Modulation of Glycine Affinity for NMDA Receptors by Extracellular Ca^{2+} in Trigeminal Neurons

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Glycine and the divalent cation Ca^{2+} play key roles in regulating the activity of excitatory amino acid NMDA receptor channels. There is accumulating evidence that the concentration of glycine at the synaptic cleft is below a saturated level. We examined the effect of external Ca^{2+} on NMDA responses in various concentrations of glycine in isolated trigeminal neurons. We found that external Ca^{2+} potentiated NMDA responses and this potentiation occurred only when glycine sites were unsaturated. Since single-channel conductance decreases in the high external Ca^{2+} solution, the observation cannot be explained by an increase in Ca^{2+} influx through the channels. Studying the dose–response curves for glycine in different Ca^{2+} solutions, we found that the apparent dissociation constant (EC_{50}) for glycine decreases with increasing external Ca^{2+} concentrations. Kinetics studies of glycine binding to NMDA receptors indicated that external Ca^{2+} causes a decrease in the off rate of the glycine binding, while having no effect on the on rate. Our analyses suggest that the apparent glycine affinity increases by about 3.7 times in Ca -containing solution. Thus, external Ca^{2+} contributes to the unusually high glycine affinity for NMDA receptors and may have a role in regulating the NMDA receptor channel activities during intensive or sustained neuronal stimulation.

[Key words: NMDA receptors, glutamate receptors, ion channels, glycine binding, calcium, trigeminal neurons, dorsal horn neurons, nociception]

Glutamate and aspartate are the major excitatory transmitters participating in the synaptic transmission in the CNS, including medullary and spinal dorsal horns (Jahr and Jessell, 1985; Mayer and Westbrook, 1987b; Gu and Huang, 1989; Murase et al., 1989; Yoshimura and Jessell, 1990; Chen and Huang, 1991). The NMDA receptor channel is one of the postsynaptic targets for these excitatory amino acids (Mayer and Westbrook, 1987b). The transition of an NMDA channel to the open state requires that glycine binds to a high-affinity site on the NMDA receptor (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988;

Vyklicky et al., 1990). Once opened, NMDA channels are highly permeable to Ca^{2+} (MacDermott et al., 1986; Jahr and Stevens, 1987; Mayer and Westbrook, 1987a; Ascher et al., 1988). The entry of Ca^{2+} initiates many cellular processes, including long-term potentiation (LTP) in the hippocampus (Lynch et al., 1983; Malenka et al., 1988; Regehr and Tank, 1990; Madison et al., 1991; Ben-Ari et al., 1992; Malenka et al., 1992), prolonged discharge (windup) in the spinal cord (Dickenson, 1990; Woolf and Thompson, 1991), and sustained enhancement of NMDA responses in trigeminal neurons (Chen and Huang, 1991, 1992). Externally applied glycine was found to potently enhance NMDA-evoked neuronal activity in many *in vivo* preparations (Salt, 1989; Thomson, 1990; Budai et al., 1992; Thiels et al., 1992). This suggests, contrary to the earlier view (Fletcher and Lodge, 1988), that the endogenous glycine concentration in the synaptic cleft is below a saturated level (for review, see Ascher, 1990). Although divalent cations may affect ^3H -glycine binding (Marvizon and Skolnick, 1988), the mechanism by which extracellular Ca^{2+} regulates NMDA receptor activity in different external concentrations of glycine has not been explored. We therefore studied the effects of external Ca^{2+} on NMDA responses in various concentrations of glycine in isolated trigeminal neurons. Our results show that the extracellular Ca^{2+} enhances the apparent affinity of glycine for the NMDA receptor, thus potentiating NMDA responses only in subsaturated concentrations of glycine.

The preliminary results have appeared in an abstract (Gu and Huang, 1991b).

Materials and Methods

Trigeminal neurons were isolated from 9–16-d-old Long-Evans rats according to the method previously described (Huang, 1989; Gu and Huang, 1991a). In brief, the lower medulla and upper cervical regions of the spinal cord were rapidly removed from the animal and put in an ice-cold, oxygenated dissecting solution. The solution consisted of (mM) NaCl (120), KCl (10), CaCl_2 (1), MgCl_2 (6), glucose (10), and PIPES (10) (pH 7.15). The osmolarity was between 305 and 315 mosm. The tissue was cut into 250–300 μm horizontal slices with a vibratome slicer and incubated in the dissecting solution at 34.5°C for 15–30 min. The slices were then transferred to an enzyme solution consisting of dissecting solution and 0.75 mg/ml protease III (Sigma). After a 10 min incubation period at 34.5°C, tissue slices were washed with enzyme-free dissecting solution and stored at room temperature. Immediately before an experiment, the spinal trigeminal nuclei in the caudal medulla were isolated from a tissue slice with a scalpel. Neurons were isolated by triturating the tissue with a series of fire-polished Pasteur pipettes.

Whole-cell and single-channel currents were recorded using the patch-clamp technique. Whole-cell currents were filtered at 3 kHz and sampled every 200 μsec . Single-channel activities were recorded from cell-attached and outside-out patches; these signals were filtered at 1 kHz and sampled every 100 μsec . The external solution contained (mM) NaCl

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Figure 1. External Ca^{2+} potentiates I_{NMDA} in subsaturated concentrations of glycine. Whole-cell I_{NMDA} evoked by $100 \mu\text{M}$ NMDA was recorded at -60 mV in $0.2 \mu\text{M}$ glycine, $3 \mu\text{M}$ glycine, and $3 \mu\text{M}$ glycine plus $10 \mu\text{M}$ 7-Cl-Kyn solutions. Ca^{2+} enhances NMDA responses in $0.2 \mu\text{M}$ glycine and in $3 \mu\text{M}$ glycine plus 7-Cl-Kyn, but affects the responses very little in $3 \mu\text{M}$ glycine. These results are from a single neuron.

