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SELECTIVE ANTAGONISM OF AMINO ACID-INDUCED AND SYNAPTIC EXCITATION IN THE CAT SPINAL CORD

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SUMMARY

1. The effects of D-α-aminoadipate (DαAA), D-α-aminosuberate (DαAS) and other excitatory amino acid antagonists have been compared on the excitatory responses of neurones of the cat spinal cord to acetylcholine, a range of glutamate-related amino acids and stimulation of appropriate excitatory synaptic pathways. The ionophoretic technique was used for administration of excitants and antagonists.

2. DαAA and DαAS had little or no effect on acetylcholine-induced excitation of Renshaw cells. Responses of either Renshaw cells or dorsal horn neurones in the spinal cord to excitatory amino acids were depressed in the order: N-methyl-D-aspartate (NMDA), L-homocysteate, D-glutamate, ibotenate > D-homocysteate, L-aspartate, D-aspartate > L-glutamate, kainate and quisqualate.

3. These effects are consistent with the existence of different excitatory amino acid receptors, one type being sensitive to the actions of the antagonists, and activated predominantly by the NMDA group of excitants, with other receptors being relatively insensitive to DαAA and DαAS and activated predominantly by quisqualate and kainate. On this hypothesis, many amino acids are assumed to have mixed actions on DαAA-sensitive and -insensitive receptors.

4. 2-Amino-4-phosphonobutyrate (2APB) and L-glutamic acid diethyl ester (GDEE) produced different patterns of antagonism of excitatory amino acid-induced responses from those observed with DαAA and DαAS. Neither substance was as potent as DαAA or DαAS as an excitatory amino acid antagonist.

5. Both DαAA and DαAS selectively antagonized synaptic excitation of Renshaw cells evoked by dorsal root stimulation without affecting cholinergic excitation of these cells evoked by ventral root stimulation. These latter responses were selectively antagonized by dihydro-β-erythroidine (DHβE). DαAA also antagonized synaptic excitation of unidentified dorsal horn neurones of the spinal cord evoked by dorsal root stimulation. Neither GDEE (particularly) nor 2APB were as effective as DαAA or DαAS as depressants of synaptic excitation.

6. Taken in conjunction with the results of *in vitro* studies on the specificity of action of DαAA and related substances, these observations suggest that certain synaptic excitations in the spinal cord are mediated by an excitatory amino acid transmitter, and that this transmitter interacts with receptors which are activated selectively by NMDA, less selectively by other amino acids, including L-aspartate, and probably only slightly by quisqualate, kainate and (exogenous) L-glutamate.

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INTRODUCTION

Investigation of the possibility that excitatory amino acids function as synaptic transmitters in the mammalian central nervous system has long been handicapped by the lack of suitable antagonists for either amino acid-induced or synaptic excitation. Among the various substances that have been proposed as antagonists of excitatory amino acids on mammalian neurones are L-glutamic acid diethyl ester (GDEE) (Haldeman, Huffman, Marshall & McLennan, 1972) and 1-hydroxy-3-aminopyrrolidone-2 (HA-966) (Davies & Watkins, 1973) but the specificity of these compounds has been questioned (Curtis, Duggan, Felix, Johnston, Tebēcis & Watkins, 1972; Curtis, Johnston, Game & McCulloch, 1973; Zieglgänsberger & Puil, 1973; Altmann, Ten Bruggencate, Pickelmann & Steinberg, 1976). More recently it has been reported that certain longer chain mono- and diamino dicarboxylic acids have the ability to antagonize the excitatory actions of amino acids in the mammalian and amphibian central nervous system (Biscoe, Davies, Dray, Evans, Francis, Martin & Watkins, 1977*a*; Hall, McLennan & Wheal, 1977). These substances have little or no action on excitatory responses produced by acetylcholine, substance P or noradrenaline (Biscoe *et al.* 1977*a*; Biscoe, Evans, Francis, Martin, Watkins, Davies & Dray, 1977*b*; Biscoe, Davies, Dray, Evans, Martin & Watkins, 1978; McLennan & Hall, 1978; Evans & Watkins, 1978; Collingridge & Davies, 1979), whilst being highly selective with respect to their effects on responses produced by different excitatory amino acids. For instance, D-α-aminoadipate and related compounds have been shown to depress responses to L-aspartate more than responses to L-glutamate. The responses to the aspartate analogue, N-methyl-D-aspartate (NMDA), were extremely sensitive to these agents, whereas responses to the glutamate analogue, kainate, were relatively insensitive (Biscoe *et al.* 1977*a*, *b*, 1978; Evans, Francis & Watkins, 1978; Evans & Watkins, 1978). These substances are also selective antagonists of synaptic excitation, depressing dorsal root-evoked responses of Renshaw cells and dorsal horn neurones in the spinal cord of the mouse and cat, whilst having no effect on the cholinergic excitation of Renshaw cells evoked by ventral root stimulation (Biscoe *et al.* 1977*b*, 1978) or on cholinergic transmission in the rat isolated superior cervical ganglion (Evans & Watkins, 1978). Such results not only provide strong evidence for amino acid-mediated synaptic excitation, but also indicate the existence of different types of excitatory amino acid receptors.

The present paper describes the effects of two of these selective antagonists, D-α-aminoadipate and D-α-aminosuberate, on amino acid-induced and synaptic excitation in the cat spinal cord. In addition, the effects of these substances have been compared with those of two other antagonists of the actions of excitatory amino acids, L-glutamic acid diethyl ester (GDEE) and DL-2-amino-4-phosphonobutyrate (2APB). This latter substance has recently been proposed as an effective antagonist of the action of L-glutamate in crayfish muscle (Dudel, 1977) and in the hippocampus of the rat (White, Nadler, Hamberger, Cotman & Cummins, 1977).

Some of these results have been reported in preliminary form (Davies, Evans, Francis & Watkins, 1979*a*, *b*).

METHODS

Experiments were performed on cats anaesthetized with pentobarbitone sodium (35 mg/kg I.P. initially, supplemented when necessary by 5 mg/kg I.V.). Arterial blood pressure was monitored continuously via a carotid cannula and experiments were terminated if the systolic blood pressure fell below 90 mmHg. Body temperature was maintained at 37 °C by means of a thermostatically controlled heating blanket.

