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L-Proline depolarizes rat spinal motoneurones by an excitatory amino acid antagonist-sensitive mechanism

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1 Isolated spinal cords prepared from neonatal rats were used to examine the effects of L-proline (L-Pro).

2 L-Pro (1–8 mM) depolarized ventral and dorsal roots in a dose-dependent manner with one sixth of the potency of L-glutamate (L-Glu). L-Pro was four times more potent than D-Pro. Prolonged application of L-Pro produced a plateau depolarization of motoneurones with no apparent fade.

3 Omission of calcium ions from the medium potentiated the depolarizing actions of L-Pro, L-Glu and quisqualate.

4 L-Pro was antagonized by concentrations of 2-amino-5-phosphonovalerate (25 μM), γ-D-glutamylglycine (100 μM) and Mg^{2+} ions (1 mM) that depressed responses to N-methyl-D-aspartate (NMDA). The NMDA receptor-mediated component of the response to L-Pro was estimated to be 60–70%.

5 These data suggest that L-Pro should be considered as a possible excitatory neurotransmitter and that, because L-Pro is a neutral compound, excitatory amino acid receptors may not require an agonist to possess two anionic groups and one cationic group.

Introduction

Investigations into the neuroactive properties of endogenous amino acids since the pioneering studies of Curtis, Watkins and co-workers (Curtis *et al.*, 1960, Curtis & Watkins, 1960; 1963) have provided compelling evidence that excitant amino acids are extensively utilized as neurotransmitters in the mammalian CNS (Curtis & Johnston, 1974; Johnson 1978; Nistri & Constanti, 1979; Cotman & Nadler, 1981; Watkins & Evans, 1981; Fonnum, 1984). The receptors that mediate the excitatory actions of amino acid transmitters have been categorized into at least three classes according to their most specific agonist ligands, N-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (QA) (Evans & Watkins, 1981; Watkins & Evans, 1981; McLennan, 1981). The strongest evidence favours a transmitter role for glutamate and aspartate (Cotman & Nadler, 1981; Watkins & Evans, 1981), but several other amino acids have been proposed to interact with glutamate/aspartate-sensitive receptors, e.g. homocysteate (Evans *et al.*, 1979; Collins & Brown, 1986) and quinolinate (Stone & Connick, 1985).

Neuroactive actions of L-proline (L-Pro) have been reported by a number of groups. A glycine-like depressant action was seen in studies of spinal neurones (Curtis & Johnston, 1974) although inhibition of cerebellar Purkinje neurones was not blocked by strychnine (Felix & Künzle, 1974). In contrast, an interaction with L-glutamate (L-Glu) receptors has been suggested in other studies. Evidence for antagonism of glutamate receptors was provided by Van Harreveld (1980) and Segal (1976) while a partial agonist action of L-Pro appeared to underlie antagonism of glutamate in chick retina (Van Harreveld, 1979;1984; Van Harreveld & Reuter, 1981) and hippocampal neurones (Van Harreveld & Strumwasser, 1981). However, Ault & Nadler (1984) proposed only an excitatory (depolarizing) action of L-Pro on hippocampal pyramidal cells, sensitivity to γ-D-glutamylglycine suggesting a stimulation of excitatory amino acid receptors. Unlike L-Glu, however, L-Pro does not consistently excite CNS neurones (Zarzecki *et al.*, 1975; Segal, 1976). Because of the possible physiological and pharmacological importance of a neuroexcitatory action of L-Pro, we have examined directly the depolarizing effect of L-Pro upon rat spinal neurones and compared the effect of L-Pro to other excitatory amino acids.

Methods

Preparation of spinal cord tissues

Spinal cords were dissected from 3–6 day old rats as described by Otsuka & Konishi (1974), hemisected by cutting along the ventral fissure, and mounted so that d.c. potentials could be recorded from the L4 or L5 spinal roots (Evans & Watkins, 1978). The preparations displayed spontaneous activity, and segmental dorsal root-evoked ventral root potentials were recorded (Figure 1). In some preparations the dorsal root d.c. potential was recorded from the dorsal root adjacent to that stimulated (Figure 1). In four cases, isolated L5 dorsal roots were mounted so that the proximal end of the root was superfused with medium (Evans, 1980; Agrawal & Evans, 1986). Upward deflections on the chart records reflect depolarization of the motoneurones or afferent fibres from which potentials were measured. The preparations were superfused by the use of a roller pump at approximately 1.0 ml min^{-1} with medium of composition (mM): NaCl 118, KCl 3, $NaHCO_3$ 24, $CaCl_2$ 2.5, NaH_2PO_4 2.1, D-glucose 12, which was maintained between 25°C and 26°C.

