Mechanism for the Channel-Opening Reaction of Strychnine-Sensitive Glycine Receptors on Cultured Embryonic Mouse Spinal Cord Cells†

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ABSTRACT: The strychnine-sensitive glycine receptor, a member of a superfamily of proteins involved in chemical reactions that regulate signal transmission between cells of the nervous system, forms an anion-specific transmembrane channel in response to glycine binding. A rapid-reaction technique, a cell-flow method with a 10-ms time resolution, was adapted for measurements with cultured embryonic mouse spinal cord cells containing glycine receptors. Whole-cell current responses resulting from the opening of glycine receptor channels were measured at pH 7.4, 22–24 °C, and transmembrane voltages of –40 and –75 mV. Two different receptor forms, A_α and A_β, were detected. At saturating glycine concentrations, an average of 70% of the whole-cell current amplitude was associated with form A_α and 30% with A_β. The constants pertaining to the minimum mechanisms that account for the concentration of the two open-channel receptor forms over a 100-fold range of glycine concentration were determined by cell-flow measurements of the current amplitudes and of the falling (desensitizing) rate of the current. The dissociation constant of the site controlling channel opening was 220 μM on the basis of three binding sites for A_α and 380 μM on the basis of two binding sites for A_β. The channel-opening equilibrium constant, Φ^{-1}, was 170 for A_α and 110 for A_β. The rate coefficients for desensitization, α and β, associated with these two forms have maximum values of 0.7 and 0.1 s^{-1}, respectively. The rates at which the receptors recovered from desensitization were also measured, using a double-flow mixing device, and were found to be 0.06 s^{-1} for A_α and 0.02 s^{-1} for A_β. In the presence of 100 μM glycine, the apparent dissociation constant for the inhibitor picrotoxinin from receptor form A_α was 80 μM, and that from A_β was 460 μM. This suggests that A_β contains β-subunits (58 kD), because this subunit confers picrotoxinin insensitivity to glycine receptors (Pribilla, I., et al. (1992) *EMBO J. 11*, 4305). In the case of one receptor form (A_α), the chemical mechanism and its constants led to two measurements that could be assessed by an independent method, the single-channel current-recording technique: (i) the fraction of receptor channels open at a given glycine concentration $(\overline{AL_n})_o$ and (ii) the rate coefficient for desensitization. In the presence of 200 μM glycine and at a transmembrane voltage of –75 mV, the chemical kinetic measurement and the single-channel current-recording technique gave values for α of 0.8 and 0.6 s^{-1}, respectively. The value of $(\overline{AL_n})_o$ appears to be the same at –40 and –75 mV. The $(\overline{AL_n})_o$ value obtained in presence of 200 μM glycine is 0.9 when measured by the cell-flow technique at –40 mV. The electrophysiological technique gave a value of 0.7 at –75 mV and showed that receptor form A_α has a channel conductance of 48 pS.

The strychnine-sensitive glycine receptor, a member of a superfamily of proteins (Betz, 1990a,b; Stroud et al., 1990) involved in chemical reactions that regulate signal transmission between cells of the nervous system, forms an anion-specific transmembrane channel in response to glycine binding. The mechanism that controls the concentration of open receptor channels is of interest because this concentration, together with the inorganic ion concentration and the channel conductance, determines the rate at which inorganic ions cross the membrane. This rate plays an important role in determining the transient change in transmembrane voltage (Planck, 1890) that triggers signal transmission in neurons (Kandel et al., 1991). In this paper, we describe channel-opening mechanisms for two forms of the glycine receptor expressed on cultured embryonic mouse spinal cord cells.

A major difficulty in investigating the mechanism of receptor-mediated reactions is that the concentration of open receptor channels decreases with time in the continuous presence of neurotransmitter as a new receptor form is produced with both altered ligand binding and biological activity, an example of a general process called desensitization. When it was observed that desensitization of a neurotransmitter receptor can occur in the millisecond time region (Hess et al., 1979), rapid chemical reaction techniques were developed suitable for investigation of these membrane-bound receptors that must be studied in intact membrane vesicles or cells [Hess et al., 1979; reviewed in Hess et al. (1983, 1987) and Hess (1993)]. These techniques allow one to determine the ligand-binding properties and the rate constants pertaining

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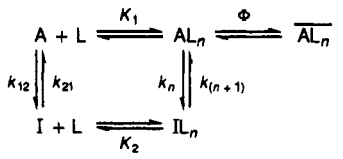
Scheme 1



to the active form of the receptor before it desensitizes (Hess et al., 1983). A minimum reaction scheme for the acetylcholine receptor in the electric organ of *Electrophorus electricus* was determined using rapid chemical reaction techniques (Cash & Hess, 1980; Hess et al., 1983). It accounts for the concentration of open receptor channels and for desensitization over a 5000-fold range of acetylcholine concentration. Quench-flow investigations of the GABA receptor in membrane vesicles prepared from the rat brain (Cash & Subbarao, 1987a–c, 1989) and cell-flow investigations (Hess et al., 1987) of the acetylcholine receptor in clonal mammalian muscle (Udgaonkar & Hess, 1987) and neuronal (Matsubara & Hess, 1992) cells and of GABA receptors in cultured mouse embryonic cortical cells (Geetha & Hess, 1992) led to a similar reaction scheme. We also considered this reaction pathway as a model for our investigatino of glycine receptors in cultured embryonic mouse spinal cord cells. The minimum mechanism shown in Scheme 1 was formulated for the *E. electricus* acetylcholine receptor (Cash & Hess, 1980) and is based on a scheme originally proposed by Katz and Thesleff (1957).

In Scheme 1, A represents the receptor in its active form and I the inactive, desensitized receptor form. L represents the activating ligand and the subscript the number of ligand molecules bound to the receptor protein. $\overline{AL_n}$ represents the open-channel form of the receptor through which inorganic ions exchange across the cell membrane. K_1 and K_2 are the intrinsic dissociation constants of the ligand for the active and desensitized receptor forms, respectively. We assume that each site on the active receptor form has the same affinity for ligand (K_1) and that each site on the inactive form has the same affinity for ligand (K_2). Φ^{-1} is the channel-opening equilibrium constant (Cash & Hess, 1980), and k_{12}, k_{21}, k_n, and k_{n+1} are the rate constants for the interconversion between active and inactive receptor forms.

A cell-flow technique (Udgaonkar & Hess, 1987; Hess et al., 1987) that allows one to equilibrate cell surface receptors with activators or inhibitors within 10 ms was used in the present study.

More than one glycine receptor form is expressed in rat spinal cord cells (Akagi & Miledi, 1988), cultured mouse spinal cord cells (Twyman & Macdonald, 1991), and acutely dissociated rat hypothalamic neurons (Akaike & Kaneda, 1989). Many isoforms of the glycine receptor transmembrane subunits have been cloned from rat brain and mammalian spinal cord (Grenningloh et al., 1987, 1990; Kuhse et al., 1990a,b; Schmieden et al., 1993). Receptors formed from different combinations of subunits have different properties when they are expressed in HEK 293 cells (Pribilla et al., 1992; Bormann et al., 1993) or *Xenopus* oocytes (Schmieden et al., 1989, 1992).

In this paper we describe a study of the channel-opening mechanism of glycine receptors expressed on cultured embryonic mouse spinal cord cells. A technique was developed to obtain nearly spherical vesicles (about 10 μm in diameter) from cells that are firmly attached to surfaces by long processes. Our approach differs from previous studies of glycine receptors in several respects. (1) The observed current was corrected for desensitization that occurs while the receptors equilibrate with glycine in the cell-flow experiments (Hess et al., 1987). (2) The results for each receptor form were analyzed separately to determine (i) the glycine dissociation constant of the receptor site controlling channel opening, (ii) the equilibrium constant for channel opening, and (iii) the rate constants for desensitization. (3) The results from chemical kinetics measurements were compared to results obtained by an independent method, the single-channel current-recording technique (Neher & Sakmann, 1976). We describe channel-opening mechanisms for both receptor forms that account for the concentration of open receptor channels and for the desensitization rates of each form over a 100-fold range of glycine concentration. We also show that there is a slow step in the channel-opening reaction that was not observed for the acetylcholine receptor (Matsubara et al., 1992).

EXPERIMENTAL PROCEDURES

Materials. All general chemical reagents were purchased from Sigma, Fisher, or Mallinckrodt. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) was purchased from Research Organics, Inc., and glycine (Ultrapure grade) from Boehringer Mannheim Biochemicals. All cell culture reagents and receptor activators and inhibitors were purchased from Sigma except as noted below.

Glycine was dissolved in the extracellular solution (see below); solutions less than 100 μM glycine were made fresh before use. Picrotoxinin was dissolved in absolute ethanol and diluted at least 500-fold to make the glycine plus picrotoxinin solutions.

Cell Culture. Spinal cord neurons were prepared as previously described (Ransom et al., 1977) from 12-day-old CFW or CD-1 mouse embryos (Charles River Laboratories). Neurons were kept in culture for 7–22 days at 36 °C, 5% CO_2, in Minimal Essential Media (MEM, from GIBCO BRL) with supplements as listed in Hoch et al. (1989) with the following modifications. Sometimes serum (from GIBCO BRL or Hazelton Biological) was dialyzed against 0.15 M NaCl overnight before use (Schrann et al., 1990). Nerve growth factor, sodium bicarbonate, and antibiotics were not added to the media, but 2.7 mM glutamine, 1 mg/mL putrescine·2HCl, and 20 nM sodium selenite (from GIBCO BRL) (final concentrations) were added (Romijn et al., 1984). On the second day after plating, 0.5 mL of media was added to each 35-mm dish (Falcon). On the third day, 41 mM 5′-fluoro-2′-deoxy-β-uridine and 102 mM uridine (final concentrations) were added to each dish. Starting on the sixth day after plating and twice a week thereafter, 1 mL of media in each dish was replaced with media containing dialyzed horse serum and supplements but without fetal bovine serum.

