Mechanisms for Picrotoxinin and Picrotin Blocks of $\alpha_2$ Homomeric Glycine Receptors

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Contrary to its effect on the $\gamma$-aminobutyric acid type A and C receptors, picrotoxin antagonism of the $\alpha_2$ homomeric glycine receptors (GlyRs) has been shown to be non-use-dependent and nonselective between the picrotoxin components picrotoxinin and picrotin. Picrotoxinin antagonism of the embryonic $\alpha_2$ homomeric GlyR is known to be use-dependent and reflects a channel-blocking mechanism, but the selectivity of picrotoxin antagonism of the embryonic $\alpha_2$ homomeric GlyRs between picrotoxinin and picrotin is unknown. Hence, we used the patch clamp recording technique in the outside-out configuration to investigate, at the single channel level, the mechanism of picrotin- and picrotoxinin-induced inhibition of currents, which were evoked by the activation of $\alpha_2$ homomeric GlyRs stably transfected into Chinese hamster ovary cells. Although both picrotoxinin and picrotin inhibited glycine-evoked outside-out currents, picrotin had a 30 times higher $I_C^{90}$ than picrotoxinin. Picrotin-evoked inhibition displayed voltage dependence, whereas picrotoxinin did not. Picrotoxinin and picrotin decreased the mean open time of the channel in a concentration-dependent manner, indicating that these picrotoxin components can bind to the receptor in its open state. When picrotin and glycine were co-applied, a large rebound current was observed at the end of the application. This rebound current was considerably smaller when picrotoxinin and glycine were co-applied. Both picrotin and picrotoxinin were unable to bind to the unbound conformation of the receptor, but both could be trapped at their binding site when the channel closed during glycine dissociation. Our data indicate that picrotoxinin and picrotin are not equivalent in blocking $\alpha_2$ homomeric GlyR.

Glycine and GABA$^4$ are the most important inhibitory neurotransmitters in the adult central nervous system. The glycine receptors (GlyRs) belong to the cysteine-loop family of ligand-gated ion channels. The GlyR is a pentameric transmembrane protein complex, which forms an anion-selective channel. So far five different subunits have been cloned in mammals, one $\beta$ subunit and four $\alpha$ subunits ($\alpha_1$-$\alpha_4$) (1). Each subunit is composed of a large external N-terminal domain and four transmembrane domains termed M1–M4, with the M2 transmembrane domain forming the pore of the channel (1). These subunits can be associated in two different ways to form functional receptor channels. The homomeric receptors consist of five $\alpha$ subunits, whereas the heteromeric receptors are a combination of two $\alpha$ and three $\beta$ subunits with the agonist-binding site at the interface of the $\alpha$ and the $\beta$ subunits (2). Although the plant alkaloid picrotoxin (PTX) was first used to discriminate between functional GABA$\alpha$$_2$Rs and GlyRs, it is now well established that PTX can inhibit both cation-selective (nicotinic acetylcholine receptor and 5-hydroxytryptophan receptor type 3) and anion-selective (GABA$\alpha$$_2$R, GABA$\alpha$$_3$R, GlyR, and GluCl) receptor channels (3–7). The action of PTX has been extensively studied on GABA$\alpha$$_2$Rs, on GABA$\beta$$_2$Rs, and on GlyRs (see for review 8, 9). The PTX inhibitory mechanism can vary between ionic channel receptors of the cysteine-loop family, and it ranges from an open channel blocker on some GABARs to an allosteric competitive antagonist on GlyRs (8). The number of binding sites of PTX, the location of these sites, and the inhibitory mechanism of this compound are still under debate (8), but there are several lines of evidence to indicate that PTX can bind within the channel pore. The 2$’$–6$’$ amino acid residues that line the pore in the M2 domain are likely to be involved in the PTX effect (5, 7, 10–12) or to form the binding site for PTX at least in ionotropic GABA receptors (13). The proposal that the location of the PTX-binding site is within the channel pore of GlyRs was recently reinforced. Indeed, it has been shown that PTX can be trapped on its binding site when the channel closes in $\alpha_1$ homomeric GlyRs with an $\alpha_2$ subunit R271C mutation (14). This was also the case in wild-type $\alpha_2$ homomeric GlyRs (9).

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4. The abbreviations used are: GABA, $\gamma$-aminobutyric acid; GABA$\alpha$$_2$R, $\gamma$-aminobutyric acid type A receptor; GABA$\alpha$$_3$R, $\gamma$-aminobutyric acid type C receptor; ANOVA, analysis of variance; CHO, Chinese hamster ovary; Me$_2$SO, dimethyl sulfoxide; GlyR, glycine receptor; $I$-$V$, current-voltage; PTX, picrotoxin; PXN, picrotoxinin; PTN, picrotin; SSEs, sums of squared errors.

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In fact, PTX is an equimolecular complex of picrotoxinin and picrotin (15), which differ only by a single group, with picrotin having a hydrophilic elongated end. Picrotin is inactive in inhibiting the GABA<sub>A</sub>R and GABA<sub>C</sub>R, indicating that the inhibitory effect of PTX is related to picrotoxinin (15, 16). This is not the case for α<sub>2</sub> homomeric GlyRs. Picrotoxinin and picrotin are equally potent in inhibiting α<sub>1</sub> homomeric GlyR activation (17). The main structural difference in the putative PTX-binding site in GABA<sub>A</sub>Rs or GABA<sub>C</sub>Rs and in α<sub>1</sub> homomeric GlyRs is located at the 2<sup>′</sup> pore-lining position. GABA<sub>A</sub>Rs and GABA<sub>C</sub>Rs contain an alanine residue instead of a glycine residue at the 2<sup>′</sup> pore-lining position. This could facilitate the discrimination between the two PTX components. As the α<sub>2</sub> homomeric GlyR 2<sup>′</sup> residues are also alanine, it is possible that α<sub>2</sub> homomeric GlyR could also discriminate between picrotoxinin and picrotin. This would imply that the blocking mechanism of PTX previously described on this GlyR subtype (9) could be mediated by picrotoxinin only.

The α<sub>2</sub> homomeric GlyR subunit is an embryonic receptor form (18, 19) and plays an important role during synaptogenesis and cell differentiation. Furthermore, the α<sub>2</sub> homomeric GlyR has slow kinetic properties and opens mainly with a single large conductance state (100–120 pS) (20); this property makes this receptor a good model for analyzing the effects of picrotoxinin and picrotin on GlyR kinetics. Therefore, we investigated the effectiveness of picrotoxin in inhibiting α<sub>2</sub> homomeric GlyRs and their mechanisms of inhibition of the activation and deactivation kinetics of the α<sub>2</sub> homomeric GlyRs. The receptor was expressed in Chinese hamster ovary (CHO) cells. The single GlyR channel currents were recorded using outside-out patch clamp recordings, and they were evoked using an ultra-fast flow application system.

We showed that picrotoxin is considerably more potent than picrotin in inhibiting α<sub>2</sub> homomeric GlyRs, indicating that the inhibitory effect of PTX we previously described (9) is mainly because of picrotoxinin. Although picrotoxinin blocks homomeric α<sub>2</sub> GlyR activation in a voltage-independent manner, picrotin-evoked inhibition appeared to be more voltage-dependent. Both picrotoxinin and picrotin can bind to the receptor in both the open channel conformation and the liganded closed state. They can both be trapped at their binding site when the channel closes during glycine molecule dissociation.

The blocking mechanism for picrotoxinin can be well predicted by the kinetic model previously proposed to describe PTX-evoked inhibition of α<sub>2</sub> homomeric GlyR (9). The blocking mechanism for picrotin is probably different. The kinetic model used to predict the blocking mechanism for picrotoxinin poorly predicted the experimental results obtained with picrotin. A relatively simpler kinetic model better predicted the blocking mechanism for picrotin.

**MATERIALS AND METHODS**

**Cell Culture**—Chinese hamster ovary cells (CHO-K1, ATCC number CCL61) were maintained in a 95% air, 5% CO<sub>2</sub> humidified incubator at 35 °C in Dulbecco’s modified Eagle’s medium supplemented with 0.11 g/liter sodium pyruvate, 6 g/liter d-glucose, 10% (v/v) heat-inactivated fetal bovine serum (all from Invitrogen). Cells were passaged every 5–6 days (up to 20 times). For electrophysiological recordings, cells were seeded onto glass coverslips coated with poly-L-ornithine (0.1 mg/ml). Glycine receptor α<sub>2</sub> subunit cloning and transection were performed as described previously (20).

**Outside-out Recordings**—Outside-out recordings (21) were done under direct visualization on α<sub>2</sub> GlyR-transfected CHO cells with the use of Normaski optics (×40 immersion lens; Nikon Optiphot). Cells were continuously perfused at room temperature (20–22 °C) with oxygenated bathing solution (2 ml/min) containing (in mM) the following: NaCl 147, KCl 2.4, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 2, HEPES 10, glucose 10 (pH 7.2, osmolarity 320 mosM). Patch clamp electrodes (5–10 megohms) were pulled from thick wall borosilicate glass with an outer diameter of 1.5 mm and inner diameter of 0.86 mm (Harvard Apparatus, Kent, UK) in multiple steps using a Brown-Flaming puller (Sutter Instrument Co., Navato). They were fire-polished and filled with (in mM) the following: CsCl 130, MgCl<sub>2</sub> 4, Na<sub>2</sub>ATP 4, EGTA 10, HEPES (pH 7.2, osmolarity 290 mosM). Currents were recorded using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) and stored using a digital recorder (PCM-R300, Sony, Tokyo, Japan). Recordings were filtered at 10 kHz using an eight-pole bessel filter (Frequency Devices, Haverhill, MA), sampled at 50 kHz and stored on a PC computer using pClamp software 6.03 (Axon Instruments). The membrane potential was held at −50 mV throughout the experiment, except when examining the I-V relationship. Patch currents represent the average of several trials as further specified in the figure legends or in the text.

**Drug Delivery**—Outside-out single channel currents were evoked using a fast-flow operating system (22, 23). Control and drug solution were gravity-fed into two channels of a thin-wall glass theta tube (2 mm outer diameter; Hilgenberg, Malsfeld, Germany), pulled, and broken to obtain a 200-μm tip diameter. The outside-out patch was positioned (45° angle) 100 μm away from the theta tubing, to be close to the interface formed between the flowing control and drug solutions. One lumen of the theta tube was connected to reservoirs filled with solutions containing glycine and/or blockers. The solution exchange was performed by rapidly moving the solution interface across the tip of the patch pipette, using a piezo-electric translator (model P245.30, Physik Instrument, Waldbronn, Germany). Concentration steps of glycine lasting 1–1000 ms were applied with an interval of ≥10 s. Exchange time of 10–90% (<100 μs) was determined before each set of experiments by monitoring the change in the liquid junction potential evoked by the application of a 10% diluted control solution to the open tip of the patch pipette (23). For the experiments requiring fast solution exchange between three different conditions (see Figs. 8 and 9), we used a homemade multibarreled application system composed of three horizontally aligned quartz tubes (inside diameter 2.5 mm; outside diameter 3.5 mm; Polymicro Technologies). Solution exchange was achieved by lateral movements, using an SF-77B fast-step perfusion system (Warner Instruments, Hamdell, CT). The complete solution change was achieved in 200–300 μs. Glycine, PTX, picrotoxinin, and picrotin were from Sigma. Stock solution of PTX, picrotoxinin, and picrotin was prepared in dimethyl sulfoxide (Me<sub>2</sub>SO) and then diluted to an appropriate concentration with the above extra-
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cellular solution just before use. The final concentration (v/v) of Me₂SO was not higher than 0.3%, which had no effect on α₂ homomeric GlyRs, as verified by control experiments (data not shown).

