Br. J. Pharmac. (1975), 55 519-526

VENTRAL ROOT RESPONSES OF THE HEMISECTED AMPHIBIAN SPINAL CORD TO PERFUSED AMINO ACIDS IN THE PRESENCE OF PROCAINE

R.H. EVANS & J.C. WATKINS
Department of Pharmacology, Medical School, University Walk, Bristol BS8 1TD

1 The use of the procaine-blocked hemisected spinal cord preparation to identify the primary action of amino acids and their antagonists on amphibian motoneurones is described.

2 Apart from an anomalous effect of glycine, the responses of frog spinal motoneurones to amino acids were shown to be similar to those of mammalian spinal neurones.

3 In the presence of procaine, γ-aminobutyrate (GABA), taurine and β-alanine caused a hyperpolarizing response, measured in ventral roots, whereas L-glutamate and, to a lesser extent, glycine caused depolarization.

4 Picrotoxin and bicuculline specifically blocked ventral root responses to GABA; strychnine blocked responses to taurine and β-alanine but not responses to L-glutamate, glycine or GABA.

Introduction

Putative amino acid transmitters have been shown to have characteristic effects on the electrical activity of the hemisected amphibian spinal cord preparation as recorded from ventral and dorsal roots (Curtis, Phillis & Watkins, 1961). However, these actions of superfused amino acids on ventral root activity are complicated by indirect effects arising through actions on neurones which synapse with motoneurones or which are part of complex pathways terminating on motoneurones. To eliminate such indirectly mediated effects we have superfused preparations with procaine (1 mM) thereby eliminating all regenerative activity within the hemicord. The results show that, under these conditions, direct comparison can be made of the primary response of motoneurones to amino acids and of the potency of amino acid antagonists. In the course of the investigation, the use of procaine was compared with that of tetrodotoxin (Konishi & Otsuka, 1974) and high magnesium ion concentrations (Barker & Nicoll, 1973) both of which achieve a similar result. The preparation is discussed in terms of its usefulness for predicting amino acid agonist and antagonist activity on mammalian central nervous tissue.

Methods

Preparation of hemicords

Hemicords were set up in a superfusion trough as described by Curtis *et al.* (1961). Most experiments were carried out with hemicords from *Rana temporaria* but in some experiments hemicords from *Bufo bufo* were used. The latter preparations were somewhat easier to handle because of the longer spinal roots, but responses were similar in the two species, and frogs were more readily available. After being hemisected and placed in perfusion medium at 4° C, the preparations were found to be active for up to 8 days. Preparations were normally used within 4 days.

Electrical recording

Ventral roots VIII or IX were passed through a mixture of liquid paraffin/petroleum jelly and placed in contact with a chlorided silver wire. Potentials were recorded between this electrode and a similar one placed in contact with the Ringer solution. The perfusion medium was earthed via another Ag/AgCl electrode. Slow records of potential changes were made with a Vitatron 1 mV single channel ink writing pen recorder. Faster records of activity in ventral roots were made on photographic paper with a Telford oscilloscope camera and a Tectronix 502A oscilloscope. The records shown are representative of observations on at least 3 hemicords. An upward deflection on the records indicates an increase in negativity of the proximal region of the ventral root.

To stimulate the preparation, dorsal roots VIII or IX were passed through liquid paraffin/petroleum jelly and placed in contact with bipolar

silver electrodes through which were passed supramaximal square wave pulses (50 V, 0.05 ms duration).

Composition of perfusion medium

The preparations were superfused at a rate of approximately 5 ml/min with a solution of the following composition (mM): NaCl 111, KCl 2, NaH_2PO_4 1, $CaCl_2$ 2, $NaHCO_3$ 10, glucose 12, tris base 10. The pH of this mixture was adjusted to 7.9 with 11.3 M HCl and the preparation was maintained at $12.5 \pm 0.2^\circ C$. This solution was found to buffer adequately all the drug and amino acid solutions which were used; oxygenation was found to be unnecessary.

