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Activity of 5,7-Dichlorokynurenic Acid, a Potent Antagonist at the *N*-Methyl-D-aspartate Receptor-Associated Glycine Binding Site

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SUMMARY

5,7-Dichlorokynurenic acid (5,7-DCKA), one of the most potent excitatory amino acid receptor antagonists yet described, binds to a strychnine-insensitive glycine binding site located on the *N*-methyl-D-aspartate (NMDA) receptor complex (K_i = 79 nM versus [^{3}H]glycine). 5,7-DCKA (10 μM) antagonized the ability of NMDA to stimulate the binding of the radiolabeled ion channel blocker *N*-[^{3}H][1-(2-thienyl)cyclohexyl]-piperidine ([^{3}]TCP). Glycine was able to overcome this effect and in the presence of 5,7-DCKA enhanced [^{3}H]TCP binding to antagonist-free levels. 5,7-DCKA completely and noncompetitively antagonized several NMDA receptor-mediated biochemical and electrophysiological responses. Thus, micromolar concentrations of 5,7-DCKA inhibited NMDA-stimulated elevation of cytosolic calcium in cultured hippocampal neurons, cGMP accumulation in cerebellar slices, and norepinephrine release from hippocampal slices. The glycine antagonist could also block the action of synaptically released agonist, as shown by its ability to inhibit the increase in the magnitude of the population spike that follows tetanic stimulation of the hippocampus *in vitro* (long term potentiation). Inclusion of glycine or D-serine prevented all these effects of the antagonist. 5,7-DCKA was a potent anticonvulsant when administered intracerebroventricularly to mice. As in the *in vitro* experiments, the dose-response curve for the antagonist was shifted rightward in a parallel fashion when D-serine was coinjected. This spectrum of activity displayed by a compound acting at the glycine binding site suggests that the therapeutic utility of glycine antagonists will be similar to those proposed for other types of glutamate receptor antagonists.

Glycine, by binding to a strychnine-insensitive site, greatly enhances ion flux mediated by NMDA-type glutamate receptors (1, 2). This has led to the conception of glycine as a modulator of NMDA neurotransmission. The concentrations of glycine that alter NMDA responses *in vitro* are well within the range of glycine concentrations occurring *in vivo* (3). Recently, it has been suggested that glycine may be obligatory for NMDA receptor activation. The best evidence for this hypothesis comes from patch-clamp experiments using mRNA-injected *Xenopus* oocyte preparations expressing rat brain NMDA receptors (4–6). NMDA responses are dependent upon exogenous glycine and extrapolate to zero as glycine concentrations are reduced. It has been difficult to duplicate these findings using nervous tissue, presumably because the levels of tissue-contained glycine are sufficient to saturate the glycine binding site. One obvious way to address this question would be to remove the influence of glycine by using a glycine antagonist. We report here the effects of a potent and highly selective glycine antagonist in a variety of tests of NMDA receptor activation. Our results with this useful tool are consistent with previous reports in suggesting a requirement for glycine in NMDA receptor activation.

Experimental Procedures

Materials. 5,7-DCKA, 7-chlorokynurenic acid, and HA-966 were synthesized at Merrell Dow Research Institute (Cincinnati, OH). Unlabeled CPP was obtained from Research Biochemicals (Natick, MA). 6,7-Dichloroquinoxaline-2,3-dione was obtained from Tocris Neuramin (Essex, England). Radiochemicals used were obtained from DuPont New England Nuclear (Boston, MA) and are as follows: [^{3}H]CPP (30.7 Ci/mmol), [^{3}H]TCP (47.6 Ci/mmol), [^{3}H]glycine (43.5 Ci/mmol), [^{3}H] kainate (58.0 Ci/mmol), [^{3}H]AMPA (27.6 Ci/mmol), and [^{3}H]NE (10 Ci/mmol). Cell culture reagents used were DMEM, laminin, and poly-D-ornithine (all from Sigma Chemical Co., St. Louis, MO), newborn calf serum (Whittaker Bioproducts, Walkersville, MD), and glass coverslips (0.07-mm thick, 31-mm diameter; Biophysica Technologies, Baltimore, MD). Fura-2/AM was from Molecular Probes (Eugene, OR). All other reagents were obtained from Sigma unless otherwise specified.

NMDA receptor binding assays ([^{3}H]CPP, [^{3}H]glycine, and [^{3}H]

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; TCP, *N*-[1,(2-thienyl)cyclohexyl]-piperidine; 5,7-DCKA, 5,7-dichlorokynurenic acid; NE, norepinephrine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; $[Ca]_i$, cytosolic free calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; GABA, γ-aminobutyric acid; LTP, long term potentiation; fura-2/AM, fura-2 acetoxymethyl ester.

TCP) were performed using pooled tissue obtained from the rat hippocampus and cerebral cortex and washed using Triton X-100, as described previously (7).

[^{3}H]CPP binding. [^{3}H]CPP binding was performed as described by Murphy *et al.* (8). Frozen membranes were thawed at room temperature and washed three times using 30 ml of ice-cold assay buffer. Assays (1 ml) were conducted in scintillation minivials containing 10 nM [^{3}H] CPP, 200 μg of membrane protein, 50 mM Tris·HCl buffer (pH 7.40 at 25°), and various concentrations of test compounds (pH adjusted to 7.40) or 1 mM unlabeled glutamate (nonspecific binding). Following a 15-min incubation at 25°, vials were centrifuged 10 min at 46,000 × *g*. Supernatants were decanted and the pellets were superficially washed three times with 2 ml of ice-cold assay buffer. Pellets were solubilized overnight in 4 ml of Ready Protein (Beckman). Specific binding accounted for 76% of the total binding, which was 11,000 to 20,000 dpm/assay vial.

