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Kynurenic Acid Analogues with Improved Affinity and Selectivity for the Glycine Site on the *N*-Methyl-D-aspartate Receptor from Rat Brain

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SUMMARY

The glycine site on the *N*-methyl-D-aspartate (NMDA) subtype of receptors for the excitatory neurotransmitter glutamate is a potential target for the development of neuroprotective drugs. We report here two chemical series of glycine site antagonists derived from kynurenic acid (KYNA), with greatly improved potency and selectivity. Disubstitution with chlorine or bromine in the 5- and 7-positions of KYNA increased affinity for [^{3}H]glycine binding sites in rat cortex/hippocampus P_2 membranes, with a parallel increase of potency for antagonism of NMDA-evoked responses in the rat cortical wedge preparation. The optimal compound was 5-I,7-Cl-KYNA, with an IC_{50} for [^{3}H]glycine binding of 29 nM and an apparent K_b in the cortical wedge preparation of 0.41 μM. Reduction of the right-hand ring of 5,7-diCl-KYNA reduced affinity by 10-fold, but this was restored by substitution in the 4-position with the *trans*-phenylamide and further improved in the *trans*-benzylamide. The optimal compound was the *trans*-phenylurea (L-689,560), with an IC_{50} of 7.4 nM and an apparent K_b of 0.13 μM. Both series of compounds displayed a high degree of selectivity for the glycine site, having IC_{50} values of >10 μM versus radioligand binding to the glutamate recognition sites of NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate receptors and the strychnine-sensitive glycine receptor. Selectivity versus AMPA receptor-mediated responses was also apparent in the rat cortical wedge and in patch-clamp recordings of cortical neurons in culture. Experiments using [^{3}H]dizocilpine (MK-801) binding indicated that 5,7-diBr-KYNA, 5,7-diCl-KYNA, 5-I,7-Cl-KYNA, and L-689,560 all behaved as full antagonists and were competitive with glycine. Patch-clamp recordings of cortical neurons in culture also indicated that NMDA-induced currents were antagonized by competition for the glycine site, and gave no evidence for partial agonist activity. pK_i values for 5,7-diBr-KYNA and L-689,560 in these experiments were 7.2 and 7.98, respectively, similar to the affinities of these compounds in the glycine binding assay. The high affinity and selectivity of these new derivatives make them useful tools to investigate the function of the glycine site on the NMDA receptor.

The NMDA subtype of receptors for the excitatory neurotransmitter glutamate possesses two distinct amino acid recognition sites. One site is specific for acidic amino acids such as glutamate, aspartate, or NMDA itself (1), and the other is selective for certain neutral amino acids such as glycine, serine, and alanine (2). Occupation of both amino acid recognition sites appears to be required for receptor activation (3), and thus glutamate and glycine act as "coagonists." Compounds that compete with glycine for its recognition site are noncompetitive antagonists of NMDA- or glutamate-induced responses (4, 5) and have been shown to antagonize NMDA receptor-mediated responses in both physiological (6) and pathophysiological (7, 8) situations.

The idea that NMDA receptor antagonists may have therapeutic potential has arisen from the evidence that an overactivation of this receptor subtype occurs in a variety of neurodegenerative diseases, e.g., epilepsy and stroke (9–11). Selective NMDA receptor antagonists include drugs that block the receptor-associated ion channel, e.g., dizocilpine (MK-801) (12, 13), and compounds that compete at the glutamate recognition site, the so-called competitive antagonists, e.g., CPP, CGS19755, and CGP37849 (14). The realization that glycine is important for NMDA receptor function has prompted the search for selective glycine site antagonists, both to produce improved tools for further evaluation of the functional rele-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; KYNA, kynurenic acid; AMPA, α-2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CPP, DL-(2-carboxypiperazine-4-yl)propyl-1-phosphonate; CGS19755, DL-(*cis*)-4-phosphonomethylpiperidine-2-carboxylate; CGP37849, DL-ε-2-amino-4-methyl-5-phosphono-3-pentenoate; ACBC, 1-amino-1-carboxycyclobutane; HA-966, 3-amino-1-hydroxypyrrolid-2-one; 5,7-diCl-KYNA, 5,7-dichlorokynurenic acid; 7-Cl-KYNA, 7-chlorokynurenic acid; 5,7-diBr-KYNA, 5,7-dibromokynurenic acid; 5-I,7-Cl-KYNA, 5-iodo,7-chlorokynurenic acid; SDS, sodium dodecyl sulfate; L-689, 560, (±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline.

vance of this site and to compare their therapeutic potential with that of the existing classes of NMDA receptor antagonists.

Several structural types are now known to act as glycine site antagonists. These include quinoxaline-2,3-diones (15, 16), 6,7-dichloroquinoxalic acid (17, 18), and 5-chloroindole-2-carboxylate (5). In addition, HA-966 (8, 19, 20) and its analogues (21, 22) and certain cyclic derivatives of glycine, e.g., ACBC (23), appear to act as low efficacy partial agonists. KYNA is a naturally occurring substance and was one of the first glycine site antagonists identified (24, 25). KYNA is a broad spectrum excitatory amino acid antagonist, possessing additional affinity for the glutamate recognition site of the NMDA receptor and for AMPA receptors (26). Substitution of a chlorine in the 7-position yields a compound (7-Cl-KYNA) with improved affinity and selectivity for the glycine site (4), which has proved to be a useful tool in the investigation of the functions of this site.

As a result of a detailed study of the structure-activity relationships of KYNA analogues (27, 28), we have identified compounds with improved affinity and selectivity as glycine site antagonists. Here we report the properties of two chemical series, substituted KYNA derivatives and derivatives of carboxytetrahydroquinoline, in radioligand binding and electrophysiological experiments. A preliminary account of some of this work has appeared (29).

