Br. J. Pharmac. (1978), **62**, 171–176

THE EFFECTS OF AMINO ACIDS AND ANTAGONISTS ON THE ISOLATED HEMISECTED SPINAL CORD OF THE IMMATURE RAT

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1 Records of ventral and dorsal root polarity of the isolated hemisected spinal cord of the 3–9 day old rat showed that respective dose-dependent depolarizations of motoneurones (VR responses) and primary afferent terminals (DR responses) were produced by both acidic and neutral amino acids in the presence of procaine (1 mM) or tetrodotoxin (0.1 μM).

2 Of the four neutral amino acids, γ-aminobutyrate (GABA), glycine, taurine, and β-alanine, GABA was the most effective in producing DR responses and glycine the most effective in producing VR responses. Only taurine depressed the electrical activity recorded from ventral roots.

3 The DR responses produced by GABA, β-alanine and taurine were all antagonized by bicuculline (5 μM) and picrotoxin (5 μM). Bicuculline was more selective than picrotoxin in antagonizing VR responses produced by GABA.

4 Strychnine (1 μM) antagonized VR responses produced by glycine β-alanine and taurine without affecting responses produced by GABA. DR responses to the neutral amino acids were unaffected by strychnine.

Introduction

The use of the hemisected spinal cord of the newborn rat as a pharmacological preparation has been described by Otsuka & Konishi (1974) who compared the actions of L-glutamate and substance P on motoneurones of this tissue (Konishi & Otsuka, 1974). The preparation offers a convenient *in vitro* system for examination of the effects of drugs on central mammalian receptors. In the present paper, the effects of a range of amino acids on both motoneurones and afferent terminals of this preparation are described. In addition, a comparison has been made of the antagonism of these effects by picrotoxin, bicuculline and strychnine.

Methods

Preparation of hemicords

Animals were taken 3–9 days after birth (body weight 8–20 g) and anaesthetized with urethane (2.5 g/kg). After decapitation the spine was removed and placed in a dish containing 0.5% w/v urethane in Ringer solution at 15–20°C in order to dissect out and hemisect the spinal cord. The hemicords were mounted medial surface downwards on four layers of coarse absorbent paper (disposable nappy liner, Bowater Scott, Babette) on a brass block covered with plastic film and the exposed surface of the hemicord was covered with a single layer of nappy liner. Water at 20 ± 0.5°C was passed through the brass block. A constant flow pump was used to pass Ringer solution through a jacket at the same temperature. The Ringer solution dripped onto the surface of the hemicord adjacent to the roots (L4 or 5) from which recordings were made. The brass block was inclined slightly and the perfused solution dripped off the lower end of the brass block after passing away from the tissue down a strip of filter paper. This arrangement was adjusted so that each successive drop of the perfused solution moistened the surface of the hemicord and drained away without forming a pool around the tissue.

Electrical recording

The electrodes consisted of silver wires chlorided by immersion in molten silver chloride. Each chlorided wire was sealed into a glass tube and this assembly was pushed into a polythene tube containing agar (0.5% w/v) in Ringer solution and terminating with a cotton wick. The root to be recorded from was placed in contact with the cotton wick of the electrode and insulated from the Ringer solution with

a mixture of liquid paraffin and petroleum jelly (1:1, w/w) applied through a syringe. Potentials were recorded between this electrode and a similar one placed in contact with the perfused Ringer solution. The Ringer solution in contact with the hemicord was connected to earth through a similar electrode.

The dorsal root corresponding to the ventral root recorded from was stimulated through two stainless steel wires with supramaximal pulses (0.05 ms duration, 30 Volts). The dorsal root recordings were made from the root immediately above or below the stimulated segment. Upward deflection shown on the records indicates increase in positivity of the distal recording electrode on the spinal roots and corresponds to depolarization of motoneurones or primary afferent terminals.

Ringer solution

The composition of the Ringer solution was as follows: (mM) NaCl 118, KCl 2, $NaHCO_3$ 24, $CaCl_2$ 2.5 and glucose 12. This solution was gassed with a mixture of 95% O_2 and 5% CO_2 and the flow rate was maintained at 0.8 ml/minute. When it was desired to block synaptic activity and thereby eliminate indirect actions of perfused substances, procaine hydrochloride (1 mM) or tetrodotoxin (0.1 μM) was included in the Ringer solution. Test substances were dissolved in Ringer solution and were introduced through the constant flow pump instead of the normal Ringer solution. Amino acids were perfused in 2 ml test doses. The pH of the gassed Ringer solution was 7.4.

