Noncatalytic Inhibition of Cyclic Nucleotide–gated Channels by Tyrosine Kinase Induced by Genistein

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ABSTRACT   Rod photoreceptor cyclic nucleotide–gated (CNG) channels are modulated by tyrosine phosphorylation. Rod CNG channels expressed in *Xenopus* oocytes are associated with constitutively active protein tyrosine kinases (PTKs) and protein tyrosine phosphatases that decrease and increase, respectively, the apparent affinity of the channels for cGMP. Here, we examine the effects of genistein, a competitive inhibitor of the ATP binding site, on PTKs. Like other PTK inhibitors (lavendustin A and erbstatin), cytoplasmic application of genistein prevents changes in the cGMP sensitivity that are attributable to tyrosine phosphorylation of the CNG channels. However, unlike these other inhibitors, genistein also slows the activation kinetics and reduces the maximal current through CNG channels at saturating cGMP. These effects occur in the absence of ATP, indicating that they do not involve inhibition of a phosphorylation event, but rather involve an allosteric effect of genistein on CNG channel gating. This could result from direct binding of genistein to the channel; however, the time course of inhibition is surprisingly slow (>30 s), raising the possibility that genistein exerts its effects indirectly. In support of this hypothesis, we find that ligands that selectively bind to PTKs without directly binding to the CNG channel can nonetheless decrease the effect of genistein. Thus, ATP and a nonhydrolyzable ATP derivative competitively inhibit the effect of genistein on the channel. Moreover, erbstatin, an inhibitor of PTKs, can noncompetitively inhibit the effect of genistein. Taken together, these results suggest that in addition to inhibiting tyrosine phosphorylation of the rod CNG channel catalyzed by PTKs, genistein triggers a noncatalytic interaction between the PTK and the channel that allosterically inhibits gating.

KEY WORDS: cyclic guanosine 5′-monophosphate • protein kinase • rod outer segment • photoreceptor • phosphorylation

INTRODUCTION

Cyclic nucleotide–gated (CNG)[1] channels play a central role in both visual and olfactory sensory transduction. CNG channels are directly activated by the binding of cyclic nucleotides to the channel protein. Recent studies show that the sensitivity of CNG channels to cyclic nucleotides is not fixed, but rather can be modulated by other cellular factors. Transition metals (Ildefonse and Bennett, 1991; Karpen et al., 1993; Gordon and Zagotta, 1995), diacylglycerol analogues (Gordon et al., 1995b), and the local anesthetic tetracaine (Fodor et al., 1997a,b) all bind to rod photoreceptor CNG channels and alter their apparent affinity for cGMP. In addition, Ca^{2+} modulates the sensitivity of CNG channels, but it acts in an indirect manner. Thus, Ca^{2+} decreases the apparent affinity for cGMP (increases the $K_{1/2}$ value) by binding to calmodulin and other unidentified Ca^{2+}-binding proteins, which then interact with the CNG channel protein (Hsu and Molday, 1993; Chen et al., 1994; Gordon et al., 1995a).

We have recently shown that rod CNG channels can also be modulated by tyrosine phosphorylation (Molokanova et al., 1997). Rod CNG channels expressed in *Xenopus* oocytes exhibit a spontaneous increase in cGMP sensitivity after patch excision, and this is reversed by application of ATP. These changes in cGMP sensitivity are blocked by specific inhibitors of protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs), respectively. These results imply that the channel is associated with PTKs and PTPs that remain active for many minutes after patch excision. Additional studies (Molokanova Maddox, Luetje, and Kramer, manuscript submitted for publication) show that mutagenesis of a specific tyrosine in the α subunit of the rod CNG channel greatly reduces modulation, suggesting that the crucial phosphorylation site is located in the channel protein itself.

In this paper, we study the effects on CNG channels of genistein, a broad-spectrum PTK inhibitor isolated from legumes (Akiyama et al., 1987). PTKs have a conserved binding site for ATP and an additional distinct site for binding of their protein substrate (Ullrich and Schlessinger, 1990). Genistein is a competitive inhibitor with respect to ATP in the kinase reaction and a

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[1]*Abbreviations used in this paper:* CNG, cyclic nucleotide–gated; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase.

noncompetitive inhibitor with respect to the peptide substrate, suggesting that genistein specifically interacts with the ATP-binding site. Several other proteins that possess ATP-binding sites are similarly influenced by genistein. Thus, genistein competes for ATP-binding sites on histidine kinase (Huang et al., 1992) and topoisomerase II (Markovits et al., 1989), inhibiting these enzymes, and on the cystic fibrosis transmembrane conductance regulator, potentiating activation of this ion channel (Weinreich et al., 1997; Wang et al., 1998).

This paper shows that genistein inhibits the rod CNG channel, above and beyond its inhibitory effect on tyrosine phosphorylation. The simplest explanation for this inhibition would involve a direct binding of genistein to the CNG channel. However, unlike all of the established direct targets for genistein action, CNG channels do not appear to contain ATP binding sites. Examination of the amino acid sequence of the rod channel α subunit does not reveal conserved ATP-binding domains (Kaupp et al., 1989), and the only known physiological effects of ATP on CNG channels occur through its participation in phosphorylation reactions (Molokanova et al., 1997). Hence, we have considered the possibility that genistein does not bind directly to the channel, but rather acts indirectly by binding to an accessory protein that then binds to the CNG channel. Since our previous studies indicate that the expressed CNG channel is closely associated with PTKs, we considered the possibility that genistein inhibition involves a noncatalytic effect of the PTK. Remarkably, we observe that the effect of genistein on the channel is suppressed by erbstatin, another PTK inhibitor, and by a nonhydrolyzable ATP analogue, suggesting that the receptor for genistein that mediates inhibition of the rod CNG channel is indeed a PTK. Hence, we propose that PTKs affect rod CNG channels in two ways: (a) by allosterically regulating channel gating, and (b) by catalyzing phosphorylation of the channel protein.

MATERIALS AND METHODS

Expression and Recording from Rod CNG Channels Expressed in Xenopus Oocytes

A cDNA clone encoding the bovine rod photoreceptor CNG channel α subunit (Kaupp et al., 1989) was used for in vitro transcription of mRNA, 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 115 mM NaCl, 5 mM EGTA, 1 mM EDTA, and 10 mM HEPES, pH 7.5, NaOH. This also served as the standard bath solution and cGMP perfusion solution. EDTA was not included in solutions containing the Mg^{2+} salt of ATP or its analogues. After formation of a gigaohm seal, inside-out patches were excised and the patch pipette was quickly (<30 s) placed in the outlet of a 1-mm diame-

ter tube for application of cGMP and other agents. We used a perfusion manifold containing up to eight different solutions that is capable of solution changes within 50 ms. Most patches contained 100–200 channels. To calculate the apparent affinity of CNG channels to cGMP, a series of four to five cGMP concentrations was applied to the patch. Steady state cGMP-activated currents were normalized to saturating responses and fit to the Hill equation to determine the apparent affinity ($K_{1/2}$ value) for cGMP.

PTK inhibitors (genistein, erbstatin, lavendustin A) were prepared as concentrated stock solutions in water or DMSO, and aqueous solutions containing the final concentrations were prepared for use as needed. The final concentration of DMSO did not exceed 0.1%, which had no effect on CNG channels or their modulation. Cyclic GMP, ATP, and AMP-PNP were purchased from Sigma Chemical Co., genistein, daidzein, erbstatin (stable analogue), and lavendustin A were purchased from Alexis Corp.

