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COMPETITIVE ANTAGONISTS AND PARTIAL AGONISTS AT THE GLYCINE MODULATORY SITE OF THE MOUSE *N*-METHYL-D-ASPARTATE RECEPTOR

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SUMMARY

1. Kynurenate (Kyn), 7-chlorokynurenate (7-Cl-Kyn), 3-amino-1-hydroxy-pyrrolid-2-one (HA-966) and D-cycloserine are known to bind to the glycine site that modulates the *N*-methyl-D-aspartate (NMDA) response of vertebrate central neurones. The effects of these compounds were investigated with patch-clamp and fast-perfusion techniques on mouse cortical neurones in primary culture in an effort to establish whether they act as antagonists, partial agonists and/or inverse agonists of glycine. A fast drug application method allowed the study of both steady-state and transient responses.

2. The analysis of steady-state responses indicates that the main effects of Kyn and 7-Cl-Kyn are those expected from competitive antagonists of glycine, with a dissociation constant of 15 μM for Kyn, and of 0·3 μM for 7-Cl-Kyn. Concentration jumps indicate that at all concentrations of glycine, and in particular in the absence of added glycine, the blockade by Kyn and 7-Cl-Kyn develops at a rate which is close to the rate of dissociation of glycine from its binding site and is independent of antagonist concentration.

3. The main effects of D-cycloserine and of HA-966 are those of partial agonists of high and low efficacy, respectively. In the absence of added glycine, D-cycloserine always produced a potentiation, while HA-966 produced either a potentiation or an inhibition. This can be explained by assuming the presence of a variable level of contaminating glycine. With both D-cycloserine and HA-966, concentration jumps produced biphasic relaxations in which the onset rate of the slow component was, here again, close to the rate of dissociation of glycine from its binding site.

4. These results can be interpreted by assuming that (1) Kyn and 7-Cl-Kyn are competitive antagonists of glycine, (2) HA-966 and D-cycloserine are partial agonists, (3) in the absence of added glycine some glycine is present in the extracellular solution and (4) the response in the total absence of glycine is very small or negligible.

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INTRODUCTION

During the last three years, the observation that the NMDA responses of cultured neurones are modulated by a glycine-sensitive site (Johnson & Ascher, 1987*a*) has been extended to a variety of other experimental preparations (reviewed by Thomson, 1990). During the same period, the search for glycine antagonists has been increasingly successful. The first antagonist identified, kynurenate (Kyn), had been widely used since the initial observations of Perkins & Stone (1982) as a 'non-specific' antagonist of excitatory amino-acid-induced depolarizations, because at relatively high concentrations (hundreds of micromolar) it blocked both 'NMDA' and 'non-NMDA' responses to L-glutamate. Kessler, Baudry, Terramani & Lynch (1987) observed that Kyn displaced glycine from the 'strychnine-insensitive' binding site known to be associated with the NMDA receptor. This effect occurred at concentrations (tens of micromolar) that have little effect on glutamate binding, which suggested that at low concentrations Kyn blocked the NMDA response via a competitive antagonism of glycine at the modulatory site on the NMDA receptor. This hypothesis was supported by further studies which indicated that Kyn had a dissociation constant of 15–35 μM for the glycine site (Watson, Hood, Monahan & Lanthorn, 1988; Kessler, Terramani, Lynch & Baudry, 1989). In parallel, physiological studies of the Kyn–NMDA interactions were performed using various indices of NMDA receptor activation: currents in voltage-clamped neurones (Ascher, Henderson & Johnson, 1988*b*; Mayer, Westbrook & Vyklicky, 1988), depolarizations in hemisected spinal cord (Birch, Grossman & Hayes, 1988*a*, *b*) or cortical wedges (Fletcher, Millar, Zeman & Lodge, 1989), 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) binding (Watson *et al.* 1988; Kloog, Lamdani-Itkin & Sokolovsky, 1990) and Ca^{2+} fluxes (Reynolds, Harris & Miller, 1989). All these experiments showed that, at low micromolar concentrations, the inhibition produced by Kyn could be largely overcome by increasing the glycine concentration, while higher concentrations of Kyn revealed an additional inhibitory effect of this compound at the NMDA binding site itself.

Soon after the first reports of Kyn action, a simple derivative of this compound, 7-chlorokynurenate (7-Cl-Kyn), was found to have similar effects but with a much higher potency at the glycine site (Kemp, Foster, Leeson, Priestley, Tridgett, Iversen & Woodruff, 1988). At about the same time, it was found that HA-966, which had long been known to antagonize NMDA responses but which had remained an enigma because of the absence of any structural analogy with other NMDA antagonists (Evans, Francis & Watkins, 1978), probably owed its action to glycine antagonism since its blocking action on the NMDA response could be overcome by increasing the extracellular glycine concentration (Fletcher *et al.* 1989; Foster & Kemp, 1989). However, even at very high concentrations, HA-966 never produced a complete block of the NMDA response (Foster & Kemp, 1989; Reynolds *et al.* 1989; Danysz, Fadda, Wroblewski & Costa, 1989; Singh, Donald, Foster, Hutson, Iversen, Kemp, Leeson, Marshall, Oles, Priestley, Thorn, Tricklebank, Vass & Williams, 1990; Kloog *et al.* 1990) which led Foster & Kemp (1989) to suggest that HA-966 could be a partial agonist (Stephenson, 1956) of low efficacy, the potentiating effect of which would only be detectable in the presence of very low glycine concentrations.

More recently, new compounds acting at the glycine site have been identified (see Thomson, 1990), among which some, such as D-cycloserine (Hood, Compton & Monahan, 1989; Monahan, Corpus, Hood, Thomas & Compton, 1989; McBain, Kleckner, Wyrick & Dingledine, 1989) and 1-amino-cyclopropane carboxylic acid (Nadler, Kloog & Sokolovsky, 1988; Marvizón, Lewin & Skolnick, 1989), appear to be partial agonists of relatively high efficacy.

The work presented below was initiated soon after the first report of Kessler *et al.* (1987) and was aimed at characterizing the action of Kyn in cultured neurones bathed in a medium in which the glycine concentration was, in principle, well controlled. Our initial experiments readily confirmed that the inhibition produced by Kyn was reduced by glycine, but they also indicated that Kyn blocked the NMDA response in the absence of added glycine, suggesting that Kyn could act as an 'inverse agonist' (Ascher *et al.* 1988*b*). However, the more complete study presented below, in which we analysed the relaxations following concentration jumps as well as the steady-state responses, and in which we controlled more accurately the glycine contamination, has led us to conclude that Kyn, as well as 7-Cl-Kyn, are pure competitive antagonists of glycine, while HA-966 and D-cycloserine are partial agonists of low and high efficacy, respectively.

METHODS

All experiments were performed on primary cultures of cortical and diencephalic neurones taken from 15- to 16-day-old mouse embryos as previously described by Ascher, Bregestovksi & Nowak (1988*a*). The cells were maintained in primary culture for up to 10 weeks.

The whole-cell and outside-out configurations of the patch-clamp method (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were used to study macroscopic and single-channel currents activated by excitatory amino acids. Recording pipettes with resistances of 4–6 MΩ were coated with Sylgard, lightly fire-polished, and filled with (in mM): CsF, 120; CsCl, 20; EGTA, 10; HEPES, 10. The pH was adjusted to 7·2 using CsOH. Fluoride was chosen as the major anion because its use increased the longevity of whole-cell and patch recordings (Fernandez, Fox & Krasne, 1984); no differences in NMDA receptor properties have been observed when Cl^- is replaced by F^- in the pipette solution (Johnson & Ascher, 1987*a*). Except where stated otherwise, cells and patches were clamped at a holding potential of −50 mV. Current signals were recorded, using a List EPC-7 patch-clamp amplifier, on a Racal FM tape-recorder and a Gould chart recorder (Brush 280). Single-channel currents were replayed from magnetic tape, filtered, digitized and analysed with a Minc PDP 11/23 computer. Analysis was as described by Takeda & Trautmann (1984) and Ascher *et al.* (1988*a*).

During the period of recording, cells were bathed in a solution of the following composition (in mM): NaCl, 140; KCl, 2·8; $CaCl_2$, 1; HEPES, 10; and tetrodotoxin (0·2 μM). The pH was adjusted to 7·2 using NaOH. In addition, drugs were applied by means of the multi-barrel, fast-perfusion technique (Johnson & Ascher, 1987*a*). The composition of the solutions flowing through the fast-perfusion system was identical to that of the bathing solution except that drugs were added in known concentrations to the fast-perfusion solutions. The barrels were positioned within 100 μm of the cell under study and the solutions pumped at a rate of 0·1 ml min^{-1}. Initially the cell or patch was superfused with solution flowing from the control barrel. Change of the solution flowing around the cell or patch was accomplished by rapidly (movement duration of about 60 ms) moving the barrels such that the cell or patch lay in the path of the fluid flowing from one of the other barrels. By this means a cell or patch could be superfused with bathing solution or a solution containing a drug before a change to a solution that lacked the drug or to which other drugs had been added. The minimum speed at which this system can change solutions at NMDA receptors was measured by changing from 10 μM-glycine to 10 μM-glycine plus 10 μM-NMDA. A jump of NMDA concentration was used because its kinetics are much faster that those of glycine. The change took place

with an average time constant of 55 ms, indicating that the solution change must take place with a time constant at least this fast (J. W. Johnson & P. Ascher, in preparation). All experiments were performed at room temperature (16–26 °C).

