

Picrotoxin Accelerates Relaxation of GABA_C Receptors

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ABSTRACT

Picrotoxin is a plant alkaloid that is often used to block the activity of neuronal GABA and glycine receptors. However, the mechanism by which picrotoxin inhibits these receptors is still in debate. In this study, we investigated the picrotoxin inhibition on perch- ρ subunits expressed heterologously in *Xenopus laevis* oocytes, and on native GABA_C receptors of perch bipolar cells. Both competitive and noncompetitive mechanisms were observed for picrotoxin inhibition of the GABA_C receptor. In oocytes expressing the $\rho 1A$ subunit, terminating simultaneously the coapplication of GABA and picrotoxin induced a large rebound of membrane current. In addition, picrotoxin significantly accelerated the kinetics of GABA responses, particularly in the relaxation (offset) phase of GABA currents. Both

current rebound and the large acceleration of GABA relaxation were unique to picrotoxin inhibition and were not observed with the competitive antagonist (1,2,5,6-tetrahydropyridin-4-yl)-methylphosphinic acid or the allosteric modulator zinc. The change in kinetics induced by picrotoxin was also observed on receptors formed by other GABA ρ subunits, as well as on the GABA_C receptors of retinal bipolar cells. Based on these observations, we proposed a model in which picrotoxin binds to the GABA_C receptor in both channel open and closed states. Overall, this model provides a remarkably good approximation of the experimental findings we observed for picrotoxin inhibition of GABA_C receptors. These results support an allosteric mechanism of picrotoxin inhibition of ligand-gated chloride channels.

GABA and glycine are the main inhibitory neurotransmitters in the nervous system. GABA activates three types of receptors (GABA_A, GABA_B, and GABA_C) on neuronal membranes; GABA_A and GABA_C (or GABA_{AO}) receptors function as anion channels that are permeable to chloride, whereas GABA_B receptors are metabotropic receptors that are linked through second messengers to potassium or calcium channels (Barnard et al., 1998; Costa, 1998; Qian and Ripps, 2001). Receptors activated by glycine also gate chloride channels, and thus both GABA and glycine receptors provide mechanisms for fast synaptic inhibition in neuronal tissues (Moss and Smart, 2001).

It has been recognized for decades that picrotoxin, a plant alkaloid, inhibits ligand-gated chloride channels (Bormann, 1988; Pribilla et al., 1992; Macdonald and Olsen, 1994). However, its binding site on these receptors has not been identified, and the inhibitory mechanism is still debated (Macdonald and Olsen, 1994; Luddens et al., 1995; Zhorov and Bregestovski, 2000). There are two hypotheses proposed to explain picrotoxin inhibition of GABA receptors. One hypothesis suggests that picrotoxin acts as a noncompetitive channel blocker for these receptors. This concept is supported by the fact that picrotoxin

inhibits various anion-selective, ligand-gated channels independent of the receptor type. For example, besides GABA and glycine receptors, picrotoxin also effectively inhibits invertebrate chloride currents gated by glutamate (Cleland, 1996), acetylcholine (Yarowsky and Carpenter, 1978), and dopamine (Magoski and Bulloch, 1999). In addition, Inoue and Akaike (1988) noticed noncompetitive and apparent use-dependent qualities of picrotoxin inhibition in GABA_A receptor-mediated responses on frog sensory neurons. Support for picrotoxin as a channel blocker is also provided by the finding that mutations in the intracellular half of the M2 segment (channel forming domain) of the subunit protein significantly affect the sensitivity of GABA receptors to picrotoxin (Gurley et al., 1995; Wang et al., 1995b; Zhang et al., 1995; Dibas et al., 2002). Furthermore, an anion-selective channel formed by a synthetic four-helix bundle protein composed of M2 segments from the glycine receptor can also be blocked by picrotoxin (Reddy et al., 1993).

However, there are other effects of picrotoxin that cannot be explained by a simple channel-block mechanism. Single channel activity of GABA_A receptors recorded from rat sympathetic neurons suggests picrotoxin binds preferentially to agonist-bound forms of the receptor and stabilizes the channel in closed states (Newland and Cull-Candy, 1992). In addition, it has been demonstrated that there is a competitive component of picrotoxin inhibition of homomeric $\alpha 1$ glycine receptors (Lynch et al., 1995) and GABA_C receptors (Qian and Dowling, 1994; Wang et al., 1995b; Zhang et al.,

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ABBREVIATIONS: TPMPA, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid; G_nR*, open channel; G_nR, closed channel.

1995). These results suggest an allosteric mechanism for picrotoxin inhibition.

Although picrotoxin is widely used to inhibit neuronal GABA receptor activity, its effects on the kinetics of GABA-elicited responses have not been fully explored. No study, to our knowledge, has examined the effects of picrotoxin on relaxation of GABA-elicited whole-cell membrane currents. Here, we investigate the mechanism of picrotoxin inhibition using the GABA_C receptor as a model system. Although the nomenclature is still in debate (Barnard et al., 1998), GABA_C receptors referred to in this article are defined as those receptors formed at least in part by GABA_ρ subunits and the bicuculline- and benzodiazepine-insensitive GABA receptors such as those present on retinal neurons (Qian and Ripps, 2001). These GABA_C receptors are sensitive to picrotoxin inhibition, and their current responses display very slow response kinetics, with offset time constants on the order of seconds (Qian and Ripps, 2001). This unique feature makes the GABA_C receptor an ideal model system for investigating the action of picrotoxin on response kinetics. We found evidence that picrotoxin greatly accelerates the GABA response elicited from various types of GABA_C receptors. This is in contrast to the competitive antagonist (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA), which has no effect, and the allosteric modulator zinc, which only slightly accelerates the GABA responses. Fitting the recorded responses to a mathematic model, our data indicate that picrotoxin could bind the receptor in both channel open and closed conformations. These results support an allosteric mechanism for picrotoxin inhibition of GABA_C receptors.

Materials and Methods

All experimental procedures adhered to the Guidelines for the Care and Use of Laboratory Animals formulated by the National Institutes of Health and were approved by the Animal Care Committee of the University of Illinois at Chicago College of Medicine.

