0026-895X/90/060780-05$02.00/0

MOLECULAR PHARMACOLOGY, 37:780-784

ACCELERATED COMMUNICATION

Differential Modulation of the Associated Glycine Recognition Site by Competitive *N*-Methyl-D-aspartate Receptor Antagonists

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Received January 8, 1990; Accepted March 8, 1990

SUMMARY

The competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonopentanoate and two other five-atom linkage (C-5) ω-phosphono-α-amino acid analogs reduced [^{3}H]glycine binding, in a dose-dependent manner, to a maximum of 45–55%, whereas seven-atom linkage (C-7) analogs had significantly less effect. The IC_{50} of the C-5 antagonists for the inhibition of [^{3}H]glycine binding closely paralleled their potency both in displacing NMDA-selective L-[^{3}H]glutamate binding and in negatively modulating (+)-[^{3}H]5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate ([^{3}H]MK-801) binding. Additionally, reduction of glycine binding by the C-5 antagonists was reversed by both NMDA receptor agonists and C-7 competitive NMDA antagonists, providing evidence that the site of action of these C-5 antagonists is the NMDA recognition site, resulting in indirect modulation of the glycine site. These data imply a functional coupling between the NMDA and associated glycine recognition sites and, furthermore, suggest a differential interaction of C-5 and C-7 competitive NMDA antagonists with the NMDA receptor complex.

The concept of the NMDA receptor as a complex consisting of several interrelated recognition sites has evolved over the past few years. The NMDA recognition site has been pharmacologically characterized in some detail with the availability of specific and potent competitive antagonists (for review, see Refs. 1 and 2). These competitive ω-phosphono-α-amino acid antagonists primarily fall into two structural classes, with a five-atom linkage (C-5 antagonists) or seven-atom linkage (C-7 antagonists). Compounds in each class inhibit both agonist (L-[^{3}H]glutamate) (3) and antagonist ([^{3}H]CPP) (4) binding to the NMDA recognition site with a similar stereospecificity (the D-isomers are more potent than the corresponding L-isomers) and have been shown via functional analyses to be competitive antagonists of the NMDA receptor (2, 5).

Further analysis of these phosphonoamino acid antagonists provides evidence for an indirect modulation of the interactions at the NMDA receptor-coupled neutral amino acid recognition site specifically labeled with [^{3}H]glycine. AP5 has been shown to reduce [^{3}H]glycine binding (6–8) to the NMDA receptor-associated recognition site in an L-glutamate-reversible manner, whereas the C-7 antagonists AP7 and CPP have significantly less effect (8). Subsequent studies have shown that a C-5 antagonist (CGS 19755)-induced reduction in [^{3}H]glycine binding occurs through an alteration in affinity without a change in the number of recognition sites (9). The present study extends these findings to include additional C-5 and C-7 phosphonoamino acid analogs and further reports that structurally restricted C-7 antagonists, while having little effect alone, can reverse the inhibition of [^{3}H]glycine binding induced by C-5 antagonists.

Materials and Methods

Strychnine-insensitive [^{3}H]glycine binding to the NMDA receptor-associated recognition site was performed using Triton X-100-washed SPM prepared from rat forebrain (30–45-day-old male Sprague-Dawley; Sasco, St. Charles MO) as described previously (8). The endogenous levels of glycine and glutamate in these SPM preparations were below detectable levels (<100 nM), as measured by high pressure liquid chromatographic analysis. The assay was initiated by the addition of 0.2–0.4 mg of SPM to an incubation containing 10 nM [^{3}H]glycine (49 Ci/mmol; New England Nuclear, Boston MA) and various concentrations of the appropriate test compounds in a total volume of 1 ml, with all additions made in 50 mM Tris acetate, pH 7.4. Following a 10-min incubation at 2° (conditions sufficient for equilibrium), the bound radioactivity was separated from the free by either centrifugation

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate; AP7, 2-amino-7-phosphonoheptanoate; CGS 19755, (*cis*)-4-phosphonomethyl-2-piperidine carboxylate; SPM, synaptic plasma membranes; MCP, 4-methylphosphono-2-carboxypiperazine; SC 46643, 4-(methylphosphono)phenylglycine; AP5, 2-amino-5-phosphonopentanoate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate.