Current responses through CNG channels were obtained with an Axopatch 200A patch clamp (Axon Instruments), digitized, stored, and later analyzed on a 486 PC using pClamp 6.0 software. Membrane potential was held at −75 mV in all experiments. 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: $I/I_{max} = 1/[1 + (K_{1/2}/A)^N]$, using a nonlinear least squares fitting routine (Origin; Microcal Software, Inc.), where A is the cGMP concentration and N is the Hill coefficient. To estimate the K_i for genistein, we used a modified Hill equation: $I_b/I_{max} = [1 − (I_{b(max)}/I_{max})]/[1 + (K_i/B)^N] + I_{b(max)}/I_{max}$, where B is the concentration of blocker, I_b and $I_{b(max)}$ are the currents activated by saturating cGMP in the presence of a given blocker concentration, and a saturating blocker concentration, respectively. Variability among measurements is expressed as mean ± SEM.

Recording from CNG Channels from Rod Outer Segments

Water-phase tiger salamanders (*Ambystoma tigrinum*) maintained in a temperature-controlled aquarium (16°C) on a 12/12 light/dark cycle were used in all experiments at the same time of day. Animals were dark adapted for 1 h and anesthetized in an ice-cold solution containing 1 g/liter of 2-amino benzoic acid for 20 min before decapitation and removal of eyes under dim red light. Eyes were placed in saline containing (mM) 155 NaCl, 2.5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 glucose, and 10 HEPES, pH.7.5. Retinas were removed and gently triturated to yield isolated rods or rod outer segments. Borosilicate glass pipettes (3–5 MΩ) were filled with standard patch solution (see above) and used to obtain excised inside-out patches from the outer segment.

RESULTS

Effect of Genistein and Other PTK Inhibitors on CNG Channels

Rod CNG channels expressed in *Xenopus* oocytes are modulated by changes in tyrosine phosphorylation state, catalyzed by PTKs and PTPs that are native to the oocyte membrane (Molokanova et al., 1997). Phosphorylation lowers the cGMP sensitivity of the channel, whereas dephosphorylation increases cGMP sensitivity. Hence, after a membrane patch is excised from the oocyte, the channels gradually become dephosphorylated, resulting in a dramatic increase in cGMP sensitivity (decrease in $K_{1/2}$). When patches are excised into a solution that

contains ATP, there is little spontaneous change in cGMP sensitivity, apparently because dephosphorylation by tyrosine phosphatases is balanced by phosphorylation by PTKs. We have shown that addition of PTK inhibitors blocks phosphorylation and shifts this equilibrium towards dephosphorylation, resulting in an increase in sensitivity to cGMP (Molokanova et al., 1997).

To learn more about modulation of the rod CNG channel by PTKs, we examined the effects of genistein, a broad-spectrum PTK inhibitor. Fig. 1 A shows that genistein, like two other PTK inhibitors (lavendustin A and erbstatin), causes an increase in cGMP sensitivity (decrease in $K_{1/2}$) when applied in the presence of ATP. In the absence of ATP, genistein, like the other

inhibitors, had no effect on cGMP sensitivity, whether it was applied at 10 (Fig. 1 B) or 1 min ($n = 12$; data not shown) after patch excision. Presumably, when ATP is absent, the PTK is not enzymatically active, precluding the inhibitors from affecting the phosphorylation state, and hence the cGMP sensitivity of the channels.

In contrast to their effects on cGMP sensitivity, lavendustin A and erbstatin have no effect on the current elicited by saturating cGMP. Thus, both in the presence (Fig. 1 C) and absence (Fig. 1 D) of ATP, these agents had no effect on the maximal current. However, genistein does have an effect. Addition of 10 μM genistein inhibits the maximal current by ∼50% in the presence and by 85% in the absence of ATP. Thus
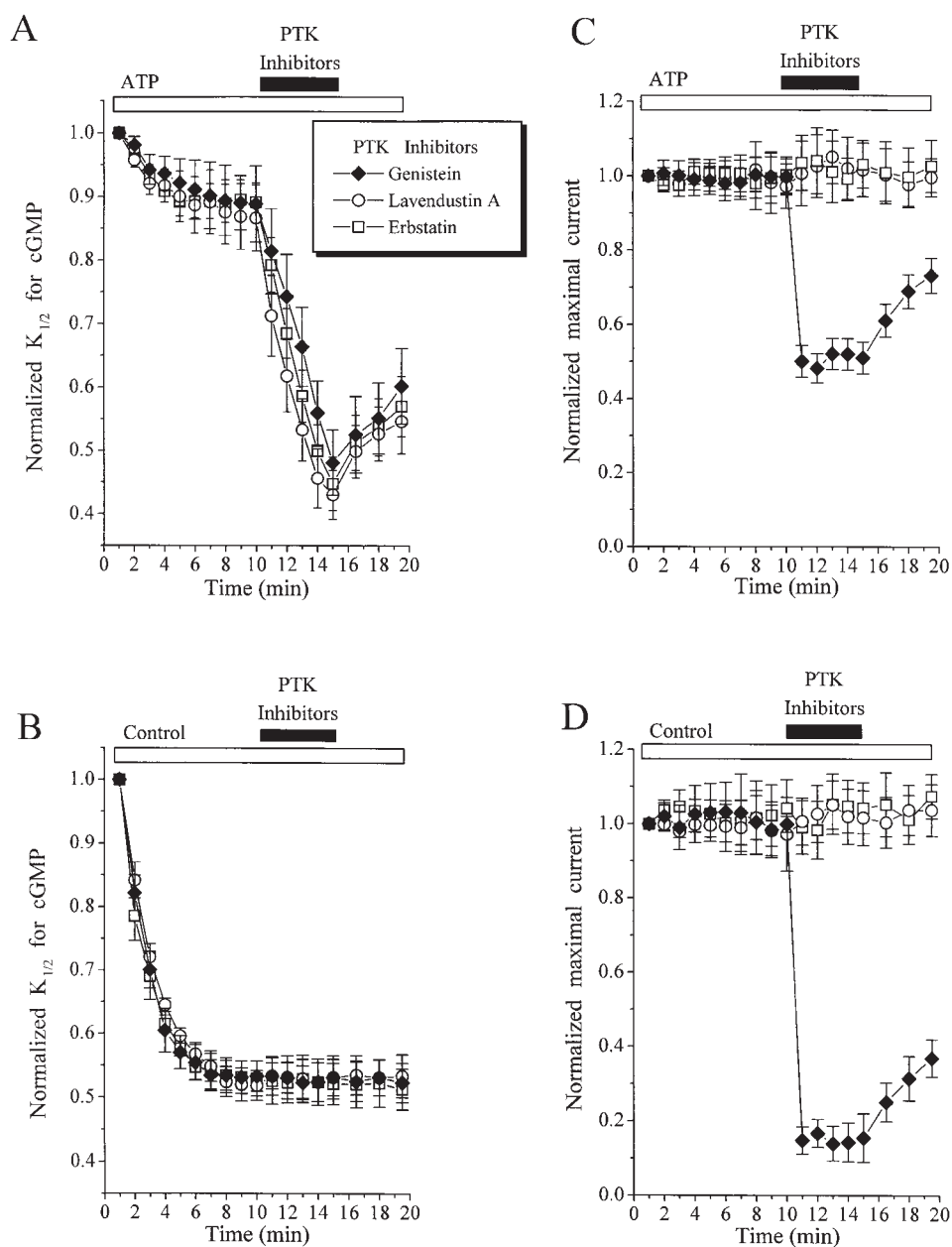