[^{3}H]TCP binding. [^{3}H]TCP binding was measured using a more extensively washed membrane preparation. On the day of assay the Triton-washed membranes were thawed at room temperature and resuspended in 30 ml of 25° assay buffer. This suspension was incubated for 15 min at 25° in a shaking water bath and then centrifuged at 4°. This resuspension and incubation procedure was repeated two more times. This modified wash procedure was found to be necessary to demonstrate effects of exogenous glutamate and glycine. Assays were performed in glass tubes containing 2 nM [^{3}H]TCP, 100 μg of membrane protein, and unlabeled ligands as indicated, in a final volume of 0.5 ml of 5 mM Tris-acetate (pH 7.4) buffer. Nonspecific binding was defined as that remaining in the presence of 100 μM unlabeled phencyclidine. Following a 2-hr incubation at 25°, samples were filtered through glass fiber (GF/B) filter strips presoaked in 0.1% (v/v) polyethyleneimine and were washed three times with 3 ml of ice-cold assay buffer. These assay parameters were chosen to maximize the magnitude of NMDA- and glycine-induced increases in [^{3}H]TCP binding and do not necessarily represent equilibrium conditions. Specific binding, in the absence of added amino acids, was about 65% of total binding, which generally was 800 dpm/assay tube.

[^{3}H]Glycine binding. [^{3}H]Glycine binding was measured using the more extensively washed membrane preparation described above for [^{3}H]TCP binding. Assays were conducted in minivials, incubated for 30 min on ice in 0.5 ml of 50 mM HEPES-KOH (pH 7.4) containing 20 nM [^{3}H]glycine, 100 μg of membrane protein, unlabeled ligands as indicated, and 100 μM strychnine. Nonspecific binding was defined by inclusion of 100 μM D-serine. Bound ligand was isolated by centrifugation, as described above. Specific binding accounted for 75% of total binding, which was generally 19,000 dpm/assay vial.

[^{3}H]AMPA binding. [^{3}H]AMPA binding was performed as described by Honoré and Drejer (9). Assay vials contained 0.5 mg of membrane protein, 5 nM [^{3}H]AMPA, 100 mM KSCN, various concentrations of test compounds, 2.5 mM $CaCl_2$, and 30 mM Tris·HCl (pH 7.4 at 4°), in a final volume of 0.5 ml. Nonspecific binding was defined using 1 mM L-glutamate. Specific binding accounted for >85% of total binding. Following incubation on ice for 60 min, bound ligand was collected by centrifugation.

[^{3}H]Kainate binding. [^{3}H]Kainate binding was performed as described by London and Coyle (10). Assay vials contained 0.5 mg of membrane protein (prepared as above for [^{3}H]AMPA binding), 5 nM [^{3}H]kainate, various concentrations of test compounds, and 50 mM Tris·HCl (pH 7.4 at 4°), in a final volume of 1 ml. Nonspecific binding was defined using 100 μM unlabeled kainic acid. Specific binding accounted for >80% of the total binding. Following incubation on ice for 60 min, bound ligand was collected by centrifugation.

Cyclic GMP accumulation. NMDA-stimulated cGMP accumulation was measured in neonatal rat cerebellar slices as described by Baron *et al.* (7). Cerebella were removed from 8-day-old rats and pooled in ice-cold saline. The tissue was chopped into 400-μm slices using a McIlwain tissue chopper. The stage was then rotated 90° and the tissue was chopped a second time. Sliced tissue was removed with a Teflon-coated spatula and divided between two vials, each containing 15 ml of oxygenated Krebs buffer (in mM: 108 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.0 KH_2PO_4, 25 $NaHCO_3$, 11.1 D-glucose, 10 HEPES-NaOH, pH 7.35, with 0.001% phenol red). The vials were tightly capped, wrapped with Parafilm, and vortexed for 5 sec. The vials were placed in a shaking water bath in a horizontal orientation and incubated at 37° for 2 hr. During this period the vials were removed at 30-min intervals, the slices were allowed to settle, and the medium was replaced with 15 ml of fresh buffer. At the end of this period, the slices were washed four times with 20 ml of well oxygenated assay buffer (in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.18 KH_2PO_4, 25 $NaHCO_3$, 11.1 glucose, 10 HEPES-NaOH, pH 7.35). Assays were performed in stoppered polypropylene tubes, in a final volume of 500 μl containing 25 μl of gravity-packed slices (200 to 400 μg of membrane protein), various test compounds, and, where indicated, 15 μM NMDA. Studies measuring antagonism utilized a 10-min preincubation with the antagonist before agonist challenge. Before NMDA addition, the assay tubes were cooled on ice and uncapped and the agonist was added. The tubes were gassed with 95% O_2/5% CO_2, recapped, and incubated for 5 min at 37°. Assays were stopped on ice using 50 μl of 44 mM EDTA. The tubes were then heated in a boiling water bath for 4 min, homogenized, and centrifuged. The supernatants containing the cGMP were transferred to separate tubes. Pellets containing membrane protein were dissolved in 2 ml of 0.1 N NaOH and assayed for protein content using a dye-binding method (Bio-Rad). Levels of cGMP were quantitated using a commercially available tritium-based radioimmunoassay kit (Amersham), according to the manufacturer's instructions. Levels of cGMP were quantitated using a commercially available radioimmunoassay kit (Amersham), according to the manufacturer's instructions, and were expressed as pmol/mg of protein.

[^{3}H]NE release. [^{3}H]NE release was measured using hippocampal slices superfused in a chamber system described previously (11). Tissues from two rats (Sprague-Dawley males, 250–350 g) were used for each individual experiment. The paired hippocampi were removed and sliced along three angles at 0.25 mm, using a McIlwain tissue chopper. The slices were then incubated for 10 min in 5 ml of a Krebs Ringer bicarbonate buffer (in mM: 118 NaCl, 4.85 KCl, 2.5 $CaCl_2$, 1.15 KH_2PO_4, 25 $NaHCO_3$, 11.1 glucose, pH 7.3). All solutions also contained 100 μM pargyline and were constantly bubbled with 95% O_2/5% CO_2. [^{3}H]NE was added at approximately 0.1 μM and the incubation was continued for an additional 20 min. After washing, the slices were aliquoted between eight superfusion chambers set to a volume of 0.25 ml. The chambers were superfused at a rate of 0.5 ml/min for 30 min at 37° to achieve a stable baseline efflux of radioactivity. During the experiments, efflux from the chambers was collected in 5-min fractions directly into scintillation vials. Slices were removed from each chamber and solubilized with Protosol (Dupont NEN) at the termination of each experiment. The radioactivity in each fraction and in the solubilized slices was determined, as dpm, in a Beckman LS 5801 liquid scintillation counter following addition of Formula 989 scintillation cocktail (Dupont NEN).

