Mechanisms for Picrotoxin Block of \( \alpha_2 \) Homomeric Glycine Receptors*

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It is well known that the convulsant alkaloid picrotoxin (PTX) can inhibit neuronal \( \gamma \)-aminobutyric acid (GABA) and homomeric glycine receptors (GlyR). However, the mechanism for PTX block of \( \alpha_2 \) homomeric GlyR is still unclear compared with that of \( \alpha_1 \) homomeric GlyR, GABA\(_A\), and GABA\(_C\) receptors. Furthermore, PTX effects on GlyR kinetics have been poorly explored at the single-channel level. Hence, we used the patch-clamp technique in the outside-out configuration to investigate the mechanism of PTX suppression of currents carried by \( \alpha_2 \) homomeric GlyRs stably transfected into Chinese hamster ovary cells. PTX inhibited the \( \alpha_2 \) homomeric GlyR current elicited by glycine in a concentration-dependent and voltage-independent manner. Both competitive and noncompetitive mechanisms were observed. PTX decreased the mean open time of the GlyR channel in a concentration-dependent manner, suggesting that PTX can block channel openings and bind to the receptor in the open channel conformation. When PTX and glycine were co-applied, a small rebound current was observed during drug washout. Application of PTX during the deactivation phase of glycine-induced currents eliminated the rebound current and accelerated the deactivation time course in a concentration-dependent manner. PTX could not bind to the unbound conformation of GlyR, but could be trapped at its binding site when the channel closed during glycine dissociation. Based on these observations, we propose a kinetic Markov model in which PTX binds to the \( \alpha_2 \) homomeric GlyR in both the open channel state and the fully liganded closed state. Our data suggest a new allosteric mechanism for PTX inhibition of wild-type homomeric \( \alpha_2 \) GlyR.

Glycine and GABA\(^3\) are the main inhibitory neurotransmitters in the central nervous system. The glycine receptor (GlyR) is a pentameric transmembrane protein complex, which forms an anion-selective channel (1). Five different subunits have hitherto been cloned in mammals, one \( \beta \) subunit and four \( \alpha \) subunits (\( \alpha_1 \)–\( \alpha_5 \)), which are associated with two different ways of forming functional receptors: the homomeric configuration composed of five \( \alpha \) subunits (1) and the heteromeric configuration comprising two \( \alpha \) subunits and three \( \beta \) subunits (2). In the adult brain, GlyR is primarily involved in fast inhibitory synaptic transmission in the brainstem and spinal cord.

It is now well established that picrotoxin (PTX), a plant alkaloid, which was first used to discriminate GABAergic from glycineric currents, can also strongly inhibit the homomeric GlyR subtypes, whereas the \( \alpha/\beta \) heteromeric GlyR subtype is much less sensitive to PTX (3). Although the action of PTX has been extensively studied both on GABA\(_A\) and GABA\(_C\) receptors and on GlyRs (4), the one or more binding sites of this compound and its inhibitory mechanism are still under debate. There are lines of evidence indicating that PTX binding and/or inhibitory mechanisms are related to amino acid residues in the transmembrane domain TM2 forming the pore of the ionic channel (3, 5–13). A series of studies on the GABA\(_A\) \( \alpha \), GABA\(_A\) \( \beta \), invertebrate glutamate receptor Cl\(^–\) channel, and GlyRs has established that a ring of 6-\(^{\prime}\)-threonines within the pore is invariably required for PTX sensitivity (4). Recently, it has been accurately demonstrated that PTX is likely to bind in the channel pore of the homomeric \( \alpha_1 \) GlyR (14). Although PTX could be trapped when the GlyR channel closed in the \( \alpha_1 \) subunit R271C mutation, this was not the case for wild-type GlyR (14), and it is unlikely that PTX can act as an open channel blocker on this GlyR subtype. In fact, the inhibitory mechanism of PTX can differ between anionic receptor-channel family subtypes, and it ranges from open channel blocker to allosteric competitive antagonist (4). Although in some preparations PTX inhibition of GABA\(_A\) \( \alpha \) appeared to be use-dependent and noncompetitive (15), suggesting an open channel block mechanism for PTX inhibition, analysis of PTX-evoked inhibition of the single-channel activity of GABA\(_A\) \( \alpha \) recorded from rat sympathetic neurons indicates that PTX inhibition is not use-dependent (16). In this study, the authors proposed that PTX preferentially binds to the agonist-bound conformation of the receptor and stabilized the channel in the closed state (16). This was also demonstrated with GABA\(_C\) receptors from isolated retinal bipolar cells and from oocytes expressing the GABAR \( \alpha \) subunit (17). A competitive inhibitory component of PTX inhibition has been described for homomeric \( \alpha_1 \) GlyRs. Its potency decreased as agonist concentration increased (18). But PTX is unlikely to modify glycine binding, and it was proposed that PTX rather acts as an allosteric inhibitor by altering the coupling between agonist binding and channel gating (18). Although extensive efforts have been made to determine the molecular mechanism of PTX inhibition of GlyRs, paradoxically little work has been done on the single-channel effects of PTX on GlyR kinetics. It has only been shown that at low concentrations (1–30 \( \mu \)M) PTX decreased the probability of predominantly high conductance with homomeric \( \alpha_1 \) GlyRs. In contrast, at higher concentrations PTX induced flickering closings in both heteromeric \( \alpha_1/\beta \) GlyRs and homomeric \( \alpha_1 \) GlyRs (19, 20).

The \( \alpha_2 \) homomeric GlyR subunit has been identified as an embryonic...
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receptor form (21, 22) and plays an important role during synaptogenesis and cell differentiation. It is more adapted to sustained and slow paracrine neurotransmitter release (23) as observed in the fetal brain (24). It has recently been shown that PTX is as potent on homomeric α2 GlyRs (25) as on homomeric α1 GlyRs (3, 18, 19). Furthermore, α2 homomeric GlyR has slow kinetic properties (23) and opens mainly with a single large conductance state (100–120 pS), which makes this receptor a good model for analyzing the effect of PTX on GlyR kinetics.

Therefore, we investigated here the mechanism of inhibition of PTX on the activation and deactivation kinetics of the homomeric α2 GlyR expressed in Chinese hamster ovary (CHO) cells as a model system, by means of outside-out patch-clamp recordings using an ultra-fast flow application system. We showed that PTX inhibited α2 homomeric GlyRs in a concentration-dependent and voltage-independent manner, and that PTX could bind to the receptor in both the open channel conformation and the fully liganded closed state. We also demonstrated that PTX could be trapped at its binding site when the channel closed during glycine dissociation. This complex mechanism can be predicted by a simple kinetic model in which glycine can dissociate while PTX remains bound.

MATERIALS AND METHODS

Cell Culture—Chinese hamster ovary cells (CHO-K1, ATCC number CCL61) were maintained in a 95% air-5% CO2 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 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 α2 subunit cloning and transfection were performed as previously described (23).

Outside-out Recordings—Outside-out recordings (26) were done under direct visualization on α2 GlyR-transfected CHO cells with the use of Nomarski optics (∼×40, immersion lens, Nikon Optiphoto). Cells were continuously perfused at room temperature (20–22 °C) with oxygenated bathing solution (2 ml/min) containing (in mM): NaCl 147, KCl 2.4, CaCl2 2, MgCl2 2, HEPES 10, glucose 10 (pH 7.2, osmolarity of 320 mosm). Patch-clamp electrodes (5–10 MΩ) 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., Novato, CA). They were fire-polished and filled with (in mM): CsCl 130, MgCl2 4, Na2ATP 4, EGTA 10, HEPES 10 (pH 7.2, osmolarity of 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 10 or more trials as specified in the figure legends or the text unless otherwise noted.