FIGURE 1. Comparison of the effects of PTK inhibitors on the apparent affinity for cGMP ($K_{1/2}$) and maximal current (I_{max}) through rod CNG channels. (A) Effect of PTK inhibitors on the $K_{1/2}$ for cGMP in the presence of ATP. Note that all three inhibitors reduce the $K_{1/2}$ for activation by cGMP. (B) None of the inhibitors affected the $K_{1/2}$ for cGMP in the absence of ATP. The effect of PTK inhibitors on $K_{1/2}$ is consistent with inhibition of a phosphorylation reaction. (C) Effect of PTK inhibitors on the maximal current elicited by saturating (2 mM) cGMP in the presence of ATP. Note that genistein, but not the other inhibitors, reduced I_{max}. (D) Genistein inhibition of maximal current is even larger in the absence of ATP. The effect of genistein on I_{max} is inconsistent with inhibition of a phosphorylation reaction. PTK inhibitor concentrations used in these experiments were (μM) 10 genistein, 10 lavendustin A, and 25 erbstatin. Data represents mean ± SEM ($n = 8$–12 patches for each protocol).

genistein more effectively inhibits maximal current in the absence than in the presence of ATP. These observations indicate that genistein, distinct from the other inhibitors, must do more than simply prevent tyrosine phosphorylation.

Genistein Preferentially Inhibits Closed CNG Channels

We more closely examined the effects of genistein on the maximal current of rod CNG channels expressed in *Xenopus* oocytes. Excised inside-out patches typically contained hundreds of CNG channels that could be saturated by application of 2 mM cGMP, generating large currents that reach steady state within 100 ms (Fig. 2 A). In the presence of 100 μM genistein, the steady state current elicited by cGMP was much smaller (40% of control) and activated over a more complex time course (Fig. 2 B). A fraction of the total current (4.5%), labeled "residual current," activates over the normal rapid time course. The remaining current activates very slowly, with a time course that could be fit with a single exponential with a time constant of 10.6 s at 100 μM genistein. The observation that pretreatment with genistein slows activation elicited by cGMP suggests that genistein can interact with the CNG channel in its closed state.

Genistein can also inhibit the rod CNG channel after it has been activated by cGMP (Fig. 2 C). In fact, the steady state level of inhibition is the same regardless of the order of application of genistein and cGMP. Genistein slowly suppresses the saturating current with a time course that can be fit with a single exponential with a time constant of 7.3 s. The inhibition of closed CNG channels also develops slowly (data not shown), and the time course can be fit with a single exponential of 8.9 s. The slow time constant of genistein inhibition suggests that rather than interacting directly with the channel, genistein may bind to a distinct protein that can allosterically influence channel gating. Daidzein, an inactive analogue of genistein (Akiyama et al., 1987), has no effect on rod CNG channels (Fig. 2 D). Moreover, we observed no effect of genistein or daidzein on rat olfactory CNG channels (Dhallan et al., 1990) expressed in *Xenopus* oocytes ($n = 10$ patches).

To investigate the interaction between genistein and closed CNG channels, patches were pretreated with various concentrations of genistein before application of cGMP, as illustrated in Fig. 2 B. We found that the magnitude of the residual current is inversely related to the genistein concentration (Fig. 3 A). Therefore, the residual current appears to reflect the fraction of the current not blocked by genistein. At saturating concentrations of genistein (>100 μM), the residual current was nearly eliminated, indicating complete inhibition of closed channels by genistein.

In contrast to its effect on closed channels, genistein was less effective at inhibiting CNG current once it had been activated by cGMP (Fig. 3 B). Even at high con-
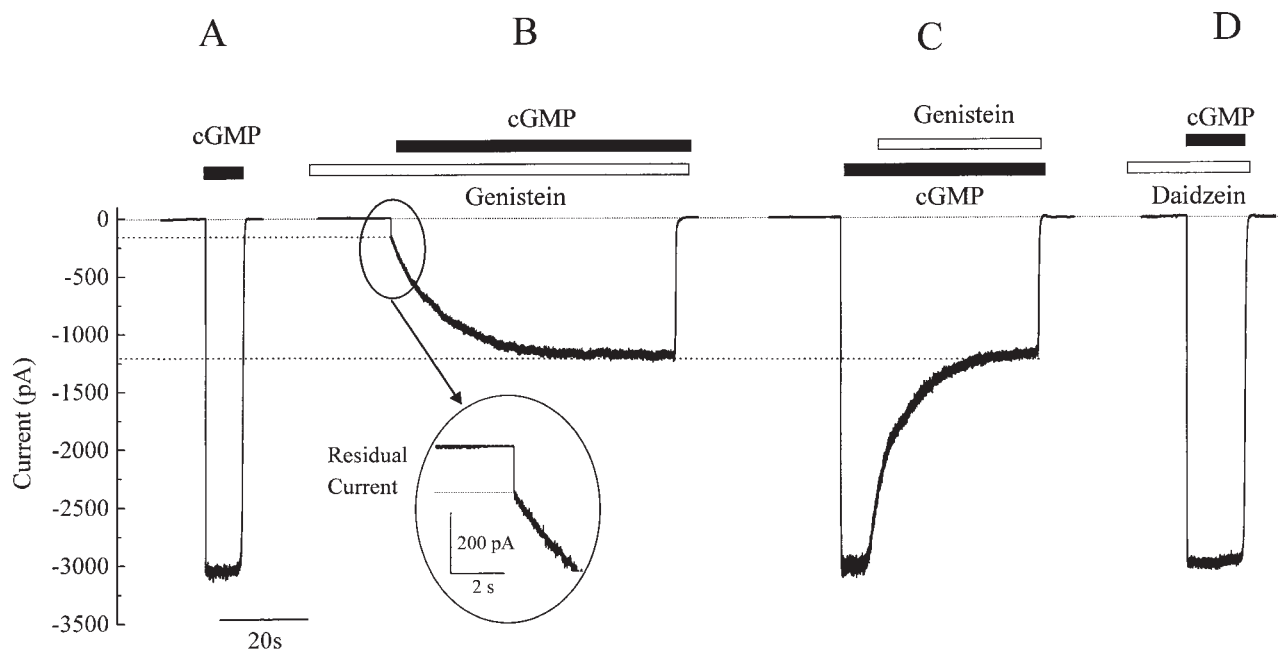

FIGURE 2. Genistein slows activation and reduces maximal current through rod CNG channels. (A) Response of rod CNG channels in an excised patch to saturating (2 mM) cGMP. (B) Response to saturating cGMP after preexposure to 100 μM genistein for 1 min. Inset shows residual current not inhibited by genistein. (C) Partial inhibition of maximal CNG current activated by saturating cGMP. (D) The inactive genistein analogue daidzein has no effect on the maximal current.