(140), KCl (4), glucose (10), HEPES (10) (pH 7.4), and the indicated concentrations of CaCl_2 and glycine. The internal solution contained (mM) Cs methanesulfonate (125), CsCl (15), glucose (10), BAPTA (11), CaCl_2 (1), and HEPES (10) (pH 7.2). The free intracellular Ca^{2+} concentration was calculated to be $1.6 \times 10^{-8} \text{ M}$. Magnesium-ATP (5 mM), leupeptin ($400 \mu\text{M}$), and GTP ($100 \mu\text{M}$) were added to the internal solution to prevent rundown. All of the chemicals were ultrapure grade. NMDA forms a complex with Ca^{2+} ; therefore, the effective concentrations of NMDA were adjusted according to the method previously described (Gu and Huang, 1991a). The dissociation constant for the Ca^{2+} -NMDA complex, measured with a Ca^{2+} -sensitive electrode, was $120 \pm 13 \text{ mM}$ ($n = 4$). To prevent the activation of glycine-sensitive Cl^- channels, $10 \mu\text{M}$ strychnine was included in the external buffer solutions. At this concentration, strychnine did not change either the amplitude of NMDA-activated currents (I_{NMDA}) or the modulatory property of glycine on NMDA responses (Bertolino and Vicini, 1988). In the external solutions containing no added glycine, I_{NMDA} was observed, thus indicating the presence of background glycine. The concentration of the background glycine, measured by high-performance liquid chromatography (HPLC), was $20\text{--}30 \text{ nM}$ and did not increase when Ca^{2+} was added. The amino acids were delivered to the cell being recorded using the fast perfusion technique (Gu and Huang, 1991a). The speed of solution change was determined by changing the Na^+ concentration in the external solution from 140 to 10 mM after kainate application. Since the kainate currents did not desensitize, the rate of decrease in kainate responses reflected the rate of solution change. The time constant for the solution exchange was between 14 and 25 msec. Experimental results are expressed as mean $\pm$ SE. The least χ^2 method was used to fit the theoretical equations to the experimental data.

Results

External Ca^{2+} potentiates I_{NMDA} in subsaturated concentrations of glycine

Figure 1 shows the effect of external Ca^{2+} on I_{NMDA} measured at -60 mV in two different concentrations of glycine. As the external Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) was raised from 1 to 10 mM in $0.2 \mu\text{M}$ glycine, the peak current increased by 40% and

Figure 2. *a*, Slow Ca^{2+} -dependent inactivation of NMDA responses is present in both low and high glycine solutions. NMDA responses were recorded as 1.5 sec $100 \mu\text{M}$ NMDA pulses were applied every 60 sec to the cell. The peak current amplitude was plotted as a function of time in 2 and 20 mM Ca^{2+} . The time periods for 20 mM Ca^{2+} applications are indicated by the bars above the data points; the glycine content of the solution is indicated by the bars below. In $0.3 \mu\text{M}$ glycine, increasing $[\text{Ca}^{2+}]_o$ initially resulted in a larger NMDA response. The response decreased progressively with successive NMDA applications. In $3 \mu\text{M}$ glycine, the NMDA responses decreased as the $[\text{Ca}^{2+}]_o$ was switched from 2 to 20 mM . The responses gradually increased when the $[\text{Ca}^{2+}]_o$ was switched back to 2 mM . The smaller responses in $3 \mu\text{M}$ glycine in comparison with that at the start of the experiment result from the rundown of the I_{NMDA} . *b*, External Ca^{2+} depresses NMDA responses in the high glycine solution even when the current amplitude is small. *Left*, The I_{NMDA} were evoked in a solution containing $100 \mu\text{M}$ NMDA, $0.3 \mu\text{M}$ glycine, and $2 \mu\text{M}$ 7-Cl-Kyn. *Right*, In the same cell, the current was evoked in a solution containing $10 \mu\text{M}$ NMDA and $3 \mu\text{M}$ glycine. Increasing $[\text{Ca}^{2+}]_o$ potentiated NMDA responses in low glycine, but decreased the responses in the saturated glycine. The data in *a* and *b* were obtained from different cells.

the steady state current (measured at the end of 1.5 sec of NMDA application) increased by 100%. When we raised the external glycine concentration to a saturated level ($3 \mu\text{M}$), NMDA responses, compared to $0.2 \mu\text{M}$ glycine, were potentiated. However, an increase in $[\text{Ca}^{2+}]_o$ at $3 \mu\text{M}$ glycine resulted in little change in peak NMDA response and a slight decrease in the steady state NMDA response. If we then lowered the effective concentration of glycine by simultaneously applying $3 \mu\text{M}$ glycine and a competitive glycine antagonist, 7-chlorokynurenic acid (7-Cl-Kyn) (Kemp et al., 1988; Henderson et al., 1990; Vyklicky et al., 1990), to the cell, I_{NMDA} was again potentiated by increasing $[\text{Ca}^{2+}]_o$. These results were consistently observed in a total of 72 cells. Thus, external Ca^{2+} enhanced NMDA responses only when a less than saturated concentration of glycine was present in the external solution.