Cell Preparation. Immediately before a measurement, cells were washed with extracellular buffer containing 142 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 8.1 mM KCl, 10 mM glucose, and 10 mM Hepes, pH 7.4, with NaOH. Sometimes cells were then treated with 0.25% collagenase (Type IV) in 40 mM sucrose, 35 mM glucose, 124 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 9 mM Hepes, pH 7.3, for 5–30 s and again washed with extracellular buffer. This treatment facilitated vesicle formation for some cell preparations. Vesicles were obtained from the cell bodies of the neurons by forming a seal between the recording electrode and the cell in the whole-cell mode (Hamill et al., 1981) and then gently lifting the membrane with the recording electrode (pipet) until it pinched off from the cell and formed a vesicle. The vesicles attached to the tip of the electrode typically had a diameter of about

10 μm. It is possible to know and adjust the solution composition inside the vesicles. The method is similar to one used by Sather et al. (1992) except that our vesicles did not contain the cell nucleus. The spherical vesicle was clearly visible under the microscope. The measured capacitance of the vesicle membrane (1–3 pF) was in good agreement with the value (2.2 pF) we calculated for a 10-μm-diameter vesicle using an empirical formula derived for small, round cells (Pusch & Neher, 1988). This indicated that the vesicle was in the whole-cell mode. We calculated a time constant for equilibration of the intracellular buffer in the pipet with that in the vesicle of 2–7 s by using a second empirical formula (Pusch & Neher, 1988). This time constant is in good agreement with theoretical calculations of Oliva et al. (1988). Before and between each measurement, the cells were washed for 4 min with extracellular solution flowing over the cells at a rate of 1 cm/s.

In control experiments, cells were removed from the culture dish by first using a glass pipet to cut or loosen the axon and all the dendrites of a neuron from the dish and the surrounding cells. A second pipet was then used to form a seal on the cell body in the whole-cell mode and to pick up the cell. These cells were round, and some had one or two short dendrites still attached. The cell body was approximately 15 μm in diameter, and the membrane capacitance varied from 3–8 pF, depending on the size of the cell and the number of dendrites still attached. The data from cells were collected and treated as described for the data from vesicles.

Whole-Cell Current Recording. The whole-cell variant of the single-channel current-recording technique was used as described (Hamill et al., 1981; Marty & Neher, 1983) together with a List L/M-EPC7 amplifier (Sigworth, 1983). The electrode solution contained 140 mM CsCl, 1 mM $CaCl_2$, 10 mM EGTA, 2 mM Na_2ATP, 4 mM $MgCl_2$, and 10 mM Hepes, pH 7.4, with NaOH, and the bath solution contained 142 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 8.1 mM KCl, 10 mM glucose, and 10 mM Hepes, pH 7.4, with NaOH. Electrodes were prepared on a List L/M-3P-A pipet puller and fire-polished using a Narishige MF-83 polisher. Typical values for the electrode resistance were 1.5–3 MΩ, for the cell capacitance 1–3 pF, and for the series resistance 1.5–2 times the electrode resistance. Most measurements were made at a transmembrane voltage of –40 mV, room temperature (22–24 °C), and pH 7.4. To ensure a good voltage clamp (the voltage drop across the series resistance should be less than 10% of the holding voltage during maximum current flow), series resistance compensation was used as necessary to cancel up to 50% of the series resistance as described by Sigworth (1983). Since some whole cells did not contain any dendrites and since the results with and without the dendrites were the same, we assumed that the dendrites on the cells we used did not significantly affect the voltage clamp. The current amplitudes were typically between 0.5 and 2.0 nA at a holding voltage of –40 mV. The experimental error in measurements made with the same glycine concentrations on the same cell was 10–15%. The whole-cell current signal from the patch clamp amplifier was passed through a low-pass R-C filter (Krohn-Hite 3322) with a cutoff frequency of 1.2 kHz (–3-dB point) and then digitized at a 30–200-Hz sampling frequency on an AST computer with a Labmaster DMA board (Scientific Solutions) using the Clampex program in the pClamp software package from Axon Instruments. The sampling frequency depended on the rate of receptor desensitization. It was chosen so that the decay phase of the current trace contained at least half of the 2000 points collected for each measurement. The data were stored on floppy disks and analyzed on a Northgate 386 microcomputer using GENPLOT version 1.01 (Computer Graphics Service, Ithaca, NY).

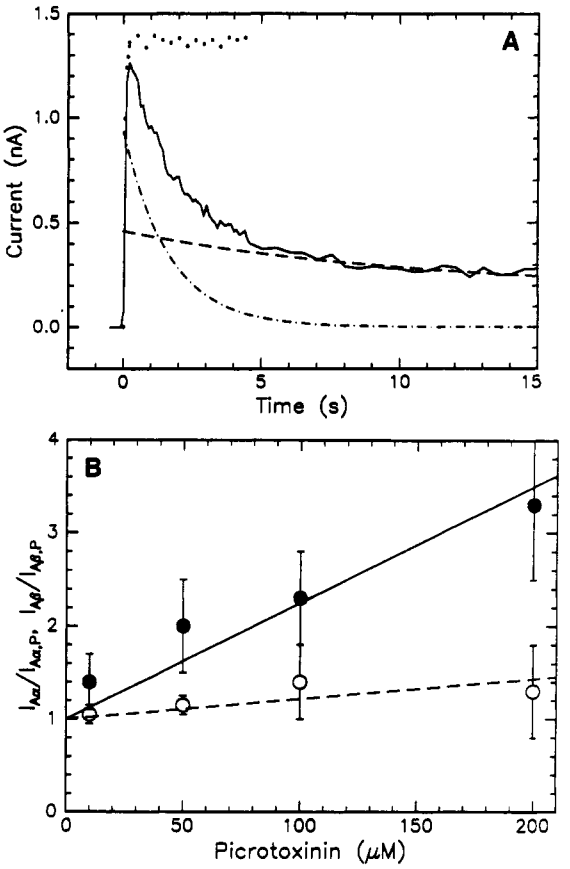


FIGURE 1: Cell-flow experiments (see Experimental Procedures) with vesicles made from embryonic mouse spinal cord cells. The current amplitude is a measure of the number of receptors in the cell membrane in the open-channel form. The measurements were made at a transmembrane voltage of –40 mV at pH 7.4 and 22–24 °C. (A) A current measurement made in the presence of a 100 μM glycine solution flowing at 1 cm/s from the flow device. The observed current (solid line) reaches its maximal value within 200 ms. Here, 66% of the current decays with a rate coefficient α of 0.6 s^{-1}, 21% of the current decays with a rate coefficient β of 0.09 s^{-1}, and 13% of the current does not decay during 5 min. The large dots parallel to the time axis represent the current corrected for desensitization that occurs during the rising phase of the current (eq 1; Udgaonkar & Hess, 1987). The curves below the whole-cell current represent the current versus time curves for the rapidly (– · –) and slowly (– – –) desensitizing receptor forms. (B) Inhibition of both receptor forms by picrotoxinin at –40 mV at pH 7.4 and 22–24 °C. Picrotoxinin (10–200 μM) with 100 μM glycine was applied to vesicles, and the ratios of the amplitudes of each component in the absence (I_{A_α} and I_{A_β}) and presence ($I_{A_\alpha,P}$ and $I_{A_\beta,P}$) of picrotoxinin were plotted versus picrotoxinin concentration. The apparent dissociation constants ($K_{I(app)}$) determined from eq 2 for picrotoxinin for A_α (●) and A_β (○) were 80 ± 8 and 460 ± 125 μM, respectively. The symbols represent the mean values of 3–6 measurements from three or four different cells.

A least-squares program (Bevington, 1969) was used to fit all the data. A linear prediction method (Millhauser et al., 1989) was used as an independent technique to evaluate the exponentials of the multiexponential decays of the current (Figure 1A). Because the whole-cell current differed from cell to cell, all measurements from different cells were normalized to the current obtained at 100 μM glycine. For some cells, the current amplitudes at low glycine concentrations (10–30 μM) were first normalized to measurements of the current amplitudes obtained at 40 μM glycine. To compare the single-channel and whole-cell recording results, the whole-

cell responses were measured using the same intracellular buffer (see below) inside the electrode as was used in single-channel current recordings at a transmembrane voltage of −75 mV. This was important for comparing the rate constants for desensitization, which are voltage-dependent for the glycine receptor (Akaike & Kaneda, 1989; Faber & Korn, 1987).

Rapid Application of Ligand Solution. The cell-flow method for measuring the concentration of open receptor channels prior to desensitization in single cells and the technique for changing the composition of the solution emerging from a U-tube flow device have been described (Krishtal & Pidoplichko, 1980; Clapham & Neher, 1984; Udgaonkar & Hess, 1987; Matsubara & Hess, 1992; Geetha & Hess, 1992). In the present experiments, a vesicle attached to a whole-cell current-recording electrode was placed 100 μm from the porthole of a flow device that had a diameter of 150 μm. The flow rates of the extracellular solutions emerging from the flow device were between 1 and 3 cm/s, and the time required for the current to reach its maximal value at high ligand concentrations (>200 μM glycine) at the higher flow rates was 20–50 ms. The time resolution of our flow device was 10 ms. This was determined by two methods. (1) We monitored the conductance during a solution exchange (Maconochie & Knight, 1989). (2) Glutamate was flowed over the cells, and the time required for glutamate receptor responses to reach their maximum amplitude was measured because they open faster (Trussell et al., 1988) than the rate of solution change.