Release in each 5-min interval was expressed as a fraction of the total radioactivity in the slices during that period. To determine stimulated release, the basal efflux of radioactivity in the fraction before stimulation was subtracted from each of the subsequent three fractions, which were then summed. The effect of inhibitors on release was determined using an S_n/S_1 paradigm, in which four of eight chambers were exposed to the test compound after the first pulse with NMDA (S_1). The release in subsequent stimulations ($n = 2$ to 4) was normalized by dividing through by the fractional release in S_1. Ratios for drug-treated slices were divided by the same values for control slices to give percentage of control release.

Measurement of cytosolic calcium in cultured neurons. NMDA-evoked changes in $[Ca]_i$ were monitored in single, cultured, hippocampal pyramidal neurons, using microspectrofluorimetry. Before cell culture, paraffin "feet" were attached to acetone-washed glass cover slips by placing three drops of molten Paraplast embedding

medium in a triangular pattern on one surface of the cover slip. The cover slips were then coated successively with poly-D-ornithine and then laminin. Cells of fetal rat hippocampi (E17) were dissociated in trypsin and suspended in DMEM containing 10% neonatal calf serum. Approximately 1.2×10^5 cells were plated on each slip, with the paraffin feet oriented upwards. The cells were then cultured 24 hr to allow adhesion. The slips were removed from the dishes and residual medium was decanted. The slips were then inverted (feet down) over a confluent layer of rat cortical astrocytes that had been switched from their growth medium (DMEM with 10% serum) to N2 defined medium (as in Ref. 12, except prepared in DMEM) approximately 4 hr before use. Cover slips containing cells (grown 4 to 12 days *in vitro* without any change of medium) were placed in new plates and loaded with 2 μM fura 2/AM for 30 min at 37° in assay buffer (in mM: 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.44 KH_2PO_4, 0.64 Na_2HPO_4, 3 $NaHCO_3$, 5.5 glucose, 20 HEPES, pH 7.35). Single-cell $[Ca]_i$ was monitored as the ratio of emitted light (>485 nm) at each of two excitation wavelengths (350 and 390 nm). Ratio values were acquired at 10 Hz but are displayed as the average of blocks of 10 values. Drugs were dissolved in assay buffer and applied by superfusion (0.9 ml/min) at room temperature.

LTP in hippocampal slices. LTP was studied in rat hippocampal slices *in vitro*, as described by Mueller *et al.* (13). Following a 1-hr equilibration period, the slices were submerged in buffer and continuously superfused (2 ml/min). Test stimuli (10 to 40 V) were applied at 30-sec intervals to the Schaffer collaterals via a nichrome bipolar electrode. Population spikes were recorded from the CA1 region using a 1–2 MΩ NaCl-filled glass microelectrode. LTP was induced by a tetanizing stimulus consisting of three (1-sec duration, 100 Hz, 100 μsec, 3-sec intertrain interval) pulse trains. Drugs were added to the perfusion media 15 to 45 min before tetanization. In preliminary experiments, we measured the effect of various concentrations of 5,7-DCKA on population spike amplitude. Concentrations of the antagonist of 4 μM and less were without significant effect on this parameter, whereas 8 μM produced a slight attenuation and 40 μM gave complete suppression. Concentrations were selected (less than or equal to 4 μM) that would block LTP without affecting the population spike.

Anticonvulsant testing. NMDA receptor blockade was inferred from the ability of an antagonist to protect mice from the effects of a convulsant stimulus. Quinolinic acid-induced seizures were measured using CD-1 mice, as described by Baron *et al.* (7). Protection from sound-induced seizures was measured using DBA/2J audiogenic seizure-susceptible mice. Drugs (in 3 μl of 0.9% saline, final pH = 7.3) were administered intracerebroventricularly with a 10-μl Hamilton syringe to groups of 8 to 10 male 18-day-old DBA/2J mice. No drug-related behavioral alterations were observed. Seizures were induced 5 min later by a 30-sec exposure to a 110-dB 11-kHz tone. The glycine mimetic D-serine, in certain cases, was coadministered with the drug or vehicle (2.4 μmol for CD-1 mice, 300 nmol for DBA/J2 mice). D-Serine alone was without effect. In addition, coadministered D-serine was unable to alter the anticonvulsant EC_{50} of the competitive glutamate antagonist CPP.

Data analysis. Data were analyzed using programs written in RS/1, version 4.0 (BBN Software Products), and run on a VAX computer system. Receptor binding and functional response data were plotted as either percentage of inhibition or response magnitude versus the logarithm of the test drug or agonist concentration and were fit using nonlinear regression analysis. We employed a logistic function of the form: $f(x) = B + M(X^n)/(K^n + X^n)$, where B is basal response (cGMP experiments with B fixed at 0 for inhibition data), M is the calculated maximal response (e.g., a variable representing the maximal NMDA-stimulated cGMP accumulation but fixed at 100 in competition binding experiments and in experiments examining antagonist inhibition of functional responses), X is the concentration of the test drug, K is the apparent affinity of the test drug or agonist (e.g., EC_{50} in functional assays using agonists and IC_{50} in competition experiments), and n is a slope factor. Radioimmunoassay standard curves were fit using the equation described above for interpreting competition binding data. Inhibition values were then calculated for each unknown sample and cGMP content (pmol) was computed by solving the equation for X. K_i values were obtained from IC_{50} values using the Cheng-Prusoff correction procedure (14). We have neglected, in these calculations, the contribution of residual endogenous amino acids to the concentration of the radioligand (e.g., [^{3}H]glycine).