A

B

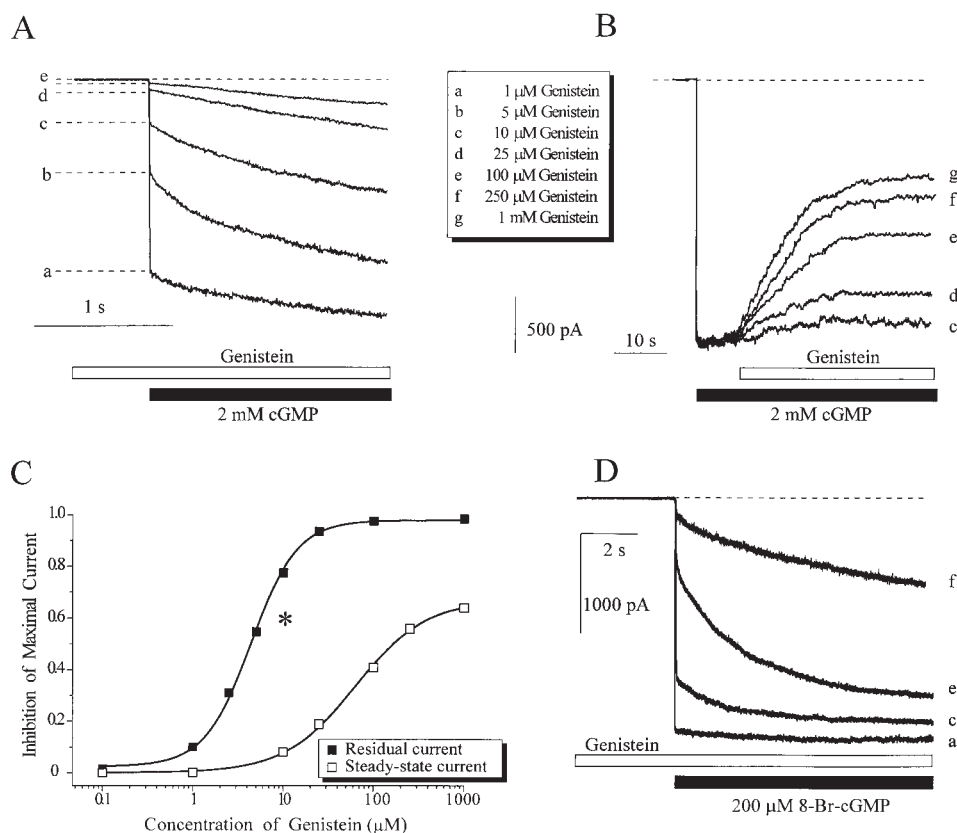| a	1 μM Genistein
b	5 μM Genistein
c	10 μM Genistein
d	25 μM Genistein
e	100 μM Genistein
f	250 μM Genistein
g	1 mM Genistein

C

D

FIGURE 3. Closed channels are more sensitive to genistein inhibition than are activated channels. (A) Inhibition of closed channels by preexposure to various concentrations of genistein for 1 min. Closed channels were activated by application of 2 mM cGMP. Residual currents at five different genistein concentrations are indicated by dotted lines, and the genistein concentrations are indicated by letter to the right of each trace. In A, B, and D, the letter refers to the genistein concentration key. Note that the amplitude of the residual current is inversely proportional to the genistein concentration. (B) Inhibition of steady state CNG current, maximally activated by saturating cGMP (2 mM). The effects of five different genistein concentrations are indicated by letter to the right of each trace. (C) Dose–inhibition curves of the effect of genistein on closed (residual current as in A) and maximally activated channels (steady state current, as in B). Continuous curves show fits of the data to the Hill equation (see MATERIALS AND METH-ODS). The asterisk depicts inhibition of current activated by saturating cGMP, after preexposure to genistein (10 μm) in conjunction with a saturating concentration of cAMP (20 mM). Note that preexposure to cAMP has only a minor effect on genistein inhibition ($n = 6$ experiments). (D) Inhibition of native CNG channels from salamander rod outer segments after preexposure to various concentrations of genistein for 1 min. The effects of four different genistein concentrations are indicated by letter to the right of each trace.

centrations of genistein (e.g., 1 mM), inhibition of steady state CNG current was incomplete. A comparison of the effect of genistein on the nonactivated CNG channels and the fully activated CNG current for this experiment is shown in Fig. 3 C. Inhibition of the closed channel by genistein was complete and had an apparent K_i of 4.1 μM, which was determined by measuring the residual current. Inhibition of the fully activated current by genistein was incomplete, with a K_i of 72.5 μM, which was determined by measuring the steady state current in the presence of genistein. These results suggest that genistein has a much higher affinity for the closed than for the open CNG channel.

Activation of the rod CNG channel by cGMP involves distinct ligand binding and channel gating steps. Like cGMP, cAMP binds to the rod channel, but it is much less effective at causing channel opening. To distinguish whether the reduced apparent affinity of genistein in the presence of cGMP is due to bound ligand per se, or to the conformational changes associated with channel opening, we investigated the effect of genistein on channels exposed to saturating cAMP. In these experiments, genistein was applied to the patch in conjunc-

tion with 20 mM cAMP, which should have saturated the cyclic nucleotide binding sites. Then, the cAMP was rapidly replaced with cGMP to measure the residual current. Our results showed that saturating cAMP had only a small effect on the apparent affinity of genistein, much less than that observed with saturating cGMP (Fig. 3 C).

We also examined the effect of genistein on native CNG channels from rod outer segments from salamander retina. As we observed for the expressed channel, application of 100 μM genistein on inside-out patches containing native channels reduced the magnitude of the cGMP-activated current and slowed the activation kinetics (Fig. 3 D). Thus, even though the native channel contains α and β subunits (Kaupp et al., 1989; Chen et al., 1993; Körschen et al., 1995), it is inhibited by genistein similarly to the inhibition of the expressed channel, which contains only α subunits.

Our results suggest that genistein is not a competitive inhibitor of the cyclic nucleotide binding site on the rod channel. The observation that saturating concentrations of genistein incompletely inhibit the steady state CNG current is consistent with this conclusion.

Moreover, the extent of inhibition does not decrease as cGMP concentration is increased beyond the level required to saturate the channel. Hence, inhibition is the same in the presence of 2 and 20 mM cGMP, both for low (10 μM) and high (100 μM) concentrations of genistein (Fig. 4 A). Thus, rather than interfering with cyclic nucleotide binding, genistein appears to inhibit the rod CNG channel by allosterically affecting channel gating. Unlike tetracaine, which elicits a voltage-dependent inhibition of CNG channels by binding within the pore of the channel, genistein inhibits the rod channel in a voltage-independent manner (Fig. 4 B).

Adenosine Triphosphates Decrease Genistein Inhibition

The inhibition of CNG current by genistein described above occurs in the absence of ATP and therefore cannot involve a phosphorylation reaction. However, we noticed that ATP reduces the ability of genistein to inhibit the channels. Fig. 5 A shows that ATP shifts the dose–response curve for genistein inhibition to the right, such that a higher concentration of genistein is required to inhibit CNG current activation by cGMP. Analysis of these curves (Fig. 5 B) shows that in the ab-

sence of ATP, the K_i for genistein inhibition of CNG current activation by cGMP was 4.1 ± 0.7 μM, whereas in the presence of 1 mM ATP, the K_i was shifted to a higher value (10.6 ± 1.3 μM). The Hill coefficient for genistein inhibition was the same in the presence and absence of ATP (2.0 ± 0.2 and 1.9 ± 0.2, respectively). To determine whether the ability of ATP to alter the K_i for genistein involves a phosphorylation reaction, we used a nonhydrolyzable ATP analogue adenylylimidodiphosphate (AMP-PMP), which cannot be used as a substrate in phosphorylation reactions, but which does bind to ATP binding sites in proteins. Like ATP, AMP-PNP reduced the ability of genistein to inhibit the CNG current. Addition of AMP-PNP shifted the K_i to 9.3 ± 0.9 μM without altering the Hill coefficient.

