

Glycine decreases desensitization of *N*-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus* oocytes and is required for NMDA responses

(excitatory amino acids/neurotransmitter/glutamate receptor)

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Contributed by Michael V. L. Bennett, December 29, 1989

ABSTRACT In *Xenopus* oocytes injected with rat brain mRNA, as in neurons, glycine greatly potentiated responses of the *N*-methyl-D-aspartate (NMDA) type of excitatory amino acid receptor. Injected oocytes generated a partially desensitizing inward current in response to NMDA with 30 nM added glycine. As the added glycine concentration was increased from 30 nM to 1 μ M, the NMDA response was increased and exhibited less desensitization. The relationship between the NMDA peak response and added glycine concentration indicated a single component response with apparent affinity of 0.29 μ M and a Hill coefficient of 0.77. The desensitized response was also fit by the Hill relation with a lower affinity but similar coefficient. The time course of desensitization at 500 μ M NMDA was exponential with a time constant (350 msec) that was independent of glycine concentration between 0.03 and 0.3 μ M. At higher glycine concentration a slower component of decay ($\tau = 1.4$ sec) was observed. This component was enhanced by increasing the extracellular Ca^{2+} . NMDA without added glycine evoked a small transient response. However this response was suppressed completely by prewashing with the glycine antagonist 7-chlorokynurenic acid, suggesting that it may have been due to glycine contamination. The dose-response relation for low concentrations of glycine indicated that the measured level of glycine contamination accounted for these responses. These results indicate that glycine has at least two actions at the NMDA receptor: it enables channel opening by the agonist and decreases desensitization.

Of the receptors activated by the excitatory amino acid neurotransmitter glutamate, the *N*-methyl-D-aspartate (NMDA) receptor-channel complex has attracted considerable attention due to its proposed roles in physiological as well as pathological phenomena. The NMDA receptor complex has been implicated in long-term potentiation (1–3), learning (4), developmental structuring (5, 6), hypoxic damage (7), epilepsy, and kindling (8). The NMDA receptor has a number of regulatory sites including a binding site within the channel for Mg^{2+} that blocks the agonist-induced current in a voltage-dependent manner (9, 10), a binding site (also within the channel) for phencyclidine (PCP) and other PCP receptor ligands (11, 12), an inhibitory site for Zn^{2+} (13, 14), and a high-affinity site for glycine (15, 16). Glycine acts at this site to potentiate NMDA-induced currents. At the single channel level in the steady state, glycine increases the frequency of channel opening by NMDA agonists without significantly altering the mean open time or single channel conductance (15). Glycine does not affect either NMDA receptor affinity or Hill coefficient (17).

The NMDA receptor-channel complex is expressed in *Xenopus* oocytes after injection of mRNA isolated from rat brain (16–18) or from the clonal cell line NCB-20 (19). The characteristics of these receptors are typical of neuronal NMDA receptors. NMDA-induced currents are inhibited competitively by D-2-amino-5-phosphonovaleric acid (APV) and noncompetitively by Zn^{2+} , Mg^{2+} , and PCP and related compounds. Block by Mg^{2+} is voltage dependent, being greater at more inside negative potentials (16, 20). Block by PCP is voltage and also use dependent in that it is greatly speeded in the presence of agonist (16, 17, 20). In addition, the NMDA-induced responses are dramatically potentiated by the presence of glycine (16–19, 21). Thus, the known features of the NMDA receptor are reproduced by this expression system.

In the course of our earlier experiments, we observed that with slow application, glycine was required for any NMDA response (ref. 16; see also ref. 21). However, with rapid application, a small inward current was generated at the onset of NMDA application in the presence of 10 nM added glycine or without added glycine (19). An initial interpretation of these results was that NMDA receptors desensitize to a greater extent at low glycine levels, a phenomenon recently reported for cultured hippocampal neurons (22). Also, these data raised the possibility that transient responses might occur in the absence of glycine and that failure to record them was due to desensitization during slow application of NMDA. In the present report we document that desensitization of NMDA receptors is reduced by glycine. However, this desensitization does not account for the absence of responses without added glycine; rather the responses that are sometimes seen in “zero glycine” appear to be due to glycine contamination.

