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ANTAGONISM OF EXCITATORY AMINO ACID-INDUCED RESPONSES AND OF SYNAPTIC EXCITATION IN THE ISOLATED SPINAL CORD OF THE FROG

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1 A range of compounds has been tested for excitatory amino acid agonist or antagonist activity and for effects on synaptic activity on isolated hemisected spinal cords of frogs.

2 L-Monoamino dicarboxylic acids of chain length up to 8 carbon atoms (L-α-aminosuberate) were all agonists.

3 Within a series of D-monoamino dicarboxylic acids, and with diamino dicarboxylic acids (mainly unresolved mixtures of diasteroisomers), there was a progression from agonist activity, for compounds of chain length equal to or shorter than glutamate, to antagonist activity, for compounds of longer chain length. D-α-Aminosuberate (DαAS) was the most potent antagonist.

4 The antagonist actions of these substances showed a Mg^{2+}-like selectivity with respect to depolarizations produced by different excitants. N-methyl-D-aspartate (NMDA) was the most susceptible agonist and quisqualate and kainate the least susceptible. Responses to other excitatory amino acids, including L-glutamate and L-aspartate, showed intermediate sensitivity to the antagonists.

5 A parallelism was observed between the relative potencies of mono- and diamino dicarboxylic acids as NMDA antagonists and their relative potencies as depressants of synaptic responses.

6 The results support the concept of different types of excitatory amino acid receptors, with NMDA and its antagonists acting predominantly on one type. These NMDA receptors are probably transmitter receptors activated by an excitatory amino acid transmitter.

Introduction

Excitatory amino acid antagonists are required for studies on synaptic transmission in the vertebrate central nervous system where L-glutamate and L-aspartate, and possibly other acidic amino acids, may function as excitatory transmitters (Curtis & Watkins, 1965; Curtis & Johnston, 1974; Johnson, 1978; Watkins, 1978). Strong evidence for the existence of different types of excitatory amino acid receptors in the mammalian and amphibian central nervous systems was recently provided by the observation that low concentrations of Mg^{2+} can profoundly reduce or abolish responses to some amino acids while having little or no effect on responses produced by other amino acids (Evans, Francis & Watkins, 1977; Davies & Watkins, 1977). Several organic antagonists, including D-α-aminoadipate (DαAA), (±)-α,ε-diaminopimelate, [(±)-α,ε-DAP], and 3-amino-1-hydroxy-2-pyrrolidone (HA-966), also exerted differential antagonist effects on responses of mammalian and amphibian spinal neurones to excitatory amino acids, this selectivity allowing of a similar division of receptors to that suggested by the effects of Mg^{2+}. In particular, N-methyl-D-aspartate (NMDA) appeared to be a selective agonist of one type of receptors that are antagonized both by Mg^{2+} and by the organic substances, and an association between these NMDA receptors and synaptic excitation was suggested by the finding that NMDA antagonists also depressed synaptically-evoked responses of spinal neurones (Biscoe, Davies, Dray, Evans, Francis, Martin & Watkins, 1977a; Biscoe, Evans, Francis, Martin, Watkins, Davies & Dray, 1977b; Biscoe, Davies, Dray, Evans, Martin & Watkins, 1978; Evans, Francis & Watkins, 1978). Since NMDA antagonists had little or no effect on depolarizing responses induced by carbachol, noradrenaline or substance P in the isolated spinal cord of the immature rat (Evans & Watkins, 1978), nor on cholinergic synaptic transmission in the rat isolated superior cervical ganglion (Evans & Watkins, 1978a) or cat spinal cord (Biscoe *et al.*, 1977b), it

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seems likely that NMDA receptors are transmitter receptors activated by an excitatory amino acid transmitter.

The present paper describes the structure-activity relations of mono- and diamino dicarboxylic acids and compares the effects of some of these compounds with those of previously reported excitatory amino acid antagonists on responses of the isolated spinal cord of the frog. Excitatory amino acid receptors in this preparation are similar to those in the mammalian central nervous system (Biscoe, Evans, Headley, Martin & Watkins, 1976). Preliminary accounts of this work have been published (Davies, Evans, Francis & Watkins, 1979a, b).

Methods

Stimulation and recording techniques

Experiments were performed on isolated hemisected spinal cords of frogs (*Rana pipiens* or *R. temporaria*). Since freshly dissected hemicords are often refractory, the preparations were routinely stored at 4°C overnight and used the following day (Evans & Watkins, 1975). Motoneurone responses to dorsal root stimulation (DR-VRPs) were recorded from the corresponding ventral root. Potentials were recorded between an electrode placed in contact with the distal end of the ventral root and another in contact with the proximal end of the root via the superfusion solution. Increase in positivity of the distal electrode, shown by upward deflection on the records, reflects depolarization of motoneurone cell bodies and/or processes evoked by supramaximal dorsal root stimulation or by amino acids added to the superfusion medium.

Solutions

The superfusion medium contained (mM): NaCl 111, KCl 2, $CaCl_2$ 2, Tris 10, glucose 12, adjusted to pH 7.5 with HCl; the temperature was maintained at 12 ± 1°C. This solution was dripped over the tissue at a rate of 1.0 to 1.5 ml/min. Tetrodotoxin (TTX, 10^{-7} M) was also included in the medium where it was desired to minimize indirect actions of perfused substances on motoneurones; spontaneous and electrical activity were usually abolished within 1 h after beginning the flow of this medium.

Monoamino dicarboxylic acids and (±)-2-amino-4-phosphonobutyric acid (2APB) were dissolved in one equivalent of NaOH solution to form concentrated solutions of the mono-sodium salts (pH 7) which were added to the medium to produce the required concentration. Except for some experiments with (±)-α,ε-DAP, when this substance was dissolved directly in the medium, diamino dicarboxylic acids were dissolved in 2 equivalents of NaOH and after adding appropriate volumes of these alkaline (disodium salt) solutions to the medium, the pH of the medium was readjusted to 7.5 with HCl. 3-Amino-1-hydroxy-2-pyrrolidone (HA-966) and L-glutamic acid diethylester (GDEE) hydrochloride were dissolved directly in the medium, pH adjustment (NaOH) being sometimes necessary in the case of GDEE hydrochloride. Excitatory amino acids, dissolved in the control medium, were applied in 2 ml test doses and antagonists were superfused continuously in the medium.