Results

5,7-DCKA (MDL 29,814) was a potent competitor of the strychnine-insensitive binding of [^{3}H]glycine to rat brain membranes (K_i = 79 ± 23 nM, four experiments), with an approximate 500-fold selectivity for the glycine binding site versus the NMDA-type glutamate binding site (K_i = 37 ± 12 μM, six experiments against [^{3}H]CPP). A comparison of 5,7-DCKA with known glycine antagonists is shown in Table 1. The effect of 5,7-DCKA on [^{3}H]CPP may be of an allosteric nature, because competition curves were unusually shallow (slope = 0.37 ± 0.06, six experiments) but approached 1 (slope = 0.83 ± 0.05, three experiments) in the presence of 100 μM glycine. Glycine was without effect on the affinity of 5,7-DCKA for [^{3}H]CPP binding sites (K_i = 48.8 ± 9.4 μM in the presence of 100 μM glycine) or on the specific binding of this radioligand (specific binding in the presence of 100 μM glycine was 122 ± 15% of that observed in its absence). 5,7-DCKA had little affinity for either the quisqualate- or kainate-preferring subtypes of glutamate receptors, with K_i values of 41 ± 4 and 245 ± 29 μM versus [^{3}H]AMPA and [^{3}H]kainate binding, respectively (three experiments).

The phencyclidine analogue [^{3}H]TCP has been suggested to bind to a site present on the open NMDA-operated channel (7, 15). As a consequence, NMDA and glycine, which facilitate channel opening, dramatically enhance [^{3}H]TCP binding (Fig. 1). In the presence of 5,7-DCKA, NMDA was unable to increase [^{3}H]TCP binding, whereas maximal levels of glycine-stimulated binding were unaffected. In control membranes (six experiments), basal binding was 130 ± 29 fmol/mg of protein and increased to 633 ± 127 and 617 ± 79 fmol/mg of protein in the presence of 1 mM NMDA or glycine, respectively. In the presence of 10 μM 5,7-DCKA (six experiments), basal binding decreased to 56 ± 27 fmol/mg of protein and 1 mM NMDA did not affect binding (48 ± 17 fmol/mg of protein; $p > 0.05$ versus

TABLE 1

Relative potency of compounds to inhibit strychnine-insensitive [^{3}H] glycine binding

[^{3}H]Glycine binding was measured to rat cerebral cortical plus hippocampal membranes. Triplicate assay tubes (0.5 ml) contained 20 nM [^{3}H]glycine, 100 μg of membrane protein, 100 μM strychnine, 50 mM HEPES-KOH, pH 7.4, and seven concentrations of the unlabeled test compound. Nonspecific binding was defined using 100 μM D-serine. Following a 30-min incubation on ice, assays were terminated by centrifugation. Results are means ± standard errors of three to eight independent experiments.

Compound	K_i
	μM
5,7-DCKA	0.08 ± 0.02
7-Chlorokynurenic acid	0.36 ± 0.05
6,7-Dichloroquinoxaline-2,3-dione	0.77 ± 0.19
D-Cycloserine	0.9 ± 0.1
Kynurenic acid	16.5 ± 3.6
HA-966	16.2 ± 3.0
Indole-2-carboxylic acid	108.0 ± 2.0
Glycine	0.18 ± 0.02
D-Serine	0.55 ± 0.21
L-Serine	36.0 ± 4.0

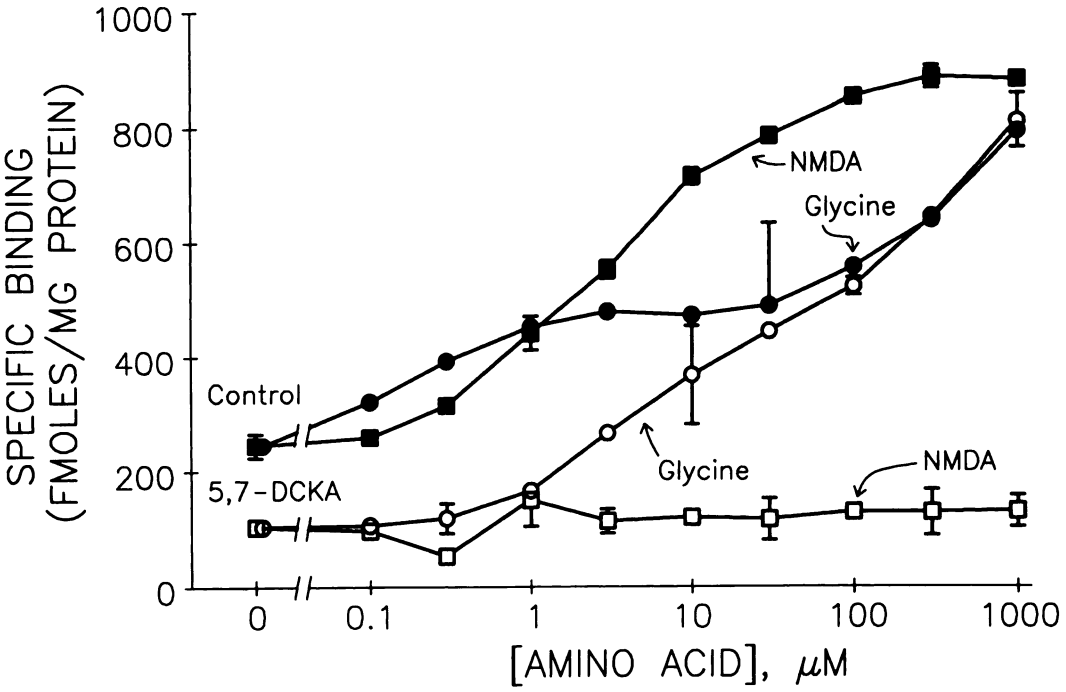

Fig. 1. 5,7-DCKA blocks NMDA-induced increases in [^{3}H]TCP binding. Specific binding of the open channel blocker [^{3}H]TCP (2 nM) to well washed brain membranes was measured either in the absence of added amino acids (basal binding) or with various concentrations of NMDA (*squares*) or glycine (*circles*). *Open symbols*, in the presence of 10 μM 5,7-DCKA; *closed symbols*, control membranes. Shown is a representative experiment repeated five times. *Points*, mean ± standard error of specific binding of triplicate determinations. *Error bars* are omitted when smaller than the *symbols*.