MATERIALS AND METHODS

Poly(A)⁺ RNA was prepared from whole brains of adult rats as described (16, 17, 19). In brief, total RNA was extracted by modification of the method of Ullrich *et al.* (23) involving guanidinium isothiocyanate (Fluka) and a CsCl density gradient. Poly(A)⁺ RNA was then purified by oligo(dT)-cellulose chromatography, dissolved in water, and stored at $-70^{\circ}C$ until use.

Oocytes were collected from ovarian lobes of anesthetized *Xenopus laevis*. The layer of follicular cells was removed mechanically after 2 hr of incubation at 22°C in a Ca^{2+} -free ND96 medium supplemented with sodium pyruvate (2.5 mmol/liter) to which penicillin (100 units/ml), streptomycin

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Abbreviations: NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; APV, D-2-amino-5-phosphonovaleric acid; 7-Cl-KA, 7-chlorokynurenic acid.

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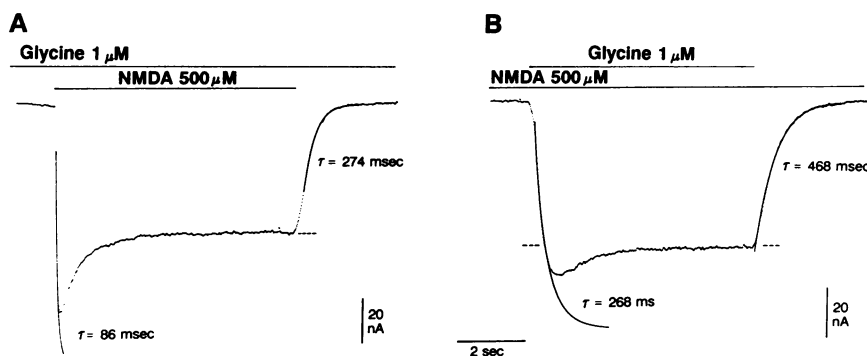


FIG. 1. Speed of response to NMDA and glycine depends on which is applied first. (A) Inward current when 500 μM NMDA and 1 μM glycine were applied some seconds after 1 μM glycine application. (B) Inward current in response to the same concentrations of NMDA and glycine after NMDA application. In both cases, the holding potential was set at -40 mV to reduce any current-dependent decay. Rates of increase of inward currents were fitted by single exponentials. The time constant was shorter for pretreatment with glycine and the peak was somewhat greater. Deactivation of the response was faster when the wash solution was 1 μM glycine than when it was 100 μM NMDA. In both cases, the steady-state response was of the same amplitude (dashed line, calibrations differ).

(1 mg/ml), and collagenase (2 mg/ml) (Sigma, type IA) had been added. The vitelline membrane was left intact. Stage V and VI oocytes were selected (24) and injected with 50 ng of mRNA. Oocytes were maintained at 16°C in Leibowitz's L-15 medium (Sigma) supplemented with 5 mM HEPES buffer (pH 7.6), penicillin (100 units/ml), and streptomycin (1 mg/ml). After 5–8 days, oocytes were placed in a recording chamber (about 0.1 ml in volume) and perfused with Mg^{2+} -free amphibian Ringer's solution (116 mM NaCl/2.0 mM KCl/0.5 mM CaCl_2 /10 mM HEPES buffer, pH 7.2). All compounds were prepared from stock solutions and bath-applied in this medium. APV was purchased from Cambridge Research Biochemicals (Harston, U.K.); NMDA, L-glutamate, and glycine were from Sigma. The glycine antagonist 7-chlorokynurenic acid (7-Cl-KA) was kindly supplied by R. Keith (ICI Americas, Wilmington, DE).

Cells were voltage-clamped at a holding potential of -60 mV (unless otherwise stated) with two electrodes filled with 1 M KCl (1–1.5 M Ω). Responses were digitized and analyzed by a computer equipped with plotting and exponential fitting programs (ASYSTANT, McMillan).

The large size of the oocyte makes rapid solution changes difficult. To estimate how fast the receptors were activated, the concentration of NMDA was changed from 0 to 500 μM in the presence of 1 μM glycine; an inward current developed with a time constant of <100 msec. Deactivation of NMDA-induced current by washing could be obtained in <1 sec (relaxation time constant = 200–400 msec). The speed and size of the initial response depended on orientation of the oocyte. The fastest rising responses with the largest peaks were obtained with the animal pole of the oocyte facing the

upstream end of the perfusion trough, as NMDA receptors are generally localized in this region (25).