Addition of amino acid and drug solutions

The preparations were superfused at constant rate by means of a Watson Marlow MHRE 22 flow inducer. Amino acids and other drugs were dissolved in perfusion medium and placed in sample bottles in the rack of a Unicam SP4OP sample applicator. These solutions were added to the preparation by means of automatic pinchcocks which halted the flow of perfusion medium and allowed the drug solutions to flow onto the preparation. The period of perfusion of added substances was 40 s except where otherwise indicated.

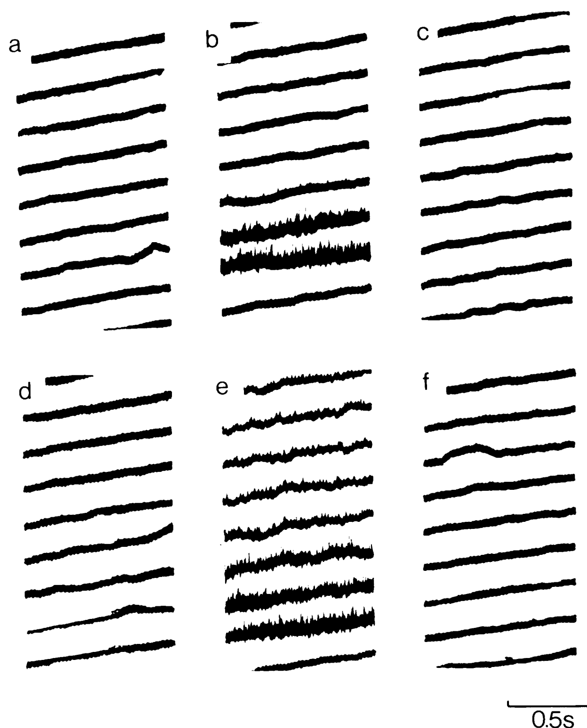

Figure 1 Effect of GABA (0.25 mM) and L-glutamate (0.5 mM) on spontaneous activity in ventral root of hemisected frog spinal cord. (a) and (c), 1 min before and 3 min after perfusion of L-glutamate respectively. (b) Recording during perfusion of L-glutamate (peak depolarization 0.42 mV). (e) Recording during perfusion of GABA (peak depolarization 1.17 mV). (d) and (f) 1 min before and 3 min after perfusion of GABA respectively. Each d.c. record progresses from the bottom to the top. Both amino acids were applied for a period of 45 seconds.

Results

Ventral root responses of unblocked hemicords

In the absence of procaine, ventral root responses to perfused amino acids were generally similar to those previously reported (Curtis *et al.*, 1961; Tebēcis & Phillis, 1969; Barker & Nicoll, 1973). L-Glutamate invariably caused depolarization of motoneurones (Figures 2 and 3) accompanied by a burst of repetitive firing of ventral root fibres during the early stages of the action (Figure 1). The action of γ-aminobutyrate (GABA) was biphasic. An initial depolarization, which is particularly marked in Figure 3, was associated with repetitive spike discharges similar to those produced by L-glutamate (Figure 1) and this was succeeded by a hyperpolarization. At high doses of GABA the initial depolarization was not observed. Taurine and β-alanine (Figure 2) also produced hyperpolarizing changes without, however, any preceding depolarizing phases. Glycine (Figures 2 and 3), at the same concentration as those which produced easily measurable effects with the other three amino acids, often had no effect, but at higher doses invariably caused depolarization.

Ventral root responses of procaine-blocked hemicords

In the presence of procaine the responses measured in the ventral root to GABA, β-alanine and taurine were always hyperpolarizing and the responses to L-glutamate were always depolarizing (Figure 2). The responses to glycine were invariably depolarizing under the standard conditions described (Figure 2) but could be converted to a hyperpolarization by a variety of means; this will form the basis of a separate communication.