Glycine has been shown to reduce the inactivation of NMDA responses (Mayer et al., 1989; Benveniste et al., 1990; Vyklicky et al., 1990). In $0.2 \mu\text{M}$ glycine, the I_{NMDA} showed a noticeable glycine-dependent inactivation (Fig. 1). In 1 mM Ca^{2+} , the ratio of the steady state to peak NMDA responses ($I_{\text{ss}}/I_{\text{peak}}$) was 0.39 ± 0.03 ($n = 6$). In 10 mM Ca^{2+} , the I_{NMDA} was larger; the inactivation was less pronounced [$I_{\text{ss}}/I_{\text{peak}} = 0.59 \pm 0.03$ ($n =$

Figure 3. The potentiation of the NMDA responses is Ca^{2+} specific. When external Ba^{2+} was switched from 2 to 20 mM, NMDA responses were reduced in both 0.2 and 3.0 μM glycine solutions. The concentration of NMDA was 100 μM . The data were obtained from the same cell.

6)]. In saturated glycine concentration (3 μM), the NMDA responses inactivated only slightly (Fig. 1) as the result of Ca^{2+} -dependent (Legendre et al., 1993) or glycine-independent (Sather et al., 1990) inactivation. $I_{ss}/I_{\text{peak}} = 0.90 \pm 0.03$ ($n = 12$) in 1 mM Ca^{2+} . I_{ss}/I_{peak} decreased in 10 mM Ca^{2+} [0.84 ± 0.03 ($n = 12$)]. In the presence of 7-Cl-Kyn and 3 μM glycine, the glycine-dependent inactivation of I_{NMDA} reappeared. The ratio I_{ss}/I_{peak} was 0.26 ± 0.02 ($n = 12$) in 1 mM Ca^{2+} . The inactivation again decreased with increasing $[\text{Ca}^{2+}]_o$ [$I_{ss}/I_{\text{peak}} = 0.36 \pm 0.05$ ($n = 6$)]. Thus, the extracellular Ca^{2+} appears to increase the effectiveness of glycine.

Potentiation by Ca^{2+} does not depend on the slow Ca^{2+} -dependent inactivation of NMDA responses

To test if the perfusion rate of the external solution was too slow to allow an accurate measurement of the peak current, the time constant for the solution exchange, measured according to the method described, was reduced from 25.0 ± 3.5 to 14.0 ± 3.0 msec ($n = 4$). Similar results as those in Figure 1 were obtained (data not shown).

We then determined whether the slow Ca^{2+} -dependent inactivation of I_{NMDA} (Vycklicky et al., 1990; Legendre et al., 1993) affected our results (Fig. 2a). The currents were recorded at -65 mV as 100 μM NMDA was repeatedly applied to the cell. In 0.3 μM glycine, the NMDA response was potentiated as the $[\text{Ca}^{2+}]_o$ was first switched from 2 to 20 mM. The current decreased with successive applications of NMDA and leveled off to a value lower than that recorded in 2 mM Ca^{2+} . The time taken to decay to 63% of the control level was 1.15 ± 0.12 min ($n = 6$). In 3 μM glycine, the NMDA response did not increase with increasing $[\text{Ca}^{2+}]_o$, but declined with repeated NMDA applications. The decay time was similar to that in 0.3 μM glycine [1.20 ± 0.20 min ($n = 6$)]. Therefore, the slow inactivation of NMDA receptor channels was observed in both glycine concentrations.

Slow Ca^{2+} -dependent inactivation results primarily from an increase in intracellular Ca^{2+} concentration (Legendre et al., 1993). Since I_{NMDA} is larger in 3 μM glycine than that in 0.3 μM glycine, the Ca^{2+} entry and thus the rate of its inactivation in 3 μM glycine solution is higher. To ensure that the faster rate of slow inactivation in the high glycine did not obscure our results, we lowered the current amplitude in 3 μM glycine by using a smaller concentration of NMDA (Fig. 2b). Under this experi-

Figure 4. The Ca^{2+} modulation of NMDA responses does not result from an increase in Ca^{2+} influx through NMDA receptor channels. *a*, The I - V relationships of peak I_{NMDA} in 1 and 10 mM Ca^{2+} solutions showed that external Ca^{2+} potentiated NMDA responses at both positive and negative potentials in 0.3 μM glycine. *b*, In 3 μM glycine, the responses changed little with changing $[\text{Ca}^{2+}]_o$. The data are the average values from eight cells. For each cell, the current at different potentials was normalized with respect to the $-I_{\text{NMDA}}$ measured at -60 mV in 1 mM Ca^{2+} and low glycine solution. The standard errors, when larger than the size of the data points, are shown by error bars. In both glycine concentrations, the reversal potentials were 5.0 mV in 1 mM Ca^{2+} and 13.5 mV in 10 mM Ca^{2+} solutions.

mental condition, the current evoked by 10 μM NMDA in 3 μM glycine was less than that evoked by 100 μM NMDA in 0.3 μM glycine. An increase in $[\text{Ca}^{2+}]_o$ still enhanced the response in the low glycine and depressed the NMDA response in the high glycine solution. The same results were observed in five cells. Thus, the modulation of NMDA receptor channels by external Ca^{2+} cannot be attributed to a change in the slow Ca^{2+} -dependent inactivation.