Chemicals and drugs

L-Homocysteic acid and *N*-methyl-D-aspartic acid were prepared by Dr J.C. Watkins and quisqualic acid was a gift to Dr Watkins from Professor Takemoto, Tohoku University.

Results

Effects of acidic amino acids

Figure 1 shows that in the absence of electrical stimulation, hemicords showed marked spontaneous dorsal root (DR) and ventral root (VR) activity, while dorsal root stimulation evoked potential changes in the corresponding ventral root (DR-VRP) and adjacent dorsal root (DR-DRP). The addition of procaine (1 mM) caused a hyperpolarizing shift in the d.c. potential recorded from both roots followed by blockade of evoked and spontaneous activity (Figure 1c).

Preparations were stable for 12 h but, unlike the frog spinal cord (Evans & Watkins, 1975), they did not survive for periods longer than 24 hours. L-Glutamate and related amino acids caused depolarizing DR and VR responses, the mean potency of the compounds on the VR response relative to L-glutamate being as follows (number of preparations in parentheses): quisqualate 410(4), kainate 380(3), N-methyl-D-aspartate 100(3), L-homocysteate 23(13), L-glutamate 1, L-aspartate 1(6). These relative potencies are similar to those reported from experiments on rat spinal neurones *in vivo* and frog motoneurones *in vitro* (Biscoe, Evans, Headley, Martin & Watkins, 1976). When a range of excitant amino acids was applied in concentrations that gave approximately equal VR responses, it was found that the corresponding DR responses were unequal, suggesting different relative potencies of the amino acids between primary afferent terminals and motneurones. For example, kainate gave a smaller and L-homocysteate a larger DR response than L-glutamate when all three produced equal VR responses. This effect is illustrated in Figure 1c and it was also observed in two other preparations on which this range of amino acids was tested.

To explore the possibility that some of these excitant amino acids may have produced all, or at least

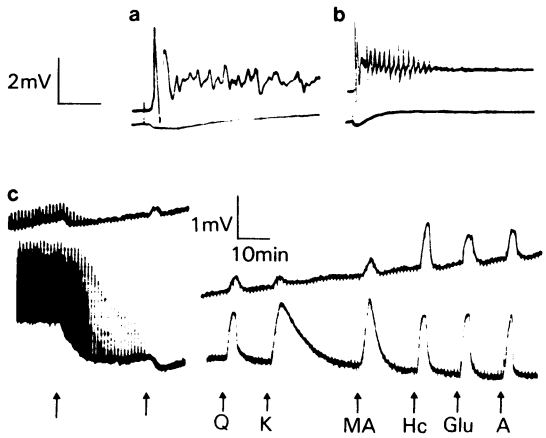

Figure 1 Effect of excitant amino acids on hemisected spinal cord of immature rat. (a) Potentials evoked by dorsal root stimulation of five day old hemisected rat spinal cord (1/min). Upper trace, corresponding ventral root. Lower trace, dorsal root adjacent to stimulated root. Horizontal calibration 40 ms. (b) Potentials as in (a). Horizontal calibration 200 ms. (c) Pen recording of polarity of dorsal root (upper trace) and ventral root (lower trace). At first arrow procaine 1 mM was introduced into the Ringer solution. At the second arrow 2 ml of procaine 10 mM was introduced followed 10 min later by the amino acids in 2 ml doses: quisqualate 2 μM (Q), kainate 2 μM (K), N-methyl-D-aspartate 10 μM (MA), L-homocysteate 25 μM (Hc), L-glutamate 1 mM (Glu) and L-aspartate 1 mM (A).