Materials and Methods

Membrane preparation. For each preparation, 25 male Sprague-Dawley rats (150–250 g) were killed by cervical dislocation [in accordance with the Animals (Scientific Procedures) Act 1986],[2] and their cerebral cortices and hippocampi were rapidly dissected and placed immediately in 0.32 M sucrose, 5 mM Tris-acetate buffer (pH 7.4), on ice. All further procedures were carried out at 4°. The tissue was weighed and homogenized in 10 volumes (with respect to original tissue weight) of 0.32 M sucrose, 5 mM Tris-acetate buffer (pH 7.4), using a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min, the supernatant was collected, and the pellet was homogenized in a small volume of the sucrose solution. After centrifugation at 1000 × *g* for 10 min, the second supernatant was combined with the first and centrifuged at 17,000 × *g* for 20 min. The resulting P_2 pellet was resuspended by homogenization in 20 volumes (with respect to original tissue weight) of 5 mM Tris-acetate buffer (pH 8.0) and was stirred on ice for 1 hr, to facilitate lysis of synaptosomes. The suspension was centrifuged at 50,000 × *g* for 30 min, the supernatant was discarded, and the pellet was frozen for at least 18 hr at −20°. The pellet was thawed, resuspended by homogenization in 180 ml of a solution of 0.1% saponin in 5 mM Tris-acetate buffer (pH 7.0), and centrifuged at 50,000 × *g* for 30 min. The pellet was resuspended in 180 ml of 5 mM Tris-acetate buffer (pH 7.0) and centrifuged at 50,000 × *g* for 30 min. The final pellet was resuspended in a small volume of 5 mM Tris-acetate buffer (pH 7.0), to give a protein concentration of 5–10 mg/ml [determined by the method of Lowry *et al.* (30)], and aliquots were stored at −20°. On the day of the binding assay, the required amount of membrane preparation was thawed and washed by homogenization in approximately 10 times the volume of 5 mM Tris-acetate buffer (pH 7.0) and centrifugation at 50,000 × *g* for 60 min or, for the determination of [^{3}H]dizocilpine binding, a cycle of three washes with the same volume of the buffer and centrifugation at 50,000 × *g* for 30 min. Finally, the pellet was homogenized in the required volume of assay buffer.

[^{3}H]Glycine binding. Assays were carried out in polypropylene tubes containing 150 μg of P_2 membrane protein, 50 nM [^{3}H]glycine (47.5–51.3 Ci/mmol; DuPont/NEN), and 50 mM Tris-acetate buffer (pH 7.0), in a final volume of 0.5 ml. Drugs were dissolved in either assay buffer or dimethylsulfoxide (final assay concentration of 1%, which did not affect the specific binding) and were added to the membranes on ice before initiation of the incubation by addition of the radioligand. Nonspecific binding was determined in the presence of 1 mM glycine. Tubes were incubated at 4° for 30 min before centrifugation at 50,000 × *g* for 5 min. The supernatant was discarded, and the pellet and sides of the tubes were superficially washed with 2 × 2 ml of assay buffer. Pellets containing bound radioactivity were solubilized overnight in 0.5 ml of 2% SDS, and 0.4 ml was added to 10 ml of scintillation fluid, for quantification of radioactivity using a β counter.

[^{3}H]Dizocilpine binding. Assays were carried out in polypropylene tubes containing 150 μg of P_2 membrane protein, 2 nM [^{3}H]dizocilpine (22.5 Ci/mmol; DuPont/NEN), 100 μM NMDA, and 5 mM Tris-acetate buffer (pH 7.4). Drugs were dissolved in either assay buffer or dimethylsulfoxide (final assay concentration of 1%, which did not affect the specific binding) and were added to the membranes at room temperature before initiation of the incubation by addition of the radioligand. Nonspecific binding was determined in the presence of 100 μM dizocilpine. Tubes were incubated at room temperature for 60 min before filtration through Whatman GF/B filters (presoaked in assay buffer), using a Brandel cell harvester, with three 2-ml washes of ice-cold 5 mM Tris-acetate buffer (pH 7.4). The filters were added to 10 ml of scintillation fluid, for quantification of radioactivity using a β counter.

Other radioligand binding assays. [^{3}H]AMPA and [^{3}H]kainate binding to the rat cortex/hippocampus P_2 membranes was determined by modifications of the methods of Honoré and Drejer (31) and Simon *et al.* (32), respectively. Membranes (150 μg of protein) were incubated at room temperature for 30 min with 36 nM [^{3}H]AMPA (29.2–60 Ci/mmol; DuPont/NEN) or 16 nM [^{3}H]kainate (58 Ci/mmol; DuPont/NEN), in 50 mM Tris-acetate buffer (pH 7.0). KSCN (100 mM) was included in the [^{3}H]AMPA binding experiments and, for both assays, 1 mM L-glutamate was included for determination of nonspecific binding. After centrifugation at 50,000 × *g* for 5 min, the supernatant was discarded and the pellet was washed superficially with 2 × 2 ml of 50 mM Tris-acetate buffer (pH 7.0), at 4°. The pellet was solubilized in 0.5 ml of SDS, and radioactivity was determined by β counting. [^{3}H]CPP and [^{3}H]CGS19755 binding was determined by modifications of the methods of Murphy *et al.* (Refs. 33 and 34, respectively). Rat cortex/hippocampus P_2 membranes (150 μg of protein) were incubated with 10 nM [^{3}H]CPP (20.1–40 Ci/mmol; DuPont/NEN) or [^{3}H]CGS19755 (33–53.3 Ci/mmol; DuPont/NEN) in 50 mM Tris-acetate buffer (pH 8.0) for 15 min at 4°, in a final volume of 0.5 ml. L-Glutamate (1 mM) was included to determine nonspecific binding. Membrane-bound radioactivity was isolated by the centrifugation procedure described above and was quantified by β counting after solubilization overnight in 2% SDS. [^{3}H]Strychnine binding to rat spinal cord/brainstem membranes was conducted as described by Young and Snyder (35), using a final concentration of [^{3}H]strychnine (27.3 Ci/mmol; Amersham) of 2 nM.

