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Characterization of the binding of [^{3}H]-CGS 19755: a novel N-methyl-D-aspartate antagonist with nanomolar affinity in rat brain

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1 CGS 19755 (*cis*-4-phosphonomethyl-2-piperidine carboxylic acid), a rigid analogue of 2-amino-5-phosphonopentanoic acid (AP5), is one of the most potent competitive N-methyl-D-aspartate (NMDA) antagonists described. Using Triton-treated crude synaptic membranes from rat brain, binding studies indicated that [^{3}H]-CGS 19755 bound with high affinity and selectivity to the NMDA-type excitatory amino acid receptor.

2 [^{3}H]-CGS 19755 binding was saturable, reversible, heat-labile, pH-dependent and linear with protein concentration. Specific binding represented 80–85% of the total amount bound.

3 Using a centrifugation assay, saturation experiments revealed two distinct binding components with K_d values of 9 and 200 nM, and corresponding B_{max} values of 0.55 and 1.00 pmol mg^{-1} protein. In contrast, a single binding component with a K_d value of 24 nM and an apparent B_{max} value of 0.74 pmol mg^{-1} protein was observed with a filtration assay.

4 Competition experiments in which both assay techniques were used, showed that [^{3}H]-CGS 19755 selectively labels the NMDA receptor. The most active inhibitors of [^{3}H]-CGS 19755 binding were L-glutamate and CGS 19755 (IC_{50} values = 100 nM).

5 In the centrifugation assay, a number of excitatory amino acids were found to generate shallow inhibition curves, and computer analysis indicated the presence of two binding components. The quisqualate receptor ligand AMPA (D,L-α-amino-3-hydroxy-5-methylisoxazole-4-propionate), kainic acid and the non-competitive NMDA antagonists, such as phencyclidine, tiletamine and MK-801, were without activity.

6 The high affinity binding obtained with [^{3}H]-CGS 19755 by use of filtration techniques thus permits the more rapid evaluation of compounds as potential NMDA antagonists and agonists. Therefore, this rigid analogue of AP5 is a more suitable radioligand for NMDA receptors than [^{3}H]-CPP (3-(±)-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid), the corresponding analogue of 2-amino-7-phosphonoheptanoic acid (AP7).

Introduction

Pharmacological studies related to the role of excitatory amino acids (EAAs) in mammalian tissue have increased considerably with the development of selective compounds and new techniques to examine EAA subtypes. These advances have allowed for the development of a more molecular basis of study to complement the electrophysiological approach which has provided much of the research impetus in this area (see Cotman & Iversen, 1987; Lehmann *et al.*, 1987a).

The N-methyl-D-aspartate (NMDA) receptor subtype has received particular attention due, in part, to the availability of various antagonists, such as 2-amino-5-phosphonopentanoic acid (AP5) and 2-amino-7-phosphonoheptanoic acid (AP7), that are selective for this receptor (Foster & Fagg, 1984; Watkins & Olverman, 1987) and the availability of radioligands such as [^{3}H]-L-glutamate (L-Glu) to characterize the receptor. Although the ability to delineate between NMDA receptors and EAA uptake systems has been resolved, radioligands for the NMDA receptor have been plagued by lack of

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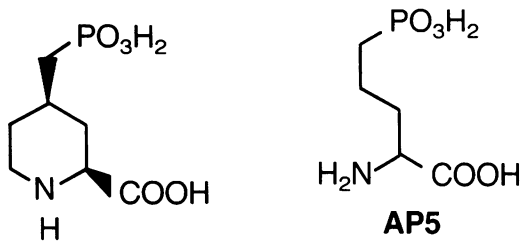

Figure 1 Structure of 2-amino-5-phosphonopentanoic acid (AP5) and *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755).

specificity and low affinity with concomitant rapid dissociation rates.