Drug Delivery—Outside-out single-channel currents were evoked using a fast-flow operating system (27, 28). 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 PTX. The solution exchange was performed by rapidly moving the solution interface across the tip of the patch pipette, using a piezoelectric 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 (28). For the experiments requiring fast solution exchange between three different conditions (see Fig. 8), we used a homemade multibarreled application system composed of three horizontally aligned quartz tubes (inside diameter, 0.25 mm; outside diameter, 0.35 mm; Polymicro Technologies). Solution exchange was achieved by lateral movements, using a SF-77B fast-step perfusion system (Warner Instruments, Hamdell, CT). The complete solution change was achieved in 200–300 μs. Glycine and PTX were from Sigma. Stock solution of PTX was prepared in dimethyl sulfoxide and then diluted to an appropriate concentration with the above extracellular solution just before use. The final concentration (v/v) of dimethyl sulfoxide 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 (Equation 1),

\[
\frac{I}{I_{\text{Con}}} = \frac{1}{1 + (\text{PTX/IC}_{50})^n},
\]

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_H is the Hill coefficient. For each concentration tested, the amplitude of the current, I, was measured at the steady-state level. The activation time constants of glycine-evoked currents in the presence and absence of PTX 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 sum of squared errors of the fits (28, 29).

For single-channel analysis, patches with one channel were included only if channel activity was stable over sweeps. First latencies, open and closed time durations were measured manually using Axograph 4.9 software. First latency distributions were created using standard histogram techniques (30). 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 (31). The number of exponential components was determined by comparing the sum of squared errors of the fits.

Kinetic Modeling Programs—To obtain a kinetic model for PTX effects on GlyR behavior, glycine-evoked currents in the absence and presence of PTX were analyzed off-line, and the inhibitory effect of PTX 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 sum of squared errors (SSSEs) between the experimental data and a given
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Application of 10 \( \mu \)M PTX and 300 \( \mu \)M glycine, when the two compounds were simultaneously withdrawn, a small transient membrane current was observed before current relaxation (Figs. 1A and 2A). Fig. 1B shows the concentration-response curves for PTX co-applied with two different concentrations of glycine obtained in twelve outside-out experiments. Concentration-response curves were fitted with the Hill equation (see "Materials and Methods") and in the presence of 300 \( \mu \)M glycine gave an IC\(_{50} \) (half-maximum inhibition) and a Hill coefficient of 2.7 \( \pm \) 0.2 \( \mu \)M and 0.8 \( \pm \) 0.04, respectively. When glycine concentration was increased to 30 \( \mu \)M, the concentration-response curve was shifted to the right and the IC\(_{50} \) value increased to 6.4 \( \mu \)M. The Hill coefficient value was not modified (0.8 in the presence of 30 \( \mu \)M glycine). This glycine concentration-dependent shift in PTX IC\(_{50} \) was previously described for \( \alpha_1 \), homomeric GlyR and was thought to reflect a competitive inhibitory mechanism (18). However, it should be noted that PTX is unlikely to be a true competitive antagonist, because increasing the glycine concentration from 30 to 100 \( \mu \)M only slightly reduced the inhibitory effect of 10 \( \mu \)M PTX. The percentage inhibition of PTX-evoked outside-out current was 57 \% \( \pm \) 2\% (\( n = 12 \)) and 50 \% \( \pm \) 3\% (\( n = 8 \)) in the presence of 30 \( \mu \)M glycine and of 100 \( \mu \)M glycine, respectively.

PTX-induced Inhibition Is Not Voltage-dependent—The small rebound current observed during GlyR deactivation immediately after the termination of co-application of 10 \( \mu \)M PTX and glycine (Figs. 1A and 2A) could reflect the recovery from PTX open channel block as described for the open-channel block effect of acetylcholine on nicotinic receptors (32, 33). If PTX acts as a classic fast open channel blocker, the inhibitory effect of this alkaloid must be voltage-dependent. To test this hypothesis, the voltage dependence of PTX-induced glycine current inhibition was examined. Typical examples of 300 \( \mu \)M glycine-evoked currents (1-s application step) at \( V_r \) of +50 and −50 mV with and without co-application of 10 \( \mu \)M PTX are shown in Fig. 2A. In this example PTX inhibited glycine-evoked currents both at positive and at negative holding potentials. Voltage dependence of PTX inhibition on glycine-evoked currents was analyzed by constructing I-V curves from 300 \( \mu \)M glycine-evoked currents in the absence and presence of 10 \( \mu \)M PTX at holding potentials ranging from −70 mV to +70 mV (Fig. 2B). As shown in Fig. 2B, the steady-state current of the responses evoked in the absence or presence of PTX varied linearly at negative potentials and rectified at positive potentials. Adding PTX to glycine solution did not significantly change the reversal potential (\( V_r \)) of the glycine-evoked currents (unpaired \( t \) test, \( p > 0.1 \)). \( V_r \) was ∼3 mV with glycine and ∼4 mV with glycine plus PTX. The voltage-independent nature of PTX block was revealed by plotting the percentage of block evoked by co-application of 10 \( \mu \)M PTX and 300 \( \mu \)M glycine at holding potentials ranging from +70 mV to −70 mV (Fig. 2C). Over this potential range PTX reduced the amplitude of glycine currents to the same extent (∼70\%), indicating that PTX might not bind to a site within the membrane field of \( \alpha_2 \) homomeric GlyR. Nevertheless, PTX is weakly charged at neutral pH and the lack of a voltage-dependent block does not exclude the possibility that PTX can bind within the channel pore as recently suggested (14).

PTX Accelerates the Deactivation Kinetics of Glycine-evoked Current—A previous study has shown that PTX applied immediately after GABA accelerates the deactivation kinetics of GABA\(_{A}\)-R (17). To determine if PTX has any effect on GlyR deactivation kinetics, we first compared the deactivation phase of the outside-out currents evoked by co-application of glycine and PTX or in the continuous presence of PTX before, during, and after glycine application (Fig. 3A). When compared with responses evoked by simultaneous application of 300 \( \mu \)M glycine and 10 \( \mu \)M PTX, the continuous presence of PTX dramatically accelerated the deactiva-

FIGURE 1. Concentration-dependent inhibition of \( \alpha_2 \) homomeric GlyR by PTX. A, outside-out patch clamp recordings showing inhibition of 300 \( \mu \)M and 30 \( \mu \)M glycine-activated currents evoked by the indicated concentrations of co-application of PTX in CHO cells transfected with the \( \alpha_2 \) GlyR subunit. Each trace represents the average of 15–30 responses. Note that when the PTX concentration was >1 \( \mu \)M the small transient rebound current was always induced during the withdrawal of the two drugs. The left and right traces were obtained from two different patches. The thick line represents the application of drugs. B, PTX inhibition curves for 300 \( \mu \)M (○) and 30 \( \mu \)M (□) glycine-evoked responses. Currents were normalized to the responses in the absence of PTX. Each point is the average of values from 5–12 cells. In most instances multiple concentrations (three) of PTX were applied to the same cell. Data were fitted with the Hill equation (see “Materials and Methods”) giving an IC\(_{50} \) of 2.7 \( \pm \) 0.2 \( \mu \)M and a Hill coefficient of 0.8 \( \pm \) 0.04 for 300 \( \mu \)M glycine, and an IC\(_{50} \) of 6.4 \( \pm \) 0.6 \( \mu \)M and a Hill coefficient of 0.8 \( \pm \) 0.05 for 30 \( \mu \)M glycine.