Electrophysiological experiments. The rat cortical wedge preparation introduced by Harrison and Simmonds (36) was used to evaluate the effects of compounds on NMDA- and AMPA-induced depolarizations, as described by Kemp *et al.* (37). Voltage-clamp recordings were made from rat cortical neurons in culture, using the whole-cell patch technique of Hamill *et al.* (38), with a rapid-perfusion apparatus, as described in detail by Priestley *et al.* (39).

Data analysis. For the radioligand binding experiments, curves were fitted and IC_{50} or EC_{50} values (concentration of compound giving 50% inhibition or enhancement of specific binding), I_{max} values (maximum percentage of inhibition of specific binding), and Hill coefficients were determined using an iterative curve-fitting procedure in Research System 1 (Bolt, Beranek, and Newman, Inc., Cambridge, MA.). In the cortical wedge experiments, the concentration of antagonist producing a 2-fold shift to the right of the NMDA concentration-response curve (apparent K_b) was calculated from measurements of the "dose-ratio" at

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the midpoint of NMDA concentration-response curves, in the presence and absence of antagonist. Care was taken that measurements were made from relatively small rightward shifts that did not produce a marked flattening of the NMDA concentration-response curve but were close to 2-fold. Assuming that the NMDA receptor cannot be activated when the glycine site is occupied by an antagonist (3, 20), a 2-fold shift to the right of the NMDA concentration-response curve produced by a glycine site antagonist reflects 50% glycine site occupancy. Antagonists were allowed to equilibrate for at least 30 min before the NMDA concentration-response curve was repeated. In the patch-clamp experiments, the inhibition curves were fitted to the equation $100 - 100/(1 + (IC_{50}/x)^{nH})$, and from the IC_{50} values K_i values were obtained using the Cheng-Prusoff equation.

Materials. Radiochemicals were purchased from DuPont/NEN or Amersham, as indicated. The KYNA and tetrahydroquinoline analogues were synthesized within the Medicinal Chemistry Laboratories of the Merck, Sharp and Dohme Neuroscience Research Centre, as described previously (27, 28). All other chemicals and solvents were of the highest purity available from commercial sources.

Results

[^{3}H]Glycine binding. To assess directly their affinity for the glycine site on the NMDA receptor, compounds were tested as inhibitors of the strychnine-insensitive binding of [^{3}H]glycine to washed P_2 membranes from rat cerebral cortex/hippocampus (40). In the KYNA series, affinity was increased, compared with 7-Cl-KYNA, for the 5,7-diCl- and 5,7-diBr- analogues, with 5-I,7-Cl-KYNA having highest affinity (Fig. 1; Table 1). As shown in Fig. 2 and Table 2, reduction of the right-hand ring of 5,7-diCl-KYNA caused a 10-fold decrease of affinity, but this was restored by substitution at the 4-position with the *trans*-phenylamide and further improved by homologation to the benzyl derivative. The optimal compound was the *trans*-phenylurea (L-689,560), with an IC_{50} of 7.4 nM. All of these analogues gave complete inhibition of specific [^{3}H]glycine binding, with Hill coefficients not significantly different from 1 (Tables 1 and 2).

Specificity in radioligand binding assays. The specificity of the compounds was tested in radioligand binding assays for other amino acid recognition sites (Table 3). In general, 5,7-diCl-, 5,7-diBr-, and 5-I,7-Cl-KYNA were weak inhibitors, with IC_{50} values of 100 μM or more. In the [^{3}H]CPP binding assay, at 100 μM the diCl- and diBr- analogues of KYNA gave approximately 50% inhibition and, in [^{3}H]CPP and [^{3}H]CGS19755 binding assays, L-689,560 (100 μM) inhibited by 65% and 52%, respectively. In the [^{3}H]AMPA binding assay, 5,7-diCl-KYNA and 5-I,7-Cl-KYNA both had IC_{50} values of approximately 100 μM, similar to that of 7-Cl-KYNA, whereas

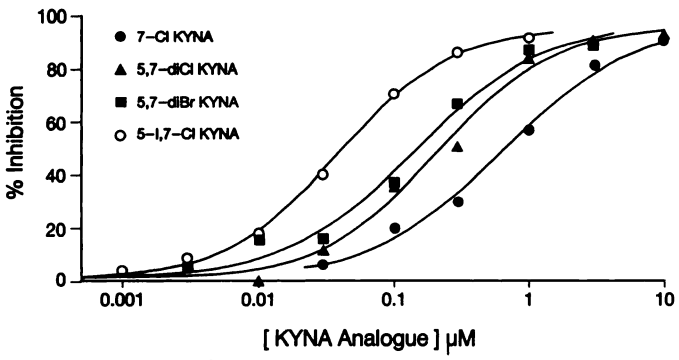
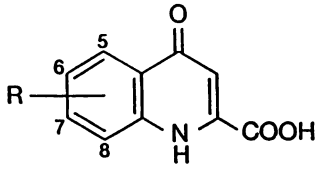


Fig. 1. Inhibition of [^{3}H]glycine binding to rat cortex/hippocampus P_2 membranes by KYNA analogues. Each *curve* is from a single experiment.

TABLE 1

Inhibition of [^{3}H]glycine binding to rat cortex/hippocampus P_2 membranes by KYNA analogues

IC_{50} values are the geometric mean (− standard error, + standard error) and I_{max} (percentage of maximum inhibition) and n_H (Hill coefficient) values are the arithmetic mean ± standard error of n observations. For K_d and B_{max} values for [^{3}H]glycine binding under these conditions, see Ref. 53. Specific binding was typically 70–80% of total [^{3}H]glycine binding.