Subunit Expression and Current Recording from *Xenopus laevis* Oocytes. Gravid *X. laevis* females were purchased from Xenopus I (Dexter, MI) and housed in climate-controlled, light-cycled rooms at the University of Illinois at Chicago Biological Resources Laboratory. Ovarian lobes were removed under surgical anesthesia (0.02% 3-aminobenzoic acid ethyl ester; Tricaine) and incubated with constant agitation for 1 h in a calcium-free Ringer's solution containing collagenase (2 mg/ml) to remove the follicular layer. Stage V and VI oocytes were selected and kept at 16°C in a Ringer's solution containing 100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 10 mM glucose, at pH 7.4. Plasmids containing perch- ρ subunits that were linearized with BamHI, and capped mRNAs were synthesized with SP6 RNA polymerase using the mMessage mMachine (Ambion, Austin, TX) according to the manufacturer's instructions. Each oocyte was injected with 50 nl of mRNA (0.5 mg/ml), and after 2 to 5 days of expression, GABA-activated currents were recorded with the cell held at -70 mV using a two-microelectrode voltage-clamp amplifier (GeneClamp 500; Axon Instruments Inc., Union City, CA). The recording electrodes were filled with 3 M KCl and had resistance about 1 M Ω measured in Ringer's solution. A gravity flow system was used to deliver drugs to the preparation. Each superfusion channel was separately controlled by a solenoid manifold and operated under computer command. The recording chamber holding the oocyte had a volume of about 20 μ l, and with a flow rate of 2.5 ml/min, the solution changing rate was 0.31 s based on measurements of the response to 25 mM potassium (Qian et al., 1997).

Patch-Clamp Recording from Isolated Retinal Bipolar Cells. Solitary bipolar cells isolated from the retina of hybrid bass (*Morone chrysops* crossed with *Morone saxatilis*) were used in this

study. The cell isolation and recording procedure were the same as published previously (Qian and Ripps, 1999). In brief, animals were anesthetized with Tricaine (0.02%) and sacrificed by double pithing. The retina was isolated from the hemisectioned eyeball and incubated for 40 min in an enzyme solution made up of Leibovitz's L-15 culture medium (Invitrogen, Carlsbad, CA) containing 2 mg/ml papain (Calbiochem, San Diego, CA) and 1 mg/ml *l*-cysteine. Enzyme activity was arrested by washing the tissue five times in fresh L-15 medium. After trituration through a sterile pipette, aliquots of the supernatant containing dissociated cells were placed in 35-mm culture dishes containing 3 ml of the L-15 medium and stored in an incubator at 16°C. Bipolar cells were readily identified by their characteristic morphology (Qian and Dowling, 1995), and those with prominent cell bodies were selected for study.

Membrane currents were recorded using whole-cell voltage-clamp (Hamill et al., 1981). During the recording, cells were bathed in a Ringer's solution containing 145 mM NaCl, 2.5 mM KCl, 2.4 mM CaCl₂, 1.5 mM MgCl₂, 10 mM glucose, and 5 mM HEPES, pH 7.6, and the pipettes were filled with an intracellular solution containing 124 mM CsCl, 1 mM CaCl₂, 11 mM EGTA, 2 mM MgCl₂, and 10 mM HEPES, pH 7.4. Macroscopic currents were recorded with the cells clamped at -60 mV. Because both GABA_A and GABA_C receptors are present on white perch bipolar cells, bicuculline was added to the bath solution to selectively block GABA_A receptor activity. A Perfusion Fast-Step system (Warner Instrument, Hamden, CT) was used to deliver drugs onto the cell. The solution changing rate, measured by liquid junction potential, was 25 ms.

Data Analysis and Modeling. Data analysis was performed with pCLAMP software (Axon Instruments Inc.). The time constants (τ) of the individual deactivation curves were calculated using the Clampfit program to fit the falling phase of the "off" response to a first-order exponential function. For the GABA-induced responses, the data were fit by the equation

$$\frac{R_X}{R_{\max}} = \frac{X^{n_H}}{X^{n_H} + EC_{50}^{n_H}} \quad (1)$$

where R_X is the response induced by GABA at a concentration X , R_{\max} is the maximum GABA response, EC_{50} is the GABA concentration at which a half-maximum response is induced, and n_H is the Hill coefficient. To study inhibition by the antagonists, the data were fit by the equation

$$\frac{R_X}{R_C} = 1 - \frac{X^{n_H}}{X^{n_H} + IC_{50}^{n_H}} \quad (2)$$

where R_X is the response in the presence of X concentration of antagonist, R_C is the control response (i.e., the GABA response in the absence of the antagonist), IC_{50} is the antagonist concentration at which half of the GABA response is blocked, and n_H is the Hill coefficient. The dose-response curves were fit to the Hill eq. 1 or 2 with the program Origin (OriginLab Corp., Northampton, MA). For each experiment, data were collected from five to eight cells and are presented as means \pm 1 S.D.

Mathematical modeling of the action of picrotoxin on GABA-evoked responses was performed with Matlab (MathWorks Inc., Natick, MA) by digitally solving differential equations derived for each step in the model shown in Fig. 5 (Colquhoun and Hawkes, 1981). An automated search program was developed under Matlab to achieve the minimal square error of means between calculated and recorded responses. Each parameter in the model was varied independently with 2 times original value in parameter space searched in each iteration. The loop continued until all the parameters converged. Three different sets of seed (initial parameter values) were used for each fit, and when the optimized parameters meet within a margin of 5%, the average was used as an estimate of the best rate. Responses from six oocytes were used for the modeling study. For each cell, GABA activation parameters (k_1 , k_2 , α , and β) were determined from GABA-elicited response in Ringer's solution. Param-

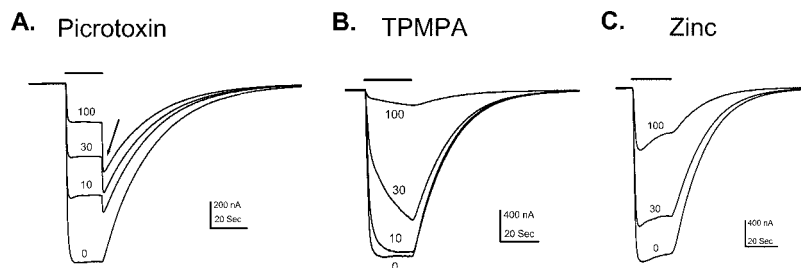


Fig. 1. Inhibition of GABA-elicited currents of perch- $\rho 1A$ receptor expressed in *Xenopus* oocytes. A, GABA (10 μM) activates a sustained inward current on oocytes expressing perch- $\rho 1A$ subunits. When picrotoxin (concentration indicated above each trace in micromolar) is coapplied with GABA, the GABA-induced current is reduced. When the two drugs are withdrawn simultaneously, there is a membrane current rebound (arrow) that is progressively greater as the concentration of picrotoxin is increased. B, TPMPA inhibits GABA-elicited responses. The GABA-induced current is reduced by coapplication of TPMPA. C, zinc also blocks GABA-elicited responses. Note, no rebound of membrane current is detected at the termination of application of either TPMPA or zinc.

eters related to picrotoxin inhibition were calculated from traces elicited by coapplication of GABA with 10, 30, and 100 μM picrotoxin. Each model was judged by comparing the residual error between the recorded response and that predicted by the model and by a comparison of other aspects of picrotoxin inhibition observed on oocyte and those predicted by the model [i.e., GABA dose-response curves in the presence of picrotoxin, and IC_{50} and Hill coefficient values of picrotoxin inhibition (Tables 3 and 4)].

