

Whole cell and single channel analysis of the kinetics of glycine-sensitive N-methyl-D-aspartate receptor desensitization

Chris G. Parsons, Xiangang Zong & Hans D. Lux

Department of Neurophysiology, Max-Planck Institute for Psychiatry, am Klopferspitz 18a, 8033 Planegg-Martinsried, Germany

- 1 The kinetics of glycine-sensitive, N-methyl-D-aspartate (NMDA) receptor desensitization were investigated in cultured neurones with the patch clamp technique.
- 2 The degree of fast NMDA-receptor desensitization was inversely related to glycine concentration. Thus, increasing concentrations of glycine from 30 nM to 2.5 μ M potentiated desensitized NMDA responses ($873\% \pm 101\%$) to a greater degree than peak responses ($260\% \pm 27\%$).
- 3 The desensitization was due to a decrease in the affinity of glycine for the strychnine-insensitive, glycine modulatory site (glycine_B site) following activation of the NMDA-receptor complex. Thus, the A50 for glycine in potentiating peak responses (77 nM, 95% confidence limited 58- 104 nM) was five fold lower than that for. plateau responses (399 nM, 340-468 nM).
- 4 The rate of desensitization was related to glycine concentration such that a reciprocal plot of desensitization rate ($1/\tau$ s⁻¹) against glycine concentration had a slope of 9.5×10^6 M⁻¹ S⁻¹
- 5 Recovery from desensitization following step increases in glycine or L-alanine concentration in the continuous presence of NMDA (200 μ M) reflected the association kinetics of the glycine_B agonist used.
- 6 The rate and degree of NMDA receptor desensitization was independent -of holding potential.
- 7 NMDA receptor desensitization was also evident at the single channel level.
- 8 The glycine_B antagonist 7-chlorokynurenic acid (7-Chl-Kyn 3 and 10 μ M) concentration-dependently induced an identical form of desensitization in the presence of 1 μ M glycine,
- 9 In contrast,. the competitive NMDA antagonist (\pm)-amino-phosphonovaleric acid (APV 30 to 300 μ M) concentration-dependently antagonized and slowed the onset kinetics of NMDA responses.

Keywords: Desensitization; N-methyl-D-aspartate (NMDA); glycine; patch clamp; electrophysiology; superior colliculus cultured neurones

Introduction

Although the phenomenon of N-methyl-D-aspartate (NMDA) receptor desensitization has been extensively documented, the nature of the processes that underlie it remain a matter of controversy. At least five types of desensitization have been reported to date. Glycine-sensitive desensitization was first reported by Mayer et al. (1989) in cultured mouse hippocampal neurones and subsequently confirmed for NMDA receptors expressed in *Xenopus* oocytes (Lerma et al., 1990). Three forms of Ca²⁺-sensitive desensitization have also been reported; one shows similarities to a pH-sensitive Na⁺ current (Grantyn & Lux, 1988), another seems to be dependent on activation of a Ca²⁺-sensitive Cl⁻ conductance (Leonard & Kelso, 1990) and another may be related to a direct influence of Ca²⁺ on NMDA receptors (Clark et al., 1990; Zilberter et al., 1991). In addition, calcium-and glycine-independent desensitization has been observed in outside-out patches of mouse cortical neurones (Sather et al., 1992). Ca²⁺-sensitive desensitization has a relatively slow time course of decay ($t \sim 2$ s), the magnitude of which is dependent on extracellular Ca²⁺ concentration. Glycine-sensitive desensitization has a much faster time course ($\tau \sim 100$ -500 ms) and is most readily observed in the absence of Ca²⁺-sensitive desensitization i.e. in the presence of low concentrations of extracellular Ca²⁺ (< 0.2 mM). Glycine is a coagonist at the NMDA receptor complex and its binding to a specific, strychnine-insensitive recognition site (hereafter termed the glycine_B site) is an absolute prerequisite for channel activation by agonists at the NMDA recognition site

(Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Kushner et al., 1988). The degree of fast NMDA receptor desensitization is inversely related to glycine concentration. Thus, NMDA responses desensitize to a plateau of around 10 to 20% of the peak response in the presence of low nM concentrations of glycine whereas little desensitization occurs at glycine concentrations of around 10 μ M. Furthermore, glycine_B antagonists such as 7-chlorokynurenic acid (7-Chl-Kyn) concentration-dependently induce an identical form of desensitization in the presence of μ M concentrations of glycine (Benveniste et al., 1990b; Henderson et al., 1990; Kemp & Priestley, 1991). It has been suggested that the desensitization is due to a decrease in the affinity of the glycine_B site following binding of agonists at the NMDA recognition site as the potency of glycine in potentiating the plateau response was 5 fold less than its potency in potentiating the peak response (Lerma et al., 1990). In this respect it is important to note that the rate of desensitization seen at low nM concentrations of glycine is similar to the dissociation rate of glycine from glycine_B sites. As such, it has been suggested that the true rate of desensitization may in fact be much faster but masked by the slow dissociation kinetics of glycine (Benveniste et al., 1990a). The purpose of this study was to characterize further the kinetics of glycine-sensitive desensitization to test this hypothesis.

Methods

- Patch clamp recordings were made from cultured superior colliculus neurones (10 to 21 days in vitro) in whole cell and single channel outside-out modes with the aid of an EPC-7 amplifier (List). Electrodes were pulled with a horizontal

1 Author for correspondence at present address: Merz + Co. GmbH & Co., Eckenheimer Landstraße 100-104, 6000 Frankfurt am Main 1, Germany.

puller (DMZ) and had an internal tip diameter between 1.2 and 1.6 μm and a tip resistance of 4 to 10 M Ω . Cells were continuously superfused via one of eight channels of a fast superfusion system (Konnerth *et al.*, 1987). Test substances were then applied by rapidly switching channels-complete exchange of the superfused solution was achieved within < 10 ms (confirmed by the speed of block of voltage-activated calcium channels when switching to a solution containing cadmium). The application of solutions and the synchronized online electronic acquisition of data (sample rate 8 kHz) were controlled by the PDP 11 programme INTESV (Zucker, MPI Munich). Subsequently, AUTESP for IBM (Zucker, MPI Munich) was utilized to filter digitally the samples at 2.5 kHz and semi-automatically analyse the data offline. Kinetics fits to the data were made by a least squares routine in AUTESP. Only results from stable cells were accepted for inclusion in the final analysis.

Superior colliculi were obtained from rat embryos (E20 to E21) and were then transferred to calcium- and magnesium-free Hank's buffered salt solution (Gibco) on ice. Cells were mechanically dissociated in 0.05% DNAase/0.3% ovomucoid (Sigma) following a 15 min preincubation with 0.66% trypsin/0.1% DNAase (Sigma). The dissociated cells were then centrifuged at 18 g for 10 min, resuspended in minimum essential medium (MEM, Gibco) and plated at a density of 200,000 cells cm^{-2} onto poly-L-lysine (Sigma)-precoated plastic petri dishes (NUNC). The cells were nourished with NaHCO_3 /HEPES-buffered MEM supplemented with 5% foetal calf serum and 5% horse serum (Gibco) and incubated at 37°C with 5% CO_2 . The MEM was completely exchanged with MEM containing a low concentration of ketamine

(20 μM , Sigma) to block the toxic effects of glutamate present in serum, following inhibition of further glial mitosis with cytosine- β -D-arabinofuranoside (20 μM Sigma) after about 7 days *in vitro*. Thereafter the MEM was partially exchanged twice weekly.

