Br. J. Pharmac. (1982), **77**, 363–373

THE USE OF LOW CONCENTRATIONS OF DIVALENT CATIONS TO DEMONSTRATE A ROLE FOR N-METHYL-D-ASPARTATE RECEPTORS IN SYNAPTIC TRANSMISSION IN AMPHIBIAN SPINAL CORD

PETER A. SMITH

Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

1 Synaptic potentials and the responses of frog spinal cord to various acidic amino acids were examined by means of the sucrose gap recording technique.

2 Divalent cations (50–250 μM) specifically antagonized responses evoked at N-methyl-D-aspartate (NMDA) receptors by N-methyl D,L aspartic acid (NMDLA). The rank order of potency was $Ni^{2+} > Co^{2+} \gg Mg^{2+} > Mn^{2+}$. Responses to glutamate and aspartate were relatively insensitive to these concentrations of divalent cations.

3 The rank order of potency for divalent ions (1 mM) for antagonism of synaptic transmission in bullfrog sympathetic ganglia was $Mn^{2+} \gg Co^{2+} > Ni^{2+} \gg Mg^{2+}$. Thus synaptic transmission in ganglia was especially sensitive to Mn^{2+} whereas NMDLA responses were especially sensitive to Co^{2+} and Mg^{2+}.

4 It was possible to depress selectively the dorsal root-dorsal root potential (DR-DRP) and dorsal root-ventral root potential (DR-VRP) of frog spinal cord using low doses of Co^{2+} or Mg^{2+} which did not affect VR-DRP (ventral root-dorsal root potential). It was not possible to produce this selective depression of DR-DRP and DR-VRP with Mn^{2+}, as this cation non-selectively depressed all responses.

5 These results suggest that: (i) divalent cations do not antagonize NMDLA responses by blocking Ca^{2+} channels which may mediate the response; (ii) postsynaptic NMDA receptors are activated by a neurotransmitter involved in the DR-DRP and DR-VRP pathways but not by any neurotransmitters involved in the VR-DRP pathway; (iii) the neurotransmitter activating NMDA receptors in amphibian spinal cord may be an aspartate-like substance rather than aspartate itself or glutamate.

Introduction

The acidic amino acids, L-glutamate and L-aspartate, are putative excitatory neurotransmitters in the central nervous system (CNS) (Krnjević, 1974; Puil, 1981). Three different types of receptors for excitatory amino acids have been characterized on the basis of their sensitivity to various agonists. These are N-methyl-D-aspartate (NMDA) receptors, quisqualate receptors and kainate receptors (Watkins & Evans, 1981). Certain lines of evidence support a role for calcium ions in the electrogenesis of responses to acidic amino acids (Sonnhof & Bührle, 1981; Padjen & Smith, 1981; Puil, 1981; but see also Shapavolov, Shiriaev & Velumain, 1978 and McDonald & Wojtowicz, 1980; 1981). Calcium influx may be especially important for responses evoked by DL-homocysteic acid, an agonist thought to act on NMDA receptors (Heinemann & Pumain, 1981). Recently, Co^{2+} and Mg^{2+} have been shown to be selective and highly potent antagonists at NMDA receptors (Ault, Evans, Francis, Oakes & Watkins, 1981). Since these ions are known to block calcium channels (Katz & Miledi, 1967; Alvarez-Leefmans, DeSantis & Miledi, 1979) the question arises as to whether their blockade of NMDA responses results from an action on a Ca^{2+} ionophore or at some other point between receptor activation and response. To address this question, the relative potencies of Ni^{2+}, Co^{2+}, Mn^{2+} and Mg^{2+} for blocking Ca^{2+} channels was compared with their relative potency in antagonizing responses at NMDA receptors. N-methyl-D,L-aspartate (NMDLA) was used to activate these receptors. The relative effects of divalent ions as Ca^{2+} channel blockers was measured by examining their ability to block synaptic transmission in sympathetic ganglia. This process depends on the presynaptic influx of Ca^{2+} ions to promote transmitter release (Blackman, Ginsborg & Ray, 1963). The results described below indicate that Mn^{2+} was much more effective than Co^{2+} or Mg^{2+} in blocking Ca^{2+} channels, but was relatively ineffective in blocking

0007-1188/82/100363–11 $01.00

NMDLA responses. On the other hand, Co^{2+} and Mg^{2+} were potent antagonists of NMDLA responses in doses that had minimal effect on Ca^{2+} channels. This suggests that divalent ions block NMDLA responses by an action at some site other than a Ca^{2+} ionophore associated with the NMDA receptor (cf. Davies, Evans, Francis & Watkins, 1979; Ault *et al.*, 1980).