R	IC_{50}	I_{max}	n_H	n
	nM	%		
7-Cl	552 (360, 847)	93.8 ± 5.0	1.01 ± 0.05	4
5,7-diCl	213 (160, 283)	100.4 ± 2.6	0.83 ± 0.13	4
5,7-diBr	90.6 (73.2, 112)	96.0 ± 2.7	0.73 ± 0.08	4
5-I,7-Cl	29.0 (23.9, 35.1)	93.5 ± 1.7	1.23 ± 0.19	3

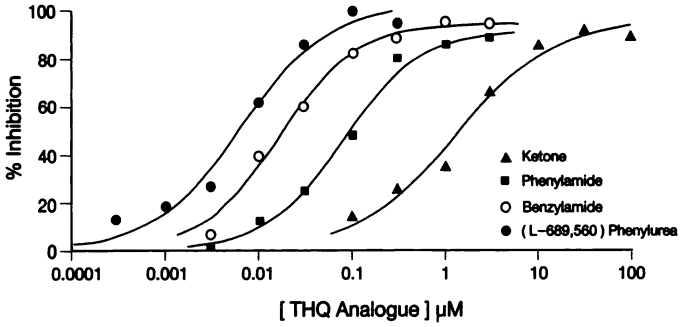
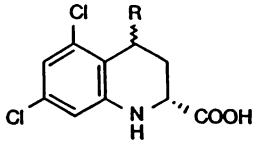


Fig. 2. Inhibition of [^{3}H]glycine binding to rat cortex/hippocampus P_2 membranes by tetrahydroquinoline analogues. Each *curve* is from a single experiment.

TABLE 2

Inhibition of [^{3}H]glycine binding to rat cortex/hippocampus P_2 membranes by tetrahydroquinoline analogues

See Table 1 for the derivation of values.



R	IC_{50}	I_{max}	n_H	n
	nM	%		
=O	2310 (1390, 5150)	99.2 ± 6.1	1.09 ± 0.32	3
trans-NHCOPh	101 (87.0, 117)	96.9 ± 5.1	1.26 ± 0.24	3
trans-NHCOCH$_2$Ph	13.6 (10.4, 17.8)	95.6 ± 3.2	1.06 ± 0.08	3
trans-NHCONHPh (L-689,560)	7.41 (6.94, 7.91)	101.5 ± 2.0	0.93 ± 0.09	3

5,7-diBr-KYNA and L-689,560 were weaker. On this basis, 5-I,7-Cl- and 5,7-diBr-KYNA are 3000- and 1000-fold selective, respectively, for the glycine site versus these related amino acid recognition sites, and L-689,560 is 10,000-fold selective for the glycine site.

[^{3}H]Dizocilpine binding. Dizocilpine blocks the open state of the NMDA receptor ion channel (12, 13), and the association

TABLE 3

Activities of KYNA analogues and L-689,560 at amino acid recognition sites

Values are percentage of inhibition at a concentration of 100 μM and are the mean ± standard error of at least three observations, except in the case of 5-I,7-Cl-KYNA where $n = 1$, due to limited availability of this compound.

Radioligand	Inhibition				
	7-Cl-KYNA	5,7-diCl-KYNA	5,7-diBr-KYNA	5-I,7-Cl-KYNA	L-689,560
	%				
[^{3}H]CPP	31.5 ± 4.6	57.5 ± 3.1	55.8 ± 1.4	NT[a]	65.4 ± 2.6
[^{3}H]CGS19755	3.0 ± 11.9	14.6 ± 13.5	7.7 ± 12.0	NT	52.4 ± 3.0
[^{3}H]AMPA	101[b,c]	104 (83.8, 129)[b]	34.8 ± 1.4	46.3	9.9 ± 3.5
[^{3}H]Kainate	>1000[b,c]	22.4 ± 3.2	26.8 ± 5.9	19.1	19.7 ± 2.6
[^{3}H]Strychnine	2.7 ± 16.1[d]	6.0 ± 0.3	7.5 ± 3.1	3.8	21.0 ± 12.0

[a] NT, not tested.
[b] IC_{50} value (μM) [geometric mean (− standard error, + standard error)].
[c] Results from Kemp *et al.* (4).
[d] Increase of specific binding.

Therefore, under nonequilibrium conditions, the binding of [^{3}H]dizocilpine can be used to study the interaction of agonists and antagonists at the amino acid recognition sites. In the presence of 100 μM NMDA, [^{3}H]dizocilpine binding to rat cortex/hippocampus P_2 membranes was enhanced by glycine, with an EC_{50} value of 56 (52, 62) nM (geometric mean (− standard error, + standard error); three experiments), and inhibited in a concentration-dependent manner by 7-Cl-KYNA (Fig. 3; Table 4), presumably reflecting competition with endogenous glycine in the membrane preparation. The inhibition was virtually complete, indicating that 7-Cl-KYNA is a full antagonist, because the partial agonists D-cycloserine (43) and (+)-HA-966 (44) gave incomplete inhibition under similar conditions. The inhibition of [^{3}H]dizocilpine binding was progressively reversed by increasing concentrations of glycine (Fig. 3), causing a 10-fold shift in the 7-Cl-KYNA IC_{50} value with addition of 1 μM glycine (Table 4). 5,7-DiCl-, 5,7-diBr-, and 5-I,7-Cl-KYNA also inhibited [^{3}H]dizocilpine binding, with an order of potency similar to that observed in the glycine binding experiments (Fig. 4; Table 4). Like 7-Cl-KYNA, these analogues also gave virtually complete inhibition of [^{3}H]dizocilpine binding, and addition of 1 μM glycine produced an approximate

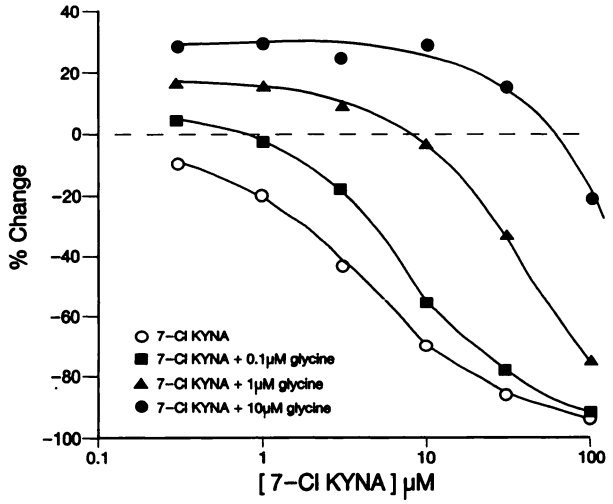


Fig. 3. Inhibition of [^{3}H]dizocilpine binding to rat cortex/hippocampus P_2 membranes by 7-Cl-KYNA and its reversal by glycine. Assays were carried out in the presence of 100 μM NMDA. *Curves* are from a single experiment, which was repeated twice with similar results. Note the progressive shift to the right of the 7-Cl-KYNA inhibition curve with increasing glycine concentration.