Application of drugs

Drugs were applied to the spinal cords by two methods. Agonists were normally applied in short pulses by adding 2–10 μl of a neutralized stock solution to 2 ml of medium to achieve a desired drug concentration, and injecting the medium immediately upstream of the superfusion pump so that it displaced normal medium along narrow bore Teflon tubing towards the reservoir. Antagonists, or media of altered composition, were superfused continuously by replacing the reservoir of medium. Care was taken to ensure that, after changing the composition of superfusion media, equilibrium was reached before assessing alterations in responses to agonists. Usually tetrodotoxin (10^{-7} M) was included in the medium (Evans & Watkins, 1978). The amplitudes of depolarizing responses were measured from the baseline preceding agonist application. Where possible, agonist responses were measured before and after washout of an antagonist under study. The degree of inhibition was calculated by comparing the amplitude of agonist response in the presence of an antagonist to the mean of responses prior to, and after washout of, the antagonist.

Materials

Compounds were acquired from Sigma Chem. Co. (St. Louis, MO, U.S.A.) except for (−)-2-amino-5-phosphonovalerate ((−)-2-APV) which was purchased from Cambridge Research Biochemicals (New York, NY, U.S.A.).

Results

Depolarizing action of L-proline

In three preparations, superfusion of 2 ml pulses of L-Pro (1–8 mM) produced a dose-dependent depolarization of both ventral and dorsal roots, with a rapid onset and offset of comparable time course to equivalent doses of L-Glu (Figure 1). The relative sensitivity of dorsal roots, compared to ventral roots, was similar for both L-Pro to L-Glu (Figure 1). Depolarizing responses to L-Pro were also recorded in the presence of tetrodotoxin (TTX), although, in common with other depolarizing agonists, the amplitudes of the potentials were reduced (Figure 1). Blockade of regenerative activity reduces possible indirect depolarization due to excitation of intraspinal neurones and the subsequent release of transmitters, therefore other experiments were performed in the presence of TTX. Only ventral root potentials were usually studied.

L-Pro (1–8 mM) depolarized ventral roots in a dose-dependent manner (Figures 1 and 2), the responses being reproducible within experiments and similar between preparations (Figure 2). L-Glu was approximately 6 times more potent than L-Pro, whilst D-Pro and other substituted analogues had decreased potency (Table 1).

Prolonged application of L-Pro generated a depolarizing response that reached a plateau within 5 min and did not exhibit notable fade (Vyklický *et al.*, 1982). If L-Pro were acting as a weak partial agonist, depolarizations induced by L-Glu should be greatly inhibited during prolonged superfusion of L-Pro. However, responses to submaximal doses of L-Glu and noradrenaline (NA) were, on average, reduced to similar degrees ($15 \pm 12\%$, $n = 4$, and $12 \pm 11\%$, $n = 3$, respectively) by 4 mM L-Pro.

Studies using receptor antagonists

(±)-2-Amino-5-phosphonovalerate (2-APV; 25 μM) and γ-D-glutamylglycine (γ-DGG; 100 μM) preferentially reduced the responses to NMDA while L-Pro was somewhat less sensitive to these antagonists (Figure 3 and Table 2). In four experiments, L-Pro dose-response curves were studied in the absence and then the presence of 25 μM (±)-2-APV (Figure 2). The degree of antagonism by 2-APV was similar (45–55%) for all the doses studied (Figure 2). To estimate the NMDA receptor-mediated component of the response to L-Pro, (−)-2-APV was used at a dose (100 μM) that would be predicted to occupy 95% of