Data Analysis—Outside-out currents were analyzed off-line on a G4 Macintosh using Axograph 4.9 software (Axon Instruments). The concentration-inhibition curve of PTX was fitted using the Hill Equation 1,

\[
\frac{I}{I_{\text{Con}}} = \frac{1}{1 + \left(\frac{[\text{PTX}]}{IC_{50}}\right)^n}
\]

(Eq. 1)

where \( I \) is the response in the presence of PTX; \( I_{\text{Con}} \) is the control response (i.e. the glycine response in the absence of PTX); \( IC_{50} \) is the PTX concentration at which half of the glycine response is blocked, and \( n \) is the Hill coefficient. For each concentration tested, the amplitude of the current, \( I \), was measured at the steady-state level.

To determine the voltage dependence of the inhibition of glycine-evoked currents by picrotoxinin and picrotin, voltage ramps were generated using pCLAMP6 software from −100 to +100 mV to be applied to outside-out patches (\( V_{H} = −50 \) mV). The voltage ramps (300-ms duration) were applied during the stable phase of the glycine-evoked responses in the presence and absence of alkaloids. A voltage ramp was applied in the absence of drug and the elicited current was subtracted from the current measured in the presence of drugs.

The activation time constants of glycine-evoked currents in the presence and absence of picrotoxinin or picrotin were estimated by fitting the onset of the responses with a sum of exponential curves using Axograph 4.9 software. Decay time constants were obtained by fitting the first 750 ms of the decay phase with a sum of exponential curves using Axograph 4.9 software (Axon Instruments). The presence of one or more exponential components was tested by comparing the sums of squared errors (SSEs) of the fits (23, 24).

For single channel analysis, patches with one channel were included only if channel activity was stable over sweeps. Open and closed time durations were measured manually using Axograph 4.9 software. For display purposes, open and closed time histograms show the distributions as log intervals with the ordinate on a square root scale. These distributions were fitted with the sum of several exponential curves. The fit was optimized with the least square method (25). The number of exponential components was determined by comparing the SSEs of the fits.

Kinetic Modeling Programs—To obtain a kinetic model for effects on GlyR behavior, glycine-evoked currents were analyzed off-line, and the inhibitory effect of picrotoxinin and picrotin on GlyR kinetics was mathematically modeled using the chemical kinetic modeling programs included in the Axograph 4.9 software package (Axon Instruments). This program first calculated the change in the number of channels in each given state for given rate constants, and then systematically varied the rate constants to give the minimum SSEs between the experimental data and a given model transient (24). Outside-out responses from 12 patches evoked by the application of glycine in the absence or presence of picrotoxinin or picrotin were used for kinetic modeling analysis. Models were compared using the resulting SSE values of the fit.

Averaged data are expressed as mean ± S.E., except when stated otherwise. Statistical significance of the data was assessed by means of Student’s \( t \) test or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post tests when significance was reached.

RESULTS

Concentration-dependent Inhibition of α₂ Homomeric GlyR by Picrotoxinin and Picrotin—We first analyzed the relative potencies of picrotin and picrotoxinin in inhibiting GlyR activity in terms of the outside-out current evoked by glycine applications to outside-out patches containing α₂ homomeric GlyR from CHO cells stably expressing the α₂ GlyR subunit. Fig. 1, A1–A4, illustrates the inhibitory effect of the same concentration (3 μM; near the IC₅₀ for PTX inhibition curve) (see 9) of PTX, picrotin, and picrotoxinin on inward currents (holding
potential ($V_{m}$) = −50 mV) evoked by 300 μM glycine (near the EC$_{50}$ for glycine-evoked response) (20). In these experiments, glycine was co-applied with PTX, picrotin or picrotoxinin. As shown in Fig. 1, A2–A4, co-application of 3 μM picrotin failed to inhibit the current activated by 300 μM glycine, whereas 3 μM picrotoxinin or 3 μM PTX inhibited about half of the glycine-elicited current (Fig. 1, A3 and A4). The percentage block was $3.4 \pm 2.5\%$ (n = 9) for 3 μM picrotin and 50.0 ± 3.5% (n = 7) and 54.7 ± 5.7% (n = 8) for 3 μM picrotoxinin and 3 μM PTX, respectively. The effect of picrotin and picrotoxinin was reversible after washout.

This difference between picrotin and picrotoxinin in their effectiveness in inhibiting glycine-evoked current is related to a difference in potency between these two drugs. Fig. 1B shows the concentration-response curves for picrotin and picrotoxinin co-applied with 300 μM glycine. For comparison, we also show the concentration-response curve for PTX that we published previously (Fig. 1B, gray curve) (9). Concentration-response curves were fitted with the Hill equation (see “Materials and Methods”) giving an IC$_{50}$ (half-maximum inhibition) and a Hill coefficient for picrotoxinin of 2.4 ± 0.2 μM and 0.79 ± 0.05, respectively. These values were not significantly different (unpaired t test, $p > 0.1$) from values we previously published for PTX (IC$_{50}$ = 2.7 ± 0.2 μM and Hill coefficient = 0.8 ± 0.04) (9).

When compared with picrotoxinin, the concentration-response curve for picrotin showed a >30-fold higher IC$_{50}$. The IC$_{50}$ value and Hill coefficient for picrotin were 117.3 ± 14.3 μM and 0.89 ± 0.09, respectively. These results indicate that, unlike what is observed on GABA$_{A}$Rs, picrotin can inhibit α$_{2}$ homomeric GlyRs. But these two PTX components were not equally effective in blocking α$_{2}$ homomeric GlyR, which contrasts with α$_{1}$ homomeric GlyR (17). According to the concentration-response curves shown in Fig. 1B, GlyR inhibition evoked by PTX is likely to be mainly mediated by picrotoxinin. Indeed, the IC$_{50}$ values for PTX and picrotoxinin are identical. This is not surprising because PTX has a molecular weight (602.57) twice that of picrotoxinin (292.28) and picrotin (310.29).

Hence, PTX is an equimolecular mixture of picrotoxinin and picrotin. If picrotoxinin and picrotin were equally active on α$_{2}$ homomeric GlyR, the IC$_{50}$ value for PTX would be lower (half of) than the IC$_{50}$ values for picrotoxinin and for picrotin if they were both equally active. This was the case for α$_{1}$ homomeric GlyR (17).

To determine whether picrotoxinin and/or picrotin inhibitory effects are also dependent on glycine concentrations, as observed for PTX inhibition (9), we compared the blocking effects of picrotoxinin (10 μM) and picrotin (300 μM) on 300 μM and 30 mM glycine-activated currents (Fig. 2, A and B). These concentrations of picrotoxinin and picrotin were chosen to obtain an inhibition of glycine-evoked current above the IC$_{50}$ of the two compounds. The percentage block evoked by 10 μM picrotoxinin of the responses elicited by a saturating concentration of glycine (30 mM) was 47.4 ± 5.1% (n = 8), which is significantly different from its block observed on 300 μM glycine-elicited responses (78.9 ± 3.5%, n = 9; unpaired t test, $p < 0.01$) (Fig. 2C). The percentage block of 30 mM and 300 μM glycine was co-applied with PTX, picrotin or picrotoxinin. As shown in Fig. 1, B1, responses to 30 mM glycine (control) and to co-application of 300 μM picrotoxin were equally active on homomeric GlyR, which contrasts with α$_{1}$ homomeric GlyR. The concentration-response curves for picrotoxinin and picrotin were 117.3 ± 14.3 μM and 0.89 ± 0.09, respectively. These results indicate that, unlike what is observed on GABA$_{A}$Rs, picrotin can inhibit α$_{2}$ homomeric GlyRs. But these two PTX components were not equally effective in blocking α$_{2}$ homomeric GlyR, which contrasts with α$_{1}$ homomeric GlyR (17). According to the concentration-response curves shown in Fig. 1B, GlyR inhibition evoked by PTX is likely to be mainly mediated by picrotoxinin. Indeed, the IC$_{50}$ values for PTX and picrotoxinin are identical. This is not surprising because PTX has a molecular weight (602.57) twice that of picrotoxinin (292.28) and picrotin (310.29).

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A1

Picrotoxin

300 μM glycine
+50 mV
-50 mV
Control

glycine+3 μM PXN

A2

Picrotin

300 μM glycine
+50 mV
-50 mV
Control

glycine+100 μM PTN

B1

3 μM PXN

Percent change of block

Holding potentials (mV)

B2

100 μM PTN

Percent change of block

Holding potentials (mV)

C1

Amplitude (pA)

Voltage (mV)

300 mM glycine

+3 μM PXN

+100 μM PTN

C2

3 μM PXN

Percent change of block

Holding potentials (mV)

C3

100 μM PTN

Percent change of block

Holding potentials (mV)

FIGURE 3. Voltage-dependent inhibition of glycine response by picrotoxinin and picrotin. A1 and A2, responses to 300 μM glycine (control) and to co-application of 300 μM glycine with either 3 μM picrotoxinin (A1) or 100 μM picrotin (A2) at V_h of +50 and −50 mV. Note that the rebound current at +50 mV is larger than that at −50 mV in the presence of 100 μM picrotin. Each trace represents the average of 10–12 trials. The thick line represents the application of 300 μM glycine, A1 and A2 were obtained from different patches. B1 and B2, summary results for percentage block by either 3 μM PXN (B1) or 100 μM PTN (B2) at V_h = +50 and −50 mV. NS, not significant. Data were averaged from six patches for both picrotoxinin and picrotin. B1, there was no significant difference in the intensity of block evoked by 3 μM PXN at V_h = −50 and at V_h = +50 mV (Student’s paired t test p > 0.1). B2, in contrast, a significant difference between the percentage block by 100 μM PTN was obtained between V_h = −50 and V_h = +50 mV (Student’s paired t test, p = 0.01). C1, example of a recording showing current evoked by a voltage ramp from −100 to +100 mV in the presence of 300 μM glycine, in the presence of glycine + 3 μM PXN, and in the presence of glycine + 100 μM PTN (ramp duration = 300 ms; V_h = −50 mV). The current evoked by the voltage ramp in the absence of glycine was subtracted from the experimental current obtained in the presence of glycine. C2 and C3, plot of the percentage block by co-application of 3 μM PXN and 300 μM glycine (C2) or 100 μM PTN and 300 μM glycine (C3) at every 10 mV as a function of the holding potentials from −100 to +100 mV. Data were averaged from six patches and fitted by linear regression giving slope factors of 0.007 and 0.069 for 3 μM PXN and 100 μM PTN, respectively. *, statistical significance p < 0.05.

Voltage Dependence of Picrotoxin-induced Inhibition and Picrotin-induced Inhibition—In our previous study (9), we showed that PTX-induced inhibition on α₂ homomeric GlyR is not voltage-dependent. As opposed to what was observed for PTX or picrotoxin inhibition (Fig. 3A1), a large rebound current was observed during GlyR deactivation immediately after the termination of co-application of >30 μM picrotin and glycine (Fig. 3A2). Such a large rebound current was described for the open channel block effect of acetylcholine on nicotinic receptors (26, 27). It is therefore possible that picrotin could act as a fast open channel blocker on α₂ homomeric GlyR. In such a hypothesis, the inhibitory effect of picrotin might be voltage-dependent, which should not be true for picrotoxinin.