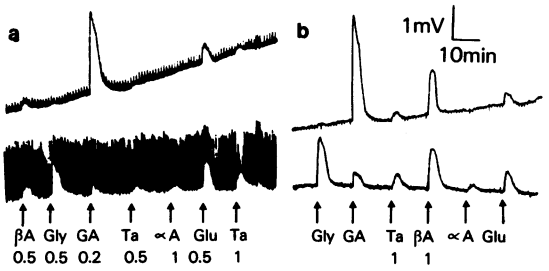

Figure 2 Effect of neutral amino acids and L-glutamate (Glu) on dorsal (upper trace) and ventral (lower trace) root polarity of five day old hemisected rat spinal cord. Dorsal root stimulation 1/minute. (a) Unblocked response; (b) 3 h after introduction of procaine hydrochloride 1 mM. β-Alanine (βA), glycine (Gly), γ-aminobutyrate (GA), taurine (Ta), L-α-alanine (αA); 2 ml doses, concentration (mM) shown under each response.

part, of their DR responses by releasing GABA or other endogenous neutral amino acids, the effect of picrotoxin 0.1 mM was tested in two hemicords on VR and DR responses to the series of excitant amino acids shown in Figure 1. This level of picrotoxin was found to have no effect on DR and VR responses evoked by these excitant amino acids.

Low concentrations of Mg^{2+} have been shown to antagonize VR responses specifically produced by N-methyl-D-aspartate and L-homocysteate (Evans, Francis & Watkins, 1977). During the present experiments it was found that Mg^{2+} (1 mM) also specifically antagonized DR responses to these amino acids.

Neutral amino acids

Glycine and GABA cause membrane hyperpolarization of mammalian motoneurones *in vivo* (Curtis, Hösli, Johnston & Johnston, 1968) and GABA, taurine and β-alanine hyperpolarize frog motoneurones *in vitro* (Evans & Watkins, 1975; Nicoll, Padjen & Barker, 1976). All four amino acids depolarize primary afferent terminals of amphibian spinal cords (Barker, Nicoll & Padjen, 1975), while GABA similarly depolarizes primary afferent terminals of the isolated immature rat spinal cord (Otsuka & Konishi, 1976a) and both GABA and glycine also depolarize motoneurones in this preparation (Otsuka & Konishi, 1976b). These latter findings were confirmed in the present experiments; the depolarizing DR and VR responses produced by neutral amino acids are shown in Figure 2. The mean potency ratios relative to GABA were as follows (number of preparations tested in parentheses): VR response, taurine 0.7(7), β-alanine 1.5(10), glycine 4(15), GABA 1; DR response, taurine 1/80(5), β-alanine 1/25(8), glycine 1/170(11), GABA 1. Thus glycine was the most potent on ventral roots and GABA the most potent on dorsal roots. The threshold level for primary afferent depolarization produced by GABA was 1 μM and that for motoneurone depolarization produced by glycine was 50 μM. The action of large doses of GABA on the DR response was unusual in that the depolarizations faded rapidly from the maximum as seen in Figure 2 and Figure 3c.

Of the four neutral amino acids tested, only taurine depressed spontaneous activity recorded in ventral roots (Figure 2).

Comparison of the antagonism of neutral amino acid-induced responses by bicuculline, picrotoxin or strychnine

Strychnine is considered to be a specific glycine antagonist and bicuculline and picrotoxin relatively specific GABA antagonists as determined by application of these compounds frrom micropipettes onto single central mammalian neurones *in vivo* (see Curtis & Johnston, 1974; Krnjević, 1974). Similar conclusions were reached in studies on amphibian spinal cord *in vitro* (Barker *et al.*, 1975, Evans & Watkins, 1975; Nicoll *et al.*, 1976), except that strychnine does not antagonize glycine-induced depolarization of primary afferent terminals (Barker *et al.*, 1975) or motoneurones (Evans, Francis & Watkins, 1976). Table 1 gives dose-ratios for antagonism obtained at different concentrations of the antagonists when applied to 14 hemisected immature rat spinal cord preparations. Log dose–response plots for GABA and glycine on VR responses and for GABA only on DR responses were measured before and after attainment of equilibrium with antagonist solutions. At least 30 min was necessary to reach equilibrium. In these experiments procaine (1 mM) was present in the Ringer solution.

Although the effects of these antagonists were reversible (Figure 3) the life time of the preparation (approximately 16 h) did not allow recovery from antagonism to be observed in the experiments listed in Table 1, particularly when two antagonist concentrations were used. However, responses to standard doses of GABA or glycine declined by less than 10% over 12 h in the case of a preparation that was not treated with any antagonist.

Picrotoxin and bicuculline were more potent antagonists of DR responses than of VR responses; dose-ratios for antagonism of GABA-induced DR responses were always higher than those for VR responses (Table 1). This difference is illustrated by comparison of Figure 3a and b for picrotoxin and the upper and lower trace of Figure 3 for bicuculline. It was also noted that picrotoxin and bicuculline showed no selectivity towards DR responses produced by either GABA, β-alanine or taurine.