Pharmacological comparisons

All comparisons of agonist and antagonist activity were made in TTX-containing medium.

Agonist potencies were compared by estimating equi-effective concentrations of L-glutamate and the agonists. Initial experiments showed that log dose-response plots for excitant amino acids were parallel for depolarizations within the range 0 to 3 mV. Molar potency ratios (test agonist/L-glutamate) were obtained by matching depolarizations of 0.5 to 2 mV produced by the agonist with depolarizations produced by L-glutamate (contact time 80 s). Such values were obtained from three or more preparations with each agonist. Certain differences were apparent between the values so obtained for some agonists (for example, L-aspartate and D-glutamate) compared with those obtained for the same compounds in an earlier comparison (Biscoe *et al.*, 1976). These differences are probably attributable to the shorter contact time of the amino acids with the tissue in the early investigation (30 s) and to the use, in that study, of procaine (1 mM) as the blocking agent instead of TTX. Procaine has been found to have slight depressant effects on amino acid-induced depolarizations varying in degree with respect to different agonists (Evans, Francis & Watkins, unpublished observations).

Comparisons of antagonist activity were made by two methods. Dose-ratios for antagonism were estimated by matching submaximal depolarizations produced by excitatory amino acids in the presence and absence of antagonists. This method is only suitable for agonists acting on a single type of receptor. Since most of the agonists appeared to act on more than one type of receptor, the susceptibility of the agonists to a particular antagonist, and the differences in potency between different antagonists, were assessed from the relative depressant effects of the antagonists on similar agonist-induced responses. Agonist-induced depolarizations were measured in sequences of 2 to 4 amino acids per hemicord, all responses (1 to 3 mV) being matched to ±5% before addition of the antagonist. Depolarizations produced in the presence of an antagonist were measured 10 to 30

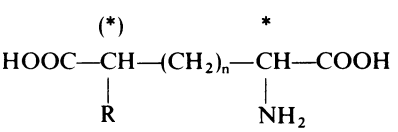
min after introduction of the antagonist, responses being relatively stable within this period. Values obtained in the presence of an antagonist were expressed as a percentage of the values measured in the absence of the antagonist.

Uptake of amino acids in frog spinal cord

Hemicords (15 to 25 mg) were prepared as described for electrophysiological experiments except that the medium also contained 75 μM sucrose. Following equilibration of each hemicord in 1 ml medium at 12°C for 15 min, the hemicords were transferred to fresh medium (1 ml) at 12°C and equilibrated, with gentle agitation, for 15 min. To each tube was then added 30 μl of a solution containing 0.05 μCi [U-^{14}C]-L-glutamate (17 mM) or [U-^{14}C]-L-aspartate (17 mM), 0.25 μCi of [6,6′(n)^{3}H]-sucrose (3 Ci/mmol), and either a potential inhibitor (85 mM) or control medium, the inhibitor solution being added to one hemicord and the solution lacking the inhibitor to the corresponding hemicord (so that each cord always provided both 'control' and 'test' hemicords). After gentle swirling to effect complete mixing, uptake was allowed to proceed at 12°C for 15 min with gentle agitation. The cords were then collected by suction on paper filters (Whatman No. 1), washed with 2 × 1 ml volumes of medium at 12°C and the filter circles and cords transferred to scintillation vials. The tissue was covered with 0.5 ml 1 M hyamine hydroxide in methanol solution and the vials were tightly capped and warmed at 45°C for 18 h. After the addition of 10 ml scintillation fluid (PPO, 0.4%; POPOP, 0.01%, in 7:3 toluene/methoxyethanol) the vials were counted on a Packard 3320 scintillation counter. Tritium counts were used to correct the measured ^{14}C uptake for the estimated amino acid content of extracellular fluid ('sucrose space').

Monoamino and diamino dicarboxylic acids

The main compounds studied have the general formula:



where R = H for the monoamino dicarboxylic acids and R = NH_2 for the diamino analogues. An additional member of the monoamino series is aminomalonic acid (HOOC—CH(NH_2)—COOH), which has the shortest chain, while aspartic acid (n = 0) and glutamic acid (n = 1) are described by the general formula above (R = H), and follow next in the series. Except for aminomalonic acid, which has a centre of symmetry, these substances exist in D and L forms (* = asymmetric carbon atom). Separate isomers were available for compounds of chain length up to n = 4 and DL forms (racemic mixtures) for compounds of chain length up to n = 5. The diamino compounds have an additional asymmetric carbon atom, designated (*) in the general formula, and exist in two optically active forms (DD and LL) and an optically inactive, *meso*, form (D at one end and L at the other end). In theory, the proportions of the three isomers produced by the usual (non-stereospecific) routes of synthesis are 1:1:2 for the DD, LL and *meso*- forms respectively, though these proportions may be altered in recrystallization or other purification procedures involved in their isolation from reaction mixtures. The relative amounts of the three forms in our optically inactive synthetic compounds are unknown. These compounds were available up to n = 6. Separate forms were available only for α,ε-diaminopimelic acid (n = 3).

D-α-*Aminoadipic acid* One molecular equivalent of solid DL-α-aminoadipic acid (Sigma) was dissolved in a warmed (60°C) aqueous solution of D-lysine (1 mmol/ml), the solution was filtered, and 2 volumes of hot methanol were added to the filtrate. After removal of an initial flocculent precipitate, ether was added at room temperature until the point of incipient turbidity, and successive crops of crystals were obtained at room temperature and/or at 4°C, by addition of more ether to filtrates after removal of each crop. Crops with $[\alpha]_D$ − 22° to −25° (c, 0.5–0.7 in 6 N HCl) were predominantly D-lysine-D-α-aminoadipate, and those with $[\alpha]_D$ 0 to −3° were predominantly D-lysine-L-α-aminoadipate. Free D-α-aminoadipic acid was obtained by passage of the appropriate salt, dissolved in 2 M aqueous pyridine, through a column of BioRad AG 50 W, 200–400 mesh pyridinium form (approx. 5 ml resin per 500 mg salt), and washing the column with approximately 4 bed volumes of 2 M aqueous pyridine solution. After evaporation of the pyridine solution the white residue (100 mg) was recrystallized from 2.5 ml hot 2 M pyridine by the addition of 7 ml glacial acetic acid plus 30 ml ethanol, giving a product with $[\alpha]_D$ − 25.1° (c, 0.7 in 6 N HCl). Another sample of D-α-aminoadipic acid ($[\alpha]_D$ − 25.0°) was synthesized by a stereospecific route according to the method of Rudinger & Farkešová (1963).