Results

Picrotoxin Inhibition of GABA-Mediated Responses.

The homomeric receptors formed by GABA ρ subunits are highly sensitive to picrotoxin inhibition. Figure 1A illustrates the inhibitory effects of picrotoxin on GABA-activated currents recorded from an oocyte expressing the perch- $\rho 1A$ subunit. In these experiments, GABA (10 μM) was coapplied with picrotoxin at concentrations indicated by the numbers above each

trace. Note the extremely slow kinetics that characterize the GABA-evoked responses, especially the long time course for the off responses after the termination of GABA application (Qian et al., 1998). In the absence of picrotoxin (trace zero), superfusion of 10 μM GABA elicited an inward current of about 1.2 μA in this cell, and it returned slowly toward baseline after the termination of a 20-s GABA pulse. The magnitude of the GABA current response was progressively reduced by coapplication of increasing concentrations of picrotoxin. Interestingly, when the two drugs were simultaneously withdrawn, membrane current increased transiently (Fig. 1A, arrow). The magnitude of this rebound current increased with increasing concentrations of picrotoxin, but after reaching its peak, the membrane currents gradually returned to baseline with a similar time course as the GABA-elicited currents.

The current rebound that followed termination of the coapplied drugs is unique for picrotoxin. As shown in Fig. 1, B and

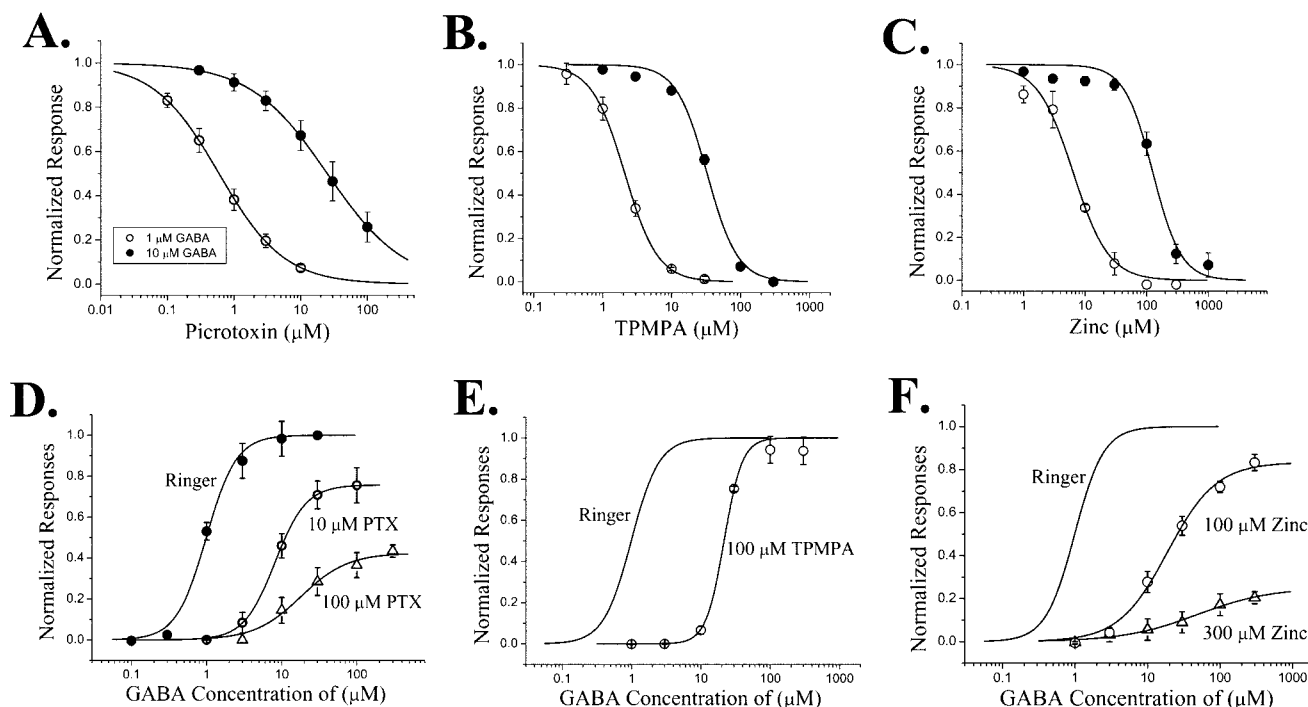


Fig. 2. Inhibition curves for picrotoxin, TPMPA, and zinc. A to C, inhibition curves determined with 1 and 10 μM GABA for picrotoxin, TPMPA, and zinc, respectively. Currents were normalized to the response obtained in the absence of any antagonist. In each case, sensitivity to antagonist varied with the concentration of GABA tested. A large value of IC_{50} was obtained when measured with high GABA concentration (see text for details). D to F, GABA dose-response curves in the presence of picrotoxin, TPMPA, and zinc, respectively. Both picrotoxin and zinc shifted the GABA dose-response to the right along the abscissa and reduced the maximum response in a concentration-dependent manner. On the other hand, TPMPA only caused a right shift of the GABA dose-response curve without significantly reducing the maximum responses.

C, both the GABA_C receptor competitive antagonist TPMPA (Ragozzino et al., 1996) and the allosteric modulator zinc (Chang et al., 1995; Wang et al., 1995a), when coapplied with GABA, inhibited GABA-elicited membrane currents in a concentration-dependent manner. However, even at high concentration (100 μ M), simultaneous withdrawal of GABA with these antagonists did not produce a rebound in the membrane current. Instead, the membrane current decayed back to baseline and followed a similar time course as the GABA-elicited response in Ringer's solution. Similar results were observed on five oocytes with TPMPA and four cells for zinc inhibition.

The sensitivity to picrotoxin on ρ 1A receptors was dependent on GABA concentration. Figure 2A shows picrotoxin inhibition data obtained with two concentrations of GABA; responses were normalized to the maximum amplitudes elicited by GABA alone. When tested with 1 μ M GABA, the IC₅₀ value of picrotoxin inhibition was about 0.59 μ M. However, with a 10-fold higher concentration of GABA, the picrotoxin inhibition curve was shifted to the right and the IC₅₀ value increased to 25 μ M. The dependence of picrotoxin sensitivity on GABA concentration suggests an apparent competitive inhibitory mechanism on the GABA_C receptor. Similar behavior was observed for TPMPA and zinc inhibition. As shown in Fig. 2B, the IC₅₀ values for TPMPA inhibition on the perch- ρ 1A receptor also varied with GABA concentration. When tested with 1 μ M GABA, the IC₅₀ value for TPMPA was 2.1 μ M, and the value increased to 32.6 μ M when tested with 10 μ M GABA. Figure 2C

illustrates zinc inhibition curves tested with high and low GABA concentrations. The IC₅₀ value for zinc increases from 6.4 μ M in the presence of 1 μ M GABA to 125.6 μ M with 10 μ M GABA.