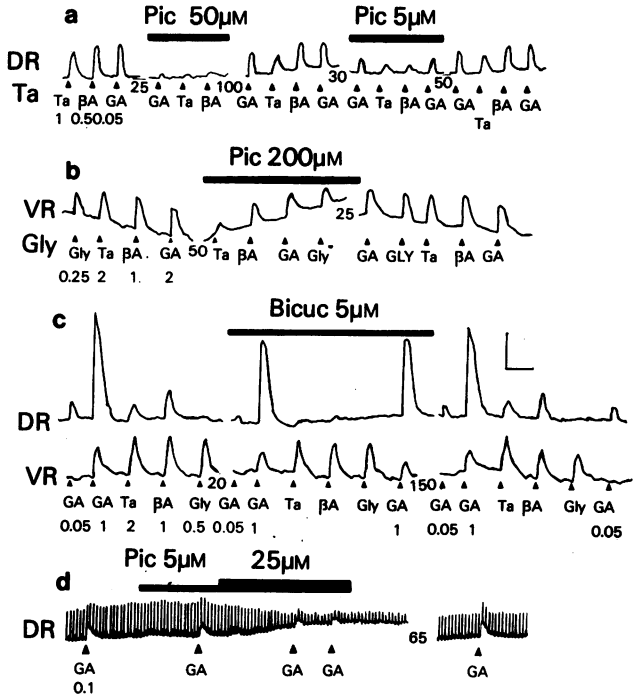

Figure 3 Effect of picrotoxin (Pic) and bicuculline (Bicuc) on depolarizations produced by taurine (Ta), β-alanine (βA), γ-aminobutyrate (GA) and glycine (Gly). Amino acid concentration (mM) shown beneath records. DR, dorsal root record. VR, ventral root record. (a), (b) and (c) separate preparations recorded in the presence of procaine 1 mM. (d), Recorded in the absence of procaine with electrical stimulation (1/min) of adjacent dorsal root. Intervals between traces shown in minutes. Calibration (a), (b) and (c) vertical 1 mV, horizontal 10 min; (d) vertical 0.93 mV.

Table 1 Dose-ratios for antagonism of responses of motoneurones (ventral root (VR), γ-aminobutyrate (GABA) and glycine) and primary afferent terminals (dorsal root (DR), GABA only) by picrotoxin, bicuculline and strychnine.

Picrotoxin		*5*	*25*	*50*	*100* μM
Glycine	VR	1.5		1.8(2)	
GABA	DR	3.8	3.8	14.5 ± 8.5(3)*	6.9
	VR	2.4	2.4	3.3 ± 1.9(3)	3.3
Bicuculline		*5*	*25*	*50*	*100* μM
Glycine	VR	1	1.3(2)	1.5	2.2
GABA	DR	2	7.4 ± 1.4(3)	6.3	15.0 ± 17(4)†
	VR	2	4.2 ± 1.8(3)	6.3	7.0 ± 3.2(4)
Strychnine		*1*	*4*	*10* μM	
Glycine	VR	9.0 ± 1.2(3)	18(2)	28 ± 30(3)‡	
GABA	DR	2.2 ± 0.8(3)	2.2(2)	2.2(2)	
	VR	1.1 ± 0.2(3)	1.0(2)	1.2 ± 0.4(3)	

Dose-ratios were measured from the parallel displacement of log dose-response plots. Number of preparations in parentheses. Standard deviations of dose-ratios are given for results from three or more preparations.

*, † and ‡; the lowest dose-ratios recorded in each of these groups were 8.1, 5.0 and 10.0 respectively.

Bicuculline was the more potent and specific of the two GABA antagonists, because dose-ratios for bicuculline antagonism of GABA-induced VR responses were higher than those for picrotoxin and, whereas bicuculline showed some selectivity towards antagonism of GABA-induced VR responses, taurine-induced VR responses were depressed more by picrotoxin than were GABA-induced responses (Figure 3). The selectivity observed with bicuculline on VR responses disappeared at higher concentrations (100 μM).

The DR-DRP was depressed by 25 μM picrotoxin (Figure 3d) and 5 μM bicuculline had a similar effect. These concentrations of the antagonists were also effective in blocking the DR response induced by neutral amino acids (Figure 3a and c, upper trace).