The rigid analogue of AP7, CPP (3-(±)(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid) was recently shown to be the first radioligand that selectively labels the NMDA receptor with reasonably high affinity (K_d value = 200 nM; Murphy *et al.*, 1987a). Although this represented a 5 fold improvement in affinity as compared to [^{3}H]-L-Glu (Foster & Fagg, 1987), the use of [^{3}H]-CPP still required centrifugation isolation techniques to obtain reproducible results and high percentage specific binding.

Recently, CGS 19755 (*cis*-4-phosphonomethyl-2-piperidine carboxylic acid), a constrained analogue of AP5 (Figure 1), has been found to be a potent, competitive NMDA-type receptor antagonist (Lehmann *et al.*, 1987c). The compound potently inhibited NMDA-evoked release of [^{3}H]-acetylcholine from rat striatal slices (Lehmann *et al.*, 1987c) and blocked NMDA- and audiogenic-induced seizures (Bennett *et al.*, 1987). Like other NMDA receptor antagonists (Boast *et al.*, 1987), CGS 19755 prevented ischaemia-induced hippocampal cell damage in gerbils (Jarvis *et al.*, 1987; Boast *et al.*, 1988). Furthermore, CGS 19755 inhibited the binding of [^{3}H]-CPP with an IC_{50} value of 50 nM, but was found to be inactive in the kainate and quisqualate EAA receptor binding assays and had no activity ($IC_{50} > 10\,\mu$M) in binding assays for 21 other putative neurotransmitter receptors (Lehmann *et al.*, 1987b).

In the present study, [^{3}H]-CGS 19755 was evaluated as a potential NMDA receptor ligand with crude synaptic membranes (CSMs) from rat brain used as a receptor source with the aim of developing a binding assay that was amenable to the use of the more rapid filtration isolation procedure. Portions of this work have been presented in abstract form (Murphy *et al.*, 1988).

Methods

Tissue preparation

Crude synaptic membranes (CSMs) were prepared as described by Enna & Snyder (1977) and modified by Murphy *et al.* (1987b). Briefly, male Sprague-Dawley rats (Tac: (SD), Taconic Farms, Germantown, NY) were decapitated and forebrains (whole brain minus brain stem and cerebellum) removed and homogenized in 15 volumes of ice-cold 0.32 M sucrose with a Teflon pestle and glass homogenizer (Type B; A.H. Thomas, Phila., PA). The homogenate was centrifuged (1000 *g*, 10 min) in a Beckmann J2-21M centrifuge (4°C) and the supernatant collected and recentrifuged (20,000 *g*, 20 min). The resultant pellet was dispersed in ice-cold distilled water with a Brinkmann Polytron (setting 6, 30 s) followed by centrifugation (8,000 *g*, 20 min). The supernatant and soft, buffy uppercoat layer of the pellet were collected and centrifuged (48,000 *g*, 20 min), followed by Polytron resuspension of the CSM pellet in ice-cold buffer (50 mM Tris-HCl, pH 7.6). To facilitate the removal of endogenous EAA receptor ligands such as Glu, the CSM suspensions were incubated with the detergent Triton X-100 (0.04%; Eastman Kodak Co., Rochester, NY) at 37°C for 15 min followed by centrifugation (48,000 *g*, 10 min; Fisher *et al.*, 1986; Murphy *et al.*, 1987a,b). The detergent was removed with two tissue washes (Polytron resuspension in buffer, followed by centrifugation) and the CSM pellets frozen at −70°C until use.

Radioligand binding

On the day of the assay, the CSMs were washed an additional two times, with final resuspension of the pellet in 50 mM Tris-HCl buffer, pH 8.0 at 4°C to yield a final protein concentration of 200–400 μg ml^{-1} as determined by the Bio-Rad assay in which bovine serum albumin was used as standard (Bradford, 1976).