TABLE 4

Inhibition of [^{3}H]dizocilpine binding to rat cortex/hippocampus P_2 membranes by KYNA analogues and L-689,560

IC_{50} values are the geometric mean (− standard error, + standard error) and I_{max} and n_H values are the arithmetic mean ± standard error of n observations. For 5-I,7-Cl-KYNA, the individual values from two experiments (due to limited availability of compound) are shown. All experiments were carried out in the presence of 100 μM NMDA, with a [^{3}H]dizocilpine concentration of 2 nM. Typical values for specific binding (mean ± standard error, $n = 3$) were control, 218 ± 34.9 fmol/mg of protein; +1 μM glycine, 528 ± 13.0 fmol/mg of protein.

	IC_{50}	I_{max}	n_H	n
	μM	%		
7-Cl-KYNA				
Control	2.71 (2.27, 3.24)	92.3 ± 1.7	1.19 ± 0.06	7
+1 μM Glycine	28.8 (19.6, 42.2)	100 ± 0.7	1.19 ± 0.10	3
5,7-diCl-KYNA				
Control	0.330 (0.277, 0.392)	91.9 ± 1.7	1.13 ± 0.07	3
+1 μM Glycine	4.80 (4.59, 5.02)	97.9 ± 1.6	1.10 ± 0.10	3
5,7-diBr-KYNA				
Control	0.174 (0.146, 0.207)	90.1 ± 2.8	1.22 ± 0.12	3
+1 μM Glycine	3.01 (2.89, 3.14)	99.0 ± 0.7	1.02 ± 0.02	3
5-I,7-Cl-KYNA				
Control	0.125, 0.076	95.0, 93.4	1.3, 1.6	2
+1 μM Glycine	0.784, 0.881	97.9, 97.3	1.2, 1.3	2
L-689,560				
Control	0.008 (0.007, 0.009)	82.2 ± 4.7	1.12 ± 0.22	3
+1 μM Glycine	0.078 (0.076, 0.081)	95.4 ± 1.1	1.00 ± 0.02	3

10-fold shift in their IC_{50} values (Table 4). L-689,560 was the most potent inhibitor of [^{3}H]dizocilpine binding, giving >90% maximal inhibition, and its inhibition curve was shifted to the right by the addition of 1 μM glycine, producing a 10-fold reduction in the IC_{50} value (Fig. 4; Table 4). These results indicate that inhibition of [^{3}H]dizocilpine binding by these compounds is mediated by competition at the glycine site and that they do not appear to possess partial agonist properties.

Rat cortical slice experiments. The rank order of potency for both series of compounds as NMDA antagonists on rat cortical slices was the same as that for inhibition of [^{3}H]glycine binding (Table 5). For the KYNA analogues, 5-I,7-Cl-KYNA was the most potent compound, followed by 5,7-diBr-KYNA and 5,7-diCl-KYNA. Among the 4-substituted carboxytetrahydroquinolines, the phenylurea (L-689,560) was the most potent compound, followed by the benzylamide and the phenylamide. L-689,560 was the most potent compound overall, with an apparent K_b of 0.13 μM. At concentrations approximately 10-fold greater than their apparent K_b values, 5,7-diBr-KYNA, 5,7-di-Cl-KYNA, and L-689,560 (Fig. 5) produced marked flattening of the NMDA concentration-response curves, similar to

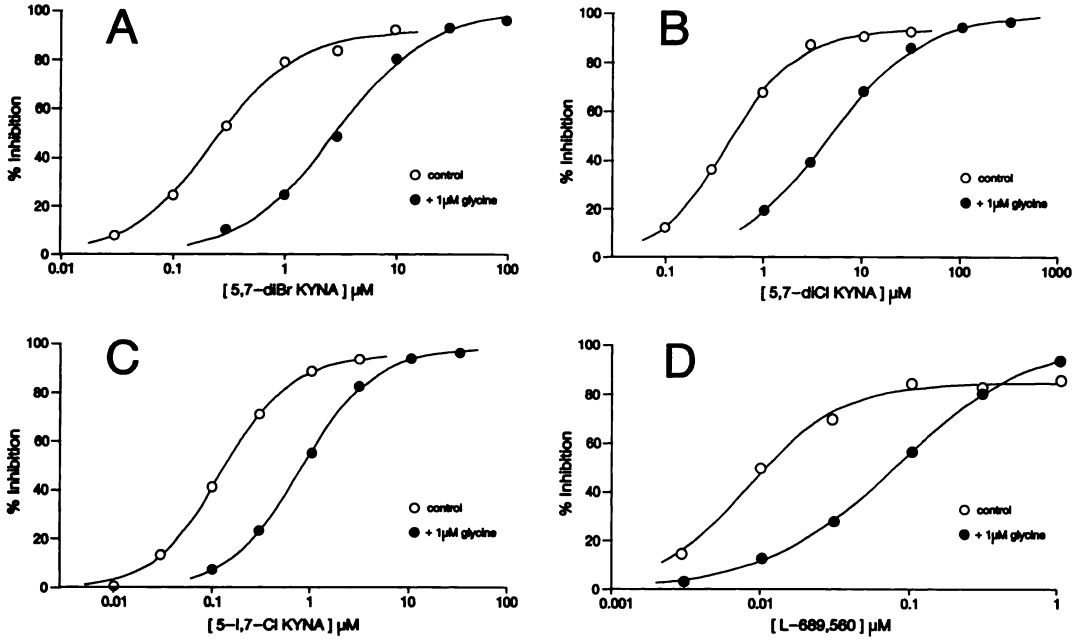


Fig. 4. Inhibition of [^{3}H]dizocilpine binding to rat cortex/hippocampus P_2 membranes by KYNA analogues and L-689,560. Assays were carried out in the presence of 100 μM NMDA. Each *curve* is from a single experiment and was obtained in the absence or presence of 1 μM glycine. A, 5,7-diBr KYNA; B, 5,7-dicl KYNA; C, 5-I,7-Cl KYNA; D, L-689,560.

