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ACCELERATED COMMUNICATION

Interaction of 6-Cyano-7-nitroquinoxaline-2,3-dione with the *N*-Methyl-D-aspartate Receptor-Associated Glycine Binding Site

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SUMMARY

The interaction of newly described antagonist of the non-NMDA glutamate receptor 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) with the glycine site of the NMDA receptor complex has been investigated. In whole-cell patch recordings from hippocampal neurons maintained in culture, currents induced by *N*-methyl-D-aspartate (NMDA) were dependent on extracellular glycine. Responses to both NMDA (30 μM) and kainate (20 μM) were reduced by CNQX (10–30 μM). The antagonism by CNQX of NMDA, but not kainate, receptor-mediated responses could be reversed by increasing the concentration of glycine in the external medium. Glycine concentration-response curves constructed in the presence of 30 μM NMDA were shifted to the right by CNQX, suggesting that CNQX was competing with glycine for the glycine binding site. However, even at high concentrations of glycine (300 μM) the maximal NMDA current obtained in the presence of CNQX (10–30 μM) was not restored to control levels. Because CNQX had no effect on responses produced by supramaximal concentrations of NMDA (500 μM) and glycine (300 μM), it is suggested that CNQX also interacts with the NMDA recognition site. The antagonism of currents induced by NMDA was not dependent on the membrane potential, and the rapid onset and offset of the block suggested that there was little or no use dependence. Radioligand binding experiments were performed using [^{3}H]glycine to label the strychnine-insensitive glycine regulatory site of the NMDA receptor complex in guinea pig brain frontal cortex membranes. CNQX displaced [^{3}H]glycine binding in a concentration-dependent manner (IC_{50} = 5.7 μM). Scatchard analysis of the inhibition showed a decrease in the affinity (increase in K_d) of [^{3}H]glycine binding, but no change in the number of binding sites (B_{max}) in the presence of 5 μM CNQX, suggesting a competitive interaction. These data provide evidence that CNQX antagonizes NMDA receptor-mediated responses by competing with glycine for a modulatory site associated with the NMDA receptor complex. Furthermore, the results indicate that CNQX may not be as selective an antagonist for non-NMDA receptors as initially described, although its selectivity will depend on the concentration of the NMDA receptor ligand and may be enhanced by increasing the extracellular concentration of glycine.

Receptors in the vertebrate central nervous system that mediate the actions of the excitatory amino acid transmitter L-glutamate have been broadly divided into two classes, NMDA and non-NMDA (1). Although selective NMDA receptor antagonists have been available for some time (2), only recently have specific antagonists become available for non-NMDA receptors (3). In the original report, two compounds, DNQX and CNQX, were observed to markedly depress the excitation of spinal cord neurons induced by the non-NMDA receptor agonists kainate and quisqualate, with little effect on responses produced by NMDA (3). In addition, these compounds showed a higher affinity for non-NMDA than for NMDA receptor binding sites (3). Electrophysiological (4, 5) and release (6) studies utilizing *in vitro* preparations have since confirmed these findings and suggested that CNQX interacts competitively with non-NMDA receptors.

However, there are reasons to believe that the quinoxalinediones may be interacting with the NMDA receptor system in a more complex manner. In quantitative pharmacological studies on neonatal rat spinal cord preparations, DNQX produced a shift to the right in the NMDA concentration-response curve, which was accompanied by a depression of the maximal response at high antagonist concentrations, suggesting a noncompetitive inhibition (7). A similar noncompetitive block of NMDA receptor-induced responses has been observed with kynurenic acid (7–9) and 7-chlorokynurenic acid (10). Based on the observations that both these compounds could displace [^{3}H]glycine from a strychnine-insensitive binding site (10, 11) and that an increase in the extracellular glycine concentration

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitro-quinoxaline-2,3,-dione; TTX, tetrodotoxin; CPP, 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-2-hydroxyethyl)-1-piperazineethanesulfonic acid.

could partially reverse the antagonism of NMDA receptor-induced responses (10, 12, 13), it was suggested that the antagonism occurred through an interaction with the NMDA receptor-associated modulatory glycine site (14), at which the binding of glycine is essential for NMDA channel activation (15).

The studies described above suggest a mechanism through which CNQX may interfere with NMDA receptor-mediated responses. The present study has been designed to investigate this possible interaction. Firstly, recordings were made from neurons grown in culture, a preparation that allowed precise control of the extracellular glycine concentration (10, 13, 16) and, therefore, facilitated the detection of antagonism at the glycine site. Secondly, [^{3}H]glycine binding studies on guinea pig brain frontal cortex membranes were utilized in order to evaluate the interaction of CNQX with the NMDA receptor complex.