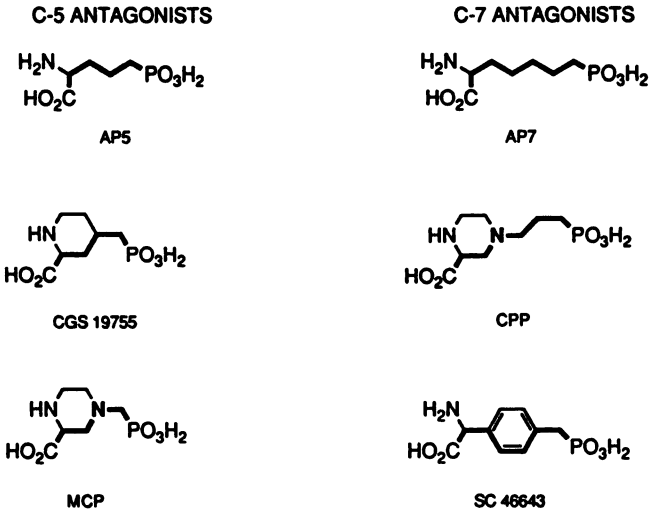


Fig. 1. Structures of C-5 and C-7 competitive NMDA antagonists. *Highlighted linkages* indicate distance, either four (C-5) or six (C-7) atoms, between functional groups.

(12,000 × *g* for 15 min at 4°) or vacuum filtration through Whatman GF/B filters using a Brandel MB-18 harvester. The K_d and B_{max} values for [^{3}H]glycine were similar using either separation technique (K_d = 0.16 ± 0.04 μM; B_{max} = 9.8 ± 1.9 pmol/mg). The radioactivity associated with the SPM was quantitated using liquid scintillation counting. IC_{50} values for the phosphonoamino acid inhibition of [^{3}H]glycine binding were determined using the LIGAND program, with nonspecific binding calculated in the analysis. The IC_{50} is defined as the concentration that causes 50% of the maximal inhibition of [^{3}H]glycine binding observed for each antagonist. Typically, 10–20 μM levels of the C-5 phosphonoamino acids tested inhibited [^{3}H]glycine binding maximally (45–55% inhibition of total specific [^{3}H]glycine binding, which was determined using 100 μM glycine to define nonspecific binding; specific binding represented 80–90% of the total radioligand bound). L-[^{3}H]Glutamate binding to the NMDA recognition site was performed as previously described (3).

Modulation of [^{3}H]MK-801 binding was performed using Triton X-100 (0.04% v/v)-treated rat forebrain SPM, which had been extensively washed as previously described (8). Assay incubations were at 25° for 30 min and contained 5 nM [^{3}H]MK-801, 5 nM L-glutamate, and various concentrations of the phosphonoamino acid antagonists in 50 mM Tris acetate, pH 7.4. Under the conditions utilized in this study, [^{3}H]MK-801 binding does not reach equilibrium. These nonequilibrium conditions allow quantitation of the effects of competitive NMDA receptor antagonists, which have been shown to alter the association rate of ligands interacting with the phencyclidine recognition site (10). The assay was terminated by rapid filtration, using a Brandel MB-48 harvester, through Whatman GF/B filters pretreated with 0.05% polyethylenimine, and the samples were washed four times with 2.0 ml of cold buffer. Radioactivity associated with the filter was determined by liquid scintillation counting, as described above. Nonspecific binding was defined using 60 μM MK-801 and represented 20–30% of the total radioactivity bound in the presence of 5 nM L-glutamate.

MCP was prepared by the reaction of 2-carboxyethylpiperazine with diethylphosphonomethyltriflate in the presence of 1 equivalent of triethylamine. The triester was hydrolyzed by refluxing overnight in 6 N HCl, and the product was purified by ion exchange chromatography. NMR, mass spectral, and elemental analyses were consistent with the structure.