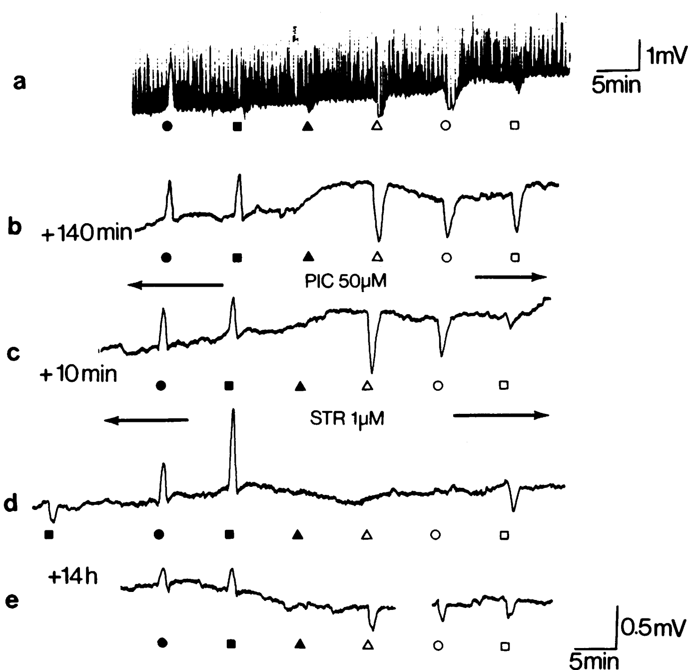

Figure 2 Comparison of the effects of amino acids on ventral root responses. (a) In the absence of procaine; (b)-(e) in the presence of procaine (1 mM); (c) effect of picrotoxin (PIC, 50 μM); (d) effect of strychnine (STR, 1 μM). Continuous d.c. record. L-glutamate, 0.25 mM (●); glycine, 1 mM (■); α-alanine, 1 mM (▲); β-alanine, 0.5 mM (△); taurine, 0.5 mM (○); GABA, 0.5 mM (□).

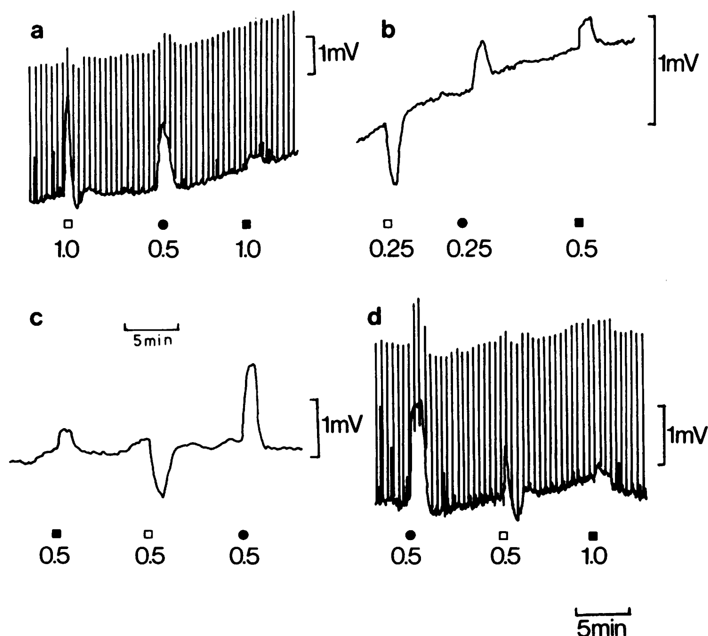

Figure 3 Effect of procaine on the ventral root response to amino acids of the toad spinal cord (d.c. record). Dorsal root stimulated at 2/minute. (a) Before addition of procaine to perfusion medium; (b) 100 min after adding procaine (1 mM) to perfusion medium; (c) another preparation in the presence of procaine (1 mM); (d) same preparation as (c), 100 min after washout of procaine. Glycine (■); GABA (□); glutamate (●). Amino acid concentration (mM) indicated on record.

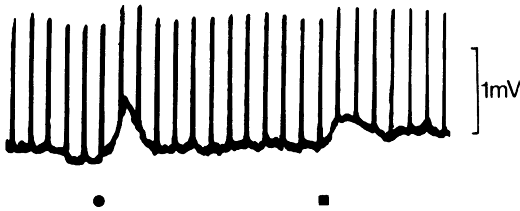

Figure 4 Effect of L-glutamate, 2.5 mM (•) and glycine, 10 mM (▪) on the polarity of frog ventral root (L VIII) measured *in vivo*. The exposed spinal cord of the cerebrally pithed animal was superfused at 1.5 ml/min and at the symbols the flow of the perfusion medium was interrupted and amino acid solution was superfused for 40 seconds. Dorsal root stimulated 2/minute.