control basal by Neuman-Keuls multiple range test). In the combined presence of 1 mM glycine and 5,7-DCKA, specific binding was 653 ± 115 fmol/mg of protein ($p > 0.05$ versus control plus glycine by Neuman-Keuls test). Similar results were obtained when glycine was replaced with D-serine or when glutamate was substituted for NMDA. This contrasts with the effects of glutamate antagonists, where NMDA increases [^{3}H]TCP binding to control (drug-free) levels but glycine is ineffective (7). These results suggest a corequisite role for NMDA and for glycine in stimulating [^{3}H]TCP binding.

Channel opening was studied more directly by measuring NMDA-elicited elevation of $[Ca]_i$ (7, 16) in single, cultured, hippocampal pyramidal neurons. NMDA-elicited responses were facilitated by glycine and under optimal conditions amounted to 5- to 10-fold of the resting value of $[Ca]_i$. We observed a variation in the dependence on exogenous glycine of NMDA-evoked increases in $[Ca]_i$ as a function of days in culture. The response to 20 μM NMDA was measured after 4, 8, or 11 days in culture, in the absence and presence of 10 μM glycine. Fractional responses occurring in the absence of added glycine were 17 ± 5% (three experiments) 50 ± 11% (three experiments), and 76%, respectively. At all ages, 5,7-DCKA could block 100% of the response and glycine alone evoked no response (data not shown). These results suggest the ontogeny of intrinsic glycine-providing cells in culture, coinciding with the appearance of glial cells, and are similar to the results obtained by Huettner (17). Therefore, like in most other *in vitro* preparations used to study NMDA receptor-mediated responses, we cannot precisely control the glycine concentration in the vicinity of the receptor and, thus, this model is primarily of qualitative utility. As shown in Fig. 2, this inhibition by 5,7-DCKA was readily surmountable by glycine (10 μM) but not by NMDA (up to 10 mM). 5,7-DCKA, when applied alone, had no discernible effect on $[Ca]_i$, a finding consistent with the antagonist lacking inverse agonist properties (see Discussion). On the other hand, it may reflect a lack of significant receptor activation in the absence of added NMDA.

A consequence of NMDA receptor-mediated calcium influx is an increase in cGMP content of neonatal rat cerebellar slices (7, 18, 19). Activation of the guanylate cyclase enzyme is mediated by production of a diffusible factor derived from metabolism of either arginine (20 21) or arachidonic acid (22, 23). 5,7-DCKA completely inhibited the cGMP response to 15 μM NMDA, with an IC_{50} value of 3.5 ± 0.9 μM (four experiments). As shown in Fig. 3, inhibition by 5,7-DCKA was noncompetitive, because maximal response values for NMDA were preferentially reduced in the presence of the antagonist. In the presence of 0, 5, or 30 μM 5,7-DCKA, calculated maximum NMDA-elicited responses were 42.5, 34.7, and 1.9 pmol/mg of protein, respectively (Fig. 3A). By comparison, NMDA-induced maximal responses were not affected (55.2, 63.5, and 67.4, pmol/mg of protein, respectively) in a parallel experiment utilizing these concentrations of antagonist plus 1 mM glycine (Fig. 3B).

NMDA receptor-mediated depolarization leads to the release of a variety of neurotransmitters (24–26). In preloaded superfused slices of the rat hippocampus, superfusion with NMDA (25 μM, S_1 interval) elicited a calcium-dependent 4- to 5-fold increase in the release of [^{3}H]NE (Fig. 4). As with the cGMP accumulation, the glycine antagonist was able to completely block this NMDA-mediated response (IC_{50} = 2.9 ± 0.28 μM; four experiments). As shown in Fig. 4, glycine was able to restore the NMDA-elicited release of [^{3}H]NE in a concentration-dependent manner. Comparison of S_2/S_1 ratios for slices receiving superfusion with 5 μM 5,7-DCKA versus those receiving buffer alone indicated that the antagonist reduced [^{3}H]NE release to 31.7 ± 2.1% of control values. Similarly, calculation of S_n/S_1 ratios for slices receiving the NMDA test pulse in the presence of glycine alone (control) or in combination with 5 μM 5,7-DCKA indicated that glycine increased the level of release observed in the presence of the antagonist. Release was 43.1 ± 3.3, 90.4 ± 3.1, and 119 ± 4.0% of control values in the presence of 1, 10, or 100 μM glycine, respectively. Release in