Numerous precautions were taken to minimize glycine contamination in external solutions. Bathing solution was made fresh every day from frozen 20 × concentrated stock solution. All drug stock solutions were stored frozen, and kept on ice after being thawed for use. Drug stock solutions were diluted at least thirtyfold into bathing solution immediately before drug applications. Despite these precautions, evidence of significant glycine contamination sometimes appeared. Additionally, in some experiments, clean glassware was soaked overnight in double-distilled water before being used to prepare and hold solutions, and all containers were covered during experiments. With these precautions, glycine contamination was generally reduced to quite low levels (see Results).

Single- or double-exponential curves were fitted to whole-cell current relaxation data either using the curve-fitting routines of the AXESS data analysis programs (Axon Instruments) and an IBM AT computer, or by eye using a Minc PDP 11/23. Concentration–response data were fitted with the equations described in the text using an iterative technique based on the Simplex algorithm (Caceci & Carcheris, 1984). Best fit was defined as the set of free parameter values that maximized the correlation coefficient between the data points and the fit line. Where appropriate, results have been presented as the mean ± standard deviation.

Drugs used were: 2-amino-5-phosphonovaleric acid (APV), *N*-methyl-D-aspartic acid (NMDA) and quisqualic acid (all from Cambridge Research Biochemicals); kainic acid, Kyn and tetrodotoxin (all from Sigma); 3-(+)-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid (CPP), 7-Cl-Kyn and HA-966 (from Tocris); glycine (Prolabo). Some of the HA-966 was a gift of Dr Graham Fagg (Ciba-Geigy).

RESULTS

The steady-state responses

The NMDA responses in the absence and in the presence of glycine

In the absence of added glycine the current produced by NMDA was usually very small, even when the concentration of NMDA was raised from the usual value of 10 μM to 100 μM (except in early experiments in which no special care was taken to eliminate glycine from the perfused solution). Although some response was always measurable, it was sometimes 2–3 orders of magnitude smaller than the response in high glycine concentrations and was visible only because of the high signal-to-noise ratio of patch-clamp recording. These results resemble those of Kleckner & Dingledine (1988) and support the idea that the NMDA response is negligible in the complete absence of glycine.

In most experiments NMDA was applied for periods of 20 s separated by 20 s intervals. The responses showed some desensitization (usually slight when NMDA was applied at 10 μM, more marked when NMDA was applied at 100 μM). In the Ca^{2+}-containing solutions used, desensitization onset and desensitization recovery have both complex time courses due to the superposition of Ca^{2+}-insensitive and Ca^{2+}-sensitive desensitizations (Clark, Clifford & Zorumski, 1988; Mayer, Vyklický & Clements, 1989; Benveniste, Clements, Vyklický & Mayer, 1990*a*; Vyklický, Benveniste & Mayer, 1990; Sather, Johnson, Henderson & Ascher, 1990) but in most conditions the steady-state value was reached before the end of the 20 s application of NMDA, and recovery from desensitization was complete after a 20 s washing period.

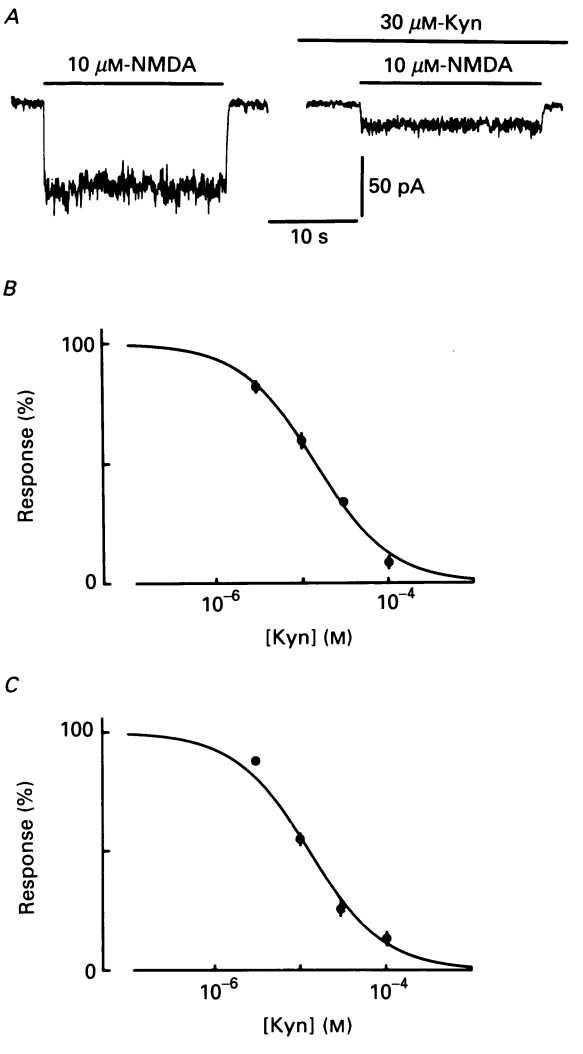

Fig. 1. Depression by Kyn of whole-cell currents evoked by NMDA in the absence of added glycine. *A*, inward currents evoked in a single cell (holding potential = −50 mV) by application of 10 μM-NMDA in the absence or presence of 30 μM-Kyn. NMDA was applied for the periods indicated; Kyn was present before and during the second application of NMDA. *B*, the amplitude of the response to 10 μM-NMDA is plotted *versus* the concentration of Kyn added to the perfusion solution. Each point represents the mean ± S.D. of data from three to six cells. The amplitudes of the responses have been normalized to the response to NMDA in the absence of Kyn. The IC_{50} for Kyn was calculated by fitting the data with the relation: % response = 100 $IC_{50}/(IC_{50}+[Kyn])$. In this figure the IC_{50} is 14·5 μM. *C*, same as in *B* except with 100 μM-NMDA. The IC_{50} is 12·7 μM.

The effects of kynurenate on NMDA responses in the absence and in the presence of added glycine

The effects of Kyn on steady-state NMDA responses were analysed at various concentrations of glycine with the objective of evaluating the dissociation constant,

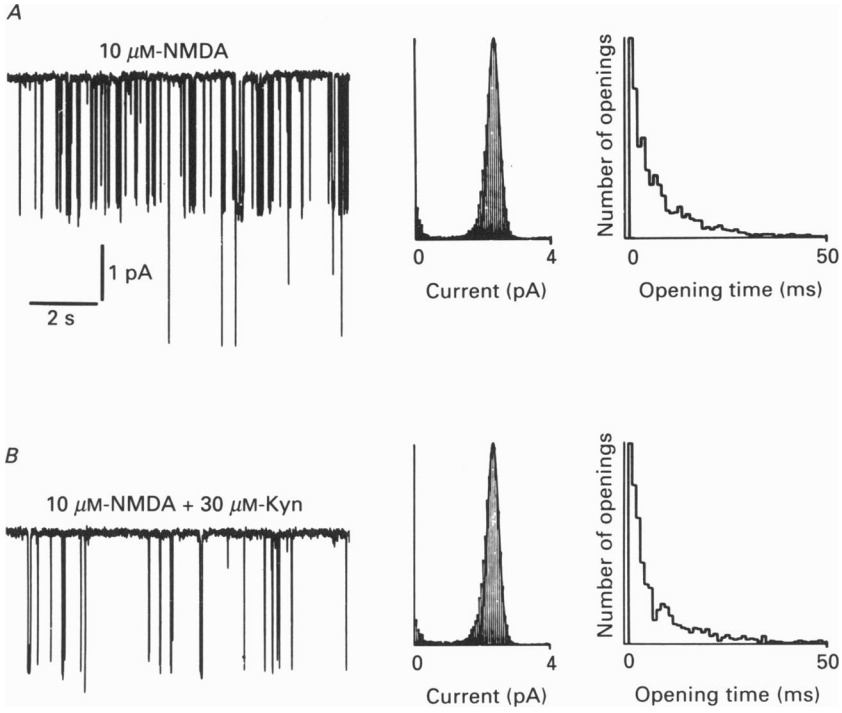

Fig. 2. Depression by Kyn of the NMDA response in an outside-out patch. Holding potential, −50 mV. Data were obtained in the presence of 10 μM-NMDA (*A*) or 10 μM-NMDA plus 30 μM-Kyn (*B*). Left, representative single-channel currents recorded on a slow time base. Centre, amplitude histograms of the single-channel currents fitted with Gaussian curves. Right, open-time histograms. For analysis currents were filtered at 2 kHz and digitized at 5 kHz. The mean single-channel conductance and arithmetic mean open time in this experiment were, respectively: in the absence of Kyn, 46·8 pS and 5·82 ms and, in the presence of Kyn, 47·0 pS and 5·52 ms.