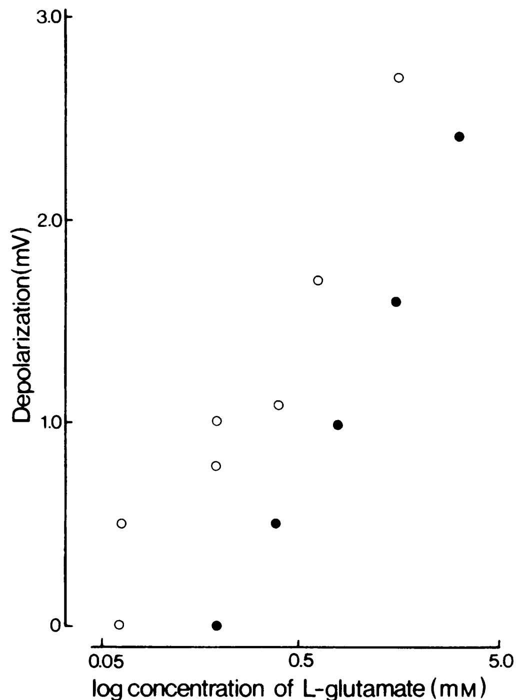

Figure 5 Relationship of concentration of L-glutamate to depolarization measured in ventral root of hemisected frog spinal cord. (o) Normal Ringer (procaine-free); (•) in the presence of procaine (1 mM).

The depressant effect of procaine on the electrical activity of hemicords was reversible as shown by Figure 3 in which the actions of L-glutamate, GABA and glycine are compared. These effects of the three amino acids are typical of potential changes which were observed in over 30 preparations. The finding that glycine depolarized preparations on which GABA had a hyperpolarizing action contrasts with the known hyperpolarizing action of glycine on mammalian motoneurones (Werman, Davidoff & Aprison, 1968, and Curtis, Hösli, Johnston & Johnston, 1968). Glycine also had a depolarizing action when it was applied to amphibian spinal cords *in vivo* (Figure 4) and this indicates that it was unlikely to have been the *in vitro* experimental conditions which caused glycine to produce the observed depolarization.

Effects of amino acids on the polarization of ventral roots were found to be dose-dependent. Figure 5 shows a dose-response plot obtained for L-glutamate. It can be seen that the dose-response relationship is shifted to the right on treatment of the preparation with procaine, as would be expected from the depression of the indirect excitation of motoneurones by the superfused glutamate.

Procaine has a depressant action on conductance changes at the post-junctional membrane of frog skeletal muscle (Maeno, 1966). To determine if procaine had any such effects on amino acid responses apart from those due to blockade of regenerative activity, the action of amino acids was observed on preparations which were treated with tetrodotoxin (10^{-7} M) or $MgSO_4$ (20 mM). Tetrodotoxin has no effect either on post-junctional conductance changes at the frog neuromuscular junction (Katz & Miledi, 1967) or on glutamate-induced depolarization of cat spinal motoneurones (Curtis, Duggan, Felix, Johnston, Tebēcis & Watkins, 1972). In the present experiments, the effect of tetrodotoxin and procaine were compared by measurement of the decrease in response to a standard dose of glutamate which occurred in the presence of these agents. A mean response of 58% (s.d. = 6) of the control response occurred in 5 tissues treated with tetrodotoxin (10^{-7} M); this compares with a mean value of 49% (s.d. = 14) for 7 tissues which were treated with procaine (1 mM). Amino acid responses obtained in the presence of $MgSO_4$ were similar to those obtained in the presence of procaine or tetrodotoxin. However, whereas procaine caused no depression of responses to L-glutamate in preparations which were treated with either tetrodotoxin or high Mg^{++} concentration, addition of $MgSO_4$ (20 mM) to procaine-blocked hemicords caused slight depression of responses to L-glutamate (Figure 6). In this experiment the control perfusion solution was made iso-osmotic with the $MgSO_4$ solution by the incorporation of sucrose (40 mM) into the control solution.