To test this hypothesis, the voltage sensitivity of the picrotoxinin- and picrotin-induced inhibition of the glycine current was examined at different holding potentials (V_h). Typical examples of 300 μM glycine-evoked currents (1-s application step) at V_h of +50 and −50 mV with and without co-application of 3 μM picrotoxinin or 100 μM picrotin are shown in Fig. 3, A1 and A2, respectively. Both picrotoxinin and picrotin inhibited glycine-evoked currents at both positive and negative V_h. As already stated above, a transient rebound current could clearly be observed before current relaxation when co-application of 100 μM picrotin and 300 μM glycine terminated simultaneously (Fig. 3A2). Interestingly, the rebound current at a positive holding potential was relatively larger than the one observed at a negative holding potential, which indicates that the picrotin-induced inhibition on α₂ homomeric GlyR could be voltage-dependent. Accordingly, a significant difference was observed between the percentage block evoked by 100 μM picrotin at V_h of +50 and at −50 mV (paired t test, p < 0.05; Fig. 3B2), although there was no significant difference

glycine-elicited responses by 300 μM picrotin was also significantly different (unpaired t test, p < 0.01); it was 36.3 ± 3.8% (n = 8) for 30 mM glycine and 63.7 ± 3.6% (n = 8) for 300 μM glycine (Fig. 2D). These results indicate that although picrotoxinin and picrotin have different efficiencies, they share some common features, as a competitive-like inhibitory mechanism on α₂ homomeric GlyR.

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between the percentage block at $V_H$ of $+50$ and $-50$ mV by 3 μM picrotoxinin (paired $t$ test, $p > 0.1$; Fig. 3B1).

To further characterize the voltage dependence of picrotoxinin and picrotin inhibition of glycine-evoked current, the current-voltage (I-V) relationship of 300 μM glycine-evoked currents in the absence and presence of 3 μM picrotoxinin or 100 μM picrotin was analyzed from -100 mV to +100 mV using ramp voltage clamp protocols (see “Materials and Methods”) (Fig. 3C1). The averaged percentage block by co-application of 3 μM picrotoxinin and 300 μM glycine ($n = 6$) or 100 μM picrotin and 300 μM glycine ($n = 6$) was calculated every 10 mV (Fig. 3C2). As shown in Fig. 3C3, picrotoxinin block was voltage-dependent, whereas picrotoxinin block was not (Fig. 3C2). Picrotin block increased with voltage. This is consistent with an open channel block mechanism on an anionic channel with a chloride equilibrium potential close to 0 mV (see “Materials and Methods”). Nevertheless, the voltage dependence of the block evoked by picrotin was relatively modest when compared with what is known, for example, for the open channel block of acetylcholine on nicotinic receptors (26, 27). Indeed, for picrotin, the block increased with a limiting slope of exponential-fold/730 mV of depolarization, although it was e-fold/32 mV for acetylcholine block on nicotinic receptors (26, 27). The differential voltage dependences of picrotoxinin- and picrotin-inhibited inhibitions could suggest that picrotin goes deeper than picrotoxinin within the membrane electric field.

Picrotoxinin and Picrotin Accelerate the Deactivation Kinetics of Glycine-induced Current—PTX accelerates the deactivation kinetics of $\alpha_2$ homomeric GlyR in a concentration-dependent manner (9). According to the data presented above, it is likely that picrotoxinin can also accelerate the deactivation kinetics of $\alpha_2$ homomeric GlyR. This could also be the case for picrotin if this PTX component can block the GlyR channel. To determine whether the two PTX components picrotoxinin and picrotin have similar effects on GlyR deactivation kinetics, we first compared the deactivation phase of responses evoked by a short (1 ms) step of a saturating concentration of glycine (30 mM) in the continuous presence of different concentrations of picrotoxinin or picrotin. As shown in Fig. 4A1, a continuous application of picrotoxinin accelerated the deactivation time course of the response. As the picrotoxinin concentration increased, the deactivation time constants decreased in a concentration-dependent manner (Fig. 4A2). The decay phase of outside-out currents evoked by 1-ms application of 30 mM glycine was well fitted with a single exponential function, giving a deactivation time constant ($\tau_{\text{decay}}$) of 129 ± 11 ms ($n = 11$). In the presence of continuous picrotoxinin, $\tau_{\text{decay}}$ significantly decreased to 79 ± 6 ms for 1 μM picrotoxinin ($n = 9$), 57 ± 3 ms for 3 μM picrotoxinin ($n = 11$), and 29 ± 2 ms for 10 μM picrotoxinin ($n = 11$) (ANOVA test, $p < 0.001$). Interestingly, these values are not significantly different from those obtained for PTX at similar concentrations, as reported previously (9) (unpaired $t$ test, $p > 0.05$).

As shown in Fig. 4B1, picrotin application also decreased the decay time duration of currents evoked by 1-ms application of 30 mM glycine, but this effect was observed at higher concentrations (≥10 μM) than for picrotoxinin. The decay phase in the presence of picrotin could also be fitted with a single exponential function, giving a significant lower $\tau_{\text{decay}}$ for picrotin concentrations >3 μM (ANOVA test, $p < 0.001$; Fig. 4B2); $\tau_{\text{decay}}$ was decreased to 92 ± 4 ms in the presence of 10 μM picrotin ($n = 6$), to 58.8 ± 4.2 ms for 30 μM picrotin ($n = 9$), to 30.7 ± 1.9 ms for 100 μM picrotin ($n = 7$), and to 19.6 ± 1.8 ms for 300 μM picrotin ($n = 6$).

The deactivation phase of the responses evoked by a short concentration pulse of agonist reflects channel reopening before the agonist can dissociate from its binding site(s). In a
simple kinetic model with several liganded closed states as described for homomeric α₂ GlyRs (9, 20), and in the absence of any open channel blocker or inhibitory drugs promoting closed states from the open state, the deactivation time constant is a good approximation to the mean burst duration. For homomeric α₂ GlyRs, the mean burst duration during deactivation depends on the mean open time of the channel, on the mean closed time, on the number of openings, and on the number of closures (see Ref. 9 for detailed explanation). Slow blockers must shorten the openings on average and limit reopening of the channel during relaxation. Such slow blockers will appear to speed up relaxation as observed previously for PTX (9). Faster blockers should evoke a biphasic relaxation (28). In our experiments, both picrotoxinin and picrotin decreased in a concentration-dependent manner the deactivation time constant of the current evoked by a short concentration step of glycine (Fig. 4). In the experiments described above, the relaxation of currents evoked by a short concentration step of glycine in the presence of picrotoxinin or of picrotin could be fitted by a single exponential curve. This favors the hypothesis of slow blocker-like mechanisms for both compounds. If this were true, both picrotoxinin and picrotin should decrease the mean open time of the channel to the same extent. Nevertheless, the picrotin concentration must be increased 10 times to obtain an effect similar to picrotoxinin on the deactivation time constant of the glycine-evoked currents. Accordingly, this could be achieved either by a lower association rate for picrotin than for picrotoxinin, and/or a larger number of reopenings within a burst in the presence of picrotin compared with picrotoxinin, because the deactivation time constant reflects the mean open burst duration (28).

To determine the microscopic determinants of the decrease in the decay time constant observed in the presence of picrotoxinin and picrotin, we analyzed the open time and closed time distributions in single receptor burst of openings in response to short (1 ms) concentration pulses of glycine near GlyR saturation (30 mM) in the absence and presence of 10 μM picrotoxinin or 30 μM picrotin. To perform this analysis, patches with a single functional GlyR were selected (i.e. patches that did not display superimposed openings in response to a saturating concentration of agonist; see Ref. 20). Single openings and closures were manually detected and measured using a filter cut-off frequency of 5 kHz. In control conditions, GlyR opens in bursts of long openings interrupted by very short closures (Fig. 5A1). In the continuous presence of 10 μM picrotoxinin or 30 μM picrotin (Fig. 5, B1 and C1), the single opening duration appeared to be shortened, but more flickering of channel openings was observed in the presence of picrotin.

Opening and closing time constants were estimated by pooling measurements made on single channel responses obtained from 3 to 9 patches. The open time histograms were best fitted by single exponential curves both in control conditions and in the continuous presence of picrotoxinin or picrotin (Fig. 5, A2, B2, and C2). In control conditions, the mean open time was 47.9 ms, which is consistent with the values we previously obtained (9, 20). The mean open time was decreased to 9.3 ms in the presence of 10 μM picrotoxinin. This was closely similar to what we previously observed in the presence of PTX (6.1 ms) (9). But although 30 μM picrotin had a lower effect than 10 μM picrotoxinin on the deactivation time constant of glycine-evoked current (Fig. 4), the mean open time was decreased to 2.9 ms in the presence of 30 μM picrotin, which was lower than the decrease observed in the presence of 10 μM picrotoxinin.

In control conditions, the closed time distribution was best fitted by a single exponential curve with a closed time constant \( \tau_c = 0.23 \) ms (Fig. 5A3), as described previously for homomeric α₂ GlyRs (9, 20). In the presence of picrotoxinin (10 μM), the closed time distribution was best fitted by the sum of two exponential curves giving time constants of \( \tau_{c1} = 0.24 \) ms and \( \tau_{c2} = 5.72 \) ms (Fig. 5B3), which are also very similar to the values obtained for PTX (\( \tau_{c1} = 0.23 \) ms and \( \tau_{c2} = 5.76 \) ms) (9). The closed time distribution was also best fitted by the sum of two exponential curves in the presence of picrotin (30 μM) giving \( \tau_{c1} = 0.29 \) ms and \( \tau_{c2} = 1.76 \) ms (Fig. 5C3). As suggested previously (9), the longer closed time is likely to reflect an additional recovery pathway from picrotoxinin- or picrotin-evoked open channel block.

The ensemble-averaged currents obtained by averaging single channel responses (108 trials for 30 mM glycine, 117 trials in the continuous presence of 10 μM picrotoxinin, and 77 trials for 30 μM picrotin) indicated a \( \tau_{decay} \) similar to that observed for macroscopic glycine currents in the absence and presence of continuous picrotoxinin or picrotin (Fig. 5, A1, B1, and C1, lower parts). \( \tau_{decay} \) was 158.8 ms for the averaged current in the absence of blocker. It was 35.5 and 53.4 ms, respectively, for the averaged current in the presence of continuous picrotoxinin or in the presence of continuous picrotin. Altogether these results indicate that the decrease in the deactivation time constant evoked by picrotoxinin and by picrotin could mainly be due to a decrease in the mean open time of the channel. To explain our observation of a shorter open time constant and a longer \( \tau_{decay} \) in the presence of 30 μM picrotin, compared with the values obtained in the presence of 10 μM picrotoxinin, one must assume that channel reopenings occur to a larger extent during the deactivation phase of glycine-evoked current in the presence of picrotin. This was indeed exemplified in Fig. 5C1.