D-(*and* L-)α-*Aminosuberic acids* DL-α-Aminosuberic acid (Hase, Kiyoi & Sakakibara, 1968) was acetylated and the product treated with acylase I from hog kidney, 1500 units/mg (Sigma) as described by Greenstein & Winitz (1961) for the pimelic acid analogue.

The L-isomer so obtained had $[\alpha]_D$ + 21.7° (c, 0.6 in 6 N HCl). Due to incomplete enzymatic action the residual acetyl derivative, after acid hydrolysis, yielded a product which contained D and L forms in the ratio of about 4:1. D-α-Aminosuberic acid ($[\alpha]_D$ − 22.8°; c, 0.5 in 6 N HCl) was obtained both from this product and from DL-α-aminosuberic acid by treatment with L-amino acid oxidase.

Other unsubstituted monoamino and diamino dicarboxylic acids were obtained as follows: D- and L-α-aminopimelic acids, prepared by resolution of the DL form (Greenstein & Winitz, 1961), DL-α-aminoazelaic acid, synthesized by modification of the method of Hase *et al.* (1968) for DL-α-aminosuberic acid; (±)-α,γ-diaminoglutaric acid, synthesized (Elberson, 1958); (±)-α,δ-diaminoadipic acid, (±)-2,7-diaminosuberic acid, (±)-2,8-diaminoazelaic acid and (±)-2,9-diaminosebacic acid, synthesized (Toi, Mori & Izumi, 1960); DD- and LL-α,ε-diaminopimelic acids, prepared by the methods of Wade, Birnbaum, Winitz, Koegel & Greenstein (1967) and Rhuland, Work, Denman & Hoare (1955); (±)-α,ε-diaminopimelic acid, purchased from the Sigma Chemical Co.

Other substances

The sources of other substances used in this study were as follows: N-methyl-D-aspartic acid and D- and L-homocysteic acids, synthesized (Watkins, 1962); L-glutamic acid diethyl ester hydrochloride, prepared by esterification of L-glutamic acid and freed from traces of unchanged amino acid and monoester contaminants by ion exchange chromatography and recrystallization; 2-amino-4-phosphonobutyric acid purchased from Calbiochem; L-α-aminoadipic acid purchased from Koch-Light; [U-^{14}C]-L-glutamate and [U-^{14}C]-L-aspartate, purchased from the Radiochemical Centre, Amersham; quisqualic acid and HA-966 were gifts from Professors T. Takemoto (Tohoku) and I. Bonta (Rotterdam), respectively.

Results

Agonist activity

All substances were tested for their ability to cause motoneuronal depolarization as recorded from ventral roots and, for compounds showing such action, their potencies relative to L-glutamate were determined. In the monoamino series, L and D compounds showed different profiles of activity with respect to chain length (Figure 1). With the L-isomers highest activity was associated with L-aspartate (L-α-aminosuccinate) and L-glutamate (L-α-aminoglutarate), but a second peak of somewhat weaker activity was associated with L-α-aminopimelate and L-α-aminosuberate. In the D series, there was only a single peak of depolarizing activity associated with D-aspartate and D-glutamate, with D-α-aminoadipate showing very weak activity, and the other two homologues being virtually inactive (potency < 0.02 relative to L-glutamate). In the case of diamino compounds, depolarizing activity was shown only by diaminosuccinic acid (*meso* form) and diaminoglutaric acid (mixture of DD, LL and *meso* forms). This latter substance was approximately 4 times more potent than either L- or D-glutamate, but the relative potencies of the individual diastereoisomers is unknown.

Antagonist activity

It was considered important to test potential antagonists on responses produced by a variety of excitants because of the evidence provided by the differential effects of Mg^{2+} (Evans *et al.*, 1977) that different excitants act on different receptors. The excitants studied included the putative transmitters L-glutamate and L-aspartate and two analogues, kainate and NMDA, which have been proposed to act on 'glutamate-preferring' and 'aspartate-preferring' receptors, respectively (Johnston, Curtis, Davies & McCulloch, 1974; McCulloch, Johnston, Game & Curtis, 1974). The two other excitants routinely used were quisqualate, a potent excitant of unusual structure (Shinozaki & Shibuya, 1974; Biscoe *et al.*, 1976) and L-homocysteate, the ω-sulphonic acid analogue of L-glutamate. Antagonists of excitatory amino acid-induced depolarizations were also tested for their effects on spontaneous synaptic activity and on DR-VRPs.

In comparison of antagonist potency it is important to use an agonist which acts only on receptors sensitive to the antagonist. Preliminary experiments suggested that NMDA is such an agonist (Evans *et al.*, 1978). This is supported by the data of Figure 2 which shows that increasing concentrations of (±)α,ε-DAP produced parallel displacements of the NMDA dose-response plot consistent with the Gaddum–Schild equation (see Barlow, 1964), indicating competition between agonist and antagonist for the same receptors. Dose-ratios for antagonism of NMDA-induced responses by selected antagonists are given in Table 1. Dose-ratios obtained with the other agonists listed in Table 1 are different from those obtained with NMDA, suggesting the involvement of more than one receptor type. The most potent amino acid antagonist was D-α-aminosuberate (DαAS); this substance produced 50% depression of NMDA-induced responses at 22 μM, with a threshold concentration of around 1 μM.