Materials and Methods

Electrophysiology. Primary cultures of hippocampal neurons were prepared using a method modified from that described by Jahr and Stevens (17). Hippocampi were dissected from brains of 1–3-day-old neonatal Long-Evans rats, chopped into small blocks (<1 mm^3), and transferred into a papain solution (20 units·ml^{-1}; Worthington Biochemical Corp., Freehold, NJ), in which they were incubated with constant agitation for 30 min at 37°. The tissue was then dissociated into a single-cell suspension in a complete growth medium (minimum essential medium with Earle's salts, 20 mM glucose, 50 units·ml^{-1} penicillin/streptomycin, 5% heat-inactivated fetal calf serum, and 1:1000 serum extender) that contained 2.5 mg·ml^{-1} bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and 2.5 mg·ml^{-1} trypsin inhibitor (Sigma), using a fire-polished Pasteur pipette. The suspension was plated onto glass cover slips, coated with poly-D-lysine (0.1 mg·ml^{-1}; Collaborative Research, Bedford, MA) and collagen (0.5 mg·ml^{-1}; Biomedical Technologies, Stroughton, MA). One half of the growth medium was exchanged every 2–3 days. When the astrocytes were 80% confluent (4–7 days), 5 μM cytosine arabinoside (Sigma) was added to prevent further division of glial cells. After 7 days in culture, neurons were killed by incubation for 30 min in a balanced salt solution containing 100 μM glutamate (Sigma). This provided a confluent non-neuronal background-feeder layer on which newly dissociated neurons could be plated at low density, thereby minimizing synaptic contacts and reducing TTX-resistant transmitter release.

Tight-seal whole-cell patch recordings were obtained at room temperature (25°) from neurons maintained in culture for 1–3 weeks, using an Axoclamp-2A amplifier in discontinuous voltage-clamp mode (switching frequency, 10–15 Hz) or an Axopatch-1B patch-clamp amplifier. The currents were low-pass-filtered (300–1000 Hz) and recorded on a Gould chart recorder or digitally sampled at 50–200 Hz. Unless otherwise stated, cells were voltage-clamped at −60 mV. Patch pipettes were filled with an intracellular solution containing, in mM: Cs-gluconate, 160; CsCl, 10; NaCl, 10; EGTA, 1; Mg-ATP, 1.5; HEPES, 5; adjusted to pH 7.4 with CsOH. During recording the cells were continuously superfused (2–5 ml·min^{-1}) with an external solution containing, in mM: NaCl, 165; KCl, 3; $CaCl_2$, 2; HEPES, 5; adjusted to pH 7.4 with NaOH. TTX (0.5 μM; Calbiochem, San Diego, CA), to reduce spike-triggered synaptic activity, and picrotoxin (100 μM; Sigma) and strychnine (1 μM; Sigma), to block inhibitory currents, were added to the external medium. Solutions containing NMDA (Tocris, Buckhurst Hill, UK and Cambridge Research Biochemicals, Valley Stream, NY) and kainate (Sigma), with or without CNQX (Tocris and Cambridge Research Biochemicals) and glycine (Bio-Rad, Richmond, CA), were prepared in the external medium and applied close (<100 μm) to the cell under study through multibarrelled perfusion pipettes, which enabled rapid switching between solutions, similar to that described by Johnson and Ascher (14). One of the barrels always contained external solution alone, which ensured a rapid wash-out after drug application. Solution changes were complete in less than 200–250 msec, as judged by the time to peak response to a nondesensitizing concentration of NMDA/glycine (see Results).

[^{3}H]Glycine binding assay. A crude membrane preparation was obtained by homogenizing frontal cortex from frozen guinea-pig brains (Pel-Freeze, Rogers, AZ) in 10 volumes (w/v) of 0.32 M sucrose. The homogenate was centrifuged (1000 × *g*, 10 min, 4°). The nuclear pellet was discarded and the supernatant was centrifuged (20,000 × *g*, 20 min, 4°). The pellet was again resuspended to a protein concentration of 2.5 mg·ml^{-1}, using the method of Bradford (18), and was stored in 20-ml aliquots at −70° for at least 48 hr before use.

On the day of the assay, the crude membranes were thawed and resuspended in assay buffer (50 mM Tris/citrate, pH 7.2). The membranes were then centrifuged (20,000 × *g*, 20 min) and resuspended in assay buffer to a protein concentration of 2.5 mg·ml^{-1}. In order to facilitate the removal of endogenous ligands, the membrane preparation was then treated with 0.1% Triton X-100 and 0.1 EDTA on ice for 20 min. The detergent treatment was terminated by centrifugation (20,000 × *g*, 20 min). Before the assay, the membranes were washed an additional three times with assay buffer. The washed membranes (150–200 μg) were incubated with 4 nM [^{3}H]glycine (43.5 Ci/mmol; Dupont NEN, Boston, MA) and unlabeled drug or buffer in a final volume of 0.2 ml. After incubation on ice for 30 min, the reaction was terminated by rapid filtration at 4° using no. 32 glass filters (Schleicher and Schuell, Keene, NH) pretreated with 0.05% polyethylenimine. The filters were washed with 5 ml of ice-cold assay buffer and radioactivity was measured by liquid scintillation counting. Nonspecific binding, measured in the presence of 1 mM glycine, varied between 5 and 15% of the total [^{3}H]glycine binding. Saturation data were evaluated by Scatchard analysis using EBDA (19) and LIGAND (20) data analysis programs.