The spinal cord was exposed by lumbar laminectomy between L1 and S1 and was sectioned at L1. The L7 and S1 dorsal and ventral roots were sectioned close to their entry through the dura and their central ends were mounted on bipolar silver stimulating electrodes. The exposed cord and adjacent tissue was covered with paraffin oil (maintained at 37 °C) contained in elevated skin flaps.

The activity of single spinal neurones was recorded extracellularly from the centre barrel of a seven-barrelled glass micro-electrode. The recording barrel contained 4 M-NaCl whilst another barrel frequently contained 1 M-NaCl and was used for current balancing or for the ejection of Na^+ or Cl^- to test for ionophoretic current effects. Each of the remaining barrels contained one of the following compounds which were ejected from the electrode using conventional microiontophoretic techniques: acetylcholine Cl (0·5 M), Na L- and D-glutamate (0·2 M, pH 7·0), Na L- and D-aspartate (0·2 M, pH 7·0), Na L- and D-homocysteate (0·2 M, pH 7·0), Na N-methyl-D-aspartate (NMDA) (0·05 M in 0·15 M-NaCl, pH 7·0), Na kainate (0·02 M in 0·18 M-NaCl, pH 7·0), Na D, L- and DL-α-aminosuberate (D, L and DLαAS) (0·2 M, pH 7·0), Na D- and L-α-aminoadipate (D and LαAA) (0·2 M, pH 7·0), glutamic acid diethyl ester hydrochloride (GDEE) (0·2 M, pH 3·5), Na DL-2-amino-4-phosphonobutyrate (2APB) (0·2 M, pH 7·0), Na quisqualate (0·02 M in 0·13 M-NaCl), Na ibotenate (0·05 M in 0·1 M-NaCl), dihydro-β-erythroidine (DHβE) (0·01M in 0·165 M-NaCl).

Neuronal recordings were obtained from Renshaw cells and unidentified dorsal horn neurones. Renshaw cells were identified by their characteristic response to stimulation of L7 or S1 ventral root and dorsal horn neurones were located by advancing the micro-electrode through the dorsal horn whilst either stimulating L7 or S1 dorsal root or allowing an excitant amino acid to diffuse (retaining current zero) from one barrel of the micro-electrode assembly. The firing frequency of spinal neurones was electronically counted and displayed on a pen-recorder trace. The counted pulses were also fed into a small computer (Neurolog) which was used to compile peristimulus time histograms of synaptic events. Excitant substances were administered in an automatically time-controlled sequence, up to three different excitants being ejected in a cycle. The currents ejecting the excitants were adjusted to produce approximately equal frequencies of neuronal spike discharge. Once stable control responses had been established the effects of one of the proposed antagonists were determined. The peak heights of the responses to excitants during the ejection of a proposed antagonist were compared with those produced during the control period. Changes in peak firing of 10 % or more were considered genuine if recovery of the response occurred on terminating the ejection of the substance under test.

Sources of amino acids

N-Methyl-D-aspartic acid (NMDA), L-homocysteic acid and D-homocysteic acid were prepared as previously described (Watkins, 1962). D-α-Aminoadipic acid, and D- and L-α-aminosuberic acids were obtained as described by Evans, Francis, Hunt, Oakes & Watkins (1979). Other substances were the purest obtainable commercial products or were obtained as gifts.

RESULTS

Effects of DαAA on the chemical excitation of spinal neurones

Renshaw cells. Recordings were made from forty-six Renshaw cells, of which twelve had a resting discharge. DαAA (5–50 nA, ejected for 2–6 min) reduced this discharge in five and enhanced it in two neurones. These effects of DαAA were not accompanied by any change in spike amplitude and were rapidly reversible on terminating the ejecting current.

The effects of DαAA ejected with currents of 5–90 nA (mean 26 nA) for 2–9 min (mean 4 min) on the excitatory responses to acetylcholine, L-aspartate, L-glutamate, kainate and NMDA are summarized in Table 1. DαAA had little or no influence on

TABLE 1. Effects of DαAA on the excitatory responses of Renshaw cells to acetylcholine, L-aspartate, L-glutamate, kainate and NMDA

Excitant	Effect of DαAA‡			
(current nA range and mean ± S.E.)	Total no. cells tested	No. cells response depressed (mean ± S.E. % reduction)	No. cells response enhanced (mean ± S.E. % increase)	No. cells unaffected
Acetylcholine (0–57, 10·3 ± 1·8)	46	9 (16·4 ± 2·3)	6 (36·6 ± 8·0)	31
L-Aspartate (10–130, 55·4 ± 3·9)	36	33 (66·3 ± 4·3)§	0	3
L-Glutamate (20–150, 60·3 ± 5·6)	27	14 (47·5 ± 8·8)	1 (20)	12
NMDA (2–40, 24·8 ± 2·4)	13	13 (76·6 ± 6·6)‖	0	0
Kainate (2–40, 28·6 ± 2·6)	13	3 (46·6 ± 26·6)	1 (20)	9

‡ Ejection current: 26 ± 3 nA (mean ± S.E.).

§ In tests on the same neurones, responses to L-aspartate were depressed significantly more than responses to L-glutamate ($P < 0·001$, paired t test).

‖ In tests on the same neurones, responses to NMDA were depressed significantly more than responses to kainate ($P < 0·001$, paired t test).

the excitatory responses induced by acetylcholine in the majority of neurones. However, responses of the same cells to excitatory amino acids were differentially reduced. In particular, excitatory responses to NMDA were markedly depressed in all thirteen neurones tested whereas responses of the same neurones to kainate were mostly unaffected. This preferential effect of DαAA on responses to NMDA is illustrated in Fig. 1. DαAA also reduced responses to L-aspartate to a greater extent than the responses to L-glutamate. Thus, in only fourteen of twenty-seven neurones were the responses to L-glutamate depressed by DαAA whereas L-aspartate-induced responses were depressed in thirty-three of thirty-six cells. Moreover, the mean depression (where observed) was greater for L-aspartate-excited than for L-glutamate excited cells (Table 1 and Fig. 1). However, the differentiation by DαAA between these two amino acids was not as marked as that between NMDA and kainate.

