0026-895X/91/030337-05$03.00/0

MOLECULAR PHARMACOLOGY, 40:337-341

ACCELERATED COMMUNICATION

Desensitization of *N*-Methyl-D-aspartate Receptors in Neurons Dissociated from Adult Rat Hippocampus

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Received May 1, 1991; Accepted June 14, 1991

SUMMARY

Desensitization of the *N*-methyl-D-aspartate (NMDA) receptor-channel complex was studied in isolated rat hippocampal neurons using a fast drug application system. 1) Desensitization rate was slower at more negative membrane potentials and when external [Ca^{2+}] was lowered. 2) In the presence of 10 μM glycine, 2-amino-5-phosphonovalerate neither induced desensitization nor prevented recovery from it. 3) Preincubation in 500 μM aspartate or 10 μM glycine alone elicited desensitization only weakly or not at all. 4) Aspartate appeared to bind at its receptor site in the absence of glycine, and vice versa. It is proposed that, for the NMDA receptor, channel opening is necessary for the occurrence of desensitization and, thus, that desensitization involves structural changes in the channel-lining section of the protein rather than the glycine or NMDA binding sites.

The NMDA receptors are the most well studied of the glutamate receptors, largely due to their involvement in a variety of physiological processes (see, for example, Ref. 1). The receptor-channel complex has an agonist recognition site and a separate binding site for glycine, which regulates activity of the ionic channel (2–4). Activation of the channel by NMDA is greatly potentiated in the presence of glycine (3). On activation, NMDA receptor channels undergo desensitization (4–8). It is, however, not clear whether desensitization is dependent on the NMDA binding site, the glycine binding site, or both or, moreover, whether channel opening is necessary for its development. Such questions are important in considerations of which parts of the protein are involved in determining different functional attributes of the receptor, such as the desensitization process.

Such questions have previously been considered for the nicotinic AChR. Katz and Thesleff (9) first suggested that desensitization results from a discrete conformational change of the receptor. It has been shown that nicotinic antagonists such as tubocurarine (10), pancuronium (11), or gallamine (11) have a similar affinity for desensitized receptors. In contrast, a few antagonists (termed "metaphilic antagonists") were found to have a higher affinity for normal or desensitized receptors (10, 11). It has since been shown, using direct structural measurements (12), that AChR desensitization is associated with quite large structural rearrangements of the δ and, to some extent, the γ subunits of the protein complex, with relatively little change in the agonist-binding α subunits.

In contrast to the AChR, virtually no information is available on the structural changes that presumably underlie NMDA receptor desensitization. In this paper, we have studied NMDA receptor desensitization using a fast perfusion system to generate responses to NMDA in isolated hippocampal neurons. The experiments were designed to test whether binding of agonists (aspartate or NMDA) or the antagonist AP5 at the NMDA recognition site, or binding of glycine at the glycine recognition site, influences desensitization. The results suggest that the Ca^{2+}-sensitive component of NMDA receptor desensitization occurs without major changes at the NMDA or glycine recognition sites and that receptor activation is necessary for desensitization to occur.

Materials and Methods

Isolated hippocampal neurons from 2–3-week-old rats were obtained by procedures described elsewhere (6) and stored at room temperature (22–24°) in solution containing (in mM): NaCl, 125; KCl, 3.75; KH_2PO_4, 1.25; $MgCl_2$, 1.3; $CaCl_2$, 1.8; glucose, 10; HEPES/NaOH, 5; and $NaHCO_3$, 26 (pH 7.3 when bubbled with 95% O_2/5% CO_2). Normal solution contained (in mM): NaCl, 150; KCl, 5; $CaCl_2$, 1.8; glucose, 10; and HEPES/NaOH, 10 (pH 7.3). Drugs were dissolved in the normal solution at the following concentrations: aspartate, 500 μM (Reanal); NMDA, 200 μM (Sigma); glycine 10 μM (Serva); and AP5, 100 μM (Cambridge Research Biochemicals). Intracellular solution contained (in mM): KF, 100; Tris-HF, 30 mM (pH 7.3).