Potentiation of the NMDA responses is Ca^{2+} specific

We next determined whether the potentiation could occur for other divalent cations. I_{NMDA} was examined in Ca^{2+} -free solutions containing 2 or 20 mM BaCl_2 . In both low and high glycine, increasing the external Ba^{2+} reduced the NMDA responses (Fig. 3). The peak currents decreased by $66.2 \pm 17.0\%$ ($n = 3$) in 0.2 μM glycine and by $63.6 \pm 12.0\%$ ($n = 3$) in 3 μM glycine. Thus, the modulation of NMDA responses by Ca^{2+} results from a specific interaction between Ca^{2+} and the NMDA-glycine receptor complex, rather than from a nonspecific ionic effect. The intracellular Ca^{2+} is unlikely to be involved in the modulation of NMDA response because the NMDA responses were still enhanced when $[\text{Ca}^{2+}]_o$ was changed from 0.5 to 2 mM in the presence of a high concentration of the Ca^{2+} chelator BAPTA (11 mM) in the cells.

An increase in Ca^{2+} influx through the NMDA receptor channel cannot account for the modulation

The current-voltage (I - V) relationships of NMDA responses are shown in Figure 4. The reversal potential (E_{rev}) of the NMDA responses in different Ca^{2+} was not affected by external glycine; the E_{rev} of the average responses ($n = 8$) shifted from 5 mV to 13.5 mV as $[\text{Ca}^{2+}]_o$ increased from 1 to 10 mM in both 0.3 μM and 3 μM glycine. In 3 μM glycine, the amplitude of I_{NMDA} did not significantly change with the increasing $[\text{Ca}^{2+}]_o$ (Fig. 4b). In 0.3 μM glycine, the responses increased with Ca^{2+} at both negative and positive holding potentials (Fig. 4a). Taking into account the reversal potential change in different $[\text{Ca}^{2+}]_o$, the av-

Figure 5. Single NMDA receptor channel conductance decreases with an increase in $[Ca^{2+}]_o$ at negative potentials. *a*, Single-channel activities measured from cell-attached patches with 10 μM NMDA at an apparent V_{app} of -10 mV. V_{app} was defined as $(V_{patch} - V_{rest})$. Currents flowing into the cells appeared as downward deflections. In 2 μM glycine, the channel currents (*i*) decreased from 2.8 pA to 2.4 pA as $[Ca^{2+}]_o$ increased from 1 to 5 mM. The histogram of the patch was shown to the right of each trace. In 0.3 μM glycine, the main current level was 2.9 pA in 1 mM Ca^{2+} , and 2.3 pA in 5 mM Ca^{2+} (data not shown). *b*, Single-channel currents plotted as a function of V_{app} in 2 μM glycine. The measured reversal potentials were about 60 mV in 1 mM Ca^{2+} and 65 mV in 5 mM Ca^{2+} . With the average resting potential of these cells measured around -55 mV, the estimated E_{rev} were 5 and 10 mV. The data points are the average values from four patches in each Ca^{2+} concentration. Standard errors are indicated by error bars. The single-channel conductance (γ) was calculated according to the formula $[i/(V_{app} - 55 - E_{rev})]$. At potentials $< E_{rev}$, γ was 42 pS in 1 mM Ca^{2+} and 30 pS in 5 mM Ca^{2+} . At potentials $> E_{rev}$, γ was 45.0–47.5 pS in 1 mM Ca^{2+} and 26.7–38.6 pS in 5 mM Ca^{2+} . *c*, Single-channel activities measured at -70 mV from outside-out patches. In 2 μM glycine, the γ , averaged from three patches, was 30 pS ($E_{rev} = 10$ mV), and 22.8 pS in 10 mM Ca^{2+} ($E_{rev} = 13.5$ mV).

erage peak conductance increased by 30.0% at -60 mV and by 42.4% at $+60$ mV ($n = 6$). Because the Ca^{2+} equilibrium potential should be more positive than $+60$ mV, the portion of the current carried by Ca^{2+} at the positive potentials shown was

all inward. If the potentiation of NMDA responses in the low glycine resulted from additional Ca^{2+} influx through the channels, then the outward I_{NMDA} would be smaller in the high Ca^{2+} solution. This was not observed. Thus, the potentiation of

NMDA responses by Ca^{2+} was not the result of changing reversal potentials, or of additional Ca^{2+} influx.

The analyses of single NMDA receptor channels in cell-attached patches indicated that the current level for the main conductance state (which occurred more than 90% of the time) (Fig. 5a) and for the subconductance state (data not shown) decreased with increasing $[\text{Ca}^{2+}]_o$. The I - V curves of the single NMDA channels in 0.3 and 2 μM glycine solutions are given in Figure 5b. The single-channel conductance (γ) decreased from 42 pS in 1 mM Ca^{2+} to 30 pS in 5 mM Ca^{2+} ($n = 4$) at potentials below E_{rev} (Fig. 5b). We also compared the γ in different external Ca^{2+} in the same outside-out patches (Fig. 5c). In 5 mM Ca^{2+} , at -70 mV, γ was 30.6 pS, similar to those obtained from cell-attached patches. In 10 mM Ca^{2+} , γ decreased further to 22.8 pS. Thus, γ in 10 mM Ca^{2+} is 56% of the γ in 1 mM Ca^{2+} . Since the probability of a channel being open (p_o) decreased with time as the result of the slow Ca^{2+} -dependent inactivation of the channels, a reliable measurement of p_o was difficult to obtain.