K_{Kyn}, and to test the hypothesis proposed by Kessler *et al.* (1987) that Kyn is a competitive antagonist of glycine.

In the nominal absence of glycine, Kyn applied at concentrations of 3–300 μM depressed the whole-cell inward currents evoked by NMDA (Fig. 1*A*). The antagonism was reversed on wash-out of the drug. Figure 1*B* and *C* illustrates the dependence of the blockade on the Kyn concentration. These data were pooled from a series of experiments similar to that illustrated in Fig. 1*A*, performed at NMDA concentrations of 10 and 100 μM. The values of the Kyn concentrations producing half-maximal inhibition (IC_{50}) obtained from experiments using 10 and 100 μM-NMDA were 14·5 and 12·7 μM respectively. The absence of a significant difference

between these two values indicates that in these experiments Kyn was not acting as a competitive antagonist of NMDA.

The blocking action of non-competitive NMDA antagonists that appear to act as channel blockers, such as Mg^{2+}, phencyclidine and MK-801, is voltage-dependent and, for the last two compounds, use-dependent (see MacDonald & Nowak, 1990). In contrast, the depression by Kyn of responses to NMDA exhibited neither voltage dependence nor use dependence. The degree of blockade of responses to 10 μM-NMDA produced by 20 μM-Kyn was similar over the voltage range -80 to $+50$ mV (three experiments). When repeated applications (5×20 s applications at 20 s intervals) of 10 or 100 μM-NMDA were made in the presence of 20 μM-Kyn (Kyn being applied 15 s before the second NMDA application), no evidence for use dependence of the block was observed.

To further examine the interaction between Kyn and NMDA we determined the characteristics of the single-channel currents evoked by NMDA in the absence and presence of Kyn. Outside-out patches were superfused alternately with solutions containing NMDA (10, 30 or 100 μM) alone or NMDA (10, 30 or 100 μM) plus Kyn (30 μM). Each solution was applied for at least 30 s and the first 5 s of each application excluded from the analysis to avoid complications due to the slow development of the responses in low glycine concentrations (Johnson & Ascher, 1987*b*) and to the presence of desensitization (Sather *et al.* 1990). In the four patches analysed, Kyn produced a marked decrease in the frequency of channel opening (Fig. 2). Kynurenate did not significantly reduce the single-channel current of the main conductance state ($2{\cdot}31 \pm 0{\cdot}31$ pA, $n = 4$, for NMDA alone and $2{\cdot}35 \pm 0{\cdot}27$ pA for NMDA plus Kyn), the value of which corresponded to that previously described (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Ascher *et al.* 1988*a*). The ratio of the mean burst length in NMDA plus Kyn to that in NMDA alone was $0{\cdot}94 \pm 0{\cdot}09$ ($n = 4$). The range of mean burst lengths was 5·0–11·8 ms in NMDA plus Kyn and 5·8–12·8 ms in NMDA alone, which is similar to the range found in previous experiments (Ascher *et al.* 1988*a*). These data are consistent with an antagonistic action of Kyn at the glycine site.

As expected from a competitive antagonist of glycine, Kyn became increasingly less effective in reducing the NMDA response as the concentration of glycine was raised. When the glycine concentration–response curve was compared before and after addition of Kyn, the main effect of Kyn was to produce a shift to the right (Fig. 3). In the case of Fig. 3*B*, the concentration of glycine producing a half-maximal response (EC_{50}) shifted from 0·17 μM in the absence of Kyn to 0·43 μM in the presence of 30 μM-Kyn. The Kyn dissociation constant, K_{Kyn}, can be calculated from the shift in apparent glycine affinity using the equation

$$K_{Kyn} = [Kyn]K_{Gly}/(EC_{50} - K_{Gly}), \quad (1)$$

where K_{Gly}, the glycine dissociation constant, is the glycine concentration producing a half-maximal response in the absence of Kyn, and EC_{50} is the glycine concentration producing a half-maximal response in the presence of Kyn at concentration [Kyn]. Three experiments similar to that illustrated in Fig. 3*B* indicated that the EC_{50} measured in the absence of glycine was $0{\cdot}14 \pm 0{\cdot}03$ μM, and that it shifted to $0{\cdot}44 \pm 0{\cdot}15$ μM in the presence of 30 μM-Kyn and to $1{\cdot}01 \pm 0{\cdot}7$ μM in the presence of

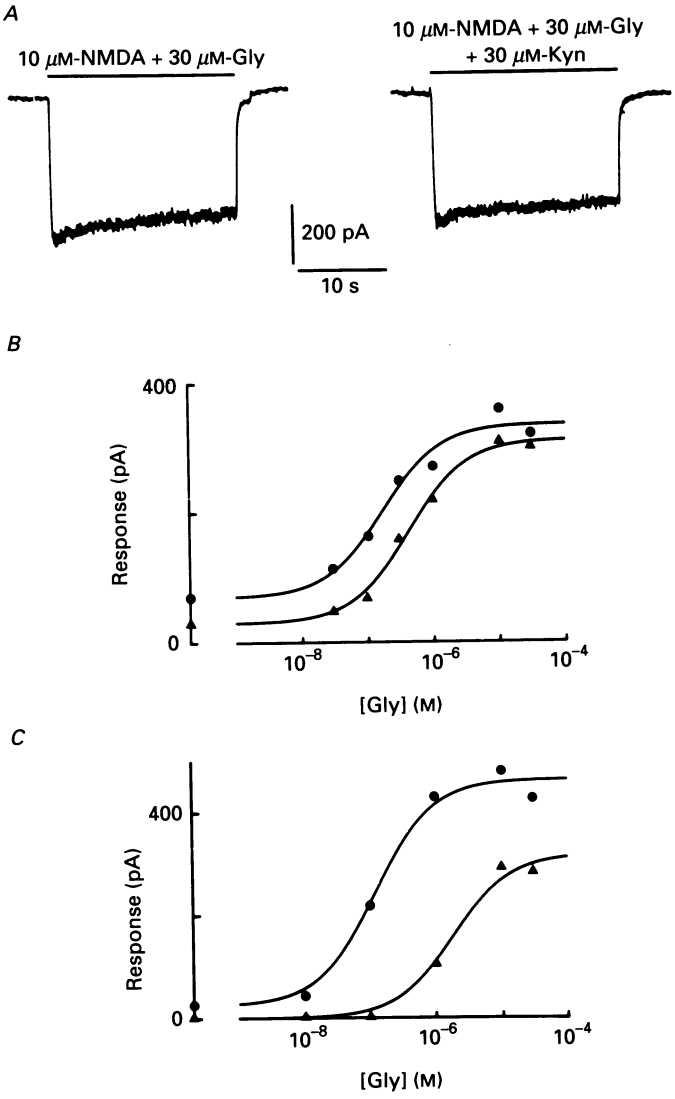

Fig. 3. Kynurenate produced a rightward shift of the concentration–response curve for glycine potentiation of whole-cell currents evoked by NMDA. *A*, in the presence of a high concentration of glycine (30 μM), 30 μM-Kyn produced only a small depression (12%) of the inward current evoked in a single cell (holding potential = −50 mV) by 10 μM-NMDA. *B*, glycine concentration–response curves for the potentiation of 10 μM-NMDA in the absence (●) or presence (▲) of 30 μM-Kyn were fitted with an equation of the form

$$R = R_0 + R_{max}([Gly]/(EC_{50} + [Gly])),$$

where R is the response amplitude, R_0 is the response amplitude in the nominal absence of glycine, R_{max} is the maximum response and EC_{50} is the apparent dissociation constant for glycine. The parameters left free during the fit were R_{max} and EC_{50}. In this figure the glycine EC_{50} in the absence of Kyn is 0·17 μM and in the presence of 30 μM-Kyn is 0·43 μM. *C*, same as part *B* except from a different cell and the Kyn concentration was 100 μM. The EC_{50} of glycine in the absence of Kyn is 0·12 μM and in the presence of 100 μM-Kyn is 1·83 μM.

100 μM-Kyn. Equation (1) leads to values of K_{Kyn} of 14 μM in the first case, and of 16·1 μM in the second, which is similar to the values (14·5 μM, 12·7 μM) calculated from the depression of the response to 10 or 100 μM-NMDA in the absence of added glycine (Fig. 1). This suggests that in the experiments of Fig. 1 the error due to glycine contamination was not substantial.