Results

In hippocampal neurons, voltage-clamped at −60 mV, fast perfusion of NMDA in the presence of glycine induced inward currents that exhibited some desensitization over the first few seconds and then attained a 'steady state' level (Fig. 1A). The desensitizing phase of the response was reduced by increasing the frequency or number of applications of NMDA/glycine or by slowing the exchange of solutions. The rapidly fading component was attenuated also at low glycine concentrations (Fig. 1A), although this effect was not systematically studied. Because the steady state component remained stable over the course of the experiment, the actions of CNQX were examined on this phase of the response. Glycine concentration-response curves were constructed by measuring the steady state current produced by 30 μM NMDA in varying concentrations of glycine. Over the range 30 nM to 300 μM, glycine potentiated NMDA receptor-mediated responses. From the logistic curve, the half-maximally effective concentration of glycine was estimated as 244 nM, and the slope was 1.06 (Fig. 1B). The glycine enhancement was insensitive to strychnine (1 μM) and, under the experimental conditions employed, perfusion of glycine alone at concentrations up to 300 μM had no effect on the holding potential. Conversely, in the absence of any added glycine, NMDA elicited little or no current (Fig. 1A).

The sensitivity of NMDA/glycine receptor-mediated currents to CNQX was examined by including this antagonist in the perfusion pipettes. CNQX, at concentrations of 10 and 30 μM, reduced the steady state current induced by NMDA (30 μM)/glycine (1 μM) by 38.4 and 76.6%, respectively. CNQX alone, at concentrations up to 100 μM, produced no change in the holding current, although, in cells in which TTX-resistant

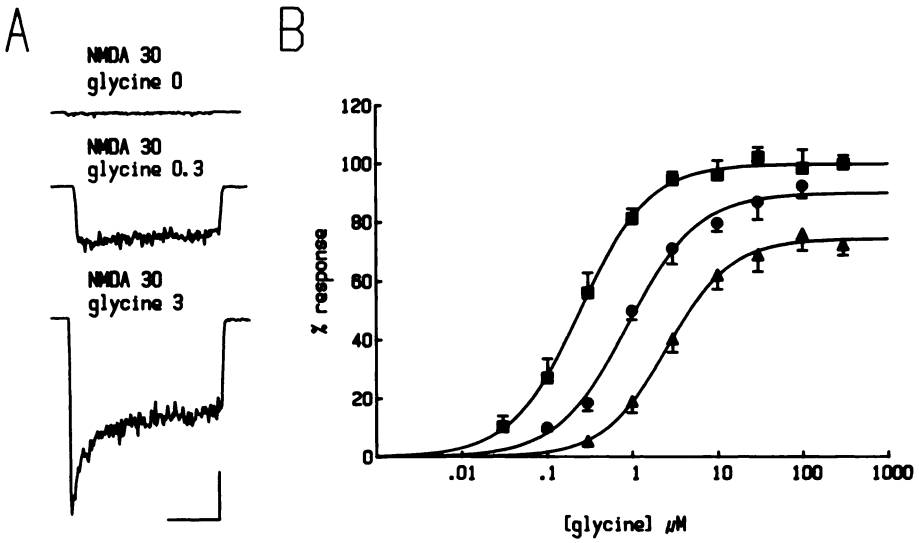

Fig. 1. The effect of glycine on NMDA receptor-mediated currents in hippocampal neurons in the presence and absence of CNQX. A, Currents induced by 13-sec applications of 30 μM NMDA with the addition of 0, 0.3, or 3 μM glycine. B, Glycine concentration-response curves constructed with a constant concentration of NMDA (30 μM) in the presence (10 μM, ●; 30 μM; ▲) or absence (■) of CNQX. The *curves* were constructed from a population of neurons, voltage-clamped at −60 mV; in each cell 3–5 separate points were obtained with reference to a standard (3 μM glycine). The data were fitted to the logistic equation by a least-squares method. *Error bars* indicate the standard deviation. *Calibration bars* in A are 280 pA and 4 sec.

synaptic activity was present, these events were antagonized by CNQX. The antagonism of NMDA currents by CNQX was reversed at higher concentrations of glycine (3–300 μM) (Fig. 2A). In effect, the glycine concentration-response curves were shifted to the right in the presence of CNQX (Fig. 1B). Glycine concentrations up to 300 μM, however, failed to completely reverse the antagonism of NMDA receptor-induced responses by CNQX, apparent from the glycine concentration-response curves as a reduction in the maximum NMDA current (Fig. 1B). This depression was more marked for higher concentrations of CNQX. Thus, for 10 and 30 μM CNQX, the maximum NMDA current was depressed by 10 and 26%, respectively. To test whether this effect of CNQX was due to an interaction with the NMDA recognition site, the concentration of NMDA was increased to 500 μM in the presence of supramaximal concentrations of glycine (300 μM). Under these conditions, the block of the NMDA/glycine receptor-induced current could be completely overcome (Fig. 2B).