Currents were recorded in the whole-cell mode at a membrane potential of −100 mV (unless otherwise stated), using the loose-patch technique as previously described (13, 14). Solutions were exchanged rapidly, as previously described, allowing concentration clamp within

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AChR, acetylcholine receptor; AP5, 2-amino-5-phosphonovalerate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

10–15 msec (14, 15). Current records were stored on magnetic tape and analyzed with an IBM PC/XT computer, using the statistical software STATGRAPHICS (STSC, Inc.).

Results

Desensitization. In most neurons tested, the application of a solution containing 500 μM aspartate or 200 μM NMDA plus 10 μM glycine (aspartate plus glycine) induced inward currents, which almost completely desensitized within 2 sec in the continued presence of the agonist (Fig. 1, *inset*). These currents were completely blocked by AP5 (100 μM), indicating that the response was due to the activation of NMDA receptor channels. (Note that the aforementioned drug concentrations were used throughout the study.) The characteristics of the desensitization are very similar to those reported by other groups (5, 6) (but not Ref. 4). Responses to aspartate plus glycine rose to a peak in 15–25 msec (presumably dependent on rate of solution exchange) and decayed in a voltage-sensitive manner (Fig. 1). The ratio of the steady state to peak current was somewhat varied (0.24 ± 0.09, $n = 28$, mean ± standard error for aspartate; 0.19 ± 0.07, $n = 28$, mean ± standard error for NMDA) but not voltage sensitive.

Effect of external Ca^{2+} concentration of desensitization. The desensitization was strongly dependent on extracellular calcium levels. In the five cells tested, dropping the Ca^{2+} concentration in the perfusion medium from 1.8 to 0.18 mM resulted in much slower and relatively less complete desensitization, especially at more negative potentials (Fig. 2). The desensitization time constants and steady state to peak ratios listed in the figure legend refer to the data shown. In two cells, however, desensitization was completely abolished at voltages of −60 to −100 mV. Perfusion of cells with low Ca^{2+} solution did not significantly affect input resistance of the cells. Note that all other experiments in this study were performed in the presence of 1.8 mM Ca^{2+} in the external solution.

Effect of AP5 on desensitization. In six cells, continuous application of aspartate plus glycine resulted in the usual desensitizing response. The perfusion was changed to AP5 plus glycine and after 20–30 sec the aspartate plus glycine was again applied using the fast application system. The response to application of aspartate plus glycine was almost as large as the control response (Fig. 3), demonstrating, firstly, that the recovery from AP5 was as fast as the solution change (20 msec) and, secondly, that the presence of AP5 did not prevent recovery from desensitization. Moreover, when AP5 plus glycine were subsequently applied, no further desensitization occurred (data not shown). Thus, AP5 was shown to be an easily reversible blocker of the response, but its binding to the NMDA agonist recognition site neither caused nor prevented recovery from desensitization. Rapid recovery from AP5 action (τ, 62 msec) has been previously described (16).

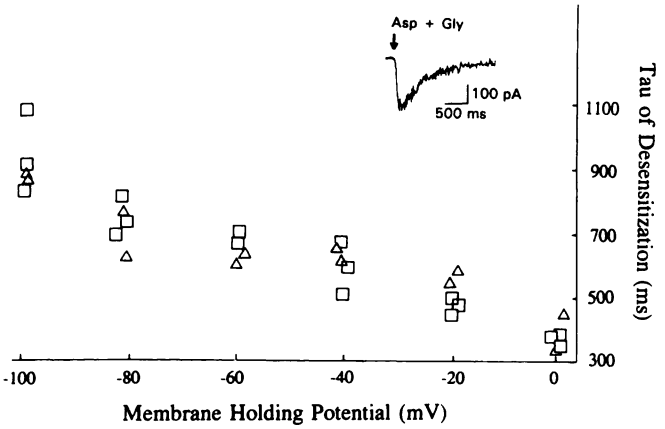

Fig. 1. Voltage sensitivity of desensitization decay rates. Rates of desensitization (Tau) were measured in five cells, at membrane voltages ranging from 0 to −100 mV, after application of NMDA (200 μM) (△) or aspartate (500 μM) (□) in the presence of 10 μM added glycine. The rate of desensitization was found to be voltage dependent, independent of which NMDA receptor agonist was used. *Inset*, typical response to aspartate plus glycine at a membrane holding potential of −100 mV. Note that the reversal potential of these responses is approximately +10 mV. Such responses were completely blocked by addition of AP5 (100 μM) to the perfusion medium.

