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TWO TYPES OF STEADY-STATE DESENSITIZATION OF *N*-METHYL-D-ASPARTATE RECEPTOR IN ISOLATED HIPPOCAMPAL NEURONES OF RAT

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SUMMARY

1. Whole-cell voltage-clamp recordings were made from rat isolated hippocampal neurones. Aspartate (Asp) and/or glycine (Gly) were applied by a method in which the external solution could be changed within 30 ms and thereafter held constant.

2. Asp and Gly applied together at maximal concentrations (5 mM and 10 μM, respectively) evoked an inward current due to activation of *N*-methyl-D-aspartate (NMDA) receptors. The current peaked and then declined to a steady state during the application. The time constant of desensitization (τ) was about 1 s when the agonists were applied soon after the onset of whole-cell recording. The desensitization became more rapid ($\tau = 0{\cdot}3$ s) and more complete during the first 15 min of recording, and thereafter remained stable; the amplitude of the peak response did not change throughout. In solutions containing 10 μM-Gly, Asp had an apparent K_d of 51 μM at the peak of response and 20 μM measured at the steady state. The steady-state current was 14% of the peak current.

3. Asp was applied after a conditioning exposure of the cell of Gly (from 1 to 50 μM), together with the same Gly concentration. The maximum current evoked by the application of Asp was increased while increasing Gly in the conditioning solution, with no change in the apparent K_d for Asp at the peak of Asp-activated response.

4. Various concentrations of Asp (plus 10 μM-Gly) were applied after a conditioning exposure to Asp (which alone was without effect). The maximum current induced by Asp applications was only 28% of that observed without conditioning Asp application, but the apparent K_d was unchanged (about 57 μM).

5. Test solution containing maximal concentrations of Asp and Gly was applied after conditioning exposure to both Asp (varying concentrations) and Gly (10 μM). Complete desensitization was caused by 200 μM-Asp. The apparent K_d for Asp to induce desensitization (8·7 μM) was less than the K_d as an agonist (51 μM).

6. Test solution containing maximal concentrations of Asp and Gly was applied after conditioning exposure to both Gly (varying concentrations) and Asp (5 mM). Complete desensitization was caused by 1 μM-Gly. The apparent K_d for Gly to induce desensitization (120 nM) was less than the K_d as a co-agonist (about 1 μM).

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7. The results imply that Asp alone induces desensitization of the NMDA receptor manifested as a reduced maximal response with no change in apparent K_d, whereas a combination of both co-agonists induces a desensitization of the NMDA receptor which is accompanied by an increase in affinity of receptor for both co-agonists.

INTRODUCTION

Glutamate receptors are widespread in the mammalian central nervous system, mediating excitatory synaptic transmission and events involving plasticity in different brain regions, particularly in the hippocampus (Collingridge, Herron & Lester, 1988; Cotman, Monaghan & Ganong, 1988; Collingridge & Singer, 1990). According to pharmacological and biochemical data they are subdivided into three major classes: *N*-methyl-D-aspartate (NMDA) receptors, selectively activated by NMDA and aspartate (Asp) and blocked by D-2-amino-5-phosphonovaleric acid, kainate receptors and quisqualate receptors (Watkins & Evans, 1981; Mayer & Westbrook, 1987; Monaghan, Bridges & Cotman, 1989).

Glycine (Gly) and some other amino acids in low micromolar concentrations selectively potentiate the responses to NMDA (Johnson & Ascher, 1987). This unique action is mediated through the binding of Gly at a strychnine-insensitive allosteric site on the NMDA receptor–channel complex (Monahan, Corpus, Hood, Thomas & Compton, 1989; White, Kirsten & Frank, 1989; Kessler, Terramani, Lynch & Baudry, 1989). At the single-channel level this potentiation was revealed as an increase in opening frequency without changes in mean open time or the conductance of channels (Johnson & Ascher, 1987; Mayer, Westbrook & Vyklicky, 1988). However, some recent findings suggest the Gly is absolutely necessary for the activation of NMDA receptors (Kleckner & Dingledine, 1988; Foster & Kemp, 1989; Huettner, 1989; Lerma, Zukin & Bennett, 1990) and may be regarded as an NMDA 'co-agonist'. Thus, in isolated cells which could be studied at lower level of Gly contamination (e.g. freshly isolated neurones (Chizhmakov, Kiskin, Krishtal & Tsyndrenko, 1989, 1990; Mody, Salter & MacDonald, 1989) or *Xenopus* oocytes injected with mammalian brain mRNA (Verdoorn, Kleckner & Dingledine, 1987; Kleckner & Dingledine, 1988; Lerma, Kushner, Zukin & Bennett, 1989; Lerma *et al.* 1990), the response to NMDA or its agonist alone were usually absent or very small. The 'co-agonist' role of Gly was further strengthened by the findings that selective competitive antagonists of Gly, 7-chlorokynurenic acid (Kemp, Foster, Leeson, Priestley, Tridgett, Iversen & Woodruff, 1988; Lerma *et al.* 1990; Vyklicky, Benveniste & Mayer, 1990) and indole-2-carboxylic acid (Huettner, 1989), completely blocked NMDA-activated responses recorded without addition of exogenous Gly. As the onset of such block was independent of antagonist concentration, it was concluded that these substances are competitive Gly antagonists, rather than inverse agonists (Henderson, Johnson & Ascher, 1990).

In experiments with rapid agonist delivery, ionic currents elicited by NMDA and its agonists show a marked decline due to desensitization (Chizhmakov *et al.* 1989, 1990; Mayer, Vyklicky & Clements, 1989); the kinetics are slower than those described for the non-NMDA currents (Kiskin, Krishtal & Tsyndrenko, 1986; Trussell & Fischbach, 1989; Tang, Dichter & Morad, 1989). Mayer *et al.* (1989) (see also Lerma *et al.* 1990; Sather, Johnson, Henderson & Ascher, 1990) have found that

Gly regulates NMDA receptor desensitization: increases in Gly concentration from 30 nM to 1 μM both decreased desensitization of NMDA-activated currents and increased the rate of recovery from desensitization. It has been suggested that the Gly-dependent decrease in the time spent by the NMDA receptor in the desensitized state underlies the potentiating effects of Gly on central neurones (Mayer *et al.* 1989). In contrast, desensitization was found to be independent of the Gly concentration, and Gly did not reverse desensitization in small cells and excised outside-out membrane patches (Sather *et al.* 1990) or in acutely isolated central neurones (Shirasaki, Nakagawa, Wakamori, Tateishi, Fukuda, Murase & Akaike, 1990); this raises the possibility that NMDA receptor desensitization and the potentiating effect of Gly may be regulated separately.

Steady-state desensitization is estimated as a decreased response to saturating concentrations of agonist after pre-incubation in agonist-containing solutions sufficiently long to achieve dynamic equilibrium conditions (Katz & Thesleff, 1957; Kiskin *et al.* 1986; Trussell & Fischbach, 1988). We used this experimental paradigm in the study of the steady-state desensitization of NMDA receptors in freshly isolated perfused hippocampal neurones to distinguish the properties of steady-state desensitization produced by Asp alone and by the combined action of Asp and Gly. In a preliminary report (Chizhmakov *et al.* 1990) we showed that Asp decreases sensitivity to subsequent application of Asp and Gly, and this type of desensitization is prevented by kynurenate, the selective antagonist of Gly at the modulatory site (Birch, Grossman & Hayes, 1988; Kemp *et al.* 1988; Williams, Stone, Burton & Smith, 1988).