Although the sensitivity of the three antagonists exhibited a similar dependence on GABA concentration, each compound had different effects on the GABA dose-response relations. Figure 2D shows the GABA dose-response curves for the perch- ρ 1A receptor measured in the presence of various picrotoxin concentrations. Two effects are observed: 1) the GABA dose-response curves were shifted to the right along the abscissa by picrotoxin in a concentration-dependent manner; and 2) there was a reduction in the maximum response elicited by GABA in the presence of picrotoxin. These results suggest that both competitive and noncompetitive mechanisms exist for picrotoxin inhibition of GABA_C receptors (Qian et al., 1998).

On the other hand, TPMPA proved to be a competitive antagonist of GABA_C receptors. Only a rightward shift of the GABA dose-response curve was observed in the presence of TPMPA (Fig. 2E), and no significant reduction of the maximum GABA-elicited current was observed. It has been reported that zinc acts as an allosteric modulator of the receptor formed by human ρ 1 subunits and that zinc inhibition contains both competitive and noncompetitive mechanisms (Chang et al., 1995; Wang et al., 1995a). We observed a

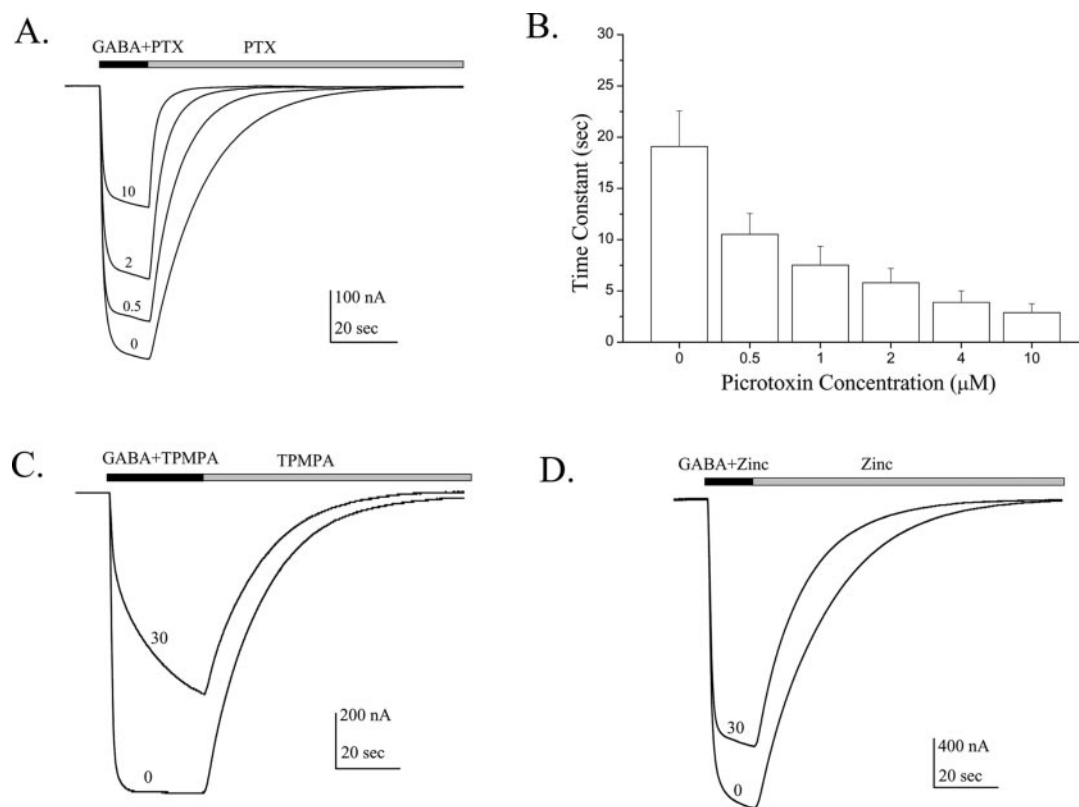


Fig. 3. Picrotoxin, but not TPMPA or zinc, greatly accelerates the offset response of GABA-evoked currents from perch- ρ 1A receptors. A, current traces of GABA-elicited responses in the presence of various picrotoxin concentrations (shown above each trace in micromolar). When no picrotoxin is present, the GABA-elicited current returned slowly to the baseline after GABA application was terminated. In the presence of picrotoxin, there was a concentration-dependent increase in the rate of the GABA response decay. B, bar graphs showing results for the decay time constants obtained with five different concentrations of picrotoxin. Analysis of variance analysis indicated significant statistical difference ($p < 0.001$) among data groups. C, current traces of GABA-elicited responses in Ringer's solution and in the presence of 30 μ M TPMPA. The GABA offset response in the presence of TPMPA followed a similar time course as those in Ringer's solution. D, current traces of GABA-elicited responses in Ringer's solution and in the presence of 30 μ M zinc. Zinc only slightly accelerated the GABA offset response.

similar effect on perch- $\rho 1A$ receptors; i.e., zinc reduced both the sensitivity and maximum response to GABA (Fig. 2F).

Picrotoxin Accelerates Relaxation of GABA-Elicited Responses. The presence of picrotoxin significantly altered the kinetics of the GABA-induced responses. In particular, the rate at which the GABA-elicited responses returned to baseline was greatly accelerated by picrotoxin. Figure 3A shows typical current traces elicited from perch- $\rho 1A$ receptors by 10 μM GABA (trace 0) or by coapplication of 10 μM GABA with varying concentrations of picrotoxin (0.5–10 μM). Termination of GABA in the presence of picrotoxin caused a rapid return of membrane current; the rate of return increased with rising concentrations of picrotoxin. To quantitate this effect, we measured the time constants for the current decay (summarized in Fig. 3B). As the picrotoxin concentration increased, the time constant of the GABA offset current progressively decreased. Pairwise comparisons of the time constants in Ringer's solution and in the various concentrations of picrotoxin were significantly different.

The large acceleration of GABA relaxation is unique to picrotoxin. After a similar protocol, we tested the effects of TPMPA and zinc on the time course of GABA offset responses. As shown in Fig. 3C, in the presence of 30 μM TPMPA, the GABA offset response followed a similar time course as that observed in Ringer's solution. The time con-

stant of the GABA offset response in the presence of TPMPA was 0.98 ± 0.03 ($n = 4$) of that measured in Ringer. The allosteric modulator zinc accelerated the GABA relaxation only slightly (Fig. 3D). In the presence of 30 μM zinc, the time constant of the GABA offset response was 0.76 ± 0.08 ($n = 4$) of the value measured in Ringer's solution.