TABLE 5

Antagonism of NMDA-induced depolarization of rat cortical slices by analogues of KYNA and tetrahydroquinoline

Apparent K_b values were calculated as described in the text and are the mean ± standard error of *n* observations.

R	Apparent K_b	*n*
	μM	
KYNA analogues		
5,7-diCl	1.9 ± 0.06	3
5,7-diBr	0.9 ± 0.18	8
5-I,7-Cl	0.41 ± 0.03	3
Tetrahydroquinoline analogues		
=O	13.9 ± 0.6	5
trans-NHCOPh	1.20 ± 0.05	3
trans-NHCOCH$_2$Ph	0.53 ± 0.07	6
trans-NHCONHPh (L-689,560)	0.11 ± 0.02	5

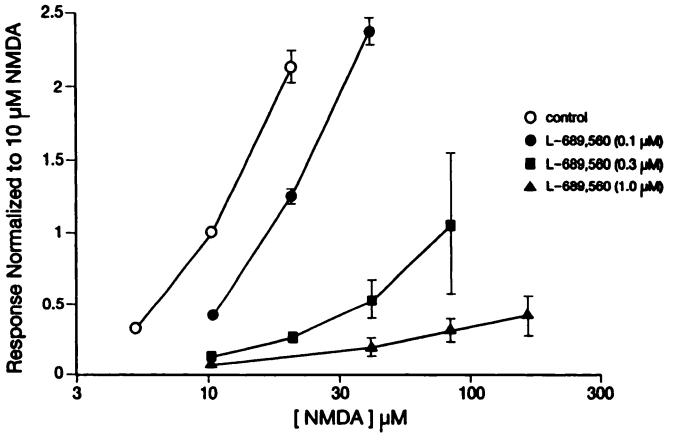


Fig. 5. Inhibition of NMDA-evoked depolarizations by L-689,560 (0.1, 0.3, or 1 μM) in the rat cortical wedge preparation. The responses were normalized with respect to that produced by 10 μM NMDA, the mean ± standard error of which was 0.71 ± 0.12 mV (nine experiments). For each antagonist concentration, three experiments were performed. Note the progressive flattening of the NMDA concentration-response curve with increasing concentrations of L-689,560.

5,7-di-Cl-KYNA, and L-689,560 (Fig. 5) produced marked flattening of the NMDA concentration-response curves, similar to that observed with 7-Cl-KYNA (4). At the highest concentrations tested, 5,7-diBr-KYNA (10 μM), 5,7-diCl-KYNA (30 μM), and L-689,560 (1 μM) had no antagonist effect on depolarizations evoked by AMPA or quisqualate, producing a <2-fold shift to the right of their concentration-response curves.

Whole-cell patch-clamp experiments. On whole-cell voltage-clamped neurons from rat cortex in primary culture, the three disubstituted KYNA analogues produced an inhibition of NMDA responses in the presence of glycine. This antagonist action could be reversed by increasing the concentration of glycine but not NMDA (Fig. 6A). More detailed studies were carried out with 5,7-diBr-KYNA, and its selectivity for the non-NMDA glutamate receptor was examined. This compound produced a concentration-dependent inhibition of NMDA (30 μM) responses evoked in the presence of 300 nM glycine. The combined data from five cells are illustrated in Fig. 7. The pIC_{50} (mean ± standard error) value for 5,7-diBr-KYNA was 6.7 ± 0.05, and the slope of the inhibition curves (mean ± standard error) was 1.03 ± 0.04. Using the Cheng-Prusoff equation, this gives a pK_i (mean ± standard error) for the glycine site of 7.2 ± 0.05. 5,7-DiBr-KYNA was much weaker as an inhibitor of kainate (30 μM)-induced responses (Fig. 7), with a pIC_{50} of 4.1 ± 0.02 and a slope factor of 1.4 ± 0.07. This results in a pK_i for non-NMDA receptors of 4.2 ± 0.02. Thus, 5,7-diBr-KYNA shows a 1000-fold selectivity for the glycine site on the NMDA receptor versus non-NMDA receptors on these cultured neurons.

L-689,560 also gave a concentration-dependent inhibition of NMDA (30 μM) responses evoked in the presence of glycine (300 nM), which was not overcome by raising the NMDA