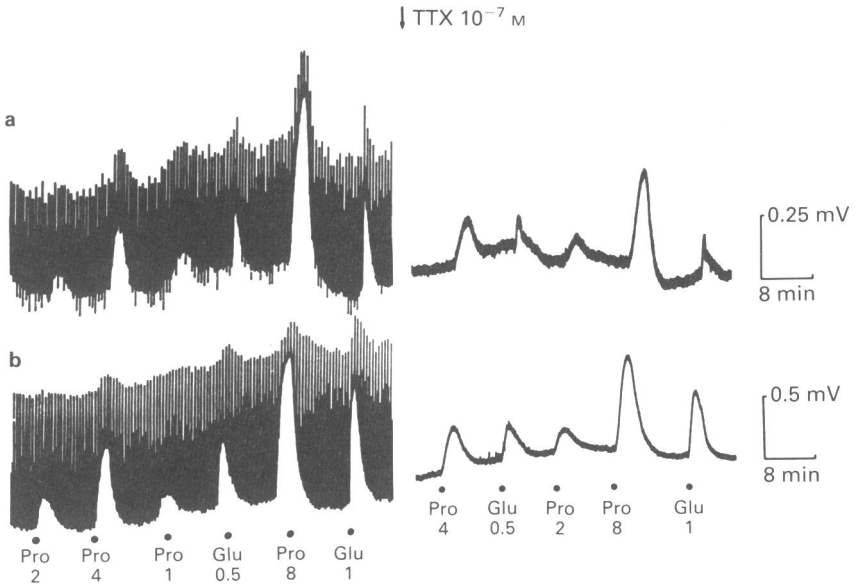

Figure 1 Depolarizing action of L-proline (Pro) and L-glutamate (Glu) upon dorsal and ventral roots of the isolated spinal cord of the neonatal rat. Chart recordings of the d.c. dorsal (a) and ventral (b) root potentials reflect spontaneous activity and evoked potentials (high amplitude deflections) subsequent to stimulation of the dorsal root in the same lumbar segment from which the ventral root recording was made (L4 or L5). Depolarization is in an upward direction and reflects a more positive potential of the recording electrode at the distal end of a spinal root with reference to the indifferent electrode in contact with the superfusion medium. The stimuli were at a rate of 2 min^{-1} with a pulse width of 40 μs at a maximally effective current strength; 2 ml boluses of the compounds were superfused over the spinal cord at a rate of 1 ml min^{-1} at the stated millimolar concentrations. At the point indicated, medium containing tetrodotoxin (TTX) was superfused at 10^{-7} M in order to block regenerative activity (referred to as TTX-blocked). L-Pro and L-Glu still produced consistent dose-dependent depolarizations in the presence of TTX although the amplitudes of these responses were reduced when compared to the non-TTX-blocked cord.

NMDA receptors. This concentration of 2-APV inhibited responses to L-Pro by 65 ± 7%, $n = 3$ with only a small concomitant reduction of QA-induced depolarizations (6 ± 7%, $n = 3$).

Table 1 Relative molar potencies of some amino acids compared to L-proline

Compound	*Relative potency*	n
L-Proline	1.0	6
L-Glutamate	6.0 ± 1	4
D-Proline	0.24 ± 0.1	4
trans-4-Hydroxy-L-proline	0.33 ± 0.01	3
cis-4-Hydroxy-L-proline	0.12 ± 0.04	3

Determinations of the potencies of compounds relative to L-Pro for the ability to depolarize ventral roots in the presence of tetrodotoxin were made from bracketing assays. The number of spinal cords in which the relative potencies (means ± s.e.means) were assessed is indicated by *n*.

γ-DGG (100 μM) reduced the responses of all the test compounds examined to some degree (Figure 3b and Table 2). Superfusion of γ-DGG at a concentration of 1 mM, which did not antagonize 0.5 or 0.75 mM γ-aminobutyric acid (GABA) ($n = 3$), inhibited responses to QA to a greater degree than at 100 μM, 43 ± 8% ($n = 3$) compared to 15 ± 5% ($n = 8$), but the antagonism of L-Pro was only increased from 58.5 ± 4% ($n = 7$) to 69 ± 6% ($n = 4$).