Effects of Picrotoxin on Other GABA_C Receptors. Because both the kinetics and the amplitude of the inhibitory current contribute significantly to the shape of the neuronal signal conveyed across the synapse, we next explored whether a similar acceleration of GABA responses by picrotoxin could also be observed on other GABA_C receptors. Specifically, we determined the effect of picrotoxin on the time constant of the off response for the receptors formed by various perch- ρ subunits that we had previously cloned from a perch retinal cDNA library (Qian et al., 1998). A similar but abbreviated protocol was used in this study. For the receptors formed by GABA ρ subunits, the kinetics of the GABA offset response was largely independent of the GABA concentration (Qian et al., 1998). In these experiments, a GABA concentration eliciting a submaximum response was used, and a picrotoxin concentration was chosen to inhibit 50% of these GABA-elicited responses. As shown in Fig. 4A, picrotoxin reduced the time constants of the offset currents for each of the perch GABA_C receptors (plotted on a logarithmic scale).

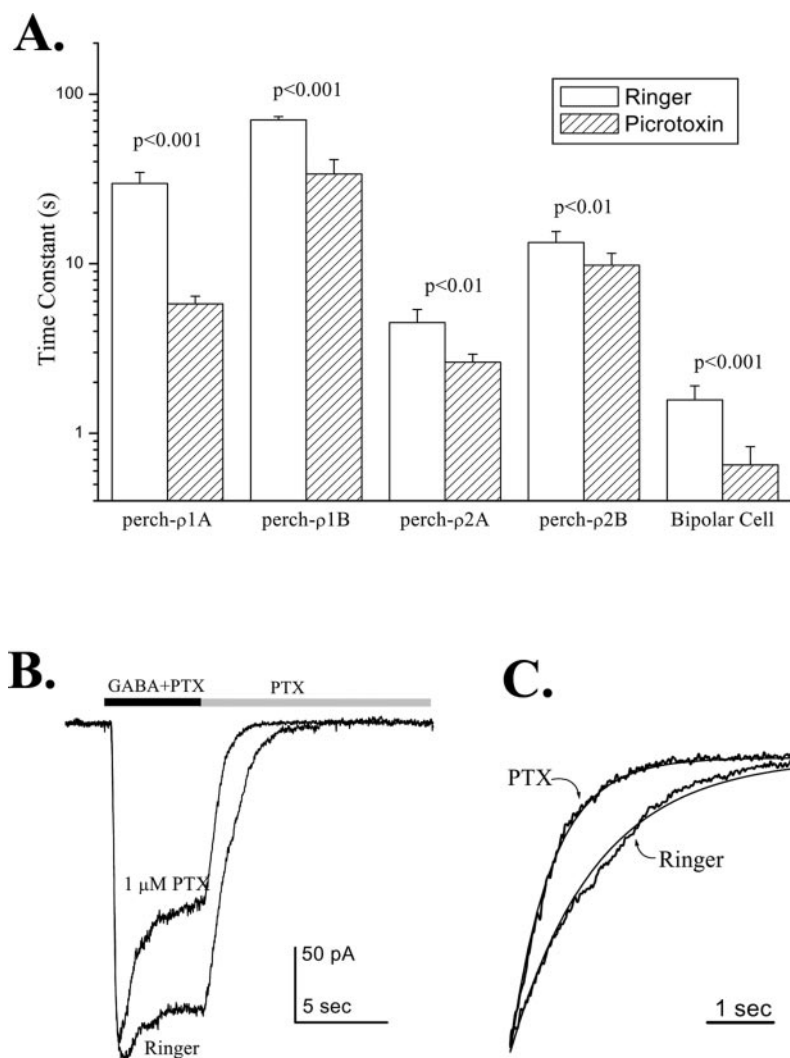


Fig. 4. Picrotoxin accelerates the GABA relaxation on GABA_C receptors. **A**, time constants of the GABA offset responses recorded in Ringer's solution and in picrotoxin for various GABA ρ receptors, as well as GABA_C receptors on retinal bipolar cells. The GABA concentration was 10 μM for perch- $\rho 1A$, - $\rho 2A$, and - $\rho 2B$ receptors, 2 μM for perch- $\rho 1B$ receptor, and 30 μM for bipolar cells. Picrotoxin (10 μM for perch- $\rho 1A$, 25 μM for perch- $\rho 1B$, 2 μM for perch- $\rho 2A$ and - $\rho 2B$ receptors, and 1 μM for bipolar cells) reduced the time constant for both GABA ρ receptors and native GABA_C receptors on bipolar cells. Note the time constants are plotted on a logarithmic scale, and the distance between values measured in Ringer and in picrotoxin reflects the fold change in the time constant. Statistical p values (Student's t test) shown above each set of data compares the time constants measured in Ringer's solution with those in picrotoxin (scale bars, ± 1 S.D.). **B**, GABA_C receptor-mediated currents from a hybrid bass bipolar cell. Bicuculline (200 μM) was used to block the activation of GABA_A receptors. The traces show whole-cell current responses to the application of 30 μM GABA and to the combination of GABA with 1 μM picrotoxin. GABA offset response is accelerated in the presence of picrotoxin. **C**, normalized GABA offset responses and replotted on an expanded time scale to better illustrate the effect of picrotoxin.

Among them, picrotoxin produced the largest (5-fold) reduction in the time constant of the GABA-offset response on perch- ρ 1A receptors; for the other ρ subunits, the decay rates were accelerated between 1.4- and 2.1-fold (Fig. 4A).

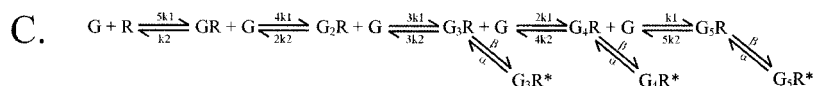
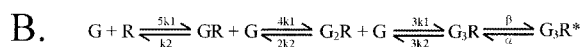
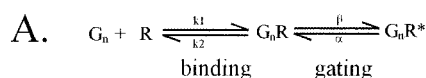
Having shown that picrotoxin could significantly accelerate the GABA responses of GABA_C receptors expressed in oocytes, we next tested whether the native GABA_C receptors present on retinal neurons showed a similar behavior. Figure 4B shows an example of a GABA_C receptor-mediated response recorded in Ringer's solution from an individual bipolar cell isolated from the hybrid bass retina; the cell was incubated in 200 μ M bicuculline to block the activity of the endogenous GABA_A receptors. As shown previously (Qian and Ripps, 1999), coapplication of 1 μ M picrotoxin with 30 μ M GABA reduced by about half the GABA-elicited current. In addition, the GABA offset response was accelerated in the presence of picrotoxin. This is shown more clearly in Fig. 4C, where each response is normalized to its initial amplitude and plotted on an expanded time scale. Superimposed on the current traces are single exponential decay functions (thin continuous lines). Note that they provide good approximations for the decay phase of the GABA-elicited responses. The time constants of the GABA offset responses measured in Ringer's solution, or in picrotoxin, are presented in the bar graphs to the right of Fig. 4A. The reduction in the time constants from 1.6 s in Ringer to 0.65 s in picrotoxin reflects the approximately 2.4-fold increase in the decay rate of the GABA responses in the presence of picrotoxin. The large difference in the response kinetics of native GABA_C receptors on bipolar cells compared with those from homomeric GABA ρ receptors expressed in *X. laevis* oocytes might reflect the incorporation of the GABA_A receptor γ ₂ subunits in the native GABA_C receptors, as we have reported previously (Qian and Ripps, 1999; Qian and Pan, 2002).