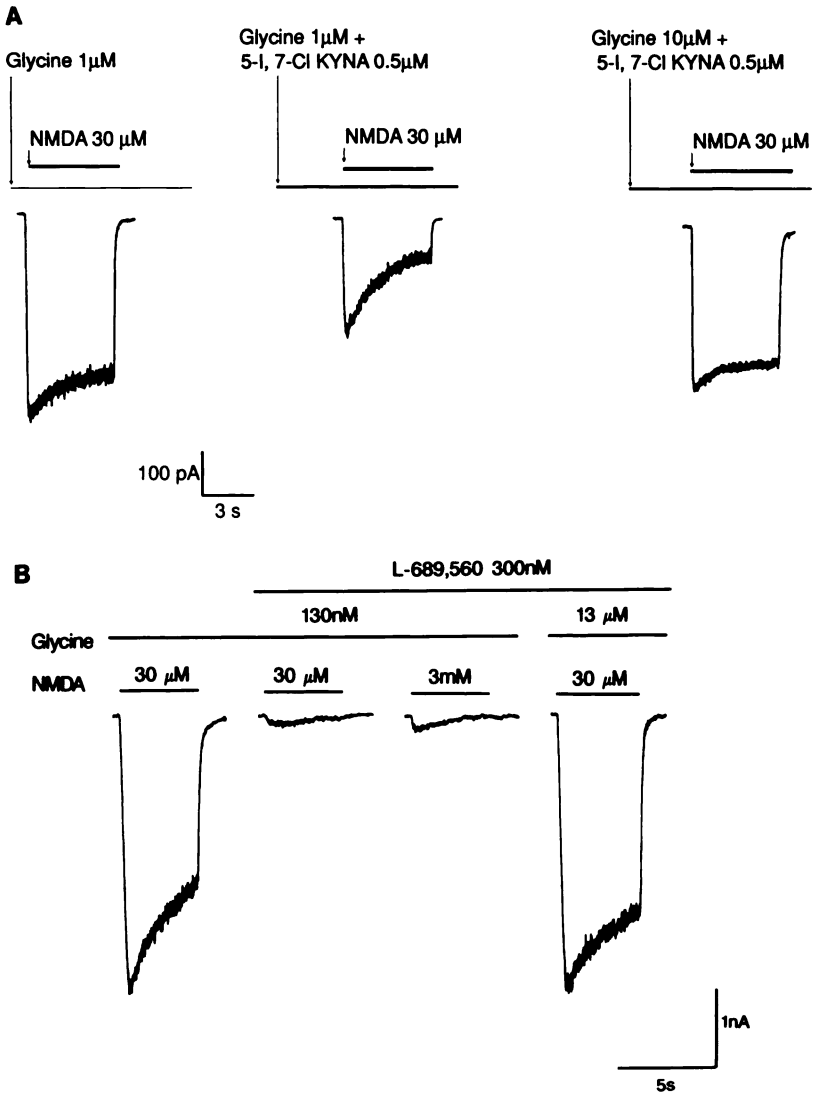


Fig. 6. A, Inhibition of NMDA-evoked currents in rat cortical neurons in culture by 5-I,7-Cl-KYNA and its reversal by glycine. Cells were voltage-clamped in the whole-cell patch configuration, and currents were evoked by 5-sec applications of NMDA. Note reversal of antagonism by 5-I,7-Cl-KYNA by raising of the glycine concentration to 10 μM. The increase in fade of the NMDA response in the presence of 5-I,7-Cl-KYNA is probably the result of the block of glycine-sensitive desensitization and is similar to that seen with other glycine site antagonists of this class (see Ref. 54). B, Inhibition of NMDA-evoked currents by L-689,560. Note reversal of antagonism by raising of the glycine concentration but not by raising of the NMDA concentration.

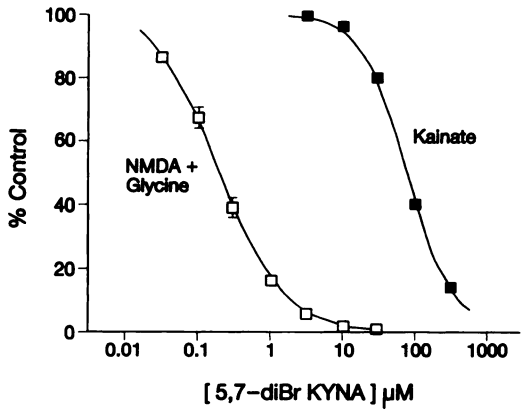


Fig. 7. Inhibition of NMDA- and kainate-evoked currents in rat cortical neurons in culture by 5,7-diBr-KYNA. Currents were evoked by 5-sec applications of NMDA (30 μM) in the presence of 300 nM glycine (□) or 30 μM kainate (■). Values are the percentage of the control response in the presence of 5,7-diBr-KYNA and are the mean ± standard error of five cells for each agonist. In most cases, error bars are smaller than the size of the symbols. Currents were measured at steady state.

concentration but was by increasing the glycine concentration (Fig. 6B). Fig. 8 shows the combined data from four cells, giving a pIC_{50} for L-689,560 of 7.46 ± 0.08 and a slope of the inhibition curve of 1.26 ± 0.20. From the Cheng-Prusoff equation, this gives a pK_i for the glycine site of 7.98 ± 0.08.

Discussion

Derivatization of KYNA has produced compounds that are potent and selective antagonists at the glycine site of the NMDA receptor. In the two chemical series examined here, the optimal compounds were 5-I,7-Cl-KYNA (IC_{50} = 29 nM) and L-689,560 (IC_{50} = 7.8 nM), which were both full antagonists with a high degree of specificity for the glycine site, relative to other amino acid recognition sites.

As noted previously (4), 7-substitution of KYNA led to an increase of affinity and selectivity for the glycine site. An increase of affinity also resulted from 5-substitution, and 5,7-disubstitution had an additive effect (27). Recent reports from Baron *et al.* (45) and McNamara *et al.* (46) also indicated that 5,7-diCl-KYNA has a higher affinity for the glycine site than does 7-Cl-KYNA. The radioligand binding experiments revealed that 5,7-disubstituted KYNA analogues retained excel-