Resensitization Experiments. A double-flow mixing device based on the flow device of Carbone and Lux (1987) was constructed for use in resensitization and strychnine preincubation experiments. It had a 15-ms time resolution, as determined when the solution exchange rate was measured by detecting the change in conductance of two solutions at the tip of an open electrode. Two tubes delivered solutions to the mixer, and one tube removed solution from it. The flow rate of the solutions entering and emerging from the flow device was 50 μL/min. The flow rate was 1 cm/s at the outlet. Electronic valves controlled the solution exchanges. A cell was attached to the current-recording electrode and placed less than 50 μm from the outlet of the mixer. The current amplitudes before desensitization of the rapidly ($I_{A_\alpha\ \infty}$) and slowly ($I_{A_\beta\ \infty}$) desensitizing components were measured by flowing 1 mM glycine over a cell. The receptors were then desensitized by applying 1 mM glycine to the cell for 5 min. The current remaining (I_{nondes}) was less than 10% of the total cell current ($I_{A_\alpha\ \infty} + I_{A_\beta\ \infty}$). The receptors were then allowed to recover from desensitization for varying periods of time (5–150 s) with buffer flowing over the cell. The number of receptors that resensitized in the presence of buffer alone for 3–5 min was measured by flowing 1 mM glycine over the cell again and measuring the current amplitudes $I_{A_\alpha\ t}$ and $I_{A_\beta\ t}$. It is assumed that in the presence of 1 mM glycine, channel opening is much faster than receptor resensitization.

Single-Channel Recording. The method of Twyman and Macdonald (1991) was used. The extracellular solution was the same as that given above. The intracellular solution contained 153 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, and 10 mM Hepes, pH 7.4, with CsOH. The single-channel current measurements were made in the outside-out mode (Hamill et al., 1981), and the data were stored on a video casette. The data were then passed through a low-pass R-C filter (Krohn-Hite 3322) with a cutoff frequency (−3-dB point) of 1.2 kHz, which resulted in an average base-line deviation that was 20 times less than the unit amplitude of the main-state single-channel current. After being filtered, the data were digitized on an AST personal computer using the Fetchex program of the pClamp software package (Axon Instruments) at a 10-kHz sampling frequency, which was greater than 5 times the cutoff frequency of the low-pass filter. The data were then transferred to a Convex Computer (Material Science Center, Cornell University) and analyzed using an automated analysis program (IPROC) developed by Sachs et al. (1982) and modified by Udgaonkar (1986). The fraction of the burst length during which the receptor was in the open-channel state (P_o) was determined using IPROC. A burst of channel activity was accepted for analysis only if it lacked overlapping open states and if it was separated from the next burst by at least 3 times the mean intraburst closed time (Neher, 1983).

To obtain the conductance of the open channels, histograms of the single-channel current amplitudes were constructed. A parameter in IPROC determines the current amplitude range of valid open states. In the experiments with the glycine receptor, it was possible to set this parameter so that the range of valid open states spanned the amplitude of a channel with one conductance plus or minus three standard deviations from the mean, without counting the amplitudes due to the channel with a different conductance. The data file was then analyzed a second time with the parameter reset to analyze the channels with the other conductance.

RESULTS

Figure 1A shows a whole-cell current measurement made in the presence of 100 μM glycine with a vesicle from a single spinal cord neuron using the cell-flow technique. The current (solid line) reaches a maximum value within 200 ms. Two exponentials plus a constant term are required to fit the decaying phase of the current, and the rate coefficients for receptor desensitization, α and β, have values of 0.6 and 0.1 s^{-1}, respectively. In Figure 1, the dashed/dotted line shows the current associated with the fast desensitization rate, and the dashed line shows the current associated with the slow desensitization rate (plus the constant term). The same number of components was present in current traces recorded using vesicles from cells grown in media described by Ransom et al. (1977) (data not shown).

Calculation of the Corrected Current Amplitude, I_A. Because some receptor proteins begin to desensitize before the current reaches a maximum amplitude, the current amplitude corrected for desensitization was calculated using eq 1 (Udgaonkar & Hess, 1987; Hess et al., 1987). I_A is the

$$I_A = (e^{\alpha \Delta t} - 1)\sum_{i=1}^{n}(I_{obs})_{\Delta ti} + (I_{obs})_{tn} \qquad (1)$$

current amplitude corrected for desensitization, where I represents the current and A refers to the active, nondesensitized receptor form (Scheme 1). $(I_{obs})_{\Delta ti}$ is the observed current during the ith time interval, and $(I_{obs})_{tn}$ is the observed current during a time interval equal to or greater than the time to reach the maximum current. α is the rate coefficient for desensitization, and Δt is the sampling time interval, which was 5 ms in these experiments.

The decay of the glycine receptor current response contains two desensitizing components and a component that does not desensitize in 5 min (see Figure 1A). Therefore, the slowly desensitizing and nondesensitizing components were subtracted from the observed current before the current correction

equation (eq 1) was used, and only the rapidly desensitizing component was corrected to give $I_{A\alpha}$ [see Geetha and Hess (1992) for details]. This procedure is valid because the slowly desensitizing receptor form desensitizes less than 1% during the rising phase of the current. The dots parallel to the time axis in Figure 1A represent the current corrected for desensitization. For the glycine receptor, the current correction is small because the response decays more slowly than the responses of other neurotransmitter receptors studied (Udgaonkar & Hess, 1987; Geetha & Hess, 1992; Matsubara & Hess, 1992). When the time required for the current to reach its maximum value is less than 100 ms, the observed amplitude equals the corrected amplitude, even at the highest desensitization rates observed.

At saturating glycine concentrations, 70% of the current is associated with A_α and 30% with the slowly desensitizing receptor form A_β. Thirty percent of the current associated with A_β does not desensitize within 5 min. The nondesensitizing component is considered a part of receptor form A_β, based on the following information. Picrotoxinin, the active component of picrotoxin, is a noncompetitive antagonist of $GABA_A$ receptors (Olsen, 1982) and an inhibitor of some glycine receptors (Pribilla et al., 1992). It is a more potent inhibitor of the glycine receptor form A_α than of A_β (Figure 1B). The current amplitudes of both the A_α and A_β receptor forms in the absence (I_{A_α} and I_{A_β}) and presence ($I_{A_\alpha,P}$ and $I_{A_\beta,P}$) of picrotoxinin were measured, and $I_{A_\alpha}/I_{A_\alpha,P}$ and $I_{A_\beta}/I_{A_\beta,P}$ were determined at a constant glycine concentration (100 μM) but at different picrotoxinin concentrations. The data were plotted according to the equation (Shiono et al., 1984):

$$\frac{I_A}{I_{A,P}} = 1 + \frac{[P]}{K_{I(app)}} \qquad (2)$$

[P] is the picrotoxinin concentration, and $K_{I(app)}$ is the apparent dissociation constant. I_A and $I_{A,P}$ correspond to I_{A_α} measured in the presence and absence of picrotoxinin, respectively, for receptor form A_α, and to I_{A_β} in the presence and absence of picrotoxinin, respectively, for receptor form A_β. $K_{I(app)}$ for receptor forms A_α and A_β was 80 and 460 μM, respectively (Figure 1B). The current due to the nondesensitizing receptor is included in the measurements of I_{A_β}, and we conclude, therefore, that the $K_{I(app)}$ value for picrotoxinin is similar for the slowly desensitizing and nondesensitizing receptor forms. We therefore assume that the sum of the amplitudes of the slowly desensitizing and nondesensitizing components reflects the concentration of receptor form A_β. At 10 and 200 μM picrotoxinin, the receptors were preincubated for 30 s, and the same current amplitudes were obtained as in experiments in which picrotoxin and glycine were equilibrated together with the cell surface receptors using the flow device. This indicates that the rate of picrotoxinin binding to the receptor is not responsible for the difference in $K_{I(app)}$ values obtained.

Each receptor form is sensitive to strychnine, a specific inhibitor of the glycine receptor (Young & Snyder, 1973), and preincubation of the receptors with 20 nM strychnine for 10 s or longer reduces the amplitude of each component to half its original value (data not shown). Preincubation of the cells with 200 nM strychnine for 2 s or longer abolishes all the glycine-activated current (including the nondesensitizing component associated with receptor form A_β, I_{nondes}) (data not shown).

Taurine, an amino acid that activates the glycine receptor (Curtis et al., 1968), produces a larger current response from receptors containing only adult α1-subunits (a specific transmembrane subunit) than from those containing only neonatal α2-subunits (Schmieden et al., 1992). The response of each receptor form to 3 mM taurine was identical to the response to 1 mM glycine in our experiments (data not shown).

Evaluating the Constants in Scheme 1. The relationship between I_A, a measure of the concentration of open receptor channels, and the constants pertaining to the mechanism shown in Scheme 1 was derived for the acetylcholine receptor from the electric organ of *E. electricus* (Cash & Hess, 1980; Hess et al., 1983), and the equation was then adapted to apply to cell-flow measurements (Udgaonkar & Hess, 1987). When the initial ligand concentration is much larger than the number of moles of receptor molecules in the membrane,

$$I_A = I_M R_M \frac{L^n}{L^n + \Phi(L + K_1)^n} = I_M R_M (\overline{AL_n})_o \qquad (3a)$$

$(\overline{AL_n})_o$ is the fraction of receptor molecules in the open-channel form, L represents the molar concentration of ligand, n is the number of ligand-binding sites, and Φ^{-1} is the channel-opening equilibrium constant. K_1 is the dissociation constant of glycine from the receptor sites. We assumed the sites to be equivalent. I_M is the current due to 1 mol of open receptor channels, R_M represents the number of moles of receptors in the cell membrane controlling channel opening, and $I_M R_M$ corresponds to the whole-cell current that would be observed if all the receptor channels opened in the presence of a saturating concentration of ligand. The constants K_1, n, Φ, and $I_M R_M$ in eq 3a can be evaluated. Equation 3a can be rewritten in linear form (Cash & Hess, 1980):

$$\left[\left(\frac{I_M R_M}{I_A}\right) - 1\right]^{1/n} = \Phi^{1/n} + \Phi^{1/n}\left(\frac{K_1}{L}\right) \qquad (3b)$$

A least-squares computer program was used to evaluate the constants. Several independent measurements were made (see below) to assess this evaluation.