Binding and receptor activation by aspartate or glycine alone. In order to test whether either glycine or aspartate alone could cause desensitization, it was first necessary to establish whether they could bind independently. This is difficult to assess directly with electrophysiological techniques, but it was possible to obtain some data pertaining to this issue (Fig. 4). Neurons were first perfused with aspartate alone, which caused no response. On changing to a pure glycine solution, a brief current was seen, which returned to control levels within 200 msec. Note that the decay time constant of this current (51 msec) was considerably less than that in the continued presence of aspartate plus glycine (738 msec) and, thus, presumably reflects dissociation of aspartate from its receptor. This suggests that, despite the fast washout, enough aspartate was apparently bound to receptors to allow the occurrence of a brief response as glycine was washed in. Similarly, when glycine was perfused alone no response was seen, and changing to a pure aspartate solution again resulted in a brief response (229 msec) (Fig. 4, *center*). Note that the latter response always decayed more slowly than when the solutions were applied in reverse order. This presumably reflects the fact that aspartate has a lower affinity for the receptor. Moreover, the glycine concentration is about 10 times the saturating concentration, whereas this aspartate concentration is far from saturating in this preparation. Consequently, glycine is more resistant to washout and a much higher proportion must be washed out to decrease the effect. Similar results were obtained in all five cells tested.

Dependence of desensitization on the combined presence of aspartate and glycine. The final series of experiments was designed to test whether either glycine binding or NMDA binding alone was sufficient to cause desensitization. Fig. 5A shows currents elicited by application of aspartate plus glycine before and after 10-, 20-, and 30-sec preincubation in glycine-containing solution. Currents elicited after preincubation with glycine (Fig. 5A, *right*) had amplitudes very similar to those of control currents (Fig. 5A, *left*). Thus, glycine alone was not sufficient to cause desensitization. In contrast, responses to aspartate plus glycine after 10-, 20-, and 30-sec preincubation in aspartate-containing solution (Fig. 5B, *right*) were slightly smaller in amplitude (8–17%, $n = 17$) than control currents. In another five cells, NMDA was substituted for aspartate, with similar results. Thus, preincubation in glycine-containing solution did not elicit desensitization, whereas preincubation in aspartate (or NMDA)-containing solutions elicited a small amount of desensitization even in the absence of added glycine. Note that, although the presence of low levels of glycine in the solution cannot be excluded, the level was not