RESULTS

Concentration-dependent Inhibition of \( \alpha_2 \) Homomeric GlyR by PTX—We first analyzed the ability of PTX to inhibit GlyR activity in terms of the outside-out current evoked by glycine applications to patches containing recombinant \( \alpha_2 \) homomeric GlyR from CHO cells stably expressing the \( \alpha_2 \) GlyR subunit. Fig. 1A illustrates the inhibitory effect of different concentrations of PTX on inward currents (\( V_r \) = −50 mV) evoked by 300 \( \mu \)M glycine (left traces, near the EC\(_{50} \) for glycine-evoked response, see Ref. 23) and 30 \( \mu \)M glycine (right traces). In these experiments glycine was co-applied with PTX at concentrations indicated by the number below each trace. The co-application of PTX and glycine caused a concentration-dependent reduction of the current amplitude. The effect of PTX was reversible after washout. Interestingly, after co-

model transient (29). Outside-out responses from 12 patches evoked by the application of glycine in the absence or presence of PTX 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.

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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 sites. In a simple kinetic model with several liganded closed states 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. This is the case for homomeric α2 GlyRs (23). For homomeric α2 GlyRs the mean burst duration during deactivation depends on the mean open time of the channel, the number of openings, and the number of closures. The mean burst duration $\tau_B = N_c/\alpha + N_i/(\beta + k_{off})$, where $\beta$ is the opening rate constant, $\alpha$ the closing rate constant, $k_{off}$ the dissociation rate constant for glycine, $N_c$ the number of openings per burst ($N_c = 1 + \beta/k_{off}$), and $N_i$ the number of closures per burst ($N_i = \beta/k_{off}$) (34). In the case of a simple fast open channel block mechanism with no other way than the open state for the channel to escape from the block, the mean open time of each opening within a burst must decrease but the mean burst length must be increased, which slows down the relaxation (34). In contrast, 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. Besides these two extreme blockers, an intermediate blocker must evoke a biphasic relaxation (34). In our experiments, PTX decreased in a concentration-dependent manner the deactivation time constant of the current evoked by a short concentration step of glycine (Fig. 3B). In this case the relaxation can still be fitted by a single exponential curve, which favors the hypothesis of slow blocker-like mechanisms for PTX. If so, PTX must also decrease the mean open time of the channel to the same extent as the deactivation time constant of the glycine-evoked currents.

To determine the microscopic determinants of the decrease in the decay time constant, we have analyzed the open time and closed time distributions in single receptor bursts of openings in response to short (1 ms) concentration pulses of glycine near GlyR saturation (30 μM) in the absence and presence of PTX. 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. 23). 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. 4A). In the presence of continuous 10 μM PTX, the single opening duration appeared to be shortened (Fig. 4B). Opening and closing time constants were estimated by pooling measurements made on these single-channel responses obtained from 7–9 patches (23). The open time histograms were best fitted by single exponential curves both in control conditions and in the presence of continuous PTX (Fig. 4B and C). In control conditions, the mean open time was 48.4 ms, which is consistent with the value we obtained previously (23). The mean open time was decreased to 61.1 ms in the presence of 10 μM PTX. In control conditions, the closed time distribution was best fitted by a single exponential curve with a closed time constant $\tau_c = 0.27$ ms, as previously described for homomeric α2 GlyRs (23). In the presence of PTX (10 μM) the closed time distribution was best fitted by the sum of two exponential curves giving a $\tau_{c1} = 0.23$ ms, which is very similar to the closed time constant in the control conditions. A second closed time was detected in the presence of PTX with a time constant $\tau_{c2} = 5.76$ ms (Fig. 4F). This longer closed time is likely to reflect an additional recovery pathway from PTX-evoked open channel block.

The ensemble-averaged currents obtained by averaging single channel responses (116 trials for 30 μM glycine, 202 trials for continuous 10 μM PTX) indicated a $\tau_{decay}$ similar to that observed for macroscopic
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FIGURE 3. Acceleration of the decay phase of glycine-activated currents by the continuous presence of PTX. A1, current traces activated by 300 $\mu$M glycine (control), by co-application of 300 $\mu$M glycine and 10 $\mu$M PTX (plus 10 $\mu$M PTX), and by 300 $\mu$M glycine in the continuous presence of 10 $\mu$M PTX (plus continuous PTX) when PTX was maintained during, before, and after glycine application. Each trace represents the average of 12–15 responses. Note that the glycine-elicteded currents in the control condition and with co-application of PTX returned slowly to the baseline after termination of glycine application, whereas the continuous presence of PTX eliminated the rebound current and significantly accelerated the decay phase. The thick line represents the application of 300 $\mu$M glycine. A2, normalized decay phase of glycine responses replotted on an expanded time scale to illustrate better the acceleration of the deactivation time course by continuous 10 $\mu$M PTX. The decay phase was best fitted with a single exponential function giving a decay time constant of 114 ms for control response, 116 ms for co-application of glycine and PTX, and 27 ms for continuous presence of PTX. Similar results were obtained from another 12 cells. 

To obtain more precise information on the block mechanism of PTX, we analyzed the effect of increasing PTX concentration on the mean open time of the GlyR channel. Such analysis will also give us information on the blocking rate constant of PTX (34). We analyzed the mean open time of the GlyR channel. Such analysis will also give us information on the kinetics of the receptor channel (23). The effects of PTX on the rising phase of macroscopic averaged currents evoked by glycine on $\alpha_2$ homomeric GlyRs were therefore analyzed (Fig. 6). A series of 10–25 trials evoked with $\approx10$-s intervals was used to generate macroscopic currents as shown in Fig. 6A. The duration of the applications was adjusted to obtain a steady-state current. The rising phases of the outside-out currents evoked by the application of 300 $\mu$M glycine were best fitted with the sum of two exponential curves at all PTX concentrations tested (Fig. 5, A–C). As expected, PTX decreased the mean open time in a concentration-dependent manner. The mean open time for the control response was 50.1 ms (66 trials from 3 patches, Fig. 5A). In the presence of continuous PTX, the mean open time decreased to 26.2, 16.2, 6.3, and 2.7 ms, for 1 $\mu$M PTX (44 trials from 3 patches), 3 $\mu$M PTX (52 trials from 3 patches), 10 $\mu$M PTX (47 trials from 3 patches), and 30 $\mu$M PTX (22 trials from 1 patch), respectively (Fig. 5, B–D). These results indicate that PTX inhibition can be related to an open channel blocker mechanism (34).

To obtain an approximation of the binding rate constant for PTX, the reciprocals of the mean open times were plotted as a function of the PTX concentration as shown in Fig. 5D. Binding ($k_{on}$) and closing ($\alpha$) rate constants were calculated from the relationship $1/\tau_a = [PTX]k_{on} + \alpha$ (34), where $\tau_a$ is the mean open time and [PTX] is the PTX concentration. The linear fit to the data gave $k_{on} = 11.6 \mu M^{-1} s^{-1}$ and an $\alpha$ value of 27.9 s$^{-1}$.