Figure 3 shows the dependence of NMDA antagonist activity on chain length for D-α-amino dicarboxylic acids and for mixtures of diastereoisomers of diamino

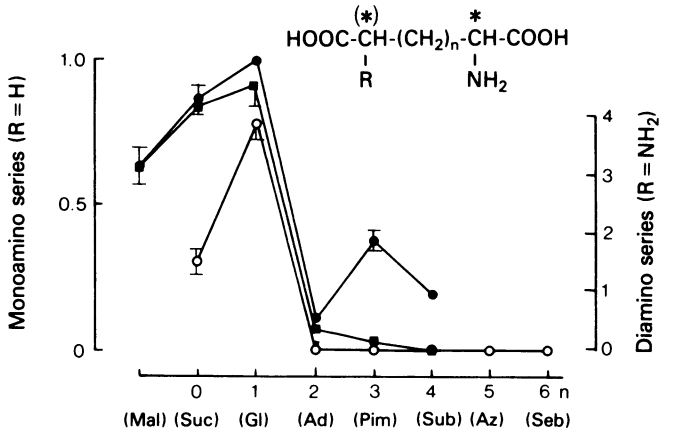


Figure 1 Relative agonist potencies for excitatory amino acid-induced depolarization of frog motoneurones. The abscissa scale gives values of *n* for the general formula shown; the names of the homologous dicarboxylic acids, from which the monoamino (R = H) and diamino (R = NH_2) compounds are derived are given under the corresponding value of *n*—Suc, succinate; Gl, glutarate; Ad, adipate; Pim, pimelate; Sub, suberate; Az, azelaate; Seb, sebacate. Mal represents α-aminomalonate, the structure of which (HOOC—CH(NH_2)—COOH) is not described by the general formula. (●) L-Monoamino; (■) D-monoamino; (○) diamino (DD + LL + *meso*) dicarboxylic acids. For example, for $n = 0$ (Suc), the above symbols represent L-α-aminosuccinate (L-aspartate), D-α-aminosuccinate (D-aspartate) and (±)-α,β-diaminosuccinate, respectively. The ordinates give values of relative potencies (L-glutamate = 1.0); left hand side for the two monoamino series, right hand side for the diamino series. Bars represent s.e. mean (3 to 10 preparations); where no bars are shown they are covered by the symbols.

dicarboxylic acids. Several of these substances also depressed DR-VRPs, and their relative potencies in producing this effect paralleled their relative potencies as antagonists of NMDA-induced responses (Figure 3). An example of the depression of DR-VRPs by DαAA, D-α-aminopimelate (DαAP) and DαAS is shown in Figure 4. DαAS was the most potent depressant while DαAP was less potent than either the shorter or longer chain analogues. The duration of the effect after washout of the substances paralleled chain length, DαAS having the most prolonged action. A dose-response plot for the depressant action of DαAS on DR-VRPs is shown in Figure 5; the threshold concentration of this substance for depression of synaptic activity was less than 5 μM.

Table 2 compares the antagonist effects of various members of the two groups of substances on responses to a range of agonists and on DR-VRPs. The same pattern of depression of agonist responses was observed with all the antagonists of Table 2 except that quisqualate induced responses were enhanced by DαAA as also evident from the data of Table 1.

As also indicated by the results of Tables 1 and 2, the antagonist activity of DL-α-aminoadipate, DL-α-aminopimelate and DL-α-aminosuberate resided mainly in the D forms of these substances; although some depressant activity was also shown at higher concentrations by the L forms, such effects were associated with depolarizing shifts in the baseline ventral root potential. Limited data were also obtained on stereospecificity within the diamino series. When tested on amino acid-induced responses and

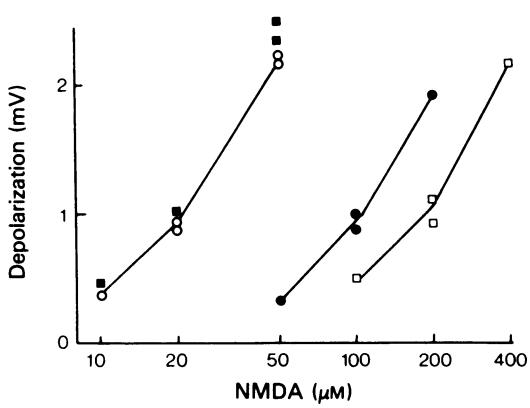


Figure 2 Dose-response curves for motoneurone depolarization produced by N-methyl-D-aspartate (NMDA). (○) Control; (●) and (□) during superfusion with medium containing 0.5 and 1.0 mM (±)-α,ε-diaminopimelate, respectively; (■) 30 min after washout of the antagonist. Abscissa scale, NMDA concentration, μM. Ordinate scale, depolarization, mV. The medium contained 10^{-7} M tetrodotoxin. Agonist applications, 2 min.

DR-VRPs, *meso*-α,ε-DAP (Work, Birnbaum, Winitz & Greenstein, 1955) was approximately equi-active with the mixture of all three diastereoisomers of this substance and also with the racemic mixture of DD and LL forms (Work *et al.*, 1955). Small samples of the pure DD and LL isomers of α,ε-DAP were obtained by paper chromatography of the racemate (Rhuland *et al.*, 1955). The DD form (0.25 mM) was approximately twice as potent as the original racemate while the LL isomer was inactive at the same concentration. Thus, it can be concluded that the antagonist potency of the three forms of α,ε-DAP follow the order: DD > *meso* ≫ LL.

Effects of other amino acid antagonists

It was of interest to compare the antagonist actions of the mono and diamino dicarboxylic acids with those of previously reported excitatory amino acid antagonists, HA-966 (Davies & Watkins, 1973), GDEE (Haldeman, Huffman, Marshall & McLennan, 1972) and DL-2-amino-4-phosphonobutyrate (2APB). The latter substance has been reported to inhibit L-glutamate-induced responses and synaptic excitation in crayfish muscle (Dudel, 1977) and evoked field potentials in rat hippocampal slices (White, Nadler, Hamberger, Cotman & Cummins, 1977).

The effects of these three substances as antagonists of amino acid-induced depolarization of motoneurones are shown in Table 3. The values for HA-966 (0.5 mM), which are partly reproduced from a preliminary communication (Evans *et al.*, 1978), indicate that this substance closely resembles DαAA (Table 1) in its effects. 2APB (1.0 mM) had weaker effects showing the same general pattern of antagonism, with the notable exception that kainate-induced responses were antagonized to approximately the same extent as responses to NMDA. GDEE (2 to 10 mM) had only very weak and somewhat inconsistent effects on TTX-blocked spinal cords, these effects showing no clear selectivity (Table 3), while in unblocked preparations the weak effects of these high concentrations of GDEE seemed again generally to resemble those of low concentrations of mono and diamino dicarboxylic acids. In contrast to HA-966 and GDEE, neither of which caused marked changes in the level of ventral root polarization, 2APB had a slight but definite depolarizing action (potency approximately 0.05 that of L-glutamate) in accordance with observations in the mammalian central nervous system (Curtis & Watkins, 1965).

