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Characterization of the Binding of [^{3}H]L-689,560, an Antagonist for the Glycine Site on the *N*-Methyl-D-aspartate Receptor, to Rat Brain Membranes

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SUMMARY

The binding characteristics of [^{3}H]L-689,560 [(±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline], a selective antagonist for the glycine site on the *N*-methyl-D-aspartate receptor, have been evaluated using rat cortex/hippocampus P_2 membranes. Specific [^{3}H]L-689,560 binding was saturable, having a K_d of 2.97 nM and a B_{max} of 4.15 pmol/mg of protein. The B_{max} value was not significantly different from that obtained for [^{3}H]glycine in the same membrane preparation, and L-689,560 and glycine were found to be mutually competitive. The specific binding of [^{3}H]L-689,560 (1 nM) represented 96 ± 0.02% (four experiments) of total binding. Association experiments at 4° revealed that [^{3}H]L-689,560 reached equilibrium in 120 min, with a $t_{1/2}$ of 40 min. The dissociation of [^{3}H]L-689,560 was slow at 4° ($t_{1/2}$ = 118 min), allowing the use of filtration to separate free from bound radioactivity. Both association and dissociation curves were best fitted by a double-exponential function, suggesting the presence of two components. Comparison of IC_{50} values obtained using [^{3}H]glycine and [^{3}H]L-689,560 binding for 21 glycine site ligands (including agonists, partial agonists, and antagonists, with affinities spanning 5 orders of magnitude) showed a 1:1 correlation, with a correlation coefficient of 0.97. This suggests that efficacy does not have a large influence on the affinity of glycine site ligands when an agonist or antagonist radioligand is used. Ligands for other amino acid recognition sites did not directly inhibit [^{3}H]L-689,560 binding. [^{3}H]L-689,560 is an improved radioligand for the glycine site that will facilitate further investigations of its properties.

The NMDA receptor-ionophore complex, one of the subtypes of receptors for the neurotransmitter L-glutamate, consists of several functional domains, each with separate ligand binding sites (1, 2). NMDA receptor function can be manipulated pharmacologically by compounds acting through distinct recognition sites, which include one for acidic amino acids such as glutamate and NMDA, one for neutral amino acids such as glycine, and one within the ion channel for dissociative anesthetics such as PCP and ketamine. Studies with selective NMDA antagonists have been instrumental in the definition of a role for this receptor in both physiological and pathophysiological mechanisms (for reviews, see Refs. 2–5).

Evidence for a strychnine-insensitive glycine recognition site was first provided by Johnson and Ascher (6), who demonstrated that glycine markedly potentiated NMDA receptor-mediated responses in cultured neurons. Kleckner and Dingledine (7) have since shown that, when steps are taken to remove glycine from the receptor, little or no NMDA receptor-mediated response can be obtained and, hence, they proposed that glycine is an absolute requirement for NMDA receptor activation and that glutamate and glycine may be "coagonists" at the receptor. Since 1987, when the glycine effect was first described (6), several different types of glycine site antagonists have been reported. These include quinoxaline-2,3-diones (8, 9), 6,7-dichloroquinoxalic acid (10, 11), and 5-chloroindole-2-carboxylate (12). HA-966, which was one of the first substances reported as an excitatory amino antagonist (13), is now known to be a low efficacy partial agonist of the glycine site (14, 15). *Cis* substitution of a methyl group in the 4-position of HA-966 leads to increased affinity for the glycine site but reduced agonist efficacy (16, 17). The cyclic glycine derivatives ACC (18) and ACBC (19) have also been described as partial agonists at the glycine site. The naturally occurring substance KYNA was one of the first glycine site antagonists identified (8, 20).

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HA-966, 3-amino-1-hydroxypyrrolid-2-one; ACC, 1-aminocyclopropyl-1-carboxylate; AMPA, α-2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; TCP, 1-(2-thienyl)cyclohexylpiperidine; *trans*-ACPD, (*trans*)-1-aminocyclopentyl-1,3-dicarboxylic acid; PCP, phencyclidine; 5,7-diCl-KYNA, 5,7-dichlorokynurenic acid; 7-Cl-KYNA, 7-chlorokynurenic acid; L-689,560, (±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline; ACBC, 1-amino-1-carboxycyclobutane; 5I,7Cl-KYNA, 5-iodo,7-chlorokynurenic acid; KYNA, kynurenic acid; CPP, DL-(2-carboxypiperazine-4-yl)propyl-1-phosphonate; MNQX, 5,7-dinitroquinoxaline-2,3-dione.

Substitution of a chlorine in the 7-position resulted in a more selective glycine site antagonist, 7-Cl-KYNA, which was the first glycine site antagonist to be described with submicromolar affinity (21). Substitution of 7-Cl-KYNA in the 5-position produced further improvements in affinity, without loss of selectivity, yielding glycine site antagonists with nanomolar affinity, e.g., 5,7-diCl-KYNA (22–24) and 5I,7-Cl-KYNA (23, 24).