The effect of glycine concentration on the two receptor forms present in mouse spinal cord cells (Figure 1A) was analyzed to evaluate the parameters that determine the formation of transmembrane channels. The values of I_{A_α} (Figure 2A) and I_{A_β} (Figure 2B) were determined as a function of a 100-fold range of glycine concentration (10 μM to 1 mM) in experiments such as the one illustrated in Figure 1A. All I_A values were normalized to the value obtained at 100 μM glycine because the concentration of receptors, as measured by the whole-cell current, differs from cell to cell. The values of I_{A_α} and I_{A_β} at this glycine concentration were taken to be 0.62 and 0.33 nA, respectively, which were the average current amplitudes obtained with the vesicles used for the measurements shown in Figure 2.

The I_{A_α} or I_{A_β} values were fitted to eq 3b. Each individual measurement of I_{A_α} or I_{A_β} was included as a separate point during the fitting process. A fit of the I_{A_α} values to eq 3b with $n = 2$ yielded a value for K_1 of 1.5 mM and for Φ^{-1} of 1000 ($\chi^2 = 0.6$). A fit of the I_{A_α} values to eq 3b with $n = 3$ gave a χ^2 value of 0.3 (Figure 2A, inset). The I_{A_β} values fit well to eq 3b with $n = 2$ and a χ^2 of 0.02 (Figure 2B, inset). Hill plots (Hill, 1910) of the data in Figure 2 gave a Hill coefficient n_H of 2.5 ± 0.07 for I_{A_α} (Figure 3A) and 1.73 ± 0.07 for I_{A_β} (Figure 3B), in agreement with the assignment of $n = 3$ for I_{A_α} and $n = 2$ for I_{A_β}. Adding the current due to the nondesensitizing component to I_{A_α} did not decrease the value of n_H below 2 (data not shown). There was no improvement in the fit of the I_{A_α} values when we used a model with nonequivalent binding sites. The values for the constants (K_1,

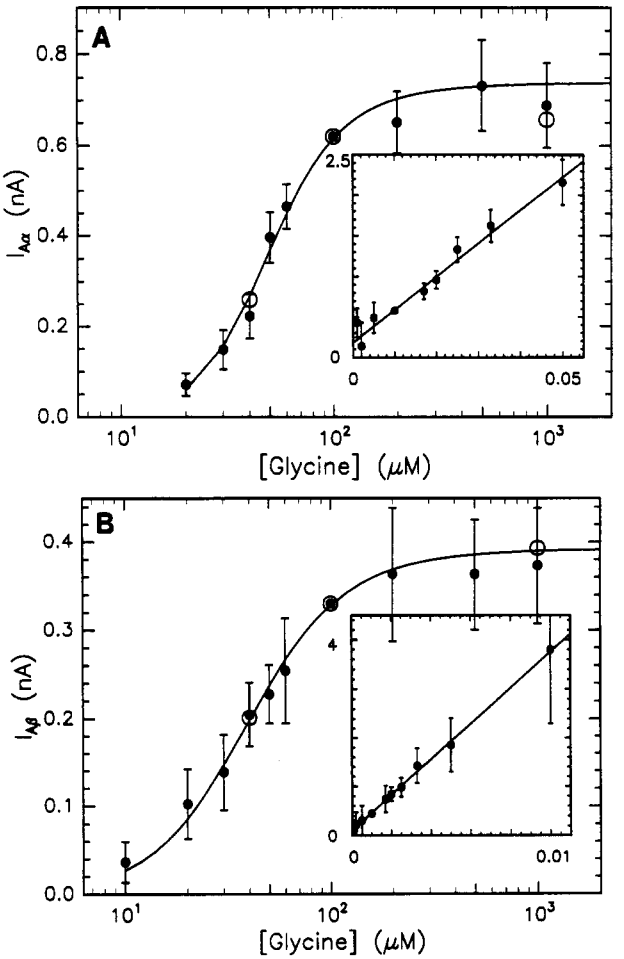


FIGURE 2: Relationship between I_A and glycine concentration at −40 mV at pH 7.4 and 22–24 °C. The corrected current amplitudes measured with each vesicle were normalized to the I_A values obtained in the presence of 100 μM glycine. Table 1 lists the parameters used for computing the solid line ($I_M R_M$, K_1, Φ) using eq 3a with $n = 3$ and 2 for parts A and B, respectively. The solid line was computed at glycine concentration intervals of 10 μM. The solid circles refer to experiments with vesicles and the open circles to experiments with whole cells. The insets show the same data replotted according to eq 3b with $n = 3$ and 2 for parts A and B, respectively. The values of $I_M R_M$ for receptor forms A_α and A_β were 0.74 ± 0.02 and 0.40 ± 0.03 nA, respectively, and were obtained by use of a nonlinear least-squares program and eq 3b. (A) I_{A_α} versus glycine concentration. The x- and y-axis labels for the inset are $((I_M R_M/I_{A_\alpha}) - 1)^{1/3}$ and 1/[glycine] (μM), respectively. (B) I_{A_β} versus glycine concentration. The x- and y-axis labels for the inset are $((I_M R_M/I_{A\beta}) - 1)^{1/2}$ and 1/[glycine] (μM), respectively. The data were obtained from measurements made with 20 vesicles (each vesicle from a different cell); two or three measurements at each glycine concentration were made with each vesicle, and measurements were made at 2–5 glycine concentrations per vesicle. The relative error in measurements made with the same vesicle was ±15% for I_{A_α} and ±25% for I_{A_β}. The solid circles show the mean values, and the error bars represent plus or minus standard deviations. The open circles represent the mean values of 5–15 measurements from 4–8 whole cells with a standard deviation of 15–30% of the mean values. The whole-cell data are not shown in the insets.

Φ, and $I_M R_M$) used to calculate the solid lines in Figure 2 using eq 3a are listed in Table 1; they were determined from fits of the I_{A_α} and I_{A_β} values to eq 3b (Figure 2, insets) with $n = 3$ and 2, respectively.

Evaluation of the Rate Coefficients for Receptor Desensitization, α and β. The relationship between ligand concentration and the rate coefficient for desensitization based on Scheme 1 can be derived with the following assumptions. The concentration of ligand, L, is much larger than R_M, and the reaction leading to the open-channel form, AL_n, is rapid

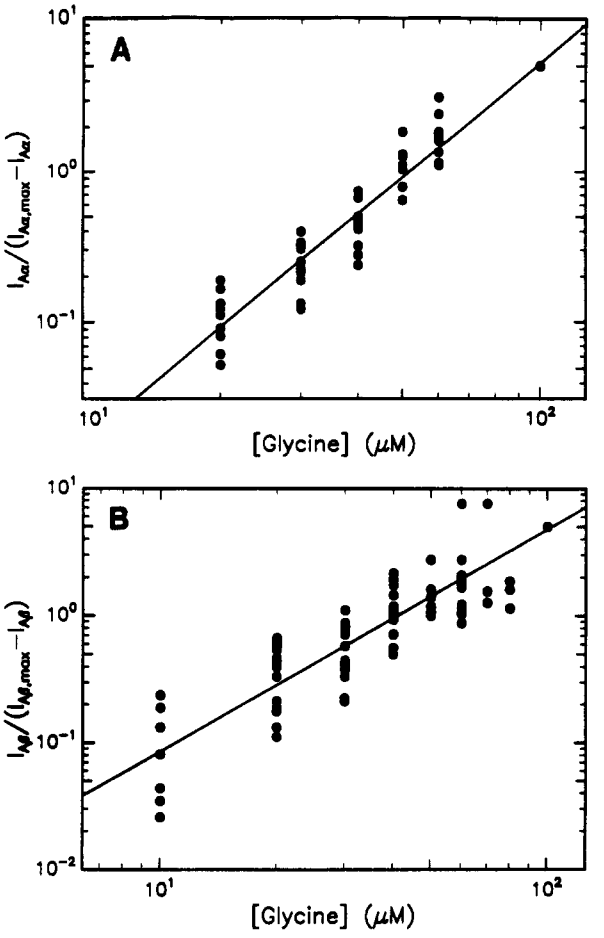


FIGURE 3: Hill plots of I_{A_α} and I_{A_β}. The data in Figure 2 were transformed by computing $I_A/(I_{A,max} - I_A)$ (Colquhoun & Ogden, 1988). $I_{A,max}$ is computed using $I_{A,max} = (I_M R_M)/(1 + \Phi)$, where the $I_M R_M$ and Φ values are shown in Table 1. The slope and intercept of the Hill plots were determined from a least-squares fit of the data. (A) $I_{A\alpha}$ data obtained from the experiments shown in Figure 2A. The values of $I_M R_M$ in this plot is 0.74, the slope of the line is 2.5 ± 0.07, and the intercept is −4.3 ± 0.1. For values of $I_M R_M$ ranging from 0.68 to 0.87 nA, the slope was 2.2–2.6. (B) I_{A_β} data obtained from the experiments shown in Figure 2B. The value of $I_M R_M$ is 0.4, the slope of the line is 1.73 ± 0.07, and the intercept is −2.8 ± 0.12. The solid points show each individual I_{A_α} and I_{A_β} measurement.

compared to the formation of the inactive, desensitized receptor species, IL_n (Scheme 1). We further assume that the rate constant, k_n, for the conversion of receptor forms saturated with ligand (AL_n in Scheme 1) to inactive forms dominates the rate constants for desensitization (Aoshima et al., 1981; Udgaonkar & Hess, 1987):

$$\alpha,\beta = \frac{k_n \Phi L^n}{L^n + \Phi(L + K_1)^n} \qquad (4)$$

Figure 4 shows that the glycine concentration dependence of the desensitization rate coefficients α and β is consistent with eq 4 with $n = 3$ and 2, respectively. This equation gives the relationship between the rate coefficients and glycine concentration in terms of only three constants: k_n, K_1, and Φ for receptor forms A_α and A_β.