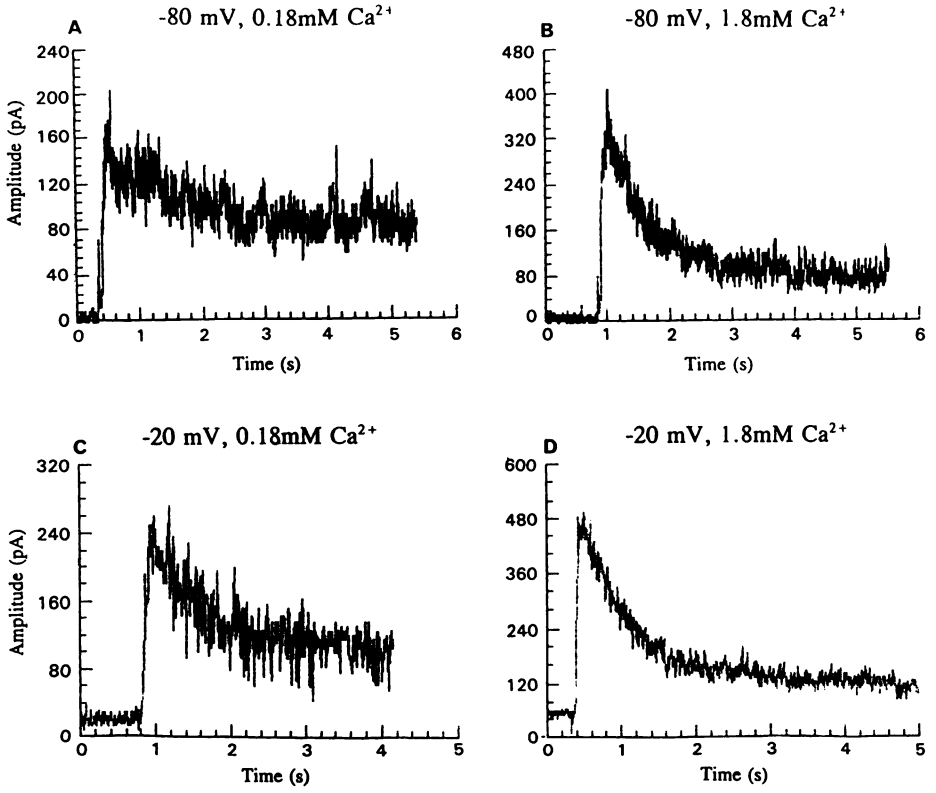

Fig. 2. Effect of external Ca^{2+} concentration on desensitization at different voltages. A and B, Membrane holding potential, −80 mV. A, Low external [Ca^{2+}] (0.18 mM); τ, 1301 msec; ratio of steady state to peak current, 0.59. B, Higher external [Ca^{2+}] (1.8 mM); τ, 727 msec; ratio of steady state to peak current, 0.21. C and D, Membrane holding potential, −20 mV. A, Low external [Ca^{2+}] (0.18 mM); τ, 714 msec; ratio of steady state to peak current, 0.44. D, Higher external [Ca^{2+}] (1.8 mM); τ, 512 msec; ratio of steady state to peak current, 0.18. The results refer to measurements in the same cell. Qualitatively similar results were found in all five cells tested. Note that, in constant external [Ca^{2+}], the ratio of steady state to peak current was not voltage dependent. Presumably the relatively small amplitude of the peak currents at −80 mV, compared with those at −20 mV, results from a small amount of contaminant Mg^{2+} in the solutions.

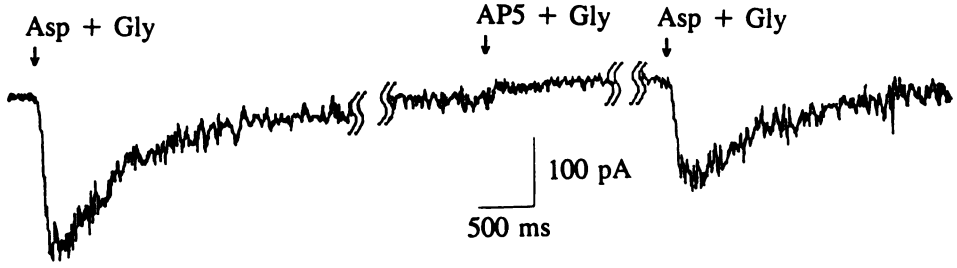

Fig. 3. AP5 does not elicit or prevent recovery from NMDA receptor desensitization. *Left*, the control response to application of aspartate plus glycine. The response desensitized over the next 1–2 sec. *Center*, the solution was changed to AP5 plus glycine, and the steady state current returned to baseline. *Right*, application of aspartate plus glycine immediately resulted in a response only slightly smaller than the control response in amplitude and showing similar desensitization. *Arrows*, time of solution changes.

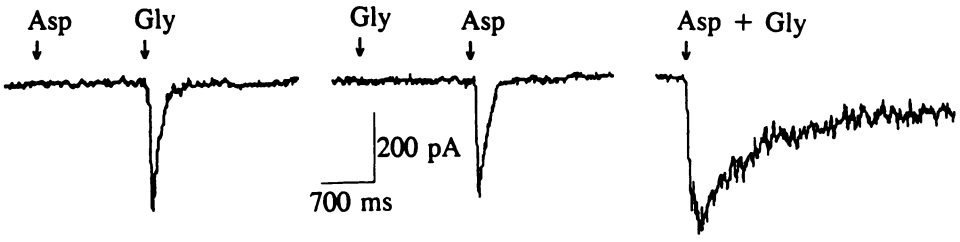

Fig. 4. Separate binding of aspartate and glycine. *Left*, response to fast change of perfusion solution from aspartate (500 μM) alone to glycine (10 μM) alone. *Center*, response to fast change of perfusion solution from glycine alone to aspartate alone. *Right*, control response to aspartate plus glycine in the same cell. *Arrows*, time of solution changes. Note the fast washout of responses in the *left* (51 msec) and *center* panels (229 msec), compared with the slower time course of desensitization shown in the *right* panel (738 msec).

sufficient to result in detectable currents in response to aspartate unless glycine was added to the solution.