High affinity, strychnine-insensitive binding sites for [^{3}H] glycine in rat forebrain membranes were originally identified by Kishimoto *et al.* (25). Autoradiographical experiments demonstrated that the distribution of strychnine-insensitive [^{3}H] glycine binding sites in rat brain sections (26) closely paralleled that of NMDA receptors labeled by L-[^{3}H]glutamate (27). Such studies provided evidence that these glycine sites are associated with the NMDA receptor. [^{3}H]Glycine was the first radioligand to be used to investigate the glycine site on the NMDA receptor complex (10, 28, 29). However, the presence of an uptake system for glycine and its interactions with a strychnine-sensitive inhibitory receptor limit its usefulness in characterizing the glycine site. The agonist D-[^{3}H]serine (30), the partial agonist [^{3}H]ACC (31), and, more recently, the first radiolabeled glycine site antagonist, [^{3}H]5,7-diCl-KYNA (32), have also been described as glycine site radioligands. All of the aforementioned compounds, however, have the disadvantage of rapid dissociation rates, therefore requiring centrifugation techniques in order to separate bound from free radioactivity adequately.

Recently, a series of (*trans*)-2-carboxy-4-substituted tetrahydroquinolines, which are potent glycine site NMDA receptor antagonists, have been described (23). L-689,560, which inhibits [^{3}H]glycine binding with an IC_{50} of 7.8 nM (23), has subsequently been radiolabeled. In the present study, we have evaluated the binding characteristics of [^{3}H]L-689,560 (Fig. 1) as a radioligand for the glycine site on the NMDA receptor. A preliminary account of some of this work has appeared (33).

Materials and Methods

Membrane preparation. For each preparation, 25 male Sprague-Dawley rats (150–250 g) were killed and their cerebral cortices and hippocampi were rapidly removed and placed into 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 remaining pellet was homogenized in a small volume of the sucrose buffer. 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 resultant 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 then centrifuged at 50,000 × *g* for 30 min, the supernatant was discarded, and the pellet was frozen at −20° for at least 18 hr. The pellet was thawed, resuspended by homogenization in 180 ml of a solution of 0.1% saponin in 5 mM Tris-acetate (pH 7.0), and centrifuged at 50,000 × *g* for 20 min. The supernatant was discarded, and the pellet was resuspended, by homogenization, in 180 ml of saponin-free 5 mM Tris-acetate (pH 7.0) and centrifuged at 50,000 × *g* for 20 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.* (34)], and aliquots were stored at −20°. On the day of the binding assay, the required amount of membrane suspension was thawed, washed by homogenization in approximately 10 times the volume of 5 mM Tris-acetate (pH 7.0), and centrifuged at 50,000 × *g* for 60 min. Finally, the pellet was resuspended by homogenization in the required volume of assay buffer.

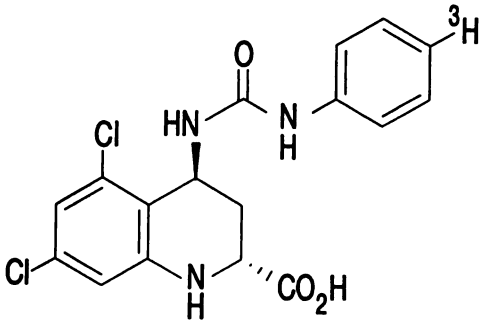

Fig. 1. Structure of [^{3}H]L-689,560.

Standard assay procedure for [^{3}H]glycine binding. Assays were performed 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). Drugs were dissolved in either assay buffer or dimethylsulfoxide (final assay concentration of 1%) 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, to separate free radioactivity from bound. The supernatant was discarded and the pellet was superficially washed with 2 × 2 ml of ice-cold assay buffer. Pellets were solubilized overnight in 0.5 ml of 2% sodium dodecyl sulfate, and then 0.4 ml of the resulting solution was added to 10 ml of scintillation fluid, for quantification of radioactivity using a β counter.

Standard assay procedure for [^{3}H]L-689,560 binding. Assays were performed in polypropylene tubes containing 100 μg of P_2 membrane protein, 1 nM [^{3}H]L-689,560 (28.37 Ci/mmol; Amersham), and 50 mM Tris-acetate buffer (pH 7.0). Drugs were dissolved in either assay buffer or dimethylsulfoxide (final concentration of 1%) 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 45 or 120 min before filtration through Whatman GF/B filters (presoaked in assay buffer), using a Brandel cell harvester (three 5-ml washes with ice-cold 50 mM Tris-acetate buffer, pH 7.0). The filters were added to 10 ml of scintillation fluid, for quantification of radioactivity using a β counter.

Data analysis. Inhibition curves were analyzed for a one-site model by the equation $\% I = \% I_{max}/1 + (IC_{50}/[L])^{n_H}$, where I is inhibition, IC_{50} is the concentration of ligand giving 50% inhibition of specific binding, $[L]$ is the ligand (inhibitor) concentration, and n_H is the Hill coefficient. Association curves were fitted by either a single exponential function $[a - a \cdot e^{(-x/t_{1/2})}]$ or a double-exponential function $[(a_1 + a_2) - (a_1 \cdot e^{(-x/t_{1/2}')} + a_2 \cdot e^{(-x/t_{1/2}'')})]$, where a is the level of specific binding. Dissociation curves were fitted by either a single-exponential function $[a \cdot e^{(-x/t_{1/2})}]$ or a double-exponential function $[a_1 \cdot e^{(-x/t_{1/2}')}] + [a_2 \cdot e^{(-x/t_{1/2}'')}]$. Calculations were performed with the data manipulation and analysis software RS1 (Bolt, Beranek, and Newman, Inc., Cambridge, MA).