To obtain more precise information on the block mechanism of picrotoxinin and picrotin, we analyzed the effect of increasing picrotoxinin or picrotin concentrations on the mean open time of the GlyR channel. Such analyses were also intended to gather information about the blocking rate constants of picrotoxinin and of picrotin (28). We analyzed the open time distributions in single receptor bursts of openings in response to 300 μM concentration pulses (1 s) of glycine. Opening time constants were analyzed by pooling measurements made on 8 – 61 sweeps from 1 to 3 patches. Histograms of the open durations within bursts in the absence and presence of picrotoxinin or picrotin were constructed and best fitted by single exponential curves at all picrotoxinin or picrotin concentrations tested (Fig. 6). As expected, both picrotoxinin and picrotin decreased the mean open time in a concentration-dependent manner. The mean open time for the control response was 51.7 ms (61 trials from three patches; Fig. 6A). In the presence of picrotoxinin, the mean open time decreased to 19.6, 8.5, 2.9, and 1.0 ms for 3 μM picrotoxinin (61 trials from two patches), 10 μM picrotoxinin (52 trials from two patches), 30 μM picrotoxinin (42 trials...
from one patch), and 100 μM picrotoxinin (29 trials from two patches), respectively (Fig. 6, B1–B2 and D). In the presence of picrotin, the mean open time decreased to 26.6, 7.8, 2.9, and 1.2 ms for 3 μM picrotin (23 trials from two patches), 10 μM picrotin (23 trials from one patch), 30 μM (18 trials from two patches), and 100 μM picrotin (14 trials from one patch), respectively (Fig. 6, C1–C2 and D). These results indicate that both picrotoxinin and picrotin inhibition can be related to an open channel block mechanism (28).

To obtain an approximation of the binding rate constants for picrotoxinin and picrotin, the reciprocals of the mean open times were plotted as a function of the picrotoxinin or picrotin concentration as shown in Fig. 6D. Binding (k_on) and closing (α) rate constants were calculated from the relationship 1/τ_o = [P]k_on + α (20), where τ_o is the mean open time, and [P] is the picrotoxinin or picrotin concentration. The linear fit to the data gave k_on and α values of 9.8 μM^{-1} s^{-1} and 26.2 s^{-1} for picrotoxinin and 8.1 μM^{-1} s^{-1} and 26.1 s^{-1} for picrotin. These results demonstrate that binding and closing rate constants are quite similar for picrotoxinin and picrotin. This further suggests that the longer τ_decay for picrotin compared with picrotoxinin can only be explained by a larger number of channel reopenings during the deactivation of glycine-evoked currents.

Differential Effects of Picrotoxinin and Picrotin on the Activation Kinetics of Glycine-evoked Current—Our previous study demonstrates that continuous application of PTX slows down the activation of saturating concentrations of glycine-activated current. Therefore, the effects of picrotoxinin and picrotin on the rising phase of macroscopic averaged currents evoked by a saturating concentration of glycine (30 mM) on α_2 homomeric GlyRs were analyzed (Fig. 7). In control conditions, the rising phase of the current evoked by 30 mM glycine was well fitted with the sum of two exponential curves in 8 of 10 patches (in the other two patches the rising phase was fitted with a single exponential function; see Ref. 20). Our analysis of picrotoxinin and picrotin effects focused on
FIGURE 6. Both picrotoxin and picrotin decreased the mean open time of GlyR in a concentration-dependent manner. A–C2, open time duration histograms obtained in control conditions (300 μM glycine, 1-s application; A), in the continuous presence of 3 μM and 10 μM picrotoxin (B1 and B2), and 3 μM picrotin and 10 μM (C1 and C2) are shown as a function of log intervals with the ordinate on a square root scale. D, reciprocals of the mean open times were plotted as a function of the PXN or PTN concentration, and the binding (k_on) and closing (k_off) rate constants were calculated from the relationship 1/τ = [P]k_on + k_off, where τ is the mean open time and [P] is the picrotoxin or picrotin concentration. Mean open times were obtained by pooling single channel currents from 61 trials in three different experiments for controls, 29–61 trials in 1–2 different experiments for each concentration of picrotoxin, and 8–23 trials in 1–2 different experiments for each concentration of picrotin.

those eight patches. Fig. 7, A1 and B1, shows the activation phase of the responses evoked in the same patch by 30 mM glycine, by co-application of 30 mM glycine with 10 μM picrotoxin or 300 μM picrotin, and by 30 mM glycine in the continuous presence of 10 μM picrotoxin or 300 μM picrotin. Similarly to what was observed for 10 μM PTX (9), the activation phase of the glycine response was obviously slowed down in the continuous presence of 10 μM picrotoxin. A continuous application of 300 μM picrotin also slowed down the activation phase of the glycine response, although to a lesser extent than 10 μM picrotoxin. As shown in Fig. 7, A2 and A3, the time constants for the glycine response in the control conditions were τ_fast = 0.36 ± 0.04 ms (82 ± 3%) and τ_slow = 2.1 ± 0.3 ms (18 ± 3%; n = 8). Simultaneous application of 30 mM glycine and 10 μM picrotoxin did not significantly change the rising time constants and relative area of the rising time constants, giving τ_fast = 0.28 ± 0.02 ms (81 ± 5%) and τ_slow = 2.0 ± 0.4 ms (19 ± 5%; n = 8) (paired t test, p > 0.1). However, when 10 μM picrotoxin was continuously applied before, during, and after 30 mM glycine successive concentration steps (application frequency 0.1 Hz), the rising phase of the first glycine-evoked response was unchanged, although it was slowed down for the next responses (data not shown). This picrotoxin effect on the rising phase of glycine-evoked responses mimicked that of PTX. In this case, both τ_fast and τ_slow were significantly increased (paired t test, p < 0.01; Fig. 7A2) as follows: τ_fast = 2.4 ± 0.5 ms (37 ± 11%) and τ_slow = 10.1 ± 1.2 ms (63 ± 11%; n = 8). Note also that the relative area of τ_fast was significantly decreased, whereas that of τ_slow was significantly increased (paired t test, p < 0.01; Fig. 7A3). The same measurements were performed for 300 μM picrotin. As shown in Fig. 7, B2 and B3, the time constants for the glycine response in control conditions (30 mM glycine) were τ_fast = 0.35 ± 0.03 ms (83 ± 2%) and τ_slow = 2.1 ± 0.3 ms (17 ± 2%; n = 8). Simultaneous application of 30 mM glycine and 300 μM picrotin did not significantly change the rising time constants of the glycine-evoked response (paired t test, p > 0.1), although τ_fast had a significantly higher relative area (paired t test, p < 0.05) as follows: τ_fast = 0.25 ± 0.09 ms (91.1 ± 3%) and τ_slow = 2.98 ± 0.8 ms (8.9 ± 3%; n = 8). When 300 μM picrotin was continuously applied before, during, and after 30 mM glycine successive concentration steps (application frequency = 0.1 Hz), the rising phase of the first glycine-evoked response was unchanged, although it was slowed down for the next responses, as also mentioned for picrotoxin (data not shown). The rising phase of the glycine response in the continuous presence of 300 μM picrotin gave τ_fast = 0.48 ± 0.04 ms (77 ± 4%) and τ_slow = 5.2 ± 0.7 ms (23 ± 4%; n = 8), which were significantly increased (paired t test, p < 0.01) compared with those in control conditions (Fig. 7B2). The relative area of τ_fast and τ_slow in the presence of 300 μM picrotin was, however, not changed when compared with control (paired t test, p > 0.1; Fig. 7B3).

Finally, we observed that both τ_fast and τ_slow were significantly faster in the presence of continuous 300 μM picrotin than in the continuous presence of 10 μM picrotoxin (paired t test, p < 0.01). This could indicate that recovery from block is faster in the presence of picrotin (see below).

Recovery from Picrotoxin or Picrotin Block Requires Channel Reopening—The lengthening of the rise time of glycine-evoked outside-out current was also observed in the presence of PTX on α2 homomeric GlyR, and it was proposed to reflect recovery from channel block (9) rather than being the consequence of PTX binding to the unliganded receptor as proposed for GABA_A R in the crayfish muscle (29). To determine whether the lengthening of the rise time of glycine-evoked outside-out
As shown in Fig. 9A, the block evoked by picrotin and its recovery time course were faster than for picrotoxinin. In the 11 outside-out patches tested, the time course of picrotin block was best fitted by a bi-exponential curve with time constants \( \tau_{\text{fast}} = 0.5 \pm 0.1 \text{ ms (69.4 \pm 3.8\%)} \) and \( \tau_{\text{slow}} = 6.9 \pm 1.7 \text{ ms (30.6 \pm 3.8\%)} \). In these patches, the current amplitude again increased progressively at the end of picrotin application, with a time course best fitted by a bi-exponential curve with recovery time constants \( \tau_{\text{fast}} = 0.64 \pm 0.08 \text{ ms (63.9 \pm 5.1\%)} \) and \( \tau_{\text{slow}} = 14.2 \pm 3.1 \text{ ms (36.1 \pm 5.1\%)} \).

If picrotin or picrotin bind to the unliganded receptor, picroxin or picrotin preincubation should result in a lengthening of the rise time of the glycine-evoked response. This was not the case. When 10 \( \mu M \) picrotin or 100 \( \mu M \) picrotin was applied immediately before (time interval <0.1 ms) the application of a saturating concentration of glycine (10 mM), they did not change the amplitude of the glycine-evoked outside-out currents or their rising phase (Figs. 8B and 9B). The activation time constants were \( \tau_{\text{fast}} = 0.50 \pm 0.06 \text{ ms (90.4 \pm 4.7\%)} \) and \( \tau_{\text{slow}} = 1.97 \pm 0.52 \text{ ms in control conditions, and } \tau_{\text{fast}} = 0.50 \pm 0.05 \text{ ms (80.6 \pm 8.6\%)} \) and \( \tau_{\text{slow}} = 1.71 \pm 0.19 \text{ ms with 10 \( \mu M \) picrotin preincubation, or } \tau_{\text{fast}} = 0.55 \pm 0.14 \text{ ms (86.8 \pm 9.2\%)} \) and \( \tau_{\text{slow}} = 2.83 \pm 1.25 \text{ ms with 100 \( \mu M \) picrotin preincubation.} \)

These results make it unlikely that the lengthening of the rising phase of glycine-evoked current we observed in the continuous presence of picroxin or picrotin results from picroxin or picrotin binding to unliganded \( \alpha_2 \) GlyR. These results are also consistent with our study that the activation time course of the first response evoked by glycine application in the continuous presence of picroxin or picrotin was unchanged. As suggested previously for PTX, the lengthening of the rise time we observed for the following responses in the continuous presence of picroxin or picrotin could be explained by picroxin or picrotin binding to GlyRs when channels are open and then being trapped at their binding sites after glycine washout.