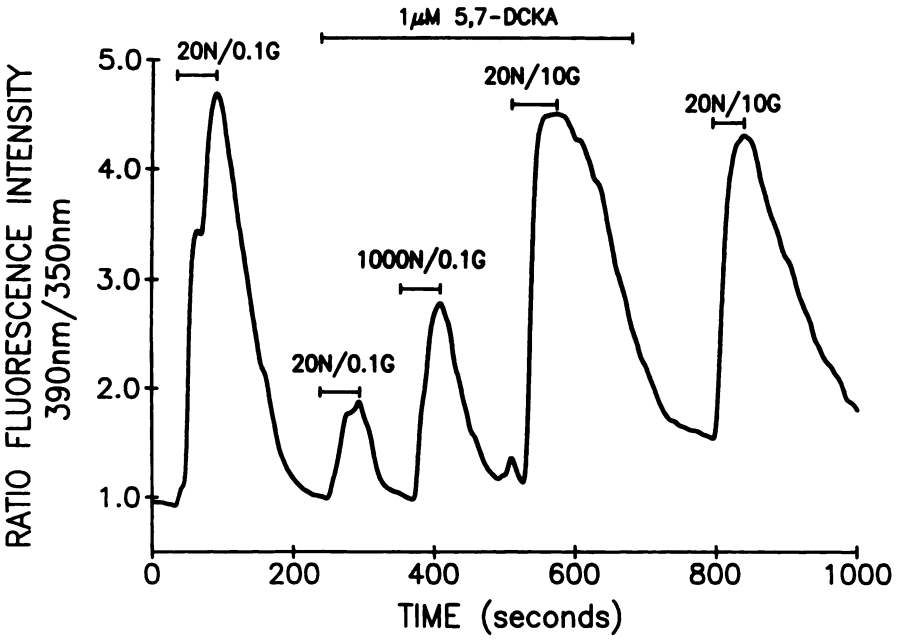

Fig. 2. 5,7-DCKA application results in a blockade of NMDA-evoked increases in $[Ca]_i$ in hippocampal pyramidal neurons. Calcium levels were measured spectrofluorometrically using fura-2 in single, cultured, hippocampal pyramidal neurons. Periods of drug exposure are indicated by *horizontal bars*. Concentrations of NMDA (*N*) and glycine (*G*) are in μM. Shown is a representative tracing of the response of an 11-day cultured neuron (repeated in seven different cells). Coapplication of 20 μM NMDA plus 0.1 μM glycine produced a 5-fold increase in $[Ca]_i$. Inclusion of 1 μM 5,7-DCKA reduced this response by 80%. Inhibition was not surmountable by increasing the NMDA concentration to 1 mM but control response magnitude was restored by increasing the concentration of coapplied glycine to 10 μM.

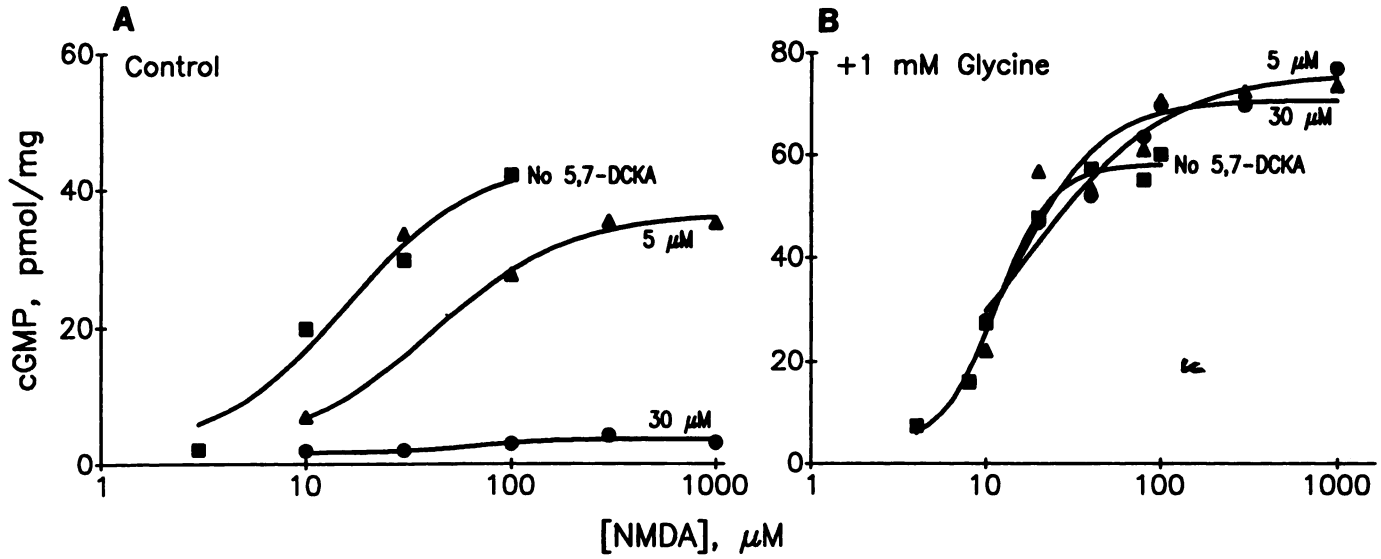

Fig. 3. 5,7-DCKA is a noncompetitive inhibitor of NMDA-stimulated cGMP accumulation in cerebellar slices. A, Neonatal rat cerebellar slices were preincubated with 0, 5, or 30 μM 5,7-DCKA and then challenged with NMDA (4 μM to 1 mM). Cyclic GMP accumulation was measured by radioimmunoassay and results were normalized for protein content. Results are means of triplicate determinations. *Solid lines*, computer-drawn fit of the experimental data. B, In a separate experiment, cGMP accumulation was measured in the presence of 1 mM glycine with other additions as in A. Glycine in control experiments had no direct effect on cGMP levels.

the presence of either 10 or 100 μM glycine was not significantly different from control values ($p > 0.05$, two-tailed t test). Similar results were obtained using D-serine.

The ability of both competitive and noncompetitive NMDA antagonists to block the induction of LTP suggests that this response also requires calcium influx through an NMDA-gated channel (27, 28). As shown in Fig. 5, 5,7-DCKA (2 μM) produced a roughly 75% attenuation of the acquisition of LTP (Fig. 5b) in hippocampal slices. In experiments not shown, 5,7-DCKA at 0.2 and 4 μM produced roughly 25 and 100% attenuation, respectively. Coincubation of the slices with D-serine (100 μM) completely prevented the LTP-blocking effect of 2 μM 5,7-DCKA (Fig. 5c). Thus, glycine is apparently required for NMDA receptor activation even when one examines the response to synaptically released glutamate.