The values of the constants, k_n, K_1, and Φ, that account for the effect of glycine concentration on the desensitization rate coefficients and that were used to compute the solid lines in Figure 4 are also shown in Table 1. The values determined by measuring the effect of glycine concentration on the rate of the falling phase of the current (Figure 4), a measure of

Table 1: Constants Determining the Formation of Glycine Receptor Transmembrane Channels in Embryonic Mouse Spinal Cord Cells at 22–24 °C, pH 7.4, and –40 mV[a]

constant	A_α, rapidly desensitizing receptor form[b]	A_β, slowly desensitizing receptor form
n (number of ligand-binding sites)	3	2
K_I (μM) (dissociation constant of site controlling channel opening), determined by cell-flow measurements of		
(i) current amplitudes	230[c]	380[d]
(ii) falling rate of the current	200[e]	360[f]
$(1 + \Phi)^{-1}$ (maximal fraction of receptors in the open-channel form), determined by cell-flow measurements of		
(i) current amplitudes	0.99[c]	0.99[d]
(ii) falling rate of the current	0.99[e]	0.98[f]
$I_M R_M$ (nA) (observed maximal current amplitude)	0.74	0.40
rate coefficient for desensitization at saturating glycine concentrations (s^{-1})[g]	0.7	0.1
rate coefficient for desensitization at 200 μM glycine and –75 mV (s^{-1})		
(i) from SCCR[h] measurements[i]	0.6 ± 0.5[j]	
(ii) from cell-flow experiments	0.77 ± 0.09[k]	
P_0 at 200 μM glycine		
(i) from SCCR measurements	0.7 ± 0.2[j]	
(ii) from cell-flow experiments[l]	0.9 ± 0.1	
average number of glycine receptors per cell	390	250[m]

[a] The value of $I_M R_M$ determined for A_α using the single-channel current-recording method was 0.95 at –75 mV. [b] Average error in K_I and $I_M R_M$ was ±20%, and that in Φ was ±100%. [c] Constants were determined from the effect of a 50-fold range of glycine concentration on I_{A_α} using eq 3b. [d] Constants were determined from the effect of a 100-fold range of glycine concentration on I_{A_β} using eq 3b. [e] Constants were determined from the effect of a 50-fold range of glycine concentration on the rate coefficient α using eq 4 (k_n = 100 s^{-1}). [f] Constants were determined from the effect of a 100-fold range of glycine concentration on the rate coefficient β using eq 4 (k_n = 4.6 s^{-1}). [g] Average error in desensitization rates was ±45%. [h] SCCR is single-channel current-recording. [i] Determined using the inverse of the burst length at –75 mV. [j] Determined using 65 bursts from two cells. [k] Determined from seven measurements made using four vesicles at –75 mV. [l] Determined from Figure 2A at –40 mV (see text for comparison of measurements at –40 and –75 mV). [m] On the assumption that the 40-pS channel is associated with A_β (see text).

desensitization, and on the current amplitudes (Figure 2) agree (Table 1).

Resensitization Experiments. We measured the rate of receptor reactivation, or resensitization, from the desensitized state to determine if there are two forms of glycine receptors present on cultured spinal cord cells. If the continuous presence of a high concentration of glycine (1 mM) forces the receptors into the desensitized state, we assume that when the inactivated receptors are placed in buffer solution alone they will lose glycine molecules before the reactivation of the glycine receptor is measured. The rate-limiting step in the reactivation reaction will then be a measure of the rate of conversion of receptor forms I to A (k_{21}) in Scheme 1. The receptors were desensitized in the presence of 1 mM glycine for 5 min and allowed to resensitize for various lengths of time (see Experimental Procedures for details). The current amplitude after resensitization was within 20% of the original amplitude for all measurements.

In measurements of the effect of glycine concentrations on the current amplitude using the cell-flow technique with the same cell, we sometimes noticed a slow decrease in the current amplitude in successive measurements with the same concentration of glycine. We have addressed this problem by alternating the current amplitude measurements with measurements at the normalizing concentration of glycine, either 40 or 100 μM (see Experimental Procedures). To increase the precision of some measurements at 100 and 150 s, only cells with current amplitudes varying less than 10% were used.

In determining the rate of resensitization, we assume that, in the presence of high concentrations of glycine (1 mM), the channel-opening reaction is rapid compared to resensitization. After resensitization of the receptor in extracellular solution for various periods of time, we applied 1 mM glycine to the cells and measured the current amplitudes associated with the rapidly and slowly desensitizing receptor forms A_α and A_β, respectively. These current amplitudes are considered to reflect the concentrations of A_α and A_β. The resensitization reaction of each receptor form was observed to follow a single exponential equation:

$$\frac{I_{A\infty} - I_{At}}{I_{A\infty} - I_{A(t=0)}} = \exp(-k_{21}t) \qquad (5)$$

$I_{A\infty}$ is the current amplitude before desensitization for either receptor form ($I_{A_\alpha\infty}$ or $I_{A_\beta\infty}$), and I_{At} is the current amplitude after t s of resensitization in buffer for either receptor form ($I_{A_\alpha t}$ or $I_{A_\beta t}$). $I_{A_\beta t}$ contains the current due to the nondesensitizing component I_{nondes}. We assume $I_{A_\alpha(t=0)}$ is zero, i.e. receptor form A_α desensitizes completely, and $I_{A_\beta(t=0)} = I_{nondes}$. k_{21} is the rate of resensitization due to the conversion of receptor form I to form A in Scheme 1. A reactivation rate of 0.06 s^{-1} for I_{A_α} (Figure 5A) and 0.02 s^{-1} for I_{A_β} (Figure 5B) was measured.

Single-Channel Current Recording. An example of a single-channel current-recording measurement made with 200 μM glycine is shown in Figure 6A. At this concentration of glycine, we observe only a single type of channel. Its conductance of 48 pA/V (48 pS) is in agreement with previous reports (Takahashi & Momiyama, 1991; Bormann, 1990; Hamill et al., 1983). Strychnine eliminated the chloride channel activity (data not shown). The results of single-channel current recordings (Neher & Sakmann, 1976) can be compared to chemical kinetic measurements made with the cell-flow technique, which utilizes an entirely different approach and methodology (Udgaonkar & Hess, 1987; Geetha & Hess, 1992; Matsubara & Hess, 1992). The cell-flow method allows one to determine the effect of a wide range of glycine concentration on the concentration of open receptor channels (as measured by I_A) and on the rate of desensitization. The cell-flow technique also allows one to determine P_o, the conditional probability that the channel is open while the receptor is in a nondesensitized state, since $(\overline{AL_n})_o$ is P_o

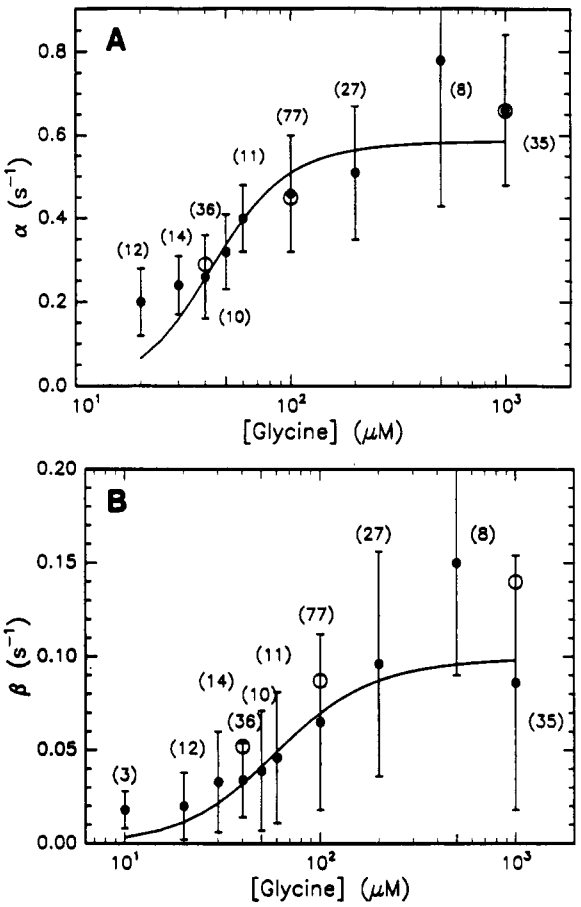


FIGURE 4: Relationship between rate coefficients for desensitization and glycine concentration. The same cell-flow measurements used to evaluate I_{A_α} and I_{A_β} in Figure 2 were used to evaluate the rate coefficients for desensitization, α and β. (A) α as a function of glycine concentration. The values of the parameters used to compute the solid line using eq 4b are $k_n = 100 \pm 10\ s^{-1}$, $K_1 = 200 \pm 30\ \mu M$, and $\Phi = 0.006 \pm 0.004$. (B) β as a function of glycine concentration. The values of the parameters used to compute the solid line using eq 5b are $k_n = 4.6 \pm 0.5\ s^{-1}$, $K_1 = 360 \pm 60\ \mu M$, and $\Phi = 0.02 \pm 0.02$. The solid symbols show the average of 3–77 measurements from 3–20 different vesicles. The error bars represent plus or minus standard deviations from the mean. The number of measurements for each point is indicated in parentheses next to the symbol. The open circles represent the mean values of 5–15 measurements of the desensitization rates from 4–8 whole cells, with a standard deviation of 7–90% of the mean values.