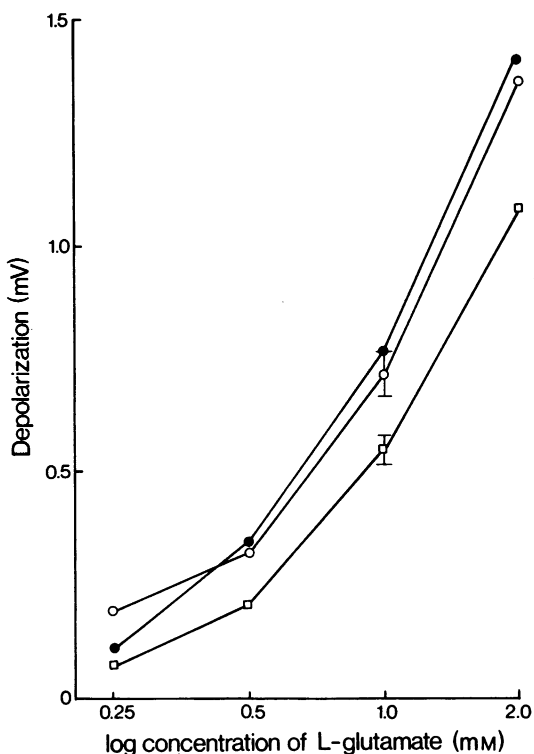

Figure 6 Depressant effect of $MgSO_4$ (20 mM) on ventral root response to L-glutamate in a procaine-blocked hemicord. (○) Control (+ 40 mM sucrose); (□) + 20 mM $MgSO_4$. (●) after washout of $MgSO_4$. Bars at 1 mM represent s.e. mean of 5 determinations.

It was of interest that the excitatory effects of acetylcholine (ACh) and carbachol, observed in unblocked preparations, were abolished by procaine (1 mM, Figure 7). This effect was not likely to have been caused by cholinolytic properties of the procaine because similar effects were also seen in preparations which were perfused with tetrodotoxin (10^{-7} M) or $MgSO_4$ (20 mM). As mentioned above, tetrodotoxin does not interfere with the local action of ACh at the neuromuscular junction (Katz & Miledi, 1967). Thus the excitatory action of ACh and carbachol on ventral root activity is most likely produced by an indirect action and not through stimulation of receptors on motoneurones. This conclusion contrasts with that of Matsuura (1971) who claimed the existence of depolarizing cholinoceptive sites on toad motoneurones. However, the experimental conditions used in that study may not have completely eliminated indirectly mediated effects.

Amino acid antagonists

Effects of strychnine and picrotoxin on ventral root potentials of the unblocked amphibian spinal cord have been reported previously (Tebēcis & Phillis, 1969; Barker & Nicoll, 1973; Barker, Nicoll & Padjen, 1975). The following results show that antagonism of amino acid induced changes in polarity of ventral roots may be specifically demonstrated in the presence of procaine.

Picrotoxin antagonized the responses produced by GABA but not those produced by L-glutamate, glycine, β-alanine or taurine (Figure 2c). The