Strychnine was the most potent and specific of the three antagonists tested in terms of discriminating between GABA- or glycine-induced VR responses. No antagonism of GABA-induced VR responses occurred at levels of strychnine which produced dose-ratios of 10 or more against glycine-induced VR responses (Table 1). However, strychnine did not discriminate between VR responses produced by glycine, β-alanine or taurine.

Discussion

Amino acids

The results show that the isolated hemisected immature rat spinal cord has similar pharmacological specificity to the spinal cord of the adult rat *in vivo*.

An anomaly in the action of the neutral amino acids on the present preparation was that they all depolarized motoneurones whereas it is known that in the mature cat spinal cord *in vivo*, glycine, β-alanine and GABA produce hyperpolarization of motoneurones (Curtis *et al.*, 1968). This motoneuronal depolarizing action of neutral amino acids may represent a physiological difference between immature and mature animals or it may be a consequence of redistribution of chloride, perhaps due to the low temperature of incubation or hypoxia, and possibly associated with a swelling of the cells after isolation of the hemicord. However, increasing the temperature of 35°C, preincubation in Ringer solution which contained methyl sulphate instead of chloride or incubation in Ringer solution made hypertonic with sucrose, did not reverse the responses.

Taurine was the only one of the four neutral amino acids tested that produced a clear depressant action on spontaneous activity. The taurine content of the brains of rats up to 12 days old is unusual in being four times higher than the levels found in adult animals (Davies & Himwich, 1973). However, glycine was the most potent neutral amino acid to produce a VR response and GABA the most potent in the case of DR responses. This would accord with the accepted role for GABA as the presynaptic inhibitory transmitter and for glycine as the postsynaptic inhibitory transmitter in the mammalian spinal cord. The rapid fade of the DR response observed with high doses of GABA compares with the effects of high doses of GABA applied electrophoretically to cortical neurones *in vivo* (Dreifuss, Kelly & Krnjević, 1969).

Antagonists of neutral amino acids

Strychnine showed a similar specificity to that observed with frog motoneurones (Evans & Watkins, 1975; Nicoll *et al.*, 1976). Responses to GABA were unaffected by strychnine and responses to glycine, β-alanine and taurine were blocked to a similar extent. There was no evidence of a strychnine-resistant glycine response as observed in the frog (Tebēcis & Phillis, 1969; Nicoll *et al.*, 1976; Evans *et al.*, 1976).

The relative contribution of blockade of pre- and postsynaptic inhibitions in the convulsant action of picrotoxin is not known, but the greater potency of picrotoxin in antagonizing DR responses compared with VR responses would suggest blockade of presynaptic inhibition to be the more important factor. Bicuculline also was a more potent antagonist of DR responses than of VR responses and this effect observed with picrotoxin and bicuculline suggests that neutral amino acid receptors on afferent terminals may be different from those on motoneurones. Such differences in neutral amino acid and antagonist receptors on pre- and postsynaptic sites have been discussed previously by Hill, Simmonds & Straughan (1973) and Nicoll *et al.* (1976).

Experiments with isolated synaptic membrane fragments have revealed competition for binding between strychnine and glycine, and between bicuculline and GABA, in rat spinal cord (Young & Snyder, 1973) and brain (Zukin, Young & Snyder, 1974) respectively. The variability of the data in Table 1 does not allow conclusions to be made regarding the mechanism of the antagonisms observed. Nevertheless, application of the relationship $K = [\text{antagonist}]/\text{dose-ratio} - 1$ (Barlow, 1964) to the data of Table 1 yields an apparent dissociation constant for bicuculline and the primary afferent GABA receptor of 5–10 μM which compares favourably with the value of 5 μM reported by Zukin, Young & Snyder (1974). In the case of strychnine and the glycine receptor, the apparent dissociation constant (0.03 μM) reported by Young & Snyder, (1973) is in less close agreement with that derived from Table 1, which yields values varying from 0.1 μM at the lowest strychnine concentration to 3 μM at the highest strychnine concentration.

This work was supported by the Medical Research Council. The author is grateful to Dr J.C. Watkins and Professor J.F. Mitchell for helpful criticism during the preparation of the manuscript and to Dr R.B. Barlow for discussion of the data on antagonists.

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(Received December 2, 1976. Revised August 1, 1977.)