HA-966 (0.5 mM) had a pronounced depressant effect on spontaneous root potentials and on DR-VRPs (Table 3) which was considerably more prolonged than that produced by the longer chain mono- and diamino dicarboxylic acids, and the magnitude of this depression appeared to be somewhat greater than that which would have been expected solely on the basis of the potency of this substance as an NMDA antagonist relative to that of the mono- and diamino dicarboxylic acids. The effects of 2APB on synaptic activity (Table 3) more closely resembled those of the longer chain mono- and diamino dicarboxylic acids; however the depolarization often accompanying the action of 2APB at concentrations above 0.5 mM was a complicating factor in this case. GDEE was a relatively weak depressant of synaptic activity, concentrations above 2 mM being required to cause measurable effects on synaptic activity. Also such effects were prolonged and usually incompletely reversible.

Table 1 Dose-ratios for antagonism of amino acid-induced depolarizations of frog motoneurones

	*Dose-ratio**				
	α-Aminosuberate		*α-Aminoadipate*		(±)-α,ε-*DAP*
	D *form*	DL *form*	D *form*	DL *form*	
Agonist	(*0.25 mM*)	(*0.5 mM*)	(*0.25 mM*)	(*0.5 mM*)	(*1 mM*)
NMDA	16.7 ± 1.2 (4)	14.9 ± 1.1 (3)	6.9 ± 0.1 (4)		9.3 ± 0.4 (5)
L-Homocysteate		9.3 ± 0.7 (3)	6.8 ± 0.1 (4)	5.5	
L-Aspartate	1.5 ± 0.1 (3)	1.3 ± 0.1 (3)	1.5 ± 0.1 (3)	1.35	
L-Glutamate	1.2 ± 0.1 (3)	1.3 ± 0.1 (3)	1.3 ± 0.2 (4)	1.25	
Kainate	1.5 ± 0.2 (3)	1.3 ± 0.1 (3)	1.3 ± 0.1 (4)		
Quisqualate		1.0 ± 0.1 (3)	0.67 ± 0.05 (4)		

NMDA, N-methyl-D-aspartate.

* Ratio of concentration of agonist in the presence of the antagonist to the concentration of the agonist in the absence of the antagonist required to produce the same motoneurone depolarization (approx. 2 mV) in TTX-containing medium.

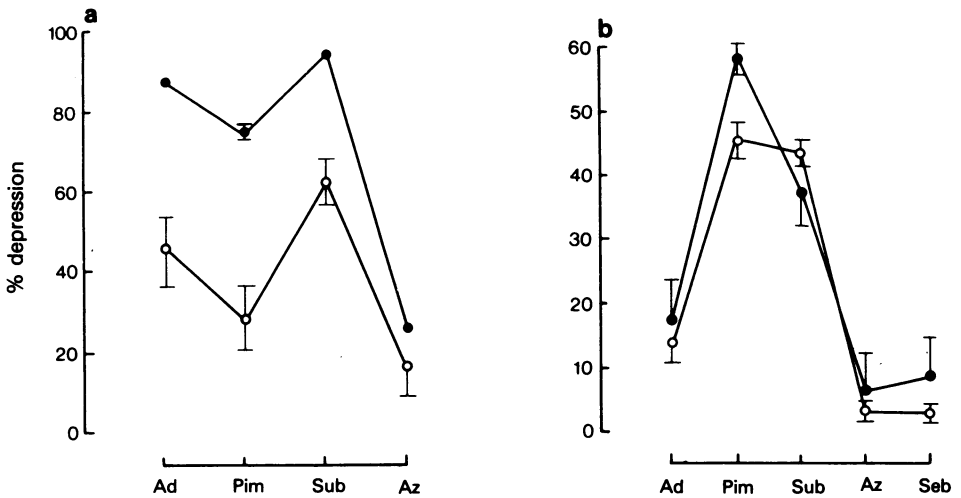


Figure 3 Comparison of depression of N-methyl-D-aspartate (NMDA)-induced and synaptically-evoked motoneurone depolarization by monoamino and diamino dicarboxylic acids. (a) Monoamino series: Ad, D-α-aminoadipate; Pim, D-α-aminopimelate; Sub, D-α-aminosuberate (all 0.25 mM); Az, DL-α-aminoazelaate (0.5 mM). (b) Corresponding diamino compounds (mixtures of diastereoisomers), 0.25 mM for NMDA-induced and 0.5 mM for synaptically evoked responses. (●) NMDA-induced depolarizations; (○) motoneurone responses to dorsal root stimulation. Bars represent s.e. mean (3 preparations); where no bars shown, they are smaller than the symbols.

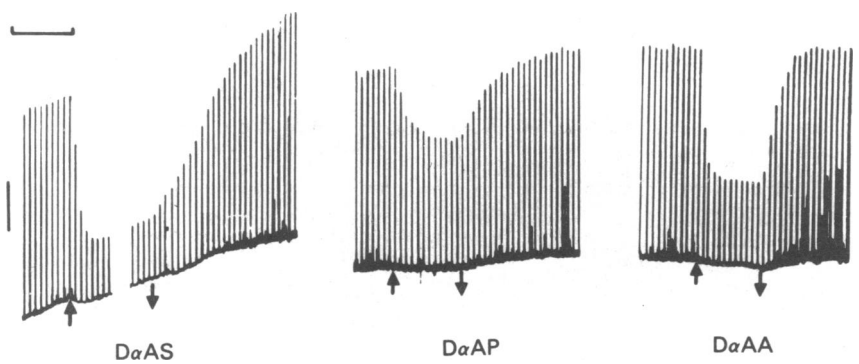


Figure 4 Depression of synaptic responses in the same preparation by D-α-aminosuberate (DαAS), D-α-aminopimelate (DαAP) and D-α-aminoadipate (DαAA). The dorsal root was stimulated at a rate of 1 pulse per min. Antagonists (all 0.25 mM) were applied during the periods indicated by the arrows. The gap in the DαAS record represents 13 min. Calibration: vertical, 1 mV; horizontal: 10 min.