To test this hypothesis, we used the same protocol that we described previously for PTX (9). First, we analyzed the effect of picroxin or picrotin on the activation phase of successive outside-out currents evoked by glycine applications (10 mM) when picroxin or picrotin was applied during the deactivation phase of the first response (post-treatment). We preferentially used patches with a high GlyR density to obtain a good description of the time course of the response on a single trace. Picroxin or picrotin was applied during 500 ms, which corresponds to the duration of the deactivation phase of glycine-evoked currents in control conditions. Glycine was then applied alone 60 s after picrotin or picroxin post-treatments and finally a last time after 10 s to determine whether the effect of picroxin or picrotin was reversible. As shown in Fig. 8C, post-treatment with 10 \( \mu M \) picroxin shortened the deactivation phase of the glycine-evoked current. The current evoked by glycine alone 60 s after the end of glycine-evoked current plus picroxin or picrotin post-treatment had an amplitude similar to that of control response (<4% decrease \( n = 5 \)). However, those currents had a significantly slower rising phase when compared with control responses. This was related to a significant increase in the rising time constants \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) of the glycine-evoked current.
PXN and PTN Inhibition of $\alpha_2$ Homomeric GlyR

**FIGURE 8. Differential recovery from picrotoxinin block.** A1, average of five traces of current obtained in response to a 600-ms step application of 10 mM glycine and transiently inhibited by a 300-ms step application of 10 $\mu$M PXN with 10 mM glycine. The dashed boxes in A1 indicate parts of the trace enlarged in A2 (left box) and A3 (right box). A2, the onset of the picrotoxinin inhibition was well fitted by a mono-exponential curve (gray dashed line) giving a time constant of 13 ms. A3, the recovery from the inhibition by PXN was best fitted by a bi-exponential curve (gray dashed line) giving time constants $\tau_{\text{fast}} = 6$ ms (38%) and $\tau_{\text{slow}} = 37$ ms (62%). B1, average of four traces showing currents evoked by a 300-ms step application of 10 mM glycine following a 300-ms step application of control solution (left black trace) or 10 $\mu$M PXN (right gray trace). Dashed boxes in B1 indicate the part of the traces enlarged in B2. B2, the onset of both responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (control preincubation) and in gray (PXN preincubation). Note the absence of effect of the PXN preincubation. C1, example of three consecutive responses to 100 $\mu$M glycine with time constants $\tau_{\text{fast}} = 0.4$ ms (86%) and $\tau_{\text{slow}} = 37$ ms (14%). C2, the onset of the picrotoxinin inhibition was well fitted by a mono-exponential curve (gray dashed line) giving a time constant of 2.5 ms. Dashed boxes indicate the part of the two first traces enlarged in C2. C2, the onset of the first and second responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (1st application) and gray (2nd application).

$\tau_{\text{fast}} (n = 5; \text{paired } t \text{ test, } p < 0.01)$ and an increased proportion of the slow component. ($n = 5; \text{paired } t \text{ test, } p = 0.051$). The activation time course of the responses evoked 60 s after picrotoxinin post-treatment was well fitted with the sum of two exponential curves, as in the control, with time constants $\tau_{\text{fast}} = 4.15 \pm 1.24$ ms (53.6 $\pm$ 7.9%) and $\tau_{\text{slow}} = 18.5 \pm 9.3$ ms. Applying glycine 10 s after the response with the slower rising phase evoked an outside-out current with activation time constants similar to that of the control (corresponding to the very first application) as follows: $\tau_{\text{fast}} = 0.42 \pm 0.05$ ms (65.6 $\pm$ 4.6%) and $\tau_{\text{slow}} = 2.31 \pm 0.39$ ms. These results clearly indicate, as observed previously for PTX (9), that the effect of picrotoxinin on the rising phase of the glycine response can persist for up to 60 s after the wash-out of glycine and picrotoxinin. It is therefore likely that picrotoxinin can be trapped at its binding site when the channel closes and/or when glycine dissociates from its binding sites.

Although continuous application of picrotin also lengthened the activation phase of glycine-evoked responses, the recovery time course of the glycine response amplitude observed at the end of picrotin application is considerably faster than that observed after picrotoxinin application, suggesting a faster dissociation rate for picrotin from its binding site. It is therefore possible that picrotin cannot be trapped when the channel closes because it would have dissociated from its binding site before the channel closes. To resolve this issue we used the same protocol as described above for picrotoxinin.

As shown in Fig. 9C, post-treatment with 100 $\mu$M picrotin greatly shortened the deactivation phase of the glycine-evoked current. As observed for picrotoxinin, picrotin post-treatment had no effect on the current amplitude of the glycine responses evoked 60 s after the end of the glycine-evoked current plus picrotin post-treatment (<4.5% of...
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Differential recovery from picrotin block. Figure 9 shows average of five traces of current obtained in response to a 600-ms step application of 10 mM glycine and transiently inhibited by a 300-ms step application of 100 μM PTN with 10 mM glycine. The dashed boxes in A1 indicate parts of the trace enlarged in A2 (left box) and A3 (right box). A2, the onset of PTN inhibition was well fitted by a bi-exponential curve (gray dashed line) giving time constants τ_fast = 0.7 ms (78%) and τ_slow = 4.8 ms. A3, recovery from the inhibition by PTN was also best fitted by a bi-exponential curve (gray dashed line) giving time constants τ_fast = 0.7 ms (72%) and τ_slow = 9.4 ms.

B1, average of five traces showing currents evoked by a 300-ms step application of 10 mM glycine following a 300-ms step application of control solution (left black trace) or 100 μM PTN (right gray trace). Dashed boxes in B1 indicate the part of the traces enlarged in B2. B2, onset of both responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (control preincubation) and in gray (PTN preincubation). Note the absence of effect of the PTN preincubation.

C1, example of three consecutive responses to 200-ms step application of 10 mM glycine where the first application was directly followed by a 500-ms step application of 100 μM PTN. The interval between each application is indicated between each trace. Note the quickening in the decay of the first glycine response during the PTN application. Dashed boxes indicate the part of the two first traces enlarged in C2. C2, the onset of the first and second responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (1st application) and gray (2nd application). Note that there is no significant difference for τ_fast and a slight lengthening of τ_slow between the first and the second application.

These results clearly indicate that picrotin can also be trapped at its binding site when the GlyR channel closes and/or when glycine dissociates from its binding sites. The lack of effect of picrotin post-treatment on τ_fast may also suggest that the likelihood of picrotin being trapped when the channel closes is lower than for picrotoxinin.

Minimal Markov Models for Picrotoxin Inhibition and Picrotin Inhibition—To account for the results obtained on picrotoxinin and picrotin inhibition, we based ourselves on the minimal Markov model previously proposed for homomeric α2 GlyR subtype (9). This model has three binding sites for glycine and three desensitization closed states and a single open state linked to the fully liganded closed state. Each desensitization state is linked to the mono-ligated closed state, the doubly liganded closed state, and the triply liganded closed state, respectively. This model is somewhat similar to the model proposed for homomeric α1 GlyR subtype (30), although only one open state linked to the fully liganded closed state decreased, n = 5). Although picrotin post-treatment had a less pronounced effect than picrotoxinin on the activation time course of these currents, the rising phase was significantly slower when compared with control responses. This was mainly related to a significant increase in the slower rising time constant τ_slow (n = 8; paired t test, p < 0.01) and to an increase in the proportion of the slow component (n = 8; paired t test, p < 0.05). The activation time course of the responses evoked 60 s after picrotin post-treatment was well fitted with the sum of two exponential curves, as in the control, with time constants τ_fast = 0.53 ± 0.05 ms (52.7 ± 4.9%) and τ_slow = 4.95 ± 0.8 ms. Applying glycine 10 s after the response with the slower rising phase evoked an outside-out current with activation time constants similar to that of the control, τ_fast = 0.42 ± 0.05 ms (65.6 ± 4.63%) and τ_slow = 2.31 ± 0.91 ms. These results clearly indicate that picrotin can also be trapped at its binding site when the GlyR channel closes and/or when glycine dissociates from its binding sites. The lack of effect of picrotin post-treatment on τ_fast may also suggest that the likelihood of picrotin being trapped when the channel closes is lower than for picrotoxinin.
state is necessary to describe homomorphic $\alpha_2$ GlyR subtype behavior (9, 20). Before testing the Markov models for picrotoxinin or picrotin inhibition, it was necessary to adjust the different rate constants of the model describing glycine-evoked outside-out currents for each control trace (9). To do so, we fitted experimental traces obtained by long application of 0.1–0.3 and 30 mM glycine (9, 20, 31). Table 1 and Table 2 summarize the averaged kinetic parameters derived from model fitting of glycine-evoked responses. This model predicts a glycine EC$_{50}$ of 250 $\mu$M and a Hill coefficient of 2.1, which are in good agreement with published experimental values (200 $\mu$M and 1.9) (9, 20). We then analyzed picrotoxinin or picrotin inhibition responses on the same traces. The different models tested were elaborated according to experimental data.

Those data showed that PTX and picrotoxinin inhibited the homomorphic $\alpha_2$ GlyR subtype in a similar way. We therefore decided to test the two kinetic models we already proposed for PTX (Fig. 10, B and C, model 1 and model 2) on picrotoxinin-evoked inhibition of homomorphic $\alpha_2$ GlyRs. As observed for PTX, the Hill coefficient of the concentration-response curve for picrotoxinin was close to 1. We therefore postulated that only one molecule of picrotoxinin binds to the receptor, as also postulated for picrotoxinin on GABA$_A$ receptors (16). Picrotoxinin is also likely to bind to the receptor in the open conformation because the mean open time of the channel was decreased when picrotoxinin concentration was increased, giving an estimated association rate constant of 9.8 $\mu$M$^{-1}$ s$^{-1}$, which is close to the association rate constant previously estimated for PTX (11.6 $\mu$M$^{-1}$ s$^{-1}$) (9). Picrotoxinin had no effect when applied immediately before glycine application (Fig. 8B1), although the activation time constants of the glycine-evoked outside-out current was increased by picrotoxinin applied during deactivation even when glycine was applied 60 s after picrotoxinin washout (Fig. 8, CI and C2). This was also the case for PTX (9). Accordingly, picrotoxinin cannot bind directly to the unliganded closed state of the receptor ($A_3$ + C). Moreover, picrotoxinin must bind within the vestibule of the channel (Fig. 10, B and C, A$_3$O to A$_3$PB), whereas it remains trapped when the channel closes (A$_4$PB to A$_4$PC) if glycine dissociates before picrotoxinin (Fig. 10, B and C). In these models, picrotoxinin remains bound when glycine dissociates from its binding sites. This was simulated by three sequential glycine-bound states ($A_3$PC to $A_2$+APC and $A_2$+APC) linked to a glycine-unbound state ($A_3$ + PC). As mentioned previously for PTX (9), adding this bound state accounts for the decrease in the deactivation time constant of the glycine-evoked current observed when picrotoxinin was applied during the relaxation phase (Fig. 8C1). In these models, each glycine-bound state related to the picrotoxinin-bound state ($A_3$PC to $A_2$+APC and $A_2$+APC) was linked to a desensitization closed state ($A_3$PD, $A_2$+APD, and $A_2$+APD), as for each glycine-bound state in the absence of picrotoxinin (see Fig. 10, B and C) (9).

The two models tested (Fig. 10, B and C, model 1 and model 2) described equally well the picrotoxinin-evoked inhibition of the homomorphic $\alpha_2$ GlyRs (9). In model 1, one step was incorporated between the fully glycine-ligated closed state $A_3$C and the corresponding fully glycine-ligated block closed state $A_3$PC. This model supposed that picrotoxinin can dissociate from its binding site only when the glycine receptor is fully liganded. Accordingly, this model contains one cycle scheme (Fig. 10B). Model 2 is somewhat similar to the kinetic scheme recently proposed for GABA$_A$ receptors (16). In this model, PTX or picrotoxinin can dissociate from its binding sites during all glycine-binding steps ($A_2$+AC, $A_4$+C, and $A_4$C; Fig. 10C). Therefore, three steps were incorporated between glycine-ligated closed states and glycine-ligated closed states on which picrotoxinin remains bound ($A_2$+PC, $A_2$+PC, and $A_4$PC; Fig. 10C).