METHODS

Cell preparation

Rats (14–21 days) were decapitated without anaesthesia and the hippocampus was removed. It was manually cut into slices (300–500 μm), in a solution containing (mM): 150, NaCl; 5, KCl; 1·25, NaH_2PO_4; 2, $CaCl_2$; 2, $MgCl_2$; 26, $NaHCO_3$; 20, glucose. Slices were placed on a nylon net in a 20–50 ml glass vessel and incubated in this solution for 40–60 min at room temperature (21–26 °C). The enzymatic treatment proceeded in the same solution with lower Ca^{2+} concentration ($CaCl_2$ 0.9 mM and ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) 1 mM) and 0·3–0·5 % protease from *Aspergillus oryzae* (Sigma) added. Similar results were obtained with the use of protease Akrezim 3 (0·3 %) from microbial origin kindly provided by Drs Yu. W. Kostenko and I. F. Mishunin (A. V. Palladin Institute of Biochemistry, Kiev, USSR). The addition of 0·1–0·2 % collagenase (Type II, Sigma) to the enzyme solutions sometimes improved results when the enzymatic treatment proved to be insufficient. The incubation in the enzyme solution proceeded at 36 °C within 50–90 min, until most slices could be separated along the pyramidal cell layer to produce viable cells. Slices were kept subsequently in enzyme-free solution containing normal Ca^{2+} concentration and used within 6 h. Throughout the entire procedure the solutions were continuously saturated with a 95 % O_2 and 5 % CO_2 gas mixture to maintain pH 7·4. For cell dissociation the slice was transferred to an extracellular solution containing (mM): 150, NaCl; 5, KCl; 2 $CaCl_2$; 2, $MgCl_2$; 10, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES); pH adjusted with 9–11 mM of NaOH to 7·4. Under low magnification the slice was carefully separated along the pyramidal cell layers CA1 and CA3 with steel needles. For experiments we used either the cells accessible from the slice surface or neurones mechanically dissociated by trituration of pieces of slices with a Pasteur pipette (tip diameter 150–300 μm). The isolated cells with small pieces of slices were transferred to a bath containing the same solution mounted on the stage of an inverted microscope. The neurones selected for investigation were 10–30 μm in diameter and preserved a small part of dendritic processes. They were usually suitable for the recording for 2–4 h.

Recording conditions

Patch micropipettes were used for recording in the whole-cell configuration. They were made from soft glass capillaries (diameter 1·8 mm) by the usual two-stage pulling procedure. After fire-polishing, the pipettes had a broad, thick-walled tip with internal diameter 2–4 μm (resistance 1–3 MΩ). No pipette coating was employed. The intracellular solution contained 100 mM-KF (or 100 mM-CsF) and 30 mM-tris(hydroxymethyl aminomethane chloride (Tris-Cl), pH 7·2. In some experiments, the intracellular solution was 130 mM-CsOH, adjusted to pH 7·2 with H_3PO_4 and with 2 mM of $MgCl_2$–ATP added. The cell was sucked into the pipette on approximately $\frac{1}{2}$–$\frac{1}{3}$ of its somatic part, and the membrane under the pipette ruptured after 1–2 min of exposure to the intracellular solution, either spontaneously or due to a suction applied to the pipette. Depolarization of neurones from holding potentials between −80 and −100 mV to −20 to −30 mV activated inward sodium and outward potassium (when using a KF intracellular solution) currents. The presence of stable voltage-gated currents was used as the index of neurone viability during the experiment.

Solutions and chemicals

All applications were performed in Mg^{2+}-free solution which contained (mM): 150, NaCl; 5, KCl; 5, $CaCl_2$; 10, HEPES–NaOH (pH 7·4). When amino acids were dissolved in this solution, the final pH was also adjusted to 7·4 with Tris-OH. All chemicals were purchased from Sigma. We used L-aspartate (Asp) in most experiments as a selective NMDA agonist (Watkins & Evans, 1981; Mayer & Westbrook, 1987) but NMDA gave similar results. When examined in the same cell, the responses to L-Asp, D-Asp and NMDA were practically identical and produced complete cross-desensitization (Chizhmakov *et al.* 1989). Special precautions to reduce Gly contamination in extracellular solutions were not taken; however, we always used freshly made solutions and discarded experiments in which Asp alone elicited currents exceeding 30–50 pA.

Concentration clamp method

Rapid application of agonists or blockers to the membrane of internally perfused neurone was performed by using a modification of the concentration clamp method (Krishtal, Marchenko & Pidoplichko, 1983) (Fig. 1*A*). The recording chamber rested on a microscope stage. It had several compartments, one filled with control extracellular solution, and others containing test extracellular solutions (typically containing one or more drugs). A V-shaped glass tube (internal diameter 0·5–1 mm) entered the solution in the chamber before bending upwards so that its orifice was beneath the surface of the solution; the other end of this tube could be connected to a source of negative pressure (25–30 cm water) by activating an electromagnetic solenoid valve (60–90 ms voltage pulse), so that the solution in the chamber rapidly filled the glass tube (flow velocity 150–250 μm ms^{-1}). The tip of the recording pipette, with attached neurone, was inserted into the orifice of the glass tube. The glass tube, connected to a Ag–AgCl electrode, and the recording pipette were mounted independently of the microscopic stage, so that they could be positioned without a loss of electrical contact in one or other compartments of the recording chamber by lowering, laterally moving, and raising the stage. Complete change of the external solution, measured as a decrease in the voltage-activated Na^+ current after application of a Na^+-free solution (equimolar substitution of the choline chloride for NaCl), occurred within 10–30 ms. After closure of the valve the neurone remained bathed in test solution until the tube and pipette with neurone were moved to another compartment and the next application occurred. The experiment started with the neurone in a fresh Mg^{2+}-free extracellular solution for 30–60 s.

The rapidity of the solution exchange was tested by experiment. Figure 1*B* shows the inward current observed when the extracellular solution was changed from control (150 mM-sodium) to a test solution containing 5 mM-Asp and 10 μM-Gly agonist in 150 mM-sodium. Figure 1*C* shows the inward current observed when the extracellular solution was changed from a control solution that contained no sodium to the same test solution. The finding that the inward currents were the same implies that the sodium concentration at the cell surface had already reached close to 150 mM by the time of the peak inward current. It was therefore inferred that the agonist concentration (in this case 5 mM-Asp and 10 μM-Gly) reached a steady-state value in a time that was less than the time-to-peak of the inward current.

Data analysis

Digitized records (512 points, sampling rate from 0·1 to 20 ms per point) were stored on a PDP 11-compatible microcomputer and plotted by using and *X*–*Y* pen recorder. The peak amplitudes

of currents were normalized to the maximal amplitude of response (I_{max}) or to the corresponding value of the test response in normal conditions. Population results are expressed as means ±S.E.M. Calculations of the theoretical dose–response dependences were performed by a least squares method. Curve fitting with exponentials was done by using the linear least squares method approximation of the logarithm of current and a comparison of theoretical curve with experimental record.