Sensitivity of responses produced by other excitatory amino acids to NMDA antagonists

It is likely that the depression of synaptic activity produced by the antagonists listed in Tables 1 to 3 reflects the antagonism of a transmitter which acts on NMDA receptors. Thus, only agonists that produce responses sensitive to these antagonists are likely to be transmitters at sites of antagonist-sensitive synaptic excitation. In addition to L-glutamate and L-aspartate, several sulphur-containing amino acids must be considered as possible excitatory transmitters in the vertebrate CNS; these include L-cysteate, L-cysteine sulphinate, L-homocysteine sulphinate and S-sulphocysteine as well as L-homocysteate (Watkins, 1978). Table 4A shows the effects of DαAA and (±)-α,ε-DAP on responses induced by these amino acids. Responses to L-homocysteate were the most

susceptible to these antagonists, while responses to L-homocysteine sulphinate were the next most sensitive. The effects of DαAA and (±)-α,ε-DAP on responses to the other sulphur-containing amino acids were similar to each other and roughly comparable in magnitude to the effects of the antagonists on responses to L-aspartate (Table 2).

A further important question relates to whether the relative sensitivities to NMDA antagonists of responses induced by different excitants can be correlated with 'glutamate-like' or 'aspartate-like' structural features. If the hypothesis of Johnston *et al.* (1974) is correct, that kainate acts preferentially on receptors that have a high affinity for substances with a spatial separation of anionic groups similar to that between the carboxylate terminals of L-glutamate in an extended conformation of that molecule, then ibotenate should have a high affinity for such receptors. The separation of the anionic moieties in the molecules of this compound, the conformation of which is relatively fixed due to the restraint imposed by the ring system, is similar to that maximally occurring in the flexible L-glutamate molecule (Johnston *et al.*, 1974; Buu, Puil & Van Gelder, 1976). Also D-glutamate, though conformationally variable, might be expected to have a greater affinity for such receptors than D-aspartate, since the maximum intercarboxyl distance is smaller in the latter than in the former

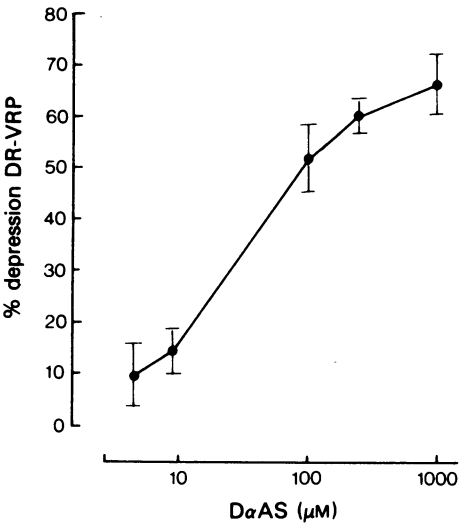


Figure 5 Antagonism of synaptically evoked motoneurone depolarization by D-α-aminosuberate (DαAS). Ordinate scale, percent depression of control motoneurone response to dorsal root stimulation (DR-VRP) measured 20 min after introduction of medium containing DαAS. Abscissa scale, DαAS concentration, μM, log scale. Bars represent s.e. mean (2 to 4 preparations).

Table 2 Depression of amino acid-induced and synaptically evoked frog motoneurone depolarizations by mono- and diamino dicarboxylic acids

	% control motoneuronal depolarization in presence of						
	*DαAA** (0.25 mM)	*DLαAA** (0.5 mM)	*DαAP* (0.25 mM)	*DLαAP* (0.25 mM)	*DαAS* (0.25 mM)	*DLαAS* (0.5 mM)	*(±)α,ε-DAP** (0.5 mM)
Agonist							
NMDA	12 ± 1 (4)	11 ± 2 (4)	25 ± 2 (3)	45 ± 1 (3)	6 ± 1 (3)	4 ± 2 (3)	14 ± 1 (4)
L-HCA	26 ± 4 (5)	25 ± 2 (5)	47	—	23 ± 1 (3)	22 ± 3 (4)	34 ± 3 (4)
L-Asp	72 ± 3 (4)	72 ± 3 (6)	74, 84	—	81 ± 2 (3)	66 ± 6 (3)	71 ± 3 (4)
L-Glu	83 ± 2 (4)	82 ± 2 (6)	86, 90	—	87 ± 4 (3)	79 ± 4 (3)	83 ± 3 (4)
KA	82 ± 3 (4)	84 ± 3 (3)	99, 95	—	75 ± 5 (3)	82 ± 2 (4)	98 ± 2 (4)
QA	128 ± 6 (4)	—	—	—	90 ± 2 (3)	99 ± 1 (3)	101 ± 4 (4)
Synaptic							
DR-VRP	55 ± 9 (5)	53 ± 4 (4)	72 ± 8 (4)	74	37 ± 5 (4)	35 ± 9 (3)	52 ± 9 (5)

The values given are the magnitudes of the depolarizations measured in TTX-containing medium in the presence of an antagonist expressed as a percentage (mean ± s.e. mean) of matched control responses measured in the absence of the antagonist. The number of experiments is given in parentheses; where this is less than 3, the individual values are shown. *Values for amino acid-induced responses previously reported (Evans *et al.*, 1978). Depression of synaptic responses, measured in TTX-free medium, was estimated 10 to 20 min after addition of the antagonist, during which period a new plateau level had usually been reached. Abbreviations: NMDA, N-methyl-D-aspartate; L-HCA, L-homocysteate; L-Asp, L-aspartate; L-Glu, L-glutamate; KA, kainate; QA, quisqualate; DR-VRP, ventral root potentials evoked by supramaximal dorsal root stimulation (1/min); DαAA and DLαAA, D- and DL-α-aminoadipate; DαAP and DLαAP, D- and DL-α-aminopimelate; DαAS and DLαAS, D- and DL-α-aminosuberate, (±)-α,ε-DAP, mixture of DD, LL and *meso* forms of α,ε-diaminopimelic acid.

molecule. Table 4B shows the effects of DαAA and (±)-α,ε-DAP on responses to L-aspartate, D-aspartate, D-glutamate and ibotenate. Contrary to prediction from the above structural arguments, responses to the latter two excitants were considerably more sensitive to the antagonists than were responses to the former two excitants.