Materials. The preparation of [^{3}H]L-689,560 was conducted by Amersham International PLC. Briefly, a solution of (*trans*)-2-methoxycarbonyl-5,7-dichloro-4-(4-iodophenyl)aminocarbonylamino-1,2,3,4-tetrahydroquinoline (35) in methanol was treated with tritium gas, in the presence of 10% palladium on charcoal. The resulting 4-(4-[^{3}H]phenyl) derivative was hydrolyzed with potassium hydroxide and purified by reverse phase thin layer chromatography, to give [^{3}H]L-689,560. The specific activity of [^{3}H]L-689,560 was 72.2 mCi/mg (28.37 Ci/mmol), and the radiochemical purity was 97%.

[^{3}H]Glycine (47.5–51.3 Ci/mmol) was obtained from DuPont-New England Nuclear. D- and L-Serine, D- and L-alanine, D- and L-cycloserine, KYNA, kainate, spermine, and spermidine were purchased from Sigma. CNQX, AMPA, and *trans*-ACPD were purchased from Tocris Neuramin (Essex, UK). Quisqualate was from Cambridge Research Biochemicals. ACC and 5-chloroindole-2-carboxylate were from Aldrich. Glycine was from FSA Lab Supplies. PCP and TCP were provided by the National Institute on Drug Abuse (Baltimore, MD). Ketamine was bought from Parke Davis in the Vetalar form (ketamine HCl, 100 mg/ml). Ifenprodil and milacemide were kind gifts from Syntholabo (Paris, France), and G. D. Searle (Chicago, IL), respectively. All other compounds were synthesized by the Medicinal Chemistry Department at the Neuroscience Research Centre of Merck Sharp and Dohme Research Laboratories (Terlings Park, UK).

Results

Preliminary experiments with [^{3}H]L-689,560 were performed to evaluate specific binding levels, using either 10 μM L-689,560 or 1 mM glycine to determine nonspecific binding. For these experiments, 10 nM [^{3}H]L-689,560 and 150 μg of membrane protein/tube were used, with a 45-min incubation at 4°. Centrifugation and filtration procedures (see Materials and Methods) were compared as methods for separating free radioactivity from bound (Table 1). Both glycine and L-689,560 were able to inhibit [^{3}H]L-689,560 binding. In the centrifugation experiments, higher levels of total and nonspecific binding were obtained; however, the values for specific [^{3}H]L-689,560 binding, as defined using 1 mM glycine or 10 μM L-689,560, were not significantly different from those obtained using filtration. Unlabeled L-689,560 tended to inhibit to a greater extent than glycine, especially in the centrifugation experiments, suggesting the existence of a low affinity, glycine-insensitive component of [^{3}H]L-689,560 binding. Filtration was used in all subsequent experiments, and 1 mM glycine was used to determine nonspecific binding.

Further experiments performed to determine optimal binding conditions (data not shown) indicated that the specific binding of [^{3}H]L-689,560 (1 nM) increased with membrane protein concentration over the range of 25–200 μg/ml, but this was not a strictly linear relationship. For this reason, a fixed concentration of membrane protein (100 μg/0.5 ml) was used for all subsequent [^{3}H]L-689,560 binding experiments. The specific binding of [^{3}H]L-689,560 had a broad pH optimum, between pH 6.0 and 8.0, and was greater at 4°, compared with 24° and 37°. The specific binding of [^{3}H]L-689,560 at 4° and at a radioligand concentration of 1 nM represented 96 ± 0.02% (four experiments) of the total binding, with typical binding values of 3818 ± 278 dpm (four experiments) for total binding and 159 ± 43 dpm (four experiments) for nonspecific binding.

Association and dissociation rates. The association of [^{3}H]L-689,560 (1 nM) at 4° reached equilibrium by 2 hr and, when fitted by a single-exponential equation, gave a $t_{1/2}$ of 35.1 ± 6.7 min (three experiments) (Fig. 2a). However, a double-exponential function provided a significantly better fit ($p < 0.05$; partial F test), giving $t_{1/2}$ values of 10.3 ± 3.3 min and 65.8 ± 7.1 min, with the fast component representing 26.4 ± 3.1% of the total specific binding. For experiments to determine the dissociation of [^{3}H]L-689,560, radioligand (1 nM) and membranes were incubated for 2 hr (at 4°), to allow equilibrium to be reached, and then the incubation mixture was diluted 10-fold with assay buffer containing 1 mM glycine. Aliquots were filtered at time points up to 210 min later. As shown in Fig. 2b, the dissociation rate was slow. Fitting a single-exponential function to the data gave a $t_{1/2}$ for dissociation of 118 ± 9.8 min (three experiments). However, a significantly better fit ($p < 0.01$; partial F test) was provided by a double-exponential function (three experiments), with $t_{1/2}$ values of 8.1 ± 2.8 min and 134 ± 15 min (Fig. 2b). The component showing fast dissociation represented 13.3 ± 3.6% of the total specific binding.

TABLE 1

Initial determinations of [^{3}H]L-689,560 binding to rat cortex/hippocampus P_2 membranes

The binding of 10 nM [^{3}H]L-689,560 to rat cortex/hippocampus P_2 membranes was determined in the absence and presence of either 10 μM L-689,560 or 1 mM glycine, using centrifugation or filtration to separate bound from free radioactivity (see Materials and Methods). Results are the mean ± standard error of four separate experiments, using four different membrane preparations.