PTX Slows Down the Activation Kinetics of Glycine-evoked Current—The activation phase of current evoked by concentration steps of agonist gives important information on the kinetics of the receptor channels (23). The activation phase of PTX inhibition by concentration steps of agonist gives important information on the kinetics of the receptor channels (23). The effects of PTX on the rising phase of macroscopic averaged currents evoked by glycine on $\alpha_2$ homomeric GlyRs were therefore analyzed (Fig. 6). A series of 10–25 trials evoked with $\approx10$-s intervals was used to generate macroscopic currents as shown in Fig. 6A. The duration of the applications was adjusted to obtain a steady-state current. The rising phases of the outside-out currents evoked by the application of 300 $\mu$M glycine were best fitted with the sum of two exponential curves giving a fast rising time constant ($\tau_{fast}$) and a slow rising time constant ($\tau_{slow}$) as previously described (23). As shown in Fig. 6B, there was no significant difference in the $\tau_{fast}$ of currents activated by 300 $\mu$M glycine alone ($\tau_{fast} = 38 \pm 5$ ms, $n = 14$), during co-applications of glycine and PTX ($\tau_{fast} = 24 \pm 5$ ms, $n = 13$) or during the continuous application of PTX, before, during and after glycine application ($\tau_{fast} = 24 \pm 4$ ms, $n = 12$) (ANOVA, $p > 0.05$). $\tau_{slow}$ in the presence of co-application of 10 $\mu$M PTX and 300 $\mu$M glycine was significantly decreased, compared with responses evoked by glycine application alone (paired t test, $p < 0.05$). These results could suggest that PTX had little or no effect on glycine binding.
PTX Inhibition of \( \alpha_2 \) Homomeric GlyR

For homomeric \( \alpha_2 \) GlyRs, \( \tau_{\text{fast}} = 1/\alpha + \beta(\text{[glycine]}^\alpha/\text{[glycine]}^\beta + r_{E_{C50}}^\beta) \), where [glycine] is the concentration of the agonist, \( n \) the Hill coefficient, and \( r_{E_{C50}} \) is the concentration of glycine that gives half of the maximum opening rate constant \( \beta \) (23). Therefore, to determine whether the opening rate constant \( \beta \) was modified by PTX or not, we analyzed the rising phase of the currents evoked by a saturating concentration of glycine (30–100 mM) in the absence and presence of PTX. For such saturating concentrations, the faster rising time constant \( \tau_{\text{fast}} \approx 1/\alpha + \beta \) and is moreover mainly controlled by \( \beta \), since \( \beta \) is >200 times faster than \( \alpha \) for homomeric \( \alpha_2 \) GlyRs (23). Measurements were performed on averaged traces of 12–25 trials. In control conditions the rising phase of the currents evoked by 30 mM glycine was well fitted with the sum of two exponential curves in 12 out of 17 patches (Fig. 6, C and D) with time constants \( \tau_{\text{fast}} = 0.27 \pm 0.03 \text{ ms} \) and \( \tau_{\text{slow}} = 3.1 \pm 0.5 \text{ ms} \) (n = 12). As shown in Fig. 6 (E and F), simultaneous application of 30 mM glycine and 10 \( \mu \)M PTX did not significantly change the rising time constants as expected if the \( \beta \) value was not modified by PTX applications (paired t test, \( p > 0.1 \)). In the presence of PTX, \( \tau_{\text{fast}} = 0.26 \pm 0.03 \text{ ms} \) and \( \tau_{\text{slow}} = 2.6 \pm 0.8 \text{ ms} \) (n = 12). Surprisingly, when 10 \( \mu \)M PTX was continuously applied before, during and after 30 mM glycine successive concentration steps (application frequency of 0.1 Hz), the rising phase of the first glycine-evoked response was unchanged, whereas it was slowed down for the next responses (data not shown). This PTX effect on the rising phase of glycine-evoked responses was analyzed on averaged traces (12–15 sweeps, Fig. 6C). In this case both \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) were significantly increased (paired t test, \( p < 0.01 \)). During continuous application of 10 \( \mu \)M PTX, \( \tau_{\text{fast}} = 2.1 \pm 0.4 \text{ ms} \) and \( \tau_{\text{slow}} = 15.2 \pm 3.0 \text{ ms} \) (n = 12). Increasing the glycine concentration to 100 mM (Fig. 6D) did not prevent this PTX effect, confirming that it cannot result from modifications in glycine binding kinetics (Fig. 6, E and F). In control conditions, the rising phase of the responses evoked by the 100 mM glycine step was better fitted by two exponential curves in 8 out of 11 patches tested (in the other patches the rising phase was fitted with a single exponential function; see Ref. 23). In this case, \( \tau_{\text{fast}} = 0.26 \pm 0.03 \text{ ms} \) and \( \tau_{\text{slow}} = 3.1 \pm 0.6 \text{ ms} \) (n = 8). With continuous application of PTX, \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) increased to 2.1 \pm 0.3 ms and 15.0 \pm 2.5 ms, respectively (n = 8). This was not significantly different from the measurements obtained with 30 mM glycine (unpaired t test, \( p > 0.1 \)).

An Increase in the First Latency Accounts for the Increase in the Two Activation Time Constants—To determine if the increase in the two activation time constants of the rising phase of the currents that we observed in the presence of continuous application of PTX reflects...
changes in GlyR behavior occurring before channel conformational changes leading to the open state, we analyzed the distribution of initial closed times leading to the first opening (first latencies) in outside-out patches containing one active GlyR (see “Materials and Methods”). Fig. 7 (A and B) shows the activation of a single receptor from the same patch in response to 200-ms step applications of 30 mM glycine in the absence (Fig. 7A) or in the continuous presence (Fig. 7B) of 10 μM PTX (application frequency, 0.1 Hz). The ensemble-averaged current obtained by averaging single channel responses (229–266 trials) had time courses similar to those observed for macroscopic currents in the absence or

FIGURE 5. PTX decreased the mean open time of GlyR in a concentration-dependent manner. A–C, open time duration histograms obtained in the control (30 mM glycine; A) and in the continuous presence of 3 μM PTX (B) and 10 μM PTX (C) are shown as a function of log intervals with the ordinate on a square root scale. Histograms were better fitted with one exponential curve. Note that mean open time was decreased by PTX in a concentration-dependent manner. D, the reciprocals of the mean open times were plotted as a function of the PTX concentration, and the binding (k_on) and closing (k_off) rate constants were calculated from the relationship \(1/\tau_o = [\text{PTX}]k_{\text{on}} + \alpha\), where \(\tau_o\) is the mean open time, and [PTX] is the PTX concentration. The linear fit to the data gave a k_on value of 11.6 μM^{-1}s^{-1} and a value of 27.9 s^{-1}. Mean open times were obtained by pooling single-channel currents (22–66 trials) in one to three different experiments for each concentration of PTX.