The data of Fig. 3 suggested that, in addition to shifting the glycine concentration–response curve to the right, 30 μM-Kyn produced a small depression of the current evoked by 10 μM-NMDA in the presence of a maximally potentiating concentration of glycine. This was confirmed in a series of experiments in which, without constructing a complete concentration–response curve, we analysed the effect of 30 μM-Kyn on the response to 10 μM-NMDA plus 30 μM-glycine. The depression produced by Kyn was $14 \pm 6\%$ ($n = 8$). The small depression by 30 μM-Kyn of the response to 10 μM-NMDA in the presence of a saturating concentration of glycine contrasts with the marked depression ($64 \pm 9\%$, $n = 5$; see Fig. 1*A* and *B*) of the response to 10 μM-NMDA produced by the same concentration of Kyn in the absence of added glycine. Thus, increasing the glycine concentration largely overcomes the depression by 30 μM-Kyn of the response to NMDA. When the Kyn concentration was raised to 100 μM there was, in addition to the larger shift to the right of the glycine concentration–response curve, a further decrease in the maximum potentiation by glycine (Fig. 3*C*). The depression of the maximum response reached $25{\cdot}8 \pm 5{\cdot}2\%$ ($n = 3$).

Kynurenate depression of the responses to quisqualate and kainate

Kynurenate has previously been used as a non-selective glutamate receptor antagonist (Perkins & Stone, 1982) and did indeed reduce the inward currents evoked by 1 μM-quisqualate or 10 μM-kainate. 100 μM-Kyn produced a 29% ($n = 2$) depression of the response to 1 μM-quisqualate and a $47 \pm 11\%$ ($n = 5$) depression of the response to 10 μM-kainate. Full concentration–inhibition curves for Kyn were not obtained due to its lower potency as an antagonist of quisqualate and kainate. The concentration range over which Kyn inhibited responses to quisqualate and kainate (30–400 μM) was similar to that over which it reduced the response to NMDA in the presence of a maximally potentiating concentration of glycine.

Effects of CPP and APV

CPP and APV are NMDA antagonists which appear to act primarily by competing for the site at which NMDA binds (Olverman & Watkins, 1989). CPP (1–10 μM) depressed the response to NMDA alone and the response to NMDA plus glycine. In contrast to what was observed with Kyn, the depression of responses to 10 μM-NMDA produced by CPP was independent of the glycine concentration (Fig. 4).

The effect of APV was at first sight similar to that of CPP inasmuch as the depression of the responses to NMDA was not overcome by increasing the glycine concentration. However, the depression was slightly smaller at high glycine concentrations. This differential effect was evaluated by analysing the ratio of the responses to NMDA (10 μM) in the presence of 10 μM-glycine and in the presence of 1 μM-glycine (Gly_{10}/Gly_1 ratio). Three antagonists (CPP, APV and Kyn) were added at concentrations (CPP: 3 μM; APV: 10 μM; Kyn: 100 μM) which produced a similar

reduction (of about 80%) of the response recorded in 1 μM-glycine. In control conditions, the Gly_{10}/Gly_1 ratio was $1{\cdot}15 \pm 0{\cdot}06$ ($n = 10$) (Johnson & Ascher, 1987*a*). It was not significantly altered by CPP (3 μM) ($1{\cdot}24 \pm 0{\cdot}27$, $n = 5$) but increased to $1{\cdot}55 \pm 0{\cdot}30$ ($n = 4$) in the presence of APV (10 μM) and to $3{\cdot}16 \pm 0{\cdot}85$ ($n = 5$) in the

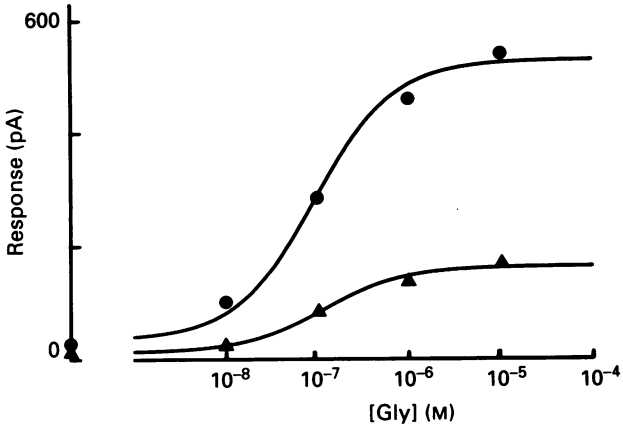

Fig. 4. The depression by CPP of the inward current evoked by NMDA could not be overcome by increasing the concentration of glycine. The graph shows glycine concentration–response curves for the potentiation of 10 μM-NMDA obtained from a single cell in the absence (●) and presence (▲) of 1 μM-CPP. The curves were fitted as described in Fig. 3. The EC_{50} of glycine in the absence of CPP was 0·095 μM and in the presence of 1 μM-CPP was 0·12 μM. Continuous lines are fitted as described in the legend of Fig. 3.

presence of Kyn (100 μM). These results are consistent with a previous report indicating that APV inhibits glycine binding up to a maximum of 20% with an IC_{50} of 0·32 μM (Kessler *et al.* 1989).

Effects of 7-chlorokynurenate on the responses to NMDA in the absence and presence of added glycine

As expected from the observations of Kemp *et al.* (1988), 7-Cl-Kyn resembles Kyn in that it inhibits NMDA responses with an IC_{50} dependent on the glycine concentration, but differs from Kyn by the much lower value of the dissociation constant. Figure 5 illustrates the results of an experiment in which the inhibition produced by 7-Cl-Kyn was studied in the presence of three glycine concentrations (0·1, 1 and 10 μM): the shift to the right is what is expected from a competitive antagonist of glycine. The dissociation constant for 7-Cl-Kyn, $K_{7\text{-Cl-K}}$, can be derived knowing the glycine dissociation constant K_{Gly}, and the IC_{50} in a known concentration of glycine, [Gly], using the relation

$$K_{7\text{-Cl-K}} = IC_{50}K_{Gly}/(K_{Gly} + [Gly]). \quad (2)$$

The pooled data from twelve experiments using various glycine concentrations were analysed by using for K_{Gly} the mean value of 0·14 μM calculated in our parallel study of the mechanism of glycine action (Johnson & Ascher, 1987*b*, and in preparation; Ascher & Johnson, 1989). The mean value of $K_{7\text{-Cl-K}}$ was $0{\cdot}23 \pm 0{\cdot}08$ μM

with similar values for 0·1 μM-Gly ($K_{7\text{-Cl-K}} = 0{\cdot}27 \pm 0{\cdot}08$ μM; $n = 6$) or 10 μM-Gly ($K_{7\text{-Cl-K}} = 0{\cdot}24 \pm 0{\cdot}05$ μM; $n = 3$).

Effects of HA-966 on the responses to NMDA in the absence and presence of added glycine

Even when stringent precautions were taken to eliminate glycine from the perfusion solutions (see Methods), the effect of HA-966 on the inward current evoked

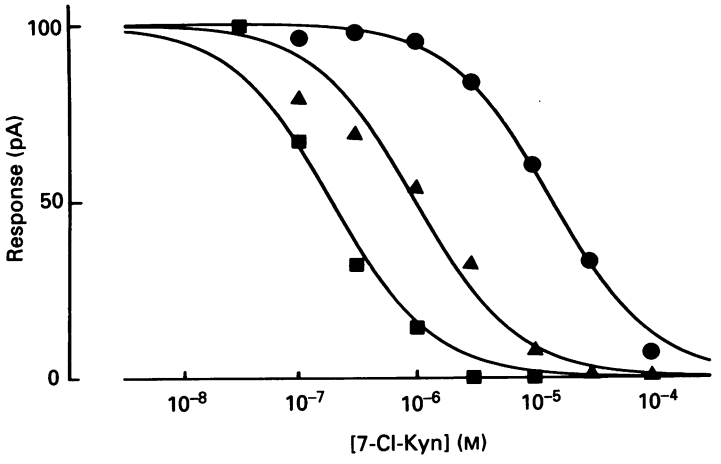

Fig. 5. Depression by 7-Cl-Kyn of the whole-cell currents evoked by 10 μM-NMDA in the presence of various concentrations of glycine. The amplitudes of the responses to 10 μM-NMDA in the presence of 0·1 (■), 1 (▲) and 10 (●) μM-glycine were measured as a function of 7-Cl-Kyn concentration. Data are normalized to the response in the absence of 7-Cl-Kyn. The IC_{50} values were 0·19 μM (0·1 μM-glycine), 0·97 μM (1 μM-glycine) and 14·2 μM (10 μM-glycine). Although the fit could have been improved by assuming more than one binding site, no attempt was made in this direction because our analysis of the glycine potentiation suggests a single glycine binding site for each receptor channel complex (Johnson & Ascher, 1987*b*, and in preparation).

by 10 μM-NMDA was found to be variable from cell to cell. In some cases the effect was a potentiation. This was not due to a direct agonist effect of HA-966 on the NMDA binding site, since when applied alone to cells at concentrations up to 300 μM, HA-966 never induced an inward current. This potentiation supports the hypothesis (Foster & Kemp, 1989) that HA-966 is a weak partial agonist of the glycine receptor. In many other cases, however, HA-966 (1–100 μM) produced an inhibition.