RESULTS

Glycine Decreases Desensitization of NMDA-Induced Currents. The response to 500 μM NMDA in the presence of 1 μM glycine was a rapidly rising inward current that decayed from a peak value to a steady state. We shall argue that the decay is largely desensitization at this and lower levels of glycine. At higher glycine levels additional mechanisms are involved that may include Ca^{2+} -dependent inactivation of NMDA channels, reducing the steady-state current, and transient Ca^{2+} activation of Cl^- channels, increasing the peak. The maximum rate of rise of responses to NMDA and glycine was obtained when NMDA and glycine were added after several seconds of preapplication of the same concentration of glycine (Fig. 1A). If NMDA were applied first, followed by NMDA plus glycine, or if NMDA and glycine were applied simultaneously, the responses were more slowly rising (Fig. 1B). To achieve the most rapidly rising responses, which was important in measuring desensitization, glycine was routinely applied 5–10 sec prior to application of NMDA together with the same concentration of glycine. The faster rate of rise observed when NMDA was applied after glycine pretreatment may reflect faster kinetics of channel opening. Deactivation of the response was also faster when NMDA was washed out in the presence of glycine than when glycine was removed in the presence of NMDA.

When the concentration of glycine was varied, the response to NMDA was larger at higher concentrations and

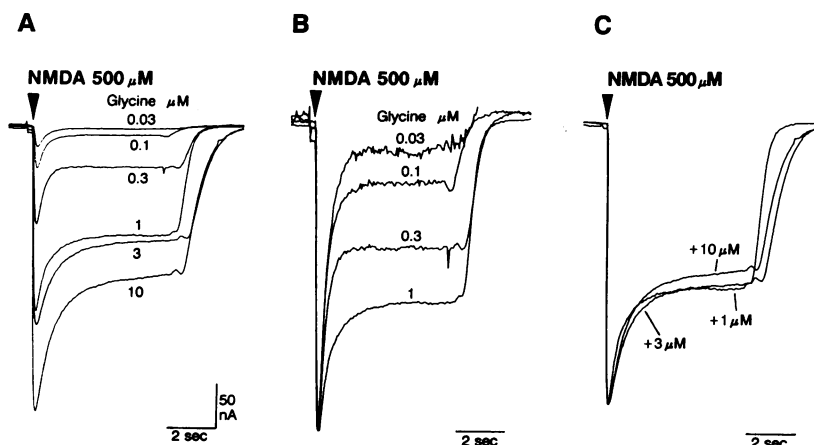


FIG. 2. Glycine increases responses to NMDA and decreases desensitization in a dose-dependent manner. Glycine was applied for 5–10 sec prior to application of 500 μM NMDA together with glycine. (A) Responses to NMDA were potentiated by glycine and also were altered in shape. (B) The same responses (to 0.03–1 μM glycine) normalized with respect to the peak. The extent to which the responses desensitized was less at higher glycine concentrations. (C) At high glycine (3–10 μM) the decay became slightly more pronounced and slower. In this and the following figures the indicated concentrations refer to added glycine.

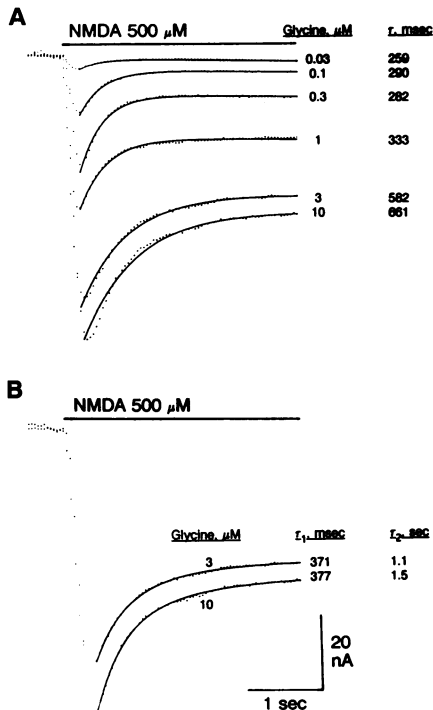


FIG. 3. Single- and double-exponential fits to the time course of decay of responses to NMDA at different concentrations of glycine. (A) Desensitization at low glycine (0.03–1 μM) was well fit by a single exponential with the indicated time constants. At higher glycine (3–10 μM) the responses were larger and attenuation of the responses was poorly fit by a single exponential. (B) The same responses at 3 μM and 10 μM glycine as in A but fitted by the sum of two exponentials. The faster time constant was close to those at lower glycine levels. The slower time constant may involve a different mechanism.

also altered in shape (Fig. 2A). The peak of the response was potentiated to a lesser degree than was the steady state—i.e., desensitization was reduced (Fig. 2B). In responses to 500 μM NMDA, increasing the added glycine concentration from 0.03 to 1 μM increased the peak by 8 ± 1.6 times (mean ± SEM, n = 5) and the steady state by 26 ± 5.3. The average ratio of potentiation of the steady-state current to potentiation of the peak current was 3.2 ± 0.3 (mean ± SEM, n = 5). Similar results were obtained at 100 μM NMDA.