To compare those two models, we adjusted experimental traces obtained by long applications of 0.3 and 30 mM glycine in the presence of 1, 3, and 10 $\mu$M picrotoxinin ($n = 12$ patches). The procedures used for the fit were identical to those described previously for picrotoxinin-evoked inhibition of homomorphic $\alpha_2$ GlyRs (9). All rate constants estimated with the homomorphic $\alpha_2$ GlyR Markov model were set as fixed parameters. The association rate constant ($k_{on}$) and the dissociation rate constant ($k_{off}$) linking the glycine-bound closed states with picrotoxinin ($A_3$+PC, $A_2$+APC, $A_2$+APC, and $A_4$PC), the desensitization rates constants, and the recovery rate constants linking the gly-

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**TABLE 1**

Kinetic parameters for picrotoxinin inhibition derived from models 1 and 2 fitting (mean ± S.E., $n = 12$)

| Model | $k_{on}$ | $k_{off}$ | $k_{att}$ | $k_{det}$ |
|-------|---------|----------|-----------|-----------|
| 1     | $0.768 \pm 0.008 \mu$M$^{-1}$ $s^{-1}$ | $0.768 \pm 0.008 \mu$M$^{-1}$ $s^{-1}$ | $1940.9 \pm 442.6 s^{-1}$ | $90.4 \pm 18.1 s^{-1}$ |
| 2     | $1045.5 \pm 411.8 s^{-1}$ | $3453 \pm 522.5 s^{-1}$ | $1579.8 \pm 683.2 s^{-1}$ | $433.8 \pm 119.8 s^{-1}$ |

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**TABLE 2**

Kinetic parameters for picrotoxinin inhibition derived from models 4 and 5 fitting (mean ± S.E., $n = 11$)

| Model | $k_{on}$ | $k_{off}$ | $k_{att}$ | $k_{det}$ |
|-------|---------|----------|-----------|-----------|
| 4     | $1502.9 \pm 322.37 s^{-1}$ | $57 \pm 17.15 s^{-1}$ | $2371.6 \pm 273.45 s^{-1}$ | $933.7 \pm 446.5 s^{-1}$ |
| 5     | $44.63 \pm 177.62 s^{-1}$ | $446.3 \pm 177.62 s^{-1}$ | $9.55 \pm 2.83 s^{-1}$ | $265 \pm 153.15 s^{-1}$ |

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cine-bound closed states plus picrotoxinin to the corresponding desensitization states (A$_2$+APD, A$_2$+PD, and A$_3$PD) were also set as fixed parameters. All other parameters were set as free parameters. In these two models, it was also necessary to constrain reactions depending on the reaction cycles (one reaction cycle in model 1 and three reaction cycles in model 2) to satisfy the principle of microscopic reversibility (29). As performed previously for picrotoxinin, the association and dissociation rate constants for picrotoxinin linking the glycine-ligated closed states with and without picrotoxinin binding (A$_2$+AC to A$_2$+APC, A$_2$+C to A$_2$+PC, and A$_3$C to A$_3$PC) were set as equivalent (9). Model 1 and model 2 provided a good prediction of our experimental results. As reported previously for PTX (9), model 2 did not give a better prediction of the experimental results. SSFs for these two models were not significantly different (ANOVA test, $p > 0.1$), which indicates that the transitions between A$_2$+AC and A$_2$+APC and between A$_2$+C and A$_2$+PC are not necessary to describe our experimental data.

The optimal averaged rate constant values for these two models are listed in Table 1. These values were closely similar to those obtained for PTX (9), confirming that the inhibition of $\alpha_2$ homomeric GlyR by PTX is likely to be related to picrotoxinin. For picrotoxinin the fits gave $k_{on1P}$ values ($k_{on1P} = 4.2 \pm 0.5 \mu M^{-1} s^{-1}$ for model 1 and $4.2 \pm 0.5 \mu M^{-1} s^{-1}$ for model 2) and $k_{off2P}$ values ($k_{off2P} = 545.7 \pm 220.9 \mu M^{-1} s^{-1}$ for model 1 and $198.5 \pm 46.5 \mu M^{-1} s^{-1}$ for model 2) similar to those previously obtained for PTX ($k_{on1P} = 4.9 \pm 0.9 \mu M^{-1} s^{-1}$ for model 1 and $5.4 \pm 0.9 \mu M^{-1} s^{-1}$ for model 2; $k_{off2P} = 483.5 \pm 121.4 \mu M^{-1} s^{-1}$ for model 1 and $278 \pm 69.4 \mu M^{-1} s^{-1}$ for model 2). This was also the case for the dissociation rate constant values $k_{off1P}$ and $k_{off2P}$ estimated for picrotoxinin (see Table 1). For PTX, $k_{off1P} = 46.9 \pm 11.9 s^{-1}$ for model 1 and $57.8 \pm 10.3 s^{-1}$ for model 2 and $k_{off2P} = 749.8 \pm 198.3 s^{-1}$ for model 1 and $327.9 \pm 83.6 s^{-1}$ for model 2 (9). As also observed for PTX (9), the affinity of picrotoxinin for the GlyR open state (model 1 $k_{off1P}/k_{on1P} = 8.21 \mu M$; model 2 $k_{off1P}/k_{on1P} = 9 \mu M$) was lower than that for the liganded closed states (model 1 $k_{off1P}/k_{on1P} = 0.97 \mu M$; model 2 $k_{off1P}/k_{on1P} = 0.93 \mu M$), the dissociation constants ($k_{off2P}/k_{on1P}$) being similar to the values obtained for PTX (model 1 $k_{off2P}/k_{on1P} = 9.6 \mu M$; model 2 $k_{off2P}/k_{on1P} = 10.7 \mu M$; model 1 $k_{off2P}/k_{on2P} = 1.6 \mu M$; model 2 $k_{off2P}/k_{on2P} = 1.2 \mu M$) (9). If we attempted to set the affinity of picrotoxinin for the open state equal to its affinity for the liganded closed states, the SSE of the fit became $3.9 \pm 0.9$ times significantly

was used for homomeric $\alpha_2$ GlyR in control conditions (without picrotoxinin or picrotin). B. model 1, picrotoxinin can bind and unbind from the fully glycine-ligated closed state or from the open state of GlyR. In this scheme, picrotoxinin is trapped when glycine dissociates from the fully liganded closed state. C. model 2, picrotoxinin can bind and unbind from all glycine-bound states, but picrotoxinin is only trapped when the receptor returns to the glycine-unbound closed state. D. model 3, picrotoxinin can bind and unbind from the GlyR open state only. Picrotoxin remains bound if continuously applied when glycine dissociates from its binding sites. In this model, as in model 4 (E) and model 5 (F), GlyRs cannot further desensitize from the glycine-bound closed states when picroxin is trapped on its binding site. E, as in model 1, model 4 picrotin can bind and unbind from the fully glycine-ligated closed state or from the open state of GlyR only. In this scheme, picrotin is trapped when glycine dissociates from the fully liganded closed state. F, model 5 is identical to model 2 except that, as in models 3 and 4, GlyRs cannot further desensitize from the glycine-bound closed states when picrotin is trapped on its binding site.

FIGURE 10. Kinetic schemes used for fitting glycine responses in the absence and presence of picrotoxinin or picrotin. The abbreviations used are as follows: A, agonist; P, picrotoxinin or picrotin; C, resting states of the receptor; D, desensitization states, and O, open states. A, this kinetic scheme
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FIGURE 11. Prediction of the experimental results for picrotoxinin by kinetic models. A, outside-out currents (gray traces) elicited by glycine (30 mM) in the absence and presence of 10 \( \mu M \) PXN were superimposed on simulated currents using model 1 (black line) \( (V_h = -50 \) mV). B, outside-out currents evoked by co-application of glycine (0.3 mM) and 0, 3, 10, and 30 \( \mu M \) PXN (gray lines) were superimposed on simulated currents using model 1. Model 1 predicts the concentration-dependent inhibition effect of PXN and the time course of glycine-evoked currents when PXN and glycine are co-applied. C, this model also predicts the shift to the right of the PXN inhibition curve when glycine concentration is increased. The concentration-response curves were obtained from theoretical currents generated using model 1. D, theoretical currents obtained with model 1 evoked by a 1-ms pulse of 30 mM glycine during the application of 0, 1, 3, and 10 \( \mu M \) PXN. Peak current amplitude was normalized. Note that this kinetic scheme predicts the PXN-evoked concentration-dependent decrease in the decay phase duration. E, simulated traces of currents showing the time course of the responses evoked by 30 mM glycine, by the co-application of 30 mM glycine and 10 \( \mu M \) PXN and by 30 mM glycine in the continuous presence of 10 \( \mu M \) PXN. F, activation phase of the responses shown in E. Model 1 predicts that the activation phase of the glycine response was slowed down in the continuous presence of PXN. G, simulated traces generated using model 1 showing that this kinetic scheme also predicts that when picrotoxinin was applied during the deactivation phase of the glycine-evoked current, and the lengthening of the rise time evoked by PXN can persist up to 60 s after washout of glycine and PXN. For time course comparisons, the control response on the left was superimposed (in gray) on the other simulated glycine-evoked currents.

higher (ANOVA test, \( p < 0.01 \)), indicating that the rate constant values for these picrotoxinin-binding steps are unlikely to be equivalent, as was observed previously for PTX (9), and as also reported for picrotoxinin-evoked GABA\(_C\) receptors inhibition (16). As discussed previously (9), it is likely that the access of picrotoxinin to its binding site could be different between the glycine-bound closed state conformation and the glycine-bound open state conformation (16). This could be related to the various large conformational changes of the receptor associated with agonist binding and channel gating (16, 32).

Fig. 11 shows examples of fitting experimental traces with model 1 for responses evoked by the co-application of 30 mM glycine and 10 \( \mu M \) picrotoxinin (Fig. 11A) or of 0.3 mM glycine and 3, 10, and 30 \( \mu M \) picrotoxinin (Fig. 11B). This model well predicts an increase in \( I_{C_{50}} \) when glycine concentration was increased (Fig. 11C), a decrease in the deactivation time constant of the currents at the end of the application of glycine (Fig. 11D), as well as a lengthening of the rising phase of the response evoked by a saturating concentration of glycine when picrotoxinin was applied continuously (Fig. 11, E and F). It also predicts that picrotoxinin does not change the rise time of glycine currents when applied just before the application of glycine (not shown). The model also accounts for the slower rising phase of the response that follows picrotoxinin application during the relaxation phase of the preceding response (Fig. 11G). Overall, these data indicate that the same kinetic model with closely similar rate constants can describe the inhibitory effects of both PTX and picrotoxinin. Those data also strongly suggest that picrotoxinin is likely to be mainly responsible for the PTX inhibitory effects previously described on homomeric \( \alpha_2 \) GlyRs (9).

In inhibiting homomeric \( \alpha_2 \) GlyRs, picrotin and picrotoxinin share some similar properties as follows. The block evoked by both picrotoxinin and picrotin depends on glycine concentration (Fig. 2). Both picrotoxinin and picrotin can slow down the activation phase of the glycine-evoked current and speed up the relaxation phase when they are applied continuously (Figs. 4 and 7). Their estimated association rate constant values from the open state are closely similar (Fig. 6D); They can both be trapped at their binding site when the channel closes during glycine dissociation.