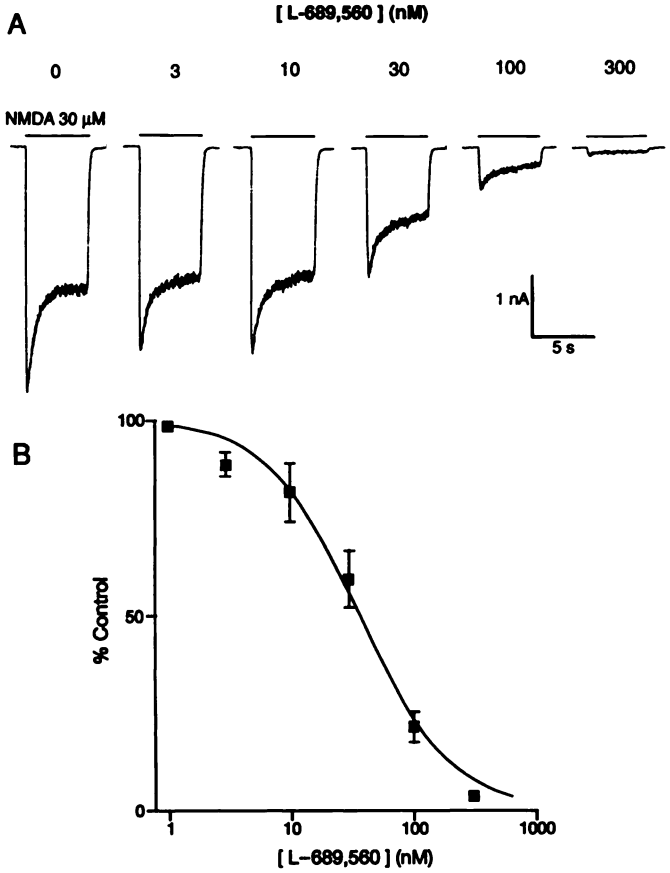


Fig. 8. Inhibition of NMDA-evoked currents in rat cortical neurons in culture by L-689,560. Currents were evoked by 5-sec applications of NMDA (30 μM) in the presence of 300 nM glycine. A, Effect of L-689,560 on currents evoked by NMDA. B, Mean inhibition curve for L-689,560 obtained from four cells; values are the percentage of control (mean ± standard error). Currents were measured at steady state.

clamp experiments, with 5,7-diBr-KYNA having a 1000-fold higher affinity for the glycine site than for the AMPA/kainate receptor. The increased selectivity was entirely due to the increased affinity of these derivatives for the glycine site itself, because certain analogues retained a weak affinity for the glutamate recognition site of the AMPA and NMDA receptors, properties that are also characteristic of substituted quinoxalines (47). Consequently, the most potent analogue, 5-I,7-Cl-KYNA, displayed 3 orders of magnitude selectivity versus other amino acid recognition sites.

Reduction of the right-hand ring of 5,7-diCl-KYNA impaired glycine site affinity; however, this was restored by *trans* substitution in the 4-position (28). The optimal compound, L-689,560, possessed low nanomolar affinity for the glycine site, with 10,000-fold selectivity versus other amino acid recognition sites. The improved affinity may have resulted from several factors, as follows: (i) the pseudo-equatorial 2-carboxyl group in the active *trans*-tetrahydroquinolines can mimic the planar 2-carboxyl group of KYNA; (ii) the 4-substituent amide carbonyl group may act as a hydrogen bond acceptor, like the 4-oxo group of KYNA; (iii) a bulk tolerance site may exist near the 4-position, which is hydrophobic and accepts the phenyl group of L-689,560 and related active compounds. In support of this latter point, Harrison *et al.* (48) and Salituro *et al.* (49) have also suggested the presence of a binding domain within the glycine site that can accept the 4-substituent of KYNA analogues.

Similar structure-activity profiles result from consideration of affinity assessed in the [^{3}H]glycine binding assay or of antagonism of NMDA responses in the rat cortical slice preparation. However, the IC_{50} values from the binding experiments are approximately 10-fold lower (i.e., higher affinity) than the apparent K_b values derived from the functional assay. We attribute this difference to a higher concentration of glycine surrounding the receptors in the cortical slice preparation. Indeed, application of glycine or other glycine site agonists to this preparation does not alter NMDA responses, suggesting that the glycine site is fully occupied under these experimental conditions (4). The noncompetitive nature of the block of NMDA responses in the cortical slice also makes absolute estimations of affinity less reliable. Consequently, the IC_{50} values derived from the binding experiments are a better reflection of the true affinity. Patch-clamp studies using rat cortical neurons in culture, employing the fast superfusion technique by which the composition of the medium surrounding NMDA receptors can be tightly controlled, indicate K_i values for glycine site antagonists close to their affinities derived from [^{3}H]glycine binding assays. Thus, the pK_i of 5,7-diBr-KYNA was 7.2 (63 nM), an estimate of affinity close to the IC_{50} value of 96 nM observed in the glycine binding experiments, and the pK_i of L-689,560 was 7.98 (10.5 nM), similar to the IC_{50} value of 7.41 nM in the binding assay.

Both the [^{3}H]dizocilpine binding and electrophysiological experiments confirm that the compounds antagonize NMDA receptors by competing at the glycine site. This is evident from the parallel shift to the right of KYNA analogue inhibition curves produced by glycine in the [^{3}H]dizocilpine binding experiments and from the reversal of antagonist effects by glycine in the patch-clamp experiments. In addition, these experiments indicate that the compounds are full antagonists, causing a complete block of the NMDA receptor that cannot be overcome by raising the NMDA concentration. This is in contrast to certain other glycine site ligands, such as HA-966 and its analogues (8, 20–22) and ACBC (23), which appear to act as low efficacy partial agonists.

In conclusion, we have identified 5,7-disubstituted analogues of KYNA and 4-substituted carboxytetrahydroquinolines with increased affinity and selectivity for the glycine site on the NMDA receptor. It is anticipated that these compounds will be useful as improved tools to probe the functions of the glycine site in both physiological and pathophysiological situations. Indeed, one of these analogues, 5,7-diCl-KYNA, has been shown to prevent seizures in mice when injected intracerebroventricularly in nanomolar amounts, an effect that is reversed by D-serine (45, 50), indicating the potential anticonvulsant properties of a glycine site antagonist. Recently, Baron *et al.* (51) have reported that [^{3}H]5,7-diCl-KYNA can label the glycine site in radioligand binding experiments. The >10-fold higher affinity of L-689,560 and its excellent selectivity for the glycine site suggested that this compound would provide an improved radioligand, and this has been confirmed in experiments with [^{3}H]L-689,560 (52, 53).

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