The onset of the depressant action of DαAA on amino acid induced responses was apparent within 1–4 min of commencing the ejection of DαAA and recovery was usually rapid on terminating the ejection (Fig. 1). None of these effects of DαAA

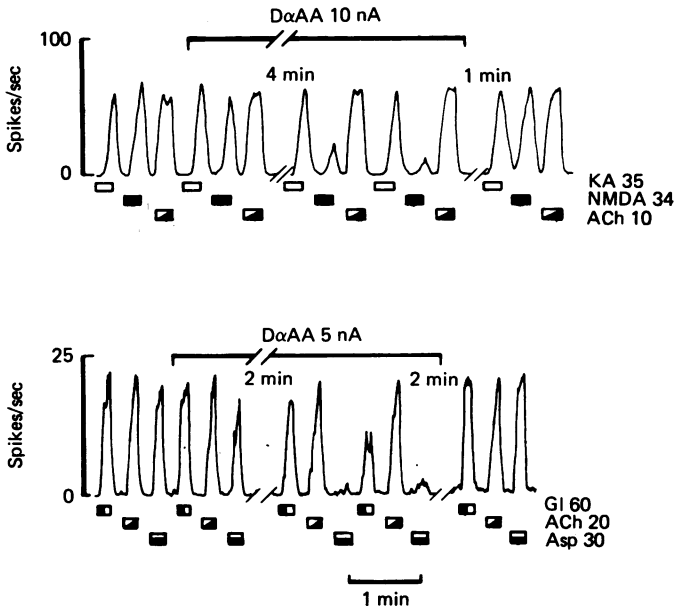

Fig. 1. Rate-meter records showing selective effects of D-α-aminoadipate (DαAA) on chemically induced excitation of Renshaw cells. In the upper record, responses to N-methyl-D-aspartate (NMDA 34 nA) were markedly reduced by DαAA 10 nA while responses to kainate (KA 35 nA) and acetylcholine (ACh 10 nA) were unaffected by this agent. In the lower record (from a different Renshaw cell), responses to L-aspartate (Asp 30 nA) were reduced more than responses to L-glutamate (Gl 60 nA) by DαAA 5 nA whereas responses to ACh 20 nA were virtually uninfluenced by DαAA. The breaks in both rate-meter records were for the time periods indicated above each record.

could be reproduced by the passage of negative current through either the NaCl or acetylcholine containing barrel of the micro-electrode.

L-α-Aminoadipate (LαAA; 20–100 nA for 2–5 min) slightly enhanced the excitatory responses induced by excitant amino acids (five cells) and initiated the firing of one neurone.

Dorsal horn neurones. When tested on seventeen dorsal horn neurones DαAA had essentially similar effects on the excitatory responses induced by amino acids to those observed on Renshaw cells. Thus, ejection of 5–40 nA of DαAA (mean 21 nA) for 2–6 min (mean 3 min) selectively and reversibly reduced responses induced by NMDA compared with those induced by kainate (five neurones) and responses to L-aspartate were depressed more than those to L-glutamate in twelve of fifteen neurones tested (Table 2).

These results indicate that DαAA has differential effects on responses to excitant amino acids. The observations were extended by comparing the ability of DαAA to reduce the excitatory effects of L-aspartate with its ability to reduce the excitatory effects produced by equally effective ejecting currents of other amino acids on the same spinal neurones. Tests were made on Renshaw cells and dorsal horn neurones and excitants have been grouped according to whether DαAA depressed their effectiveness to a greater or lesser extent than it depressed the effectiveness of L-aspartate,

TABLE 2. Effects of DαAA on the excitatory responses of dorsal horn neurones to L-aspartate, L-glutamate, kainate and NMDA

Excitant (current nA, range, mean ± S.E.)	Effect of DαAA‡ Total no. cells tested	No. cells response depressed (mean ± S.E. % reduction)	No. cells response increased (mean % increase)	No. cells unaffected
L-Aspartate (10–100, 45·9 ± 8·3)	15	15 (73·3 ± 4·3)§	0	0
L-Glutamate (20–75, 39·1 ± 7·9)	15	10 (52·5 ± 8·0)	1 (25)	4
NMDA (30–35, 31·6 ± 1·8)	5	5 (78·0 ± 7·0)	0	0
Kainate (2–20, 12·8 ± 3·3)	5	1 (20)	0	4

‡ Ejection current: 21 ± 4 nA (mean ± S.E.).

§ In tests on the same cells DαAA depressed responses to L-aspartate significantly more than responses to L-glutamate ($P < 0·001$; paired t test).

TABLE 3. Order of effectiveness of DαAA on responses to various excitants relative to its action on L-aspartate induced responses

Amino acid	Relative depression, mean ± S.E. (no. cells tested)‡	Significance (paired t test)§
N-Methyl-D-aspartate	1·3 ± 0·20 (11)	***
L-Homocysteate	1·25 ± 0·13 (8)	**
D-Glutamate	1·12 ± 0·12 (10)	*
Ibotenate	1·10 ± 0·10 (4)	*
D-Homocysteate	1·05 ± 0·22 (6)	n.s.
L-Aspartate	1·0	
D-Aspartate	0·95 ± 0·03 (9)	n.s.
L-Glutamate	0·49 ± 0·08 (35)	†††
Kainate	0·17 ± 0·06 (11)	†††
Quisqualate	0·0 (6)	†††

‡ DαAA was tested on responses of equal magnitude obtained with a series of three or four excitants including the standard, L-aspartate. The numbers refer to the mean ± S.E. of the ratios of the percentage reduction of the response to the agonist to the percentage reduction of the response to L-aspartate on the same neurones.

§ Significantly more sensitive than L-aspartate-induced responses: *** $P < 0·001$, ** $P < 0·01$, * $P < 0·05$; n.s. = not significant. Significantly less sensitive than L-aspartate-induced responses: ††† $P < 0·001$.

using the paired t test for statistical differentiation (Table 3). Responses produced by L-homocysteate, D-glutamate and ibotenate were highly sensitive to the antagonists whilst those produced by quisqualate were relatively insensitive. Those produced by D-homocysteate and D-aspartate resembled responses to L-aspartate

in having an intermediate sensitivity to the antagonist. An approximate ranking order based on the relative depression by DαAA of the responses to an excitant compared with the depression of L-aspartate-induced responses is also shown in Table 3.

This order of sensitivity of the amino acid-induced responses to antagonism by DαAA does not correlate with the order of potency of the substances as excitants which, on the basis of equally effective ejecting currents, was approximately: quisqualate > kainate > NMDA > ibotenate > D-homocysteate > L-homocysteate > L-glutamate > L-aspartate ≃ D-aspartate > D-glutamate, in accordance with previous studies (Curtis & Watkins, 1963; Johnston, Curtis, Davies & McCulloch 1974; Biscoe, Evans, Headley, Martin & Watkins 1976; Cox, Headley & Watkins, 1977).