The contents of the intracellular (electrode) solution were as follows (mM): CsCl 120, TEACl 20, EGTA 10, MgCl_2 1, CaCl_2 0.2, glucose 10, ATP 2, adenosine 3': 5'-cyclic monophosphate (cyclic AMP) 0.25. The extracellular solutions had the following basic salt composition (mM): NaCl 140, KCl 3, glucose 10, HEPES 10, CaCl_2 0.2, sucrose 4.5. In addition, neurones were pharmacologically isolated from one another by the inclusion of 0.5 to 1.0 μM tetrodotoxin to block voltage-activated sodium currents and bicuculline methiodide (25 μM) to block spontaneous γ -aminobutyric acid-mediated miniature inhibitory postsynaptic potentials. Test substances were added to this basic solution in concentrations detailed in results. In addition, the bath solution and one superfusion solution contained 1.5 mM CaCl_2 to aid formation of a 'giga Ohm seal'.

The following drugs (with sources) were used: N-methyl-D-aspartate (Sigma), glycine (Sigma), L-alanine (Sigma), 7-chlorokynurenic acid (Tocris) (\pm)-amino-phosphonovaleric acid (APV, Tocris) and bicuculline methiodide (Sigma).

Results

The first series of experiments were undertaken to investigate the effects of glycine on the absolute magnitude and degree of desensitization of responses to NMDA. Cells were exposed

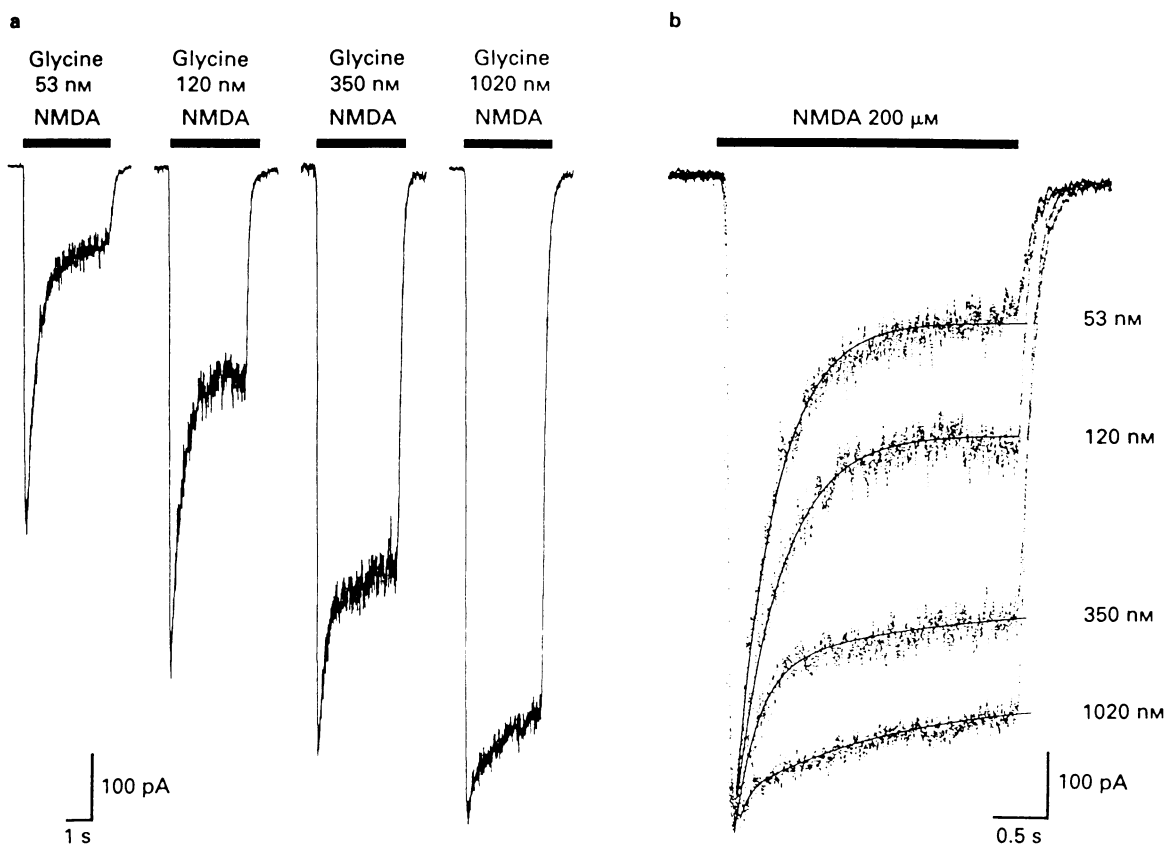


Figure 1 Desensitization of responses to N-methyl-D-aspartate (NMDA)-dependence of the magnitude and kinetics on glycine concentration. NMDA (200 μM) was applied for 2.5 s every 95 s in the continuous presence of various, corrected concentrations of glycine (see Results). (a) NMDA responses of a single cell in the presence of glycine 53 nM, 120 nM; 350 nM and 1020 nM are shown from left to right. (b) Superimposed responses following normalization of the peak response. Note the decrease in magnitude but faster kinetics of desensitization in the presence of increasing concentrations of glycine. The rate of desensitization seen with higher concentrations of glycine 350 nM and 1020 nM was best described by a double exponential fit (350 nM τ 201 and 1546 ms, 1020 nM τ 80 and 1616 ms) whereas the desensitization seen with lower concentrations was best described by a single exponential fit (53 nM τ 414ms, 120nM τ 395 ms).

to a stable concentration of glycine before, during and after application of NMDA 200 μM . Responses to NMDA in the continuous presence of glycine 53 nM showed profound desensitization ($\tau_{\text{off}} 417 \pm 12$ ms, $n = 42$) such that the plateau response at the end of a 2.5 s application of NMDA was around 35% of the peak response. Increasing the concentration of glycine from 53 nM to 1020 nM caused a progressive potentiation of current responses to NMDA which was more evident for the plateau response than for the peak response i.e. the 'degree of desensitization was inversely related to glycine concentration (Figure 1a). The change in the relative magnitude of desensitization was even more apparent when the responses were normalized such that the magnitude of the peak response was the same (Figure 1b). Plateau responses recorded with 2.5 μM glycine were $873\% \pm 101\%$ greater than those recorded with glycine 30 nM whereas this potentiation was only $260\% \pm 27\%$ for peak responses. Analysis of pooled data illustrated in Figure 2a indicates that this desensitization is due to a five fold decrease in the affinity of glycine_B sites after activation of the NMDA receptor-channel complex by NMDA with a resultant rightward shift in the glycine concentration-response curve of plateau compared to peak responses. Thus, glycine potentiated peak responses with an A_{50} of 77 nM (95% confidence limits, 58- 104 nM) whereas the A_{50} for plateau responses was 399 nM (340-468 nM). The potency of glycine in preventing desensitization was between these two values, i.e. the A_{50} was 139 nM (121- 160 nM) when the ratio of plateau over peak current was plotted against glycine concentration (Figure 2b).

It should be noted that all glycine concentration-response curves were corrected for contamination of solutions with a background glycine concentration of around 20 nM. This value was estimated by extrapolation of the linear region of the glycine concentration-response curve (10 to 100 nM) and probably accounts for, the small response seen with NMDA in the absence of added glycine (data not shown). In contrast, glycine 1 μM evoked no response in the absence of added NMDA indicating the absence of contaminating concentrations of glutamate or aspartate.

The rate of desensitization seen with glycine 30 nM was similar to the τ_{off} following step changes in glycine concentration from 1 μM to nominally zero (approx 20 nM) in the continuous presence of NMDA 200 μM ($\tau_{\text{off}} 524 \pm 16$ ms, $n = 25$) indicating the possibility that the true rate of change in the affinity of the glycine_B site is much faster but is masked by the slow dissociation kinetics of glycine from its recognition site. If this were exclusively the case, then the rate of desensitization should be independent of glycine concentration. However, a reciprocal plot of desensitization rate ($1/\tau$ s⁻¹) against corrected glycine concentration had a slope of $9.5 \cdot 10^6 \text{ M}^{-1} \text{ S}^{-1}$ indicative of an influence of glycine concentration on the rate of desensitization (Figure 3) similar to that observed by Benveniste *et al.* (1990a). They attributed this effect to a glycine-dependent increase in the rate constant for recovery from desensitization with little direct effect on the rate constant for onset of desensitization.