FIGURE 6. Slower onset of macroscopic currents activated by saturating concentration of glycine in the continuous presence of PTX. A, averaged traces of currents (n = 10–25) obtained from the same patch showing the activation phase of the responses evoked by 300 μM glycine, co-application of 300 μM glycine, and 10 μM PTX (+10 μM PTX), and by glycine in the continuous presence of 10 μM PTX (plus continuous PTX). B, summary of data (n = 12–14) obtained from the experiments shown in A. NS, not significant; *, p < 0.05. C, averaged traces of currents (n = 12–15) obtained from the same patch showing the activation phase of the responses evoked by saturating concentration of 30 mM glycine, co-application of 30 mM glycine and 10 μM PTX, and by glycine in the continuous presence of 10 μM PTX. Note that the activation phase of the glycine response was slowed down in the continuous presence of PTX. D, averaged traces of currents (n = 12–25) obtained from the same patch showing the activation phase of the responses evoked by saturating concentration of 30 mM glycine in the absence and presence of continuous 10 μM PTX, and by oversaturating concentration of 100 mM glycine in the absence and presence of continuous 10 μM PTX. Note that increasing glycine concentration to 100 mM does not change the activation time course of current response activated by 30 mM glycine in the continuous presence of PTX. E and F, summary of data (n = 8–12) obtained from the experiments shown in C and D (NS, not significant).
presence of continuous application of PTX, as previously described (see Fig. 6). As shown for macroscopic currents, the ensemble-averaged currents also exhibit a biphasic rising phase with fast and slow components, which were considerably slowed down in the continuous presence of PTX. The increase in the activation time constants observed in the presence of PTX appeared to be related to an increase in the first latency duration. The first latency cumulative distributions of the activation of a single GlyR evoked by 30 mM glycine application in the absence or presence of 10 μM PTX are shown in Fig. 7 (C and D). The first latency distributions were best fitted by the sum of two exponential functions with time constants $\tau_{\text{fast}} = 0.24$ ms (85%), $\tau_{\text{slow}} = 2.2$ ms (15%) in the absence of PTX and $\tau_{\text{fast}} = 4.7$ ms (61%), $\tau_{\text{slow}} = 18.9$ ms (39%) in the presence of continuous PTX. The corresponding ensemble-averaged current exhibited a rising phase with $\tau_{\text{fast}} = 0.33$ ms (90%), $\tau_{\text{slow}} = 2.4$ ms (10%) in the absence of PTX and $\tau_{\text{fast}} = 5.1$ ms (48%), $\tau_{\text{slow}} = 20.7$ ms (52%) in the presence of PTX, indicating that the slower activation phase of the ensemble current in the presence of continuous PTX was related to changes in GlyR conformational closed states distal from the channel open state.

Recovery from PTX Block Requires Channel Reopening—A lengthening in the rise time of responses evoked by PTX preincubation has been described for GABA-evoked outside-out currents in crayfish muscle (35). This was interpreted as the consequence of PTX binding to the unliganded receptor (35). To test this hypothesis on GlyRs, we analyzed the effects of PTX pre-treatment on current evoked by the application of 10 mM glycine alone. Because the lengthening in the current rising phase observed for GABA-evoked outside-out current is likely to reflect recovery from PTX inhibition in the presence of the agonist alone (35), we first estimated for comparison the recovery time constant of PTX inhibition by a transient application of 10 μM PTX during glycine-evoked currents. As shown in Fig. 8A, transient application of PTX evoked a fast decrease in glycine current with a time constant of 17.0 ± 2.6 ms (n = 7) (Fig. 8A). At the end of the application of PTX, current amplitude increased progressively. This recovery phase from PTX inhibition was best fitted by a bi-exponential curve with time constants $\tau_{\text{fast}} = 2.6 ± 0.4$ ms (20 ± 2%) and $\tau_{\text{slow}} = 21.7 ± 3.9$ ms (80 ± 2%) (n = 7).

When 10 μM PTX was applied immediately before (time interval of <0.1 ms) a concentration step of a saturating concentration of glycine (10 mM), it did not change the amplitude of the glycine-evoked current (10 μM PTX) or its rising phase (Fig. 8B). The activation time constants were $\tau_{\text{fast}} = 0.4 ± 0.04$ ms (81 ± 5%) and $\tau_{\text{slow}} = 2.6 ± 0.7$ ms (19 ± 5%) ms in control conditions, and $\tau_{\text{fast}} = 0.4 ± 0.04$ ms (82 ± 9%) and $\tau_{\text{slow}} = 2.4 ± 0.2$ ms (18 ± 9%) ms with PTX pre-treatment (n = 5). This was not significantly different (paired t test, p > 0.5). These data indicate that it is unlikely that PTX can bind to unliganded GlyR.

According to the results described above, the lengthening of the rise time we observed in the continuous presence of PTX is unlikely to be
due to PTX binding to unliganded GlyR. Moreover, simultaneous application of PTX and glycine had no significant effect on the rise time of the outside-out current. The only possibility of the lengthening of the rise time we observed in the continuous presence of PTX is that PTX, when applied during the deactivation of the glycine-evoked currents, modifies the activation kinetics of the next response. This hypothesis implies that...
PTX preferentially binds to GlyR in the open state and that it can remain bound after glycine washout. A similar PTX inhibitory effect was recently described for mutated R271C homomeric \( \alpha_1 \) GlyRs (14). To test this hypothesis we analyzed the effect of PTX on the rise time of successive responses evoked by glycine when this alkaloid was applied during the relaxation phase of the first response (PTX post-treatment). PTX was applied for 500 ms, which corresponds to the full time-course of the deactivation phase of the glycine-evoked current without PTX. For these experiments we selected patches with a large number of GlyRs. This allowed us to compare the rise time between individual traces. The activation time constants were measured for glycine responses evoked 60 s after PTX post-treatment. They were compared with the values obtained in control conditions. To determine if this effect of PTX was reversible, we analyzed the rise time of responses evoked 3 s after the second application of glycine alone. As shown in Fig. 8C, post-treatment with 10 \( \mu \)M PTX was sufficient to speed up the deactivation phase of glycine-evoked current. The current evoked by the application of glycine alone up to 60 s after the end of the glycine current plus PTX post-treatment had an amplitude similar to that of the control response (<5% decrease, \( n = 7 \)). Surprisingly, this current had a significantly slower rise time than that of control (\( n = 7 \), paired \( t \) test, \( p < 0.01 \)). This was due to slower rising time constants \( \tau_{fast} \) and \( \tau_{slow} \) and to an increase in the proportion of the slow component of the activation phase. The activation time course of these responses was well fitted by the sum of two exponential curves as in the control, but with time constants \( \tau_{fast} = 2.2 \pm 0.2 \) ms (38 ± 1%) and \( \tau_{slow} = 31 \pm 3 \) ms (62 ± 1%). Applying glycine after (3 s) the response with a slower rising phase (Fig. 8C) evoked a current with activation time constants very similar to control values: \( \tau_{fast} = 0.5 \pm 0.04 \) ms (85 ± 5%) and \( \tau_{slow} = 3.1 \pm 0.3 \) ms (15 ± 5%). These results clearly indicate that the lengthening of the rise time evoked by PTX can persist up to 60 s after washout of glycine and PTX. They also indicate that GlyRs must be reactivated to allow recovery from the PTX effect. It is therefore likely that PTX can be trapped at its binding site when the channel closes and/or when glycine molecules dissociate from their binding sites.

A Minimal Markov Model for PTX Inhibition—To account for the data obtained on PTX inhibition of homomeric \( \alpha_1 \) GlyR, we adopted the minimal Markov model previously proposed for this GlyR subtype (23). This model has two binding sites for glycine, two desensitized closed states and a single open state linked to the doubly liganded closed state. Each desensitized state is linked to the mono-liganded closed state and the doubly liganded closed state, respectively. But this model has some limitations, because it has a tendency to underestimate the time constant values of the rising phase of the currents evoked by glycine concentrations lower than the EC\(_{50}\) of glycine (23). We overcame this problem by adding a third binding site linked to another desensitization state (Fig. 9A), as recently proposed for homomeric \( \alpha_1 \) GlyR (36). Before testing the Markov models accounting for PTX inhibition, we first adjusted the different rate constants of the model describing glycine-evoked responses for each control trace. To do so we fitted experimental traces obtained by a long application of 0.3 mM glycine (23, 37). The average kinetic parameters derived from model fitting of glycine-evoked outside-out currents are listed in Table 1. This model predicts a glycine EC\(_{50}\) of 240 \( \mu \)M and a Hill coefficient of 2, which are in good agreement with previously published values (200 \( \mu \)M and 1.9, respectively) (23).