To further test whether the phosphorylation state of the CNG channel is important for determining the K_i for genistein, we examined CNG channels at various times after patch excision from the oocyte. During first 10 min after patch excision, the sensitivity of rod CNG channels to cGMP changes dramatically, resulting from tyrosine dephosphorylation (Molokanova et al., 1997), probably of the CNG channel protein itself (Molokanova, Maddox, Luetje, and Kramer, manuscript sub-
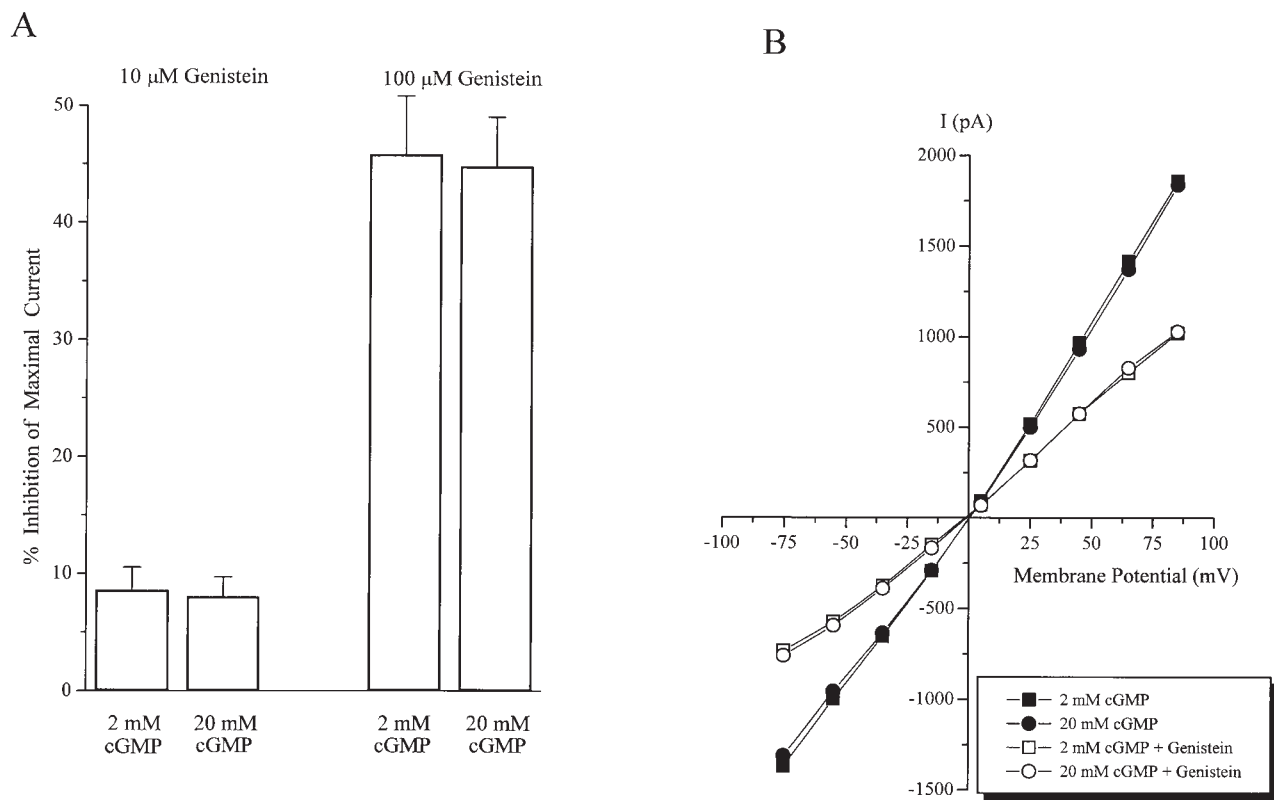

FIGURE 4. Genistein inhibition is not competitive with respect to cGMP and is not voltage dependent. (A) Inhibition of steady state CNG current by 10 and 100 μM genistein. Note that the percent inhibition of the maximal current does not change when cGMP is increased from 2 to 20 mM. (B) Current–voltage curves of CNG current activated by saturating cGMP (2 and 20 mM) in the presence and absence of 100 μM genistein. $n = 5$–8 patches for each experiment.
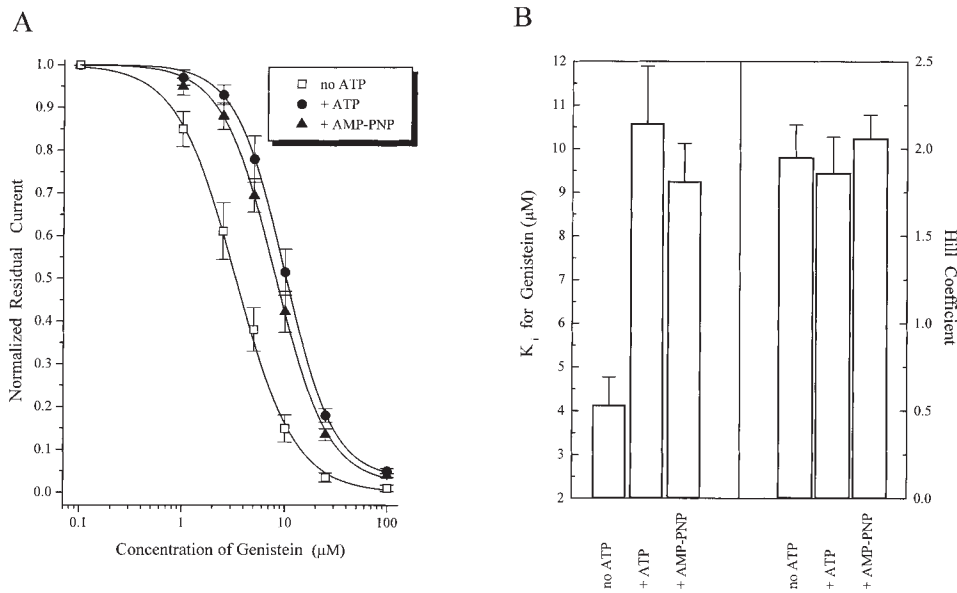
A

B

FIGURE 5. Competitive effects of ATP and AMP-PNP on genistein inhibition. (A) Dose–inhibition curves for genistein alone ($n = 13$), genistein plus 200 μM ATP ($n = 7$), and genistein plus 200 μM AMP-PNP. Continuous curves show fits to the Hill equation. (B) Histogram showing K_i and Hill coefficient values for genistein inhibition curves. Data represents mean ± SEM for all individual experiments included in A. Note that ATP and AMP-PNP increase the K_i for genistein without affecting the Hill coefficient.

mitted for publication). Hence, at 1 min after excision, a patch is likely to have a greater proportion of phosphorylated channels than at 10 min after excision. Fig. 6 shows that the K_i values for genistein at 1 and 10 min after excision do not differ, suggesting that sensitivity of the channels to genistein is not strongly dependent on phosphorylation.

Since it appears that these effects of ATP on genistein inhibition do not involve changes in phosphorylation, an alternate possibility is that ATP competes with genistein for binding to a common site. We propose that the competition between ATP and genistein concerns the ATP binding site on the PTK. According to this model, genistein, rather than having a direct effect on the rod CNG channel, indirectly regulates gating of the channel by interacting with this tightly associated protein.