Additional experiments were performed to characterize further the nature of the block of NMDA receptor-mediated currents produced by CNQX. Rapid switching between solutions containing NMDA/glycine and NMDA/glycine/CNQX indicated a rapid onset and offset of the block by CNQX, suggesting a lack of use dependence (Fig. 3). In addition, the antagonism produced by CNQX was not dependent on the holding potential over the range −80 to +60 mV. To demonstrate that the glycine reversal of the NMDA/glycine receptor block by CNQX was specific to the NMDA receptor complex,

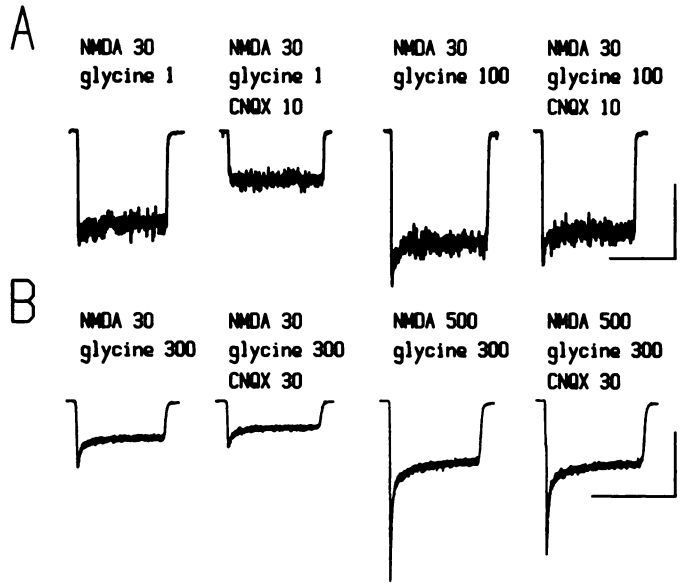

Fig. 2. Glycine reverses the antagonism of NMDA receptor-induced currents by CNQX. A, The current induced by fast perfusion of 30 μM NMDA/1 μM glycine (*left*) is reduced by 10 μM CNQX (*center left*). In the presence of 100 μM glycine, the response to 30 μM NMDA is enhanced (*center right*), although the degree of antagonism by CNQX is markedly reduced (*right*). B, The NMDA current induced at high concentrations (300 μM) of glycine (*left*) is reduced by 30 μM CNQX (*center left*). However, this residual block can be overcome by increasing the concentration of NMDA to 500 μM (*center right, right*). Calibration in A, 300 pA × 10 sec.; in B, 2 nA × 15 sec.

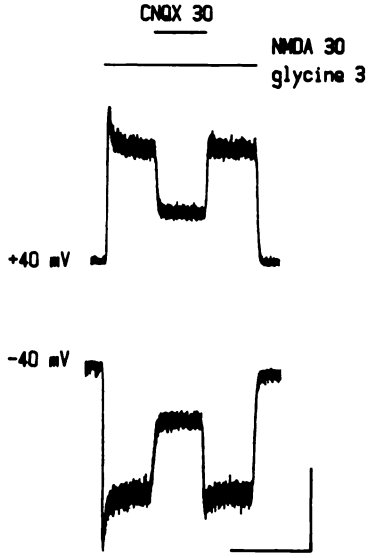

Fig. 3. The block of NMDA currents is not dependent on the membrane potential. A, Examples of currents recorded at +40 mV (*upper*) and −40 mV (*lower*) in response to switching between solutions containing 30 μM NMDA/3 μM glycine and 30 μM NMDA/3 μM glycine/30 μM CNQX. The same concentration of NMDA/glycine is maintained throughout the perfusion. Calibration is 500 pA × 20 sec.

responses produced by the non-NMDA receptor agonist kainate were studied. At holding potentials of −60 mV, inward currents induced by 20 μM kainate were substantially reduced by 10 μM CNQX, and this antagonism was found to be insensitive to increases in the concentration of glycine from 1 μM to 1 mM (Fig. 4). This observation supports the finding that the potentiating effects of glycine result from a specific interaction with the NMDA receptor complex (14).

[^{3}H]Glycine binding to a crude membrane preparation was used to label the NMDA receptor-associated glycine site (10, 11, 21, 22) and provide an additional method of examining the interaction of CNQX with the NMDA receptor complex. [^{3}H]Glycine binding was saturable and fully reversible. The binding was linear up to 800 μg of protein. Equilibrium of [^{3}H]glycine binding was reached within 30 min and was stable for 2 hr. The equilibrium dissociation constant (K_d) for glycine was 119± 20 nM. [^{3}H]Glycine binding was inhibited by D-serine (K_i = 390 ± 10 nM) and kynurenic acid (K_i = 22 ± 4 μM) but not by strychnine (3 μM), consistent with data reported previously for the NMDA receptor-associated glycine binding site (10, 11, 21). CNQX inhibited specific [^{3}H]glycine binding dose dependently and completely. An IC_{50} value for CNQX of 5.7 μM (n=5) was estimated from the logistic fit of the data (Fig. 5). Scatchard analyses of saturation isotherms of specific [^{3}H]glycine binding in the presence and absence of CNQX were also performed (Fig. 6). The results revealed that [^{3}H]glycine was binding to a single site and showed that CNQX (5 μM) decreased the affinity