Effects of D-*and* L-α-*Aminosuberate*

The effects were also investigated of D, L and DL forms of α-aminosuberate, a higher homologue of α-aminoadipate with two extra carbon atoms in the chain

TABLE 4. Effects of D-α-aminosuberate (DαAS) on responses of spinal neurones (Renshaw cells and dorsal horn neurones) to a range of excitant substances

Excitant	Total no. cells tested	Effect of DαAS‡ No. cells response depressed (mean ± S.E. % reduction)	No. cells response increased	No. cells unaffected
L-Aspartate	18	17 (78 ± 15·0)§	0	1
L-Glutamate	16	11 (50 ± 6·7)	0	5
NMDA	5	5 (74 ± 9·2)‖	0	0
Kainate	5	2 (20 ± 5·0)	0	3
Acetylcholine	14	2 (35 ± 15·0)	0	12

‡ Ejecting current: 21 ± 3 nA (mean ± S.E.).

§ In tests on the same neurones responses to L-aspartate were reduced significantly more than responses to L-glutamate ($P < 0·001$, paired t test).

‖ In tests on the same neurones responses to NMDA were reduced significantly more than responses to kainate ($P < 0·001$, paired t test).

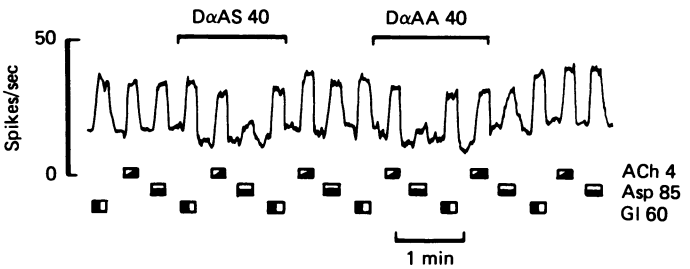

Fig. 2. Rate-meter record from a Renshaw cell illustrating the similar effects of D-α-aminosuberate (DαAS 40 nA) and D-α-aminoadipate (DαAA 40 nA) on excitation induced by acetylcholine (ACh 4 nA), L-aspartate (Asp 85 nA) and L-glutamate (Gl 60 nA). Note the reversible reduction in spontaneous firing of this cell in the presence of DαAA and DαAS.

linking the carboxyl groups. In experiments on the isolated frog spinal cord, D-α-aminosuberate (DαAS) has been shown to have effects qualitatively similar to, but more potent than those of DαAA (Davies *et al.* 1979*a*; Evans *et al.* 1979). The effects of DαAS on responses of Renshaw cells and dorsal horn neurones are shown in Table 4. In direct comparisons of DαAA and DαAS on nine Renshaw cells and four dorsal horn neurones, the effects of the two antagonists were qualitatively and quantitatively similar. An example is shown in Fig. 2. The effects of DL-α-aminosuberate (20–60 nA), tested on six Renshaw cells, were similar to those of DαAS.

L-α-Aminosuberate (LαAS; 10–30 nA for 2–5 min) enhanced the excitatory responses to kainate and NMDA and increased the rate of spontaneous background firing (four neurones); higher currents (80–100 nA) of LαAS directly excited cells (three neurones). These excitatory effects of LαAS are in accordance with the actions of this substance on the isolated spinal cord of the frog (Evans *et al.* 1979).

Comparison of DαAA with other excitatory amino acid antagonists

It is apparent from the foregoing results that DαAA and DαAS are relatively selective antagonists of excitatory amino acids. It was therefore important to compare the effects of these compounds with those of other reported amino acid antagonists. A comparison of DαAA and HA-966 has been previously reported (Biscoe *et al.* 1978). In the present work we have compared the effects of GDEE and 2APB with those of DαAA on the same neurones. The results are summarized in Table 5.

Neither 2APB nor GDEE were as effective as DαAA as antagonists of L-aspartate or NMDA-induced excitation in terms of the ejecting currents required to reduce responses to the amino acids, the magnitude of the depression produced, or the proportion of neurones in which responses to amino acids were affected (Table 5). Like DαAA, 2APB (20–80 nA for 2–7 min) had little effect on responses of eleven

TABLE 5. Comparison of the effects of 2APB and GDEE with those of DαAA on the same spinal neurones (Renshaw cells and dorsal horn neurones)

Antagonist (mean ± S.E. nA)	ACh No. depressed/ no. tested (mean ± S.E. % depression)	L-Aspartate No. depressed/ no. tested (mean ± S.E. % depression)	L-Glutamate No. depressed/ no. tested (mean ± S.E. % depression)	Kainate No. depressed/ no. tested (mean ± S.E. % depression)	NMDA No. depressed/ no. tested (mean ± S.E. % depression)
2APB (51 ± 3·9)‡	1/11 (35)	8/15 (53 ± 6·7)	6/15 (43 ± 8·6)	*4/6 (42 ± 10·3)	4/6 (50 ± 5·7)
DαAA (26 ± 3·9)	0/11 —	14/15 (57 ± 7·4)	6/15 (34 ± 4·9)	1/6 (20)	6/6 (71 ± 8·3)
GDEE (41 ± 3·6)‡	6/9 (39 ± 7·7)	10/17 (45 ± 8·3)	9/14 (53 ± 7·3)	0/9	0/9
DαAA (25 ± 3·0)	1/9 (25)	15/17 (64 ± 5·5)	6/14 (35 ± 9·5)	1/9 (20)	9/9 (76 ± 6·8)

‡ Significantly different from the corresponding DαAA ejecting current: $P < 0{\cdot}01$ (Student's t test).

Renshaw cells to acetylcholine. However, amino acid-induced responses were only reduced by 2APB in eight of a total of fifteen Renshaw cells and dorsal horn neurones whereas DαAA was effective in almost every case, and with ejection currents only one half of those administering 2APB. Unlike DαAA, 2APB did not clearly differentiate between responses to kainate and NMDA nor between responses to L-glutamate and L-aspartate. The spectrum of activity of GDEE was different from that of either DαAA or 2APB. In tests on nine neurones, GDEE (20-60 nA) did not influence neuronal responses to either kainate or NMDA. However, responses to L-glutamate and L-aspartate were reduced by this substance in the majority of neurones tested (Table 5). The potency of GDEE was less than that of DαAA in the case of L-aspartate-induced responses but there was less difference between the two antagonists with respect to their actions on L-glutamate-induced responses. In contrast to DαAA, GDEE reduced responses induced by acetylcholine in six of nine Renshaw cells. Some of the differences outlined above between the effects of 2APB, GDEE and DαAA on neuronal responses to excitants are illustrated in Fig. 3.