When 500 μM NMDA was applied in the presence of 0.03–1 μM glycine, the time course of desensitization could be described by a single exponential decay with a similar time constant for each glycine concentration (mean of 291 msec in Fig. 3A, pooled data: 350 ± 30 msec, mean ± SEM, n = 4). However, when glycine concentration was further increased to 3–10 μM, the overall decay was slowed and slightly increased in magnitude (Fig. 2C); desensitization in this range of glycine concentration was better described as the sum of two exponentials (τ₁ = 387 ± 45 msec and τ₂ = 1.4 ± 0.12 sec, n = 3, Fig. 3B). These data indicate the appearance of an additional mechanism of decay of NMDA-induced current. This kind of decay was slower and may have been current dependent, because it was larger at more hyperpolarized holding potentials (not shown). In order to determine whether the faster component of the response to 500 μM NMDA was further reduced at high glycine levels (1–10 μM), the amplitudes of the two exponentials accounting for the decay process and the steady-state to peak ratios that each would produce in the absence of the other were determined for 1, 3, and 10 μM glycine. Although the total decay in current was slightly greater at 10 than at 1 and 3 μM glycine (Fig. 2C), the faster component was not significantly reduced at the higher glycine levels (ratios: 0.73 ± 0.08 at 1 μM, 0.79 ± 0.06 at 3 μM, and 0.81 ± 0.06 at 10 μM glycine, n = 3 or 4). With 100 μM NMDA the decays at low glycine levels were somewhat slower and less marked than with 500 μM NMDA (Fig. 4A and B). The time constant was the same for 0.03, 0.1, and 0.3 μM glycine (mean ± SEM, 459 ± 19, pooled data, 5 oocytes), and more slowly developing attenuation of the response was clearly observed at higher glycine concentrations (τ = 1–2 sec for 1 μM glycine).

The relation between the peak inward current elicited by a saturating concentration of NMDA (500 μM) and added glycine concentration indicated a single component response (not illustrated; see refs. 14, 17). Hill analysis gave an apparent affinity (K_d) for glycine of 0.29 ± 0.071 μM (mean ± SEM, n = 4 oocytes) and a Hill coefficient not far from unity (0.77 ± 0.06). Although the assumptions used in deriving the Hill equation do not necessarily apply to the desensitized state, Hill analysis of the steady-state responses gave a larger K_d (1.2 ± 0.26 μM) but a similar coefficient (0.99 ± 0.06). Thus, the desensitization could be described as a change in apparent affinity for glycine with no change in cooperativity. The effect of glycine on desensitization can be illustrated by plotting the ratio of steady-state to peak NMDA