In the binding assay, an aliquot (1.0 ml) of CSMs was incubated in triplicate with 10 nM [^{3}H]-CGS 19755 (10 μl; specific activity 30–40 Ci mmol^{-1}, DuPont-NEN, Boston, MA) in the presence or absence of test compounds (10 μl) for 15 min at 4°C. Due to the difficulty of measuring CGS 19755 as a chromophore, the degree of difficulty in determining the specific activity should be noted. L-Glu (1.0 mM) was used to define non-specific binding. The amount of ligand bound was isolated from free radioactivity by either filtration or centrifugation techniques as previously described (Murphy *et al.*, 1987a). For filtration experiments, bound radioactivity was separated from free radioactivity by vacuum filtration through Whatman GF/B glass fibre filters

using a Brandel Cell Harvester (M-24R, Gaithersburg, MD) followed by two, 3 ml rinses with ice-cold buffer. The filters were placed in minivials, scintillation mixture added (Formula-989, DuPont-NEN, Boston, MA), and the radioactivity quantitated 12 h later by conventional liquid scintillation spectroscopy techniques at an efficiency of 40%.

For the centrifugation assay, the receptor-ligand complexes were isolated by centrifugation at 48,000 *g*, 10 min. The supernatant was then decanted and the pellets rapidly rinsed (2 × 3 ml ice-cold buffer). The pellets were digested in Protosol tissue solubilizer (1.0 ml; DuPont-NEN) in a sonicating water bath, after which 4 ml Formula-989 was added. To reduce the chemiluminescence produced by the Protosol, glacial acetic acid was added to the scintillation cocktail mixture at a final concentration of 1.0%. After a 30 min equilibration period, radioactivity was then determined as described above.

Saturation experiments were performed with 15–20 concentrations of [^{3}H]-CGS 19755 over the concentration range of 0.22–1280 nM in centrifugation and 0.22–640 nM in filtration assays, respectively. The data were analyzed by the iterative curve-fitting programme Lundon-1 (Lundeen & Gordon, 1986). In the competition experiments, 8–15 different concentrations of each inhibitor were incubated in triplicate with 10 nM [^{3}H]-CGS 19755. The data from 3–4 separate experiments were analyzed simultaneously by non-linear regression analysis using RS/1 (Bolt, Beranek and Newman, Boston, MA) and expressed as the mean ± s.e.mean. Whether a two-component binding model fit the data significantly better than a one-component model ($P < 0.01$) was determined by the partial F-test (Draper & Smith, 1966).

Materials

CGS 19755, CPP, AMPA and phencyclidine (PCP) were synthesized in the Chemistry Research Department of CIBA-GEIGY (Summit, NJ, U.S.A.). Tritiation of CGS 19755 was performed by DuPont-NEN (Boston, MA, U.S.A.) by reduction of the suitable precursor. All compounds and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) with the exception of the following: Tris (Ultrol-grade) and D,L-AP4 (D,L-2-amino-4-phosphonobutanoic acid; Calbiochem-Behring, La Jolla, CA, U.S.A.); D-AP5 (Cambridge Research Biochemicals, Ltd., Cambridgeshire); D,L-AP7 (Institute National de Recherche Chimique, Paris, France); (±)-quisqualate (synthetic; Research Biochemicals Inc., Natick, MA, U.S.A.). The following compounds were generous gifts: tiletamine (Warner-Lambert, Ann Arbor, MI, U.S.A.); and MK-801 ((±)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, Merck, Sharp and Dohme, Rahway, NJ, U.S.A.).

Results

Preliminary experiments with [^{3}H]-CGS 19755 in both filtration and centrifugation assays were performed to determine optimal binding conditions. The results from initial experiments indicated that these conditions were similar to those previously observed for [^{3}H]-CPP binding (Murphy *et al.*, 1987a). As found with [^{3}H]-CPP, [^{3}H]-CGS 19755 binding was enhanced in detergent-treated (0.04% Triton X-100) frozen CSMs, reversible upon addition of L-Glu (1 mM), linear with protein concentration (0.034–0.763 mg protein ml^{-1}), heat labile, and pH-dependent, with optimum binding occurring between pH 7.8–8.2 (data not shown).