	Binding	
	Centrifugation	Filtration
	dpm/mg of protein	
Total binding	29,626 ± 2,584	20,436 ± 2,816
Nonspecific binding		
1 mM Glycine	16,342 ± 1,798	2,125 ± 479
10 μM L-689,560	12,134 ± 1,318	1,429 ± 265
Specific binding		
1 mM Glycine	13,284 ± 2,013	18,311 ± 2,966
10 μM L-689,560	17,492 ± 1,649	19,007 ± 2,945

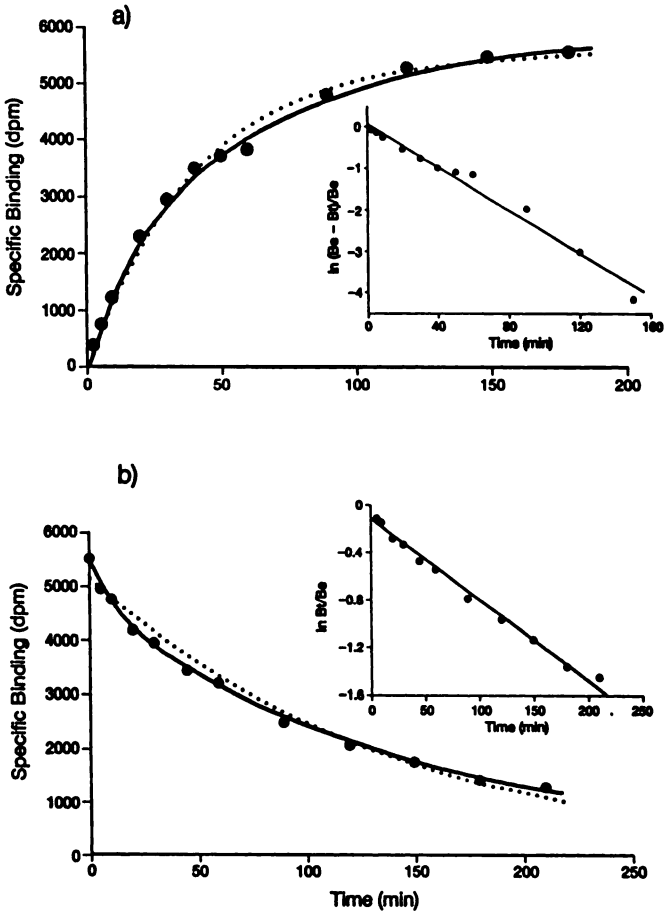

Fig. 2. Association (a) and dissociation (b) of [^{3}H]L-689,560 (1 nM) binding to rat cortex/hippocampus P_2 membranes at 4°. Both association and dissociation curves were best fitted by a double-exponential (——) rather than a single-exponential (· · · ·) function. *Insets*, logarithmic transformation of data. See text for details of methods and data analysis.

Saturation analyses. Saturation analysis of [^{3}H]L-689,560 binding was carried out over a concentration range of 0.1–1000 nM, with an incubation time of 120 min. Fig. 3a shows a typical saturation curve, and values obtained from three separate experiments are shown in Table 2. [^{3}H]L-689,560 labeled a single population of sites with high affinity. When 0.2 μM glycine was included, this resulted in a reduction of the K_d value with no change in B_{max}, consistent with a competitive interaction. The Hill coefficient of [^{3}H]L-689,560 binding determined in the presence or absence of 0.2 μM glycine was significantly greater than 1, perhaps indicating positive cooperativity. The B_{max} value for [^{3}H]L-689,560 binding was similar to that for [^{3}H]glycine binding (determined over a range of concentrations from 1 to 1000 nM) (Fig. 3b; Table 2), and inclusion of 10 nM L-689,560 reduced the K_d of [^{3}H]glycine binding but not the B_{max} value (Table 2), indicating a competitive interaction.

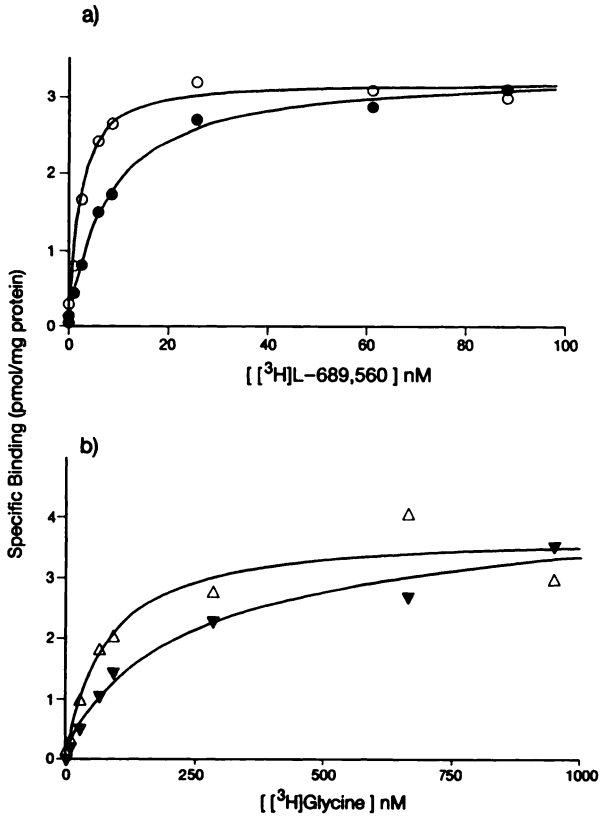

Fig. 3. Typical saturation curves for [^{3}H]L-689,560 binding to rat P_2 membranes in the absence (○) and presence (●) of 0.2 μM glycine (a) and [^{3}H]glycine binding in the absence (△) and presence (▼) of 10 nM L-689,560 (b). Experiments with [^{3}H]L-689,560 were carried out over a concentration range of 0.1–1000 nM, but for clarity the data for 0.1–100 nM are illustrated here.