Several synaptic responses may be recorded in the amphibian spinal cord by means of the sucrose gap technique (Barker, Nicoll & Padjen, 1975b; Padjen & Smith, 1980a). These include the orthodromic dorsal root-ventral root potential (DR-VRP), the dorsal root-dorsal root potential (DR-DRP) which involves primary afferent depolarization and the antidromic ventral root-dorsal root potential (VR-DRP). Experiments with 'organic' NMDA receptor antagonists have indicated that NMDA receptors may be involved in the generation of the slow, polysynaptic components of DR-DRP and DR-VRP (Evans, Smith & Watkins, 1981) but not VR-DRP (Padjen & Smith, 1980a). This conclusion is not supported by the work of Homma (1981) who demonstrated antagonism of VR-DRP with the NMDA antagonist, DL-α-aminoadipic acid. In the present study, low doses of Co^{2+} and Mg^{2+} have been used as NMDA 'receptor antagonists' so as to clarify the role of NMDA receptors in VR-DRP. The problem with this approach is that divalent cations will, of course, tend to block neurotransmitter release (Alvarez-Leefmans *et al.*, 1979). This problem was minimized by using 125 μM Co^{2+} or 250 μM Mg^{2+}. These low concentrations had no effect on VR-DRP and thus did not appear to block neurotransmitter release, but did produce marked depression of the slow (polysynaptic) components of DR-DRP and DR-VRP. This result supports the conclusion of Padjen & Smith (1980a) than an aspartate-like neurotransmitter, presumably acting on NMDA receptors, is involved in DR-DRP and DR-VRP but not VR-DRP. Since aspartate responses were relatively insensitive to divalent cations it is possible that the neurotransmitter activating NMDA receptors in DR-VRP and DR-DRP is some 'aspartate like' substance rather than aspartate itself.

Methods

The VR-DRP (ventral root-dorsal root potential), DR-DRP (dorsal root-dorsal root potential) and DR-VRP (dorsal root-ventral root potential) of the isolated hemisected spinal cord of the frog were examined by means of the sucrose gap technique as described by Barker, Nicoll & Padjen (1975a, b). Preparations were equilibrated overnight with Ringer solution at 5°C before use (Evans, Francis, Hunt, Oakes & Watkins, 1979; Ault *et al.*, 1980). For recording, hemisected spinal cords were mounted in the sucrose gap chamber and superfused with oxygenated Ringer solution at 15°–20°C. The inlet tube to the recording chamber was fitted with a tap system to permit superfusion of drug solutions. NMDA receptors were activated by superfusion of N-methyl-D,L aspartic acid (NMDLA 0.01–4mM) for 1–3 min. Indirect responses were blocked by including tetrodotoxin (TTX) (2×10^{-7} M) in the Ringer solution. Drug responses on ventral roots (VR) were then considered to result from activation of receptors located on motoneurones. Drug responses recorded in this way from dorsal roots (DR) may reflect activation of receptors located on primary afferents (Padjen & Smith, 1981; cf. Evans, 1980). Synaptic transmission in the IXth or Xth paravertebral sympathetic ganglion of the bullfrog was examined by means of the sucrose gap technique as described by Nishi & Koketsu (1968). Presynaptic B fibres in the paravertebral sympathetic chain were stimulated with supramaximal 0.5 ms pulses and the postsynaptic B spike recorded from the ramus of the IXth or Xth ganglion. All potentials were monitored on a conventional d.c.coupled electrophysiological recording system. The high impedence amplifier was connected to the recording bath by means of calomel electrodes and Ringer-agar bridges. The traces illustrated were filmed from an oscilloscope (Tektronix 564B) or recorded on a rectilinear pen recorder (Brush model 2400). The composition of the Ringer solution used in spinal cord experiments was (mM): NaCl 115, $CaCl_2$ 2, KCl 2, HEPES buffer (adjusted to pH 7.2 with NaOH) 10, and D-glucose 8. The Ringer solution used in sympathetic ganglion experiments contained (mM): NaCl 100, $CaCl_2$ 1.8, KCl 2, Tris-HCl

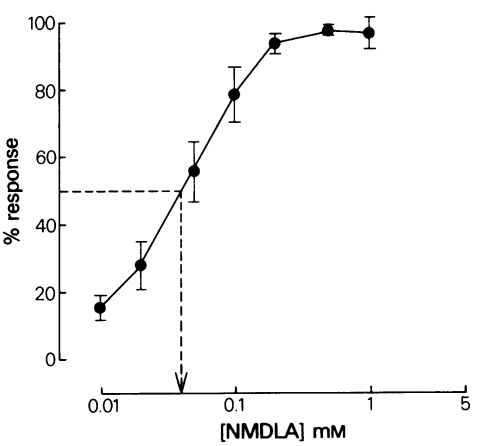

Figure 1 Normalized log dose-response curve for effect of N-methyl D,L aspartic acid (NMDLA) on ventral root (VR) of 6 preparations.

buffer pH 7.2 16 and D-glucose 8. All drugs were purchased from Sigma, St. Louis, Mo, U.S.A. Statistical data are expressed as means ± s.e. wherever possible.