Results

The goal of this study was to investigate functional coupling between the NMDA recognition site and the associated glycine site. In these experiments, three competitive NMDA receptor antagonists (structures shown in Fig. 1) each from the C-7 series [AP7, CPP, and SC 46643 (synthesized at G.D. Searle & Co.)] and the C-5 series [AP5, CGS 19755 (synthesized at G.D. Searle & Co.), and MCP (synthesized at G.D. Searle & Co.)] were initially evaluated for antagonist potency and NMDA recognition site affinity by their ability to displace L-[^{3}H]glutamate binding to the NMDA recognition site and to negatively modulate [^{3}H]MK-801 binding (Table 1). These studies indicate that all six compounds are potent displacers of L-[^{3}H]glutamate binding (K_i values of 0.16–2.3 μM) and negative modulators of [^{3}H]MK-801 binding (IC_{50} values of 0.068–1.16 μM).

The ω-phosphono-α-amino acid analogs were next evaluated for their potency in reducing [^{3}H]glycine binding (IC_{50}) to the NMDA receptor-associated glycine recognition site. At the concentrations tested, the C-5 antagonists were significantly more potent in reducing [^{3}H]glycine binding than were the C-7 antagonists, with respect to both maximal displacement and IC_{50} values. As previously reported, the C-5 antagonists displaced a maximum of 45–55% of the total specific [^{3}H]glycine binding (Table 1). The activity difference noted between the C-5 and C-7 antagonists would not be predicted based upon their potency either to displace L-[^{3}H]glutamate binding to the NMDA recognition site (K_i) or to negatively modulate [^{3}H]MK-801 binding (IC_{50}). However, the potency (IC_{50}) of the C-5 antagonists in reducing [^{3}H]glycine binding does closely

TABLE 1

Comparison of the potency of competitive NMDA antagonists in reducing [^{3}H]glycine binding with their activity at the NMDA receptor

Various concentrations of compounds were incubated with radioligand, as described in the text. Logit-log analysis was used for the determination of K_i values in the L-[^{3}H]glutamate binding assay and IC_{50} values in the [^{3}H]MK-801 binding assay. The IC_{50} values in the [^{3}H]glycine assay were defined as the concentration of compound that produced 50% of the maximal inhibition observed with the phosphonoamino acids and were calculated using the LIGAND program. The percentage of maximal inhibition refers to the maximal inhibition produced by the phosphonoamino acids as a percentage of the total specific [^{3}H]glycine binding (with nonspecific binding defined in the presence of 100 μM glycine). The results are expressed as mean values ± standard errors from at least three separate experiments, each performed in triplicate.

Compound	Linkage	L-[^{3}H]Glutamate, K_i	[^{3}H]Glycine		[^{3}H]MK-801, IC_{50}
			IC_{50}	Maximal inhibition	
		μM	μM	%	μM
D-AP5	C-5	0.42 ± 0.02	0.32 ± 0.05	48 ± 4	0.29 ± 0.03
CGS-19755	C-5	0.16 ± 0.01	0.14 ± 0.03	53 ± 3	0.068 ± 0.003
MCP	C-5	1.9 ± 0.12	0.91 ± 0.12	53 ± 4	1.16 ± 0.09
D-AP7	C-7	1.2 ± 0.2	8.3 ± 1.7	49 ± 2	0.97 ± 0.14
CPP	C-7	0.65 ± 0.12	>10	ND[a]	0.34 ± 0.03
SC 46643	C-7	2.3 ± 0.3	>10	ND	0.76 ± 0.07

[a] ND, not determined.