Discussion

Among ligand-gated ion channels, desensitization is a general phenomenon that varies in magnitude and rate of onset between different receptor types. Among the glutamate receptors, desensitization is rapid and profound in the case of non-NMDA receptors (15, 17–19) and somewhat slower and not so profound in the case of NMDA receptors (4, 6–8, 20).

In this paper, we have investigated the influence of occupation of the NMDA and glycine binding sites on NMDA receptor desensitization, in order to address the question of which parts of the NMDA receptor-channel complex undergo conformational changes during desensitization. We find that activation of both the NMDA binding site and the glycine binding site (and, hence, receptor activation) is necessary for desensitization to occur and, thus, suggest that the desensitization described may be due to changes to the channel rather than the binding sites of the receptor.

Desensitization of the response to aspartate plus glycine was

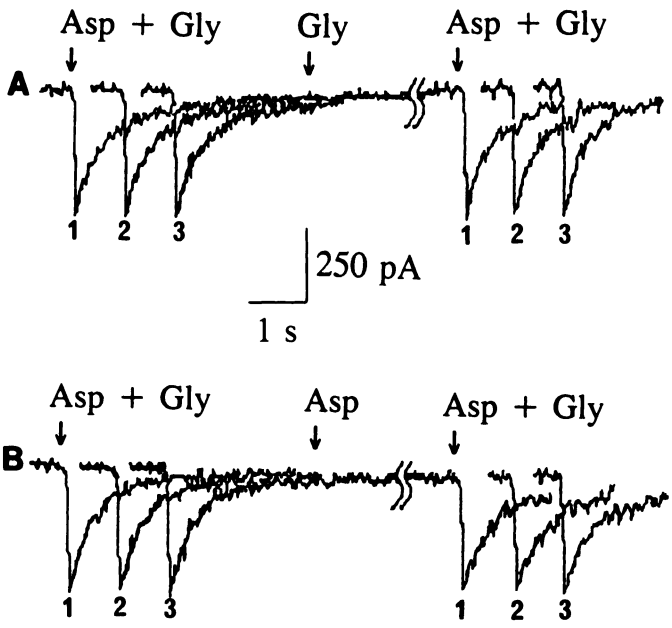

Fig. 5. Different effects of preincubation in either glycine or aspartate on desensitization of NMDA receptors. A, *Left*, control responses obtained at 10-sec intervals. *Right*, responses obtained after 10-sec (*1*), 20-sec (*2*), and 30-sec (*3*) preincubation in glycine (10 μM). B, Same experiment using preincubation with aspartate (500 μM). Although desensitization was barely observable, the responses to aspartate plus glycine after preincubation in aspartate were consistently slightly smaller (8–17%, n = 17). In these experiments, similar results were seen irrespective of whether aspartate plus glycine were washed out between the control (*left*) and perfusion of the test solution (aspartate or glycine alone) (*center*).

shown not to occur in the presence of glycine alone. Aspartate alone was shown to cause only slight desensitization (8–17%). This differs from the results of Ascher and Johnson (20), who reported that receptors "cannot be predesensitized by previous application of either glycine or NMDA" (p. 117) alone. However, as has been previously discussed, low levels of glycine may contaminate the normal solution and, indeed, occasional channel openings have been shown to occur in solutions to which no glycine has been added (3). Maintenance of desensitization was also shown to be dependent on the combined presence of aspartate and glycine. In contrast to the present study, Chizhmakov *et al.* (21) found that considerable (but still incomplete) desensitization (>50% at the peak) occurred when cells were preincubated in aspartate with no added glycine and that this effect was blocked by addition of kynurenate. The authors suggested a complex model to explain this observation, in which kynurenate acts to hold the receptor in a "glycine-sensitive conformation." However, the observation that aspartate alone induced a clearly detectable current and that the steady state nondesensitizing current was very similar whether or not the cells were preincubated suggests that the observed desensitization in the absence of added glycine could be due to a relatively high level of contaminating glycine in the control solution. Moreover, this would provide a simple explanation for the blocking effect of kynurenate.