Having established the kinetic parameters for glycine-evoked currents, we then analyzed PTX inhibition responses on the same traces. The kinetic model for PTX inhibition was elaborated according to our experimental data. According to the Hill coefficient (\( \approx 1 \)) of the concentration-response curve for PTX, we first postulated that only one PTX molecule binds to GlyR. This is consistent with what is known about PTX inhibition of GABA\(_{\text{A}}\) receptors (17), crayfish muscle GABA receptors (35), and homomeric \( \alpha_1 \) GlyR (18). PTX must interact with the fully liganded open state, because the channel mean open time was decreased when PTX concentration was increased (Fig. 5) giving an estimated association rate constant for PTX of 11.6 \( \mu \)M\(^{-1}\)s\(^{-1}\) (Fig. 5D). PTX had no effect when applied immediately before glycine, which indicates that PTX cannot directly bind to the unliganded receptor, but when PTX was applied during the deactivation phase of glycine-elicited current, the activation phase of the current was lengthened even when glycine was applied 60 s after PTX washout (Fig. 8C). This could be explained by a trapping mechanism when glycine dissociates before PTX (14). This was simulated by adding a glycine-unbound state (\( A_1 \) plus PC) linked to the sequential glycine-bound closed states (\( A_1 \) plus APC, \( A_1 \) plus A\(_2\)PC, and A\(_3\)PC) to which PTX remains bound (Fig. 9, B and C). Adding these bound states also accounted for the acceleration of the relaxation of GlyR evoked by PTX, as previously proposed for PTX-evoked GABA\(_{\text{A}}\) receptor inhibition (17). To be consistent with the GlyR model describing GABAA kinetics in the absence of PTX, each glycine-bound state associated with PTX (\( A_1 \) plus APC, \( A_1 \) plus A\(_2\)PC, and A\(_3\)PC) must be linked to a desensitization state (Fig. 9, B–D).

In the PTX block models we envisioned, PTX binds within the vestibule of the channel, which shortens channel opening (\( A_O \) to \( A_PB \); see Fig. 9B). It is then trapped at its binding site when the channel goes back to its closed-state conformation (\( A_PB \) to \( A_PC \); Fig. 9B). In these models glycine can unbind while PTX remains trapped.

Two PTX block model subtypes were tested. In model 1 (Fig. 9B), the only way for PTX to bind and unbind is from the open state. The second type of model (models 2 and model 3) supposes that the PTX binding site is not fully masked when the channel is in its bound closed conformation. This is also the case for GABA\(_{\text{A}}\) receptors (17). In model 2, one step was incorporated between the fully glycine-liganded closed states (\( A_1 \)C) and the corresponding glycine-liganded closed states plus PTX (\( A_1 \)PC). Accordingly, this model contains one cyclic scheme (Fig. 9C). This model supposes that PTX can escape from its binding site only when GlyR is fully liganded. In model 3, PTX is trapped when the receptor goes back to its unbound closed state. In this model, three steps were incorporated between the glycine-liganded closed states (\( A_2 \) plus A\(_1\)C, \( A_2 \) plus A\(_3\)C, and \( A_3 \)C) and the corresponding glycine-liganded closed states plus PTX (\( A_2 \) plus A\(_3\)PC, \( A_3 \) plus A\(_2\)PC, and \( A_2 \) plus A\(_3\)PC). Accordingly, this model contains three cyclic schemes (Fig. 9D). This model is somewhat similar to the kinetic model proposed for GABA\(_{\text{A}}\) receptors (17).

To compare the different models accounting for PTX inhibition, we fitted experimental traces obtained by long application of 0.3 mM or 30 mM glycine in the presence of 1, 3, and 10 \( \mu \)M PTX (\( n = 12 \) patches). All rate constants estimated with the control model for GlyR were set as fixed parameters. For simplicity, the glycine association rate constant (\( k_{a} \)) linking the different glycine-bound states plus PTX (\( A_1 \) plus APC, \( A_1 \) plus A\(_2\)PC, and A\(_3\)PC) to the desensitization rate constants and the corresponding recovery rate constants linking the liganded closed states plus PTX and the desensitization states (\( A_2 \) plus A\(_3\)PD, \( A_3 \) plus A\(_2\)PD, and A\(_3\)PD) were also set as fixed variables. All other parameters were set as free variables. We imposed constraints depending on the model tested. Model 1 had no constraint, but models 2 and 3 must have constrained reactions depending on the reaction cycles to satisfy the principle of microscopic reversibility (34). In model 3, the on reactions and the off reactions linking the liganded closed states with and without PTX were set as equivalent (\( A_2 \) plus \( A_3 \) to \( A_2 \) plus APC, \( A_3 \) plus \( A_2 \) to \( A_2 \) plus \( A_3 \)PC).
The simplification postulating that PTX affinity is similar for the three bound closed states of the receptor was also proposed for GABA<sub>C</sub> receptors (17). It is however important to note that when these reactions were set as independent, the fit of the experimental traces was not improved and the rate constants for these steps diverged considerably.

**Figure 9.** Kinetic schemes used for fitting glycine responses in the absence and presence of PTX. A, agonist; P, PTX; C, resting states of the receptor; D, desensitized state; and O, open state. A, this kinetic scheme was used for homomeric α<sub>2</sub> GlyR in control conditions (without PTX). B, in model 1, PTX can bind and unbind from the GlyR open state only. PTX remains bound if continuously applied when glycine dissociates from its binding sites. C, in model 2, PTX can bind and unbind from the fully glycine-ligated closed state or the open state of GlyR. In this scheme, PTX is trapped when glycine dissociates from the fully liganded closed state. D, in model 3, PTX can bind and unbind from all glycine-bound states, but PTX is only trapped when the receptor returns to the glycine-unbound closed state.
**PTX Inhibition of \(\alpha_2\) Homomeric GlyR**

**TABLE 1**  
Kinetic parameters for PTX inhibition derived from models 2 and 3 fitting (mean ± S.E., n = 12)

| Parameter | Model 2 | Model 3 |
|-----------|---------|---------|
| \(k_{on}\) | 0.77 ± 0.04 \(\mu M^{-1} s^{-1}\) | 0.77 ± 0.04 \(\mu M^{-1} s^{-1}\) |
| \(k_{off}\) | 1657.9 ± 404.5 \(s^{-1}\) | 1657.9 ± 404.5 \(s^{-1}\) |
| \(k_{cat}\) | 76.6 ± 47.4 \(s^{-1}\) | 76.6 ± 47.4 \(s^{-1}\) |
| \(d_1\) | 2853.6 ± 263.7 \(s^{-1}\) | 2853.6 ± 263.7 \(s^{-1}\) |
| \(d_2\) | 711.3 ± 309.3 \(s^{-1}\) | 711.3 ± 309.3 \(s^{-1}\) |
| \(d_3\) | 11.75 ± 2.3 \(s^{-1}\) | 11.75 ± 2.3 \(s^{-1}\) |
| \(r_1\) | 94.5 ± 44.9 \(s^{-1}\) | 94.5 ± 44.9 \(s^{-1}\) |
| \(r_2\) | 461.1 ± 123.8 \(s^{-1}\) | 461.1 ± 123.8 \(s^{-1}\) |
| \(k_{on}\_\text{p}\) | 0.1 ± 0.02 \(s^{-1}\) | 0.1 ± 0.02 \(s^{-1}\) |
| \(\alpha\) | 21.8 ± 1.3 \(s^{-1}\) | 21.8 ± 1.3 \(s^{-1}\) |
| \(\beta\) | 4875 ± 89.7 \(s^{-1}\) | 4875 ± 89.7 \(s^{-1}\) |
| \(k_{off}\_\text{p}\) | 4.9 ± 0.9 \(s^{-1}\) | 5.4 ± 0.9 \(s^{-1}\) |
| \(k_{cat}\_\text{p}\) | 46.9 ± 11.9 \(s^{-1}\) | 57.8 ± 10.3 \(s^{-1}\) |
| \(k_{cat}\_\text{p}\) | 483.5 ± 121.4 \(s^{-1}\) | 278 ± 69.4 \(s^{-1}\) |
| \(A\) | 749.8 ± 198.3 \(s^{-1}\) | 327.9 ± 83.6 \(s^{-1}\) |
| \(B\) | 3.069 ± 109 \(s^{-1}\) | 2.009 ± 108 \(s^{-1}\) |