(Udgaonkar & Hess, 1987). I_A at a given concentration of neurotransmitter is a measure of $(\overline{AL_n})_0$ (eq 3a), the fraction of channels open while the receptor is in a nondesensitized state. At low neurotransmitter concentrations at which desensitization does not abolish receptor activity, single-channel current measurements allow one to determine both P_o and the rate of receptor desensitization (Sakmann et al., 1980). Bursts of channel activity with a single conductance level (indicated by the horizontal lines in Figure 6A), separated by periods with no channel activity, were observed. Under the experimental conditions described in this paper, a period during which no channel activity was observed was taken to be 300 ms or longer to qualify as an interburst interval, because this was equal to 3 times the mean intraburst closed-time period (Neher, 1983).

We measured the length of bursts that contained the 48-pS channel and met our criteria of a burst. The mean lifetime of a burst of channel activity corresponds to the mean lifetime of the nondesensitized state of the receptor and is a measure of the rate constants leading from the active to the desensitized receptor forms (Scheme 1) (Sakmann et al., 1980). We

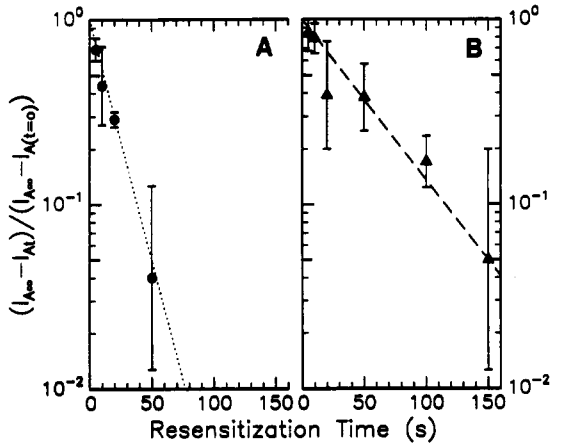


FIGURE 5: Resensitization of inactivated receptors upon removal of glycine. $I_{A\infty}$ is the current amplitude before desensitization for either receptor form ($I_{A_\alpha\infty}$ or $I_{A_\beta\infty}$). The receptors were desensitized by flowing 1 mM glycine over the cells for 5 min, and I_{A_t} is the current amplitude after t s of resensitization in buffer for either receptor form ($I_{A_\alpha t}$ or $I_{A_\beta t}$). $I_{A_\beta t}$ contains the current due to the nondesensitizing component I_{nondes}. We assume $I_{A_\alpha(t=0)}$ is zero, i.e., receptor form A_α desensitizes completely, and $I_{A_\beta(t=0)} = I_{nondes}$. (A) $(I_{A_\alpha\infty} - I_{A_\alpha t})/(I_{A_\alpha\infty} - I_{A_\alpha(t=0)})$ (●) is plotted on a semilogarithmic graph. The resensitization rate was $0.064 \pm 0.005\ s^{-1}$ (dotted line). (B) $(I_{A_\beta\infty} - I_{A_\beta t})/(I_{A_\beta\infty} - I_{A_\beta(t=0)})$ (▲) is plotted on a semilogarithmic graph. The resensitization rate was $0.020 \pm 0.002\ s^{-1}$ (dashed line). Each point shows the average of 3–8 measurements made using a total of 18 different vesicles. The error bars represent plus and minus standard deviations from the mean. For the vesicles used in these experiments, I_{A_β} was 30 ± 10% of the total current.

calculated a desensitization rate of $0.6\ s^{-1}$ at −75 mV in the presence of 200 μM glycine for the 48-pS channel; using the cell-flow technique under the same conditions, we measured a desensitization rate of $0.8\ s^{-1}$. At −75 mV and 200 μM glycine, we obtain a P_o value of 0.7 from bursts of channel activity measured using single-channel current recordings. We could also calculate P_o from measurements with the cell-flow technique at −40 mV using eq 3a. I_{A_α} at 200 μM glycine has a value of 0.65 nA (Figure 2A), and $I_M R_M$ is 0.74 nA (Figure 2A), so $(\overline{AL_n})_0 = P_o = 0.9$ (eq 3a). We observed that the ratios between I_{A_α} or I_{A_β} determined at different glycine concentrations (40, 100, and 200 μM) (e.g., the ratio between I_{A_α} at 40 μM glycine and I_{A_α} at 200 μM glycine) do not change between −40 and −75 mV (data not shown), suggesting that the value of P_o is independent of transmembrane voltage. We assume, therefore, that the comparison between P_o values determined at −40 mV by the cell-flow technique and at −75 mV by the single-channel current-recording technique is valid. The values obtained for the rate constant for desensitization and for P_o indicate therefore that the bursts of channel activity with a conductance of 48 pS are associated with receptor form A_α. These results are summarized in Table 1.

In presence of 40 μM glycine, we observed two types of receptor channels: one had a conductance of 48 pS, and the other had a conductance of 40 pS. We could not detect the 40-pS channel in the presence of 200 μM glycine. In the presence of 40 μM glycine, we did not find agreement between the length of the burst (as we defined it) of the 40-pS channel and the rate of desensitization or between the values of P_o and $(\overline{AL_n})_0$ of A_β (data not shown).

Once the conductance of a receptor channel is determined, one can calculate the number of receptor molecules in the membrane of each vesicle. $I_M R_M$ (Udgaonkar & Hess, 1987) corresponds to the whole-cell current that would be observed if all the receptor channels opened in the presence of a saturating concentration of activating ligand. The average

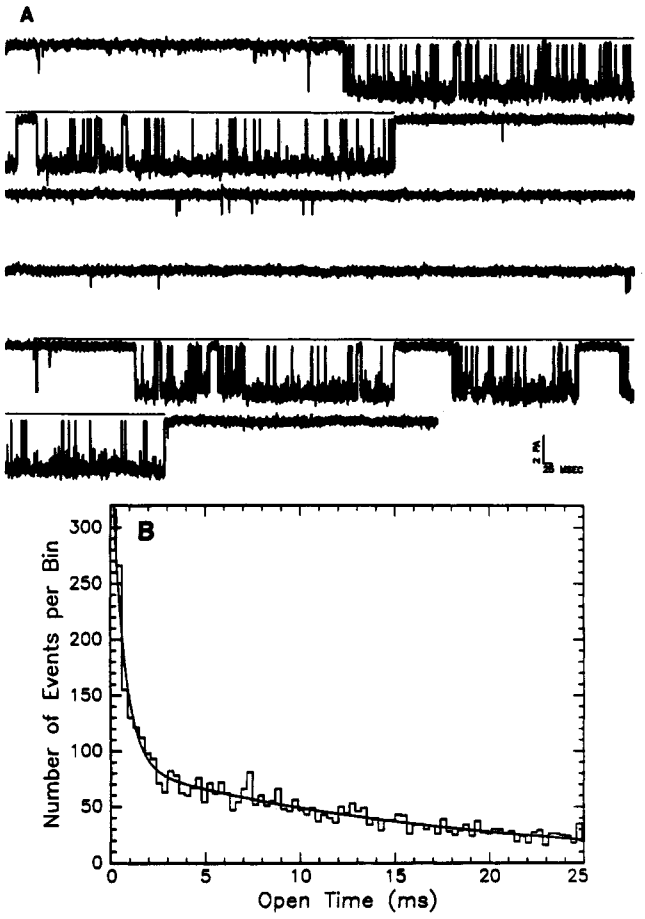


FIGURE 6: Single-channel current recordings. (A) A single-channel current trace showing individual openings of receptor channels recorded in the presence of 200 μM glycine at a transmembrane potential of –75 mV at pH 7.4 and at 22–24 °C. Two bursts of channel activity separated by a long silent period are shown. A line has been drawn over each burst of channel activity to denote the duration of the burst, and idealized data have superimposed on the channel openings. A total of 65 bursts of channel activity were measured using two cells at 200 μM glycine. The mean burst duration was 1.7 ± 1.4 s, α = 0.6 ± 0.5 s^{-1}, and P_o = 0.7 ± 0.2. (B) An open-time histogram showing openings from two outside-out patches from two different cells at –75 mV, pH 7.4, 22–24 °C, and 200 μM glycine. The histogram is represented by the sum of two exponentials, where 4507 openings were measured: 11% had a mean lifetime of 0.65 ± 0.04 ms, and 89% had a mean lifetime of 17 ± 1.5 ms. The binwidth was 0.3 ms. The openings with the shorter lifetime were assigned to a contaminating channel that opened in the absence of glycine (see Results).

number of receptor channels present in the cell membrane can then be determined by dividing $I_M R_M$ by the current that passes through a single open channel. From the value for $I_M R_M$ of 0.74 nA for receptor form A_α and a single-channel conductance of 48 pS, one can calculate a mean value of ~390 rapidly desensitizing glycine receptors in the membrane of the vesicles we obtained from mouse spinal cord cells (Table 1). If we assume that the slowly desensitizing glycine receptor has a conductance of 40 pS, we can calculate from the $I_M R_M$ value of 0.4 nA that there are ~250 slowly desensitizing glycine receptors in mouse spinal cord cell vesicles.

Open-channel histograms were represented by the sum of two exponentials. From 10 to 20% of the openings had a mean lifetime of 0.45–0.65 ms; this channel was present in outside-out patches in the absence of glycine (data not shown). The other 80–90% of the openings had a longer mean lifetime (~17 ms) (Figure 6B) and were observed only when glycine was applied to the membrane.