Effects of magnesium ions

Addition of magnesium sulphate (1 mM) to the medium reversibly depressed the responses to NMDA most effectively (Figure 3c), with QA and KA being least sensitive as previously shown (Ault *et al.*, 1980). L-Pro and L-Glu were inhibited to an intermediate degree (Table 2). In order to compare data from spinal cord and hippocampal slices, the effect of 2-APV was examined in the presence of 1 mM $MgSO_4$. In these experiments (±)-2-APV (25 μM) reduced the responses to L-Pro and NMDA by 21 ± 8% ($n = 3$) and

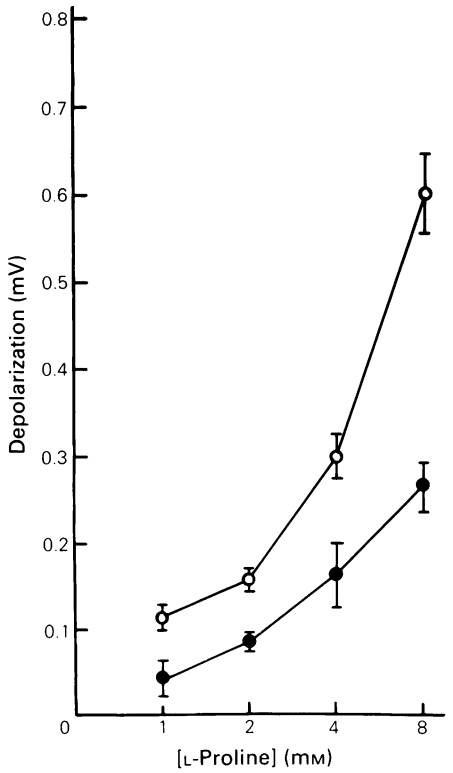

Figure 2 Dose-dependent effects L-proline (L-Pro). Data points indicate the depolarizing response of ventral roots induced by doses of L-Pro in tetrodotoxin (TTX)-blocked preparations superfused with standard medium (○) and medium to which (±)-2-amino-5-phosphonovalerate (±)-2-APV, 25 μM) had been added (●). Values indicate the mean depolarizations recorded in four TTX-blocked preparations; s.e.mean shown by vertical lines.

84 ± 4% ($n = 3$) respectively but not QA-induced depolarizations ($n = 3$).

Superfusion of calcium-free medium

In an effort to minimize mechanisms by which compounds could depolarize lumbar motoneurones indirectly, four preparations were superfused with calcium-free medium. The effect of indirectly-acting sympathomimetic compound tyramine (Trendelenburg, 1963; Ault & Evans, 1978) was compared to that of L-Pro. Superfusion of calcium-free medium produced a hyperpolarizing shift in the baseline d.c. potential and reversibly potentiated responses to L-Pro, L-Glu and quisqualate (Figure 4a). In four preparations L-Pro-induced responses were increased by 82.5 ± 28% while depolarizations induced by tyramine were variably affected with a net reduction of 20 ± 25%, ($n = 4$). Depolarizations induced by L-Glu were enhanced by 59% and 42%, and the effects of quisqualate were increased by 69 and 106% in the preparations where these agonists were also applied.

Isolated fibre studies

Isolated dorsal root fibres of the neonatal rat depolarize in response to GABA, kainate, L-Glu and, to some extent, QA (Agrawal & Evans, 1986). In this preparation one can exclude an indirect action since no synaptic terminals are present, therefore the effect of L-Pro was investigated in four experiments. As illustrated in Figure 4b, consistent depolarizing responses were recorded during the superfusion of 2 ml pulses of kainate (5–10 μM). Only very weak and inconsistent responses, however, were achieved by application of QA (10–20 μM) or L-Pro (50–4000 μM). In agreement with Agrawal & Evans (1986), the depolarizing response to L-Glu (0.25 or 0.5 mM) rapidly desensitized.

Table 2 Effects of excitatory amino acid antagonists upon depolarizations induced by L-proline (L-Pro) and other amino acids

	NMDA	*L-Pro*	*L-Glu*	*KA*	*QA*
2-APV (25 μM)	72 ± 4 (7)	37 ± 6 (10)	10 ± 7 (5)	3 ± 3 (4)	3 ± 4 (4)
γ-DGG (100 μM)	82 ± 3 (5)	58 ± 4 (7)	15 ± 7 (5)	15 ± 6 (7)	15 ± 5 (8)
Mg^{2+} (1 mM)	80 ± 3 (3)	48 ± 8 (4)	27 ± 4 (3)	11 ± 9 (3)	1 ± 6 (4)