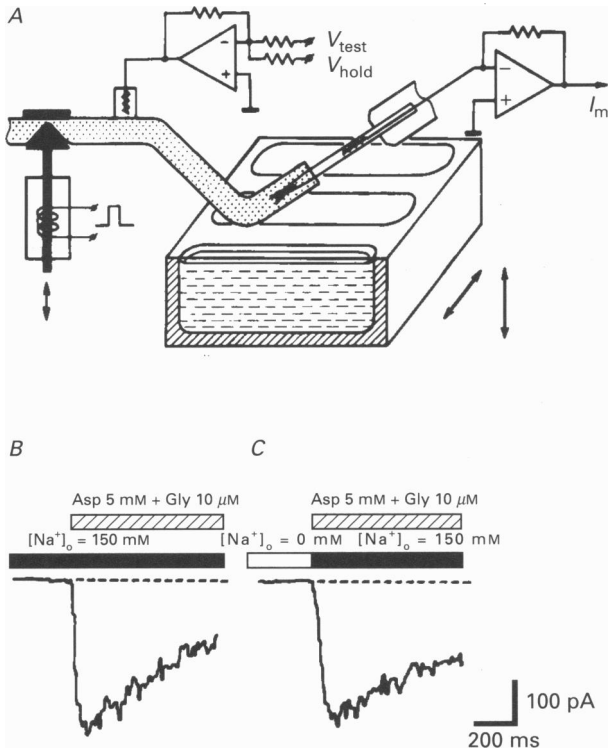

Fig. 1. Concentration clamp apparatus for the study of isolated neurones. *A*, schematic diagram of the experimental set-up. The system 'micropipette in the tube' is placed above the bath compartment with the application saline, and the bath moves upward to a fixed position. The solution flows into the tube when the valve is open. Two Ag–AgCl electrodes are connected to the conventional voltage-clamp circuitry. *B* and *C*, estimation of the rate of achieving steady-state L-aspartate (Asp) and glycine (Gly) concentrations at the response peak. Solution containing 5 mM-Asp and 10 μM-Gly was applied to the same neurone either from the normal extracellular solution (*B*), or after pre-incubation in Na^{2+}-free solution (*C*, equimolar substitution of choline chloride for NaCl). In this and subsequent figures the holding potential was −100 mV.

RESULTS

Time-dependent changes in Asp-activated currents

The application of test substances using the concentration clamp method usually began within 1–2 min after the rupture of the membrane patch. The first simultaneous application of 5 mM-Asp and 10 μM-Gly (saturating concentrations) elicited an inward current, which peaked in 100–150 ms and then declined (desensitization) to a certain steady-state level (plateau). In most experiments the decline could be fitted by a single exponential. Subsequent applications of the same

agonist solution produced responses of the same peak amplitude, but there was more rapid desensitization and, thus, a decreased amplitude of the plateau component (Fig. 2). Within 10–15 min after the beginning of perfusion, both the amplitude of the non-desensitizing component (Fig. 2*B*) and the desensitization time constant (Fig.

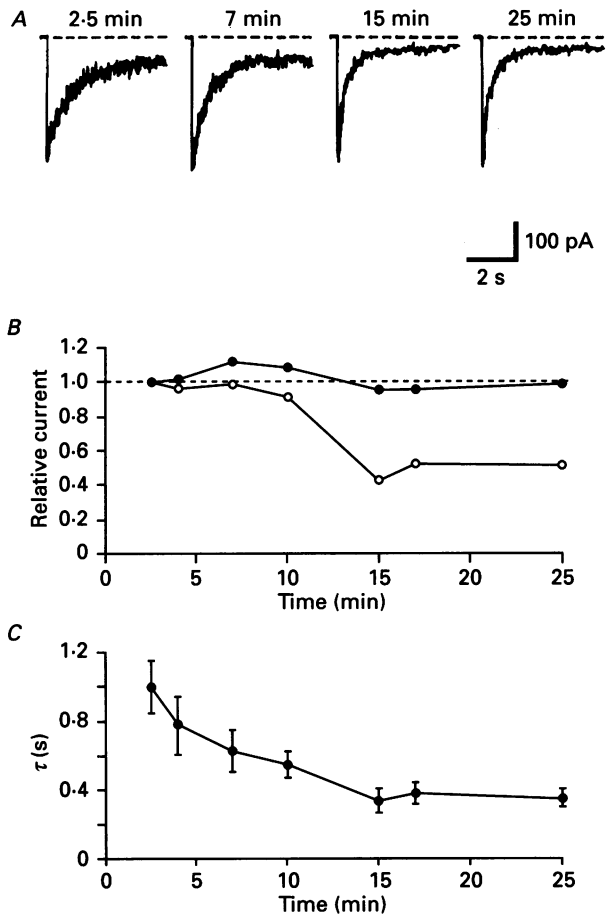

Fig. 2. Time-dependent changes in the Asp-activated response. *A*, responses to simultaneous applications of 5 mM-Asp and 10 μM-Gly taken at the times indicated above the traces. Time intervals were measured from the beginning of intracellular perfusion of the neurone. Note the gradual decrease of the steady-state component of the responses and acceleration of the desensitization rate during the first 15 min of recording. Thereafter the response remained stable. *B*, the amplitudes of peak (●) and steady-state (○) currents relative to those of the first response (2·5 min of perfusion) are plotted for the same neurone. *C*, the corresponding time dependence for mean time constants τ of the exponential response decay. The exponential approximations have been taken for different time intervals within the each current trace, and mean values from the three to four best fits are plotted. Bars indicate the S.D. of mean.

2*C*) reached stable values; therefore the experiments described were carried out more than 10–15 min after the beginning of intracellular perfusion.

The desensitization enhancement principally could be attributed to Ca^{2+} entry due to the Ca^{2+}

permeability of NMDA-activated channels (MacDermott, Westbrook, Smith & Barker, 1986; Mayer, MacDermott, Westbrook, Smith & Barker, 1987), as proposed before (Mayer & Westbrook, 1985; Mayer *et al.* 1987). In such case this effect would depend on the frequency of applications of agonists. However, the changes in desensitization were equally evident when the second

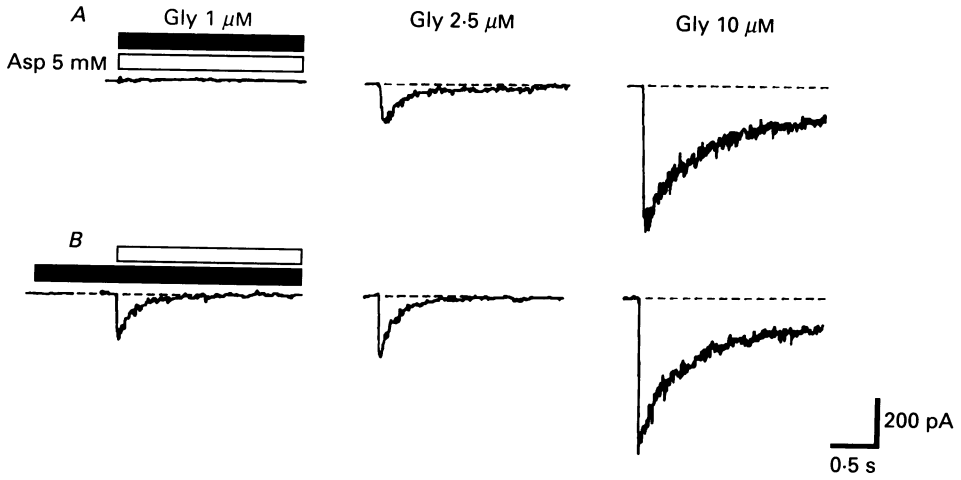

Fig. 3. Responses of an individual hippocampal neurone to 5 mM-Asp applied simultaneously with Gly (*A*) and after 30 s pre-incubation in Gly-containing solutions (*B*). The concentrations of Gly for both experimental protocols are indicated above the current traces. Note the slower onset of responses to simultaneous applications (*A*).

application of agonists was separated from the first by 15–20 min. Similar time-dependent changes were also seen at lower concentrations of Asp and Gly, as well as in experiments when neurones were perfused with phosphate-containing solutions containing Mg^{2+}–ATP (see Methods). Sometimes we observed the two-exponential or even more complex current decays ($1{\cdot}1 \pm 0{\cdot}3$ s for the time constant of the slow component and $0{\cdot}25 \pm 0{\cdot}08$ s for the fast one, $n = 6$); with increased perfusion time, both components were accelerated.