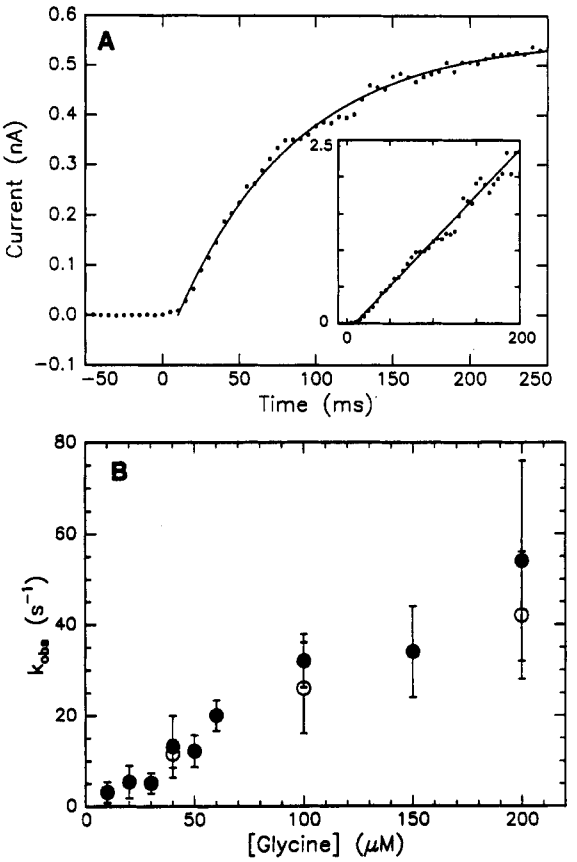


FIGURE 7: Channel-opening reaction. (A) The current-rising phase of the glycine receptor response at pH 7.4, 22–24 °C, –40 mV, and 40 μM glycine. The points show the data, and the solid line represents the fit of the current rise to a single exponential (k_{obs} = 12 s^{-1}) rate equation. The inset shows a linearized version of the data (dots) and the fit to the linearized rate equation (solid line). The labels for the x- and y-axes of the inset are time (ms) and $\log((I_{max})/(I_{max} - I_{obs}))$, respectively. I_{max} is the maximum current amplitude. The line intersects the x-axis at 9 ms, which represents the dead-time (Roughton & Chance, 1963) in the reaction (see Experimental Procdures). (B) Channel-opening rates measured at pH 7.4, 22–24 °C, and –40 mV as a function of glycine concentration. The solid circles represent the mean value of 7–46 measurements made with 3–12 vesicles. The open circles represent the mean value of 8–14 measurements made with five or six vesicles at a transmembrane voltage of –75 mV. The error bars represent the standard deviation.

Channel-Opening Reaction. We measured the rate of increase of the glycine-activated current as a function of glycine concentration because we observed that this rate is slow compared to the time resolution of our method (see Figure 7A). The rising phase of the current traces was fit to the equation:

$$I_{obs} = I_{max}(1 - \exp(-k_{obs}t)) \quad (6)$$

I_{obs} is the observed current, I_{max} is the maximum amplitude of the observed current, and k_{obs} is the rate of current increase. A single-exponential process described the current rise despite the presence of two receptor forms (Figure 7A); therefore, we assumed that the rates of current increase were similar for each receptor form. The dead-time (Roughton & Chance, 1963) of the cell-flow technique is given by the abscissa intercept when the current increase plotted as a function of time (Figure 7A) follows a single-exponential rate equation. Here, the dead-time is approximately 10 ms, which corresponds to the solution exchange time (see Experimental Procedures).

We assumed that the ligand-binding steps are faster than and in equilibrium with the channel-opening reaction and

that the channel-opening reaction is faster than the desensitization reaction. Then,

$$k_{obs} = k_{op}\left(\frac{L}{L + K_1}\right)^n + k_{cl} \quad (7)$$

This equation describes the channel-opening reaction for an n-binding-site model where k_{obs} is the observed rate of the current rise, K_1 is the dissociation constant, and k_{op} and k_{cl} are the channel-opening and -closing rate constants ($\Phi = k_{cl}/k_{op}$ in Scheme 1). Since a mixture of receptors is present, we could not evaluate k_{op} or k_{cl}. A plot of k_{obs} versus glycine concentration (Figure 7B) indicates that at low glycine concentrations k_{obs} (eq 7) has a value of 3 s^{-1}. This value reflects k_{cl} when eq 7 applies to the mechanism. k_{cl} can be calculated from the mean open time of the open channel (τ_{op}, $1/\tau_{op} = k_{cl}$; Colquhoun & Hawkes, 1983). We measured a τ_{op} of 14–17 ms for the 40- and 48-pS channels (data not shown). This gives estimates for k_{cl} of 60–70 s^{-1}, which is 20 times the value of k_{obs} determined from the cell-flow experiments (Figure 7) at low glycine concentrations. The τ_{op} values were determined at V_M values of −75 mV, and k_{obs} was generally measured at −40 mV; k_{obs} (eq 7) does not appear to depend on V_M from −40 to −75 mV (Figure 7B).

DISCUSSION

We have used a chemical kinetic technique with a 10-ms time resolution to determine the dissociation constant of the receptor site controlling channel opening (K_1), the channel-opening equilibrium constant Φ, and the rate coefficients for desensitization that determine the concentrations of open receptor channels of two different forms of the glycine receptor on cultured embryonic mouse spinal cord cells. The dependences of the current amplitude and of the desensitization rates on glycine concentration are consistent with a mechanism similar to the one that was determined for the nicotinic acetylcholine receptor in *E. electricus* vesicles and BC_3H1 and PC12 cells (Cash & Hess, 1980; Udgaonkar & Hess, 1987; Matsubara & Hess, 1992) and for GABA receptors in membrane vesicles prepared from the rat brain (Cash & Subbarao, 1987 a–c, 1989) and in mouse embryonic cortical cells (Geetha & Hess, 1992).

We showed that the properties of the glycine receptors were not affected by vesicle formation; our results from receptors on vesicles (solid circles in Figures 2 and 4) were the same as those from entire cells that had been lifted off the culture dish (open circles in Figures 2 and 4). This is of interest because an intracellular, peripheral membrane protein associates with the glycine receptor (Schmitt et al., 1987; Triller et al., 1985) and affects receptor function (Takagi et al., 1992) and receptor clustering (Kirsch et al., 1993).

In the experiments described here, the time resolution was sufficiently fast that only a small correction was required to correct the observed current for desensitization that occurs while the receptors equilibrate with ligand (eq 1) (Udgaonkar & Hess, 1987). Glycine receptor responses measured using experimental techniques with a time resolution longer than 15 ms would require significant current correction.

We observed two components in the desensitization of glycine receptors on spinal cord cells. When interpreting the cell-flow measurements in Figure 1, it is important to determine if the rapid phase of the decaying part of the current represents the consecutive conversion of one active receptor form to two desensitized forms. If it does, the total whole-cell current is proportional to the concentration of A_α receptors in the membrane. However, if the two receptor forms are not interconvertible, the current amplitudes, I_{A_α} and I_{A_β}, are proportional to the concentrations of two different receptor forms, A_α and A_β, respectively.

The rapidly and slowly desensitizing receptor forms resensitize at different rates. The observation that the rapidly desensitizing receptor form (measured by I_{A_α}) resensitizes with a faster rate than the slowly desensitizing receptor form (measured by I_{A_β}) is inconsistent with a model in which the slowly resensitizing form A_β gives rise to the rapidly resensitizing form A_α. Also consistent with the presence of two receptor forms is the 5-fold difference in affinity observed when the effects of picrotoxinin on the current amplitudes were measured.

The results described in this paper are consistent with previous reports of more than one form of the glycine receptor (Akagi & Miledi, 1988; Twyman & Macdonald, 1991; Akaike & Kaneda, 1989). Akagi & Miledi (1988) found that two different fractions of mRNA from adult rat spinal cord injected into *Xenopus* oocytes produced different types of glycine receptor. The receptors differed in their affinities for glycine and in their desensitization rates. Akaike and Kaneda (1989) used rapid-perfusion current measurements to show that glycine receptors in acutely dissociated rat hypothalamic neurons desensitize and resensitize at different rates and concluded that there were two components in the channel-opening and desensitization reactions. Glycine receptor channels with different conductances were found in cultured mouse spinal cord neurons (Hamill et al., 1983; Bormann et al., 1987; Twyman & Macdonald, 1991). Takahashi and Momiyama (1991) and Bormann (1990) observed a 46–48-pS conductance channel in most experiments but a lower conductance channel only 30–50% of the time.

Chemical kinetic measurements indicated that the two receptor forms A_α and A_β have different affinities for neurotransmitters and different desensitization rates. These are important properties in determining the rate at which inorganic ions can cross the membrane and, therefore, in receptor-controlled changes in the transmembrane voltage of a cell. We obtained intrinsic dissociation constants for A_α and A_β of 200–230 and 360–380 μM, respectively, by measuring the effect of a 100-fold range of glycine concentrations on I_{A_α} and I_{A_β} separately (using eq 3b with $n = 3$ and 2, respectively) and on α and β (using eq 4). Determination of the effect of glycine concentration on the current amplitude (Figure 2) depends on the evaluation of four constants: $I_M R_M$, Φ, K_1, and n (eq 3b). Two of these constants, Φ and K_1 (eq 4), determine the effect of glycine concentration on the desensitization rate. In separate analyses of both the current amplitude and the desensitization rate, the values of these constants agreed.