Schematic Model for Picrotoxin Inhibition. To account for the data observed with picrotoxin inhibition, we

adopted a model of picrotoxin inhibition that incorporates its effect on both agonist binding and channel gating of the GABA_C receptor (Smart and Constanti, 1986). We modeled the GABA-elicited response and picrotoxin inhibition in two sequential steps. In the first step, a simple binding and gating process was used to model GABA-activated responses (Fig. 5A). Asterisks (*) denote the receptor with its channel in the open conformation. We considered two models, three binding steps and one gating step (Fig. 5B), and five binding steps and three gating steps (Fig. 5C). Amin and Weiss (1996) have shown that, for the human ρ 1 receptor, binding of three GABA molecules is required to open the channel. A scheme similar to Fig. 5B has previously been successfully used to describe the GABA-elicited currents of the human GABA ρ 1 receptor (Chang and Weiss, 1999). On the other hand, homomeric GABA ρ receptors are thought to be pentamers, and five GABA binding sites are available on the receptor (Amin and Weiss, 1996; Qian and Ripps, 2001), and the scheme of Fig. 5C might better describe the GABA-elicited responses. We tested both models with GABA-elicited responses obtained from six oocytes. Although both models fit the current traces equally well, the model in Fig. 5C provides a much better description of picrotoxin inhibition (see below). Figure 6A illustrates an example of fitting the recorded GABA-elicited responses (thick gray lines) to the model C (thin lines). The average parameters derived from numerical model fitting of GABA-elicited responses from six oocytes are listed in Table 1.

Having fixed the parameters for GABA activation, we next examined picrotoxin inhibition responses. In this model (Fig. 5D), which is based on the GABA activation scheme shown in Fig. 5C, picrotoxin interacts with the ligand-bound GABA-receptor complex in both channel closed and channel open states (Smart and Constanti, 1986). Three pairs of parameter were used: k_3 and k_4 describe the GABA binding ability of picrotoxin-bound receptor, k_5 and k_6 indicate picrotoxin affinity to

GABA Activation



Picrotoxin Inhibition

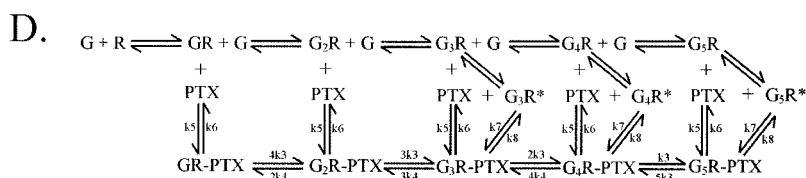


Fig. 5. Models used for fitting GABA-elicited response and picrotoxin inhibition. A, conceptual scheme for receptor activation, including binding steps and gating steps. B, three binding steps and one gating step model for GABA activation. C, five binding steps and three gating steps model for GABA activation. D, model for picrotoxin inhibition, including picrotoxin binding to ligand-bound receptor with both the channel open and closed states. GABA, receptor and picrotoxin are represented by G, R, and PTX, respectively. The asterisk (*) indicates the channel is in the open conformation.

ligand-bound channel closed receptor, and k7 and k8 illustrate binding of picrotoxin to channel open state (Table 2). A few other models were also tested in this study. 1) Model based on three-step binding and one-step gating for GABA activation (Fig. 5B) gave a less satisfactory fit than the model in Fig. 5D. The ratio of residual error derived from these two models was 2.57 ± 0.75 ($n = 6$). Furthermore, the model in Fig. 5D provided a better prediction for the Hill coefficient of picrotoxin inhibition (Tables 3 and 4). 2) Incorporating steps for picrotoxin binding to the ligand-unbound receptor provided little improvement in reducing the error (about 25%) between predicted and recorded responses. In addition, the rate constants for this step exhibited a large divergence. These results indicate that picrotoxin binding to the ligand-unbound receptor is not necessary for modeling picrotoxin inhibition on GABA ρ receptors. 3) When an additional step was introduced for picrotoxin-bound channel open state, the model yielded a poor fit to the recorded responses. The residual errors produced by this model were more than 5 times larger than those generated by model shown in Fig. 5D. 4) We also attempted to further reduce the number of free parameters in the model by considering the affinity of picrotoxin for the channel open and closed states of the receptor to be equal (i.e., eliminating parameter k7 and k8). However, such change significantly increased the residual error by $2.7 \pm$

0.5 times ($n = 6$). Because large conformational changes are required for channel gating, it is reasonable to speculate that the receptor in channel closed and open conformations exhibit different affinities for picrotoxin. For these reasons, we retained k7 and k8 in our model.

Figure 6B shows an example of fit our model (thin lines) to responses elicited by coapplication of GABA and 10, 30, and 100 μ M picrotoxin (thick gray lines). The model faithfully predicts rebound in membrane current after the termination of GABA and picrotoxin (Fig. 6B, arrow), and it reproduces the enhancement of the rebound current with increasing picrotoxin concentrations. The average parameters derived from numerical model fitting of responses elicited by coapplication of GABA and picrotoxin from six oocytes are listed in Table 2.

The model also faithfully reproduced the acceleration of GABA relaxation in the presence of picrotoxin. As shown in Fig. 6C, the model predicted responses in the presence of picrotoxin are very similar to the oocyte recordings shown in Fig. 3A. To further test the accuracy of our model for picrotoxin inhibition of GABA ρ receptors, we compared the predicted responses and the actual records from oocytes under low GABA concentrations. The predicted current responses to 1 μ M GABA exhibit both slow off and slow onset responses (Fig. 6D). Coapplication of picrotoxin reduced the amplitude of GABA-elicited current.