Asp-activated currents with or without pre-application of Gly

Solutions containing Asp and Gly were applied to the membrane of hippocampal neurone in two different ways. In the first procedure the solutions were applied simultaneously, whereas in the second case neurones were pre-incubated with Gly. The amplitude of the elicited responses depended both on the concentration of Gly and on the application procedure (Fig. 3). At Gly concentrations up to 1 μM simultaneous applications of Gly and Asp (up to 5 mM) elicited little or no response (Fig. 3*A*) although a current was observed if Gly was also present before Asp (Fig. 3*B*). At 2–5 μM-Gly, the response after pre-incubation in Gly (Fig. 3*B*) substantially exceeded the response to the simultaneous application of Asp and Gly (Fig. 3*A*). Pre-incubation in Gly resulted in larger amplitudes of Asp-activated responses at all Gly concentrations up to saturation (10 μM; Chizhmakov *et al.* 1989). At this concentration both application procedures resulted in practically identical responses. This was also true for different concentrations of Asp (50 μM, 100 μM and 1 mM). We observed qualitatively similar effects for other agonists of the Gly receptor (Johnson & Ascher, 1987; Snell, Morter & Johnson, 1988; Chizhmakov *et al.* 1989): L- and D-alanine, L- and D-serine, L- and D-proline and hydroxy-L-proline (not shown).

Thus, to achieve the maximal response, it is necessary to pre-incubate the neurone with Gly or its analogue. For Gly the pre-incubation for 30–40 s was found to be sufficient: the increase in this time to 1–2 min had no further effect.

Interaction of Asp with NMDA receptors. Dose–response relationships

Figure 4*A* demonstrates currents induced by different concentrations of Asp on the background of pre-applied Gly (5 μM). Similar experiments with various

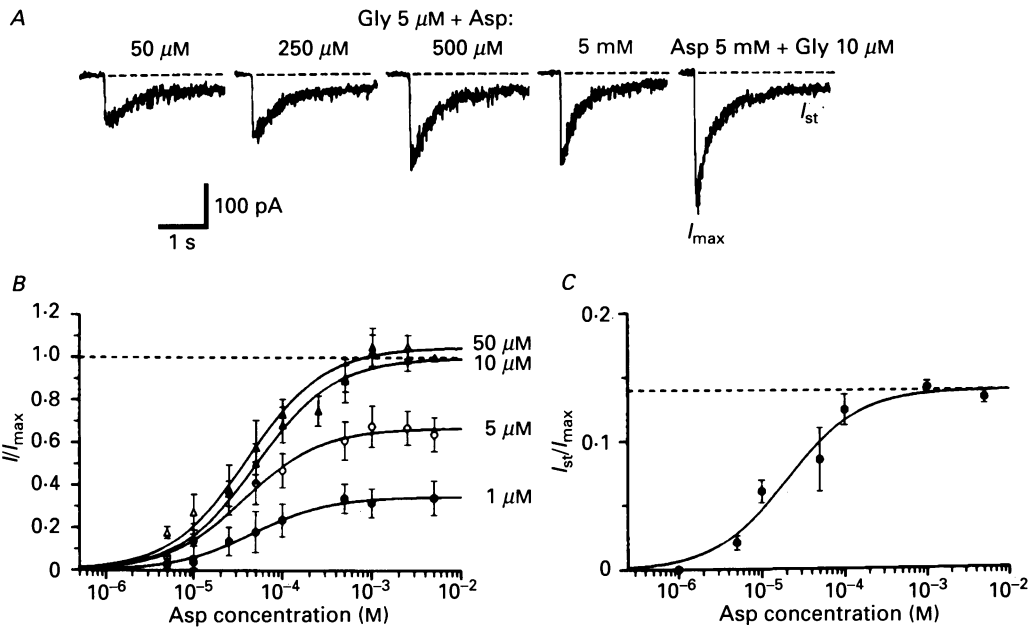

Fig. 4. Dose–response relationship for the Asp-activated responses measured at different concentrations of Gly. Pre-incubation in Gly-containing solution was used when Gly concentration was lower than 10 μM. It proceeded for 30–40 s and was succeeded by application of solutions containing the same Gly and various Asp concentrations. *A*, the responses to Asp applied on the background of 5 μM-Gly. Asp concentrations are indicated above the current traces. Starting from 500 μM-Asp, saturation of response was observed, but the peak response never reached the value obtained at 10 μM-Gly (I_{max}, right trace). *B*, the normalized dose–response dependences for the peak Asp-activated response at different concentrations of Gly. Each point represents the mean value obtained for three to five cells; bars here and in subsequent figures indicate S.E.M. Smooth curves are Langmuir isotherms with the following K_d values and saturation levels calculated to obtain the best fit using the least squares procedure: 46 μM and 0·35 for 1 μM-Gly (●), 37 μM and 0·67 for 5 μM-Gly (○), 51 μM and 1·0 for 10 μM-Gly (▲), 40 μM and 1·05 for 50 μM-Gly (△). *C*, the dose–response relationship for the steady-state current activated by Asp in different concentrations on the background of 10 μM-Gly. The amplitudes of the current, I_{st}, were normalized to the corresponding values of I_{max} for each cell ($n = 5$), and the mean values are plotted. The smooth curve is the Langmuir isotherm with a K_d value of 20 μM and saturating level of 0·14, fitting the experimental data.

concentrations of Asp were performed at other Gly concentrations (1, 10 and 50 μM). The peak amplitudes of the observed responses were normalized to the maximal response amplitude (I_{max}) obtained at saturating Asp concentration on the

background of saturating Gly concentration (10 μM). Figure 4*B* shows the dependence of peak current on Asp concentration ('activation curves') at different Gly levels. The obtained dependences were fitted by Langmuir isotherms with K_d values close to 50 μM, but with different saturation levels determined by the concentration of Gly. From these levels, the dose–response dependence for Gly at saturating Asp can also be estimated. However, this dependence with EC_{50} (half-maximal effective concentration) around 0·5–3 μM (measured in separate experiments, Chizhmakov *et al.* 1989) was different from a Langmuir isotherm, indicating possible heterogeneity of the modulatory Gly sites in this preparation (Chizhmakov *et al.* 1989).

The similar values of K_d for the peak Asp-activated currents recorded at different concentrations of Gly show that at response peak Gly does not change the affinity of Asp for the receptor (Verdoorn *et al.* 1987; Kleckner & Dingledine, 1988; Huettner, 1989; Lerma *et al.* 1989; Bonhaus, Yeh, Sharyak & McNamara, 1990; Shirasaki *et al.* 1990), and Asp does not influence the affinity of Gly to its binding site either (White *et al.* 1989; Monahan *et al.* 1989; Kessler *et al.* 1989; Bonhaus *et al.* 1990). Hence, both co-agonists initially interact with respective binding sites within the NMDA receptor independently, and the normalized peak amplitude of the response should be proportional to the fraction of receptors occupied both by Asp and Gly.