Association kinetics were examined to determine the appropriate incubation conditions. As shown in Figure 2, the association rate in the filtration assay was very rapid; specific binding reached a plateau between 2 and 10 min at 4°C and remained stable for at least 1 h. Similar results were obtained by centrifugation separation techniques. Therefore, an incubation time of 15 min was chosen for future experiments.

When dissociation of [^{3}H]-CGS 19755 was started with 1 mM L-Glu, binding was rapidly reversible as 90% of the specific binding was displaced within 2 min.

In both filtration and centrifugation assays, specific binding of 10 nM [^{3}H]-CGS 19755 represented 80–85% of the total amount bound with

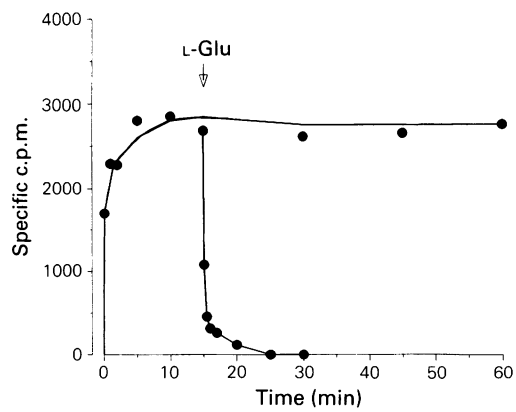

Figure 2 Time course of association and dissociation of 10 nM [^{3}H]-CGS 19755 specific binding at 4°C in the filtration assay. The figure is representative of 2–3 separate experiments. Based on the rapid association of the specific binding, a time point of 15 min was used in further experiments.

1 mM L-Glu to define non-specific binding. The values for specific binding were 190 ± 11 fmol mg^{-1} protein (mean ± s.e.mean; 3100 ± 120 total c.p.m., 640 ± 33 non-specific c.p.m., $n = 15$) and 400 ± 22 fmol mg^{-1} protein (1680 ± 55 total c.p.m., 320 ± 19 non-specific c.p.m.) in the centrifugation and filtration assays, respectively. Filter binding of [^{3}H]-CGS 19755 (10 nM) was found to be minimal and represented less than 0.1% of the total radioactivity added to tissue-containing samples.
than 0.1% of the total radioactivity added to tissue-containing samples.

Saturation curves were generated in both the centrifugation and filtration binding assays. Analysis of the data generated in the centrifugation assay indicated that a two-component binding model described the data significantly better than a one-component model. The K_d and apparent B_{max} values for the high affinity binding component were 9 ± 2 nM and 0.55 ± 0.02 pmol mg^{-1} protein, and 200 ± 28 nM and 1.00 ± 0.16 pmol mg^{-1} protein (mean ± s.e.mean) for the low affinity component, respectively (Figure 3).

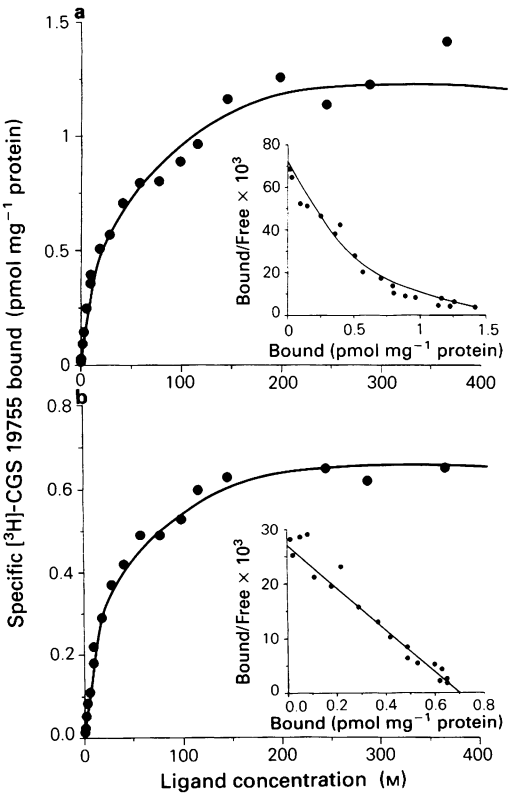

Figure 3 Representative saturation and Scatchard (inset) plots of [^{3}H]-CGS 19755 binding from (a) centrifugation and (b) filtration experiments. The details of the methodology are described in the text.