As such, further experiments with step increases in the concentration of glycine in the continuous presence of NMDA were performed to test this hypothesis. Responses to glycine 30 nM showed profound desensitization whereas responses of the same cells to glycine 350 nM exhibited much less desensitization (Figure 4a). Following desensitization of responses in glycine 30 nM a step change was made to glycine 350 nM in the continued presence of NMDA. The evoked current increased to a new plateau level with a time course faster than the desensitization ($\tau_{\text{on}} 205 \pm 9$ ms, $n = 5$) and similar to the association rate of glycine 350 nM (Benveniste *et al.*, 1990a) suggesting that the association kinetics of glycine are the rate limiting step in this process. This assumption was supported by the results of similar experiments with L-alanine which associates more quickly than glycine at the glycine_B site at the higher concentrations needed. Thus, following step changes in L-alanine concentration from 3.3 to

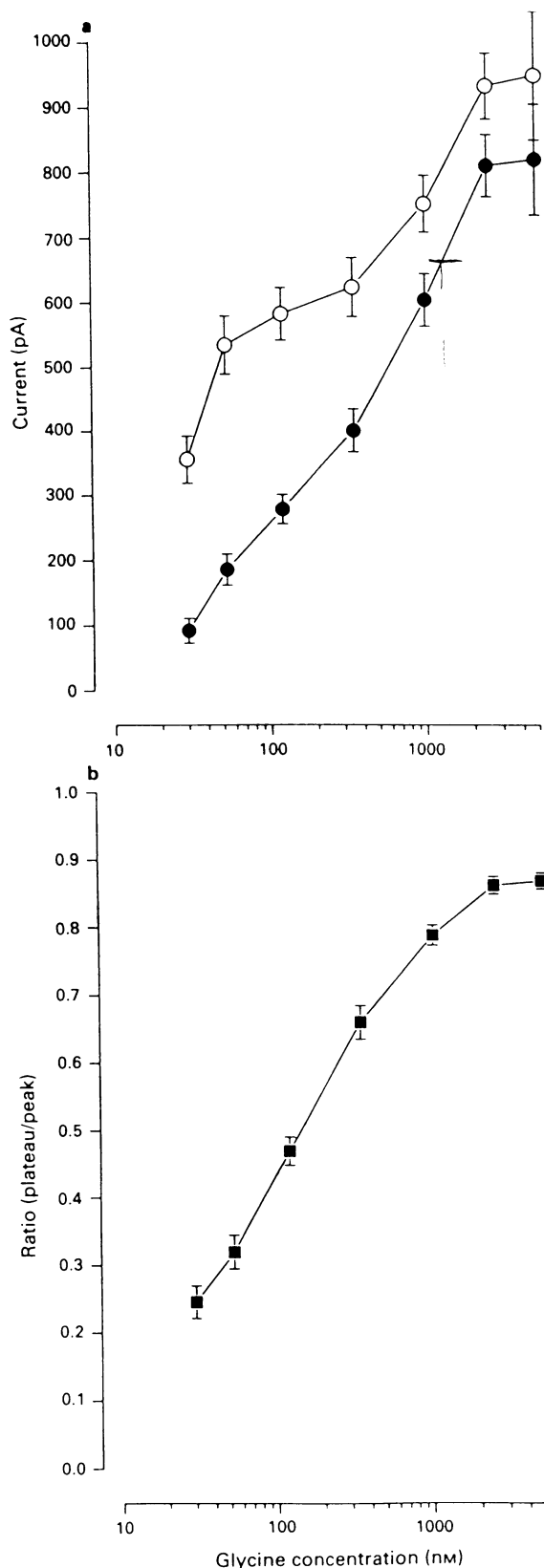


Figure 2 Concentration-dependent facilitation of peak and plateau responses to N-methyl-D-aspartate (NMDA) by glycine. NMDA (200 μM) was applied as in Figure 1. Pooled data are plotted as means (\pm s.e.mean) against corrected glycine concentration. (a) Desensitization of NMDA responses was accompanied by a rightward shift in the glycine concentration-response curve for plateau responses (●) compared to peak responses (○). (b) Responses to NMDA showed less desensitization in the presence of increasing concentrations of glycine. See Table 1 for the number of cells tested at each concentration.

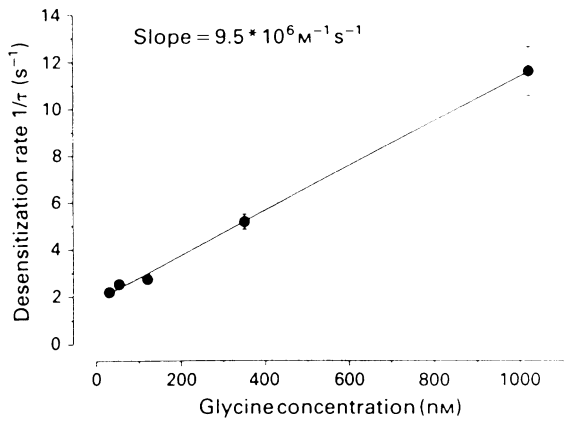


Figure 3 Glycine concentration-dependent change in the rate of N-methyl-D-aspartate (NMDA) receptor desensitization. NMDA (200 μ M) was applied as in Figure 1. The rate of desensitization seen with glycine 350 nM and 1020 nM was best described by a double exponential fit, the faster component of which is illustrated (see discussion). Pooled data are plotted as mean reciprocal desensitization rate (\pm s.e.mean) against corrected glycine concentration. Linear regression analysis was used to determine the slope of $9.51 \times 10^6 \text{ m}^{-1} \text{ s}^{-1}$.

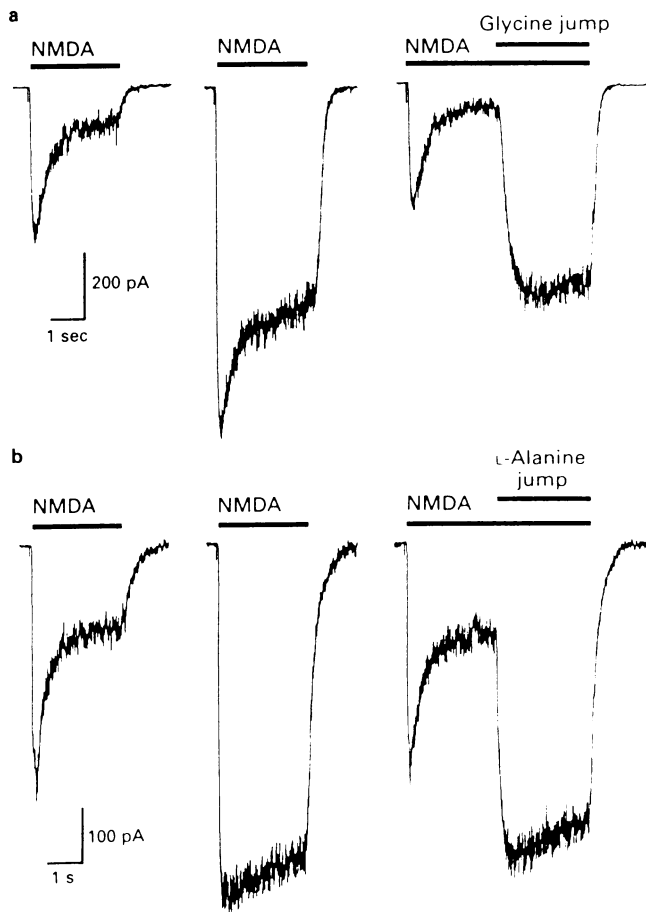


Figure 4 Recovery of N-methyl-D-aspartate (NMDA) receptor desensitization following step increases in glycine or L-alanine concentration. NMDA (200 μ M) was applied for 2.5 s (left and middle) or 5 s (right) every 95 s in the continuous presence of glycine (a) or L-alanine (b) NB: two different cells. Traces on the left show desensitization of NMDA responses in the presence of low concentrations of glycine (30 nM) and L-alanine (3.3 μ M). Traces in the middle show the facilitation of NMDA responses and decreased degree of desensitization in higher concentrations of glycine (350 nM) and L-alanine (33 μ M). Traces on the right show the recovery of desensitized responses following step increases in glycine (30 to 350 nM) or L-alanine (3.3 to 33 μ M) concentration 2.5 s after starting the application of NMDA. Note that this recovery was faster for L-alanine than for glycine.