In the presence of added glycine (0·1 or 1 μM), HA-966 only produced an inhibition which, as described by Fletcher *et al.* (1989) and Foster & Kemp (1989), could be overcome by increasing the glycine concentration. This is illustrated in Fig. 6 for a cell in which HA-966 (100 μM) produced a potentiation in the presence of a low concentration of glycine (0·01 μM), but produced an inhibition in the presence of 0·1 μM-glycine. All the effects disappeared when the glycine concentration was raised to the saturating level of 30 μM. Figure 6*D* illustrates how the glycine potentiation was altered by 100 μM-HA-966: both in the absence of added glycine and at 0·01 μM-glycine, 100 μM-HA-966 potentiated the NMDA response, while for higher con-

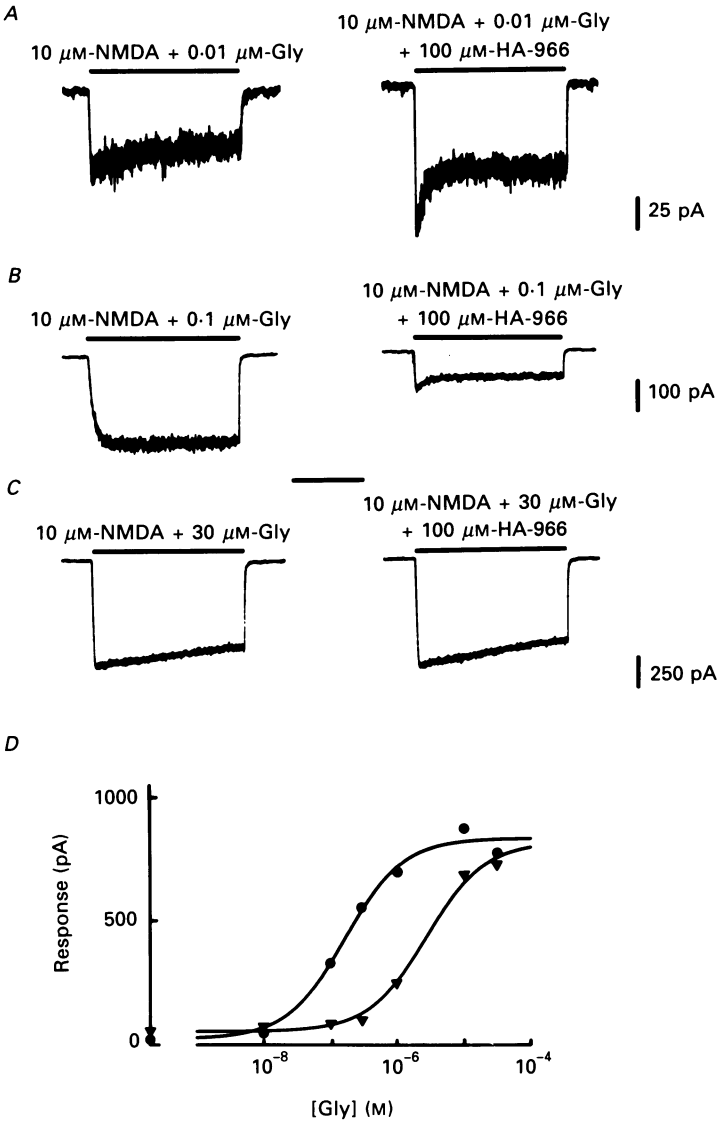

Fig. 6. Effects of HA-966 on the glycine potentiation of inward currents evoked by NMDA. *A*, *B* and *C*, depending on the concentration of glycine, HA-966 (100 μM) potentiated (0·01 μM-glycine), depressed (0·1 μM-glycine) or had no effect (30 μM-glycine) on the inward current evoked by 10 μM-NMDA. NMDA, glycine and HA-966 were applied for the periods indicated, *D*, glycine concentration–response curves for the potentiation of 10 μM-NMDA obtained from the same cell in the absence (●) and presence (▼) of 100 μM-HA-966. In nominally glycine-free solutions and at 0·01 μM-glycine HA-996 increased the response but HA-966 also caused a rightward shift of the concentration–response curve. The curves were fitted as described in Fig. 3. The EC_{50} of glycine in the absence of HA-966 was 0·165 μM and in the presence of 100 μM-HA-966 was 2·75 μM. The relaxations observed in this experiment are difficult to interpret because glycine was applied simultaneously with NMDA or NMDA plus HA-966. This implies that the rise of the current is the combined result of three simultaneous concentration changes. Despite this difficulty it is possible to assume that in the case of panel *A*, the concentration of contaminating glycine in the control barrel was low and did not change significantly

centrations of glycine the effect was an inhibition. From the shift of the concentration–response curve one can deduce a dissociation constant K_{HA} of 6·4 μM (using in eqn (1) $K_{Gly} = 0{\cdot}165$ μM and $EC_{50} = 2{\cdot}75$ μM, the values calculated from the fit curves of Fig. 6*D*). In a similar experiment, K_{Gly} was found to be equal to 0·147 μM, and the EC_{50} shifted to 0·67 μM in the presence of 30 μM-HA-966, which leads to a value for K_{HA} of 8·4 μM.

The efficacy of HA-966 appears much lower than that of glycine. The relative efficacies of the two compounds (Stephenson, 1956) were estimated in a series of five experiments in which the response to 100 μM-HA-966 was compared, on a given cell, to the response to 1 μM-glycine. The mean value of the ratio of the two responses was $0{\cdot}079 \pm 0{\cdot}014$ (mean $\pm$ S.D., $n = 5$). This is likely to be a small overestimate of the actual ratio of the efficacies since glycine at 1 μM does not saturate the receptor completely (Johnson & Ascher, 1987*a*, *b*).

When the concentration of HA-966 was increased at a given (low) glycine concentration, the effect was usually monotonous. If a potentiation was observed for the low HA-966 concentration, it increased; if an inhibition was the initial effect, it also increased. Figure 10*A* illustrates an example in which HA-966 lead to a potentiation at all concentrations; the EC_{50} was evaluated as 6·5 μM. However, in some cells in which, in the absence of added glycine, HA-966 at low concentrations produced a potentiation, it became inhibitory at higher concentrations. This effect, illustrated in Fig. 10*B*, is further considered in the analysis of relaxations.

Effects of D*-cycloserine on the NMDA responses in the absence and presence of added glycine*

D-Cycloserine was first described as a partial agonist of glycine by Hood *et al.* (1989). In experiments using TCP binding as an index of NMDA receptor activation, these authors found that the maximal effect of D-cycloserine was about 50% of that of glycine. Our experiments on the effects of D-cycloserine on NMDA-induced currents confirmed these observations. Figure 7*A* illustrates the effects of saturating concentrations of D-cycloserine (300 μM) in the absence of glycine and in the presence of glycine (10 μM) on the same cell: the response recorded in the presence of glycine is nearly twice as large as that recorded in the presence of D-cycloserine. In thirteen similar experiments the mean value of the ratio of the two responses was found to be $0{\cdot}69 \pm 0{\cdot}11$. The mean EC_{50} in four experiments similar to that illustrated in Fig. 7*B* was $5{\cdot}4 \pm 3{\cdot}3$ μM.

As expected from a partial agonist D-cycloserine, when applied in the presence of 10 μM-glycine, reduced the NMDA response if applied at high enough concentrations. The value of the IC_{50} could not be evaluated with precision but appeared to be between 10 and 50 μM.

The steady-state effects of Kyn, 7-Cl-Kyn, HA-966 and D-cycloserine support, to

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when NMDA was applied. In such a case the rising phase of the response is governed by the rise of the NMDA receptor occupancy and previous studies have shown that the corresponding relaxations are rapid. In *B*, left, the onset of the response is likely to be limited by the rise of the glycine site occupancy, which at a concentration of glycine of 0·1 μM is in the range of 0·4 s (J. W. Johnson & P. Ascher, in preparation; Benveniste *et al.* 1990*a*). In *C*, left, the onset of the response is accelerated because the glycine concentration has been increased.

a first approximation, the hypothesis that these compounds act at the glycine site as competitive antagonists (Kyn and 7-Cl-Kyn) or partial agonists (HA-966 and D-cycloserine). The data obtained at low glycine concentrations, and in particular in the nominal absence of glycine, can be incorporated into such an hypothesis by assuming a variable level of extracellular glycine contamination.