The next question to be addressed was whether only occupancy of the receptors was required or whether the channel had to be activated for the occurrence of desensitization. AP5, an agent that binds to but does not activate the NMDA receptor, cannot substitute for aspartate in causing desensitization in the presence of glycine. The observation that AP5 does not cause or maintain desensitization, despite binding to the NMDA agonist binding site, suggests that activation of the receptor channel is necessary for desensitization to occur. Further, the partial desensitization seen in the presence of aspartate alone, when rare channel openings would be expected, again correlates desensitization and channel opening. The observation, discussed above, that kynurenate blocks such desensitization, as is observed in solutions to which no glycine has been added, supports the hypothesis that desensitization under these conditions is indeed dependent on occasional activation of the glycine site by contaminating glycine.

Lastly, the effect of extracellular calcium concentration is of interest. NMDA receptor channels are permeable to calcium, and the increased rate of desensitization in high calcium is voltage sensitive, being more prominent at more negative potentials. It seems likely that, similarly to the hypothesis for AChR channels in the electroplaque (22) and the frog endplate (23), calcium accumulation at the inner surface of the NMDA receptor channel causes an increase in desensitization rate. This is supported by the study of Sather *et al.* (8), who controlled calcium concentration on the internal side of the membrane by including EGTA in the internal solution and found no effect of extracellular calcium concentration on NMDA receptor channel desensitization. This is, however, still controversial, because a similar effect of calcium on desensitization was also observed in a recent paper by Clarke *et al.* (24) and internal calcium buffering was without effect. Thus, desensitization in the present study is, in part, dependent on external calcium concentration, and this may be due to calcium flow through the channel. However, in contract to AChRs, the rate of desensitization at a calcium concentration of 1.8 mM is slower at more negative potentials. This suggests that calcium concentration cannot be the only factor and that voltage itself may influence the rate of desensitization. It is interesting to note that Clarke *et al.* (24) observed the reverse effect of voltage at low calcium concentration (0.5 mM), suggesting a possible interaction between the effects of calcium and voltage.

The subject of calcium sensitivity has also been mentioned by Mayer *et al.* (4). They primarily described experiments demonstrating that increasing extracellular glycine concentration decreases desensitization. They commented that all experiments were performed at low calcium concentration (0.2 mM) to avoid the occurrence of a glycine-insensitive desensitization, which they suggested is triggered by influx of calcium through NMDA receptor channels. In fact, at these low calcium levels and saturating glycine concentrations, Mayer *et al.* (4), similarly to the present study, saw very little desensitization. This relative lack of desensitization in the presence of low calcium and high glycine is apparently not seen in outside-out patches (8). Although the question of the effect of changing external glycine concentration is not addressed here, it seems likely that the desensitization described in the present study is the glycine-insensitive desensitization that Mayer *et al.* (4) avoided by keeping calcium levels low. Because NMDA receptor activation can lead to an increase in intracellular calcium levels, a calcium-sensitive desensitization process could play a significant physiological role.

In summary, we can hypothesize that channel opening is necessary for NMDA receptor desensitization, suggesting that desensitization involves conformational changes in the chan-

nel-lining portion of the protein rather than changes at the NMDA or glycine binding sites. This desensitization is shown to be voltage dependent in its rate and to be dependent on the external calcium concentration. It is tempting to propose that the desensitization of receptor-activated channels, which apparently does not involve changes at the agonist binding sites, may share a common mechanism with inactivation of voltage-activated channels, although the latter is generally not Ca^{2+} sensitive.

Acknowledgments

We are especially grateful to Drs. Frances Edwards and Alasdair Gibb at University College London for critical comments and suggestions and for editorial assistance on the manuscript.

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