Ventral root depolarizations were induced by the following agonists: N-methyl-D-aspartate (NMDA), L-proline (L-Pro), L-glutamate (L-Glu), kainate (KA) and quisqualate (QA). Agonist concentrations were chosen to give similar levels of depolarization. Values indicate the mean ± s.e.mean % inhibition of the agonist-induced depolarizations determined in the presence of 2-amino-5-phosphonovaleric acid (2-APV), γ-D-glutamylglycine (γ-DGG) or magnesium sulphate (Mg^{2+}). The number of separate spinal cords in which the values were measured are indicated in parentheses.

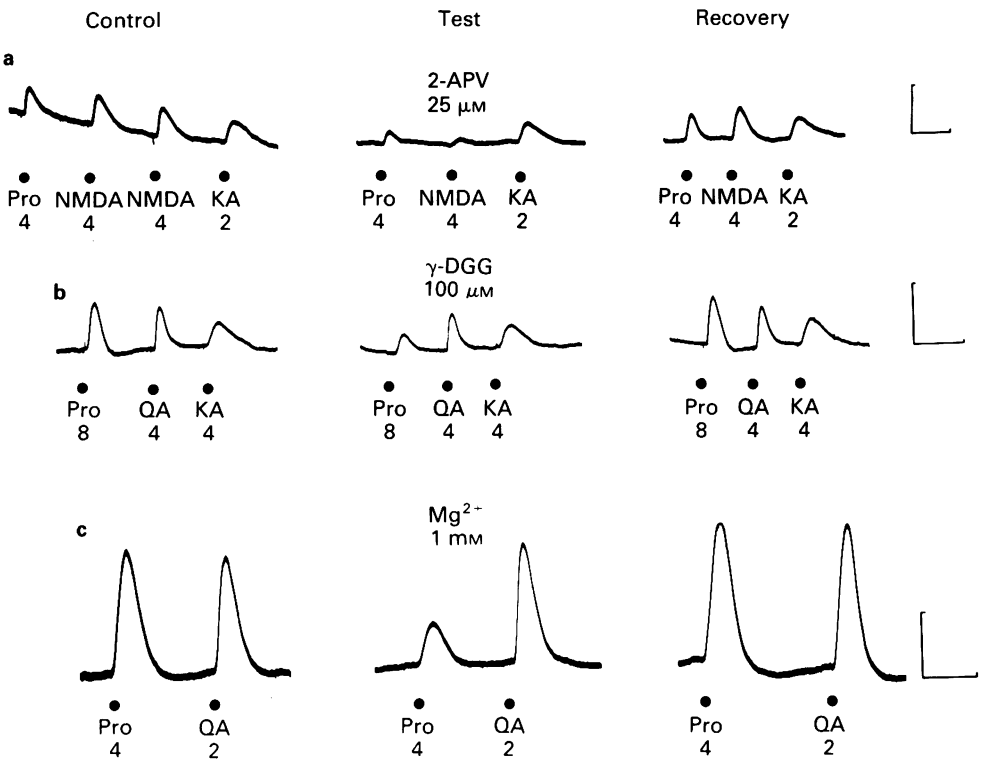

Figure 3 Effects of excitatory amino acid receptor antagonists. Depolarizations induced by L-proline (Pro) were reversibly antagonized by (±)-2-amino-5-phosphonovalerate (2-APV, 25 μM), γ-D-glutamylglycine (γ-DGG, 100 μM) and Mg^{2+} ions (1 mM) in a reversible manner. The doses indicated refer to mM concentrations of L-Pro and μM concentrations of N-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (QA). Tetrodotoxin was present in the medium. Calibrations: 0.5 mV and 8 min.

Discussion

In these experiments we have observed that millimolar doses of L-Pro induce consistent, dose-dependent depolarizations of lumbar motoneurones of the neonatal rat. This effect was stereospecific and structurally-selective since the D-isomer of proline and hydroxylated analogues were much less potent. The low potency of superfused L-Pro may be partly due to active uptake (Peterson & Raghupathy, 1972; Balcar *et al.*, 1976; Hauptmann *et al.*, 1983) in common with other amino acids (Brown *et al.*, 1980; Wood & Sidhu, 1986). L-Glu was six times more potent than L-Pro, which approximates to the relative potency in striatal tissue (Luini *et al.*, 1984). In agreement with the report of Vyclický *et al.* (1982), a depolarization of the intraspinal fibres of the dorsal roots was also noted (Figure 1).