These results confirm that [^{3}H]L-689,560 and [^{3}H]glycine label an identical population of binding sites in rat cortex/hippocampus P_2 membranes.

Pharmacological specificity. A number of glycine site ligands were tested for their ability to inhibit [^{3}H]L-689,560 binding (see Table 3). Serine, alanine, HA-966, cycloserine, and (*cis*)-4-methyl-HA-966 showed stereoselectivity consistent with binding to the glycine site on the NMDA receptor (Table 3). IC_{50} values obtained for [^{3}H]L-689,560 binding compared well with those for [^{3}H]glycine binding for agonists, partial agonists, and antagonists (Table 3; Fig. 4). All compounds gave maximal inhibition of [^{3}H]L-689,560 binding, and Hill coefficients were not significantly different from unity. Affinity values reported in Table 3 for [^{3}H]L-689,560 binding were obtained using an incubation time of 45 min. These values did not differ significantly from values obtained when binding was allowed to reach equilibrium. IC_{50} values (geometric mean ± standard error, + standard error; three experiments) obtained for the 120-min incubation were as follows: glycine: 0.234(0.228, 0.240)μM; D-serine: 0.504(0.462, 0.551)μM; (+)-HA-966: 10.3(8.23, 12.8)μM; D-cycloserine: 7.15(6.32,8.10)μM; L-689,560: 6.12(5.80, 6.46)nM; 7-Cl-KYNA: 0.357(0.338, 0.377)μM. Inhibition curves for these compounds, using both 45-min and 120-min incubation times, are shown in Fig. 4. The correlation of affinity values obtained for 21 compounds in [^{3}H]L-689,560 and [^{3}H]glycine binding experiments is shown in Fig. 5. An excellent correlation was apparent between the two sets of data, with a correlation coefficient (r) of 0.97 and a slope of 1.02. A number of other substances were tested in the [^{3}H]L-689,560 binding assay (Table 4). Among a number of monovalent and divalent cations tested, a slight inhibition of [^{3}H]L-689,560 binding was seen with 1 mM $CaCl_2$, magnesium acetate, and $MnCl_2$, but at a concentration of 10 μM these ions had no significant effect (Table 4). Compounds that act as NMDA receptor channel blockers were without effect on [^{3}H]L-689,560 binding (Table 4), as was the coagonist L-glutamate. The polyamines spermine and spermidine caused significant inhibition at 100 μM, although ifenprodil and ethanol, two compounds reported to block NMDA receptor responses, had no effect. Compounds with actions at other excitatory amino acid subtypes, i.e., AMPA, kainate, and *trans*-ACPD, had no effect at 100 μM, although quisqualate gave a small increase of [^{3}H]L-689,560 binding at 100 μM. The glycine prodrug milacemide also had no effect.

Discussion

The characteristics of [^{3}H]L-689,560 binding to rat cortex/hippocampus P_2 membranes indicate that this compound is an excellent radioligand for investigation of the glycine site on the NMDA receptor. The present studies have shown that [^{3}H]L-

TABLE 2

Summary of values from saturation experiments with [^{3}H]L-689,560 and [^{3}H]glycine

Values were derived from saturation isotherms of [^{3}H]L-689,560 (with or without 0.2 μM glycine) and [^{3}H]glycine (with or without 10 nM L-689,560) binding to rat P_2 membranes. K_d is the affinity constant (geometric mean, − standard error, + standard error). B_{max} is the maximum level of binding (mean ± standard error), and n_H is the Hill coefficient (mean ± standard error). Each set of data is the mean of three experiments, using three different membrane preparations.

	[^{3}H]L-689,560		[^{3}H]Glycine	
	Control	+0.2 μM Glycine	Control	+10 nM L-689,560
K_d (nM)	2.97 (2.14, 4.13)	7.19 (6.34, 8.15)	149 (97.1, 228)	207 (172, 250)
B_{max} (pmol/mg of protein)	4.15 ± 0.95	4.19 ± 0.86	5.59 ± 1.20	4.48 ± 0.97
n_H	1.33 ± 0.10	1.23 ± 0.02	0.89 ± 0.11	1.12 ± 0.18

TABLE 3

Inhibition of [³H]glycine and [³H]L-689,560 binding to rat cortex/hippocampus P_2 membranes by glycine site ligands

Incubation time for [³H]L-689,560 binding was 45 min. Values are the concentration of drug that inhibited 50% of specific binding (IC_{50}) and are the geometric mean (− standard error, + standard error) of at least three separate experiments.