Erbstatin Decreases Genistein Inhibition

To further test whether genistein inhibition of the rod CNG channel involves an interaction with a PTK, we used the specific PTK inhibitor erbstatin. Studies have shown that erbstatin competes with peptide substrates of PTKs, suggesting that erbstatin binds to a site on the enzyme that normally binds to target proteins (Umezawa et al., 1986; Imoto et al., 1987). If the inhibition of the rod channel by genistein is an indirect effect mediated by a PTK, then erbstatin, like ATP, should reduce the effect of genistein.

Fig. 7 A shows the effect of erbstatin on genistein inhibition of closed rod CNG channels. In this experiment, genistein was applied on closed CNG channels in the presence or absence of erbstatin, and the channels were subsequently activated by cGMP. Without erbstatin present, 100 μM genistein inhibited closed chan-

nels by >95%, resulting in a residual current that was <5% the magnitude of the maximal CNG current. With 100 μM erbstatin present, genistein inhibited closed channels by ∼80%, with a residual current 20% of maximal. Group data showing inhibition caused by different concentrations of genistein with and without erbstatin is shown in Fig. 7 B. Below saturation, increasing the concentration of genistein from 10 to 100 μm resulted in increasing inhibition, which was partly suppressed by erbstatin. However, unlike the effect of ATP, the inhibitory effect of erbstatin could not be overcome by adding additional supersaturating concentrations of genistein. Thus, increasing the concentration of genistein 10-fold further from 100 to 1,000 μM did not elicit more inhibition in the presence of erbstatin, indicating that the interaction between erbstatin and genistein is noncompetitive.

The ability of erbstatin to depress the action of genistein is more dramatic when the CNG channels are fully activated by cGMP (Fig. 8 A). Genistein inhibition of fully activated CNG current was reduced from 45.8 ± 5.7 to 5.4 ± 1.7% by pretreatment with 100 μM erbstatin (Fig. 8 B). The suppression of genistein inhibition by erbstatin reversed fully after erbstatin was removed from the superfusion solution.

DISCUSSION

Rod CNG channels expressed in *Xenopus* oocytes appear to be associated with PTKs and PTPs that are constitutively active in excised patches (Molokanova et al., 1997). Phosphorylation and dephosphorylation catalyzed by these enzymes alter the apparent affinity of the channels for cGMP. Recent studies strongly suggest that the α subunit channel protein itself is the substrate
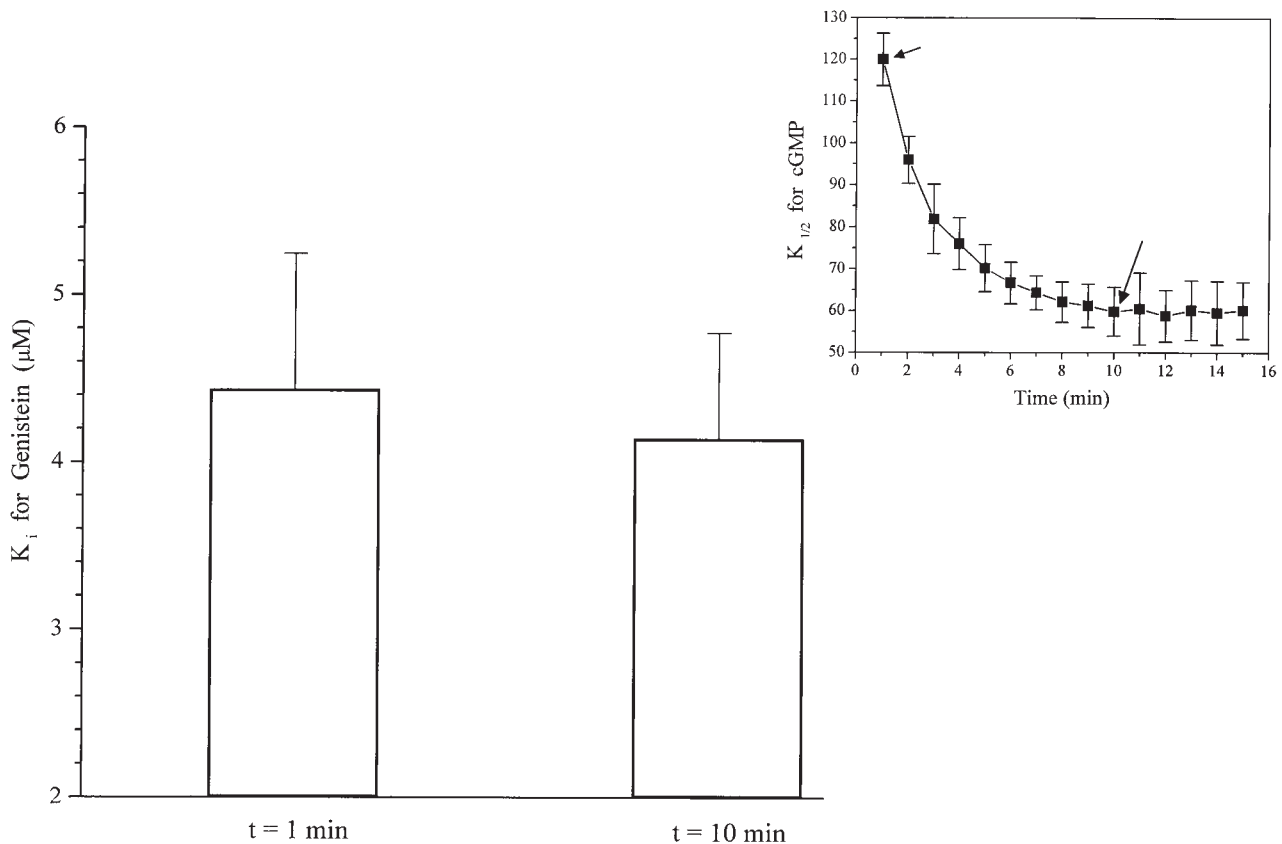
FIGURE 6. Effect of genistein on the rod CNG channels at different times after patch excision. Genistein was applied on closed rod CNG channels at different times after excision from the oocyte, and residual current was measured after application of cGMP. Bar graph represents mean ± SEM for five patches at 1 and 10 min after excision. During the first 10 min after excision, the $K_{1/2}$ for cGMP activation gradually declines (see inset), resulting from tyrosine dephosphorylation (Molokanova et al., 1997). The bar graph shows that channels are equally sensitive to genistein, independent of phosphorylation state.

for phosphorylation, with a specific tyrosine residue (Y498) in the cytoplasmic carboxyl domain being a particularly important phosphorylation site (Molokanova, Maddox, Luetje, and Kramer, manuscript submitted for publication). Ion channels often exist in tight association with protein kinases and phosphatases, such that complexes of these proteins remain intact even after reconstitution into artificial lipid bilayers (Reinhart and Levitan, 1995). A variety of channels have been found to be biochemically and functionally associated with PTKs and PTPs (Prevarskaya et al., 1995; Holmes et al., 1996; Wang and Salter, 1994; Yu et al., 1997). These accessory proteins can modulate ion channel activity, both by catalyzing changes in phosphorylation state (Wang and Salter, 1994; Aniksztejn et al., 1997) and by allosterically regulating channel gating (Holmes et al., 1996).

Our results suggest that genistein affects rod photoreceptor CNG channels expressed in *Xenopus* oocytes in two ways. First, like other PTK inhibitors, genistein prevents tyrosine phosphorylation of rod CNG channels, allowing the channels to become dephosphorylated and more sensitive to cGMP. Second, unlike other PTK

inhibitors, genistein slows activation and reduces the current elicited by saturating cGMP. This second action of genistein does not require ATP, and thus cannot involve inhibition of phosphorylation by PTKs.