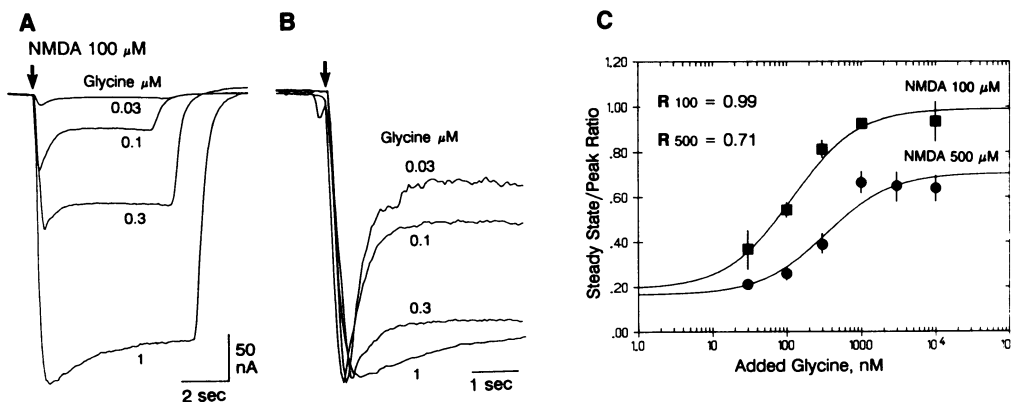


FIG. 4. Effects of glycine on decay of responses at a lower concentration of NMDA. (A) Superimposed responses to 100 μM NMDA at the indicated levels of glycine. (B) The same responses normalized with respect to the peak. Desensitization at 0.03–0.3 μM glycine was slower than with 500 μM NMDA. The time course at 1 μM glycine was still slower and may represent the second component as seen in Fig. 3B. (C) Decay of response amplitude is plotted as the ratio of steady state to peak vs. glycine concentration. Points (mean ± SEM from 5–10 oocytes) were fitted to the equation $r = R([Gly] + K_{dp}) / ([Gly] + K_{dss})$ resulting from the ratio of the Hill equations accounting for the steady state and the peak, where [Gly] is the glycine concentration, K_{dp} and K_{dss} are the apparent affinities for glycine at the peak and steady state, respectively, and R is the ratio at saturating glycine. Although NMDA desensitization was practically absent at 100 μM NMDA and 1–10 μM glycine (R = 0.99), a substantial degree of decay remained at 500 μM NMDA and 1–10 μM glycine (R = 0.71).

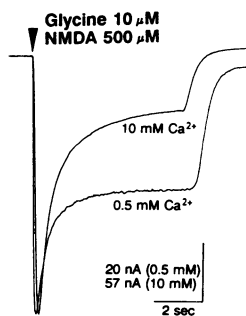


FIG. 5. Ca^{2+} promotes decay of NMDA responses. Increasing the concentration of extracellular Ca^{2+} from 0.5 to 10 mM enhanced the NMDA-induced currents (note the difference in gain), increased the extent of decay, and slowed the rate of onset.

response vs. added glycine (Fig. 4C). At 100 μM NMDA this ratio was 0.36 ± 0.09 at 0.03 μM glycine and close to unity at 1 μM glycine (0.92 ± 0.02 , $n = 10$). At 500 μM NMDA these ratios were smaller at all glycine levels and approached a maximum value of 0.67 ± 0.05 ($n = 5$) at 1 μM glycine.

Increasing the concentration of extracellular Ca^{2+} from 0.5 to 10 mM enhanced the NMDA-induced currents and increased the extent of decay (Fig. 5). Current attenuation at high Ca^{2+} developed with a slower time course, which also required a second exponential for a reasonable fit ($\tau_1 = 311$ msec, $\tau_2 = 1.6$ sec, $n = 2$). The amplitudes of fast and slow components of decay were increased. In the example shown in Fig. 5, the ratio of the fast component to the peak was increased by 48% in high Ca^{2+} , whereas the ratio of the slow component to the peak was increased by 100%. A few observations at low glycine levels also indicated that Ca^{2+} enhanced the fast component of current decay.

Is There Any NMDA Response at Zero Glycine? NMDA responses are very small at low glycine levels, and we and others have published dose-response curves for glycine at constant NMDA indicating an absolute requirement for glycine (16, 19, 21). Now, the observation that desensitization is increased at lower glycine levels suggests that brief NMDA responses might occur in the absence of glycine. However,