33 μ M desensitized responses recovered to a new plateau level at a faster rate than that seen with glycine (Figure 4b, τ_{on} 48.1 ± 9.4 ms, $n = 8$). However, in similar experiments with step changes in glycine concentration from 53 to 350 nM the rate of recovery of responses was faster than the association rate of glycine (τ_{on} 105 ± 11 ms, $n = 6$). This finding lends support to the hypothesis that glycine concentration-dependently increases the rate of recovery from desensitization. These results suggest that the rate of observed glycine-sensitive desensitization is dependent on two opposed mechanisms: (1) the slow dissociation of glycine from the glycine site following a rapid decrease in affinity subsequent to activation of the receptor complex by NMDA and (2) a glycine concentration-dependent increase in the rate of recovery from desensitization.

As desensitization only occurs subsequent to activation of the receptor complex by NMDA and glycine it seemed pertinent to examine if the rate and degree of desensitization is dependent on biophysical parameters influencing the micro-environment of, and ion flow through, the NMDA receptor channel i.e. holding potential. This was not the case. Thus, both peak and plateau components of desensitizing responses in the presence of glycine 53 nM showed a linear current-voltage relationship with a reversal potential around 0 mV (Figure 5). Furthermore, the rates of desensitization and the onset and offset rates of NMDA responses were similar over the full voltage range (-100 to $+50$ mV) tested to those measured at -70 mV ($\tau_{\text{desensitization}}$ 377 ± 32 ms; τ_{on} 16.7 ± 3.4 ms, τ_{off} 225 ± 37 ms, $n = 7$). These data also provide evidence that glycine-sensitive NMDA receptor desensitization is a property of a homogeneous population of NMDA receptors and is not dependent on the recruitment of secondary mechanisms such as Ca^{2+} -sensitive Cl^- channels.

This conclusion was supported by experiments performed to investigate if the effects of glycine on NMDA desensitization are manifested at the single channel level. Initially, the desensitization of NMDA responses in the continuous presence of glycine 53 nM were recorded in the whole cell mode (Figure 6b). Subsequently, an outside out patch was pulled from the cell to record responses of a single NMDA channel under the same conditions. Eight representative responses to NMDA from such a patch are illustrated in Figure 6a. Single channel analysis was then performed on responses after equal division into transient and plateau components i.e. the first and last 1.25 s of the response. Although averaging of single channel data from several samples indicates that desensitization is indeed exhibited at the single channel level (Figure 6c) it seems unlikely that the small decrease in mean open time and increase in mean close time without a change in the mean amplitude (see Table 2) can solely account for this effect. This probably reflects the inability of conventional single channel analysis used to take account of the long periods of channel silence observed after an initial burst of varying duration. The degree of desensitization seen at the single channel level was less when recordings were made in the continuous presence of glycine 350 nM (Figure 7).

Glycine-sensitive desensitization may represent an endogenous mechanism to differentiate between transient synaptic activation of NMDA receptors and sustained activation through increased basal levels of excitatory amino acids (EAAs). As such, the glycine site could be a promising target for pharmaceutical agents aimed at reducing the pathological consequences of sustained increases in basal EAAs and glycine known to occur following e.g. ischaemia. Indeed, the glycine_B antagonist 7-Chl-Kyn concentration-dependently induced desensitization of responses to NMDA in the continuous presence of glycine 1 μ M (Figure 7). The effect of increasing 7-Chl-Kyn was very reminiscent of that of decreasing glycine concentration i.e. the degree of desensitization was greater whereas the rate of desensitization was slower (Figure 8). This implies that the kinetics and magnitude of desensitization seen with 7-Chl-Kyn is dependent on

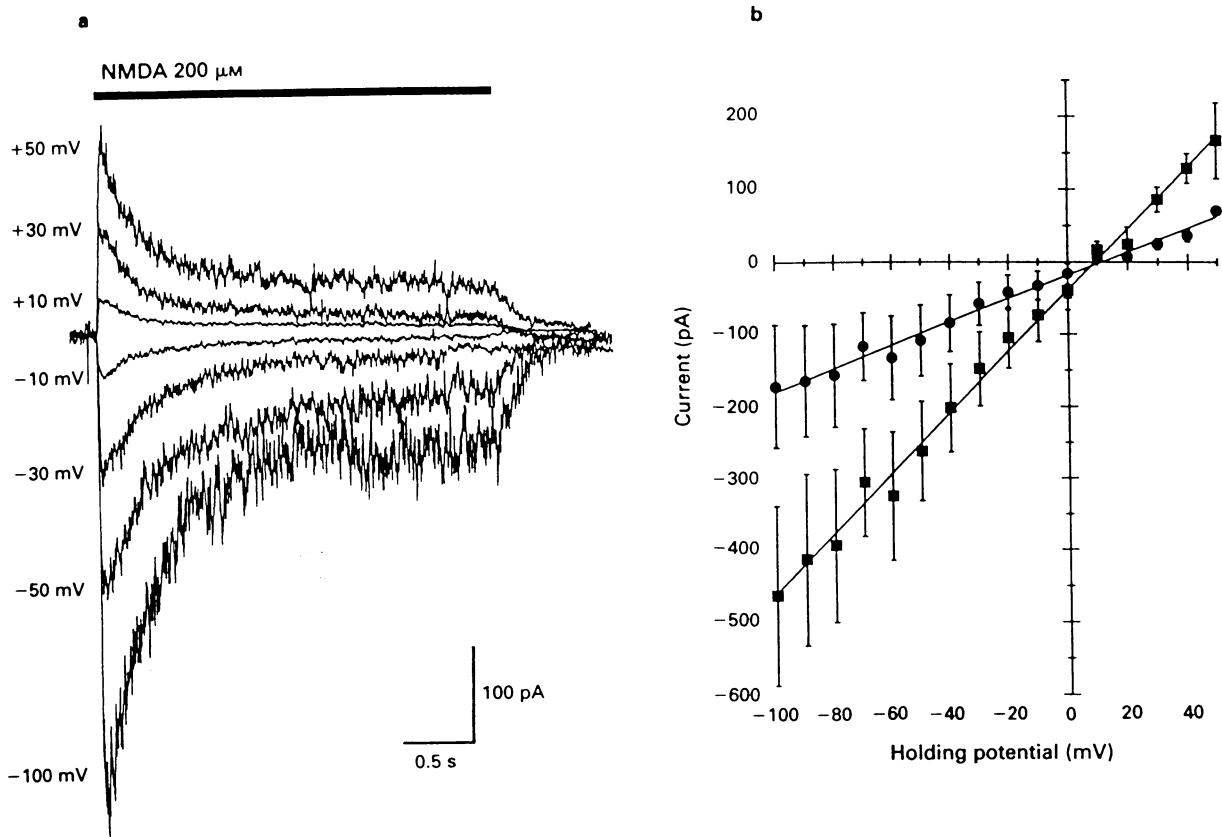


Figure 5 Voltage-independence of the rate and degree of desensitization of N-methyl-D-aspartate (NMDA) responses. (a) NMDA (200 μM) was applied for 2.5 s every 95 s in the continuous presence of glycine 53 nM and at various holding potentials (-100 mV to +50 mV). NB: step changes in holding potential were made at least 15 s before and after NMDA applications to avoid distortion of responses by voltage activated currents. (b) Pooled data showing linear current-voltage relationships for both peak (■) and plateau (●) components of responses to NMDA applied as in (a). Data show means ± s.e.mean (n = 6).