In view of the differences in the spectrum of antagonist activity of GDEE and 2APB relative to DαAA the effects of the former two substances were examined also on responses to L-homocysteate, ibotenate and quisqualate on a small number of spinal neurones. 2APB depressed excitatory responses to all three substances (six neurones), the responses to ibotenate being the most sensitive. GDEE depressed

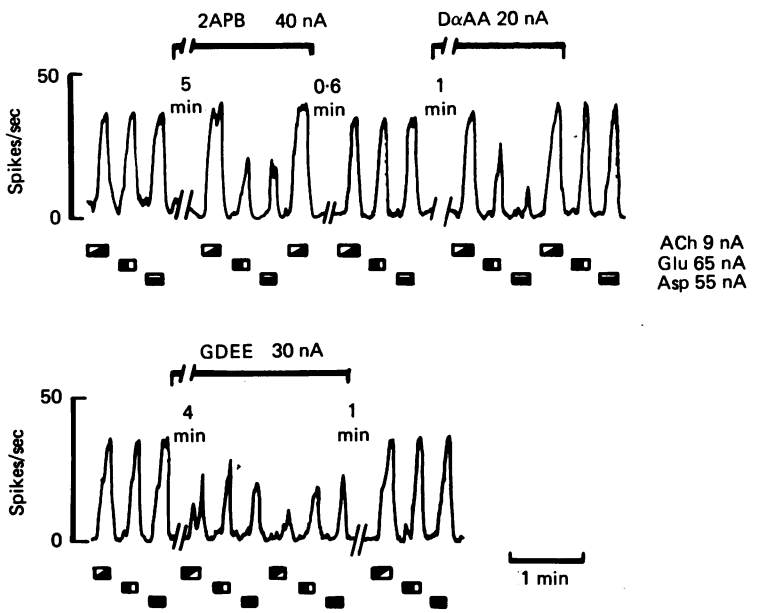

Fig. 3. This rate-meter record illustrates the effects of 2-amino-4-phosphonobutyrate (2APB), D-α-aminoadipate (DαAA) and glutamic acid diethylester (GDEE) on responses of the same Renshaw cell to acetylcholine (ACh), L-glutamate (Glu) and L-aspartate (Asp). The breaks in the rate-meter trace correspond to the time periods shown above the record; the upper and lower traces are continuous. 2APB reduced responses to Asp and Glu in parallel whilst DαAA reduced responses to Asp more than those to Glu; neither of these antagonists depressed responses to ACh. GDEE reduced responses to all three excitants, responses to ACh being the most affected.

responses to L-homocysteate and quisqualate, but responses to ibotenate were unaffected in four neurones and were enhanced in two neurones by GDEE.

Effects of antagonists on synaptic excitation of Renshaw cells and dorsal horn neurones

If amino acid antagonists are to be useful for the identification of sites of amino acid-mediated synaptic excitation, it is necessary to demonstrate selectivity in their depressant actions not only on chemically induced excitation but also on different types of synaptically evoked excitatory responses. Renshaw cells are particularly useful for assessing the specificity of antagonists of synaptic excitation since ventral root stimulation gives rise to cholinergic excitation via the terminals of motor axon collaterals (Eccles, 1963), whilst dorsal root stimulation leads to non-cholinergic excitation (Curtis & Ryall, 1966) possibly mediated by an excitatory amino acid transmitter. Experiments were conducted on twenty-four Renshaw cells, twenty of which responded to dorsal root as well as to ventral root stimulation. On eight of these cells, direct comparisons were made of the effects of DαAA and DαAS, and similar comparisons of the effects of DαAA and 2APB were made on a further eight Renshaw cells. Seven of these latter eight cells were also tested with GDEE in a

TABLE 6. Effects of antagonists on submaximal synaptic responses of Renshaw cells (RC) and dorsal horn neurones (DHN)

Antagonist (current (nA) range, mean ± S.E.)	No. of neurones	Effect on evoked responses‡					
		Decreased		Increased		Unchanged	
		DR	VR	DR	VR	DR	VR
DαAA (10–90, 38 ± 3·7)	24 RC	18 (66 ± 5·7 %)	0	0	0	2	24
	10DHN	8 (63 ± 9·9 %)	—	0	—	2	—
DαAS (10–60, 33 ± 7·0)	8RC	8 (67 ± 7·0 %)	0	0	0	0	8
	4DHN	4 (64 ± 6·1 %)	—	0	—	0	—
2APB (30–80, 60 ± 5·3)	8RC	4 (71 ± 12·5 %)	0	1 (50 %)	3 (19 ± 3·0 %)	3	5
	6DHN	3 (57 ± 9·6 %)	—	0	—	3	—
GDEE (40–80, 57 ± 11·4)	7RC	1 (55 %)	1 (30 %)	1 (157 ± 55·5 %)	2 (20 %)	3	4
	6DHN	2 (30 %)	—	0	—	4	—

‡ Synaptic responses were evoked in spinal neurones by rectangular pulses (0·1 msec ,1 Hz and submaximal intensity) applied to the ventral roots (VR) and/or dorsal roots (DR). The figures in each column show the number of neurones in which the synaptic response was increased, decreased or unchanged. The figure in parentheses refer to the mean ± S.E. % change in the number of spikes per response. These changes were calculated from either photographic records of at least fifteen oscilloscope sweeps or peristimulus histograms computed from at least twenty sweeps analysed in 250 μsec intervals. The antagonists were administered for 2–9 (mean 3·5) min.

direct comparison of the three antagonists. The results are summarized in Table 6. In addition, the effects of DαAA and DαAS were compared on the synaptic responses of four dorsal horn neurones evoked by submaximal dorsal root stimulation, and a three-way comparison between DαAA, 2APB and GDEE was made on similar responses evoked in a further six dorsal horn cells. The results of these comparisons are also included in Table 6.