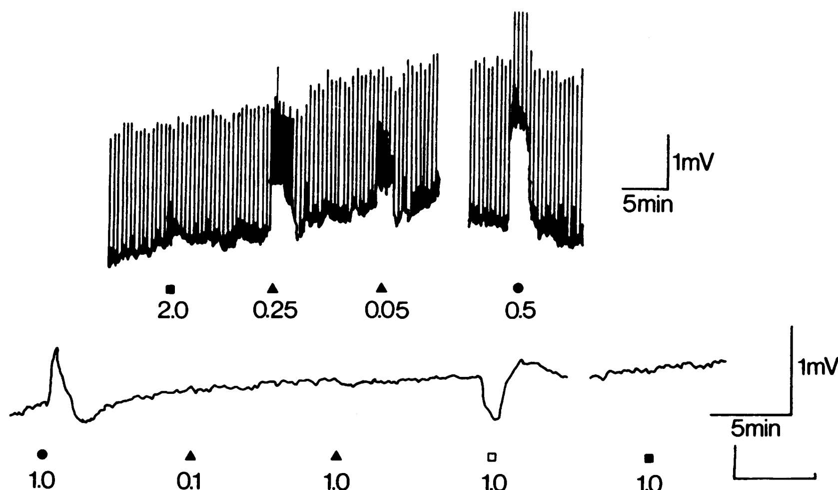

Figure 7 Effect of procaine (1 mM) on response of hemisected frog spinal cord measured in the ventral root (d.c. record) to acetylcholine (■); carbachol (▲); glutamate (●); GABA (□). Amino acid concentration (mM) indicated on record. Dorsal root stimulated at 2/minute. Lower trace in the presence of procaine (1 mM).

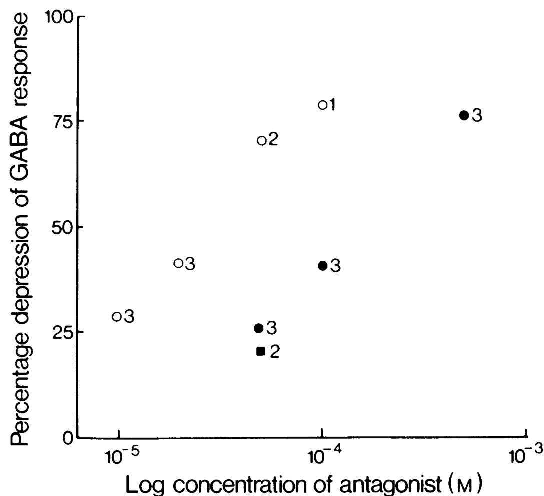

Figure 8 Comparison of picrotoxin (○), N-methyl bicuculline (●) and bicuculline (■) as antagonists of GABA in the procaine-blocked hemicord. The depression of the hyperpolarizing effect of 1mM GABA by each concentration of antagonist was measured in the ventral root. The antagonists were in contact with the preparation 5 min before addition of the test dose of GABA. The numbers suffixed to the points represent different preparations.

responses produced by taurine and β-alanine were blocked by strychnine but those produced by L-glutamate, glycine and GABA were not (Figure 2d). It should be noted here that strychnine, even in a 1 mM concentration, had no depressant effect on responses to glycine; indeed a potentiation was observed. The blockade of β-alanine and taurine responses by strychnine was much more slowly reversible than the antagonism of GABA responses by picrotoxin. Recovery from the effect of picrotoxin was usually complete after 3 hours. In the record shown in Figure 2 recovery from picrotoxin was incomplete at the time of the strychnine addition, but continued during the presence of the alkaloid. Little or no recovery of β-alanine and taurine responses was observed 3 h after washout of strychnine from hemicords. The preparation illustrated by Figure 2 was left overnight in strychnine-free medium, after which responses to β-alanine and taurine could once more be observed; at this stage, however, all the responses were somewhat attenuated.

With the method described it proved possible to compare the potencies of picrotoxin, N-methylbicuculline and bicuculline as GABA antagonists. This comparison is shown on Figure 8. In these experiments the depression of the hyperpolarization produced by a standard dose of GABA, in this case 1 mM, was measured in the presence of each concentration of antagonist. It can be seen from Figure 8 that picrotoxin was about five times more potent than bicuculline or N-methylbicuculline. Bicuculline and N-methylbicuculline appeared to be equipotent except that it was not possible to obtain accurate figures for bicuculline at concentrations higher than 0.1 mM because of its low solubility.

In the presence of procaine a saturated solution of bicuculline had no effect on ventral root responses to 1 mM glycine or L-glutamate, but depressed the response to 1 mM GABA by approximately 70%.

Discussion

It is clear from the results that, provided all regenerative activity is blocked, the isolated amphibian spinal cord preparation represents a useful system for the quantitative examination of the pharmacology of neuronal amino acid receptors. Other investigations have indicated, however, that amino acid receptors on different types of preparation may differ considerably from one another. For example, the relationship between structure and activity of excitatory substances at the insect neuromuscular junction (Clements & May, 1974) is very different from that observed on central neurones of mammalian preparations (Curtis & Watkins, 1960, 1963). The question therefore arises as to whether the amphibian preparation can provide experimental data of direct relevance to mammalian systems.