Effects of amino acid agonists and antagonists on uptake of [U-^{14}C]-L-glutamate and [U-^{14}C]-L-aspartate in frog spinal cord

Differential effects of antagonists on response to excitatory amino acids might arise not only by differential effects on the receptor level but also through varying degrees of inhibition by the antagonist of the uptake of agonists into the tissue. Although this latter mechanism probably does not apply in the case of the selective depression of NMDA-induced compared with kainate-induced responses, since neither agonist is likely to be actively accumulated (Balcar & Johnston, 1972; Johnston, Kennedy & Twitchin, 1979), nevertheless, the lesser depression of L-glutamate- and L-aspartate-induced than of NMDA-induced responses by the same antagonists could conceivably reflect such effects on transport.

To test whether any of the NMDA antagonists inhibited the uptake of [U-^{14}C]-L-glutamate (or, in

Table 3 Depression of amino acid-induced motoneurone depolarizations and ventral root potentials evoked by supramaximal dorsal root stimulation (DR-VRPs) by previously reported amino acid antagonists

	% control depolarization		
	*HA-966** (*0.5 mM*)	*2-APB* (*1.0 mM*)	*GDEE* (*5 mM*)
Agonist			
NMDA	13 ± 2 (3)	57 ± 3 (4)	93, 89
L-HCA	25 ± 4 (4)	63 ± 5 (3)	—
L-Asp	55 ± 4 (3)	91 ± 2 (3)	92, 100
L-Glu	67 ± 3 (3)	95 ± 3 (3)	100, 100
KA	83 ± 5 (3)	50 ± 3 (4)	100, 100
QA	115 ± 11 (4)	89 ± 4 (3)	—
Synaptic			
DR-VRP	24 ± 4 (3)	49 ± 6 (3)†	58^{+}

HA-966, 3-amino-1-hydroxy-2-pyrrolidone; 2APB, DL-2-amino-4-phosphonobutyrate; GDEE, L-glutamic acid diethyl ester. For other details see legend to Table 2.
*Values for amino acid-induced responses from Evans *et al.* (1978); †associated with ventral root depolarization; $^{+}$slow and only partial recovery.

Table 4 Depression by N-methyl-D-aspartate (NMDA)-antagonists of motoneurone depolarizations induced by other excitatory amino acids

		% control depolarization	
		DαAA (*0.25 mM*)	*(±)-α,ε-DAP* (*0.5 mM*)
	Agonist		
(A)	L-Homocysteate	29 ± 4 (5)	48 ± 1 (3)
	L-Homocysteine sulphinate	39 ± 7 (3)	56 ± 2 (3)
	S-Sulpho-L-cysteine	63 ± 7 (3)	66 ± 2 (3)
	L-Cysteate	59 ± 4 (3)	74 ± 6 (3)
	L-Cysteine sulphinate	60 ± 5 (3)	77 ± 6 (3)
(B)	(±)-Ibotenate	27 ± 8 (3)	29 ± 1 (3)
	D-Glutamate	29 ± 2 (3)	54 ± 4 (3)
	D-Aspartate	55 ± 5 (3)	81 ± 5 (3)
	L-Aspartate	64 ± 8 (3)	85 ± 3 (3)

For details see legend to Table 2.

some experiments, [U-^{14}C]-L-aspartate), the uptake of the radiolabelled amino acids was measured in the presence and absence of the antagonists. These uptake experiments were conducted on intact hemicords prepared in a similar way to those used for electrophysiological experiments. Under such conditions labelled L-glutamate and L-aspartate are accumulated by two saturable transport systems (Oakes, Please & Watkins, unpublished observations) similar to the 'high affinity' and 'low affinity' systems described for frog spinal cord slices (Davidoff & Adair, 1975). The ratio of the concentrations of antagonists to L-glutamate or L-aspartate in electrophysiological studies was usually in the range of only 0.25 to 0.5; however, Table 5 indicates that even with a 5 fold excess of antagonist over agonist, DαAA, DL-α-aminosuberate, (±)-α,ε-DAP, 2APB and HA-966 were without significant effect on the uptake of [U-^{14}C]-L-glutamate or [U-^{14}C]-L-aspartate. On the other hand DL-α-aminoadipate did inhibit the uptake of [U-^{14}C]-L-glutamate under these conditions, as did the agonist, L-α-aminoadipate. Longer chain agonists (L-α-aminopimelate and L-α-aminosuberate) did not significantly inhibit the uptake of [U-^{14}C]-L-glutamate.

Discussion

The study of structure-activity relations of mono- and diamino dicarboxylic acids has revealed new compounds with excitatory amino acid agonist or antagonist actions. The most effective of the new agonists was (±)-α,ε-diaminoglutarate, but it is not known how this agonist activity is distributed among the DD, LL and *meso* stereoisomers of this substance. Moderate agonist activity was also observed with L-α-aminopimelate and L-α-aminosuberate. Uptake of L-α-aminoadipate on the L-glutamate transport carrier, as shown by the inhibition of [U-^{14}C]-L-glutamate uptake by the adipate analogue, may have contributed to the weaker agonist activity of this latter compound compared with the pimelate and suberate analogues.

The antagonism produced by certain longer chain α-amino dicarboxylic acids was confined principally to the D-isomers. Although the results for the diamino series of compounds were obtained mainly with mixtures of isomers, studies with small amounts of the three separate isomers of α,ε-diaminopimelic acid suggested that the antagonism produced by members of this series likewise requires the D configuration at one

Table 5 Effects of antagonists and agonists on uptake of [U-^{14}C]-L-glutamate and [U-^{14}C]-L-aspartate on frog spinal cord

Test inhibitor (2.5 mM)	*Uptake substrate (0.5 mM)*	*Uptake (nmol g^{-1} 15 min^{-1}) Control*	*Test*	*% control*
Antagonists				
DαAA	[U-^{14}C]-L-Glu	505 ± 44 (5)	482 ± 40 (50)	95
	[U-^{14}C]-L-Asp	435 ± 26 (5)	424 ± 43 (5)	98
DLαAA	[U-^{14}C]-L-Glu	641 ± 52 (10)	511 ± 46 (10)	80**
DLαAS	[U-^{14}C]-L-Glu	594 ± 43 (5)	554 ± 78 (5)	93
(±)α,ε-DAP	[U-^{14}C]-L-Glu	561 ± 34 (5)	536 ± 59 (5)	95
2APB	[U-^{14}C]-L-Glu	508 ± 38 (10)	468 ± 26 (10)	92
HA-966	[U-^{14}C]-L-Glu	478 ± 46 (5)	482 ± 50 (5)	101
Agonists				
LαAA	[U-^{14}C]-L-Glu	550 ± 18 (9)	422 ± 15 (9)	77***
	[U-^{14}C]-L-Asp	384 ± 32 (10)	324 ± 21 (10)	84*
LαAP	[U-^{14}C]-L-Glu	495 ± 12 (5)	434 ± 33 (5)	88
LαAS	[U-^{14}C]-L-Glu	473 ± 34 (4)	433 ± 29 (4)	94
L-Asp	[U-^{14}C]-L-Glu	579 ± 38 (5)	142 ± 14 (5)	24***
L-Glu	[U-^{14}C]-L-Glu	576 ± 32 (5)	186 ± 9 (5)	32***†