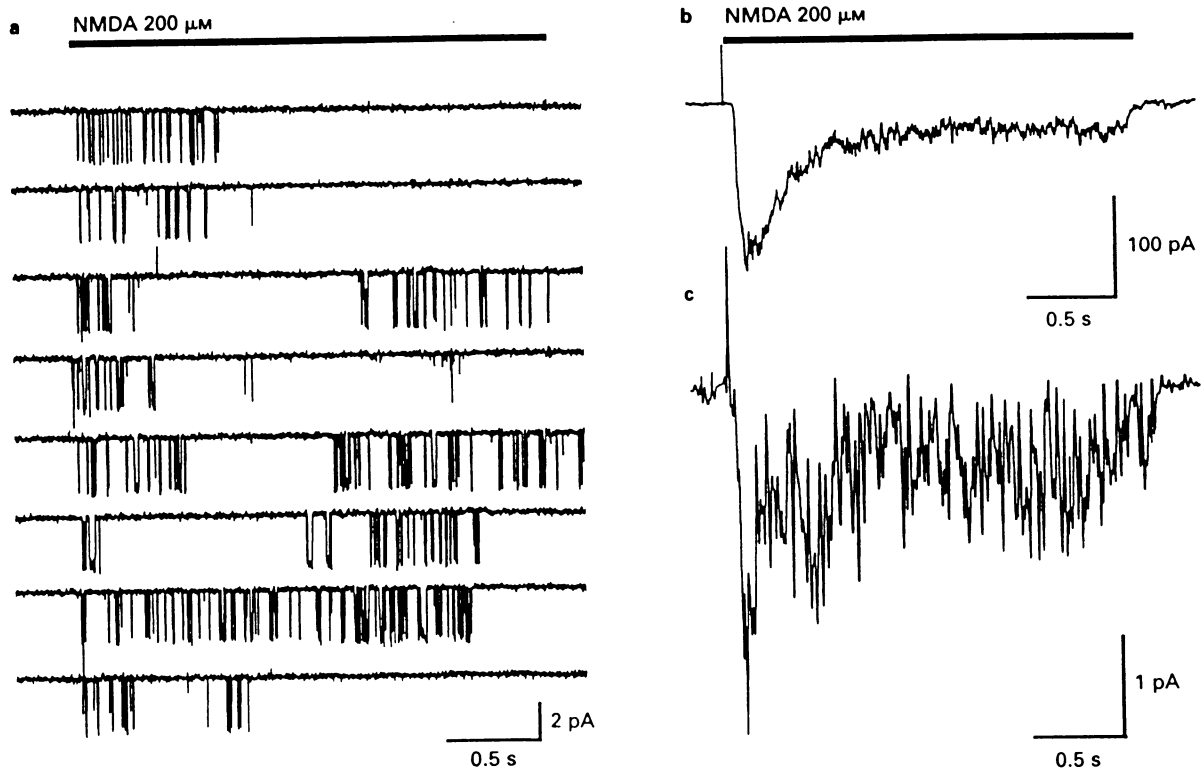


Figure 6 Glycine-sensitive desensitization of single N-methyl-aspartate (NMDA) receptor channels. Responses to NMDA 200 μM in the continuous presence of glycine 53 nM were first recorded in whole cell mode (b). Subsequently a patch with a single NMDA channel was pulled from the same cell. Representative single responses to eight applications to NMDA are illustrated. (a). Averaging of single channel responses to 33 NMDA applications (c) revealed a similar degree and kinetics of desensitization to that seen in the whole cell mode.

Table 1 Effects of glycine concentration on the degree and kinetics of NMDA receptor desensitization

Conc (nM)	n	Peak		Plateau		τ_{on}		Desen 1		Desen 2		τ_{off}	
		(pA)	s.e.mean	(pA)	s.e.mean	(ms)	s.e.mean	(ms)	s.e.mean	(ms)	s.e.mean	(ms)	s.e.mean
2520	45	934	51	813	47	24.4	0.9	n/c	n/c	n/c	n/c	103	10
1020	52	753	44	604	41	30.2	2.5	111	8	1715	212	153	13
350	46	624	45	401	34	25.6	2.5	216	10	1689	266	208	16
120	47	583	41	279	23	36.4	3.1	383	15	n/c	n/c	180	20
53	42	534	45	186	24	35.4	4.1	417	12	n/c	n/c	143	12
30	19	356	37	92	19	53.7	9.1	479	24	n/c	n/c	131	47

NMDA was applied as in Figure 1. The rate of desensitization seen with glycine 350 nM and 1020 nM was best described by a double exponential fit, both components of which are presented (Desen 1 and Desen 2). The rise and decay times of responses to NMDA are respectively given under τ_{on} and τ_{off} . n/c = not calculated.

Table 2 Glycine-sensitive desensitization of single NMDA receptor channels recorded from 6 outside out patches

Glycine (nM)	τ_{open} (ms)	Peak		Conductance (pS)	τ_{open} (ms)	Plateau		Conductance (pS)
		τ_{closed} (ms)	Conductance (pS)			τ_{closed} (ms)	Conductance (pS)	
53	3.7	24.4	54.7	3.3	34.4	53.6		
53	3.2	18.7	59.2	3.1	24.8	59.5		
53	5.4	40.4	53.3	4.3	44.1	53.9		
53	4.2	16.8	55.2	4.2	21.5	55.6		
350	3.7	28.9	56.7	2.9	28.2	54.8		
350	3.5	22.6	57.5	3.6	25.4	58.1		

NMDA (200 μ M) was applied for 2.5 s every 95 s in the continuous presence of glycine 53 nM or 350 nM and at a holding potential of -70 mV. The data from 30 to 40 NMDA applications per patch were used for conventional analysis of changes in channel kinetics after division of responses into peak and plateau components.

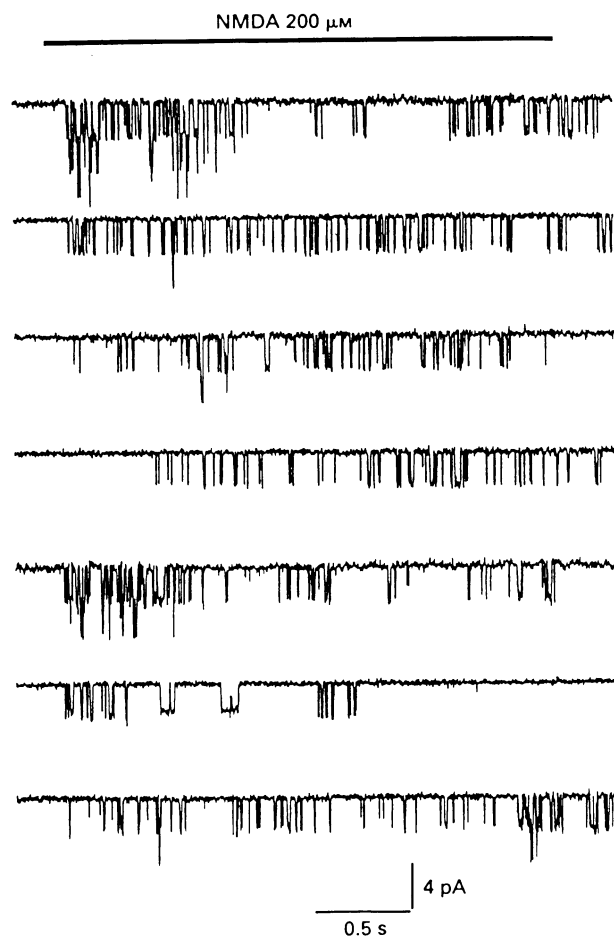


Figure 7 Desensitization of N-methyl-D-aspartate (NMDA) receptor channels is less evident at higher concentrations of glycine. Responses to NMDA 200 μ M in the continuous presence of glycine 350 nM were recorded from an outside-out patch. Representative responses to seven applications of NMDA are illustrated. Note the simultaneous activity of 2 or 3 single channels in this patch at the beginning of some responses to NMDA.