An important question is whether L-Pro acted directly or indirectly upon dorsal root terminals and motoneurones. In studies of spinal root potentials it is generally accepted that ventral root responses to applied amino acid excitants are at least partly due to the interaction of the agonists with motoneurone receptors to increase membrane conductance (Sonnhof & Bührle, 1980). The lack of a consistent effect of L-Pro upon isolated dorsal root fibres suggests that the depolarizing response of dorsal roots to L-Pro was mainly due to increased extracellular potassium activity (Vyklický *et al.*, 1982), as is the case with NMDA, quisqualate and aspartate (Evans, 1980; Curtis *et al.*, 1984).

Apart from any effect of a rise in the concentration of extracellular potassium ions, depolarization of ventral roots could arise from a direct interaction of L-Pro with motoneurone receptors or the release of another transmitter from presynaptic terminals. Superfusion of tetrodotoxin and omission of calcium ions, although eliminating action potential-dependent and calium-dependent transmitter release, cannot

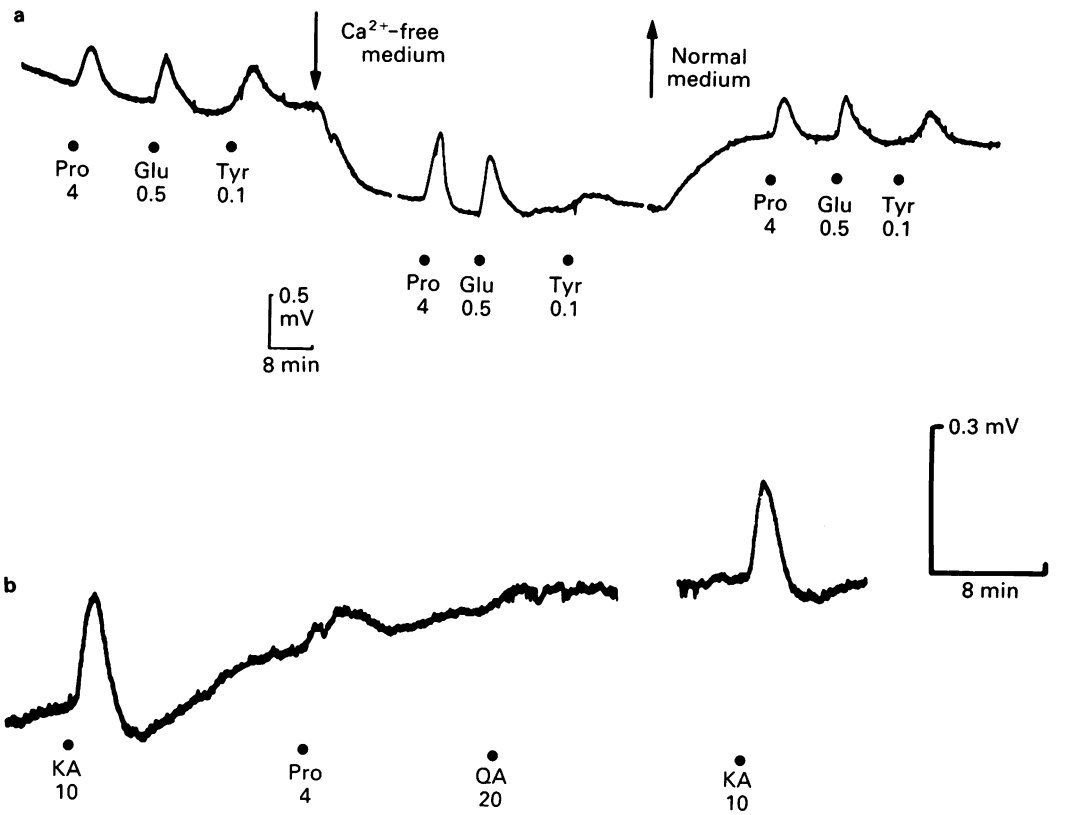

Figure 4 Superfusion of calcium-free medium and isolation of dorsal root fibres were used to minimize possible indirect actions of L-proline (L-Pro). (a) Depolarizations induced by L-Pro (Pro) and L-glutamate (Glu) were enhanced in the absence of calcium while the effect of tyramine (Tyr), an indirectly acting sympathomimetic amine was, on average, reduced. (b) Dorsal root fibres which had been isolated by sectioning the dorsal root at the point of entry into the spinal cord depolarized in response to superfusion of 10 μM kainate (KA). In contrast, only very weak depolarizing effects could be elicited by L-Pro or quisqualate (QA). Indicated doses refer to mM concentrations of L-Pro, L-glutamate and tyramine and μM concentrations of QA applied in the presence of tetrodotoxin.

effectively exclude a calcium-independent release as demonstrated with tyramine (Trendelenburg, 1963). If L-Pro did indeed release a transmitter in a manner analogous to tyramine, the antagonism by 2-APV, γ-DGG and Mg^{2+} ions suggests that it would be an excitatory amino acid. However, L-Pro inhibits rather than enhances the release of endogenous glutamate (Keller *et al.*, 1981), carriers for other amino acids do not transport L-Pro (Balcar *et al.*, 1976), and the relative potencies of the stereoisomers of proline do not correlate with their competition for uptake (Balcar *et al.*, 1976). It is thus likely that L-Pro acted, at least in part, directly upon motoneurones.

