, we predict that the effects of growth factors, such as IGF-1, on rod CNG channels should exhibit positive feedback. The positive feedback feature of modulation by

tyrosine phosphorylation may provide a mechanism for offsetting or overriding the homeostatic negative feedback mechanisms of adaptation.

REFERENCES

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

Chen T-Y, Yau K-W (1994) Direct modulation by Ca^{2+}-calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. Nature 368:545–548.

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

Dhallan RS, Yau K-W, Schrader K, Reed R (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature 347:184–187.

Fantl WF, Johnson DE, Williams LT (1993) Signaling by tyrosine kinases. Annu Rev Biochem 62:453–481.

Fuhrer C, Hall ZW (1996) Functional interaction of Src family kinases with the acetylcholine receptor in C2 myocytes. J Biol Chem 271:32474–32481.

Gordon SE, Zagotta WN (1995) Localizations of regions affecting an allosteric transitions in cyclic nucleotide-activated channels. Neuron 14:857–864.

Gordon SE, Brautigan DL, Zimmerman AL (1992) Protein phosphatases modulate the apparent agonist affinity of the light-regulated ion channel in retinal rods. Neuron 9:739–748.

Gordon SE, Downing-Park J, Tam B, Zimmerman AL (1995) Diacylglycerol analogs inhibit the rod cGMP-gated channel by a phosphorylation-independent mechanism. Biophys J 69:409–417.

Gordon SE, Varnum MD, Zagotta WN (1997) Direct interactions between amino- and carboxyl-terminal domains of cyclic nucleotide-gated channels. Neuron 19:431–441.

Goulding EH, Ngai J, Kramer RH, Colicos S, Siegelbaum SA, Axel R, Chess A (1992) Molecular cloning and single channel properties of a cyclic nucleotide-gated cation channel from catfish olfactory receptor neurons. Neuron 8:45–58.

Goulding EH, Tibbs G, Liu D, Siegelbaum SA (1993) Role of H5 domain in determining pore diameter and ion permeation through cyclic nucleotide-gated channels. Nature 364:61–64.

Goulding EH, Tibbs GR, Siegelbaum SA (1994) Molecular mechanism of cyclic-nucleotide-gated channel activation. Nature 372:369–374.

Higuchi R (1990) Recombinant PCR. In: PCR protocols, a guide to methods and applications (Innis MA, Gelfand GH, Sninsky JJ, White TJ, eds), pp 177–183. San Diego: Academic.

Holmes TC, Fadool DA, Ren R, Levitan IB (1996) Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. Science 274:2089–2091.

Hsu YT, Molday RS (1993) Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. Nature 361:76–79.

Kaupp BU, Niidome T, Tanabe T, Terada S, Bonigk W, Stuhmer W, Cook NJ, Kangawa K, Matsuo H, Hirode T, Miyata T, Numa S (1989) Primary structure and functional expression from complementary DNA of the rod photoreceptor cGMP-gated channel. Nature 342:762–766.

Koerschen HG, Illing M, Seifert R, Sesti F, Williams A, Gotzes S, Colville C, Muller F, Dose A, Godde M, Molday L, Kaupp UB, Molday RS (1995) A 240 kDa protein represents the complete β subunit of the cyclic nucleotide-gated channel from rod photoreceptor. Neuron 15:627–636.

Koutalos Y, Yau K-W (1996) Regulation of sensitivity in vertebrate rod photoreceptors by calcium. Trends Neurosci 19:73–81.

Kramer RH, Siegelbaum SA (1992) Intracellular Ca^{2+} regulates sensitivity of cyclic nucleotide-gated channels in olfactory receptor neurons. Neuron 9:897–906.

Kurahashi T, Menini A (1997) Mechanism of odorant adaptation in the olfactory receptor cell. Nature 385:725–729.

Liu DT, Tibbs GR, Paoletti P, Siegelbaum SA (1998) Constraining ligand-binding site stoichiometry suggests that a cyclic nucleotide-gated channel is composed of two functional dimers. Neuron 21:235–248.

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

Molokanova E, Trivedi B, Savchenko A, Kramer RH (1997) Modulation of rod photoreceptor cyclic nucleotide-gated channels by tyrosine phosphorylation. J Neurosci 17:9068–9076.

Monod J, Wyman J, Changeux JP (1965) On the nature of allosteric transitions: a plausible model. J Mol Biol 12:88–118.

Muller F, Bonigk W, Sesti F, Frings S (1998) Phosphorylation of mammalian olfactory cyclic nucleotide-gated channels increases ligand sensitivity. J Neurosci 18:164–173.

Nakatani K, Koutalos Y, Yau K-W (1995) Ca^{2+} modulation of the cGMP-gated channels of bullfrog retinal rod photoreceptors. J Physiol (Lond) 484:69–76.

Shabb JB, Corbin JD (1992) Cyclic nucleotide-binding domains in proteins having diverse functions. J Biol Chem 267:5723–5726.

Stryer L (1987) Visual transduction: design and recurring motifs. Chem Scr 27B:161–171.

Swarup G, Cohen S, Garbers DL (1982) Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. Biochem Biophys Res Commun 107:1104–1109.

Swope SL, Huganir RL (1994) Binding of the nicotinic acetylcholine receptor to SH2 domains of Fyn and Fyk protein tyrosine kinases. J Biol Chem 269:29817–29824.

Tibbs GR, Goulding EH, Siegelbaum SA (1997) Allosteric activation and tuning of ligand efficacy in cyclic-nucleotide-gated channels. Nature 386:612–615.

Weber IT, Steitz TA (1987) Structure of a complex of catabolite gene activator protein and cyclic AMP refined at 2.5 Å resolution. *J Mol Biol* 198:311–326.

Zagotta WN, Siegelbaum SA (1996) Structure and function of cyclic nucleotide-gated channels. Annu Rev Neurosci 19:235–263.