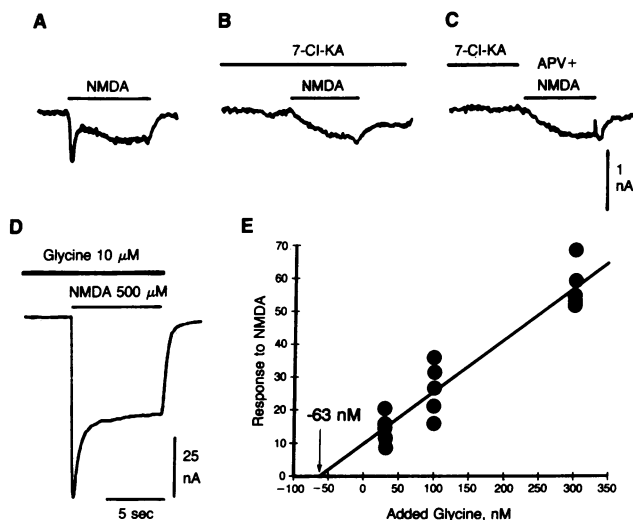


FIG. 6. Glycine is required for NMDA responses. (A) With no added glycine a small transient response developed upon application of 500 μM NMDA. (B) This small peak response was totally abolished by perfusing with a low concentration (10 μM) of 7-Cl-KA and is ascribable to glycine contamination. (C) The slowly developing inward current was not suppressed by 7-Cl-KA or by 100 μM APV, so that it was not a response mediated by the usual NMDA receptors. (D) In the same oocyte 10 μM glycine potentiated the peak NMDA response by about 90 times. (E) The relation between low glycine concentrations and peak responses to 500 μM NMDA was linear. The value obtained by extrapolating to zero response (-63 nM) was similar to the contaminating levels of glycine in the test solution measured by HPLC.

contaminating glycine is extremely difficult to avoid. Our background levels of glycine were 40–50 nM determined by HPLC. In our preparations, application of NMDA with no added glycine produced a small, brief inward current at the onset of application and a small, slowly rising maintained component (Fig. 6A). The transient was blocked by a low concentration of 7-Cl-KA (10 μM) (Fig. 6B), suggesting that it was dependent on (contaminating) glycine. The maintained component was not blocked by a high concentration of APV (100 μM) (Fig. 6C), evidence that it was not generated at conventional NMDA receptors. A similar response was reported earlier (21). The transient response was progressively increased when glycine was added, and a larger and presumably APV-sensitive sustained response occurred after the initial transient (Fig. 5D). At these low levels, the relation between glycine concentration and peak response was linear (Fig. 5E); extrapolation to zero response amplitude gave a concentration of -63 nM (five oocytes). These data are consistent with the glycine contamination level of 40–50 nM in the distilled water and NMDA solutions used, and we conclude that no response would have been obtained without contamination and without added glycine.

DISCUSSION

In this study we characterized the effects of glycine on responses to NMDA in *Xenopus* oocytes injected with adult rat brain mRNA. Glycine potentiated initial peak and steady-state responses and also decreased desensitization. Similar findings with respect to desensitization were recently reported for cultured embryonic neurons (22). Our data indicate that glycine's effect on desensitization is equivalent to a rightward shift of the relation between glycine concentration and NMDA response for steady state as compared to that for the peak. The reduction in apparent affinity for glycine of the NMDA receptor in the steady state is suggestive of an NMDA-induced conformational change to a state with reduced affinity for glycine (22).

An important issue is whether either Ca^{2+} -activated Cl^- currents or Ca^{2+} -mediated decrease in NMDA current is responsible for the apparent desensitization of NMDA-induced responses. Although Ca^{2+} influx appears to be important in the slower decay of NMDA responses at higher Ca^{2+} and current levels, it is unlikely to be involved at low glycine concentrations. At these levels increasing glycine increases the peak and steady-state current and presumably therefore Ca^{2+} influx but reduces the extent of decay, a finding consistent with true desensitization.

A further issue is whether glycine is necessary for NMDA-induced responses. In the absence of added glycine, NMDA induced a small transient response. Although precautions were taken to avoid glycine contamination (use of disposable glassware, solutions prepared daily, etc.), the basal level of glycine in the highly purified water was 40–50 nM as revealed by HPLC analysis, a level similar to those reported previously (21). The NMDA and glutamate used did not introduce additional glycine, but this normal contamination level would account for the small responses obtained in the absence of added glycine, and extrapolation of the dose-response curve to zero response predicted this level of contamination. Furthermore, these transient responses were totally suppressed by prior washing with a low concentration of 7-Cl-KA, an antagonist at the glycine binding site (26). To be sure, one cannot rule out the possibility that 7-Cl-KA acts as an inverse agonist—i.e., that it decreases NMDA responses below the level that they would have if the glycine site were unoccupied.

The proposal that glycine is required for activation of NMDA receptors was stated most emphatically in ref. 21 (but see also refs. 20, 27); however, this work did not exclude the possibility that at zero glycine NMDA channels might open and rapidly desensitize. Two aspects of our current study

make this possibility unlikely. With our recording and perfusion system we should see summed inward currents as small as 1% of the peak current at 100 msec. If the same number of channels opened at all concentrations of glycine and the channels rapidly desensitized at very low glycine, the open time would have to be <1 msec for us to see no response. Such brief openings would require desensitization considerably more rapid than that observed for quisqualate receptors in chicken neurons (6 msec time constant; ref. 28) and also much more rapid than observed for NMDA receptors in cultured neurons (e.g., ref. 22). Furthermore, in oocytes the time constant of desensitization did not change over the range 30–300 nM glycine, indicating that desensitization does not get gradually faster as glycine level is lowered. In neurons, glycine appears to accelerate recovery from desensitization with little effect on rate of onset (22). The large diameter of the oocyte precluded sufficiently rapid solution changes to measure the time course of recovery from desensitization.