It has been proposed that proline interacts with glutamate receptors as an antagonist (Van Harreveld & Fifkova, 1973), partial agonist (Shank & Freeman, 1976; Van Harreveld, 1979; Van Harreveld & Strumwasser, 1981) or agonist (Ault & Nadler, 1984). Our data are consistent with the latter proposal since antagonists of gluatamate-sensitive receptors depressed the responses to L-Pro, while combined application of L-Pro and L-Glu provided no evidence of a partial agonist action. An agonist action of L-Pro, a neutral compound, appears at first to be anomalous since studies of structure-activity series have stressed the relationship between two anionic groups and one cationic group to the excitant action of amino acids (Curtis & Watkins, 1960). Exceptions have been noted, however, in the cases of asparagine and serine (Biscoe *et al.*, 1976), although the mechanism of action of these compounds is not known at present.

(−)-2-APV, the active stereoisomer of this antagonist (Evans *et al.*, 1982), would be expected to block 95% of NMDA receptors at a concentration of 100 μM

(Harrison & Simmonds, 1985; Davies *et al.*, 1986), which indicates that approximately 60–70% of the response to L-Pro was mediated by NMDA receptors. This figure is consistent with the effect of combining 25 μM (±)-2-APV and 1 mM magnesium ions. The other well characterized receptors sensitive to L-Glu are those activated by KA and QA. However, a substantial KA-like action appears to be unlikely due to the very weak effect of L-Pro upon isolated dorsal root fibres and a significant QA receptor-mediated component is inconsistent with the observation that raising the concentration of γ-DGG to 1 mM substantially depressed the response to QA but only slightly increased the antagonism of L-Pro. Furthermore, the maximal degree of antagonsim by γ-DGG, which blocks NMDA, QA and KA receptors, was similar to 2-APV. Responses to L-Glu that are independent of NMDA, QA or KA receptors have also been noted by Collins & Surtees (1986).

The antagonism of L-Pro by 2-APV is at variance with the lack of effect reported in studies using the hippocampal slice (Ault & Nadler, 1984). This difference is probably due to the magnesium content of the medium in the latter studies and the use of high test doses of L-Pro. Cellular uptake may also influence the relative contribution of NMDA receptors (Garthwaite, 1985).

Nerve terminals can synthesize (Yoneda & Roberts, 1982; Johnson & Roberts, 1984), accumulate (Peterson & Raghupathy, 1972; Balcar *et al.*, 1976; Hauptmann *et al.*, 1983) and release (Balcar *et al.*, 1976; Nickolson, 1982) proline which has a selective (Zarzecki *et al.*, 1975; Ault & Nadler, 1984; Nadler *et al.*, 1985) and stereospecific (Ault & Nadler, 1984) neuronal action. Taken together, these data suggest that L-Pro should be considered as a possible neurotransmitter or neuromodulator in the CNS.

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