	IC_{50}	
	[³H]Glycine	[³H]L-689,560
	μM	
Agonists		
Glycine	0.18[a]	0.179 (0.159, 0.202)
D-Serine	0.57[a]	0.338 (0.247, 0.462)
L-Serine	44.3 (36.6, 53.7)	43.9 (40.4, 47.6)
D-Alanine	2.74 (1.73, 3.74)	0.835 (0.719, 0.969)
L-Alanine	44.3 (40.5, 48.1)	39.8 (33.6, 47.1)
ACC	0.042 (0.014, 0.127)	0.092 (0.034, 0.252)
Partial agonists		
(*R*)-(+)-HA-966	12.5[b]	9.67 (7.82, 12.0)
(*S*)-(−)-HA-966	339[b]	654 (494, 866)
D-Cycloserine	5.42 (5.01, 5.83)	6.99 (4.73, 10.3)
L-Cycloserine	>1000	>1000
(3*R*)-(+)-(*cis*)-4-Methyl-HA966	1.37[c]	2.72 (1.37, 5.40)
(3*S*)-(−)-(*cis*)-4-Methyl-HA966	85.7[c]	57.0 (45.2, 71.8)
ACBC	24.4 (17.0, 35.1)	31.7 (25.8, 38.8)
Antagonists		
L-689,560	0.0078[d]	0.004 (0.0025, 0.0064)
KYNA	22.3 (20.5, 24.2)	13.8 (11.2, 17.1)
7-Cl-KYNA	0.55[e]	0.315 (0.230, 0.430)
5,7-diCl-KYNA	0.213[e]	0.0643 (0.0553, 0.0749)
5-I,7-Cl-KYNA	0.029[e]	0.014 (0.011, 0.017)
5,7-Dibromo-KYNA	0.096[e]	0.060 (0.055, 0.066)
CNQX	10.8 (8.01, 13.6)	3.54 (3.06, 4.11)
MNQX	0.264 (0.191, 0.365)	0.173 (0.166, 0.181)
5Cl-Indole-2-carboxylate	146 (119, 179)	90.5 (83.9, 97.8)

[a] From Ref. 21.
[b] From Ref. 51.
[c] From Ref. 16.
[d] From Ref. 23.
[e] From Ref. 24.

689,560 selectively labels the glycine site with high affinity (K_d = 3 nM), has a slow dissociation rate (allowing the routine use of filtration to separate bound from free radioactivity), and provides accurate affinity values for glycine site ligands.

Several observations confirm that [³H]L-689,560 is a selective radioligand for the glycine site on the NMDA receptor. Firstly, the B_{max} value for [³H]L-689,560 is similar to that of [³H]glycine binding to its strychnine-insensitive site on the NMDA receptor in rat cortex/hippocampus P_2 membranes. Although the experiments using centrifugation to separate bound from free radioactivity provided a hint that [³H]L-689,560 labeled an additional glycine-insensitive site, this was not apparent in filtration experiments and presumably represents a low affinity interaction with a fast dissociation rate. Secondly, in saturation experiments with [³H]L-689,560 binding, glycine behaved as a simple competitive inhibitor, changing the K_d but not the B_{max} value, as did L-689,560 in saturation experiments with [³H]glycine binding. Thirdly, a comparison of 21 glycine site ligands in [³H]L-689,560 and [³H]glycine binding assays, with affinities spanning a range of 5 orders of magnitude, gave an excellent one-to-one correlation. Finally, a number of ligands for other recognition sites on amino acid receptors were poor inhibitors of [³H]L-689,560 binding, confirming the selectivity of the unlabeled compound reported previously (24).

The kinetics of association and dissociation of [³H]L-689,560 were complex. A double-exponential function gave a significantly better fit to both association and dissociation curves, suggesting the presence of two components. In each case, the fast component represented the smaller proportion; however, the proportions of the specific binding represented by these components of association and dissociation were close but not identical (26.4 ± 3.1% and 13.3 ± 3.6%, respectively). Calculation of kinetic constants was made difficult by the two-component association and dissociation curves. On the basis of the single-exponential fits, an association rate constant (k_1) of 0.0216 ± 0.0067 nM^{-1} min^{-1} and a dissociation rate constant (k_2) of 0.0086 ± 0.0007 min^{-1} were obtained. This gives a K_d value of 0.397 nM, which is lower than the value obtained from the saturation analysis (2.97 nM). This discrepancy may be a result of the complex kinetics observed. One explanation for the two-component kinetics would be the presence of two affinity states of [³H]L-689,560 binding; however, the saturation analyses gave no indication of this. This would suggest that, if two components of [³H]L-689,560 binding exist, their K_d values are very close to each other. An alternative possibility comes from the fact that the saturation analyses gave a Hill coefficient consistently greater than unity (1.33 ± 0.10). The Hill coefficient remained greater than 1 when experiments were carried out using a 6-hr incubation, to ensure that the lower