Recent results have indicated that, in the case of excitant amino acids, there is a remarkable degree of parallelism between excitation of rat spinal neurones and depolarization of the procaine-blocked frog spinal cord (Biscoe, Evans, Headley, Martin & Watkins, 1975; Evans & Watkins, 1975). This suggests a similarity in receptor structure for the excitatory amino acids in amphibians and mammals.

The only apparent exception to a similar parallelism between the action of inhibitory amino acids at amphibian and mammalian neurones is the effect of glycine. Whereas glycine hyperpolarizes cat spinal motoneurones, and strychnine blocks this action (Curtis *et al.*, 1968; Curtis, Hösli & Johnston, 1968), glycine depolarizes frog motoneurones and strychnine does not antagonize this response. On the other hand, strychnine does block specifically the hyperpolarizing effects of β-alanine and taurine on frog motoneurones, which parallels the antagonistic action of strychnine on both the hyperpolarization of cat spinal

neurones by β-alanine and the depression of the firing frequency of cat spinal neurones by β-alanine and taurine (Curtis *et al.*, 1968; Curtis *et al.*, 1968). Moreover, the action of GABA is specifically blocked by picrotoxin and bicuculline in mammals (Galindo, 1969. Curtis, Duggan, Felix & Johnston, 1971, respectively) and amphibians. Further work will be required to determine if any other anomalies exist, but from the present evidence it would appear that many responses of frog motoneurones will be directly relevant to mammalian systems.

The specificity of picrotoxin as an antagonist of ventral root responses to GABA (Figure 2c) contrasts with the action of this agent on dorsal root responses. Barker *et al.* (1975) found that picrotoxin was equally effective as an antagonist of dorsal root depolarizations produced by taurine, β-alanine or GABA. Thus it would seem that receptors for taurine, β-alanine and GABA on dorsal root fibres and primary afferent terminals are less specific than those of motoneurones.

The difficulties of interpretation of both the site and type of action of substances applied to unblocked tissue is exemplified by the variability in the effects of inhibitory amino acids found by other investigators (Tebēcis & Phillis, 1969). A striking example obtained in the present work was the initial excitation caused by GABA (Figure 1). This effect may be explained by an initial inhibition by GABA of interneurones which exert a tonic inhibitory influence on frog motoneurones. Alternatively, intense primary afferent depolarization produced by GABA may cause the release of excitatory transmitter (Barker & Nicoll, 1973) onto interneurones which synaptically excite motoneurones. The blockade of regenerative activity within the cord by procaine abolished this excitatory phase and revealed the direct hyperpolarizing action of GABA on motoneurones.

Glycine is now generally accepted to be the strongest candidate for post-synaptic inhibitory transmitter function in the mammalian spinal cord, although the evidence does not exclude taurine and β-alanine from playing similar roles (Curtis & Johnston, 1974). The inhibitory effects of all three amino acids are antagonized by strychnine, which also blocks short latency post-synaptic inhibition of mammalian spinal motoneurones. If strychnine also blocks post-synaptic inhibition of amphibian spinal motoneurones, then the present results would indicate that the transmitter(s) involved are more likely to be taurine and/or β-alanine, rather than glycine. However, it should be noted that strychnine potentiated the glycine-induced depolarizations of frog motoneurones (Figure 2). This observation suggests the presence of a hyperpolarizing component of the glycine action which is sensitive to strychnine, and this component may represent a glycine action on inhibitory transmitter receptors.

Moreover, the possibility cannot be excluded that GABA also acts as a post-synaptic inhibitory transmitter at motoneurones. For instance bicuculline blocks the inhibitory effects of GABA at mammalian motoneurones (Curtis *et al.*, 1971) and the present results have shown that picrotoxin and bicuculline can antagonize the hyperpolarizing effect of GABA at amphibian motoneurones. The observation of a picrotoxin sensitive component of post-synaptic inhibition at cat motoneurones (Kellerth & Szumski, 1966) supports the possibility of a post-synaptic inhibitory transmitter role for GABA in mammals. However, a picrotoxin or bicuculline-sensitive post-synaptic inhibition of motoneurones in amphibians has yet to be demonstrated.