The observation that genistein slows activation and reduces the maximal cGMP-elicited current suggests that genistein stabilizes the CNG channel in a closed state. At first glance, genistein appears to inhibit the rod CNG channel through a mechanism similar to that employed by the local anesthetic tetracaine (Fodor et al., 1997a). Tetracaine preferentially binds to the closed channel with an apparent affinity of ∼10 μM. However, tetracaine block is rapid (<100 ms) and voltage-dependent. Tetracaine is positively charged and mutagenesis studies suggest that block involves a direct interaction with a specific glutamate residue in the pore of the rod channel (Fodor et al., 1997b). In contrast, genistein inhibition is slow and voltage independent. Moreover, our evidence suggests that rather than binding directly to the channel, the genistein inhibition is indirect, involving an accessory protein that allosterically affects the CNG channel.
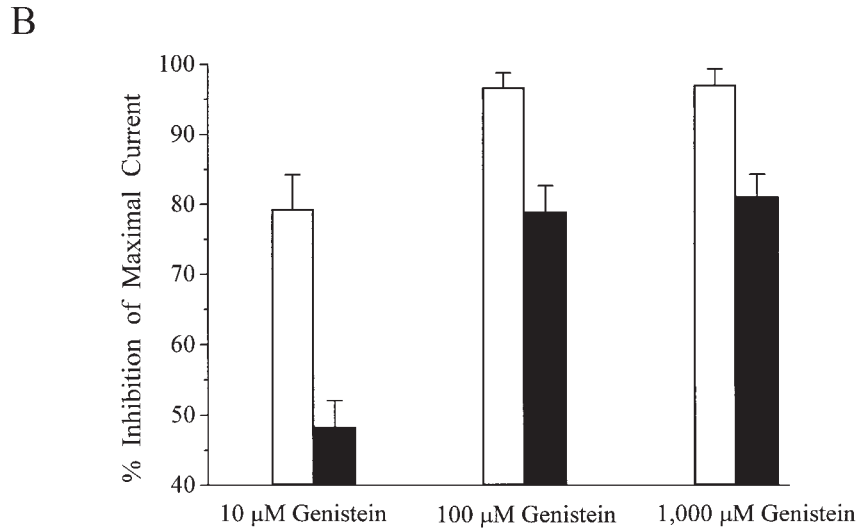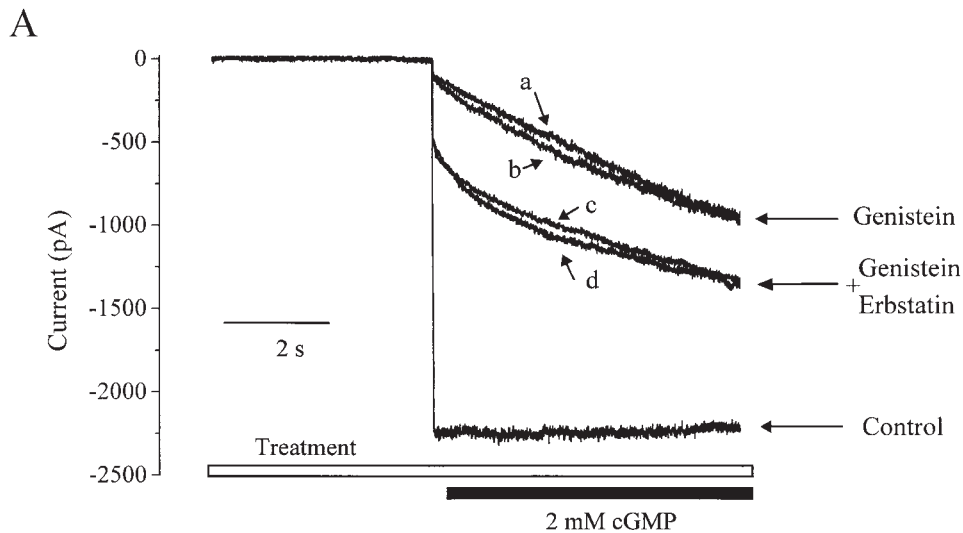
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B

FIGURE 7. Effect of erbstatin on genistein inhibition of closed channels. (A) CNG current activated by saturating (2 mM) cGMP alone (control) or after preexposure to 100 (a and c) or 1,000 (b and d) μM genistein. Preexposure to 100 μM erbstatin, as indicated, partially inhibits the effect of genistein. (B) Summary data for genistein inhibition alone (open bars) and with erbstatin (filled bars). $n = 5$ for each condition.

The evidence that genistein inhibits the rod channel by binding to an accessory protein is indirect, but nonetheless compelling. First, the effect of genistein is inhibited in a competitive manner by ATP and AMP-PNP. There is no evidence that CNG channels possess ATP binding sites, and ATP itself has no noticeable effect on CNG channels, other than being a substrate in phosphorylation reactions. Thus, if PTK inhibitors are present, ATP has no effect on the apparent affinity or maximal current elicited by cGMP (Molokanova et al., 1997), and does not alter the kinetics of activation or deactivation (our unpublished observations). In contrast, CNG channels expressed in *Xenopus* oocytes are closely associated with PTKs and, like all kinases, these proteins do possess ATP binding sites. Moreover, genistein is known to be a competitive inhibitor of these binding sites (Akiyama et al., 1987).

Second, the effect of genistein is decreased in a noncompetitive manner by erbstatin. Again, application of erbstatin in the absence of ATP fails to reveal any direct effect on the rod channel. In contrast, erbstatin is a well characterized inhibitor of PTKs. Studies have shown that erbstatin is a competitive inhibitor with respect to the substrate binding site on PTKs, whereas it has properties of both a competitive and a noncompetitive inhibitor with respect to the ATP binding site (Imoto et al., 1987; Posner et al., 1994). Our observation that erbstatin reduces genistein inhibition of CNG channels noncompetitively suggests that these two agents bind to nonoverlapping sites, consistent with previous observations about the effects of these inhibitors on PTKs.

Third, the onset of genistein inhibition for both the closed and fully activated rod CNG channel is slower
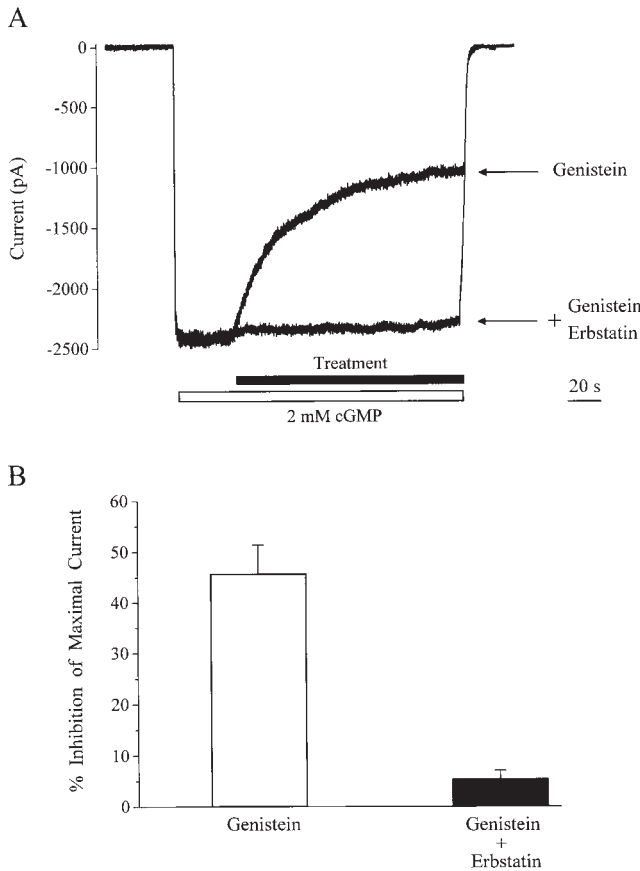
FIGURE 8. Effect of erbstatin on genistein inhibition of maximally activated channels. (A) Genistein (100 μM) inhibition of CNG current activated by 2 mM cGMP in the absence and presence of 100 μM erbstatin. (B) Summary data for genistein inhibition alone (open bars) and with erbstatin (closed bars). $n = 5$–8 for each condition.