Details of the method of comparing the uptake into paired hemicords of [U-^{14}C]-L-glutamate or [U-^{14}C]-L-aspartate in the absence and presence of possible inhibitors are given in the Methods section. LαAA, L-α-aminoadipate; LαAP, L-α-aminopimelate; LαAS, L-α-aminosuberate; for other abbreviations, see Tables 1–3. The values given are means ± s.e. mean for the number of paired hemicords given in parentheses. Significance of difference from control values (paired *t* test): *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$. †These values ignore the increase in substrate concentration produced by the addition of the 'inhibitor', L-glutamate.

end (and optimally at both ends) of the molecules. For the D series of monoamino compounds, and for the unresolved diamino compounds, a progression from agonist to antagonist activity occurs as chain length increases beyond that present in the molecule of glutamic acid. The most potent antagonists of either series were D- and DL-α-aminosuberate, which were active in μM concentrations. The duration of action after washout of the substances paralleled chain length, suggesting that lipophilicity was a major factor in determining the recovery period, as previously observed with NMDA agonists (Biscoe *et al.*, 1976). All of these antagonists exhibited a Mg^{2+}-like pattern of antagonism (Evans *et al.*, 1977) when tested against the responses produced by a range of excitants. This pattern is represented by the following decreasing order of agonist susceptibility: NMDA, L-homocysteate, L-aspartate, L-glutamate, kainate and quisqualate. Ibotenate and D-glutamate appeared to resemble L-homocysteate in susceptibility, while L-homocysteine sulphinate, L-cysteate, L-cysteine sulphinate and D-aspartate lay between L-homocysteate and kainate.

Preliminary studies suggest that Mg^{2+} and the organic antagonists act at different sites and/or by different mechanisms (Evans & Watkins, 1978b; Davies *et al.*, 1979a, b). That two such dissimilar types of antagonist should exhibit a similar spectrum of action strongly supports the existence of at least two types of excitatory amino acid receptor, one type (NMDA receptors) activated preferentially by NMDA and blocked by both classes of antagonists, and one or more other types of receptors that are relatively insensitive to both NMDA and NMDA antagonists. On this basis, quisqualate and kainate would be assumed to act predominantly on receptors of the latter type and L-homocysteate, D-glutamate and ibotenate, predominantly on receptors of the former type, with other amino acids having actions both on NMDA receptors and other receptors in varying proportion. On the same basis, a slightly greater proportion of the action of (exogeneous) L-aspartate than of (exogenous) L-glutamate would be presumed to be mediated by NMDA receptors.

Of other reported amino acid antagonists, HA-966 resembled the NMDA antagonists of the present work, while GDEE was relatively ineffective as an amino acid antagonist. 2APB, which was only available as the racemate, produced a unique pattern of antagonism characterized by moderate depressant actions on responses to both NMDA and kainate, with less effect on responses to quisqualate, L-glutamate, L-aspartate and L-homocysteate. Interpretation of this unusual action of 2APB must await an investigation of the effects of the separate D and L forms of the substance.

DαAA and HA-966 actually enhanced quisqualate-induced depolarizations. It is possible that these effects were due to inhibition of uptake of quisqualate by the two NMDA antagonists, though transport processes for quisqualate have not yet been studied. In other experiments (Watkins, Evans, Headley, Cox, Francis & Oakes, 1978 and unpublished results), we have shown that L-glutamate- and L-aspartate-induced responses are enhanced by agents and conditions that inhibit the uptake of these amino acids into frog spinal cord. However, the possibility that similar effects on amino acid transport masked the effects of NMDA antagonists on L-glutamate or L-aspartate-induced responses, with antagonism of the responses due to receptor blockade being offset by potentiation of the response due to inhibition of uptake, is unlikely. Except for DL-α-aminoadipate, where the effect could largely be accounted for by the action of the L form, none of the NMDA antagonists tested significantly inhibited the uptake into the tissue of radiolabelled L-glutamate or L-aspartate.

The location and function of the different excitatory amino acid receptors revealed by the action of NMDA antagonists requires discussion. The possibility that some kainate receptors are not involved in synaptic function arises from the finding that receptors highly sensitive to kainate and L-glutamate, but considerably less sensitive to L-aspartate, are present in isolated dorsal roots of the immature rat (Davies *et al.*, 1979b). On the other hand, NMDA receptors do appear to be important in excitatory synaptic function. Thus, in the present work all NMDA antagonists depressed synaptic activity and a parallelism was observed between the relative potencies of mono- and diamino-dicarboxylic acids as NMDA antagonists and the relative potencies of the same substances as depressants of ventral root potentials evoked by dorsal root stimulation.

In view of the specificity previously reported for these antagonists with respect to their effects on various chemically and synaptically evoked responses (Biscoe *et al.*, 1977a, 1978; Biscoe *et al.*, 1977b; Evans *et al.*, 1978; Evans & Watkins, 1978a; Davies *et al.*, 1979a) it seems likely that NMDA receptors are associated specifically with sites of acidic amino acid-mediated synaptic excitation. The possibility that the transmitter operating at these synapses is L-aspartate, as discussed in our earlier papers, is supported by the finding that DL-α-aminoadipate blocks both L-aspartate-induced responses and the synaptic activation of carp retinal neurones. These cells are far less sensitive to L-glutamate than to L-aspartate (Wu & Dowling, 1978). Nevertheless, none of the endogenous acidic amino acids that have actions sensitive to NMDA antagonists, including several sulphur-containing amino acids in addition to L-glutamate and L-aspartate, can be ruled out in the case of the frog spinal cord.

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