As indicated by Table 6, DαAA had no significant effect on any of the excitatory responses of Renshaw cells evoked by submaximal ventral root stimulation but reversibly depressed the responses to submaximal dorsal root stimulation in eighteen of the twenty cells responding to this latter stimulus. Typically, DαAA (10–90 nA for 2–5 min) reduced the number of spikes in the dorsal root evoked response by

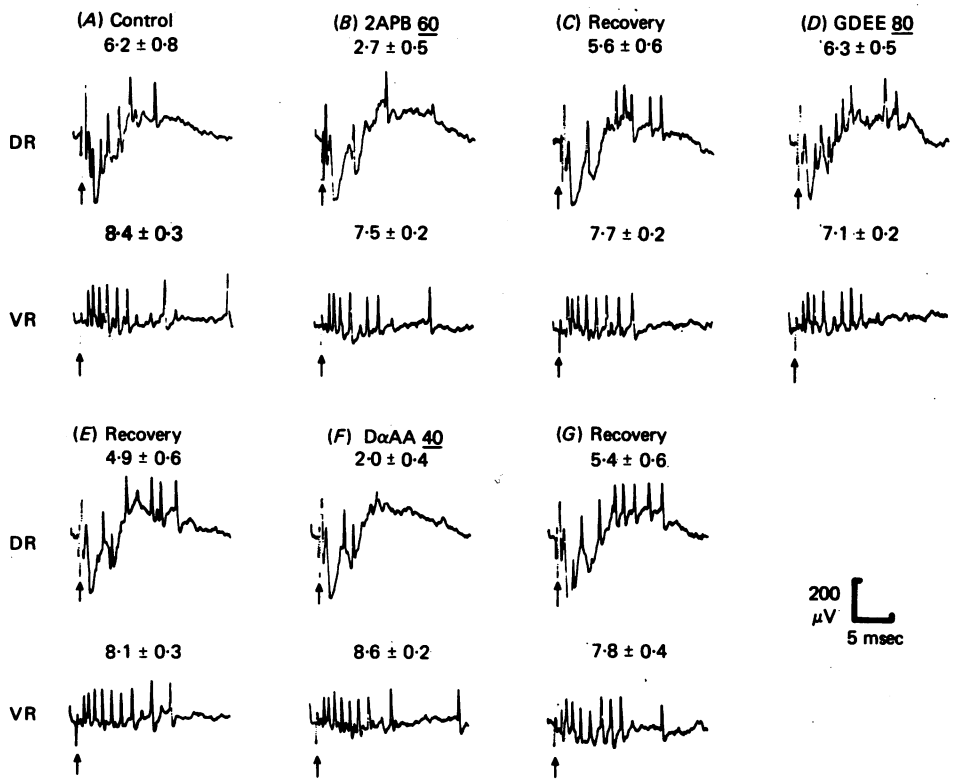

Fig. 4. Antagonist actions of 2-amino-4-phosphonobutyrate (2APB), glutamic acid diethyl ester (GDEE) and D-αaminoadipate (DαAA) on synaptically induced firing of a Renshaw cell. Records *A–G* are representative oscilloscope sweeps (retouched) of the synaptic responses of the same Renshaw cell to a constant submaximal dorsal root (DR) and ventral root (VR) stimulus. *A* was taken during the control period, *B* during the ejection of 2APB for 2 min, *C* 4 min after terminating the ejection of 2APB, *D* during the ejection of GDEE 80 nA for 4 min, *E* 2 min after terminating the ejection of GDEE, *F* during the ejection of DαAA 40 nA for 2 min, and *G* 2 min after terminating the ejection of DαAA. The numbers above each oscilloscope sweep indicate the mean ± S.E. number of spikes in fifteen responses. 2APB and DαAA significantly reduced dorsal root evoked responses ($P < 0{\cdot}001$ in both cases, Student's *t* test). The effects of GDEE on both DR- and VR-evoked responses were not significant. The arrow below each oscilloscope record indicates the position of the stimulus artifact.

about two thirds, the shorter latency spikes being the most resistant (for example the cell illustrated in Fig. 4); occasionally, however, the response was completely abolished. In contrast the ionophoretic administration of the acetylcholine antagonist DHβE (10–50 nA for 2–4 min) reversibly reduced responses evoked by ventral root stimulation in the absence of an effect on responses evoked by dorsal root stimulation (eight cells). DαAA also effectively depressed the synaptic responses in eight of ten dorsal horn neurones evoked by dorsal root stimulation whilst having no effect on the responses of the other two cells (Table 6).

DαAS had substantially the same effects as DαAA and appeared to be of similar potency to the shorter chain analogue (Table 6). The DL form of α-aminosuberate was also effective in tests on three Renshaw cells.

2APB generally resembled DαAA and DαAS in depressing dorsal root-evoked excitation of dorsal horn neurones and Renshaw cells, whilst having little effect on ventral root-evoked excitation of these latter cells. This is illustrated for a Renshaw cell in Fig. 4. In general, 2APB was less potent than DαAA in depressing dorsal root-evoked responses in both types of cell (Table 6). Thus, DαAA depressed these responses in all of the fourteen Renshaw cells and dorsal horn neurones tested with both 2APB and DαAA, whereas the phosphonate was only effective in 50 % of these cells. Moreover, the ejection currents necessary to produce these effects were lower for DαAA than for 2APB.

GDEE was a relatively poor antagonist of the synaptic responses of Renshaw cells and dorsal horn neurones with ejection currents as high as 80 nA and administration times of up to 8 min (Fig. 4 and Table 6). The synaptic responses of some Renshaw cells were actually enhanced by GDEE, this effect being most marked in the case of the dorsal root-evoked responses. DαAA depressed the dorsal root evoked responses in all of the thirteen Renshaw cells and dorsal horn neurones tested with GDEE.

DISCUSSION

The results indicate that DαAA and DαAS are effective antagonists of the responses of cat spinal neurones to certain excitatory amino acids but not to other excitatory amino acids, nor, in the case of Renshaw cells, to acetylcholine. When responses to a range of excitatory amino acids were compared with respect to their sensitivities to DαAA and DαAS, the excitants fell into three main groups. Responses induced by NMDA, L-homocysteate, D-glutamate and ibotenate were the most sensitive to the actions of the antagonists, while the least sensitive responses were those produced by quisqualate, kainate and L-glutamate. The third group produced responses of intermediate sensitivity to the antagonists and included D-homocysteate, L-aspartate and D-aspartate.