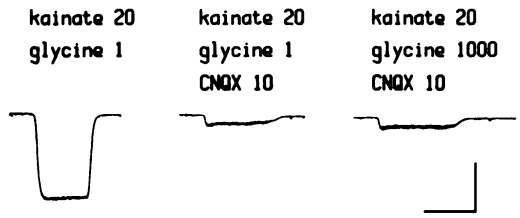

Fig. 4. The antagonism of kainate receptor-induced responses is not reversed by glycine. Fast perfusion of 20 μM kainate, like NMDA, results in an inward current in hippocampal neurons voltage-clamped at −60 mV (*left*). The response to kainate is depressed in the presence of 10 μM CNQX irrespective of the concentration of glycine, 1 μM (*center*) or 1000 μM (*right*). Calibration is 1 nA × 10 sec.

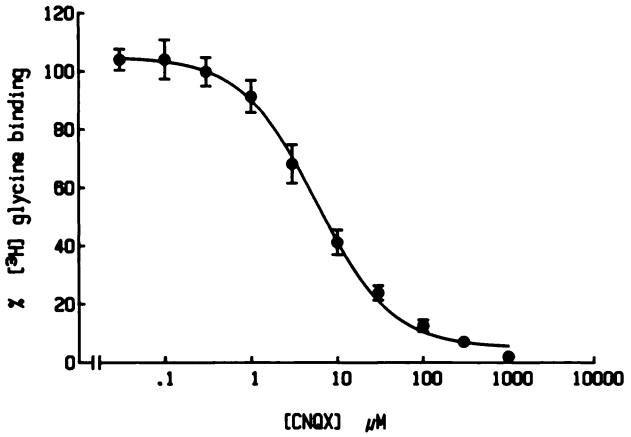

Fig. 5. CNQX inhibits the binding of [^{3}H]glycine to a crude membrane preparation. A crude preparation of guinea pig frontal cortex membranes was incubated with [^{3}H]glycine and CNQX (30 nM to 1 mM). CNQX displaced [^{3}H]glycine with an IC_{50} value of 5.7 μM. The data shown are the mean ± standard error of five experiments. The *curve* was fitted by a least squares method.

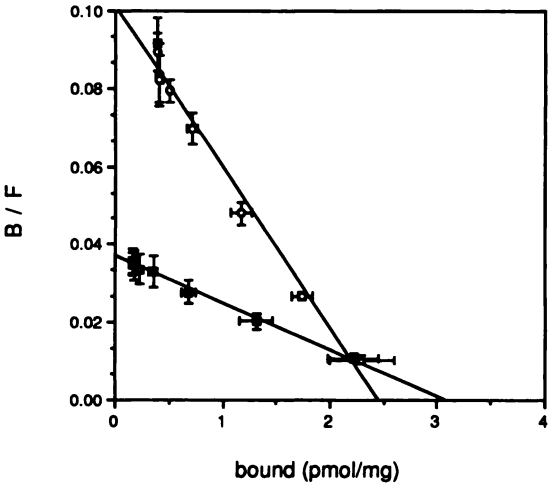

Fig. 6. Scatchard plot of [^{3}H]glycine binding, generated using EBDA (19), in the presence (○) and absence (■) of 5 μM CNQX. *Lines* were fitted by at a least squares linear regression. *B/F*, bound/free [^{3}H]glycine. The data shown are the mean ± standard error of three experiments.

(increase in K_d) of [^{3}H]glycine binding with no significant change in B_{max}. Linear regression performed on the Scatchard plot using EBDA (19) produced K_d values of 185 ± 73 and 679 ± 62 nM, and B_{max} values of 2.4 ± 0.3 and 3.2 ± 0.6 pmol·mg of protein^{-1} in the absence and presence of CNQX, respectively. Similar results were obtained when the data were analyzed using the curve-fitting program LIGAND (20); the K_d of [^{3}H]glycine binding was increased from 119 ± 20 nM to 348 ± 42 nM in the presence of CNQX, with no change in the B_{max} (2.5 ± 0.2 and 2.5 ± 0.4 pmol·mg protein^{-1}). These data suggest a competitive and reversible interaction of CNQX with the [^{3}H]glycine binding site.

Discussion

The results presented in this study suggest that the recently described non-NMDA receptor antagonist CNQX (3) in micromolar concentrations, antagonizes NMDA receptor-mediated responses, in part through a competitive interaction at the regulatory glycine site of the NMDA receptor-complex (14).

In agreement with previous studies, glycine, in nanomolar concentrations, potentiated the NMDA receptor-induced current, in a strychnine-insensitive manner (14–16). Under these conditions, compounds that interact with the regulatory glycine binding site of the NMDA receptor complex could be readily investigated.