In contrast, analysis of the filtration assay data revealed that a one-component binding model adequately described the binding data. The K_d and B_{max} values obtained were 24 ± 6 nM and 0.74 ± 0.09 pmol mg^{-1} protein, respectively (Figure 3).

Competition experiments were performed to evaluate the pharmacology of the site labelled by [^{3}H]-CGS 19755 in both the centrifugation and filtration assays. Analysis of the binding data revealed that a similar rank order of potency of compounds was obtained in both the filtration and centrifugation assays (see Table 1). L-Glu and unlabelled CGS 19755 were the most active compounds examined, exhibiting IC_{50} values of 100 and 110 nM in the filtration assay, respectively. Stereoselectivity was demonstrated by the finding that D-Glu was approximately 80 fold less active than L-Glu.

Of interest was the finding that a number of compounds exhibited Hill coefficients that were significantly less than unity in the centrifugation assay (Table 1). In the analysis of competition curves for these compounds, it was found that 30% of the binding of [^{3}H]-CGS 19755 was inhibited with high affinity by L-Glu, CPP, L-aspartate and kynurenate, whereas approximately 70% of the binding was inhibited with lower affinity (Table 2).

A number of compounds were found to be weakly active in inhibiting the binding of [^{3}H]-CGS 19755, including kynurenate, L-glutamic acid diethyl ester (L-GDEE) and quinolinic acid (Table 1). The selectivity of the radioligand for the NMDA receptor was evidenced by the weak activity of quisqualate (IC_{50} value 37–48 μM) and the inactivity of AMPA and kainate at concentrations up to 100 μM. The selectivity of [^{3}H]-CGS 19755 for the NMDA receptor was further supported by the finding that the activity of a variety of EAA ligands in inhibiting the binding of [^{3}H]-CGS 19755 correlated well with their ability to inhibit the binding of [^{3}H]-CPP ($r = 0.97$, $P < 0.01$). Other compounds that were without significant activity at a concentration of 100 μM included the dissociative anaesthetics PCP and tiletamine (Table 1), as well as the non-competitive NMDA antagonist MK-801 (Wong *et al.*, 1986).

Discussion

Due to the low affinity of known NMDA receptor ligands, it has been difficult to establish a reliable and rapid filtration assay procedure with a high percentage of specific binding (Foster & Fagg, 1984; Murphy *et al.*, 1987a). Development of the rigid AP7 analogue [^{3}H]-CPP, however, represented an improvement, though partial, over previously available ligands such as [^{3}H]-AP5 or [^{3}H]-L-Glu. The rigid AP5 analogue CGS 19755, which potently

Table 1 Inhibition of [^{3}H]-CGS 19755 binding to rat brain crude synaptic membranes by excitatory amino acid analogues