These effects are consistent with the existence of different types of excitatory amino acid receptors, one type being sensitive to the actions of the antagonists, and activated predominantly by the NMDA group of excitants, with other receptors being relatively insensitive to the antagonists and activated predominantly by quisqualate, kainate and L-glutamate. Other excitants would then be considered to act to varying extents on the NMDA-activated receptors, as reflected by their

sensitivity to NMDA antagonists, and also on other receptors which may or may not be homogeneous in nature. On this basis, a greater proportion of L-aspartate- than of L-glutamate-induced responses would be assumed to be mediated by the antagonist-sensitive receptors.

The actions of 2APB suggest that this compound is selective with respect to cholinergic and amino acid-induced excitation, acting only on the latter responses, but to be relatively non-selective with respect to the actions of different excitatory amino acids. Interpretation of the actions of 2APB are, however, complicated by the fact that only the racemate was used, and it is possible that D and L isomers have different effects. Moreover, other studies have revealed weak agonist actions of 2APB (Watkins, Curtis & Brand, 1977; Evans *et al.* 1979), though such actions were not pronounced in the present work.

GDEE produced a completely different pattern of antagonism. This substance was equally or more effective as an antagonist of acetylcholine-induced excitation of Renshaw cells than it was as an antagonist of amino acid-induced responses of these cells. Moreover, it was a generally weaker amino acid antagonist than DαAA, DαAS or 2APB except possibly in the case of L-glutamate- and quisqualate-induced responses. McLennan & Lodge (1979) have interpreted similar results as indicating the existence of a specific type of receptor activated by L-glutamate and quisqualate, but not by NMDA or kainate. However, it is possible that these latter amino acids, whose diffusion is not limited by rapid uptake (Balcar & Johnston, 1972; Johnston, Kennedy & Twitchin, 1979) may have activated distant receptors out of range of the weak antagonist action of GDEE (Davies *et al.* 1979*b*).

In direct comparison of the antagonists, neither 2APB nor GDEE (particularly) were as effective as depressants of synaptic excitation as either DαAA or DαAS. This result is in accordance with the findings of *in vitro* experiments on frog isolated spinal cord (Davies *et al.* 1979*b*; Evans *et al.* 1979). In the present work, DαAA and DαAS both depressed the dorsal root-evoked excitation of the large majority of dorsal horn neurones tested. In addition, the dorsal root-evoked excitation of Renshaw cells was depressed by these substances in the absence of an effect on the ventral root-evoked excitation of these cells. A failure of the antagonists to reach the cholinergic synapses of this latter pathway seems unlikely, since DHβE applied from another barrel of the same micropipette assemblies selectively blocked these ventral root-evoked responses. Since DαAA and related substances have little or no effect on acetylcholine-induced responses of cat Renshaw cells (present work, see also Biscoe *et al.* 1977*a*, *b*, 1978; Lodge, Headley & Curtis, 1978) or neurones in the substantia nigra (Collingridge & Davies, 1979) or thalamus (McLennan & Hall, 1978) of the rat, on carbachol or noradrenaline-induced responses in the isolated spinal cord of the immature rat (Evans & Watkins, 1978), or on substance P-induced responses in this latter preparation (Evans & Watkins, 1978), the rat substantia nigra (Collingridge & Davies, 1979) or the cat spinal cord (Davies & Dray, 1979), it seems likely that those synaptic excitations that are sensitive to DαAA and DαAS are mediated by an excitatory amino acid transmitter.

The question of the possible identity of such a transmitter has recently been discussed (Watkins, 1978; Davies *et al.* 1979*a*). The arguments in favour of L-aspartate over L-glutamate as the transmitter were based mainly on the observation that the

most readily antagonized responses were those produced by the aspartate analogue, NMDA, whilst the glutamate analogue, kainate, produced responses that were relatively resistant to NMDA antagonists (Biscoe *et al.* 1977*a*, *b*; 1978; Evans *et al.* 1978). However, the present work has indicated that other glutamate analogues, ibotenate (Johnston *et al.* 1974), D-glutamate and L-homocysteate all produce responses that are sensitive to DαAA and like-antagonists. Thus, L-glutamate cannot be excluded as the transmitter acting at DαAA-sensitive receptors. It is possible that the true effectiveness of L-glutamate at these sites is obscured by the predominant activation by the *exogeneous* amino acid of those receptors that are relatively insensitive to DαAA. These latter receptors may be extrasynaptic. A population of non-synaptic receptors that are activated by L-glutamate and kainate, but not by NMDA and only weakly by L-aspartate, has recently been identified in isolated dorsal roots of the immature rat (Davies *et al.* 1979*b*). A continuing development of specific agonists and antagonists will be required both to assess the possible importance of such non-synaptic receptors in the mammalian central nervous system and for a more discriminating identification of the transmitter(s) acting at antagonist-sensitive sites of synaptic excitation.

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REFERENCES

ALTMANN, H., TEN BRUGGENCATE, G., PICKELMANN, P. & STEINBERG, R. (1976). Effects of glutamate, aspartate and two presumed antagonists on feline rubrospinal neurones. *Pflügers Arch.* **364**, 249–255.

BALCAR, V. J. & JOHNSTON, G. A. R. (1972). Glutamate uptake by brain slices and its relation to the depolarization of neurones by acidic amino acids. *J. Neurobiol.* **3**, 295–301.

BISCOE, T. J., DAVIES, J., DRAY, A., EVANS, R. H., FRANCIS, A. A., MARTIN, M. R. & WATKINS, J. C. (1977*a*). Depression of synaptic excitation and of amino acid induced excitatory responses of spinal neurones by D-α-aminoadipate, α, ξ-diaminopimelic acid and HA-966. *Eur. J. Pharmac.* **45**, 315–316.

BISCOE, T. J., DAVIES, J., DRAY, A., EVANS, R. H., MARTIN, M. R. & WATKINS, J. C. (1978). D-α-Aminoadipate, α, ξ-diaminopimelic acid and HA-966 as antagonists of amino acid-induced and synaptic excitation of mammalian spinal neurones *in vivo*. *Brain Res.* **148**, 543–548.

BISCOE, T. J. EVANS, R. H. FRANCIS, A. A., MARTIN, M. R., WATKINS, J. C., DAVIES, J. & DRAY, A. (1977*b*). D-α-Aminoadipate as a selective antagonist of amino acid-induced and synaptic excitation of mammalian spinal neurones. *Nature, Lond.* **270**, 743–745.