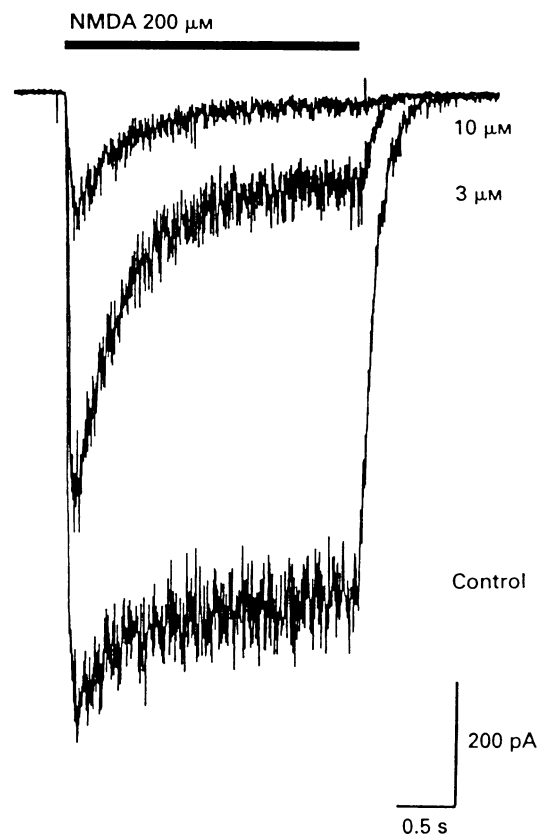


Figure 8 Desensitization of responses to N-methyl-D-aspartate (NMDA) induced by 7-chlorokynurenic acid (7-Chl-Kyn): dependence of the magnitude and kinetics on 7-Chl-Kyn concentration. Responses of a single cell to NMDA (200 μ M) in the continuous presence of glycine 1 μ M and various concentrations of 7-Chl-Kyn (0 μ M, 3 μ M and 10 μ M) have been superimposed.

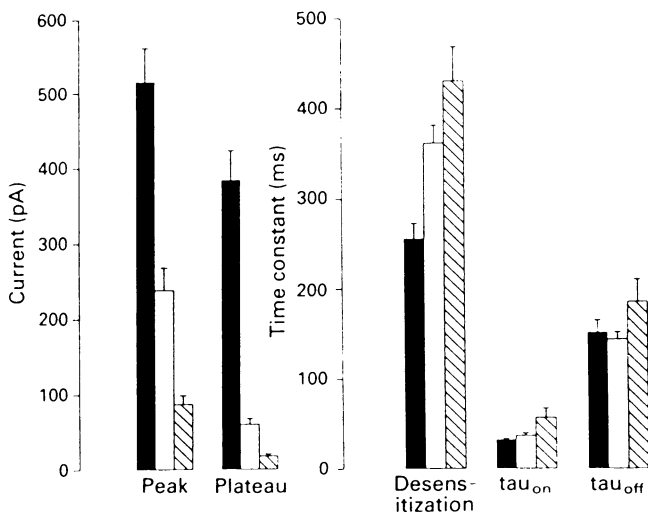


Figure 9 (a) Pooled peak and plateau responses to N-methyl-D-aspartate (NMDA, 200 μ M) in the continuous presence of glycine 1 μ M and various concentrations of 7-chlorokynurenic acid (7-Chl-Kyn). (b) Pooled kinetic analysis of responses to NMDA (200 μ M) in the continuous presence of glycine 1 μ M and various concentrations of 7-Chl-Kyn. Pooled data are presented as means \pm se-mean ($n = 9$). Filled columns = control, open columns = 7-Chl-Kyn 3 μ M; hatched columns = 7-Chl-Kyn 10 μ M

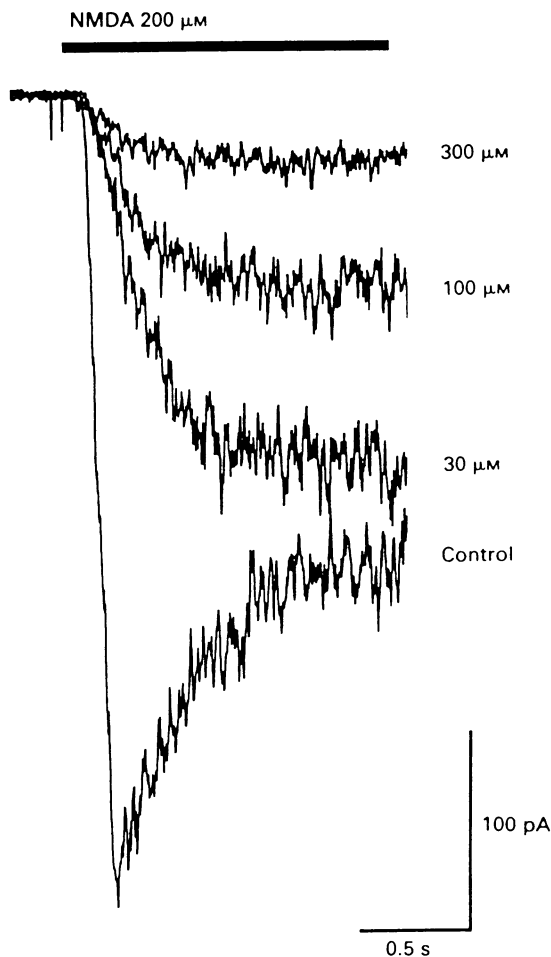


Figure 10 Concentration-dependent antagonism of and slowing in the onset kinetics of N-methyl-D-aspartate (NMDA) responses by (\pm)-amino-phosphonovaleric acid (APV). Responses of a single cell to NMDA (200 μ M) in the continuous presence of glycine 120 nM and various concentrations of APV (control, 30 μ M, 100 μ M and 300 μ M) have been superimposed.

the residual interaction of glycine with the glycine site. Moreover, τ_{on} and τ_{off} for NMDA responses were not obviously dependent on the concentration of glycine or 7-Chl-Kyn (Table 1 and Figure 9). Thus it seems improbable that glycine concentration affects the affinity of NMDA for its recognition site as this would be reflected in a consistent, concentration-dependent change in the association/dissociation kinetics of NMDA.

In contrast, the competitive NMDA antagonist APV concentration-dependently antagonized peak responses to NMDA (glycine 120 nM) more than plateau responses (Figure 10) and was associated with a concentration-dependent slowing in τ_{on} for NMDA responses which probably reflects the slow dissociation of APV from the NMDA recognition site. Thus control NMDA responses had a τ_{on} of 51 ± 2.3 ms ($n = 12$) which was slowed to 151 ± 14 ms ($n = 12$), 185 ± 14 ms ($n = 8$) and 286 ± 19 ms ($n = 5$) in the presence of APV 30 μ M, 100 μ M and 300 μ M respectively.

Discussion

The effect of glycine concentration on NMDA receptor desensitization

The magnitude, kinetics and glycine-sensitivity of the desensitization seen in this study with cultured rat superior colliculus neurones was very similar to that previously reported for cultured mouse hippocampal neurones (Mayer *et al.*, 1989; Benveniste *et al.*, 1990a) and for NMDA receptors expressed in *Xenopus* oocytes (Lerma *et al.*, 1990) and indicates receptor homology between the species and brain regions studied. Furthermore, the potency of glycine in potentiating plateau responses is in good agreement with electrophysiological studies at equilibrium (Kleckner & Dingledine, 1988; Kushner *et al.*, 1988) and functional binding studies on the facilitation of MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo-[a,d]cyclohepten-5, 10-imine maleate] binding by glycine (Reynolds *et al.*, 1987; Bakker *et al.*, 1991; Pullan *et al.*, 1991).