Accordingly, we first tested for picrotin the kinetic model we already proposed for picrotoxinin (Fig. 10B, model 1), and we hypothesized that picrotin acts as a poor agonist on the picrotoxinin-binding site. The procedures used to test the kinetic models for picrotin were identical to those described previously for picrotoxinin. This model (Fig. 10B) overestimated the speed of the activation time course of the response evoked by the simultaneous application of a nonsaturating concentration of glycine (100–300 \mu M) and of \( >100 \mu M \) picrotin. To help the fit procedure, and to obtain a better resolution of the rate constants set as free variables, we decided to fit simultaneously outside-out currents obtained, on the one hand, during the co-
application of glycine and picrotin and, on the other hand, during the application of glycine when picrotin was continuously applied. As shown in Fig. 12B, this procedure gave a poor fit of the experimental data; it overestimated the speed of the activation time course of the glycine response and underestimated the decrease in the deactivation time course of the glycine-evoked current when picrotin was applied during the relaxation phase of the glycine response. Discarding the links between the glycine-ligated closed states with and without picrotin in models 1 and 2 (leaving a single binding state for picrotin linked to the channel open state) did not improve the quality of the fit (ANOVA test, \( p > 0.1 \)). Adding another binding site linked to the open state (two mutually exclusive picrotin-binding sites) or linked to the picrotin-binding site linked to the open state (two sequential binding sites: \( A_1 O \) to \( P + A_1 P B \) and \( P + A_1 P B \) to \( A_1 P P B \)) did not improve the quality of the fit (ANOVA test, \( p > 0.1 \)).

Another hypothesis already proposed for PTX block on homomeric \( \alpha_1 \) GlyR (17) and on GABAergic chloride channels expressed on crayfish muscle (29) proposed that PTX can promote GlyR desensitization-like closed states. We tested this hypothesis using the kinetic models shown in Fig. 10, D–F (model 3, model 4, and model 5). In those models, picrotin binds within the vestibule or the pore of the channel, which, accordingly, shortens the duration of single channel openings (\( A_1 O \) to \( A_1 P B \), see Fig. 10, D–F). It is then trapped at its binding site when the channel closes (\( A_1 P B \) to \( A_1 P C \), see Fig. 10, D–F). Because, as observed for picrotoxinin, recovery from picrotin block necessitates the reactivation of the receptor by glycine application, glycine molecules might dissociate from these desensitization-like closed states, whereas picrotin remains bound (\( A_1 P C \) to \( A + A_1 P C \), \( A + A_1 P C \) to \( A_2 + A P C \), and \( A_2 + A P C \) to \( A_2 + P C \)).

Two picrotin block model subtypes were tested. In the first subtype (Fig. 10D, model 3), the only way for picrotin to bind and unbind passes through the open state. In the second subtype (Fig. 10E and F, models 4 and 5), picrotin can bind and unbind from the fully liganded bound state only (model 4, \( A_3 C \) to \( A_2 P C \)) or picrotin can bind and unbind from all glycine-bound states (model 5, \( A_2 C \) to \( A_2 P C \), \( A + A_3 C \) to \( A_2 + A P C \), and \( A_2 + A C \) to \( A_2 + A P C \)). These last two models suppose that the picrotin-binding site is not masked when the receptor is in its bound closed conformation. This has already been proposed for picrotin and picrotoxinin block on homomeric \( \alpha_1 \) GlyR (17) and for picrotoxinin block on GABA \(_{\text{A}}\) receptors (16). For simplicity, all rate constants in model 5 linking the glycine-bound using model 4. E, theoretical currents obtained with model 4 evoked by a 1-ms pulse of 30 mM glycine during the application of 0, 10, 30, and 100 \( \mu M \) PTN. Peak current amplitude was normalized. Note that this kinetic scheme predicts the PTX-evoked concentration-dependent decrease in the decay phase duration, \( F \), simulated currents showing the activation phase of the responses evoked by 30 mM glycine, by the co-application of 30 mM glycine and 300 \( \mu M \) PTN, and by glycine in the continuous presence of 300 \( \mu M \) PTN. Model 4 predicts that the activation phase of the glycine response was slowed down in the continuous presence of PTN. G, simulated traces generated using model 4 showing that this kinetic scheme also predicts that the lengthening of the rise time evoked by PTX can persist up to 60 s after washout of glycine and PTX (inset). For time course comparisons, the control response on the left was superimposed (in gray) on the other simulated glycine-evoked currents.
closed states with and without picrotin binding were set as equivalent (A_2C to A_2pC, A + A_2C to A + A_2pC, and A + AC to A_2 + APC), supposing that picrotin affinity is similar for all glycine-bound closed states. When these reactions were set as independent, the fit of experimental traces was not significantly improved (ANOVA test, \( p > 0.1 \)), and the rate constant values for these steps strongly diverged.

Model 3 did not give a better prediction of our experimental data when compared with model 2 (ANOVA test, \( p > 0.1 \)). As shown in Fig. 12C, this model failed to predict a slowdown of the activation time course of glycine-evoked outside-out currents in the presence of >100 \( \mu M \) picrotin. Models 4 and 5, on the other hand, better predicted our experimental data when compared with model 2. Model 4 gave 3.9 ± 0.6 times and model 5 4.4 ± 0.6 times significantly lower SSEs than model 2 (ANOVA test, \( p < 0.01 \)). Model 5 did not significantly improve the fit when compared with model 4 (ANOVA test, \( p > 0.1 \)), which suggests that although the transitions between the monoliganded glycine-bound closed states (A_2 + AC and A_2 + APC) and the doubly liganded glycine-bound closed states (A + A_2C and A + A_2pC) existed, they are not really necessary to describe our experimental data. The optimal averaged rate constant values obtained for models 4 and 5 are listed in Table 2. As shown there, the fits of experimental traces with model 4 or model 5 gave very similar values for picrotin association and dissociation rate constants. As already observed for the picrotoxinin block, the affinity of picrotin for the channel in the open state (for picrotin and model 4 \( k_{o2p}/k_{on1p} = 511.5 \mu M; \) for picrotoxinin and model 2 \( k_{o2p}/k_{on1p} = 486.1 \mu M \)) was lower than for the glycine-bound closed state (for picrotin and model 4 \( k_{o2p}/k_{on1p} = 11.8 \mu M; \) for picrotoxinin and model 2 \( k_{o2p}/k_{on2p} = 3.8 \mu M \)). We also attempted, as for picrotoxinin, to set the affinity of picrotin for the open state equal to its affinity for the liganded closed states. In this case, the SSE of the fit was \( \approx 4 \) times significantly higher (ANOVA test, \( p < 0.01 \)), indicating that the rate constant values for these picrotin-binding steps are unlikely to be equivalent, as was also observed for picrotoxinin.

Fig. 12 shows examples of fits of experimental traces using model 4 (thick dark lines) to outside-out currents evoked by the co-application of 30 mM glycine and 300 \( \mu M \) picrotin (Fig. 12A), the co-application of 300 mM glycine and 300 \( \mu M \) picrotin, or the application of 300 mM glycine in the continuous presence of 300 \( \mu M \) picrotin (Fig. 12B), as well as the co-application of 300 mM glycine and 10, 100, and 300 \( \mu M \) picrotin (Fig. 12C). This model predicts a large rebound current at the end of the co-application of picrotin occurring for picrotin concentrations >10 \( \mu M \) (Fig. 12C). It also predicts a picrotin \( IC_{50} \) of 81 \( \mu M \) in the presence of 0.3 mM glycine (Fig. 12D), which is in good agreement with our experimental data (\( IC_{50} = 117 \mu M \)), and an increase in picrotin \( IC_{50} \) when glycine concentration was increased (Fig. 12D). This model also predicts an acceleration of the relaxation phase of glycine-evoked currents in the continuous presence of picrotin (Fig. 12E); a slowing down of the rising phase of responses was induced by a saturating glycine concentration when picrotin was either continuously applied (Fig. 12F) or applied during the deactivation phase of the preceding response (Fig. 12G). Finally, this model also accounts for the fast doubly exponential onset of block and the fast doubly exponential recovery time course observed for picrotin-evoked inhibition within glycine application (Fig. 9A.). Simulations of a transient application of 100 \( \mu M \) picrotin during glycine-evoked outside-out currents using model 4 gave blocking time constant values \( \tau_{on1} = 0.224 \pm 0.021 \) ms and \( \tau_{on2} = 4.41 \pm 0.63 \) ms (\( n = 11 \)) and recovery time constant values \( \tau_{off1} = 0.295 \pm 0.031 \) ms and \( \tau_{off2} = 5.03 \pm 0.79 \) ms (\( n = 11 \)), which are in the range of the values measured experimentally (Fig. 9A). As also observed with the experimental data, the relative amplitude of the fast blocking time constant and the fast recovery time constant greatly varied between simulations, ranging from 41.1 to 95.2\% (70.3 ± 4.28\%; \( n = 11 \)) and from 31.9 to 98.9\% (66.2 ± 4.8\%; \( n = 11 \)), respectively.

Overall, those simulations indicate that model 4 is the minimal stochastic scheme that best predicts the picrotin inhibitory effect on homomeric \( \alpha_2 \) GlyRs. Moreover, it is important to note that models 4 and 5 cannot well predict the picrotoxinin inhibitory effect on homomeric \( \alpha_2 \) GlyRs when compared with models 1 and 2. Models 4 and 5 gave \( \pm 4 \) times significantly higher SSEs than models 1 or 2 (ANOVA test, \( p < 0.01 \)), indicating that picrotoxinin and picrotin are unlikely to share the same inhibitory mechanism on homomeric \( \alpha_2 \) GlyRs.

**DISCUSSION**

The inhibitory mechanism of PTX on ligand-gated ion channels is known to be a complex phenomenon and varies among ionotropic receptors (8). We have proposed previously a simple model that well predicted the inhibitory mechanism of PTX on wild-type homomeric \( \alpha_2 \) GlyRs (9). In this study, we demonstrated that picrotoxinin was considerably more potent (>30 times) than picrotin in blocking the activity of \( \alpha_2 \) homomeric GlyRs, indicating that PTX-evoked inhibition of \( \alpha_2 \) homomeric GlyR activity (9) is mainly mediated by picrotoxinin. The complex inhibitory mechanism of picrotoxinin can be well predicted by the simple kinetic scheme already proposed for the inhibitory mechanism of PTX on \( \alpha_2 \) homomeric GlyR. This model implied that picrotoxinin binds both to the agonist-bound closed state and to the agonist-bound open state. Our results also suggest that picrotoxinin and picrotin probably bind at different binding sites. The inhibitory mechanism of picrotin was also well predicted by a simple kinetic scheme in which picrotin can promote desensitized-like bound states. As suggested for picrotoxinin, picrotin cannot bind to the agonist-bound closed state, and both picrotoxinin and picrotin can be trapped at their binding sites while glycine dissociates from the receptor.

**Minimal Kinetic Models for Picrotoxinin and Picrotin—**The models previously proposed to describe PTX-evoked GlyR inhibition (Fig. 10, model 1 and model 2) (9) can also well predict the experimental data obtained with picrotoxinin. This was not surprising for homomeric \( \alpha_2 \) GlyRs, because our experimental data also indicated that picrotoxinin is likely to be the main effective component of PTX in blocking homomeric \( \alpha_2 \) GlyRs within the concentration range tested for PTX (see Fig. 1).