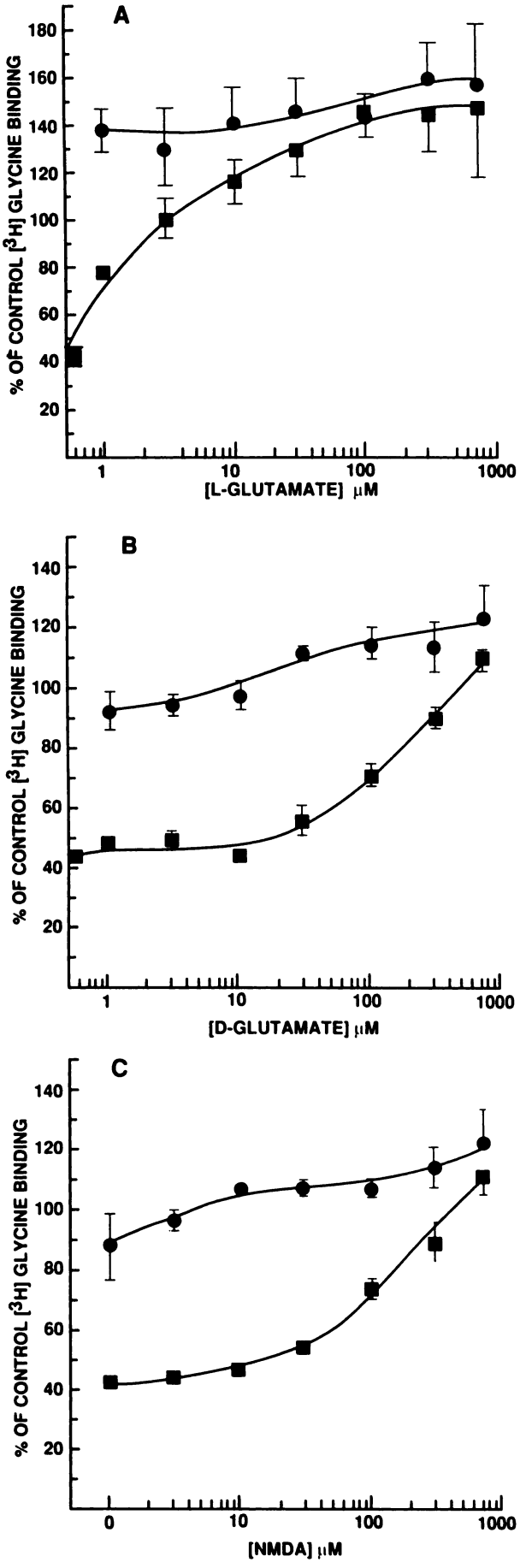


TABLE 2

Glycine modulation of the C-5 and C-7 NMDA antagonist inhibition of [^{3}H]MK-801 binding

Various concentrations of the compounds were incubated with [^{3}H]MK-801, as described in the text. Logit-log analysis was used to determine the IC_{50} values, represented as the mean ± standard error from at least four experiments, each performed in triplicate.

Compound	[^{3}H]MK-801, IC_{50}	
	Control	+3 μM Glycine
	μM	
C-5 antagonists		
D-AP5	0.29 ± 0.03	0.55 ± 0.01[a]
CGS 19755	0.068 ± 0.003	0.118 ± 0.003[b]
MCP	1.16 ± 0.09	1.51 ± 0.07[a]
C-7 antagonists		
D-AP7	0.97 ± 0.14	0.67 ± 0.05
CPP	0.34 ± 0.03	0.29 ± 0.02
SC 46643	0.76 ± 0.07	0.79 ± 0.04