Ca^{2+} increases the affinity of glycine for NMDA receptors

To determine the mechanism underlying Ca^{2+} -dependent potentiation, we studied the affinity of NMDA for its receptor in different Ca^{2+} concentrations. In 0.2–0.3 μM glycine, the maximum NMDA responses increased with external Ca^{2+} . The EC_{50} for the average peak NMDA responses ($n = 4$) was 41.0 μM in 1 mM Ca^{2+} and 42.0 μM in 10 mM Ca^{2+} (Fig. 6). There was no significant change in EC_{50} ($P > 0.05$).

We then examined the EC_{50} for glycine at a fixed concentration (100 μM) of NMDA (Fig. 7a,b). To obtain complete dose-response curves, 7-Cl-Kyn (10 μM) was added to the external solutions. This eliminated the NMDA responses in solutions that contained no added glycine and displaced the glycine dose-response curves to the right. For the average peak NMDA responses, the EC_{50} for glycine was 3.6 μM in 1 mM Ca^{2+} and 1.9 μM in 10 mM Ca^{2+} ($P < 0.05$; $n = 6$). For the average steady state NMDA responses, the EC_{50} was 9.1 μM in 1 mM Ca^{2+} and 4.3 μM in 10 mM Ca^{2+} ($P < 0.01$). Thus, Ca^{2+} increased the apparent affinity of glycine for NMDA receptors.

In addition, we analyzed the dose dependence of the 7-Cl-Kyn block in different Ca^{2+} solutions (Fig. 7c,d). If Ca^{2+} increases glycine affinity, then 7-Cl-Kyn, which competes for the glycine site, is expected to be less effective in blocking NMDA responses in the higher $[\text{Ca}^{2+}]_o$ solution. This was indeed the case. In 3 μM glycine, as the external Ca^{2+} was raised from 1 to 10 mM, the IC_{50} for 7-Cl-Kyn changed from 9 μM to 17 μM ($P < 0.01$) for peak NMDA responses and the IC_{50} changed from 2 μM to 4.8 μM ($P < 0.01$) for steady state NMDA responses ($n = 8$).

We also determined the dose-response curve for glycine in 1 mM Ca in the absence of 7-Cl-Kyn by measuring the NMDA responses in different added glycine concentrations. The actual glycine content in each solution was then determined with HPLC. The EC_{50} for glycine, determined this way, was 160 ± 20 nM ($n = 4$) (Hill coefficient = 1.1) (data not shown). The dose-response curve for glycine reached saturation around 1 μM .

To support further the conclusion that Ca^{2+} modulated the affinity of glycine, the dissociation constants of glycine in different $[\text{Ca}^{2+}]_o$ were directly estimated from the experimentally determined association and dissociation rate constants of glycine (Fig. 8). The association rates were obtained by studying the time course of NMDA responses when the external solution was rapidly switched from a solution containing 20 μM NMDA

Figure 6. Ca^{2+} does not increase the apparent affinity for NMDA. The dose-response curves for peak I_{NMDA} were measured at -60 mV in 1 and 10 mM Ca^{2+} . The data points are the average values from four cells. The standard errors are expressed as error bars. The solid lines were drawn according to the equation $I = I_o [A]^h / ([A]^h + [\text{EC}_{50}]^h)$, where I_o is the maximal current measured in 800 μM NMDA and 1 mM Ca^{2+} ; $[A]$ is the concentration of agonist (NMDA in this case); and h the Hill coefficient. The EC_{50} for peak NMDA responses was 41.0 μM in 1 mM Ca^{2+} and 42.0 μM in 10 mM Ca^{2+} ($P > 0.05$, Student's t test); the EC_{50} for steady state responses was 18 μM in both 1 and 10 mM Ca^{2+} . The h was 1.3 for peak and steady state currents in both Ca^{2+} concentrations. As a result of glycine contamination in the solutions, the glycine level in the solutions was between 0.2–0.3 μM .

with no added glycine to one with added glycine. To measure the rates accurately, the concentrations of glycine and NMDA were kept at a low level and the time constant for the solution exchange was kept at around 15.0 msec. The increases in I_{NMDA} following glycine concentration jumps could be well fitted by single exponential functions; the time constant of glycine binding (τ_{on}) at 0.2 μM added glycine was 270 ± 14 msec in 1 mM Ca and 320 ± 12 msec in 10 mM Ca ($n = 6$) (Fig. 8a). We assumed a simple model, that the NMDA receptor has one glycine site (Johnson and Ascher, 1992) and the binding of glycine is required to open an NMDA receptor channel (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Vyklícky et al., 1990). Namely,

in which R is a receptor with NMDA bound, Gly-R* is a receptor with both NMDA and glycine bound, k_+ is the association rate constant of glycine, and k_- the dissociation rate constant. Thus, $1/\tau_{\text{on}} = k_+ [\text{Gly}] + k_-$ and $1/\tau_{\text{off}} = k_-$. We plotted the $1/\tau_{\text{on}}$ versus corrected glycine concentration ([corrected gly]), taking into account the background glycine concentration of 20 nM. Consistent with the model, the $1/\tau_{\text{on}}$ was found to change linearly with the concentration of glycine (Fig. 8b). The slope of the $1/\tau_{\text{on}}$ versus [corrected gly] curve corresponding to k_+ was $1.1 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. The k_+ remained unchanged in different $[\text{Ca}^{2+}]_o$, whereas the y-intercepts of the curves shifted downward as the $[\text{Ca}^{2+}]_o$ increased. The y-intercepts, corresponding to k_- , were 1.33 sec^{-1} , 0.94 sec^{-1} , and 0.70 sec^{-1} for 1 mM Ca, 5 mM Ca, and 10 mM Ca, respectively. We also determined the dissociation rate constant of glycine (k_- or $1/\tau_{\text{off}}$) from the time course of the NMDA responses when the added glycine was rapidly