On the background of saturating concentration of Gly (10 μM) we have measured the dose–response relationship for the mean normalized amplitudes of the steady-state components of Asp-activated responses (I_{st}, Fig. 4*C*). Though variable from cell to cell, these amplitudes had a mean maximal value 0·14 ± 0·06 of the I_{max} ($n = 10$). The dependence was fitted by a Langmuir isotherm with a K_d value of 20 μM.

Steady-state desensitization of NMDA receptors induced by Asp alone

We studied steady-state desensitization of NMDA receptors induced by the Asp alone in two different sets of experiments. In the first series of experiments (Fig. 5*A*), Asp was first applied and the solution was then changed to Asp plus Gly (10 μM, a saturating concentration). In all neurones ($n = 7$), the peak amplitudes of the responses were smaller than the amplitudes of responses obtained by the same Asp and Gly concentrations without Asp pre-incubation (compare Fig. 5*A* and 5*B*), presumably reflecting desensitization to the initial Asp pre-incubation. The concentration dependence of the peak responses to Asp plus Gly (10 μM) obtained after pre-incubation in Asp was well fitted by a Langmuir isotherm (Fig. 5*D*, bottom curve); the K_d value (57 μM) was similar to that for the peak response to simultaneous application of Asp plus Gly (10 μM), and the saturation level (α) was 0·28 ± 0·13 ($n = 11$). Even after prolonged pre-incubation of the neurone in saturating Asp concentrations (5 and 10 mM) subsequent addition of Gly (10 μM) still activated an ionic current with maximal relative amplitude α. Thus, Asp alone in saturating concentration desensitizes about 70% of the Asp-bound NMDA receptors, making them insensitive to Gly.

In the second series of experiments (Fig. 5*C*), neurones were pre-incubated in solution containing different concentrations of Asp and then the solution was changed to a test solution of Asp (5 mM) and Gly (10 μM). The dependence of the test response peak amplitude on the varying concentration of Asp used for pre-incubation

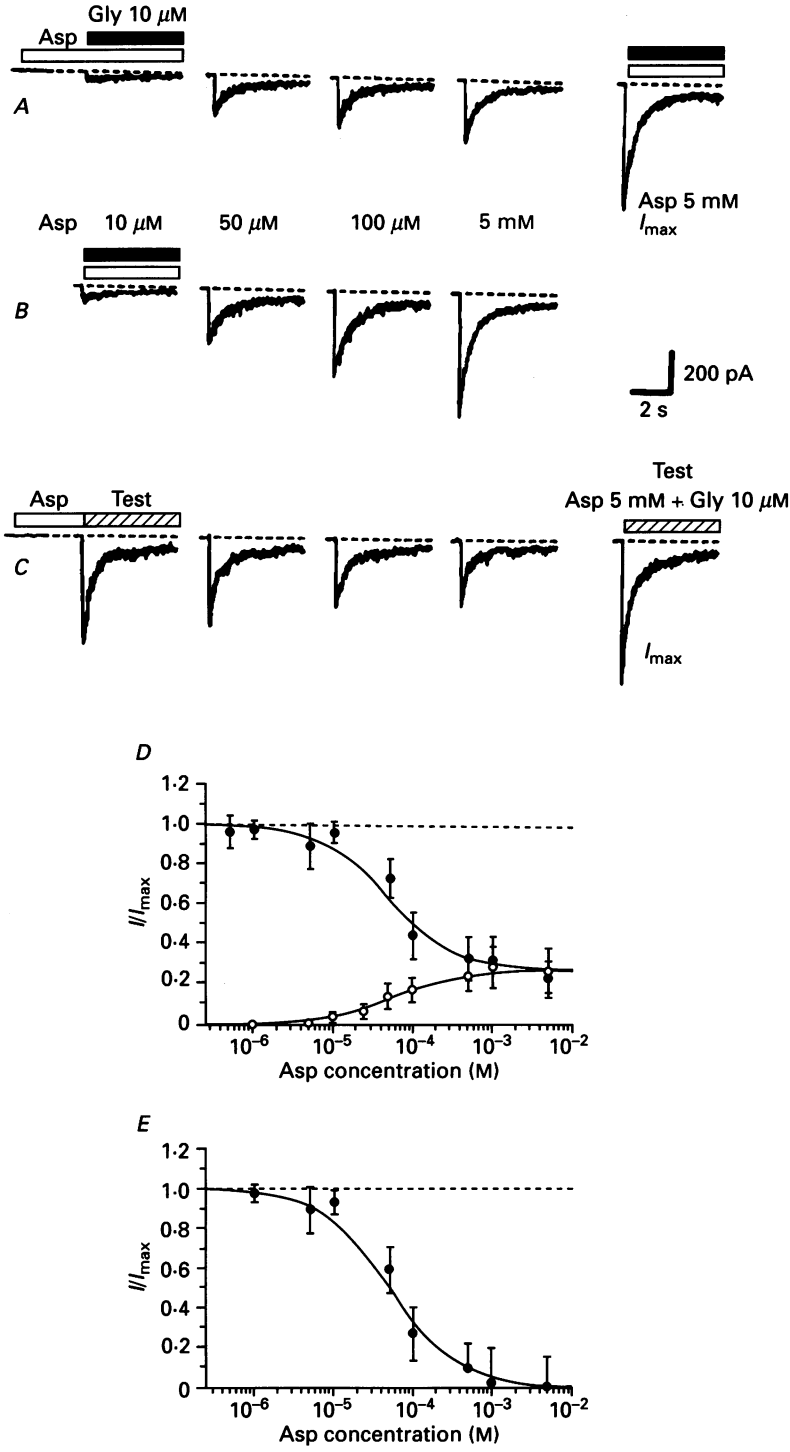

Fig. 5. Steady-state desensitization of NMDA receptors induced by Asp. *A*, the response of a hippocampal neurone to the applications of 10 μM-Gly and Asp in various concentrations (indicated below the current traces) obtained after 30 s pre-incubation in

is shown in Fig. 5*D* (top curve). The increase in Asp concentration in pre-incubation medium decreased the test response, but did not abolish it (Fig. 5*C*). The test solution should activate both receptors that are not liganded with Asp and receptors liganded with Asp, by virtue of the added Gly. This second component was measured independently in the first series of experiments, and is shown as the open circles in Fig. 5*D* (bottom curve). Thus, the current due to the activation by 5 mM-Asp and 10 μM-Gly of receptors that were not already liganded with Asp is computed as the difference between filled and open circles in Fig. 5*D*. These values are replotted as Fig. 5*E*. Figure 5*E* also shows (continuous line) the Langmuir isotherm for Asp in 10 μM-Gly (redrawn from Fig. 4*B*). The agreement with the experimental points implies that pre-incubation with Asp does not change the apparent K_d determined by a subsequent test dose.

Steady-state desensitization of NMDA receptors in the presence of Mg^{2+}

Extracellular Mg^{2+} selectively blocks the NMDA-activated ionic channels at negative membrane voltages (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Mayer, Westbrook & Guthrie, 1984; Ascher & Nowak, 1988). Neurones were pre-incubated in saturating concentration of Asp with 2 mM-Mg^{2+} to find the possible relationship between agonist-induced steady-state desensitization and cation entry into the cell (Fig. 6). The test responses were measured after the pre-incubation in Asp (Fig. 6*A*) and after pre-incubation in Asp with Mg^{2+} (Fig. 6*B*, in this experiment the Mg^{2+} was washed out directly with test solution). The comparison of test responses with controls in Fig. 6 shows that the degree of steady-state desensitization was almost identical. Thus, channel block by Mg^{2+} ions does not affect the development of Asp-induced steady-state desensitization of NMDA receptor.