Additional information about the macroscopic constants and the mechanism can be obtained from single-channel current measurements (Neher & Sakmann, 1976). The bursts are considered to reflect the lifetime of the nondesensitized receptor form (Sakmann et al., 1980). At 200 μM glycine, it was possible to analyze the length of bursts of channel activity. Good agreement between the lifetime of these bursts and the value of the desensitization rate coefficient α of receptor form A_α was obtained (Table 1). Similarly, the fraction of time the channel is open during a period of channel activity is a measure of P_o (Sakmann et al., 1980) and is directly related to the value of I_A determined in cell-flow experiments at the same concentration of neurotransmitter (Udgaonkar & Hess, 1987). I_A is determined by the constants of the mechanism: n, Φ, K_1, and $I_M R_M$ (eq 3a). Good

agreement between $(\overline{AL_n})_o$ (determined from I_{A_α}) and P_o was obtained at a glycine concentration of 200 μM (Table 1). Since, in the presence of 200 μM glycine, we observed only the channel with a conductance of 48 pS, we can assign this conductance to receptor form A_α. At 40 μM glycine, we observed receptor channels with two conductances, 48 and 40 pS. However, we did not find a correspondence between the results obtained in cell-flow experiments and single-channel current recordings at this glycine concentration. The value of I_{A_α}, which we determined by the cell-flow technique and assigned to the 48-pS channel, increases by a factor of 3 in the glycine concentration region 40–200 μM. The value of P_o, which is proportional to I_{A_α}, was determined by using the single-channel technique and remained unchanged in this concentration region. At 40 μM glycine, we were unable to find a correlation between cell-flow and single-channel current measurements of the 40-pS channel. One explanation for this is that at 40 μM glycine, our assignment of the beginning and ending of periods of channel activity may have been incorrect. The correct assignment is essential for obtaining not only the lifetime of the active receptor form but also the value of P_o. It is well known that it is possible under only some experimental conditions to define the duration of bursts of channel activity (Sakmann et al., 1980). Among the conditions that are important in defining a burst of channel activity are the number of receptor molecules being measured, the neurotransmitter concentration, and the desensitization rate.

The best-characterized receptor, the acetylcholine receptor, contains two α-subunits (Reynolds & Karlin, 1978) and requires the binding of two acetylcholine molecules before the channel opens (Katz & Thesleff, 1957; Cash & Hess, 1980). Biochemical evidence suggests that the adult glycine receptor may be composed of three α-subunits which contain the glycine-binding sites (Grenningloh et al., 1987; Schmieden et al., 1992; Kuhse et al., 1993), and two β-subunits (Langosch et al., 1988). We found that the formation of the transmembrane channel is consistent with the prior binding of at least three glycine molecules to receptor form A_α and two glycine molecules to receptor form A_β. In this regard, it is interesting to note that the Hill coefficient observed in dose–response curves of whole-cell currents in HEK-293 cells expressing glycine receptors was found to depend on the subunit composition (Bormann et al., 1993). In cell-flow experiments, the apparent dissociation constants of picrotoxinin from the slowly desensitizing receptor and from the nondesensitizing current component are the same and are almost 6 times higher than those for the rapidly desensitizing current component associated with A_α. Pribilla et al. (1992) found that when glycine receptors were expressed in HEK-293 cells, receptors containing β-subunits were less sensitive to picrotoxinin than those containing only α-subunits. The A_α receptor form probably does not consist of only α_2 subunits (α_2 is a specific α subunit found in embryonic mice and cultured cells; Kuhse et al., 1990a, 1991) because such receptors expressed in *Xenopus* oocytes have a maximum current response to taurine that is only 1/15 of the maximum response to glycine (Schmieden et al., 1992). We did not observe a strychnine-insensitive glycine receptor, which was found by Hoch et al. (1989) on cultured spinal cord cells prepared from the inbred mice strains C57BL/6 or BALB/c. Inclusion of *N*-methyl-D-aspartate (*N*-MDA) inhibitors in the cell-culture media can induce the production of some adult glycine receptors in cultured spinal cord cells (Hoch et al., 1992). Our culture medium contained one *N*-MDA receptor inhibitor (putrescine; Sacaan & Johnson, 1990). Our results may explain why no β-subunits were found when the receptors from cultured spinal cord cells were isolated and studied (Hoch et al., 1989), because only the less abundant receptor form A_β may contain this subunit. One consequence of the presence of two receptor forms with similar dissociation constants but a different number of binding sites is that at low concentrations of glycine (10–20 μM), the response of A_β dominates the glycine receptor response. At 10 μM glycine, there is not enough glycine present to cause the A_α receptors, with three binding sites, to open, but 10% of the A_β receptors do open (Figure 2). More A_α than A_β receptors are expressed on the spinal cord cells. Since A_α and A_β have similar conductances, the A_α response dominates the glycine receptor response at higher glycine concentrations. The regulation of receptor response by changing the number of binding sites but not their dissociation constants may be an important regulatory mechanism for neurotransmitter receptors.

Another feature of the channel-opening mechanism of the glycine receptor, distinguishing it from the acetylcholine receptor, is the rate at which the channel opens. In experiments with the acetylcholine receptor, processes with rate constants of less than 400 s^{-1} have not been observed (Madsen & Edeson, 1988). In the case of the glycine receptor, the maximal observed rate for the current risetime is 50 s^{-1} in cell-flow measurements in the 10–200 μM glycine range (Figure 7B). We have demonstrated that this rate does not reflect the time of mixing of the receptors with glycine (Figure 7A), and we conclude that we are measuring a rate pertaining to the channel-opening reactions. The minimum rate of the risetime at low glycine concentrations is 3 s^{-1} (Figure 7B). The channel-closing rate determined from the lifetime of the open channel (17 ms) at 200 μM glycine at –75 mV (Figure 6B) is consistent with a rate constant of $\sim$60 s^{-1} for channel closing. We ascertained that similar results were obtained at 40 μM glycine, at which both the 40- and the 48-pS channel could be observed, and that the risetime of the channel is voltage-independent between –40 and –75 mV (Figure 7B). The single-channel results agree with the results of Bormann (1990; k_{cl} = 50–200 s^{-1}) and Twyman and Macdonald (1991), who found that one component of a multiple-exponential fit to an open-time histogram has a time constant of 9–10 ms. Our observation that the channel open time is the same at 40 and 200 μM glycine and the observation by Twyman and Macdonald (1991) that the lifetime of the channels is similar at 0.5–2 μM glycine are inconsistent with short open-channel lifetimes because of channel blockage by glycine. Blockage of the acetylcholine receptor channel by acetylcholine is a popular and often invoked mechanism to account for shortened open-channel lifetimes [reviewed by Karlin (1991) and Galzi et al. (1991)].

Could the slow risetime of the current reflect a ligand-binding step? In a plot of the k_{obs} values as a function of glycine concentration (Figure 7B), the receptor:ligand dissociation constant would be reflected by the k_{obs} value obtained at low glycine concentrations. Typical bimolecular rate constants for the binding of small molecules to proteins are $>10^6$ M^{-1} s^{-1} (Hammes, 1982). The dissociation constant for glycine from either of the two receptor forms is >200 μM. Together with the on rate of $>10^6$ M^{-1} s^{-1}, the dissociation constant of 200 μM requires dissociation rates of >200 s^{-1}. The value of 3 s^{-1}, the limiting k_{obs} value for the risetime at low glycine concentrations, is too low to account for the dissociation rate of a ligand-binding step. These considerations are consistent with a model in which a rate-limiting conformational change preceeds the channel-opening reaction. The

extra slow step in the channel-opening reaction that we propose for the glycine receptor was not observed for the acetylcholine receptor (Matsubara et al., 1992).

The slow rates associated with the current risetimes are consistent with the results of Akaike and Kaneda (1989). These authors observed that the current risetime, measured in rapid-perfusion experiments with glycine receptors in freshly dissociated hypothalmic neurons, has two components. In the glycine concentration region from 10 μM to 2 mM, the rate of current rise increased from 7 to 100 s^{-1}.

Some of the measurements reported here can be compared to the results of others. Desensitization rates and the glycine concentration that gave half-maximal current amplitudes (EC_{50} value) for glycine receptor-containing cells were reported (Lewis et al., 1991; Akaike & Kaneda, 1989; Krishtal et al., 1988) from perfusion measurements. In one such experiment with a low time resolution, only a slowly desensitizing component was observed, and its EC_{50} value was reported (Lewis et al., 1991). In rapid-perfusion experiments, the EC_{50} value of the total current amplitude, which reflects both receptor forms, was determined (Akaike & Kaneda, 1989; Krishtal et al., 1988). In the transmembrane voltage range from −70 to −100 mV, EC_{50} values of 90–100 μM were obtained in those studies. In our experiments at −40 mV, an EC_{50} value of 50 μM was obtained for the total current amplitude observed in cell-flow experiments.

The approach described here differs from previous studies in several ways. (i) In cell-flow experiments, we correct the observed current for desensitization that occurs during the equilibration of the receptor with the neurotransmitter (Udgaonkar & Hess, 1976; Geetha & Hess, 1992; Matsubara & Hess, 1992). Thus we can determine the concentration of receptor forms prior to desensitization. (ii) The results for each receptor form are analyzed separately to determine the equilibrium constants for channel opening (Φ^{-1}), the dissociation constants that account for the concentration of open receptor channels, and the rate constants for desensitization. (iii) In cell-flow measurements, we determine separately the effect of glycine concentration on the amplitude and falling phase of the current. We compare the constants evaluated by these measurements to each other and, if conditions allow, to the results obtained with the single-channel technique.

A chemical kinetic approach allows the determination of constants for a mechanism that describes the concentration of open receptor channels as a function of time and over a wide range of neurotransmitter concentration. This approach also allows one to study the effects of drugs on different forms of a receptor present in a single cell. Thus, essential information for the rational design of drugs can be obtained. Rapid chemical kinetic techniques have been used to study the acetylcholine receptor in a variety of cells (Udgaonkar & Hess, 1986; Matsubara & Hess, 1992; Niu & Hess, 1993; Hess, 1993) and the GABA receptor in mouse cerebral cortical cells (Geetha & Hess, 1992; Cash & Subbarao, 1987a–c, 1989). In this paper, we describe the use of a rapid chemical kinetic technique to measure the responses of two native glycine receptor forms in a single cell. We show for each form how the constants that determine the concentration of the open channel as a function of neurotransmitter concentration differ.

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