Surprisingly, picrotin-evoked inhibition of homomeric \( \alpha_2 \) GlyRs cannot be described by the same kinetic scheme used for
picrotoxinin. Consistently, this picrotin kinetic scheme did not properly fit the experimental data obtained with picrotoxinin. It is therefore reasonable to suppose that these two alkaloids are unlikely to share the same inhibitory mechanism for homomeric α2 GlyRs. The picrotin kinetic scheme differs from the picrotoxinin kinetic scheme in that it was not necessary to add desensitization states (A3 + APD, A + APD, and A2 + APD) linked to the glycine-bound closed states plus picrotin (A3 + APC, A + APC, and A2 + PC) to predict our experimental data. Rather, such a kinetic model can be interpreted as picrotin promoting desensitization-like glycine-bound closed states, as suggested previously for picrotoxinin-evoked inhibition of homomeric α1 GlyRs and GABA_C receptors (16, 17, 29). According to models 4 and 5, it was necessary to allow these desensitization-like closed states to interconvert when glycine dissociates (A2 + PC to A + APC and A + APC to A2 + APC) to fit our experimental data. A glycine-unbound closed state (A3 + PC) in the presence of picrotin had also to be included. To our knowledge, however, such a desensitization state linked to the unbound closed state has never been described for GlyRs or ionotropic GABA receptors. Another possible interpretation of models 4 and 5 would be that picrotin binding must stabilize the receptor channel in an intermediate closed state conformation from which the receptor cannot desensitize. However, both hypotheses are equally plausible because an interaction between GlyR desensitization and channel gating is likely to occur. Indeed, the GlyR desensitization mechanism implies interactions between the M1 and M2 intracellular loop and the M2 transmembrane domain forming the channel pore of the receptor (33, 34). It is likely that M1–M2 and M2–M3 loops act in parallel to control channel activation by acting as hinges governing allosteric control of the M2 domain (35).

There are also some important differences when the estimated rate constant values between the kinetic schemes for picrotoxinin and for picrotin are compared. The picrotin dissociation rate constant from the open state and from the glycine-bound closed state is \( 100 \text{ times faster than the values obtained for picrotoxinin.} \) This indicates that the affinity of picrotin for the GlyR open state is low compared with that of picrotoxinin. This fast dissociation rate constant accounts for the lower efficacy measured for picrotin, as well as for the faster recovery time constant observed after washout of glycine and picrotin (Fig. 9 and Fig 12) compared with the picrotoxinin blockade (Fig. 8 and Fig. 11). The picrotin blockade model also predicts a considerably slower on-rate constant \((a)\) and off-rate constant \((b)\) of the transition between the picrotin-bound state A₃PB and the fully agonist-bound closed state plus picrotin A₃PC. For picrotoxinin, the estimated transition between A₃PB and A₃PC is very fast, with rate constant values \( > 10^8 \text{ s}^{-1} \). Such fast rate constant values indicate that the glycine-ligated closed state plus picrotoxinin (A₃PC) and the picrotoxinin-bound state (A₃PB) could collapse when picrotoxinin binds to the GlyR open state, as discussed previously for PTX-evoked GlyR inhibition (9). The values for the on-rate constant \((a)\) and the off-rate constant \((b)\) that we obtained for picrotin (model 4, \( a \approx 1070 \text{ s}^{-1}; \text{model 5, } a \approx 264 \text{ s}^{-1} \)) and the off rate (model 4, \( b \approx 7567 \text{ s}^{-1}; \text{model 5, } b \approx 2880 \text{ s}^{-1} \)) were closer to the ranges of the closed rate constant \((a \approx 21 \text{ s}^{-1})\) and of the open rate constant \( (\beta \approx 4935 \text{ s}^{-1}) \) of the GlyR channel itself, compared with picrotoxinin. When we attempted to set the on-rate constant \((a)\) and the off-rate constant \((b)\) values equal to the closing rate constant \((\alpha)\) and the opening rate constant \((\beta)\) values, respectively, the kinetic scheme failed to describe the picrotin experimental data. Accordingly, it is reasonable to conclude that the channel does not simply close when picrotin binds to the receptor but that picrotin binding specifically evokes a change in GlyR conformation leading to channel closure, but with slower kinetics than for picrotoxinin. It is also important to note that the off-rate constant \((b)\) between A₃PB and A₃PC is 10 times higher than the on-rate constant \((a)\) both for picrotin and picrotoxinin. Accordingly, GlyRs must remain mainly in the blocking bound state A₃PB instead of the ligand-bound closed state A₁PC during simultaneous application of glycine plus picrotoxin or picrotin.

Nevertheless, the picrotoxinin and picrotin kinetic models share similarities. As mentioned previously for PTX (9), these two groups of kinetic models differ from the model proposed for picrotoxinin-induced inhibition of GABA_C receptors (16). This is because there is an intermediate step between the picrotoxinin- or the picrotin-bound block state (A₃PB) and the fully ligand-bound closed state plus picrotoxinin or picrotin (A₃PC). A reaction cycle with four steps in models 2, 4, and 5 is physically plausible as discussed previously for PTX inhibition of homomeric α₂ GlyR (9). Accordingly, the receptor first undergoes a conformational change to a new stable state when it is fully liganded (channel opens); picrotin and picrotoxin then bind to the open conformation, and afterward the channel closes. The cycle is terminated when these compounds dissociate directly from the fully liganded closed conformation. Kinetic model subtypes for picrotoxinin and for picrotin nicely predict the previously described “competitive” and “noncompetitive” mechanisms of picrotoxinin and picrotin action on homomeric GlyRs (17). In all models tested, glycine can dissociate from its binding sites, whereas picrotoxinin or picrotin remains bound. Accordingly, an increase in glycine concentration will increase the glycine association rate between the glycine-bound closed states plus the PTX compounds (A₃PC, A₃ + APC, and A₂PC; Fig. 10); this will result in an apparently faster recovery rate of both picrotoxinin and picrotin, as mentioned previously for PTX-evoked inhibition of GlyRs (9).

Different Binding Sites for Picrotoxinin and Picrotin?—It is now recognized that picrotoxinin is likely to have a binding site lying within the channel pore between the 6′ and 2′ residues in a variety of Cys loop receptor channels (8, 13, 36). Our results using picrotoxinin are consistent with the hypothesis that there is a picrotoxinin-binding site located within the GlyR channel, as proposed previously for PTX-evoked block of homomeric α₂ GlyRs (9). In our experiments, picrotin-evoked GlyR inhibition was more sensitive to voltage than picrotoxinin-evoked GlyR inhibition, indicating that picrotin is likely to go deeper in the electrical field of the membrane, which could also suggest that picrotin binds to a different site than picrotoxinin. Other open chloride channel blockers such as ginkgolides also evoke a voltage-dependent inhibition in homomeric α₁ GlyRs (37). Their binding sites are supposed to be located at the 2′ porelining position of the GlyR channel pore. For picrotoxinin, its
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Binding site was supposed to be closer to the 6'-pore-lining residue, at least in homomeric \(\alpha_1\) GlyRs (38). The 2'-position is located deeper within the channel pore than the 6'-position (8), and this could explain the voltage-dependent inhibitory effect of picrotin when compared with the effect of picrotoxinin, if we hypothesize that the picrotin-binding site is close to the 2'-pore-lining position.

Picrotin and picrotoxinin are equally effective in inhibiting homomeric \(\alpha_1\) GlyRs (17), which was not the case for homomeric \(\alpha_2\) GlyRs. This is surprising because \(\alpha_2\) GlyR subunits are almost identical to \(\alpha_1\) GlyR subunits throughout M2 and the M2–M3 loop, except for the alanine 2'-glycine substitution. Interestingly, GABA\(_A\) Rs and GABA\(_C\) Rs, being insensitive to picrotin, also contain an alanine at the 2'-position. Hydroxyl groups of threonine 6' residues are likely to contribute important H-bonds to PTX oxygens (13), and it should also be pointed out that the amphipathic nature of glycine residue at the 2'-position in \(\alpha_1\) GlyR subunits could allow both hydrophobic interactions between apolar backbone methylene atoms and the isoprenyl group of picrotoxinin, and hydrophilic interactions between polar backbone atoms and the hydroxyl of picrotin (39). This proposal would explain the lack of discrimination between polar backbone atoms and the hydroxyl of picrotoxinin, and hydrophilic interactions between apolar backbone methylene atoms and lower main conductance level than homomeric alanine at the 2'-position.

How Many Binding Sites for Picrotoxinin and Picrotin?—Models 1 and 2 and models 4 and 5 fit equally well our experimental data with picrotoxinin and picrotin, respectively. From a statistical point of view it was reasonable to choose the simplest models to describe the inhibitory effects of picrotoxinin and picrotin. As mentioned previously for PTX-evoked GlyR inhibition, it was necessary to model picrotin and picrotoxinin binding to both ligand-bound closed states and to a ligand-bound open state to provide a reasonably good fit of our experimental data. Although unexpected if one assumes that picrotoxinin and picrotin bind within the channel, this result is consistent with what is known about the inhibition of GABA\(_A\) and GABA\(_C\) receptors by picrotoxinin. Picrotoxinin binds to these receptors with a higher affinity for the agonist-bound closed state than for the agonist-bound open state (16, 42). The model that lacked a picrotin-binding step between the ligand-bound closed states failed to predict the time course of the glycine-evoked outside-out currents in the presence of picrotin (Fig. 10, model 4). This was also the case for PTX and therefore for picrotoxinin (9).

For picrotoxinin, the kinetic models predict faster association and dissociation rate constants for the ligand-bound closed states than for the ligand-bound open state. By contrast, the picrotin kinetic models predict similar association rate constants and a slower dissociation rate constant for the ligand-bound closed states than for the ligand-bound open state. There are several possible explanations for these differences. Different association rate constant values for picrotoxinin between the ligand-bound closed states and the open state suggest that channel openings will reduce the access to the binding site of picrotoxinin. This is feasible if a change in receptor conformation results in a narrowed access to this binding site. A narrowed access to the picrotoxinin-binding site could also explain the slower rate of dissociation to the open state, as predicted by models 1 and 2. The coefficient of diffusion of an agonist partly depends on the correspondence between the effective radius of the drug molecule and the target on the receptor (43). Another possibility is that picrotoxinin binds to a specific binding site when the receptor is in the ligand-bound closed state(s) and then binds to another binding site recognized by picrotin and located deeper within the channel, when the channel is open. Although feasible, this hypothesis does not explain why the blockade of the glycine-evoked current by picrotoxinin is not voltage-dependent, in contrast to picrotin-evoked inhibition.

The highly similar slow association rate constants of picrotin in the bound closed state and in the bound open state could suggest that channel gating did not strongly influence the access of the molecule to its site. These slow association rate constants for picrotin can be related to the location of the binding site if one supposes, as discussed above, that it is located deeper within the channel pore. This is the case for the 2'-pore-lining position. If so, the picrotin-binding site is likely to reside at the end of an effective tunnel of structures (the channel pore), with steric hindrance to the drug on its path to the binding site; this will, in turn, slow down the diffusion of picrotin to its target (43). This hypothesis does not, however, explain the fast dissociation rate constant of picrotin for the open state, unless one supposes that the putative site where picrotin binds deep within the channel renders this molecule more sensitive to thermal agitation (43) because of ionic flux when the channel open. This hypothesis also implies that picrotin must have access to its binding site even when the channel is in a conformation that does not allow ionic flux. Our experimental data provide evidence that picrotoxinin and picrotin cannot directly bind to the unliganded closed conformation of the receptor, although they can bind to the ligand-bound closed conformations. Accordingly, glycine binding must evoke intermediate conformational changes of the channel prior to opening, as suggested previously (44). Finally, because the homomeric \(\alpha_2\) GlyR channel opens from the fully liganded closed state only (20, 44), which was not the case for homo-
In conclusion, the crucial insight of our study is that, contrary to what is known about the homomeric \( \alpha_2 \) GlyR, picrotoxinin and picrotin act channel blockers and can be trapped on their binding sites when glycine dissociates, their blocking mechanisms have different voltage sensitivities. This difference raises the question of the functional location of their binding sites, at least for GlyRs.

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