Steady-state desensitization of NMDA receptor under concerted action of Asp and Gly

To study the effect of Gly on the steady-state desensitization of NMDA receptors we conducted two further series of experiments with 'two-step' applications of Asp or Gly. In the first series of experiments, Asp was applied in a two-step manner on the background of a saturating concentration of Gly (10 μM, Fig. 7*A*). With pre-

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Asp. The amplitude of these responses never attained the maximum (I_{max}, right curve). *B*, the same concentrations of Asp, when applied to the same neurone as in *A*, but simultaneously with Gly, resulted in the responses always exceeding those in *A*. *C*, after 30 s pre-incubation in solutions containing the same Asp concentrations as in *A* and *B*, the responses to test solution (5 mM-Asp and 10 μM-Gly) were evoked (another neurone). The test responses decreased with the increase in Asp concentration. Right trace, the maximal test response obtained without pre-incubation. *D*, the dose–response relationship for the Asp-induced steady-state desensitization of the test response in *C* (●, top curve) and the dependence of responses to applications of Asp with 10 μM-Gly (*A*) on the pre-incubating Asp concentrations (○, bottom curve). The same saturating levels of both dependences correspond to the response to 10 μM-Gly on the background of saturating Asp. Points represent mean values obtained for four to eight cells. The top smooth curve is fitted by eye, the bottom one is a Langmuir isotherm with a K_d value of 57 μM and maximum $\alpha = 0{\cdot}28$, calculated to obtain the best fit to experimental data. *E*, subtraction of experimental points in *D* fits well the theoretically calculated dependence for the fraction of NMDA receptors free from agonist: smooth curve is obtained by subtracting from unity the dose–response curve for 10 μM-Gly, Fig. 4*B*.

incubation in Gly, the first, 'conditioning', applications of Asp in various concentrations caused inward currents; their normalized peak amplitude was taken to indicate the fraction of Asp-occupied receptors (Fig. 7*A*, left column). To allow the desensitization process to reach the steady state, the neurone was pre-incubated in

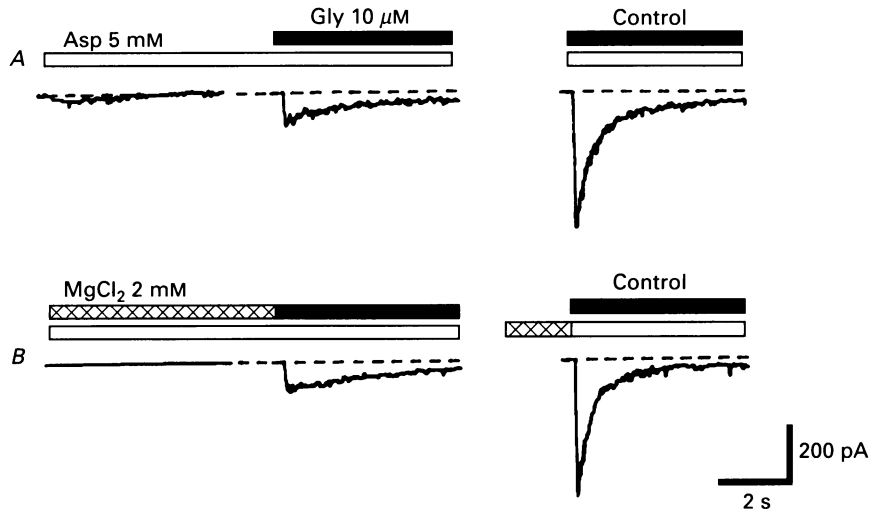

Fig. 6. Channel block by Mg^{2+} ions does not affect the Asp-induced steady-state desensitization of NMDA receptor. *A*, small amplitude of the test response to 5 mM-Asp with 10 μM-Gly after pre-incubation in 5 mM-Asp reflects the steady-state desensitization of receptors. *B*, the addition of 2 mM-$MgCl_2$ to the Asp-containing solution does not affect desensitization. The traces on the extreme right show corresponding controls performed from normal saline (*A*) and from the solution containing Mg^{2+} (*B*). They show that the offset of the blocking action of Mg^{2+} is not slower than the time of the solution change. The gaps in current traces represent pre-incubation time: 2 min (*A*) and 15 s (*B*).

conditioning Asp and saturating Gly for 30 s; the test solution (containing saturating Asp and saturating Gly) was then applied. The peak amplitudes of the test responses were measured after subtraction of the steady-state levels of conditioning currents.

The fraction of receptors occupied by Asp at the beginning of the conditioning can be estimated from the peak amplitude of the conditioning response (Fig. 7*A*, left column). If all the receptors that were unliganded by Asp were to respond to the test application, then the peak current evoked by the test application should be of the form shown in Fig. 5*E* (dashed line in Fig. 7*B*). However, the currents induced by the test application were smaller than this (Fig. 7*B*). The apparent K_d for Asp in the presence of desensitization induced by Asp and Gly was 8·7 μM, compared with about 50 μM for its effect as an agonist. Indeed, Asp with Gly induced complete desensitization at 100–200 μM, which is much lower than the saturating concentration measured from the direct effect (dose–response curve in Fig. 4*B*).

In the second series of experiments, Gly was applied in a similar two-step manner on the background of a saturating concentration of Asp (5 mM, Fig. 8*A*). In this experimental protocol, the amplitudes of test responses should be compared to the amplitude of the response elicited by an addition of 10 μM-Gly on the background of saturating Asp (I_{Asp}, see top current traces). As in the experiments with pre-

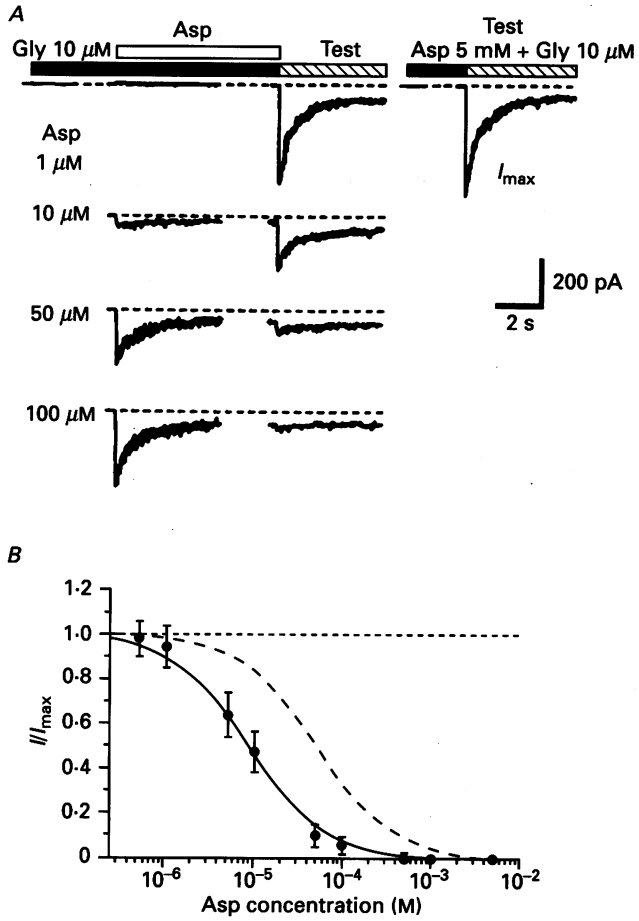

Fig. 7. Steady-state desensitization of NMDA receptors measured on the background of a saturating Gly concentration (10 μM). *A*, after pre-incubation in Gly, each conditioning application of Asp in various concentrations (indicated near corresponding traces) after 30 s incubation (gaps in current traces) was followed by the application of the test solution. The trace on the extreme right, the test application without pre-incubation in Asp represents maximal response, I_{max}. With the increase in Asp concentration in pre-incubating solution the amplitude of the test response was progressively suppressed. *B*, the peak amplitudes of the test responses were measured from the steady-state level of conditioning response and normalized to I_{max}. Mean values obtained on five cells are plotted against the Asp concentration. The smooth curve is a Langmuir isotherm, K_d = 8·7 μM, fitting the experimental data. The dashed curve is calculated from the dose–response dependence for Asp at 10 μM-Gly as in Fig. 5*D*. It represents the concentration dependence of the fraction of receptors free from Asp. The difference between the curves indicates the presence of steady-state desensitization of NMDA receptors produced by a combined action of Asp and Gly.