An examination of the antagonism of NMDA receptor-induced responses by CNQX showed that the block could be largely overcome by increasing the concentration of glycine in the extracellular medium. Similar glycine-induced reversals of the block of NMDA receptor-mediated responses have been reported for kynurenic acid (12, 13), 7-chlorokynurenic acid (10), and HA-966 (23), and the present data suggest that CNQX also belongs to this class of antagonists. Based on the findings that kynurenic acid and 7-chlorokynurenic acid depressed the NMDA current recorded in the absence of any added glycine, these compounds have been described as 'inverse agonists' (10, 12). However, in our experiments, NMDA produced little or no response in the absence of glycine, and it is not unlikely that low nanamolar traces of glycine were present in the control media, therefore precluding an investigation of inverse agonist

actions of CNQX. Thus, our results are in agreement with the suggestion that glycine is required for NMDA receptor channel activation (15). The interaction of CNQX with the NMDA receptor complex is, however, not entirely due to a simple action at the glycine site; a depression of the maximum response accompanied the shift to the right in the glycine concentration-response curve. In this respect, kynurenic acid at high concentrations was also observed to reduce the maximum NMDA current (12). The observation that, at high glycine concentrations, an increase in the concentration of NMDA could fully overcome the block suggests that the 'residual' CNQX antagonism results from an interaction with the NMDA agonist recognition site. CNQX can displace [^{3}H]CPP from the NMDA receptor ligand binding site with an IC_{50} value of 25 μM (3) and provides support for the interpretation that even relatively low concentrations of CNQX (i.e., 10 μM) may exert a direct effect at the NMDA receptor recognition site. The results from the present study support and extend the recent observation that CNQX is a competitive antagonist at the strychnine-insensitive glycine site in the neonatal rat spinal cord preparation (24). Interestingly, the data from the above study (24) indirectly suggest a second site of action for CNQX. The authors reported that 300 μM CNQX produced a 'flattening' of the NMDA concentration-response curve and that co-application of 1 mM D-serine converted the flattening of the curve into a parallel shift to the right, suggesting that the remaining CNQX antagonism may have been due to a competitive interaction at the NMDA ligand binding site.

Additional support for a direct interaction of CNQX with the glycine site is the finding that CNQX inhibited the specific binding of [^{3}H]glycine to guinea pig brain frontal cortex membranes. In these membrane preparations, [^{3}H]glycine specifically labels a strychnine-insensitive binding site that has the pharmacological characteristics of the glycine site associated with the NMDA receptor (10, 11, 21, 22). The IC_{50} value of 5.7 μM for the inhibition of [^{3}H]glycine binding by CNQX, calculated from the present experiments, agreed well with a 'K_d' value of 3 μM estimated from the shift in the glycine concentration-response curves produced by CNQX. Glutamate has been shown to enhance [^{3}H]glycine binding and, therefore, antagonists such as 2-amino-5-phosphonovalerate and CPP, which compete with glutamate for the NMDA recognition site, can also inhibit the binding of [^{3}H]glycine (11, 22). Because this study has provided evidence that CNQX can act, in part, at the NMDA recognition site, this antagonist may displace [^{3}H]glycine indirectly. However, low concentrations of this antagonist (5 μM), which should favor effects at the glycine site, decreased the affinity (increase in K_d) of [^{3}H]glycine binding with little change in B_{max}, consistent with a competitive interaction at the glycine site.

The similar IC_{50} values for the interaction of CNQX at the glycine binding site (5.7 μM) and the NMDA recognition site (25 μM; Ref. 3) suggest that CNQX does not distinguish well between these two sites. This may point to structural similarities between the NMDA and glycine binding sites and to the suggestion that the glycine binding site is a modified NMDA ligand site (15).

The lack of voltage dependence and marked use dependence of the CNQX block suggests that this compound does not behave like the NMDA channel-blocking agents phencyclidine, ketamine, and MK-801 (13, 25–27). Indeed, inasmuch as glycine has been demonstrated to increase the frequency of NMDA channel opening (14, 27), the antagonism of a channel blocker may be expected to be enhanced, not reduced, by an increase in extracellular glycine. In binding studies, glycine has been shown to potentiate the NMDA receptor ligand-induced binding of radiolabeled channel blockers (28–31).

In contrast to the marked antagonism of currents induced by NMDA, reported in this study and in oocytes (32), depression of NMDA receptor-mediated responses by low concentrations of CNQX (i.e., <10 μM) has not generally been reported in *in vitro* slice preparations (4, 5, 33, 34). The most likely explanation for these findings is that the concentration of extracellular glycine in brain slices is sufficiently high (see Ref. 14) to overcome the block at the glycine site. Because most of the above studies were conducted using CNQX at concentrations of 10 μM or less, if glycine were present in the extracellular medium in concentrations of 5–10 μM most of the antagonism by CNQX at the glycine site would be removed. It is not unreasonable to assume these levels of glycine in the extracellular space, because NMDA responses in *in vitro* preparations are unaffected, and, therefore, are presumed to be maximally potentiated, by glycine (10, 23, 24). Alternatively, the glycine site may be different in cultured neurons, although this is unlikely because the pharmacology and affinity of glycine-like compounds are similar in both isolated cells (14, 15) and *in vitro* preparations (10, 24) and binding assays (10, 11, 21, 22, 29).