One way to display glycine's effect on desensitization is to plot the steady-state to peak ratio as a function of glycine concentration (Fig. 4C). If steady-state and peak responses are each described by Hill relations with coefficients of unity, the ratio r of steady state to peak will be given by $r = R([Gly] + K_{dp}) / ([Gly] + K_{dss})$, where K_{dss} is the apparent affinity for glycine at the steady state, K_{dp} is the apparent affinity for glycine at the peak, and R is the ratio of response amplitudes at saturating glycine. The limit of this equation as glycine approaches zero is $R(K_{dp}/K_{dss})$, which is greater than zero. Experimental points were reasonably well fitted by this equation using the K_d values calculated from dose-response curves. Although the Hill equation extrapolates to zero at zero agonist concentration, this equation has been fitted to glycine modulation of desensitization of NMDA receptors measured as steady-state to peak ratios (22).

At 100 μ M NMDA, desensitization was almost abolished at saturating concentrations of glycine. Higher NMDA concentrations, and consequently higher receptor occupancy, increased and slightly speeded desensitization at low levels of glycine (30–300 nM). Even at high glycine a substantial amount of decay remained, but the process became complicated by the presence of a more slowly developing component. Since this component was potentiated by increasing the extracellular concentration of Ca^{2+} , it may represent a secondary process triggered by Ca^{2+} influx. However, the glycine-sensitive desensitization (the faster process) also appeared to be somewhat increased at higher extracellular Ca^{2+} . Ca^{2+} -induced increases of desensitization of NMDA receptors have also been observed in chicken and mouse spinal cord neurons (29–31). The mechanism by which Ca^{2+} influences desensitization of NMDA receptors is not known and deserves further study. Ca^{2+} is required for desensitization of acetylcholine responses of neuromuscular junctions (e.g., refs. 32, 33), and some mechanisms may be common to NMDA and cholinergic receptors.

The time course of glycine-sensitive desensitization of NMDA receptors is slower in oocytes than in cultured mouse hippocampal neurons. In oocytes, the time constant for onset of desensitization is about 460 msec at 100 μ M NMDA and 350 msec at 500 μ M, whereas Mayer *et al.* (22) reported time constants of 220–277 msec at 100 μ M NMDA. This difference may reflect the differences in membrane environment or in the NMDA receptor expressed in the two systems. In addition, desensitization time courses may vary between species (34). Recently it was reported that NMDA receptors in outside-out patches exhibit desensitization that is glycine and Ca^{2+} insensitive (31). This interesting result suggests that cytoplasmic factors regulate desensitization; these factors should then be common in neurons and in oocytes.

We conclude that adult rat brain NMDA-activated channels expressed in oocytes are not opened by NMDA unless glycine is present. NMDA responses showed two distinct forms of decay with different time constants. Though both seem to be affected by Ca^{2+} , only the faster component is reduced by glycine and appears to be true desensitization. In the intact nervous system, the relative degree of each will ultimately be determined by the concentration of glutamate, glycine and Ca^{2+} in the extracellular fluid.

We thank Drs. S. G. Fan, L. Kushner, and C. Samathanam for preparing mRNA and injecting the oocytes, L. Cipriani for technical assistance, Dr. R. Martín del Río for HPLC analysis of water and saline solutions, and Dr. J. A. Kessler for helpful comments on the manuscript. This research was supported in part by National Institutes of Health Grants NS 20752 and DA 04439 (to R.S.Z.) and NS 07512 and HD 04248 (to M.V.L.B.). R.S.Z. is the recipient of Research Career Development Award DA 00069 from the National Institute on Drug Abuse. J.L. held an International Research Fellowship (1F05 TW04040) from the Fogarty International Center and a Beca de Ampliación de Estudios en el Extranjero from the Fondo de Investigaciones Sanitarias (F.I.S.S.S. 951/87). M.V.L.B. is the Sylvia and Robert S. Olnick Professor of Neuroscience.

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