	Filtration		*Centrifugation*	
	IC_{50} value (μM)	*Hill coefficient*	*IC_{50} value* (μM)	*Hill coefficient*
CGS 19755	0.11 ± 0.002	0.96 ± 0.02	0.14 ± 0.005	0.81 ± 0.02
L-Glu	0.10 ± 0.005	0.80 ± 0.03	0.10 ± 0.003	0.75 ± 0.02*
CPP	0.22 ± 0.007	0.84 ± 0.02	0.15 ± 0.005	0.71 ± 0.02*
D-AP5	0.35 ± 0.015	1.08 ± 0.05	0.40 ± 0.010	0.95 ± 0.02
D-Aspartate	0.8 ± 0.05	0.87 ± 0.05	0.9 ± 0.05	0.92 ± 0.05
DL-AP7	0.9 ± 0.2	0.99 ± 0.02	1.1 ± 0.03	0.91 ± 0.02
L-Aspartate	1.4 ± 0.1	0.91 ± 0.03	1.6 ± 0.8	0.81 ± 0.02*
NMDA	4.1 ± 0.2	0.96 ± 0.04	5.6 ± 0.2	0.87 ± 0.02
D-Glu	8.1 ± 0.3	0.84 ± 0.03	8.6 ± 0.4	0.92 ± 0.04
Quisqualate	48 ± 2	0.91 ± 0.03	37 ± 1	0.87 ± 0.02
Kynurenate	190 ± 7	0.88 ± 0.03	75 ± 2	0.72 ± 0.02*
L-GDEE	680 ± 76	0.65 ± 0.03*	110 ± 57	0.57 ± 0.02*
Quinolinate	510 ± 27	0.87 ± 0.04	300 ± 16	0.80 ± 0.03
Dipicolinate	1400 ± 60	1.23 ± 0.07	1000 ± 37	1.01 ± 0.04

The following compounds were without significant activity at a concentration of 100 μM: AMPA, kainic acid, DL-AP4, MK-801, phencyclidine and tiletamine.
Values represent the mean ± s.e.mean from 3–4 different experiments as determined from computer analysis.
* Significantly different from unity, $P < 0.05$.

inhibited [^{3}H]-CPP binding (K_i = 50 nM; Lehmann *et al.*, 1987c) has been shown in the present study to bind to NMDA-type EAA receptors by both centrifugation and filtration techniques to isolate bound radioactivity.

As indicated, the pharmacology of binding with either technique is very similar. CGS 19755 was found to be equiactive with L-Glu, displaying an IC_{50} value of 110–140 nM (Table 1). CPP is approximately two fold less active with an IC_{50} value of 150–220 nM with D-AP5 and D,L-AP7 being 2 to 4 fold less active than CPP. The non-competitive NMDA antagonists PCP and MK-801 were without effect on [^{3}H]-CGS 19755 binding. The order of activity was therefore CGS 19755 = L-Glu > CPP > D-AP5 > D,L-AP7 = D-aspartate > NMDA > D-Glu ⩾ quisqualate ⩾ AMPA, kainate, PCP, MK-801. Like [^{3}H]-CPP, binding of [^{3}H]-CGS 19755 is selective for NMDA-type EAA receptors, with essentially no activity at kainate or quisqualate receptors.

In comparing the IC_{50} values obtained for CGS 19755 in the [^{3}H]-CPP binding assay and in the [^{3}H]-CGS 19755 centrifugation assay, the values were 50 nM (Lehmann *et al.*, 1987c) and 140 nM, respectively. However, assuming that under centrifugation conditions [^{3}H]-CGS 19755 predominately labels the high affinity component when using a ligand concentration of 10 nM, a K_i value of 48 nM is obtained for CGS 19755 in the [^{3}H]-CPP binding assay, and 66 nM in the centrifugation assay for [^{3}H]-CGS 19755.

Some interesting differences were observed in the binding of [^{3}H]-CGS 19755 depending on whether filtration or centrifugation separation techniques were used to isolate bound radioactivity. In the satu-

Table 2 Analysis of competition experiments resulting in Hill numbers differing from unity

	High affinity site		*Low affinity site*	
	% bound	*IC_{50}* (μM)	*% bound*	*IC_{50}* (μM)
Centrifugation:				
L-Glu	32 ± 6	0.014 ± 0.006	68 ± 6	0.250 ± 0.035
CPP	26 ± 6	0.019 ± 0.01	71 ± 6	0.340 ± 0.047
L-Aspartate	20 ± 3	0.100 ± 0.053	81 ± 4	2.7 ± 0.2
Kynurenate	31 ± 5	2.0 ± 1.1	78 ± 2	153 ± 10
L-GDEE	53 ± 3	11 ± 2	51 ± 3	780 ± 110
Filtration:				
L-GDEE	32 ± 4	34 ± 14	71 ± 4	1700 ± 230