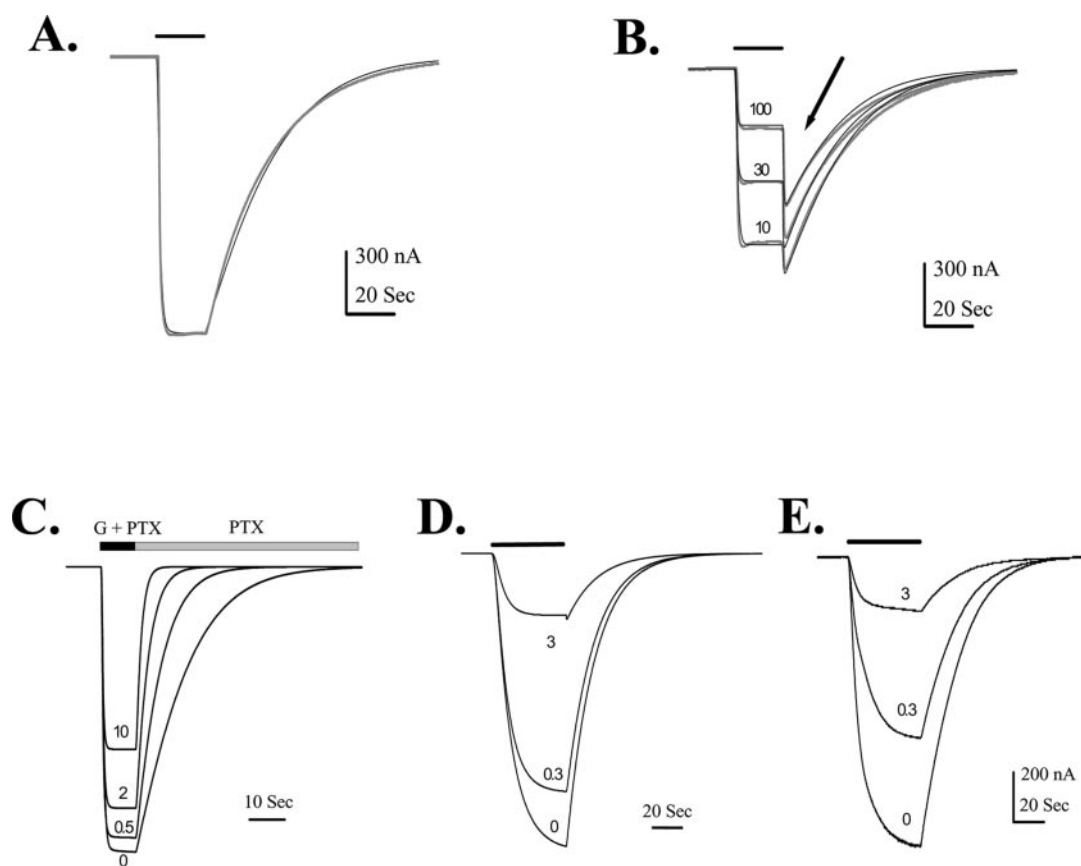


Fig. 6. Comparison of responses calculated by the model with those recorded from oocytes expressing the perch- ρ 1A subunits. A, GABA (10 μ M)-elicited current (thick gray line) superimposed with a model derived trace (thin line). B, current response to coapplication of GABA and 10, 30, and 100 μ M picrotoxin (thick gray lines), superimposed with model fit traces (thin lines). Note the rebound of current responses after removal of the picrotoxin block (arrow) is clearly predicted by the model. C, modeled GABA responses in the presence of various concentrations of picrotoxin illustrate the concentration-dependent acceleration of the off responses. D, model predicted GABA response and picrotoxin inhibition probed with 1 μ M GABA. E, current responses recorded from an oocyte expressing the perch- ρ 1A subunit. Note that the response elicited with 1 μ M GABA has a slow onset. Coapplication of picrotoxin (concentration indicated above each trace in micromolar) reduced the current response. However, unlike responses probed with high GABA concentration, no significant rebound of current was observed when the application of 1 μ M GABA and picrotoxin were terminated. Both the slowness of the GABA onset response and lack of rebound after removal of picrotoxin inhibition were predicted by the model.

Picrotoxinin Mediates Inhibition on Perch- $\rho 1A$ Receptor. Picrotoxin is an equal molar mixture of picrotin and picrotoxinin. Whereas picrotoxinin is the only active component inhibiting GABA_A responses, picrotin is equally effective when blocking certain types of glycine receptors (Lynch et al., 1995; Steinbach et al., 2000). Here, we have shown that picrotoxin inhibition on perch- $\rho 1A$ receptor contains both competitive and noncompetitive components and picrotoxin is likely to bind the ligand-bound receptor in both channel open and closed states. It is possible, therefore, that each component of picrotoxin inhibition is mediated by separate compounds present in picrotoxin (i.e., picrotin and picrotoxinin). To test this hypothesis, we evaluated the effectiveness

TABLE 3
Comparison of parameters for GABA dose-responses determined in Ringer's solution and in various picrotoxin concentrations

	Ringer's		10 μ M PTX			100 μ M PTX		
	EC ₅₀	n_H	EC ₅₀	n_H	R _{max}	EC ₅₀	n_H	R _{max}
	μ M		μ M			μ M		
Measured	0.99 \pm 0.06	2.17 \pm 0.29	8.1 \pm 0.16	2.1 \pm 0.08	0.76 \pm 0.01	18.1 \pm 4.4	1.4 \pm 0.40	0.42 \pm 0.04
Model	0.98 \pm 0.02	2.18 \pm 0.13	3.21 \pm 0.04	1.67 \pm 0.03	0.76 \pm 0.01	5.8 \pm 0.1	1.47 \pm 0.04	0.29 \pm 0.01

toxin and GABA coapplication is consistent with a noncompetitive (channel blocker) mechanism of picrotoxin action. The slow kinetics of GABA relaxation observed for GABA_C receptors make this phenomenon more prominent (Qian et al., 1998; Qian and Ripps, 2001). A similar phenomenon was observed for the GABA_C receptors present on catfish cone-driven horizontal cells (Dong and Werblin, 1996). On the other hand, the dependence of the IC₅₀ value of picrotoxin inhibition on GABA concentration (Fig. 2A) and the increase in the EC₅₀ of the GABA dose-response curve in the presence of picrotoxin (Fig. 2D) are indicative of a competitive mechanism for picrotoxin inhibition (Woodward et al., 1992).

To account for the properties of picrotoxin inhibition on GABA_C receptors, we adopted a simple model that incorporated the binding of picrotoxin to both the open and closed states of the receptor. The parameters derived from fitting the model to the recorded data yielded values for GABA binding and gating that were similar to those determined for the human $\rho 1$ receptor (Chang and Weiss, 1999). Modeling picrotoxin binding to ligand-bound receptors in both the channel open (G_nR*) and closed states (G_nR) was necessary to provide a reasonable fit. On the other hand, the binding of picrotoxin to ligand-unbound receptors was not required. The prominent rebound after terminating the coapplication of GABA and picrotoxin (Fig. 1A) is one of the signatures of an open channel blocker. If picrotoxin acted purely as a channel blocker, coapplication of GABA with picrotoxin would significantly retard the off response (Drapeau

and Legendre, 2001). However, the off response after terminating the coapplication of GABA and picrotoxin followed a similar time course as that elicited by GABA alone (Fig. 1A). In addition, modeling of picrotoxin as a pure channel blocker of the GABA_C receptor failed to predict other properties of picrotoxin inhibition, especially the competitive component.