Figure 7. Ca²⁺ increases the apparent affinity for glycine. *a* and *b*, The peak and steady state dose-response curves for glycine in different concentrations of Ca²⁺ at a constant level of NMDA (100 μM). 7-Cl-Kyn at 10 μM was added to the solution. The solid lines were drawn using the same equation as in Figure 6. For peak NMDA responses, the EC₅₀ for glycine was 3.6 μM in 1 mM Ca²⁺ and 1.9 μM in 10 mM Ca²⁺ ($P < 0.05$); for steady state responses, the EC₅₀ for glycine was 9.1 μM in 1 mM Ca²⁺ and 4.3 μM in 10 mM Ca²⁺ ($P < 0.01$). The h was 1.1. The I_0 was the maximal NMDA response in 1 mM Ca²⁺. The data points are the average values from six cells. *c* and *d*, The peak and steady state dose-response curves for 7-Cl-Kyn block. The curves were drawn using $I = I_0 [IC_{50}]^h / ([7-Cl] + [IC_{50}]^h)$. For peak NMDA responses, the IC₅₀ for 7-Cl-Kyn was 9.0 μM in 1 mM Ca²⁺ and 17.0 μM in 10 mM Ca²⁺ ($P < 0.01$); for steady state responses, the IC₅₀ for 7-Cl-Kyn was 2.0 μM in 1 mM Ca²⁺ and 4.8 μM in 10 mM Ca²⁺ ($P < 0.01$). The h was 1.0. The I_0 was the maximal NMDA response in 3 μM glycine. Each data point is the average value from seven cells.

washed from the external medium. The τ_{off} did not change significantly with corrected glycine concentration (Fig. 8*b*). The $1/\tau_{off}$ ($= k_-$) obtained from the fittings of data were $1.54 \pm 0.04 \text{ sec}^{-1}$ ($n = 14$) at 1 mM Ca, $1.10 \pm 0.04 \text{ sec}^{-1}$ ($n = 12$) at 5 mM Ca, and $0.90 \pm 0.03 \text{ sec}^{-1}$ ($n = 14$) at 10 mM Ca (Fig. 8*b*). These values were very similar to those obtained from the y-intercepts of the $1/\tau_{on}$ versus [corrected gly] curves (Fig. 8*b*). The dissociation constant of glycine (K_d) calculated using the relation k_-/k_+ was 140.0 nM at 1 mM Ca, 100.9 nM at 5 mM Ca, and 81.8 nM at 10 mM Ca.

The k_+ value ($1.1 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$) we obtained is very similar to those measured in cultured mouse hippocampal (Benveniste et al., 1990) and brain neurons (Johnson and Ascher, 1992), despite the fact that different NMDA concentrations were used among the measurements. Our k_- value in 1 mM Ca (1.5 sec^{-1}) is larger than that obtained in mouse neurons ($0.7\text{--}1.0 \text{ sec}^{-1}$) (Johnson and Ascher, 1992); the k_- value in 0.2 mM Ca (1.84 sec^{-1}), according to our calculation, is smaller than that measured in hippocampal neurons (3.07 sec^{-1}) (Benveniste et al., 1990).

Discussion

Mechanism of Ca²⁺ modulation of NMDA channels

Our results show that external Ca²⁺ enhances NMDA responses when the external concentration of glycine is at a subsaturated level. Since the change of I_{NMDA} reversal potentials by Ca²⁺ is insensitive to the external glycine (Fig. 4) and the conductance of single NMDA receptor channel decreases in the high Ca²⁺ solution (Fig. 5), the enhancement is not caused by an increase in Ca²⁺ influx through the NMDA receptor channels. External Ca²⁺ has been found to reduce NMDA responses (in the saturated concentration of glycine) and non-NMDA responses in many preparations (Mayer and Westbrook, 1987a; Ascher and Nowak, 1988; Gu and Huang, 1991a; Gibb and Colquhoun, 1992). This was attributed to the strong interactions between Ca²⁺ and its binding site(s) inside the pores of both NMDA and non-NMDA channels. As Ca²⁺ passes through a channel, it temporally occludes the channel, thus retarding the permeation of monovalent cations (Mayer and Westbrook, 1987a; Ascher and Nowak, 1988; Gu and Huang, 1991a).

a

b

Figure 8. The τ_{on} and τ_{off} of glycine. *a*, NMDA responses recorded during a step application of glycine from 0 to 0.2 μM (for τ_{on}) and rapidly washing out of glycine (for τ_{off}). The corrected glycine concentration would be 0.22 μM , taking into account the 20 nM glycine contamination in our solutions. Solid lines drawn through the data points are the theoretical fit of single exponential functions. The zero current lines are indicated by broken lines. The periods of solution application are indicated by the solid lines below. The Ca^{2+} concentration in the external solution is indicated by the number to the left of each trace. *b*, The concentration dependence of $1/\tau$. The $1/\tau_{on}$ depended linearly on the glycine concentration. The slope that corresponds to k_+ was $1.1 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. As $[\text{Ca}^{2+}]_o$ increased, the curves shifted downward but the slope of the curves remained constant. The $1/\tau_{off}$ was independent of glycine concentration. Each data point is an average value from 12–14 cells; the SE values, when larger than the data points, are expressed as error bars. The solid lines are the least χ^2 fit of the data according to the expression indicated in the text. The holding potential was at -70 mV .