Because synaptic responses in many excitatory amino acid-utilizing pathways in the vertebrate central nervous system result from co-activation of both non-NMDA and NMDA receptors (1, 35–37), one application of a selective non-NMDA receptor antagonist would be to eliminate transmission through non-NMDA receptors, thus enabling the study of NMDA receptor-mediated synaptic responses in isolation. Several studies using CNQX as a synaptic antagonist have already been undertaken (4, 5, 33, 34, 38). The results from the present study that CNQX is a reasonably potent antagonist at the NMDA receptor-associated glycine site, together with the observation that synaptically activated NMDA receptors show a similar dependency on external glycine (39), suggest that the conditions of the experiment will be important in the interpretation of data obtained using CNQX. The present evidence suggests, however, that the selectivity of CNQX for non-NMDA receptors can be enhanced, provided that it is used in low concentrations (<10 μM) in the presence of 10–100 μM glycine.

References

1. Mayer, M., and G. L. Westbrook. The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog. Neurobiol.* **28**:197–276 (1987).
2. Davies, J., R. H. Evans, A. W. Jones, and J. C. Watkins. 2-Amino-5-phosphonovalerate (2-APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci. Lett.* **21**:77–81 (1981).
3. Honore, T., S. N. Davies, J. Drejer, E. J. Fletcher, P. Jacobsen, D. Lodge, and F. E. Nielsen. Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science (Wash. D. C.)* **241**:701–703 (1988).
4. Blake, J. F., M. W. Brown, and G. L. Collingridge. CNQX blocks acidic amino acid induced depolarizations and synaptic components mediated by non-NMDA receptors in rat hippocampal slices. *Neurosci. Lett.* **89**:182–186 (1988).
5. Fletcher, E. J., D. Martin, J. A. Aram, D. Lodge, and T. Honore. Quinoxalinediones selectively block quisqualate and kainate receptors and synaptic events in rat neocortex and hippocampus and frog spinal cord *in vitro*. *Br. J. Pharmacol.* **95**:585–597 (1988).
6. Drejer, J., and T. Honore. New quinoxalinediones show potent antagonism of quisqualate responses in cultured mouse cortical neurons. *Neurosci. Lett.* **87**:104–108 (1988).
7. Birch, P. J., C. J. Grossman, and A. G. Hayes. Kynurenate and FG9041 have