Values represent the mean ± s.e.mean from 3–4 different experiments as determined by computer analysis.

ration experiments, only a single binding component (K_d value = 24 nM) was observed in the filtration assay whereas two binding components (K_d values = 9 and 200 nM) were observed in the centrifugation assay under similar conditions. The apparent B_{max} value for the higher affinity site in the centrifugation assay (0.55 pmol mg^{-1} protein) was similar to that obtained for the single site in filtration assay (B_{max} = 0.74 fmol mg^{-1} protein). It would seem, therefore, that the majority of the lower affinity (K_d = 200 nM) component of [^{3}H]-CGS 19755 binding is lost under filtration conditions and that the remaining 24 nM site is the higher affinity site. The higher K_d and B_{max} values probably reflect the presence of a small proportion of the lower affinity component. That this component is small is indicated by the inability of a two-component model to 'fit' the data.

In comparing the pharmacological profile of [^{3}H]-CGS 19755 binding by the two methodologies (Table 1), there is, in general, a good correlation between the IC_{50} values derived in the two assays even though, as noted, the filtration data may reflect a small component of low affinity binding. However, the compounds kynurenate, L-GDEE and quinolinate were significantly more active in the centrifugation assay. These data may indicate that these compounds have preferential activity at the lower affinity site that is more selectively labelled under centrifugation conditions.

The shallow inhibition curves generated by L-GDEE in the filtration assay, and by L-Glu, CPP, L-aspartate, kynurenate and L-GDEE in the centrifugation assay (Table 1) were found to be best described by a two-component binding model (Table 2). The inhibition curve generated by CGS 19755, however, was not found to be significantly fit by a two-component model, which may reflect the low degree of selectivity of the ligand for the two binding components. Whether the multiple components reflect the existence of multiple NMDA receptor subtypes or multiple states of the NMDA receptor remains to be determined, although the data presented here would appear to favour the existence of multiple states. Under centrifugation conditions, the competition curve for L-GDEE shows that high and low affinity components were present in a ratio of 50:50, whereas the other EAA compounds produced shallow competition curves generated ratios of 20–30:70–80. Since consistent ratios are normally generated when multiple receptor subtypes occur (Williams & Sills, 1989), these data may indicate the existence of multiple states of the NMDA receptor. A similar conclusion was drawn based on differences in EAA receptor agonist and antagonist profiles when autoradiographic techniques were used (Monaghan *et al.*, 1987). Further work on compounds such as L-GDEE, which evidenced multiple binding components in the filtration assay may provide more conclusive evidence for the resolution of these issues that are of considerable concern in relation to elucidating the function of NMDA receptors.

The issue of whether multiple components of the NMDA receptor exist is of particular interest as their presence may aid in the discovery of NMDA antagonists that are not associated with PCP-like dissociative effects. Most currently available NMDA receptor ligands, including both CPP (Liebman *et al.*, 1987; Lehmann *et al.*, 1987b) and CGS 19755 (Lehmann *et al.*, 1987c) have a narrow index between their ability to block NMDA receptors and elicit PCP-like dissociative effects or loss of locomotor coordination. Thus, one of the major focuses of current research in this area is to determine whether, *a priori*, all competitive NMDA-type antagonists elicit PCP-like side effects or whether, in a manner similar to that suggested for the central benzodiazepine receptor complex (Squires, 1984; Williams & Olsen, 1988), receptor subtypes may exist that mediate the different pharmacological effects of NMDA-receptor antagonists.

In summary, CGS 19755 provides an improvement on the technical aspects of the radioligand binding assay for the NMDA receptor by allowing the use of filtration techniques to permit the more rapid evaluation of potentially active NMDA compounds. Further work, however, is necessary to determine whether the two binding components in the centrifugation assay represent multiple states of the NMDA receptor or multiple NMDA receptor subtypes.

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