incubation in Asp (Fig. 5*A*), this and subsequent test responses are diminished to the same extent (α) by the steady-state desensitization induced by Asp alone. Using the same idea of measurements as in the previous series of experiments, we normalized the peak test responses to I_{Asp} and compared them with estimated concentration

dependence for the predicted fraction of Gly-free receptors. Figure 8*B* demonstrates the dependence of normalized amplitudes of test responses on Gly concentration in pre-incubating solution fitted by a Langmuir isotherm with an apparent K_d for Gly of 120 nM. The dependence was shifted towards the lower concentrations of Gly, and

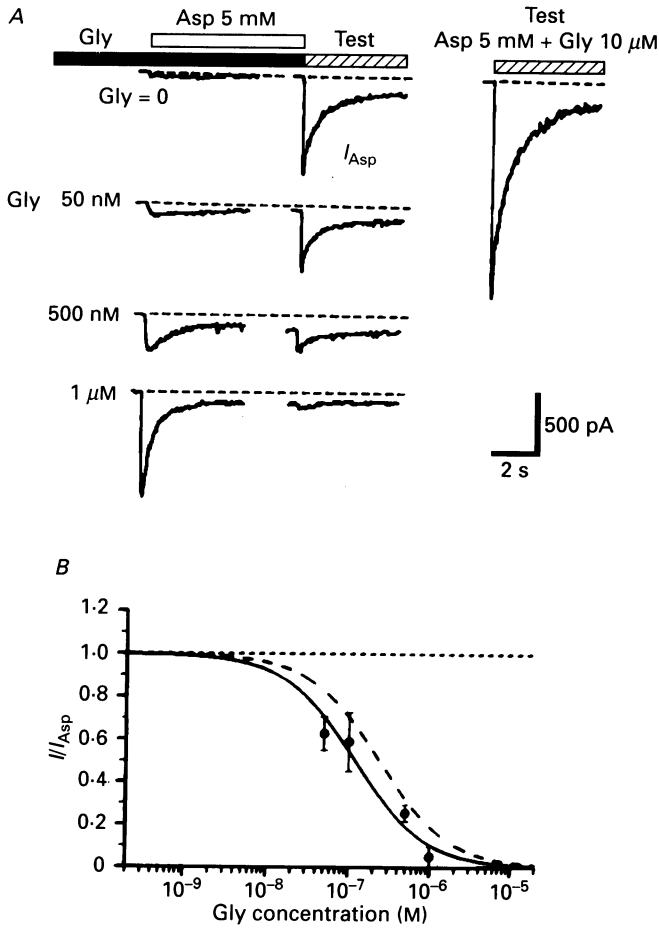

Fig. 8. Steady-state desensitization of NMDA receptors in the presence of Gly, measured on the background of saturating concentration of Asp (5 mM). *A*, pre-incubation of the neurone in Asp alone suppressed the test response to the value I_{Asp} (top traces), control without pre-incubation is represented in the trace on the right. Addition of Gly in the increasing concentrations (indicated near the current traces) further desensitized the test response. The gaps in current traces represent pre-incubation time, 30 s. *B*, the dependence of peak amplitude of test response, measured from the steady-state levels of corresponding conditioning response and normalized to I_{Asp}, on Gly concentration. The smooth curve is a Langmuir isotherm with K_d = 120 nM fitting the mean experimental data obtained for four cells. The dashed curve represents the normalized theoretical concentration dependence of the fraction of Gly-free receptors on Gly concentration, based on binding data of Monahan *et al.* 1989 and White *et al.* 1989 (K_d = 230 nM).

complete steady-state desensitization of the Asp-activated response was achieved at Gly concentrations as low as 1 μM.

DISCUSSION

The NMDA receptors display different features in a desensitized state when tested after pre-incubation of the neurone in Asp alone or in Asp with Gly. In the first set of experiments, application of saturating Gly on the background of pre-incubation in Asp (Fig. 5*A*) produced a response whose amplitude is significantly reduced as compared to the amplitude of the response to a simultaneous application of both co-agonists (Fig. 5*B*). So, it may be supposed that during pre-incubation in Asp receptors lose the ability to interact with Gly. We assume that when an Asp molecule is bound to the agonist site of an NMDA receptor and the Gly site is not occupied, the activation of the NMDA receptor does not occur, but the Gly site may acquire desensitized conformation. Our finding that kynurenate blocks the Asp-induced desensitization (Chizhmakov *et al.* 1990) is consistent with this hypothesis: while the Gly sites of NMDA receptors are protected by kynurenate, the desensitization onset under Asp may be prevented.

The second series of experiments with Asp-induced desensitization (Fig. 5*C*) may help to clarify the question whether or not receptor desensitization persists after the dissociation of the Asp–receptor molecular complex. Since we know how the Asp-bound receptors respond to the addition of maximal Gly (Fig. 5*D*, bottom curve), the remaining component of the test response to the addition of maximal Asp and Gly should reflect the response of all other receptors to both co-agonists. Figure 5*E* shows that after pre-incubation in Asp all the receptors free from agonist contribute to this component of the test response, therefore we failed to detect among them any desensitized receptors. This situation may be possible if the desensitization proceeds only while the Asp molecule is bound to the NMDA receptor, and each receptor recovers to initial non-desensitized state when the Asp–receptor complex dissociates.

A qualitatively different picture of events was observed in the steady state when the neurone is pre-incubated in Asp with Gly. On the background of Gly the transition of receptors to a desensitized state proceeds with activation and is directly seen from the decline of conditioning response. From the known receptor affinities for co-agonists at the peak of response (K_d values of 51 μM for Asp and 230 nM for Gly, Monahan *et al.* 1989 and White *et al.* 1989; our estimations gave larger values of around 1 μM for Gly, see Chizhmakov *et al.* 1989) we expected to find the whole fractions of Asp-free (Fig. 7*B*, dashed curve) or Gly-free receptors (Fig. 8*B*, dashed curve) in non-desensitized state. However, some of these receptors gave no response to the test stimulus, so the fraction actually desensitized under the action of Asp and Gly receptors seems larger than that estimated from the amplitude of the decaying component of the conditioning response (Fig. 7*B* and Fig. 8*B*). This is expressed as a decrease, after pre-incubation with both co-agonists, in apparent K_d values for Asp (8·7 μM) and for Gly (120 nM) in relation to corresponding values measured at the peak of response. In contrast, an apparent K_d value for Asp measured after pre-incubation in Asp alone (Fig. 5*E*) did not change. Thus, desensitization induced by a combined action of co-agonists could be described as an increase in apparent

affinity of receptors for co-agonists from the peak of response to the steady state. Such increase in the affinity for agonist has been proposed earlier in general models of the acetylcholine receptor desensitization (Katz & Thesleff, 1957; Changeux, Devillers-Thiery & Chemouilli, 1984).