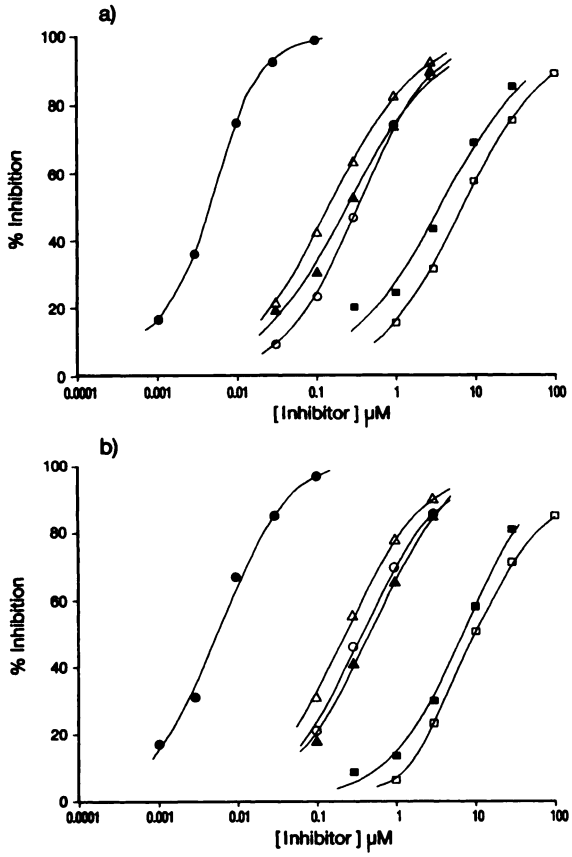

Fig. 4. Inhibition of [^{3}H]L-689,560 binding to rat cortex/hippocampus P_2 membranes by glycine site ligands. a, 45-min incubation time; b, 120-min incubation time. ●, L-689,560; △, glycine; ▲, D-serine; ○, 7-Cl-KYNA; ■, D-cycloserine; □, (*R*)-(+)-HA-966. Results shown are from a single experiment, which was repeated twice with similar results (see Table 3 and text for affinity values).

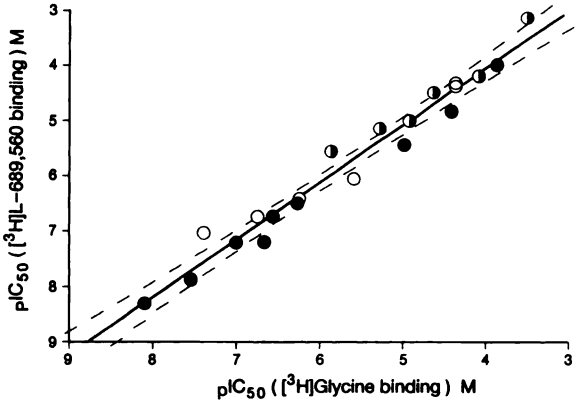

Fig. 5. Correlation of affinity values for 21 glycine site ligands obtained in [^{3}H]L-689,560 and [^{3}H]glycine binding experiments with rat cortex/hippocampus P_2 membranes. ○, Agonist; ◑, partial agonist; ●, antagonist. The slope of the regression line was 1.02, with a correlation coefficient of 0.97. – – –, 95% confidence limits.

concentrations of [^{3}H]L-689,560 had reached equilibrium (data not shown). This may suggest that positive cooperativity is present, which would indicate that multiple interacting binding sites for [^{3}H]L-689,560 may exist on a single NMDA receptor. It has been suggested that each NMDA receptor possesses two glycine recognition sites, on the basis of evidence from radioligand binding (36) and electrophysiological experiments (37). If

TABLE 4

Effect on [^{3}H]L-689,560 binding of a number of monovalent and divalent cations and compounds known to have actions at sites distinct from the glycine site

For K^+, Ca^{2+}, and Mn^{2+}, a chloride salt was used; for Mg^{2+} and Zn^{2+}, an acetate salt was used. Results are expressed as mean ± standard error of inhibition of [^{3}H]L-689,560 binding, from three separate experiments. An incubation time of 45 min was used for these experiments.

	Inhibition
	%
Monovalent ions	
Na^+ Ac^-	
1 mM	3.30 ± 3.59
100 mM	2.86 ± 5.28
Na^+ Cl^-	
1 mM	3.75 ± 4.80[a]
100 mM	4.09 ± 4.78[a]
K^+	
1 mM	1.39 ± 4.77[a]
100 mM	11.4 ± 4.82[a]
Divalent cations	
Ca^{2+}	
10 μM	1.62 ± 0.58
1 mM	21.8 ± 3.06
Mg^{2+}	
10 μM	0.80 ± 3.82[a]
1 mM	13.0 ± 10.4
Mn^{2+}	
10 μM	0.50 ± 7.07
1 mM	31.9 ± 6.27
Zn^{2+}	
10 μM	0.93 ± 9.03
1 mM	1.07 ± 3.72
NMDA receptor ion channel blockers	
Dizocilpine	
1 μM	4.30 ± 2.77
100 μM	9.77 ± 3.52
PCP	
1 μM	2.83 ± 0.89
100 μM	7.85 ± 6.39
TCP	
1 μM	2.2 ± 1.45
100 μM	1.37 ± 7.98
Ketamine	
1 μM	6.53 ± 2.46
100 μM	1.07 ± 4.29
Miscellaneous	
Kainate, 100 μM	0.24 ± 0.54[a]
AMPA, 100 μM	0.72 ± 1.43[a]
trans-ACPD, 100 μM	2.39 ± 2.02
Quisqualate, 100 μM	27.6 ± 8.88[a]
Ifenprodil, 100 μM	3.93 ± 0.80[a]
Spermine, 100 μM	51.8 ± 6.65
Spermidine, 100 μM	21.1 ± 4.73
Ethanol, 100 mM	0 ± 0.32
Milacemide, 100 μM	7.0 ± 2.72
Strychnine	
10 μM	7.2 ± 4.21
100 μM	9.6 ± 1.99
L-Glutamate, 10 μM	5.11 ± 1.34[a]

[a] Increase in binding.

positive co-operativity occurs (i.e., the binding of one molecule of L-689,560 increases the likelihood of a second molecule binding to the receptor), this may manifest itself as multiple components in kinetic experiments.