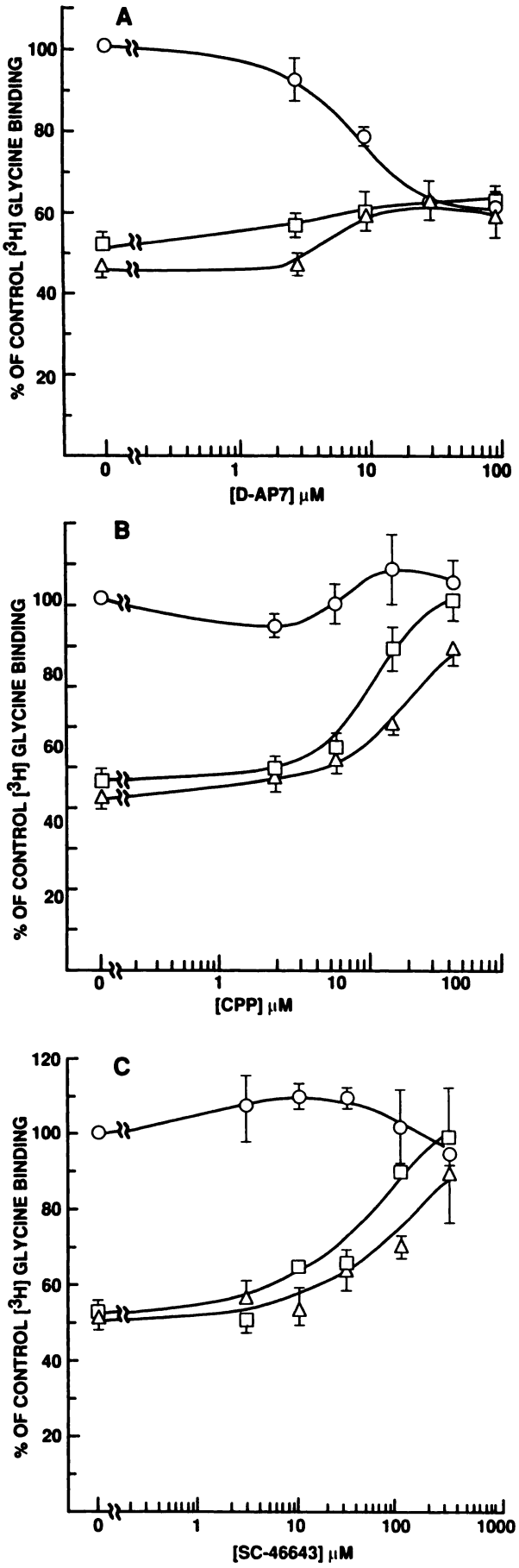
[a] $p < 0.05$.
[b] $p < 0.01$.

parallel their K_i for displacing L-[^{3}H]glutamate binding to the NMDA receptor (Table 1).

The site of action of these C-5 antagonists was addressed in reversal studies using both NMDA recognition site agonists and C-7 competitive antagonists. As shown in Fig. 2, NMDA receptor agonists reversed the inhibition of [^{3}H]glycine binding induced by the competitive antagonist CGS 19755, in a concentration-dependent manner. The relative potencies of these agonists in reversing the effect of CGS 19755 (L-glutamate > NMDA = D-glutamate) paralleled their ability to displace L-[^{3}H]glutamate binding to the NMDA recognition site. As previously observed, these three NMDA agonists also were shown to increase [^{3}H]glycine binding on their own (7) (Fig. 2). Additionally, the more structurally restricted C-7 antagonists, which were inactive (IC_{50} > 10 μM; CPP and SC 46643) at reducing [^{3}H]glycine binding, could also totally reverse the inhibitory action of the C-5 antagonists, 10 μM CGS 19755, D-AP5 (Fig. 3), and MCP (data not shown). Partial reversal was also observed with the more flexible C-7 antagonist D-AP7. A Schild analysis of CPP reversal of CGS 19755 inhibition of [^{3}H]glycine binding resulted in a slope near unity (1.07 ± 0.12; two experiments), indicating that this interaction is competitive in nature (data not shown).

To determine the functional consequences of the differential effect of the C-5 and C-7 antagonists, their potency in inhibiting [^{3}H]MK-801 binding was compared in the presence and absence of added glycine (Table 2). The results from these experiments revealed that the potency of the C-5 but not the C-7 antagonists decreased when glycine (3 μM) was added to the incubation. Thus, the C-5 antagonism of NMDA receptor activity may be augmented through its modulation of interactions at the glycine recognition site. These results are consistent with a close functional relationship between the binding site for C-5 antagonists and the glycine recognition site.

Fig. 2. Reversal of CGS 19755 reduction of [^{3}H]glycine binding by NMDA agonists. Various concentrations of the NMDA receptor agonists L-glutamate (A), D-glutamate (B), and NMDA (C) were incubated with 10 nM [^{3}H]glycine and SPM, as described in the text, either alone (●) or in the presence of 3 μM CGS 19755 (■). Values are the mean ± standard error from at least three experiments, each performed in triplicate.



Discussion

The results of this study indicate that the competitive NMDA antagonists can be classified into two categories, based upon their modulation of [^{3}H]glycine to the NMDA receptor-associated neutral amino acid modulatory site. D-AP5 and the more structurally restricted C-5 analogs (CGS 19755 and MCP) reduce glycine binding to a maximum of 45–55%, whereas the structurally restricted C-7 antagonists (CPP and SC 46643) have little effect and the flexible D-AP7 only inhibits at significantly higher concentrations.