We propose that the Ca^{2+} increases the NMDA responses by directly increasing the affinity of glycine for NMDA receptor channels because (1) Ca^{2+} increases the NMDA responses only in subsaturated glycine solution and the lack of effect of external Ca^{2+} on NMDA responses in saturated glycine can be reversed by adding the competitive antagonist 7-Cl-Kyn to the external solution (Fig. 1); (2) Ca^{2+} shifts the dose-response curves for glycine and the dose-response curves for 7-Cl-Kyn (Fig. 7); and (3) the off rates of glycine decrease with increasing $[\text{Ca}^{2+}]_o$ (Fig. 8).

To give an estimate of the change in the dissociation constant for glycine by Ca^{2+} , we fitted our results using the reaction scheme below.

R is the closed state of the receptor with NMDA bound; R* is the open state. The receptor with glycine (Gly), Ca^{2+} (Ca), or 7-Cl-Kyn (7Cl) bound is indicated separately in the scheme. We

Figure 9. Dependence of EC_{50} for glycine on external Ca^{2+} concentration. *Left*, The EC_{50} for glycine in the presence of 10 μM 7-Cl-Kyn. Data are from the experiments shown in Figure 7. The solid line was drawn according to the equation $EC_{50} = K_{gly}\{1 + ([7Cl]/K_{7Cl}) + ([Ca]/K_{Ca}) + ([7Cl]/K_{7Cl})([Ca]/K_{Ca})/\{1 + ([Ca]/\beta K_{Ca})\}$, in which $[7Cl]$ and $[Ca]$ are concentrations of 7-Cl-Kyn and Ca^{2+} , respectively; K_{gly} and K_{7Cl} are the dissociation constants for glycine and 7-Cl-Kyn. $\beta = 0.27$, $K_{Ca} = 10.75$ mM, $K_{gly} = 180.0$ nM, and $K_{7Cl} = 428.0$ nM were used for the fit. *Right*, The EC_{50} for glycine in the absence of 10 μM 7-Cl-Kyn. Data were from the experiments shown in Figure 8. The solid line was drawn according to the same equation and parameters as in the left panel, except $[7Cl]$ was set to 0.

assumed that, in addition to the binding site for NMDA, each NMDA receptor has a binding site for glycine and a separate site for Ca^{2+} . The binding of Ca^{2+} to its site reduces the dissociation constant for glycine by a factor of β .

We further assumed that 7-Cl-Kyn competes with glycine for its binding site, and the dissociation constant for 7-Cl-Kyn (K_{7Cl}) was not affected by external Ca^{2+} . The assumption was based on the analyses of K_{7Cl} calculated from the EC_{50} for glycine in the absence (EC_{50}) and in the presence ($EC_{50,7Cl}$) of 7-Cl-Kyn using the equation $EC_{50,7Cl}/EC_{50} = 1 + [7Cl]/K_{7Cl}$. The K_{7Cl} in 1 mM Ca was 405 nM, similar to those obtained in other preparations (Kemp et al., 1988; Kleckner and Dingledine, 1989). The change in K_{7Cl} with increasing external Ca was <15% ($K_{7Cl} = 451$ nM in 10 mM Ca). Thus, the shift of IC_{50} for 7-Cl-Kyn in different $[Ca^{2+}]_o$ (Fig. 7) resulted from a change in glycine affinity by external Ca^{2+} rather than from the direct Ca^{2+} action on K_{7Cl} .

The binding of two NMDA and glycine molecules was found to be required to open an NMDA receptor channel (Patneau and Mayer, 1990; Benveniste and Mayer, 1991; Clements and Westbrook, 1991) in cultured hippocampal neurons, whereas a simple one-site model was sufficient for describing the glycine binding in cultured mouse neurons (Johnson and Ascher, 1992). Although our kinetic data (Fig. 8) are consistent with a single binding site for glycine, the data were not accurate enough to distinguish between a single or multiple binding sites for glycine. A one-site model was chosen to give a qualitative comparison of glycine binding in different $[Ca^{2+}]_o$.

The theoretical apparent dissociation constants for glycine (aK_{gly}) and for 7-Cl-Kyn (aK_{7Cl}), derived according to the method previously described (Segal, 1975; Huang and Ehrenstein, 1981), are

$$aK_{gly} = K_{gly}\{1 + ([7Cl]/K_{7Cl}) + ([Ca]/K_{Ca}) + ([7Cl]/K_{7Cl})([Ca]/K_{Ca})/\{1 + ([Ca]/\beta K_{Ca})\}\},$$

$$aK_{7Cl} = K_{7Cl}\{1 + ([gly]/K_{gly}) + ([Ca]/K_{Ca}) + ([gly]/K_{gly})([Ca]/\beta K_{Ca})/\{1 + ([Ca]/K_{Ca})\}\}.$$

Assuming $K_{7Cl} = 428$ nM, the dissociation constants for glycine (K_{gly}) and for Ca^{2+} (K_{Ca}) and β were determined by fitting the

calculated EC_{50} for glycine with the experimental EC_{50} for glycine as a function of $[Ca^{2+}]_o$ in the presence (Fig. 9, left) and in the absence (Fig. 9, right) of 7-Cl-Kyn, and by fitting the calculated IC_{50} for 7-Cl-Kyn with the IC_{50} values obtained in Figure 7. The best-fitting value for K_{gly} was 180.0 nM, for K_{Ca} was 10.75 mM, and for β was 0.27. Thus, the affinity for glycine would increase about 3.7 times in the Ca-containing solution and the apparent dissociation constant for glycine decreases by about 30% when $[Ca^{2+}]_o$ changed from 2.0 to 0.5 mM (Fig. 9).