Finally, genistein inhibition of CNG channels is correlated with the effect of genistein on PTKs. The apparent affinity of genistein for the rod channel (5–80 μM) is similar to the K_i for inhibition of PTKs (2.6–18 μM; Akiyama et al., 1987; Umezawa et al., 1990). The rod CNG channel can be modulated by PTK and inhibited by genistein, whereas the rat olfactory CNG channel (Dhallan et al., 1990) expressed in oocytes exhibits neither effect (our unpublished observations). Closed rod CNG channels are more susceptible to modulation by tyrosine phosphorylation (Molokanova, Maddox, Luetje, and Kramer, manuscript submitted for publication) and are more sensitive to inhibition by genistein, than are activated channels.

Although many of the physiological effects of genistein, including the inhibition of rod CNG channels reported here, appear to involve PTKS, there have
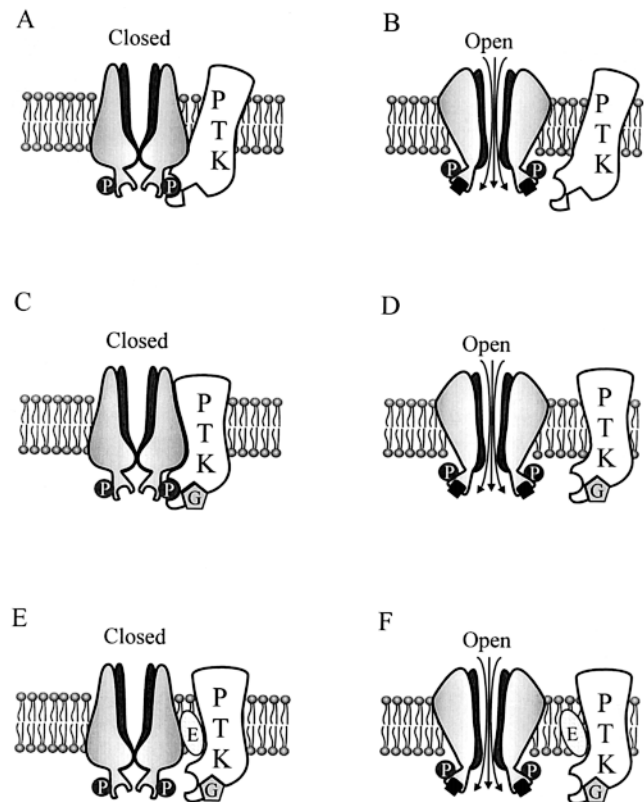

FIGURE 9. Schematic diagram of genistein inhibition. (A) The closed channel is loosely associated with a PTK that can catalyze phosphorylation, whereas the open channel (B) is not. Genistein stabilizes the interaction between the PTK and the closed channel, causing inhibition (C), whereas PTK remains unbound from the open channel (D). Erbstatin destabilizes the interaction between the PTK and the channel, decreasing genistein inhibition of the closed channel (E) and eliminating genistein inhibition of the open channel (F). Genistein is depicted as a pentagram labeled G, erbstatin is depicted as an oval labeled E, P represents tyrosine phosphorylation sites on the channel, and the black squares represent ligand (cGMP).

than would be predicted from the apparent affinity and the slow recovery rate. We find that the rod CNG channels require >60 s to recover from genistein inhibition (our unpublished observations). If the on rate of genistein inhibition were diffusion limited, as is the case for many channel blockers and allosteric regulators, an apparent affinity of genistein of 10 μM would suggest that the inhibition rate constant should be <100 ms. The rate of genistein inhibition is much slower than this value, consistent with inhibition resulting from an interaction between the channel and a second protein (a PTK) in the membrane. Despite there being a very large difference in the apparent affinity and magnitude of genistein inhibition of closed versus activated channels, the kinetics of inhibition are similar. This observation is consistent with the hypothesis that the PTK that mediates genistein inhibition diffuses in the membrane, and the on rate of inhibition is determined by the collision rate between this protein and the CNG channel, regardless of the state of the channel.

been reports of genistein actions that may not involve PTKs. It has been proposed that genistein directly blocks voltage-gated Na^+ channels in neurons (Paillart et al., 1997) and smooth muscle (Kusaka and Sperelakis, 1996), and L-type Ca^{2+} channels in heart (Chiang et al., 1996; Yokoshiki et al., 1996), in part because the blocking effect is rapid in onset and reversal and is mimicked by daidzein, which is an inactive analogue of genistein that does not inhibit PTKs. In contrast, the inhibition of rod CNG channels that we have observed is slow and is not mimicked by daidzein.

A schematic diagram depicting the action of genistein on closed and open channels is shown in Fig. 9. The closed channel is loosely associated with a PTK, which can phosphorylate specific tyrosine residues in the cyclic nucleotide binding domain (Fig. 9 A), whereas PTK cannot phosphorylate the open channel (Molokanova, Maddox, Luetje, and Kramer, manuscript submitted for publication) (Fig. 9 B). Genistein (G) indirectly affects CNG channels by binding to the PTK, causing a conformational change that stabilizes or alters its interaction with the closed channel (Fig. 9 C). The apparent affinity and efficacy of genistein inhibition are higher for the closed channel than for the open channel (Fig. 9 D). The notion that the PTK more easily dissociates from the open channel is consistent with our findings showing that activation of the channel makes it much less susceptible to tyrosine

phosphorylation. Erbstatin binds to the substrate binding site on PTKs, destabilizing the interaction between the PTK and the channel. Hence, in the presence of erbstatin, genistein inhibition is reduced in closed channels (Fig. 9 E) and eliminated in open channels (Fig. 9 F). Despite their ability to block the catalytic activity of PTKs, these effects of genistein are apparently noncatalytic, because they do not require ATP. Moreover, phosphorylated and dephosphorylated channels are equally sensitive to genistein inhibition.

In conclusion, our data suggests that when PTKs are bound to genistein, they can allosterically influence gating of the rod channel, independently of their role in catalyzing phosphorylation. Other PTKs have been shown to noncatalytically regulate gating of ion channels (Holmes et al., 1996; Zeng et al., 1998). Our finding that genistein inhibits rod CNG channels, not only in *Xenopus* oocytes but also in rod outer segments, raises the possibility that the native rod CNG channel is associated with native rod PTKs. Indeed, our previous studies have shown that the native rod CNG channel is modulated by tyrosine phosphorylation (Molokanova et al., 1997). It is possible that genistein is not the only factor capable of triggering noncatalytic inhibition of rod CNG channels; perhaps there are native ligands that have a similar effect. Hence, genistein inhibition of rod CNG channels may have a physiological correlate that is important for photoreceptor function.

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