Finally, we considered the role of glycine in NMDA receptor-mediated responses *in vivo*. As shown in Table 2, 5,7-DCKA was a potent anticonvulsant, with similar ED_{50} values against sound-induced convulsions in DBA/2J mice and quinolinic acid-induced convulsions in CD/1 mice. In both models, coinjection of D-serine with 5,7-DCKA significantly reduced the potency of the antagonist. A graphic illustration of both the protective anticonvulsant effect of 5,7-DCKA and its reversal by D-serine is revealed by the percentage of mortality in each of the sound-exposed groups, 100% for control, 0% after 2.9 nmol of 5,7-DCKA administered intracerebroventricularly, and 70% after 2.9 nmol of 5,7-DCKA plus 100 nmol D-serine.

Discussion

The interaction of glycine with the NMDA receptor was originally revealed as the ability of this amino acid to modulate

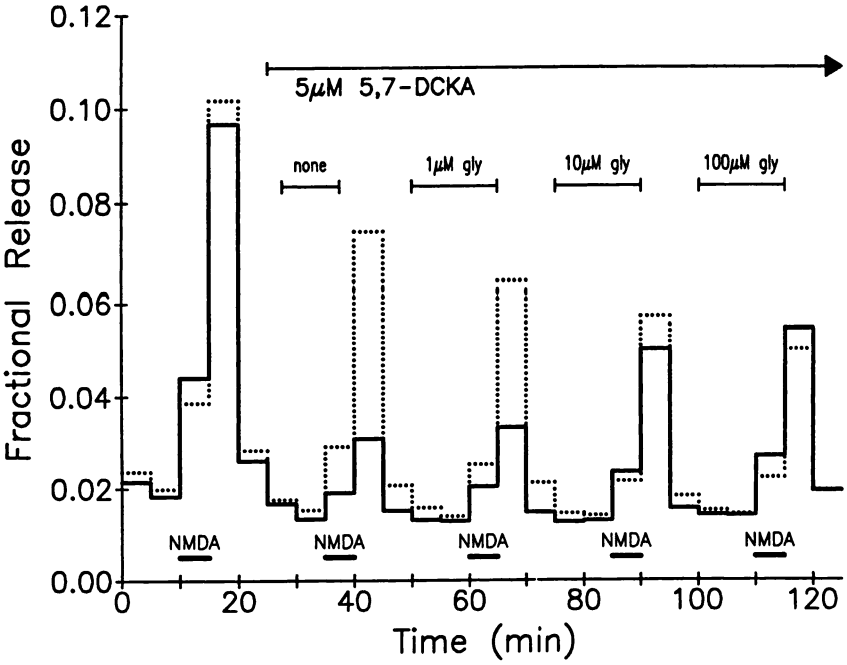

Fig. 4. Glycine reversal of 5,7-DCKA inhibition of [^{3}H]NE release. Hippocampal slices, preloaded with [^{3}H]NE, were placed in an eight-chamber superfusion apparatus and exposed in groups of four chambers. (*dotted* and *solid lines*) to 25 μM NMDA alone or in combination with 5 μM 5,7-DCKA (*dotted lines*) and glycine (both groups) where indicated. Strychnine (10 μM) was also included to inhibit non-NMDA receptor-related actions of glycine. Released [^{3}H]NE is normalized to total slice-contained radioactivity calculated at each time point. Results shown are from a representative experiment in which basal efflux was approximately 2000 dpm/5 min or 2% of total tissue radioactivity.

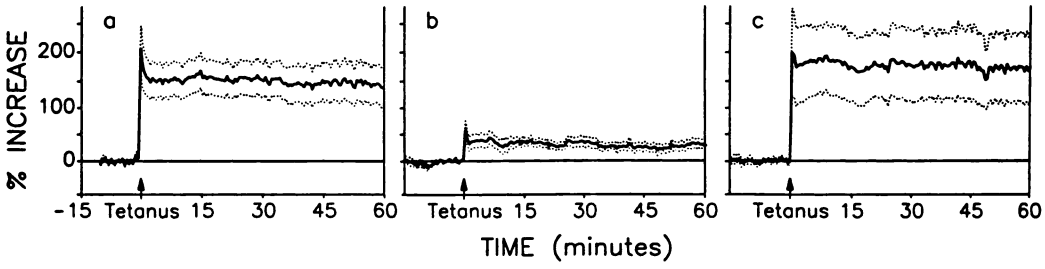

Fig. 5. Effect of 5,7-DCKA on LTP in the hippocampal slice. Percentage of change in the population spike from pretetanus baseline is plotted against time. *Solid* and *dashed lines*, mean values and 95% confidence intervals, respectively. a, With medium alone, LTP is evoked and remains stable for over 1 hr (11 experiments). b, 5,7-DCKA (2 μM) inhibits LTP when added to the perfusion medium 15–30 min before tetanization (five experiments). c, Coapplication of D-serine (100 μM) with 5,7-DCKA (2 μM) allows normal acquisition and maintenance of LTP. In control experiments, D-serine alone had no effect on the population spike or the amplitude of LTP.

TABLE 2

Anticonvulsant potency of 5,7-DCKA is reduced by D-serine

The ability of intracerebroventricularly administered 5,7-DCKA to protect against quinolinic acid-induced seizures in CD-1 mice or against sound-induced seizures in DBA/2J mice was determined. Values given are ED_{50} (nmol) and 95% confidence limits.

Convulsant agent	ED_{50}	
	Control	+D-Serine
	nmol	
Quinolinic acid (46 nmol)	3.4 (2.2–5.8)	9.49 (5.9–15.5)
Sound (110 dB)	1.9 (1.5–2.2)	4.0 (3.0–5.8)

or amplify the effects of glutamate or NMDA on ion channel opening frequency (1). Subsequent investigators have noted a more subtle capacity of glycine to reduce the time required for NMDA-elicited ion currents to recover from desensitization (5, 29). The outcome of both experimental observations would be an enhancement of NMDA-mediated ionic flux by glycine, and this would allow a significant degree of neurotransmission to occur in the absence of added glycine.