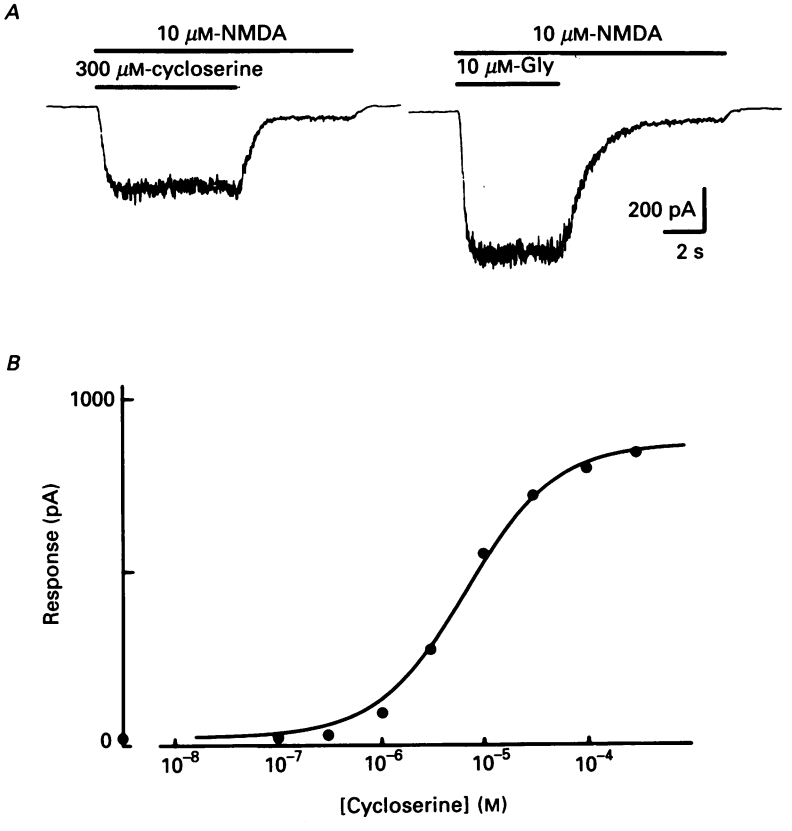

Fig. 7. Comparison of the effects of D-cycloserine and glycine on the whole-cell currents induced by 10 μM-NMDA. *A*, the response produced by 10 μM-NMDA in the presence of D-cycloserine at 300 μM is smaller than the response produced by the same concentration of NMDA in the presence of 10 μM-glycine. *B*, concentration–response curve of the potentiation produced by D-cycloserine in the nominal absence of glycine. The EC_{50} was 6·7 μM.

Relaxations observed after concentration jumps

In order to refine the above interpretation we analysed the relaxations of the currents observed after rapid 'concentration jumps' of the compounds under study. In a parallel analysis using concentration jumps of glycine (Johnson & Ascher, 1987*b* and in preparation; Ascher & Johnson, 1989), we had observed that the onset and offset of the glycine potentiation were well described by exponential functions. The time constant of the off-relaxation (0·96 ± 0·3 s, $n = 18$, J. W. Johnson & P. Ascher, in preparation) was independent of the glycine concentration preceding the concentration jump, while the time constant of the on-relaxation was shortened by

increasing the glycine concentration and eventually became faster than the time constant of the solution change. This is what is expected from a simple bimolecular reaction. As described below, the relaxations produced by concentration jumps of Kyn, 7-Cl-Kyn or HA-966 did not follow this simple pattern.

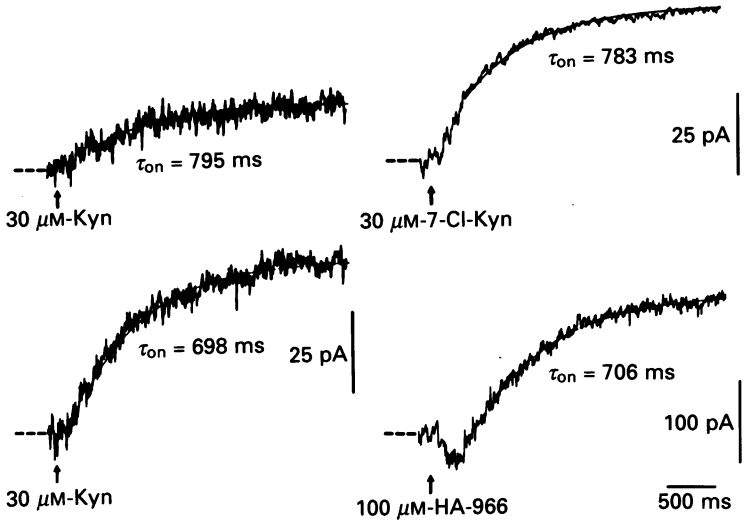

Fig. 8. 'On' time constants (τ_{on}) of the Kyn-, 7-Cl-Kyn- and HA-966-induced blockade of the inward current evoked by 10 μM-NMDA in the absence of added glycine. Cells were initially superfused with 10 μM-NMDA, and then the position of the barrels was changed such that the solution superfusing the cell contained 10 μM-NMDA plus one of the following: 3 μM-Kyn, 30 μM-7-Cl-Kyn or 100 μM-HA-966. Time of solution change is indicated by the arrow. The currents are each the average of six to ten applications of the drug. The traces for each drug were obtained from different cells. Single-exponential curves were fitted to the decay of the current as described in the Methods. Fits started 300 ms after the end of barrel movement except for the HA-966 application for which the fit was begun 300 ms after the peak of the transient inward current.

On-relaxations

The relaxations produced after the sudden application of compounds acting at the glycine site are illustrated in Fig. 8 for three experiments in which no glycine had been added to the perfused solution. Kynurenate was applied at concentrations of 3 and 30 μM, 7-Cl-Kyn at 30 μM and HA-966 at 100 μM. For the first two compounds the relaxation is entirely outward, and its main component can be fitted by an exponential with a time constant of 700–800 ms. With the presumed partial agonist HA-966 the relaxation shows both an inward and an outward component, and here again the outward relaxation can be fitted by an exponential with a time constant of about 800 ms. Thus for all three compounds the slow outward relaxations had very similar time constants, despite the fact that the dissociation constants calculated from steady-state data were very different (about 14 μM for Kyn, 0·2 μM for 7-Cl-Kyn and 7 μM for HA-966).

The speed of the slow relaxation is not sensitive to the concentration of the antagonist, as illustrated by Table 1 and Fig. 8 for Kyn, 7-Cl-Kyn and HA-966. The

time constants for the onset of Kyn blockade were not significantly modified when the Kyn concentration was raised from 1 to 100 μM. The same insensitivity, although less well documented, was observed for HA-966. The numbers in Table 1 A do suggest that the HA-966 relaxations were on average slightly slower than those induced by Kyn. This difference, however, may not be meaningful given the additional feature of the HA-966 relaxations illustrated in Fig. 8 (see below). Table 1*B* illustrates a parallel experiment in which the three compounds gave similar relaxations in a single cell.

Over a large range of concentrations, these three compounds exhibited slow and very similar rates of inhibition of the NMDA response. This suggests that some factor other than their inherent binding kinetics limits their rate of action. Because these compounds act at the glycine binding site, a plausible idea is that this other process is the unbinding of glycine. The rate at which these compounds act should approximate the rate of unbinding of glycine if their inherent kinetics are faster than the glycine unbinding kinetics, which will be the case, in particular, when the compounds are used at high concentrations. Comparison of their time constants of blockade (Table 1) with the time constant of glycine unbinding cited earlier ($0{\cdot}96 \pm 0{\cdot}3$ s) reveals a close similarity. That the blockade is also slow in the absence of added glycine is readily explained if the NMDA response requires that glycine be bound to its site and if the response observed in the absence of added glycine depends on glycine contamination (Kleckner & Dingledine, 1988). Applying the antagonist at high concentration very rapidly saturates all the non-occupied glycine binding sites without producing any effect. Later, as contaminating glycine unbinds from the receptors it was occupying, it is rapidly replaced by the antagonist. Thus the relaxation reflects the dissociation of glycine from its binding sites and its rate is expected to be independent of the antagonist used and of the antagonist concentration as long as this concentration is sufficiently high to produce rapid binding.

The outward relaxations observed after application of HA-966 were usually preceded, at least in low extracellular glycine concentrations, by an inward current (Fig. 8). This is probably due to the agonist action of HA-966: the rapid occupation of the empty sites by HA-966 leads to an increase of the NMDA response. The following decrease in the NMDA response, which has the same rate constant as glycine unbinding, is probably due to the replacement of glycine with HA-966 at sites that were occupied by background glycine at the moment of HA-966 application. As in the case of Kyn and 7-Cl-Kyn, the rate of this replacement will depend essentially on the glycine unbinding rate if the binding of HA-966 is faster than glycine unbinding.