Using the values listed in Tables 1 and 2, the model in Fig. 5D provides a good estimate of the various aspects of picrotoxin inhibition of perch- $\rho 1A$ receptors (Tables 3 and 4; Fig. 6). For example, the sensitivity (IC₅₀ value) for picrotoxin inhibition varied with GABA concentration (Fig. 2A; Table 4), and the presence of picrotoxin shifted the GABA dose-response curve to the right and reduced the maximum response (Fig. 2D; Table 3). These results indicate that the model provides a good estimate of both the competitive and noncompetitive components for picrotoxin inhibition on GABA_C receptors. Furthermore, the model predicted both the slow onset GABA response and the lack of rebound when coapplication of 1 μ M GABA and picrotoxin was terminated (Fig. 6, D and E), even though some deviation occurred with respect to the response amplitude in the presence of picrotoxin. Recording single channel GABA_C receptor activity would provide an alternative way to test our model. However, the small single channel conductance gated by the GABA_C receptor makes such a task technically difficult (Chang and Weiss, 1999).

The model also provides a good representation of the acceleration of GABA relaxation in the presence of picrotoxin.

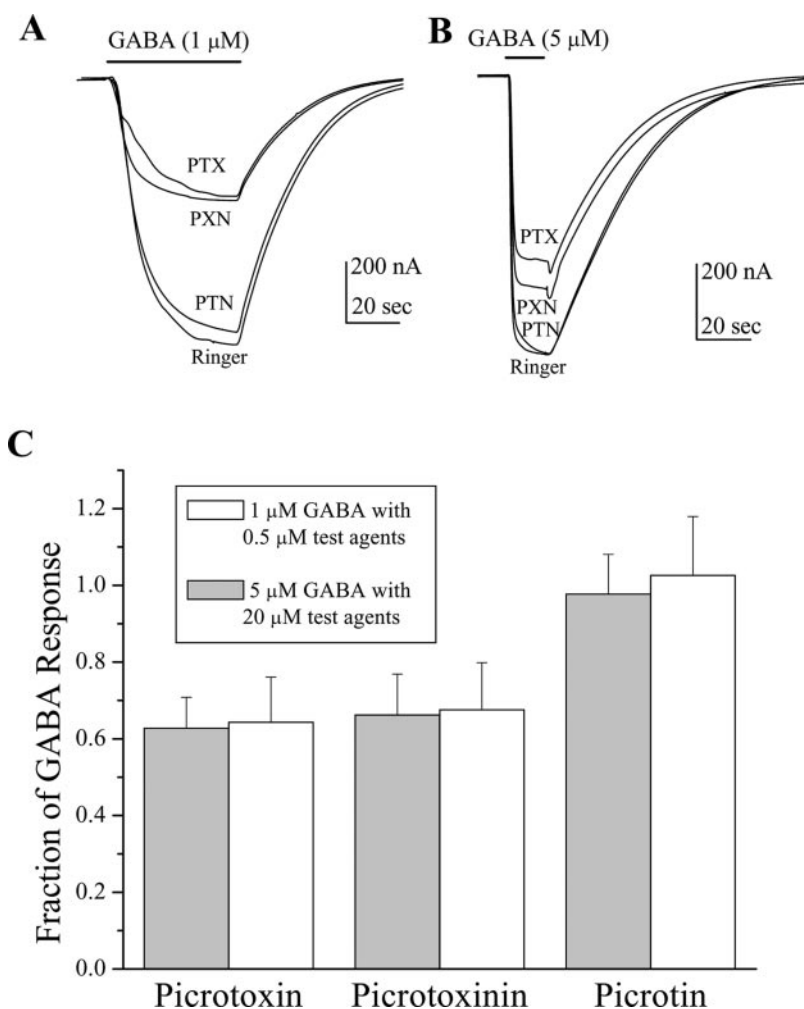


Fig. 7. Picrotoxinin inhibits perch- $\rho 1A$ receptors. A, current responses to GABA (1 μ M) and to coapplication of GABA with 0.5 μ M concentrations of either picrotoxin (PTX), picrotoxinin (PXN), or picrotin (PTN). No significant reduction in GABA-elicited responses was detected when picrotin was coapplied, whereas coapplication of GABA with picrotoxin produced amounts of inhibition comparable with picrotoxin. B, current responses with 5 μ M GABA and 20 μ M concentrations of either test agent. C, bar graph illustrates data averaged from five oocytes for inhibition of GABA-elicited responses by picrotin, picrotoxinin, and picrotoxin tested with both low and high GABA concentrations.

As shown in Fig. 6C, the traces predicted by the model nicely illustrate that the time course of the GABA off responses were significantly faster in the presence of picrotoxin, similar to those observed for GABA_C receptors (Fig. 3). For the simple kinetic model shown in Fig. 5A, the off response time constant (τ) exhibits the following relation: $1/\tau = k_2 \times (\alpha/(\alpha + \beta))$ (Bean, 1990). Thus, binding of picrotoxin to both G_nR and G_nR* contributes to the acceleration of the GABA off response. In the presence of 1 μ M picrotoxin, there was approximately 1.83-fold change in the off rate for G_nR, whereas the off rate for G_nR* was only 1.07 times that of the rate in Ringer. Therefore, the 2.5-fold reduction in the time constant of GABA off responses induced by 1 μ M picrotoxin on perch- ρ 1A receptor is mainly contributed by the enhancement of the off rate for G_nR (Fig. 3B).

Our model suggested that the affinity for picrotoxin (0.15 μ M for G_nR, and 37 μ M for G_nR*) is altered by receptor channel gating. This difference in picrotoxin affinity nicely explains the behavior of picrotoxin inhibition on GABA_C receptors. Because GABA_C receptors share a high degree of homology with GABA_A and glycine receptors, the mechanisms of picrotoxin inhibition revealed in this study might therefore be extended to these receptors. Picrotoxin inhibition of GABA_A receptors is mainly noncompetitive (Korpi et al., 2002), whereas competitive mechanisms dominate for glycine receptors (Lynch et al., 1995). These diverse actions of picrotoxin can easily be adopted in the model by altering the affinity of picrotoxin for the receptor in the open and closed states. For example, high picrotoxin affinity for channel open states of GABA_A receptors will generate predominantly noncompetitive inhibition, whereas high picrotoxin affinity for closed channel states of glycine receptors will exhibit mostly competitive inhibition. Therefore, the allosteric mechanism revealed in this study for picrotoxin inhibition of the GABA_C receptor may be extended to the actions of picrotoxin on other ligand-gated chloride channels.

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