Taken together, the data provide strong supportive evidence that a 5 fold change in the affinity of glycine_B sites after activation of the NMDA receptor-channel complex underlies the desensitization. This change was reflected by a rightward shift of the glycine concentration-response curve for plateau responses compared to peak responses. The rate of desensitization became faster with increasing glycine concentration with a slope of 9.5×10^6 M⁻¹ S⁻¹. At higher concentrations of glycine the desensitization was best described by a double exponential fit, presumably due to the appearance of slower, calcium-dependent desensitization. This effect of glycine is in good agreement with the data from Benveniste *et al.* (1990a) and has been attributed to a glycine-dependent increase in the rate constant for recovery from desensitization with little effect on the rate constant for onset of desensitization.

The similarity in the rate of desensitization in the presence of glycine 30 nM with the dissociation kinetics of glycine (see also Benveniste *et al.*, 1990a) is indicative that slow dissociation of glycine from the glycine_B site is the rate limiting step in the desensitization process at low nM concentrations of glycine. As such, one would expect the rate of desensitization with L-alanine to be much faster than with glycine due to the faster dissociation kinetics of L-alanine from the glycine site (Benveniste *et al.*, 1990b). At first site this did not seem to be the case, single exponential fits of the rate of desensitization seen with L-alanine were very similar to those obtained in the presence of low nM concentrations of glycine (most data not shown but see Figure 4b). However, it is likely that this reflects contamination of all solutions with around 20 nM glycine, a concentration similar to that estimated by Lerma *et al.* (1990) and Benveniste *et al.* (1990a). These problems confounded similar quantitative analysis of peak and plateau concentration-response curves for L-alanine.

Step increases in glycine or L-alanine concentration

Further evidence that the desensitization is due to a change in the affinity of glycine_B sites was provided by experiments where step increases in glycine or L-alanine concentration were made in the continuous presence of NMDA. The rates of recovery of desensitized responses following step changes in glycine and L-alanine concentration were similar to their respective τ_{ns} (see Benveniste *et al.*, 1990b) i.e. responses recovered more quickly following a step change in L-alanine than following a step change in glycine. However, it seems unlikely that the recovery rate of responses was exclusively dependent on the association rate of the glycine agonists used as the rate of recovery was slower following step changes in glycine from 30 nM to 350 nM than following changes from 53 nM to 350 nM. Thus, it is probable that the forward rate constant with which glycine binds to its recognition site (Benveniste *et al.*, 1990a) also influences the rate of desensitization.

Activation of the NMDA receptor complex by agonists at both the NMDA and glycine recognition sites is necessary for desensitization

It has previously been claimed that binding of aspartate to the NMDA recognition site in the absence of channel activation i.e. in the absence of glycine, is, per se, sufficient to initiate a change in the affinity of glycine_B sites (Chizhnikov *et al.*, 1990). However, contamination of solutions with low nM concentrations of glycine is unavoidable and thus invalidates the use of preincubations with an NMDA agonist in the absence of added glycine to test this hypothesis. Thus the small response to NMDA without added glycine seen in our study was almost certainly attributable to the estimated contamination of all solutions with 20 nM glycine. Although the authors claimed that such preincubations evoked no responses, the contrary was evident from the data presented and they themselves provided evidence for some degree of glycine contamination as preincubation with aspartate plus the glycine_B antagonist 7-Chl-kyn was unable to influence subsequent desensitization.

It therefore seems likely that activation of the NMDA receptor complex is necessary for desensitization. However, the desensitization seen at low nM concentrations of glycine does not seem due to changes in the conductance of NMDA channels or recruitment of secondary mechanisms such as Ca²⁺-activated Cl⁻ channels. Thus the rate and relative magnitude of desensitization seen in the presence of 53 nM glycine was not dependent on holding potential. Both peak and plateau responses showed a linear current voltage relationship with a reversal potential of around 0 mV. This is as would be predicted when both components of the current are directly attributable to ion flow through NMDA channels under the conditions used and in the absence of Mg²⁺ and provides strong evidence against a role of Ca²⁺-activated Cl⁻ channels (see Leonard & Kelso, 1990) or pH-sensitive Na⁺ channels (see Grantyn & Lux, 1988) in mediating the desensitization.

Single channel analysis

Glycine-sensitive desensitization was also observed at the single channel level. This effect was partially manifested as a small decrease in the mean open time and a small increase in the mean close time without an effect on channel conductance. However, the observed changes in these conventional parameters were too small to account exclusively for the desensitization. More pronounced changes were apparent in the pattern of firing. Single channel responses to NMDA in the presence of glycine 53 nM were characterized by an initial burst of channel activity of variable duration followed by long periods of channel inactivity. This was much less evident for single channel responses recorded in the continuous

presence of glycine 350 nM. It is tempting to speculate that these bursts occur whilst glycine remains bound to the glycine_B site for hundreds of milliseconds and that the periods of inactivity occur following dissociation of glycine.

Ca²⁺-sensitive NMDA receptor desensitization

Although these data indicate that the desensitization seen at low nM concentrations of glycine can be attributed to changes in the kinetics of a homologous species of NMDA receptors it should be noted that the second slower component of desensitization which became apparent at concentrations of glycine greater than 350 nM had a time course which was similar to that seen in the presence of 1.5 mM Ca²⁺ (data not shown). This probably reflects the appearance of Ca²⁺-sensitive desensitization following near maximal activation of NMDA receptors and probably accounts for the inability of even the highest concentrations of glycine to eliminate desensitization completely. Further studies in the complete absence of extracellular calcium and/or blockers of Ca²⁺-activated Cl⁻ channels such as flufenamic acid are required to perform quantitative concentration-response analysis at higher concentrations of glycine and to assess the nature of this Ca²⁺-sensitive desensitization.

7-Chlorokynurenic acid

This study confirmed the finding that the glycine_B antagonist, 7-Chl-Kyn, induces desensitization of NMDA responses in a manner reminiscent to that of decreasing glycine concentration i.e. the concentration-dependent increase in the magnitude of desensitization was accompanied by a slowing in kinetics. As the association/dissociation kinetic of 7-Chl-Kyn are much faster than those of glycine (Benveniste & Mayer, 1991a) it seems likely that the kinetics of the desensitization seen in the presence of 7-Chl-Kyn is dependent on the residual interaction of glycine with the glycine_B site. Moreover, the affinity of NMDA for its recognition site was not influenced by changes in glycine or 7-Chl-Kyn concentration as this would have been reflected in changes in the τ_{on} and/or τ_{off} of NMDA responses.

Buffered diffusion

It should be noted that the on and off rates of NMDA responses were slower than those reported in mouse hippocampal neurones (Patneau & Mayer, 1990) but faster than those reported for *Xenopus* oocytes (Lerma *et al.*, 1990). It has been pointed out that such differences can be attributed to slow solution exchange times. This seems unlikely as the exchange time was estimated to be under 10 ms and responses to AMPA under the same conditions were very fast (τ_{on} around 6 to 8 ms, $\tau_{desensitization}$ around 30 ms, data not shown). It seems more likely that the phenomenon of 'buffered diffusion' in the restricted extracellular space between neurones and glial cells could partially account for this difference and highlights the necessity of using young cultures with few glia when studying the on and off rates of agonists and antagonists. However, this phenomenon should not affect glycine-sensitive desensitization if this process is indeed dependent on a change in the affinity of glycine_B sites in a constant concentration of agonist.