both competitive and non-competitive antagonist actions at excitatory amino acid receptors. *Eur. J. Pharmacol.* **151**:313–315 (1988).
8. Evans, R. H., S. J. Evans, P. C. Pook, and D. C. Sunter. A comparison of excitatory amino acid antagonists acting at primary afferent C fibres and motoneurones of the isolated spinal cord of the rat. Br. J. Pharmacol. **91**:531–537 (1987).
9. Kemp, J. A., S. Grimwood, and A. C. Foster. Characterization of the antagonism of the excitatory amino acid receptors in rat cortex by kynurenic acid. *Br. J. Pharmacol.* **91**:314P (1987).
10. Kemp, J. A., A. C. Foster, P. D. Leeson, T. Priestley, R. Tridgett, L. L. Iversen, and G. N. Woodruff. 7-Chlorokynurenic acid is a selective antagonist at the glycine modulatory site of the *N*-methyl-D-aspartate receptor complex. *Proc. Natl. Acad. Sci. USA* **85**:6547–6550 (1988).
11. Kessler, M., M. Baudry, T. Terramani, and G. Lynch. Complex interactions between a glycine binding site and NMDA receptors. *Soc. Neurosci. Abstr.* **13**:760 (1987).
12. Ascher, P., G. Henderson, and J. W. Johnson. Dual inhibitory actions of kynurenate on the *N*-methyl-D-asparate (NMDA)-activated response of cultured mouse cortical neurones. *J. Physiol. (Lond.)* **406**:141P (1989).
13. Mayer, M. L., G. L. Westbrook, and L. Viklicky, Jr. Sites of antagonist action on *N*-methyl-D-aspartic acid receptors studied using fluctuation analysis and a rapid perfusion technique. *J. Neurophysiol.* **60**:645–663 (1988).
14. Johnson, J. W., and P. Ascher. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature (Lond.)* **325**:529–531 (1987).
15. Kleckner, N. W., and R. Dingledine. Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science (Wash. D. C.)* **241**:835–837 (1988).
16. Johnson, J. W., and P. Ascher. Interaction of glycine with the *N*-methyl-D-aspartate receptor. *Soc. Neurosci. Abstr.* **13**:383 (1987).
17. Jahr, C. E., and C. F. Stevens. Glutamate activates multiple single channel conductances in hippocampal neurons. *Nature (Lond.)* **325**:522–525 (1987).
18. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
19. McPhearson, G. A. A practical computer based approach to the analysis of radioligand binding experiments. *Comput. Programs Biomed.* **17**:107–114 (1983).
20. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for the characterization of ligand binding studies. *Anal. Biochem.* **107**:220–239 (1980).
21. Bristow, D. R., N. G. Bowery, and G. N. Woodruff. Light microscopic autoradiographic localization of [^{3}H]glycine and [^{3}H]strychnine binding sites in rat brain. *Eur. J. Pharmacol.* **126**:303–307 (1986).
22. Nguyen, L., D. T. Monaghan, and C. W. Cotman. Glycine binding sites reciprocally interact with glutamate binding sites at the NMDA receptor complex. *Soc. Neurosci. Abstr.* **13**:760 (1987).
23. Fletcher, E. J., and D. Lodge. Glycine reverses antagonism of *N*-methyl-D-aspartate (NMDA) by 1-hydroxy-3-aminopyrrolidone-2 (HA-966) but not by D-2-amino-5-phosphonovalerate (D-APV) on rat cortical slices. *Eur. J. Pharmacol.* **151**:161–162 (1988).
24. Birch, P. J., C. J. Grossman, and A. G. Hayes. 6,7-Dinitro-quinoxaline-2,3-dion and 6-nitro-7-cyano-quinoxaline-2,3-dion antagonise responses to NMDA in the rat spinal cord via an action at the strychnine-insensitive glycine receptor. *Eur. J. Pharmacol.* **156**:177–179 (1988).
25. Honey, C. R., Z. Miljkovic, and J. F. MacDonald. Ketamine and phencyclidine cause a voltage dependent block of responses to L-aspartic acid. *Neurosci. Lett.* **61**:131–139 (1985).
26. Huettner, J. A., and B. P. Bean. Block of NMDA activated current by the anticonvulsant MK801:selective binding to open channels. *Proc. Natl. Acad. Sci. USA*, **85**:1307–1311 (1988).
27. Bertolino, M., S. Vinci, J. Mazzetta, and E. Costa. Phencyclidine and glycine modulate NMDA-activated high conductance cationic channels by acting at different sites. *Neurosci. Lett.* **84**:351–355 (1988).
28. Bonhaus, D. W., B. C. Burge, and J. O. McNamara. Biochemical evidence that glycine allosterically regulates an NMDA receptor-coupled ion channel. *Eur. J. Pharmacol.* **142**:489–490 (1987).
29. Reynolds, I. J., S. N. Murphy, and R. J. Miller. ^{3}H-Labeled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. *Proc. Natl. Acad. Sci. USA* **84**:7744–7748 (1987).
30. Snell, L. D., R. S. Morter, and K. M. Johnson. Glycine potentiates *N*-methyl-D-aspartate-induced [^{3}H]TCP binding to rat cortical membranes. *Neurosci. Lett.* **83**:313–317 (1987).
31. Wong, E. H., A. R. Knight, and R. Ransom. Glycine modulates [^{3}H]MK-801 binding to the NMDA receptor in rat brain. *Eur. J. Pharmacol.* **142**:487–488 (1987).
32. Verdoorn, T. A., and R. Dingledine. Antagonist pharmacology of excitatory amino acid receptors expressed in *Xenopus* oocytes. *Soc. Neurosci. Abstr.* **14**:1193 (1988).
33. Neuman, R. S., Y. Ben-Ari, M. Gho, and E. Cherubini. Blockade of excitatory synaptic transmission by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) in the hippocampus *in vitro*. *Neurosci. Lett.* **92**:64–68 (1988).
34. Andreasen, M., J. D. C. Lambert, and M. Skovgaard Jensen. Direct demonstration of an *N*-methyl-D-aspartate receptor mediated component of excitatory synaptic transmission in area CA1 of the rat hippocampus. *Neurosci. Lett.* **93**:61–66 (1988).
35. Dale, N., and A. Roberts. Dual component amino acid-mediated synaptic potentials: excitatory drive for swimming in *Xenopus* embryos. *J. Physiol.* 363:35–59 (1985).
36. Collingridge, G. L., C. E. Herron, and R. A. J. Lester. Synaptic activation of *N*-methyl-D-aspartate receptors in the Schaffer collateral-commissural pathway of rat hippocampus. *J. Physiol. (Lond.)* **399**:283–300 (1988).
37. Wigstrom, H., B. Gustafsson, and Y.-Y. Huang. A synaptic potential following single volleys in the hippocampal CA1 region possibly involved in the induction of long-lasting potentiation. *Acta Physiol. Scand.* **124**:475–478 (1985).
38. Davies, S. N., C. Davies, and G. L. Collingridge. Role of excitatory amino acid receptors in synaptic transmission in area CA1 of rat hippocampus. *Proc. Royal. Soc. B.*, in press.
39. Forsythe, I. D., G. L. Westbrook, and M. l. Mayer. Modulation of excitatory synaptic transmission by glycine and zinc in cultures of mouse hippocampal neurons. *J. Neurosci.* **8**:3733–3741 (1988).

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