The increase in glycine affinity alone does not account for all of our observations. Since the single-channel conductance is reduced by increasing $[Ca^{2+}]_o$ (Fig. 5), the maximal NMDA response in the glycine dose-response curve with 10 mM Ca^{2+} should be less than that with 1 mM Ca^{2+} . Instead, the maximal responses in different Ca^{2+} solutions are nearly the same (Fig. 7a,b). One possibility may be that Ca^{2+} , in addition to changing the affinity of glycine, also affects the gating properties of NMDA channels.

Comparison of the action of Ca^{2+} on neuronal acetylcholine channels

There are several intriguing similarities between the functional properties of neuronal acetylcholine (nACh) and NMDA receptor channels. As in NMDA receptor channels, nACh receptor channels have high Ca^{2+} permeability (Decker and Dani, 1990; Mulle et al., 1992a,b; Vernino et al., 1992). In observations similar to ours, external Ca^{2+} potentiates nACh-activated currents and, at the same time, decreases the single nACh receptor channel conductance (Decker and Dani, 1990; Mulle et al., 1992a,b; Vernino et al., 1992). The nACh responses are more sensitive to the change in $[Ca^{2+}]_o$. As the $[Ca^{2+}]_o$ increases from 1 to 8 or 10 mM, nACh-activated currents increase by up to twofold (Mulle et al., 1992b; Vernino et al., 1992) while I_{NMDA} increases by about 47% (Fig. 4).

Differences between the modulation of the two channels by divalent cations do exist. For example, Mg^{2+} readily permeates through nACh channels (Decker and Dani, 1990) but is largely blocked in NMDA channels (Mayer et al., 1984; Nowak et al., 1984; Mayer and Westbrook, 1987a; Jahr and Stevens, 1990). Barium (Ba^{2+}) potentiates nACh-activated currents (Mulle et al., 1992b) whereas it reduces the I_{NMDA} (Fig. 3). Since no endogenous ligand, such as glycine, has been found to coactivate nACh receptor channels, Ca^{2+} is thought to act directly on nACh receptors (Mulle et al., 1992b; Vernino et al., 1992).

Role of Ca modulation of glycine effects

The physiological roles of modulation of glycine binding by Ca^{2+} are difficult to assess at the moment because the amplitude and duration of the changes in the concentration of Ca^{2+} at the synaptic clefts during neuronal activities are not known. One cannot, however, ignore the fact that external Ca^{2+} contributes to the unusually high glycine affinity for NMDA receptors. Furthermore, the enhancing effect of Ca^{2+} would become increasingly important during intense neuronal activity.

The $[Ca^{2+}]_o$ in the vicinity of the recording electrodes has been measured with ion-selective electrodes and was found to decrease with an application of excitatory amino acids or neuronal activity (Wadman et al., 1985; Pumain et al., 1987; Melchers et al., 1988). The extent of reduction can be large—the $[Ca^{2+}]_o$ decreases up to 95% of the baseline level during prolonged application of NMDA (Wadman et al., 1985; Pumain et al., 1987) and the $[Ca^{2+}]_o$ was found to go as low as 0.2 mM

during kindling (Pumain et al., 1987). This was attributed to a large influx of Ca^{2+} through NMDA receptor or Ca^{2+} channels, thus depleting the $[\text{Ca}^{2+}]_o$ near the synaptic cleft (Wadman et al., 1985; Pumain et al., 1987).

During intensive nerve stimulation, epileptic seizure, or anoxia, large amounts of the excitatory amino acid glutamate would be released. The $[\text{Ca}^{2+}]_o$ at the synapse drops as the result of Ca^{2+} entering both postsynaptic and presynaptic cells. The glycine affinity for NMDA receptors would decrease under this condition. Since glycine sites on NMDA receptors at the synaptic terminals in *in vivo* preparations are not saturated (Salt, 1989; Thomson, 1990; Budai et al., 1992; Thiels et al., 1992), the reduction of glycine affinity would reduce the NMDA-activated currents, thus providing a negative feedback mechanism for regulating the excitability of neuronal cells. Given the key roles that NMDA receptors play in sustained responses, such as windup (Dickenson, 1990; Woolf and Thompson, 1991), LTP (Malenka et al., 1988; Madison et al., 1991), and neurotoxicity (Choi et al., 1988), this activity-dependent modulation of neuronal excitability by extracellular Ca^{2+} could be a mechanism by which plastic changes in the nervous system are regulated.

Different subtypes of NMDA receptors have been obtained from cloning experiments (Moriyoshi et al., 1991; Meguro et al., 1992; Monyer et al., 1992). Alteration of a single amino acid residue can profoundly change the properties of these NMDA receptor channels (Burnashev et al., 1992; Mori et al., 1992). It will be interesting to determine whether the modulation of glycine affinity by external Ca^{2+} depends upon the molecular properties of NMDA receptors.

In summary, our studies show that extracellular Ca^{2+} increases the affinity of glycine for NMDA receptors, thus potentiating NMDA responses only when glycine concentration is at sub-saturated levels. The similarities in the actions of extracellular Ca^{2+} on NMDA and nACh channels suggest that extracellular Ca^{2+} , like the intracellular Ca^{2+} and the second messengers, could have an important regulatory role in the activation of ligand receptor channels.

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