The observation that the C-5 antagonists maximally inhibit 45–55% of the [^{3}H]glycine binding suggests the possibility of [^{3}H]glycine interacting at multiple sites. However, we have previously demonstrated that, under the conditions of this assay, [^{3}H]glycine binding is insensitive to strychnine (8) and is displaced in a monophasic manner by several agonists (8) and antagonists [1-aminocyclobutane-1-carboxylate (11) and 7-chlorokynurenate[1]] of the NMDA receptor-associated glycine binding site. Although the possibility that [^{3}H]glycine labels multiple recognition sites (i.e., NMDA receptor-associated and -nonassociated) in rat forebrain SPM cannot be discounted, there is little evidence in support of this concept.

We hypothesize that this C-5 antagonist-induced alteration in [^{3}H]glycine binding is not via direct action at the glycine recognition site but rather is indirect, through their action at the NMDA recognition site. Several lines of evidence are consistent with this C-5 antagonist-induced reduction in [^{3}H]glycine binding being an indirect phenomenon; these include 1) the lack of complete inhibition of [^{3}H]glycine binding by the C-5 antagonists (maximal 45–55% inhibition), 2) the similar potencies of the C-5 antagonists in both inhibiting L-[^{3}H]glutamate binding and reducing [^{3}H]glycine binding (Table 1), and 3) the reversal by NMDA recognition site agonists of this reduction in [^{3}H]glycine binding (Fig. 2).

The reversal of C-5 antagonist-induced reduction of [^{3}H]glycine binding by C-7 antagonists (CPP and SC 46643) and NMDA agonists implies a competitive interaction between C-5 antagonists, C-7 antagonists, and NMDA recognition site agonists, as has previously been demonstrated (2, 5). If the C-5 and C-7 antagonists interact with identical pharmacophores, it is difficult to explain why only the C-5 compounds negatively modulate [^{3}H]glycine binding. One possible explanation is a partial physical overlap of C-5 and C-7 sites, in which two of the three pharmacophores (α-carboxyl, amino function, and terminal phosphono moieties) are common to the two classes of antagonists whereas the third one is distinct. The possibility, therefore, exists that this third pharmacophore dictates the coupling with the glycine recognition site. Alternatively, the three interaction points may be identical, yet the orientation of the C-5 and C-7 molecules differ, resulting in different coupling with the glycine recognition site.

[1] Unpublished observations.

Fig. 3. Reversal of CGS 19755 and D-AP5 reduction of [^{3}H]glycine binding by C-7 competitive NMDA antagonists. Various concentrations of the competitive NMDA antagonists D-AP7 (A), CPP (B), and SC 46643 (C) were incubated with 10 nM [^{3}H]glycine and SPM, as described in the text, either alone (○) or in the presence of 10 μM CGS 19755 (△) or 10 μM D-AP5 (□). Values are the mean ± standard error from at least three experiments, each performed in triplicate.

It is interesting to note that the more structurally restricted analogs of AP7, although having no inhibitory effect on [^{3}H] glycine binding alone, were able to totally reverse the inhibition induced by the C-5 antagonists CGS 19755, D-AP5, and MCP. The more flexible D-AP7, on the other hand, produced a moderate decrease in [^{3}H]glycine binding alone and partially reversed the inhibition induced by CGS 19755 and D-AP5. These findings support the partial physical overlap hypothesis, with the flexible D-AP7 molecule, in addition to interacting with the C-7 site, being able to interact, albeit inefficiently, with the C-5 site and, thereby, producing an effect on [^{3}H]glycine binding somewhere between that of the rigid CPP and SC 46643 molecules and the shorter C-5 antagonists.

One may further speculate on the functional significance of these differences between C-5 and C-7 antagonists. One could speculate that the C-5 compounds, by virtue of their ability to competitively inhibit binding to the NMDA recognition site along with negatively modulating interactions at the glycine recognition site, would be more efficient antagonists of NMDA receptor function than C-7 compounds. However, careful functional analysis of the NMDA receptor antagonism produced by these two groups of phosphonoamino acid homologs is necessary to determine the physiological significance of these findings.

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