BISCOE, T. J., EVANS, R. H., HEADLEY, P. M., MARTIN, M. R. & WATKINS, J. C. (1976). Structure-activity relations of excitatory amino acids on frog and rat spinal neurones. *Br. J. Pharmac.* **58**, 373–382.

COLLINGRIDGE, G. L. & DAVIES, J. (1979). An evaluation of D-α-aminoadipate and D- (and DL-) α-aminosuberate as selective antagonists of excitatory amino acids in the substantia nigra and mesencephalic reticular formation of the rat. *Neuropharmacology* **18**, 193–199.

COX, D. W. G., HEADLEY, P. M. & WATKINS, J. C. (1977). Actions of L- and D-homocysteate in rat CNS: a correlation between low affinity uptake and the time courses of excitation by micro-electrophoretically applied L-glutamate analogues. *J. Neurochem.* **29**, 579–588.

CURTIS, D. R., DUGGAN, A. W., FELIX, D., JOHNSTON, G. A. R., TEBĒCIS, A. K. & WATKINS, J. C. (1972). Excitation of mammalian neurones by acidic amino acids. *Brain Res.* **41**, 283–301.

CURTIS, D. R., JOHNSTON, G. A. R., GAME, C. J. A. & MCCULLOCH, R. M. (1973). Antagonism of nuronal excitation by 1-hydroxy-3-aminopyrrolidone-2. *Brain Res.* **49**, 467–470.

CURTIS, D. R. & RYALL, R. W. (1966). The synaptic excitation of Renshaw cells. *Expl Brain Res.* **2**, 81–96.

CURTIS, D. R. & WATKINS, J. C. (1963). Acidic amino acids with strong excitatory actions on mammalian neurones. *J. Physiol*, **166**, 1–14.

DAVIES, J. & DRAY, A. (1979). Effects of D-α-aminoadipate on physiologically evoked responses of cat dorsal horn neurones. *Experientia* **35**, 353–354.

DAVIES, J., EVANS, R. H., FRANCIS, A. A. & WATKINS, J. C. (1979*a*). Excitatory amino acids: receptor differentiation by selective antagonists and role in synaptic excitation, In *Advances in Pharmacology and Therapeutics*, vol. 2, Neurotransmitters, ed. SIMON, P., Oxford: Pergamon. pp. 161–170.

DAVIES, J., EVANS, R. H. FRANCIS, A. A. & WATKINS, J. C. (1979*b*). Excitatory amino acid receptors and synaptic excitation in the mammalian central nervous system. *J. Physiol., Paris* (in the Press).

DAVIES, J. & WATKINS, J. C. (1973). Microelectrophoretic studies on the depressant action of HA-966 on chemically and synaptically excited neurones in the cat cerebral cortex and cuneate nucleus. *Brain Res.* **59**, 311–322.

DUDEL, J. (1977). Aspartate and other inhibitors of excitatory synaptic transmission in crayfish muscle. *Pflügers Arch.* **369**, 7–16.

ECCLES, J. C. (1963). *The Physiology of Synapses*. Berlin: Springer-Verlag.

EVANS, R. H., FRANCIS, A. A., HUNT, K., OAKES, D. J. & WATKINS, J. C. (1979). Antagonism of excitatory amino acid-induced responses and of synaptic excitation in the isolated spinal cord of the frog. *Br. J. Pharmac.* (In Press.)

EVANS, R. H., FRANCIS, A. A. & WATKINS, J. C. (1978). Mg^{2+}-like selective antagonism of excitatory amino acid-induced responses by α, ξ-diaminopimelic acid, D-α-aminoadipate, and HA-966 in isolated spinal cord of frog and immature rat. *Brain Res.* **148**, 536–542.

EVANS, R. H. & WATKINS, J. C. (1978). Specific antagonism of excitant amino acids in the isolated spinal cord of the neonatal rat. *Eur. J. Pharmacol.* **50**, 123–129.

HALDEMAN, S., HUFFMAN, R. D., MARSHALL, K. C. & MCLENNAN, H. (1972). The antagonism of the glutamate-induced and synaptic excitation of thalamic neurones. *Brain Res.* **39**, 419–425.

HALL, J. G., MCLENNAN, H. & WHEAL, H. C. (1977). The actions of certain amino acids as neuronal excitants. *J. Physiol* **272**, 52–53*P*.

JOHNSTON, G. A. R., CURTIS, D. R., DAVIES, J. & MCCULLOCH, R. M. (1974). Spinal interneurone excitation by conformationally restricted analogues of L-glutamic acid. *Nature, Lond.* **248**, 804–805.

JOHNSTON, G. A. R., KENNEDY, B. M. E. & TWITCHIN, B. (1979). Action of the neurotoxin kainic acid on high affinity uptake of L-glutamic acid in rat brain slices. *J. Neurochem.* **32**, 121–127.

LODGE, D., HEADLEY, P. M. & CURTIS, D. R. (1978). Selective antagonism by D-α-aminoadipate of amino acid and synaptic excitation of cat spinal neurones. *Brain Res.* **152**, 603–608.

MCLENNAN, H. & HALL, J. G. (1978). The action of D-α-aminoadipate on excitatory amino acid receptors of rat thalamic neurones. *Brain Res.* **149**, 541–545.

MCLENNAN, H. & LODGE, D. (1979). The antagonism of amino acid-induced excitation of spinal neurones in the cat. *Brain Res.* **169**, 83–90.

WATKINS, J. C. (1962). The synthesis of some acidic amino acids possessing neuropharmacological activity. *J. med (Pharm) Chem.* **5**, 1187–1199.

WATKINS, J. C. (1978). Excitatory amino acids. *In Kainic acid as a Tool in Neurobiology*, ed. MCGEER, E. G., OLNEY, J. W. & MCGEER, P. L., pp. 37–69, New York: Raven.

WATKINS, J. C., CURTIS, D. R. & BRAND S. S. (1977). Phosphonic acid analogues or antagonists of amino acid excitants. *J. Pharm. Pharmac.* **29**, 324.

WHITE, W. F., NADLER, J. V., HAMBERGER, A., COTMAN, C. W. & CUMMINS, J. T. (1977). Glutamate as transmitter of hippocampal perforant path. *Nature, Lond.* **270**, 356–357.

ZIEGLGÄNSBERGER, W. & PUIL, E. A. (1973). Intracellular investigations of the effect of microelectrophoretically applied glutamate antagonists upon spinal neurones of the cat. *Arch. exp. Path. Pharmak.* **277**, suppl. R89.