Off-relaxations

The relaxations observed at the end of the drug application mirrored to a first approximation those observed at the beginning of the drug application and, as could be expected, were more complex with partial agonists than with antagonists. Figure 9 illustrates the differences between the off-relaxations observed with Kyn, 7-Cl-Kyn and HA-966, in an experiment using 10 μM-NMDA and no added glycine. With Kyn and 7-Cl-Kyn, the current increased immediately after the end of the application

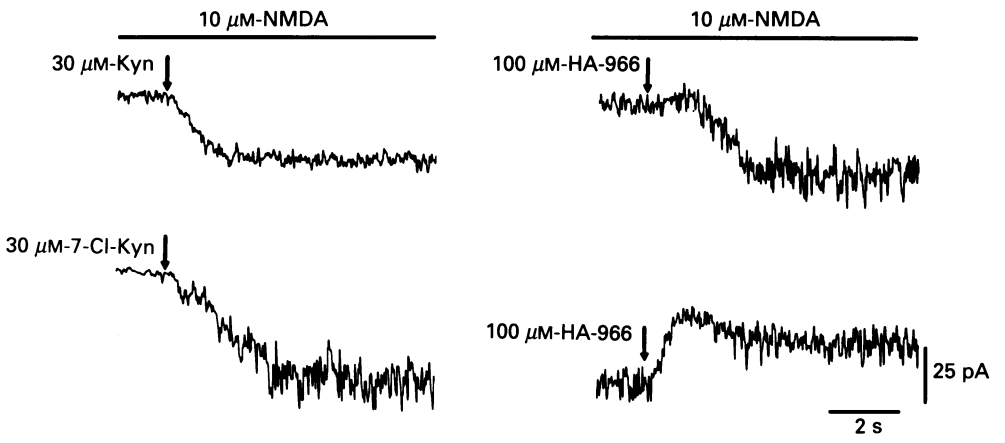

Fig. 9. 'Off' rates of blockade induced by Kyn, 7-Cl-Kyn and HA-966 on the currents evoked by 10 μM-NMDA. In the four experiments illustrated (from four different cells), NMDA (10 μM) was applied continuously in the absence of added glycine. For the 20 s preceding the arrow Kyn (30 μM) or 7-Cl-Kyn (30 μM) or HA-966 (100 μM) was applied in addition to the 10 μM-NMDA. At the time indicated by the arrow the cell was returned to a solution to which only 10 μM-NMDA had been added. The relaxations observed in the case of Kyn and 7-Cl-Kyn are slow and inward. In the case of HA-966, two patterns are observed which are related to the two patterns observed for on-relaxations. In the first (upper) record with HA-966, the steady-state effect was an inhibition, and the on-relaxation was mostly an outward relaxation which only differed from the relaxations observed with Kyn and 7-Cl-Kyn by an apparent lag after the solution change. In the second example (lower trace) the steady-state effect of HA-966 was a potentiation, and the on-relaxation (not shown) resembled that illustrated in Fig. 10*A* for 100 μM-HA-966. The off-relaxation was similarly non-monotonic.

TABLE 1. Time constants (τ_{on}, in ms) of the Kyn-, 7-Cl-Kyn- and HA-966-induced blockade of the inward currents evoked by 10 μM-NMDA in the nominal absence of glycine

Concentration	A		B		
(μM)	Kyn	HA-966	Kyn	7-Cl-Kyn	HA-966
0·3	—	—	—	1615	
1	895 (1)	—	895	1468	—
3	858±431 (3)	—	1324	1473	—
10	786±315 (6)	1110 (2)	1397	1348	—
30	638±84 (3)	890±89 (5)	—	1340*	—
100	779±300 (5)	905±189 (4)	1221	—	1330

A, data pooled from one to five cells. B, data obtained from a single cell.

τ_{on} was determined as described in the Methods and in the legend of Fig. 8. In A, values are the means±the standard deviation of the data pooled from the number of cells given in parentheses.

* The value was obtained in the presence of 50 μM-7-Cl-Kyn.

of the antagonist, at a speed which probably reflects the slow rebinding of contaminating glycine. In the case of HA-966, on the other hand, the current usually showed a biphasic evolution. In the third current trace in Fig. 9, where the steady current had been depressed by HA-966, the current increased back to its control value after the wash-out of HA-966, but only after an interval during which there

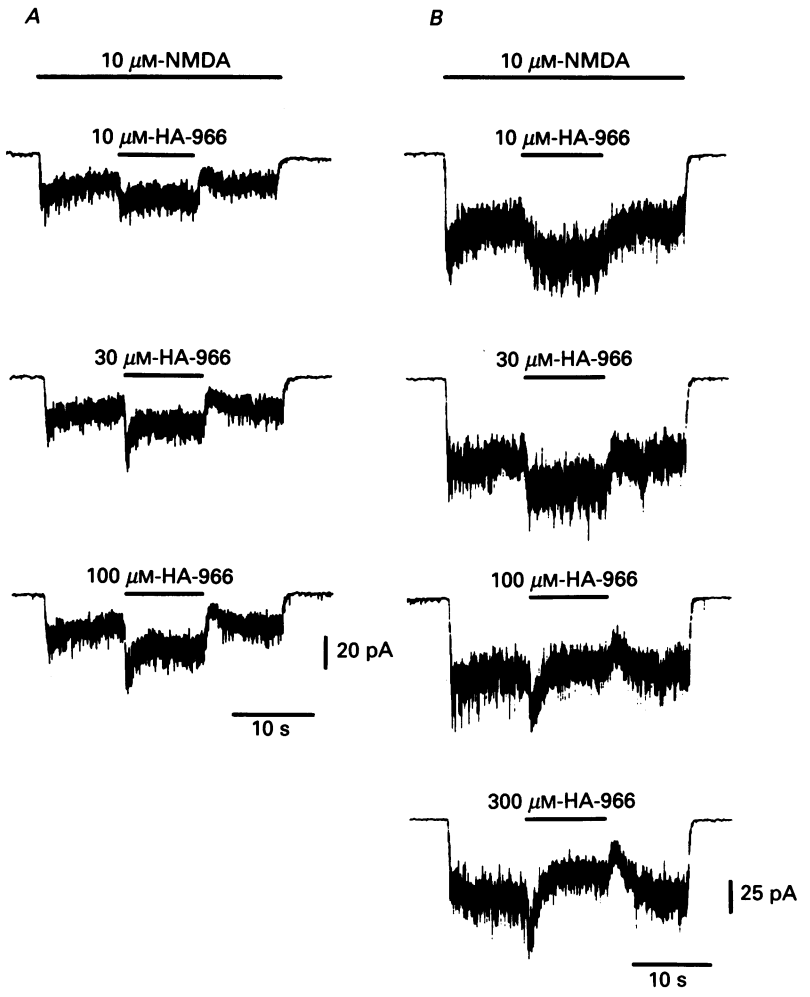

Fig. 10. Variability of the effects of HA-966 on the currents evoked by 10 μM-NMDA. HA-966 at different concentrations (10, 30, 100 and 300 μM) was applied for 10 s on a background of NMDA (10 μM) in the nominal absence of glycine. In *A*, the effect at all HA-966 concentrations is a potentiation of the NMDA response. The percentage of decay increases with the HA-966 concentration, but the steady-state net effect remains a potentiation at the highest concentration of HA-966 tested (100 μM). In *B* (different cell) the relaxations produced by the application of HA-966 always start by an inward rapid component but for low concentrations of HA-966 a second slower component is inward and leads to a steady-state potentiation, while at higher concentrations of HA-966 the slow component is outward and leads to a steady-state inhibition. Mirror images are observed at the end of the application of HA-966.

was a small decrease (outward change). In the bottom record of Fig. 9, the steady-state effect of HA-966 had been a potentiation, and accordingly the wash-out of the compound produced a decrease of the current. But, again, this decrease was polyphasic: a rapid decrease was followed by a slower rebound. In both cases, the off-relaxation reflects the summation of two processes: the relatively fast unbinding of

HA-966 and the slower rebinding of glycine. The first process leads to an outward current, the second to an inward one, and the proportions of the two currents will depend, for a given concentration of HA-966, on the level of contaminating glycine. The difference between the two records obtained with HA-966 in Fig. 9 is thus likely to reflect (like the steady-state data) the fact that the glycine level was lower in the experiment corresponding to the bottom record than in that corresponding to the next higher record.

The complex relaxations observed with HA-966 are further illustrated in Fig. 10 in two experiments in which, at low concentration, HA-966 produced a potentiation. In the experiment illustrated in the left-hand column, the biphasic behaviour illustrated in Fig. 9 is also observed at the onset of the HA-966 application, and the picture is qualitatively similar for the three concentrations of HA-966 tested (10, 30 and 100 μM). The picture is that expected from a partial agonist of low efficacy in the presence of a very low concentration of glycine. In the experiment illustrated in the right-hand column, at high concentrations the responses are again biphasic, but now the steady-state effect is an inhibition. This is not what is expected from a simple partial agonist in a very low concentration of glycine, and requires an additional hypothesis, as considered in the Discussion.

The off-relaxations observed with D-cycloserine, illustrated in Fig. 7 in the absence of added glycine, were always faster than the off-relaxations observed with glycine. In the absence of glycine, the outward relaxation must represent mostly the dissociation of D-cycloserine. The difference in the speed of the outward relaxations is likely to indicate that the dissociation of D-cycloserine is faster than that of glycine. In the presence of glycine, non-monotonic relaxations were observed which are likely to reflect the same opposing processes discussed above for the case of HA-966.

DISCUSSION

The steady-state observations presented here support the hypothesis that the four compounds studied exert their main effects by competing with glycine at its binding site. Each compound has, however, its specific pattern of action.