However, alternative explanations are possible. First, using the peak amplitude of response that does not reach a steady state may lead us to underestimate the true affinities of co-agonists. This seems to be unlikely in these experiments for the following reasons. First, the experiments shown in Fig. 1*B* and 1*C* suggest that the concentrations of Asp and Gly reach the steady-state levels at the time when ionic current reaches the peak, so an error introduced by non-equilibrated Asp and/or Gly concentration is negligible. Second, pre-incubation in Gly could ensure a correct estimation of Gly affinity. Third, the same K_d values for Asp were derived both from the peak amplitude of the response to simultaneous application of Asp and maximal Gly (Fig. 4*B*), and from the experiments with pre-incubation in Asp (Fig. 5*A* and 5*D*, bottom curve). So, it is possible to consider a presence of 'quasi-equilibrium' conditions at the peak of response. An appearance of a considerable fraction of desensitized receptors when the response reaches the peak may provide a second plausible explanation for the difference in K_d values. It also seems unlikely to us, because the changes in the kinetics of response desensitization in the course of intracellular perfusion occur at the constant peak response amplitude (Fig. 2). The peak amplitude of the response changes little if the desensitization is either much slower than activation or starts after formation of active agonist–receptor complex. Thus, desensitization of an NMDA receptor is most likely to be a consequence of receptor activation and is accompanied by an apparent increase in affinity of receptors.

The dependence of the steady-state component of NMDA response on Asp concentration (Fig. 4*C*) directly shows that, at saturating concentrations of Gly, non-desensitized receptors detected in the steady state have higher apparent affinity for Asp ($K_d = 20\ \mu M$) than the non-desensitized receptors found after pre-incubation in Asp alone (Fig. 5*D*, bottom curve). So Gly and/or receptor activation indirectly 'increases' an apparent receptor affinity for Asp. One possible way to theoretically account for this change is an introduction of a distinct receptor desensitized state which can be formed only after the receptor activation. From a desensitized state, the receptor may return to an activated state (as recently proposed by Benveniste, Clements, Vyklicky & Mayer, 1990) or may slowly transform, with a loss of one co-agonist, to either Asp–receptor or Gly–receptor initial complexes. The lifetime of a desensitized state thus may limit the time required for activated receptors to revert to the initial state. This should lead to an apparent increase in affinity for co-agonists, as forward rates of formation of activated receptor do not change whereas an apparent reverse rate decreases. From a more pronounced shift in apparent K_d value on the background of saturating Gly (Fig. 7*B*) we may suspect that the intermediate desensitized state can most likely be represented by a receptor occupied by Gly. The fraction of these receptors should be evidently less on the background of saturating Asp (Fig. 8*B*). Anyway, the fact that an increase in affinity was observed for both Asp and Gly further emphasizes the role of Gly as NMDA co-agonist in contrast to allosteric hypothesis.

The question arises whether the features of desensitization induced by Asp alone can be explained by a weak activation and subsequent desensitization of NMDA receptors due to contamination of solutions with Gly. In our experiments the desensitization is not directly regulated by Ca^{2+} or any cation entry, since the experiments with Mg^{2+} showed the absence of protection from desensitization by this antagonist (Fig. 6). Thus, the desensitization is a receptor process, which apparently has no current-dependent component. If all the solutions are equally contaminated with Gly, applying the test solution after pre-incubation in Asp (Fig. 5*E*) we should detect the shift in K_d value, as after pre-incubation in both co-agonists (Fig. 7*B*). Alternatively, if the contaminating Gly increases in pre-incubation medium proportionally to the Asp concentrations, more Asp-bound receptors should interact with Gly. Thus, by increasing Asp concentrations from 1 to 10 mM we should observe a progressive decrease of the response to subsequent addition of Gly instead of saturation (Fig. 5*A*,*D*). However, no indications of such phenomena have been found.

The fact that during the first 15–20 min of intracellular perfusion the peak response does not decrease while desensitization increases shows that there is no correlation between the facilitatory action of Gly and receptor desensitization, in contrast to original finding of Mayer *et al.* 1989 (see Sather *et al.* 1990; Shirasaki *et al.* 1990). Moreover, we show here that Gly increases te steady-state desensitization leading to the appearance of a distinct desensitized state. The differences in the mode of application of substances, in the expression of receptors in cultured and in freshly isolated cells, and in the contribution of somatic *versus* dendritic NMDA receptors, may principally account for the discrepancy (see Sather *et al.* 1990). However, the regulation of the NMDA receptor desensitization in a perfused cell by an intracellular metabolite (metabolites) is the most plausible explanation (see MacDonald, Mody & Salter, 1989). Possibly, the intracellular Ca^{2+} ions activity may serve an additional source of controversy, since to obtain non-desensitizing responses at high Gly concentrations Vyklicky *et al.* 1990, used only 0·2 mM of external Ca^{2+} and 5 mM-1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) in the intracellular solution. With fluoride or phosphate in the internal solution we were unable to decrease external Ca^{2+} below 1 mM. In our experiments the addition of 5 mM-BAPTA to intracellular solution did not measurably change the features of NMDA response desensitization. We tentatively suggest that the 'Gly-insensitive' desensitization produced by a combined action of both co-agonists is caused by a lack of some intracellular factor or by a high internal Ca^{2+} concentration and thus is not manifested in the absence of perfusion (Lerma *et al.* 1990) or at different perfusion conditions (Mayer *et al.* 1989; Vyklicky *et al.* 1990). In contrast, the 'Gly-sensitive' desensitization to the NMDA alone could be less strongly regulated. Then, when Gly concentration increases, the fraction of receptors occupied only by NMDA and capable of becoming desensitized evidently decreases. This may qualitatively explain how Gly decreases this type of desensitization. Our suggestion is supported by the finding that the 'Gly-sensitive' desensitization was less prominent either at low NMDA or high Gly concentrations (Vyklicky *et al.* 1990).

With arbitrary concentrations of Asp and Gly in the pre-incubation solution, the two types of desensitized NMDA receptors under certain intracellular conditions co-

exist and differentially contribute to the integral steady-state desensitization. In the absence of Gly, NMDA alone causes incomplete desensitization, and the receptors liganded with NMDA rapidly come back from a desensitized state. This makes possible the activation of the excitatory response by a sudden increase in Gly concentration on the background of previously released NMDA agonist (as in experiments presented in Fig. 5*A*). The dependence of these receptors on Gly concentration has an extremely low nanomolar threshold as compared to that for the inhibitory response activated by Gly (10–20 μM, Krishtal, Osipchuk & Vrublevsky, 1988). Thus, Gly in different concentrations may either evoke and maintain or inhibit neuronal excitation, acquiring a dual role in the synaptic function.

Combined action of Gly and Asp promotes a more complete second type of steady-state desensitization. This process requires receptor activation and may terminate the NMDA agonist action on the postsynaptic membrane, preventing the damage of neurones by an excessive Ca^{2+} entry which triggers the excitotoxic changes (Choi, 1987; Rothman & Olney, 1987). So, the action of Gly on desensitization introduces an additional negative feedback in the overall process of cell excitation.

In contrast to other receptors requiring no co-agonists, both the amplitude of the NMDA-activated response and the subsequent desensitization cannot be evaluated solely from the known concentration of NMDA agonist. The combined action of both co-agonists, NMDA agonist and Gly (or its analogue), results in complex and flexible regulation of neuronal excitation and related intracellular processes.

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