Our results with 5,7-DCKA complement the findings of Kleckner and Dingledine (4), Kushner *et al.* (5) and Lerma *et al.* (6). Using *Xenopus* oocyte-expressed NMDA receptors, these authors found that NMDA responses cannot be obtained in the absence of added glycine. As these authors suggest (4), the small currents observed in the absence of glycine in previous work (1, 2) can be attributed to the difficulty of totally eliminating glycine. In contrast, 'glycine-free' conditions might be approximated more easily using an antagonist of glycine such as 5,7-DCKA. Our results with 5,7-DCKA were obtained with a variety of *in vitro* and *in vivo* measures of NMDA receptor activation and in each case demonstrated that glycine is obligatory for NMDA receptor activation. Thus, 5,7-DCKA was capable of completely blocking biochemical consequences of NMDA receptor activation, inhibiting NMDA-stimulated increases in [^{3}H]TCP binding in broken membrane preparations and NMDA-elicited [^{3}H]NE release and cyclic GMP formation in brain slice preparations. It is of interest that the glycine antagonist was also able to attenuate the acquisition of LTP in hippocampal slices, because this response is dependent upon synaptically generated pulsatile release of neurotransmitter, in

distinction to the continuously applied agonist utilized in the biochemical assays. Similar results have now been reported by Bashir *et al.* (30) and Izumi *et al.* (31). As mentioned above, Mayer *et al.* (29) have reported the ability of glycine to modulate the rate of recovery of NMDA-operated ion channels from desensitization. These authors suggest that measurement of ion flux at equilibrium will erroneously lead to the conclusion that glycine is required, whereas measurement of the immediate consequences of agonist application (as in their biophysical experiments) is necessary to reveal this 'modulatory' effect of glycine. It is clear from our experiments, however, that significant neurotransmission does not occur *in vitro* or *in vivo* in the presence of 5,7-DCKA and, thus, at least from a functional perspective, there is an absolute requirement for glycine. A similar dual role of glycine has been suggested by Lerma *et al.* (6).

Previous work, utilizing less potent or less selective glycine antagonists, has also demonstrated an interference with NMDA-mediated electrophysiological responses; however, the precise nature of the blockade appeared to vary with the chemical agent used (32–36). The glycine antagonists 6,7-dichloroquinoxaline-2,3-dione, 7-chlorokynurenic acid, and indole-2-carboxylic acid produce an insurmountable blockade of NMDA-elicited responses (32–35). In contrast, HA-966 results in both glycine-reversible and NMDA-surmountable inhibition (34). Furthermore, even high concentrations of HA-966 do not produce complete inhibition of NMDA-stimulated [^{3}H]GABA release (36), Ca^{2+} influx (36), depolarization (34), ionic currents in patch-clamped neurons (17), or the binding of radiolabeled channel blockers (36, 37).[1] It has been suggested that HA-966 may have partial agonist qualities at the glycine binding site (17, 34). In contrast, 5,7-DCKA is apparently free of partial agonist effects, producing both insurmountable inhibition of functional responses and 100% inhibition of [^{3}H]TCP binding.

At present, it is not clear to what extent parallels can be made between the NMDA receptor and the $GABA_A$ receptor (for a comparison of $GABA_A$ and NMDA receptor pharmacology, see Ref. 38). Both receptor types are linked to the functioning of an ion channel and possess multiple allosteric binding sites. Activation of the $GABA_A$ receptor by its endogenous neurotransmitter, GABA, is enhanced by benzodiazepines. This effect of the benzodiazepine agonists on the $GABA_A$ receptor complex is superficially similar to the effect of glycine at the NMDA receptor observed in the original studies by Johnson and Ascher (1). However, subsequent work, described above, has suggested that glycine is necessary for channel opening and, thus, has a different role than that of the benzodiazepines in GABAergic transmission. Compounds have been identified that can occupy the benzodiazepine binding site but that do not alter the response to GABA. These have been termed "antagonists." At the other extreme, benzodiazepine site ligands, termed "inverse agonists," inhibit the response to GABA. It is tempting to speculate that a similar spectrum of activities will be defined for the NMDA receptor-associated glycine binding site. Already it is clear that glycine antagonists have differing capacities to alter the binding of glutamate-site antagonists. HA-966 can allosterically enhance [^{3}H]CPP binding (39), whereas, as shown here, 5,7-DCKA has the opposite effect. However, it is difficult to relate these qualities to the agonist, antagonist, inverse agonist scheme proposed for the GABA receptor, because significant NMDA-mediated transmission does not occur in the absence of glycine and, thus, by definition, one cannot distinguish between putative antagonists and inverse agonists. Although the present results do not allow us to resolve this question, we feel that, by discovering and characterizing new antagonists, significant distinctions will be made between the properties of glycine antagonists belonging to different chemical classes.

It is not clear to what extent NMDA receptor activation is regulated under physiological conditions by glycine availability. As discussed earlier, measurements of glycine concentrations in cerebrospinal fluid would indicate that this binding site is >95% saturated *in vivo* (3). Consistent with glycine ubiquity, there are numerous examples of *in vitro* preparations that demonstrate sensitivity to glycine antagonists but are insensitive to the direct effects of the agonist. Recently, however, direct effects of glycine site agonists have been demonstrated *in vivo* (40, 41), suggesting that glycine concentrations in the vicinity of the synapse may actually be tightly controlled. Moreover, Weiss *et al.* (42) and Roberts *et al.* (43) have demonstrated kainate- and glutamate-evoked release of glycine from striatal neurons prelabeled with [^{3}H]glycine. Taken together, these findings suggest that glycine availability may be temporally and spatially restricted to those neurons undergoing excitatory amino acid activation and that the role of glycine may be more sophisticated than a permissive stimulus but instead may be a co-transmitter at this receptor.

We have demonstrated here that 5,7-DCKA is a potent anticonvulsant when administered directly into the brain. An important implication of our results is that antagonists of the strychnine-insensitive glycine binding site will be potentially useful in the many other therapeutic areas in which NMDA receptor antagonists have been postulated to be of value.

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