The authors are grateful for grants received from the Medical Research Council and the National Fund for Research into Crippling Diseases.

References

BARKER, J.L. & NICOLL, R.A. (1973). The pharmacology and ionic dependency of amino acid responses in the frog spinal cord. *J. Physiol., Lond.*, **228**, 259-277.

BARKER, J.L., NICOLL, R.A. & PADJEN, A. (1975). Studies on convulsants in the isolated frog spinal cord. II. Effects on root potentials. *J. Physiol., Lond.*, **245**, 537-548.

BISCOE, T.J., EVANS, R.H., HEADLEY, P.M., MARTIN, M. & WATKINS, J.C. (1975). Domoic and quisqualic acids: Potent excitants of frog and rat spinal neurones. *Nature, Lond.*, **255**, 166-167.

CLEMENTS, A.N. & MAY, T.E. (1974). Pharmacological studies on a locust neuromuscular preparation. *J. exp. Biol.*, **61**, 421-442.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. & JOHNSTON, G.A.R. (1971). Bicuculline an antagonist of GABA and synaptic inhibition in the spinal cord. *Brain Res.*, **32**, 69-96.

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

CURTIS, D.R., HÖSLI, L. & JOHNSTON, G.A.R. (1968). A pharmacological study of the depression of spinal neurones by glycine and related amino acids. *Brain Res.*, **6**, 1-18.

CURTIS, D.R., HOSLI, L., JOHNSTON, G.A.R. & JOHNSTON, I.H. (1968). The hyperpolarization of

spinal motoneurones by glycine and related amino acids. *Exp. Brain. Res.*, **5**, 235-258.

CURTIS, D.R. & JOHNSTON, G.A.R. (1974). Amino acid transmitters in the mammalian central nervous system. In *Reviews of Physiology,* Vol. 69, pp. 98-188. Berlin: Springer-Verlag.

CURTIS, D.R., PHILLIS, J.W. & WATKINS, J.C. (1961). Actions of amino acids on the isolated hemisected spinal cord of the toad. *Br. J. Pharmac.*, **16**, 262-281.

CURTIS, D.R. & WATKINS, J.C. (1960). Excitation and depression of spinal neurones by structurally-related amino acids. *J. Neurochem.*, **6**, 117-141.

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

EVANS, R.H. & WATKINS, J.C. (1975). Amino acid receptors on frog spinal motoneurones. *Br. J. Pharmac.*, **54**, 238P.

GALINDO, A. (1969). GABA-picrotoxin interaction in the mammalian central nervous system. *Brain Res.*, **14**, 763-767.

KATZ, B. & MILEDI, R. (1967). Tetrodotoxin and neuromuscular transmission. *Proc. R. Soc. B*, **167**, 8-22.

KELLERTH, J.O. & SZUMSKI, A.J. (1966). Effects of picrotoxin on stretch-activated post-synaptic inhibitions in spinal motoneurones. *Acta physiol. Scand.*, **66**, 146-156.

KONISHI, S. & OTSUKA, M. (1974). The effects of substance P and other peptides on spinal neurones of the frog. *Brain Res.*, **65**, 397-410.

MAENO, T. (1966). Analysis of sodium and potassium conductances in the procaine end-plate potential. *J. Physiol., Lond.*, **183**, 592-606.

MATSUURA, S. (1971). Cholinergic transmission in the recurrent facilitatory pathway of the spinal motoneurone of the toad. *Jap. J. Physiol.*, **21**, 475-487.

TEBĒCIS, A.K. & PHILLIS, J.W. (1969). The use of convulsants in studying possible functions of amino acids in the toad spinal cord. *Comp. Biochem. Physiol.*, **28**, 1303-1315.

WERMAN, R., DAVIDOFF, R.A. & APRISON, M.H. (1968). Inhibitory action of glycine on spinal neurones in the cat. *J. Neurophysiol.*, **31**, 81-95.

(Received May 1, 1975.)