Therapeutic consequences

Glycine-dependent desensitization could have very important consequences for patho-physiological processes such as those occurring following ischaemia or in cerebrovascular dementia as regional parallel increases in the extracellular levels of glycine and glutamate have been reported following an ischaemic insult (Baker *et al.*, 1991; Globus *et al.*, 1991 a,b). This might be expected to worsen the damage caused by excessive NMDA receptor activation by preventing desen-

sitization. Systemically active glycine_B antagonists could therefore prove to be useful therapeutic tools as they might be expected to facilitate desensitization of NMDA receptors following sustained activation by pathologically raised levels of EAA. Moreover, although the NMDA component of excitatory postsynaptic currents mediated at glutamatergic synapses has a duration of several hundred milliseconds (Lester et al., 1990) its time course is still within the window of glycine-sensitive desensitization and, as such, would be expected to be relatively less affected by glycine antagonists. It is interesting to note that the same does not appear to be true for + HA-966 which is a partial agonist at the glycine_B site but suppresses peak responses to a greater degree than plateau responses and reduces the affinity of glutamate for the NMDA recognition site (Kemp & Priestley, 1991). This effect is similar to that seen with competitive NMDA antagonists (see also Benveniste & Mayer 1991 b) and both classes of antagonist might therefore be expected to influence

synaptic transmission more than pathological processes and will probably be burdened with a variety of side effects similar to those seen with uncompetitive NMDA channel blockers such as phencyclidine and MK-80 1.

In conclusion, glycine-sensitive, NMDA receptor desensitization is due to a rapid change in the affinity of glycine for glycine_B receptors following activation of the receptor-channel complex by NMDA. The rate of desensitization seen at low concentrations of glycine is largely dependent on the dissociation kinetics of glycine and is likely to be slower than the NMDA-induced change in the properties of the glycine site. At higher concentrations, the forward rate at which glycine binds to its recognition site also plays a role in determining the observed rate of desensitization.

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, Germany.

References

- BAKER, A.-J., ZORNOW, M.H., SCHELLER, M.-S., YAKSH, T.-L., SKILLING, S.-R., SMULLIN, D.-H., LARSON, A.A. & KUCZENSKI, R. (1991). Changes in extracellular concentrations of glutamate, aspartate, glycine, dopamine, serotonin, and dopamine metabolites after transient global ischemia in the rabbit brain. *J. Neurochem.*, *57*, 1370-1379.
- BAKKER, M.H.M., McKERNAN, R.-M., WONG, E.H.F. & FOSTER, A.C. (1991). ³H-MK-801 binding to N-methyl-D-aspartate receptors solubilized from rat brain - effects of glycine site ligands, polyamines, ifenprodil, and desipramine. *J. Neurochem.*, *57*, 39-45.
- BENVENISTE, M., CLEMENTS, J., VYKLYCKY, L. Jr. & MAYER, M.L. (1990a). A kinetic analysis of the modulation of N-methyl-D-aspartate receptors by glycine in mouse cultured hippocampal neurones. *J. Physiol.*, *428*, 333-357.
- BENVENISTE, M. & MAYER, M.L. (1991 a). Kinetic analysis of antagonist action at N-methyl-D-aspartate receptors. Two binding sites each for glutamate and glycine. *Biophysical J.*, *59*, 560-573.
- BENVENISTE, M. & MAYER, M.L. (1991 b). Structure-activity analysis of binding kinetics for NMDA receptor competitive antagonists - the influence of conformational restriction. *Br. J. Pharmacol.*, *104*, 207-221.
- BENVENISTE, M., MIENVILLE, J.-M., SERNAGOR, E. & MAYER, M.L. (1990b). Concentration-jump experiments with NMDA antagonists in mouse cultured hippocampal neurons. *J. Neurophysiol.*, *63*, 1373-1384.
- CHIZHMAKOV, IV., KISKIN, N.I., TSYNDRENKO, A.Y. & KRISHNAN, O.A. (1990). Desensitization of NMDA receptors does not proceed in the presence of kynurenic acid. *Neurosci. Lett.*, *108*, 88-92.
- CLARK, G.-D., CLIFFORD, D.B. & ZORUMSKI, C.F. (1990). The effect of agonist concentration, membrane voltage and calcium on N-methyl-D-aspartate receptor desensitization. *Neuroscience*, *39*, 787-797.
- GLOBUS, M.Y.T., BUSTO, R., MARTINEZ, E., VALDES, I., DIETRICH, W.D. & GINSBERG, M.D. (1991a). Comparative effect of transient global ischemia on extracellular levels of glutamate, glycine, and gamma-aminobutyric acid in vulnerable and nonvulnerable brain regions in the rat. *J. Neurochem.*, *57*, 470-478.
- GLOBUS, M.Y.T., GINSBERG, M.D. & BUSTO, R. (1991 b). Excitotoxic index - a biochemical marker of selective vulnerability. *Neurosci. Lett.*, *127*, 39-42.
- GRANTYRN, R. & LUX, H.D. (1988). Similarity and mutual exclusion of NMDA- and proton-activated transient Na⁺-currents in rat tectal neurons. *Neurosci. Lett.*, *89*, 198 - 203.
- HENDERSON, G., JOHNSON, J.W. & ASCHER, P. (1990). Competitive antagonists and partial agonists at the glycine modulatory site of the N-methyl-D-aspartate receptor. *J. Physiol.*, *430*, 189-212.
- JOHNSON, J.W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, *325*, 529-531.
- KEMP, J.-A. & PRIESTLEY, T. (1991). Effects of (+)-HA-966 and 7-chlorokynurenic acid on the kinetics of N-methyl-D-aspartate receptor agonist responses in rat cultured cortical neurons. *Mol. Pharmacol.*, *39*, 666 - 670.
- KLECKNER, N.-W. & DINGLEDINE, R. (1988). Requirements for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. *Science*, *214*, 835-837.
- KONNERTH, A., LUX, H.D. & MORAD, M. (1987). Proton-induced transformation of calcium channel in chick dorsal root ganglion cells. *J. Physiol.*, *386*, 603-633.
- KUSHNER, L., LERMA, J., ZUKIN, R.-S. & BENNETT, V.L. (1988). Coexpression of N-methyl-D-aspartate and phencyclidine receptors in *Xenopus* oocytes injected with rat brain mRNA. *Proc. Natl. Acad. Sci. U.S.A.*, *85*, 3250-3254.
- LEONARD, J.-P. & KELSO, S.R. (1990). Apparent desensitization of NMDA responses in *Xenopus* oocytes involves calcium-dependent chloride current. *Neuron*, *4*, 53-60.
- LERMA, J., ZUKIN, R.S. & BENNETT, M.V.L. (1990). Glycine decreases desensitization of N-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus* oocytes and is required for NMDA responses. *Proc. Natl. Acad. Sci. U.S.A.*, *87*, 2354-2358.
- LESTER, R.-A.-J., CLEMENTS, J.-D., WESTBROOK, G.L. & JAHR, C.E. (1990). Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents. *Nature*, *346*, 565-568.
- MAYER, M.L., VYKLYCKY, L. & CLEMENTS, J. (1989). Regulation of NMDA receptor desensitization in mouse hippocampal neurones by glycine. *Nature*, *338*, 425-427.
- PATNEAU, D.K. & MAYER, M.L. (1990). Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J. Neurosci.*, *10*, 2385-2399.
- PULLAN, L.-M., VERTICELLI, A.M. & PASCHKE-ITO, K.A. (1991). Agonist-like character of the (R)-enantiomer of 1-hydroxy-3-amino-pyrrolid-2-one (HA-966). *Eur. J. Pharmacol. - Mol. Pharmacol.*, *208*, 25-29.
- REYNOLDS, I.-J., MURPHY, S.N. & MILLER, R.J. (1987). ³H-labeled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. *Proc. Natl. Acad. Sci. U.S.A.*, *84*, 7744-7748.
- SATHER, W., DIEUDONNE, S., MACDONALD, J.-F. & ASCHER, P. (1992). Activation and desensitization of N-methyl-D-aspartate receptors in nucleated outside-out patches from mouse neurones. *J. Physiol.*, *450*, 643-672.
- ZILBERTER, Y., UTESHEV, V., SOKOLOVA, S. & KHODOROV, B. (1991). Desensitization of N-methyl-D-aspartate receptors in neurons dissociated from adult rat hippocampus. *Molec. Pharmacol.*, *40*, 337-341.

(Received August 19, 1992
Revised January 4, 1993
Accepted January 19, 1993)