As shown in Fig. 10A, model 1 failed to describe the experimental data (\(k_{on}\_\text{p}\) = 7.28 ± 1.73 \(s^{-1}\), \(k_{off}\_\text{p}\) = 72.78 ± 27.9 \(s^{-1}\), \(a\) = 2.597 × 10\(^2\) ± 1.753 × 10\(^3\) \(s^{-1}\) and \(b\) = 2.597 × 10\(^1\) ± 9.556 × 10\(^1\) \(s^{-1}\)). It always predicted a prominent peak current at the onset of the glycine-evoked current, before PTX inhibition can stabilize. This can be overcome by increasing the dissociation rate constant for PTX at a value close to the opening rate constant of the channel (33). But in this case the model predicts a large rebound current even when the association rate constant for PTX was set to maintain a good prediction of PTX IC\(_{50}\) value. This was not observed experimentally.

Model 2 (Fig. 9C) provided a better prediction of our experimental results. This model gave \(\approx 5.8 \pm 0.8\) times significant lower SSES values than model 1 (ANOVA, \(p < 0.01\)). Incorporating steps for PTX binding to the other liganded bound states (model 3; Fig. 9D) did not significantly improve the fit when compared with model 2 (ANOVA, \(p > 0.1\)), suggesting that, although such transitions could exist, they were not necessary to describe our experimental data. The optimal averaged rate constant values obtained for models 2 and 3 are listed in Table 1. As shown in Table 1, fits of experimental data with models 2 and 3 gave very similar values for PTX association and dissociation rate constants. In models 2 and 3, the affinity of PTX for the channel open state (model 2: \(k_{off1p}/ Kon1p = 9.6 \mu M\); model 3: \(koff1p/Kon1p = 10.7 \mu M\)) was found to be lower than that for the bound closed state (model 2: \(koff2p/Kon2p = 1.6 \mu M\); model 3: \(koff2p/Kon2p = 1.2 \mu M\)). When we attempted to set the affinity of PTX for the channel open state equal to that for the liganded closed states, the SSES of the fit was 3.1 ± 0.7 times significantly higher (ANOVA, \(p < 0.01\)), suggesting that the rate constant values for these PTX binding steps are unlikely to be equivalent. A similar conclusion was reported for PTX inhibition of GABA\(_{C}\) receptors (17). Because channel gating may involve large conformational changes, it is reasonable to suppose that the access of PTX to its binding site will be different when the receptor is in a bound closed conformation and in a bound open conformation (17).

Fig. 10 shows examples of fits of experimental traces using model 2 (thick dark lines) to responses evoked by the co-application of 30 mM glycine and 10 \(\mu M\) PTX (Fig. 10A) or 0.3 mM glycine and 1 and 3, and 10 \(\mu M\) PTX (Fig. 10B). The model predicts a stable current amplitude in the presence of 30 mM glycine and 10 \(\mu M\) PTX and a small rebound current at the end of the co-application of PTX and glycine occurring for PTX concentration \(\geq 3 \mu M\) (Fig. 10, A and B). The model also predicts an increase in PTX IC\(_{50}\) when glycine concentration is increased (Fig. 10C). Parameters listed in Table 1 predict a PTX IC\(_{50}\) of 3.4 \(\mu M\) and of 9.3 \(\mu M\) in the presence of 0.3 mM glycine and 30 mM glycine, respectively. This is in good agreement with our experimental data (2.7 \(\mu M\) and 6.4 \(\mu M\), respectively; Fig. 1B). When the rate constant for PTX association from the open state was set as a free parameter, it was close to 5 \(\mu M^{-1} s^{-1}\) (Table 1), which is in reasonably good agreement with our experimental measurements (11.5 \(\mu M^{-1} s^{-1}\)). This model also predicts the acceleration of the relaxation phase of the glycine-evoked current observed in the continuous presence of PTX (Fig. 10D), the lengthening of the rise time of the 30 mM glycine-evoked current (Fig. 10F) during continuous application of PTX and the lack of PTX effect on the rise time of currents evoked by 0.3 mM glycine (Fig. 10E). It also predicts the lengthening of the rise time of the glycine-evoked current when PTX was applied during the deactivation phase of the preceding response (Fig. 10G). Overall, these data indicate that model 2 characterized by the presence of two PTX binding steps, one from the fully liganded closed state and one from the open state, is the minimal stochastic scheme that best predicts PTX inhibitory effects on homomeric \(\alpha_2\) GlyRs.

**DISCUSSION**

In the present study, we demonstrated several unexpected new features for PTX inhibition of wild-type homomeric \(\alpha_2\) GlyR recorded on outside-out patches. As previously observed, PTX had both competitive and noncompetitive inhibitory effects on homomeric GlyRs. This complex inhibitory mechanism can be predicted by a simple kinetic model in which glycine can dissociate while PTX remains bound. PTX cannot bind to the GlyR unliganded-closed conformation, but our results also suggest that PTX is likely to be trapped while glycine dissociates from the wild-type homomeric \(\alpha_2\) GlyR.

**A Minimal Kinetic Model for PTX Block**—Although kinetic schemes have been proposed to describe the mechanism of PTX inhibition of GABA\(_{A}\) and GABA\(_{C}\) receptors (17, 35), this has not been the case for homomeric GlyRs. The model (model 2, Fig. 9C) we proposed to describe PTX-evoked GlyR inhibition predicts our experimental data and gives a good prediction of both competitive and noncompetitive mechanisms previously described for homomeric \(\alpha_2\) GlyRs (18). This model is, however, not identical to that recently proposed for PTX inhibition of GABA\(_{C}\) receptors (17). In the GABA\(_{C}\) model there is no intermediate step between the PTX-bound open channel state and the PTX-bound fully liganded closed state, which results in a reaction cycle with three steps only (17). Such a reaction cycle also assumes that PTX binding to the open conformation and the receptor conformational change leading to channel closure occur simultaneously. Although a reaction cycle with three steps is computationally valid, it is not physically plausible at least for PTX inhibition of homomeric \(\alpha_2\) GlyR, if one assumes that PTX must bind first before being trapped when the channel closes. This implies a reaction cycle with four steps as shown in Fig. 9. Accordingly, the receptor first undergoes a conformational change to a new stable state when it is fully liganded (channel opens), PTX binds to the open conformation and then the channel closes. The cycle is terminated when PTX dissociates directly from the fully liganded closed conformation. However, it should be noted that the estimated off-rate and on-rate constants of the change in the GlyR channel conformation after PTX binding are very fast (>10\(^4\) \(s^{-1}\)), which could indicate that the two steps (PTX binding and channel closure) collapse. If so, PTX binding might specifically evoke a fast change in GlyR conformation leading to channel closure. This is consistent with what is known about the mechanisms proposed for PTX inhibition of homomeric GlyR. It is now well established that agonist binding causes conformational changes in the extracellular ligand-binding domain, which are transmitted to the channel gate via conformational changes in the M2–M3 loop of the GlyR \(\alpha\) subunit.
subunit (4). PTX binding was recently proposed to alter GlyR M2–M3 conformational changes in a way that cannot be achieved by glycine (14). Unlike what has been postulated for the mechanism of PTX inhibition of GABA<sub>C</sub> (17), it was not necessary to assume that glycine affinity changes (<sup>k<sub>on</sub></sup> or <sup>k<sub>off</sub></sup>) in the presence of PTX would fit our experimental data. This is consistent with previously published data showing that PTX did not change glycine binding to homomeric GlyRs (18).