The IC_{50} values for inhibition of [^{3}H]L-689,560 binding by glycine site ligands were in good agreement with those reported from experiments using other glycine site radioligands, such as the agonists [^{3}H]glycine (10, 29) (present study), D-[^{3}H]serine (30), and [^{3}H]ACC (31) and the antagonist [^{3}H]5,7-diCl-KYNA

(32). Overall, the one-to-one correlation between IC_{50} values in the [^{3}H]L-689,560 and [^{3}H]glycine binding experiments for a range of agonists, partial agonists, and antagonists indicates that efficacy is not a major influence on the affinity of glycine site ligands in agonist versus antagonist binding assays. Nevertheless, antagonists tended to have IC_{50} values in the [^{3}H]L-689,560 binding assay that were 2-fold lower (i.e., higher affinity), compared with [^{3}H]glycine binding, perhaps suggesting preferential labeling of an "antagonist-preferring" state of the receptor. However, the situation is less clear cut than that for the glutamate recognition site on the NMDA receptor, where competitive antagonists have 5–10-fold higher affinity for antagonist (e.g., [^{3}H]CPP) versus agonist (L-[^{3}H]glutamate) binding sites (38).

Compounds with actions at other sites within the NMDA receptor complex had differential effects on [^{3}H]L-689,560 binding. The putative neurotransmitter L-glutamate, which has been reported to increase [^{3}H]glycine binding (10), had little effect on [^{3}H]L-689,560 binding. However, preliminary experiments indicate that competitive antagonists at the glutamate site are allosteric inhibitors of [^{3}H]L-689,560 binding (39), suggesting that occupation of the glutamate site by certain ligands influences the binding of glycine site antagonists. The polyamines spermine and spermidine inhibited [^{3}H]L-689,560 binding. Interestingly, Sacaan and Johnson (40) observed that spermine enhanced the affinity of glycine site agonists for [^{3}H] glycine binding without affecting glycine site antagonist affinity. Taken together with the present results, this may indicate that polyamine agonists promote an "agonist-preferring" state of the glycine site. The putative polyamine site antagonist ifenprodil (41) was ineffective versus [^{3}H]L-689,560 binding. Further detailed structure-activity studies are required to confirm that the inhibition of [^{3}H]L-689,560 binding by polyamines observed in the present experiments is, indeed, mediated by the polyamine site that has previously been characterized on the NMDA receptor. The channel-blocking drugs dizocilpine (MK-801), TCP, PCP, and ketamine had no effect on [^{3}H]L-689,560 binding at 100 μM, consistent with their inactivity in [^{3}H] glycine binding assays (10, 29).

Both Zn^{2+} and ethanol antagonize NMDA receptor-mediated responses (42–44) and have been suggested to interact with the glycine site (45–47). Zn^{2+} had no effect on [^{3}H]L-689,560 binding at 10 or 1000 μM. Yeh *et al.* (45) reported that Zn^{2+} inhibited [^{3}H]glycine binding with an IC_{50} value of 15 μM; however, in our own experiments Zn^{2+} gave <50% inhibition of [^{3}H]glycine binding at a concentration of 1 mM (data not shown). Therefore, we were unable to observe an effect of this divalent cation on agonist or antagonist binding to the glycine site. Methodological differences may underlie this discrepancy, e.g., Yeh *et al.* (45) used membranes that were prepared from hippocampus only and were treated with EDTA. Certain other divalent cations, namely Mn^{2+}, Ca^{2+}, and Mg^{2+}, gave a small degree of inhibition of [^{3}H]L-689,560 binding at 1 mM. Marvizon and Skolnick (48) have reported that Mg^{2+} and Ca^{2+} enhance [^{3}H] glycine binding to rat forebrain membranes, although we could not reproduce this effect in our own experiments (data not shown). Therefore, from our experiments, mono- and divalent cations do not appear to have a major influence on the glycine site, and the NMDA antagonist properties of Zn^{2+} may not result from an interaction with the glycine site. The inhibition of NMDA receptor-mediated responses by ethanol has been reported to be reversed by glycine site agonists (46, 47). At 100 mM, ethanol had no effect on [^{3}H]L-689,560 binding, indicating that it does not compete directly at the glycine site.

In conclusion, the binding characteristics of [^{3}H]L-689,560 make this the radioligand of choice for labeling the glycine site on the NMDA receptor, among currently available alternatives. Its principal advantages are (i) high affinity and a low degree of nonspecific binding, resulting in a level of specific binding that is 96% of total binding at routine radioligand concentrations; (ii) a slow dissociation rate, enabling filtration to be used as a means of separating bound from free radioactivity; and (iii) parity of IC_{50} values for glycine site ligands with those for [^{3}H]glycine and other glycine site radioligands. [^{3}H]L-689,560 will be a valuable tool for further investigation of the properties of the glycine site and, in preliminary experiments, has been used to determine the precise anatomical localization of glycine sites by autoradiography (49), to investigate allosteric interactions within the NMDA receptor complex (39), and to determine the status of the glycine site in human post-mortem brain samples (50).

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