7-Chlorokynurenate is a selective and potent competitive antagonist, and the calculated value of its dissociation constant (0·23 μM) agrees with the values calculated by Kemp *et al.* (1988) (0·3 μM), by Kleckner & Dingledine (1989) (0·35 μM) and by Benveniste, Mienville, Sernagor & Mayer (1990*b*) (0·26 μM) in similar experiments.

Kynurenate also appears to be a competitive antagonist of glycine, and here again our estimates of the dissociation constant (about 15 μM) agree with previous estimates (Kessler *et al.* 1987, 1989; Kemp *et al.* 1988; Watson *et al.* 1988; Kloog *et al.* 1990). The effect of Kyn is more complicated than that of 7-Cl-Kyn, however, because of the fact that, at concentrations higher than the dissociation constant for the glycine site, Kyn also has significant effects at the NMDA receptor site (see Mayer *et al.* 1988; Olverman, Jones & Watkins, 1988; Kessler *et al.* 1989; Kloog *et al.* 1990).

D-Cycloserine is a powerful partial agonist, since its efficacy reaches 70% of that of glycine. The calculated value of the dissociation constant is 5·4 μM. Both values

agree well with those reported by Hood *et al.* (1989) and Monahan *et al.* (1989) (40–50 % for the efficacy, 2·3 μM for the dissociation constant) and even better with those reported by McBain *et al.* (1989) (71 %, 5·3 μM).

Finally HA-966 is also a partial agonist, but has a much lower efficacy than D-cycloserine. The ratio of its efficacy to that of glycine, estimated as the ratio of the maximal response to HA-966 to the maximal response to glycine, is less than 8 %. The low efficacy of HA-966 can explain why, for low added glycine concentrations, the presence in the extracellular solution of a small amount of glycine (or glycine-like compound) may be sufficient in most experimental situations to mask the potentiation expected from HA-966. The dissociation constant of HA-966 estimated from our data, about 7·5 μM (Fig. 6), is in the range of the values deduced from other studies (17·5 μM, Foster & Kemp, 1989; 13 μM, Singh *et al.* 1990; 6–17 μM, Kloog *et al.* 1990) and somewhat smaller than the values of the EC_{50} obtained by Drejer, Sheardown, Nielsen & Honoré (1989) (25 μM) in conditions where the endogenous concentrations of glycine may have been slightly higher.

Our study of the relaxations associated with antagonist and partial agonist concentration jumps was initiated by the surprising obervation that their apparent on-rates were independent of their concentration. Because the relaxations were slow (due to the slow off-rate of glycine; see Johnson & Ascher, 1987*b*; Ascher & Johnson, 1989; Benveniste *et al.* 1990*a*, *b*), concentration jumps with the fast-perfusion system were sufficiently rapid to be considered instantaneous relative to the relaxations. The measurement of the intrinsic rates at which these compounds interact with the glycine binding site would be far more difficult for at least two reasons. First, their off-rates may be much faster than the off-rate of glycine, necessitating the use of faster concentration jumps than have been used in these experiments. Second, even if it were possible to eliminate glycine from the extracellular environment, the measurement of the inherent antagonist on- and off-rates would require the presence of both the antagonist and an agonist. The direct measurement of the inherent antagonist on-rate would be difficult because of the slow off-rate of glycine, although the use of lower affinity agonists with faster off-rates might make the measurement feasible (see Benveniste *et al.* 1990*b*). The off-relaxation also would be at best multiexponential due to the involvement of agonist binding, and thus difficult to analyse. Although these problems are not insurmountable, they have prevented us from deducing the rates of interaction with the glycine site of the drugs studied here. Some conclusions could nevertheless be drawn from the relaxation data, as discussed below.

The onset of antagonist action of Kyn and 7-Cl-Kyn has a similar speed (despite the differences in the dissociation constants of the two compounds) which is independent of the agonist concentration (1–100 μM range for Kyn, 0·3–100 μM for 7-Cl-Kyn) and is close to the mean time constant of glycine dissociation, evaluated as 960 ms on average (Ascher & Johnson, 1989; J. W. Johnson & P. Ascher, in preparation). In parallel experiments Benveniste *et al.* (1990*b*) have reported smaller values both for the time constant of glycine dissociation (331 ms) and for the time constant of 7-Cl-Kyn inhibition (261 ms) but have also noted the similarity of these two time constants and the fact that the on-rate of 7-Cl-Kyn is independent of the antagonist concentration. The fact that the onset of inhibition is limited by the

dissociation of glycine is readily explained if glycine is absolutely necessary for NMDA action (Kleckner & Dingledine, 1988) and if the speed of antagonist binding is faster than the dissociation of glycine.

The slow onset of the inhibition by high antagonist concentrations is difficult to reconcile with the hypothesis that Kyn and 7-Cl-Kyn are 'inverse agonists'. Our support of this hypothesis (Ascher *et al.* 1988*b*; see also Kemp *et al.* 1988) was based on the observation of an inhibition in the nominal absence of glycine (Fig. 1). If glycine was actually absent from the solution, the inhibition produced by Kyn would have indicated that Kyn (which we know to bind to the glycine site) had an effect opposed to that of glycine, i.e. induced a closing of the channels opened by NMDA. (As a corollary, the opening of NMDA channels in the absence of glycine would have indicated that glycine is not absolutely required for NMDA receptor activation). In this case, however, the onset of Kyn block should have been accelerated at higher concentrations since Kyn would be an 'agonist', albeit an inverse one. The fact that this was not the case is an additional argument in favour of the hypothesis that glycine is absolutely required for NMDA action (Kleckner & Dingledine, 1988) and that the responses observed in the absence of added glycine imply the presence of either some 'endogenous' glycine trapped in the restricted extracellular space, or some contaminating glycine in the experimental solutions.

The relaxations produced by the sudden application of HA-966 differed from those produced by Kyn or 7-Cl-Kyn by the fact that in many cases they displayed both an inward and an outward component. The simplest way to account for these non-monotonic (inward–outward) relaxations is to combine the hypothesis that the compounds are partial agonists (with kinetics faster than glycine kinetics) with the hypothesis that some glycine is present around the cell. When the partial agonist is added, one expects an immediate potentiation due to the rapid occupation of the vacant glycine sites before glycine has had time to significantly unbind. Then, as glycine leaves the sites it occupied, the partial agonist will partially replace glycine, causing a reduction of the response. This can result in the steady state either in a potentiation (if the level of glycine is low) or in an inhibition (if the potentiation due to glycine is already larger than the maximal potentiation produced by the partial agonist). In the same way, one expects non-monotonic off-relaxations combining the dissociation of the partial agonist and the reassociation of glycine.

While this scheme accounts for the biphasic relaxations observed, it does not explain why in some cells the steady-state effect is transformed from a potentiation to an inhibition when the concentration of HA-966 is increased (Fig. 10*B*).

One possible explanation of the dual effect of HA-966 is to assume a heterogenous distribution of endogenous glycine. The neurones used in these experiments have rather extensive dendritic arbors, parts of which are covered by glial cells. Moreover, the part of the cell body facing the glial layer covering the bottom of the culture dish is not directly exposed to the solution applied from the perfusion system. It is likely that the concentration of endogenous glycine is not homogeneous around the cell. If, in the absence of added glycine, the upper surface of the cell body is in a 'glycine-free' solution, while deeper regions are exposed to glycine, applying a partial agonist to the cell could lead to potentiation in the exposed region and to inhibition in the region in which glycine is present.

This hypothesis of a heterogeneous distribution of the glycine concentration can account for the peculiar effects of increasing the HA-966 concentration discussed above. Assuming, for simplicity, a glycine-free region and a region exposed to glycine, one expects that all HA-966 concentrations will produce at steady-state a potentiation in the glycine-free region and an inhibition in the region exposed to glycine. However, the half-maximal potentiation of the glycine-free region will be obtained with a concentration of HA-966 lower than that producing the half-maximal inhibition in the glycine-containing region. As a result, in the combined response of the two regions of the cell the relative contributions of the two components will vary with the concentration of HA-966.

Although the hypothesis of a heterogeneous distribution of endogenous glycine accounts for the patterns observed with HA-966, other explanations cannot be ruled out. For example, one could suspect that the HA-966 solution contains both a competitive glycine antagonist and a glycine agonist. The samples of HA-966 that we used contained both (+)HA-966 and (−)HA-966, but these two enantiomers appear to be both inhibitory at the glycine site, although (+)HA-966 is much more potent (IC_{50} = 13 μM) than (−)HA-966 (IC_{50} = 708 μM) (Singh *et al.* 1990). The possibility remains that HA-966 may be a pure competitive antagonist, and that the HA-966 solution be contaminated by a glycine agonist of low affinity. An alternative explanation could be that HA-966 acts at two sites, as has been proposed by Sigel & Baur (1988) for some benzodiazepines which inhibit the GABA response at low concentration and potentiate it at high concentration. In particular, HA-966 could act at low concentrations as a partial agonist binding selectively at the glycine site (producing potentiation or inhibition depending on the glycine concentration), and at high concentrations could bind at another site (e.g. the NMDA binding site) and produce an inhibition independent of the glycine concentration.

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