Models 1, 2, and 3 predict the previously described “competitive” and “noncompetitive” mechanisms of PTX action on homomeric GlyRs (18), as also described for GABA<sub>C</sub> receptors (17). The rebound current after termination of PTX and glycine co-application we observed and
the PTX concentration-dependent decrease in the GlyR mean open time of the GlyR channel are consistent with what is known about open channel blockers (noncompetitive mechanism). But in all models tested glycine can dissociate from its binding sites while PTX remains bound, as also proposed for GABA<sub>A</sub> receptors (17). This mechanism accounts for the apparent competitive PTX inhibition described for both GlyRs (18) and GABA<sub>C</sub> receptors (17). This is not surprising, because the recovery from PTX block depends on the PTX dissociation rate constant and on the different glycine-binding steps in the presence of PTX. Accordingly, an increase in glycine concentration will increase the glycine association rate between the glycine-bound closed states plus PTX (A plus A<sub>PC</sub>, A<sub>2</sub> plus APC and A<sub>PC</sub>; Fig. 9), which will result in an apparently faster PTX recovery rate. Accordingly, the simple block model of mechanism 1 also predicts a shift to the right of the PTX concentration response curve when glycine concentration is increased. The simulation of the PTX inhibition using model 1 predicted PTX IC<sub>50</sub> values of 3.0 μM and of 11.1 μM in the presence of 0.3 mM and 30 mM glycine, respectively. This is also the case for models 2 and 3.

**Location of the PTX Binding Site: Trapped or Not Trapped**—There is evidence indicating that PTX acts at the highly conserved M2 domain, because several M2 residues have been identified that, when mutated, impair PTX sensitivity (5, 6, 8–13). A series of studies on the GABA<sub>A</sub>R, GABA<sub>C</sub>R, GlyR, invertebrate glutamate receptor CI<sup>−</sup> channel, and serotonin type 3A receptor established the residues in the cytoplasmic portion of M2 (2′ and 6′ residues) as crucial determinants of PTX sensitivity (4, 13). Mutations introduced at both the 2′ and 6′ positions of M2 confer PTX resistance (11). A common feature in all of these studies is that a ring of 6′-threonines is invariably required for high PTX sensitivity (6, 8, 11, 14), and it has been suggested that the PTX-binding site probably lies close to 6′- pore-lining position of M2 (38). A recent study provided evidence supporting the hypothesis that PTX binds in the pore of the channel (14). PTX is converted into a use-dependent blocker of this GlyR subtype by mutations to R271C and K276C in the M2–M3 loop (14). This was interpreted as a disruption of the M2 structure leading to an even smaller constriction at the pore midpoint allowing PTX to be trapped when the channel closes (14). Our results also support the hypothesis that PTX can bind within the pore of the channel.

Unlike what we observed with the wild-type homomeric α<sub>2</sub> GlyR, there is no evidence that PTX can be trapped in the pore of the wild-type homomeric α<sub>2</sub> GlyR (14). Although GlyR α<sub>1</sub> and α<sub>2</sub> subunits share identical M2–M3 loops and most of the M2 amino acid residue sequence, they differ at the 2′-position (39), where glycine is present in the α<sub>1</sub> subunit and alanine in the α<sub>2</sub> subunit. α<sub>1</sub> G254 is an important determinant for PTX sensitivity (11). The 2′ residue lies in a narrow part of the pore. Although G254C mutation entirely abolished PTX sensitivity, the α<sub>2</sub> G254A mutation did not impair PTX inhibition, but the Hill coefficient of the PTX concentration-response curve was reduced (11). Moreover, the G254A mutation in the GlyR α<sub>2</sub> subunit dramatically reduces the inhibitory potency of the channel blocker cyanotriphenyl borate (40). Homomeric α<sub>1</sub> GlyR and homomeric α<sub>2</sub> GlyR subunits are functionally different. Homomeric α<sub>2</sub> GlyR openings were characterized by a larger single channel conductance (120 pS instead of 80 pS) and a considerably longer mean open time (23), suggesting that the open channel conformation differs between these two GlyR subtypes. Accordingly, it is tempting to speculate that the pore of homomeric α<sub>2</sub> GlyR is larger in the open state than that of homomeric α<sub>1</sub> GlyR. If so, PTX could go deeper within the pore, which will allow PTX to be trapped when the channel closes.

**How Many Binding Sites for PTX?**—An unexpected result obtained by fitting our experimental data with kinetic models is that, although PTX can be trapped within the channel, it can dissociate from the ligand-bound closed state(s) of the receptors. There is evidence for both GABA<sub>A</sub> and GABA<sub>C</sub> receptors that PTX binds preferentially to the agonist-bound conformation of the receptor and stabilizes the channel in the closed states (16, 17). Modeling PTX binding to ligand-bound closed states and a ligand-bound open state was necessary to provide a reasonable fit of our experimental data. Model 1 failed to predict the time course of the glycine-evoked response in the presence of PTX (see Fig. 10A). Our experimental data also provided direct evidence that PTX cannot directly bind to the unliganded closed conformation, indicating that PTX binds preferentially to the agonist-bound conformation of homomeric α<sub>2</sub> GlyRs.

Models 2 and 3 fitted our experimental data equally well. Moreover, the estimated association and dissociation rate constants for PTX were similar in the two models. Accordingly, it is statistically reasonable to choose the simplest model describing the PTX inhibitory effects. Physiologically, we cannot exclude that PTX can bind to all liganded closed states as proposed for GABA<sub>C</sub> receptors (17). In any case, both models predict a faster association and dissociation rate constant for PTX for the ligand-bound closed state than for the fully liganded open state. This is in apparent contradiction with the proposed single binding site for PTX on the GlyR (4). Our experiments provide no evidence of the presence of a second PTX binding site of different affinity. Indeed, the PTX concentration-response curve was well fitted by a single isotherm function and the Hill coefficient value is close to one.

The difference in the PTX association and dissociation rate constants between the fully liganded closed state and the fully liganded open state could also indicate that the access of PTX to its binding site depends on the GlyR channel conformation. Accordingly, it is possible to suppose that glycine binding evokes a partial GlyR channel conformational change before evoking channel openings (41), leading to partial access of PTX to its binding site. This hypothesis also raises the question of how many bound glycine molecules are necessary to evoke a partial conformational change of the channel. Our kinetic simulations cannot resolve this issue, because models 2 and 3 equally predict PTX block mechanisms.

In conclusion, the crucial insight of this study is that PTX acts as a simple channel blocker that can be trapped within the pore of the channel when glycine dissociates from its binding sites. This mechanism accounts for both the previously described competitive and noncompetitive mechanisms of PTX-evoked GlyR inhibition. It also raises the question of a complex conformational change of the GlyR channel that can unmask the PTX binding site when glycine binds to the receptor.

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