Results

Dose-response curve to NMDLA

The antagonism of NMDLA responses by divalent cations is already well documented (Evans & Watkins, 1978; Ault *et al.*, 1980; Watkins & Evans, 1981; Padjen & Smith, 1981). Since the degree of block produced by Co^{2+} increases with time (Ault *et al.*, 1980), it was not feasible to attempt to measure K_A values for Co^{2+} or other divalent cations. The ED_{50} for NMDLA from the dose-response curve for VR (motoneurones) normalized for 6 preparations was 0.04 mM (Figure 1). This dose, which elicited a clear submaximal response, was used in all subsequent experiments in which the relative potencies of divalent cations were assessed. The ED_{50} for NMDLA on DR (primary afferents) was 0.096 mM; this is to be expected since dorsal roots are generally considered to be less sensitive to acidic amino acids than ventral roots (Barker *et al.*, 1975a).

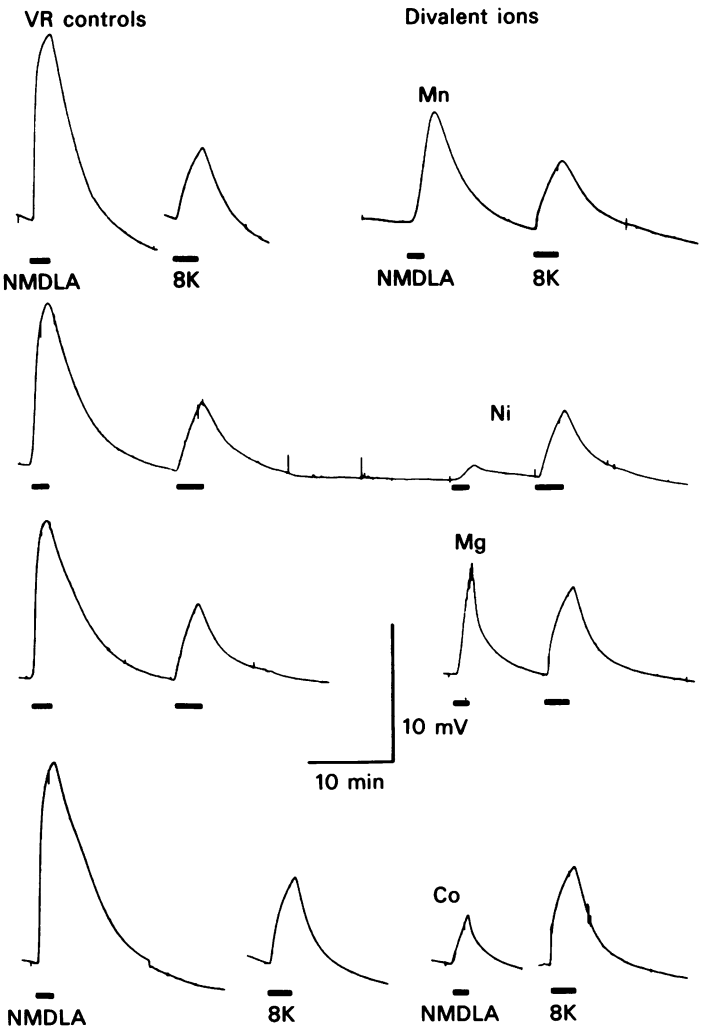

Figure 2 Sucrose gap recording from ventral roots (VR) of amphibian spinal cord. Controls: effects of NMDLA (0.04 mM) and Ringer solution containing 8 mM K (8K). Divalent ions: responses recorded after 20 min exposure ro 250 μM Mn^{2+}, Ni^{2+}, Mg^{2+}, Co^{2+}. All responses recorded from the same preparation using a rectilinear pen recorder. Black bars indicate duration of exposure to 8K or NMDLA.

Relative potencies of divalent cations in blocking NMDLA responses

Responses to 0.04 mM NMDLA were recorded from both ventral and dorsal roots before and after 20 min superfusion with Ringer solution containing various divalent cations (250 μM). On VR, the mean amplitude (% of control) of NMDLA responses recorded in the presence of various divalent cations (at 250 μM) was as follows: Ni^{2+}, 18.5 ± 4.9% ($n = 7$); Co^{2+}, 31.0 ± 4.3% ($n = 7$); Mg^{2+}, 61.2 ± 7.9% ($n = 7$) and Mn^{2+}, 78.9 ± 5.2% ($n = 7$). On DR, amplitudes were as follows in the presence of divalent cations: Ni^{2+}, 15.9 ± 4.1% ($n = 7$); Co^{2+}, 28.3 ± 4.0% ($n = 7$); Mg^{2+} 44.4 ± 5.2% ($n = 7$) and Mn^{2+}, 70.1 ± 3.3% ($n = 8$). In all preparations where all four ions were investigated the rank order of potency was $Ni^{2+} > Co^{2+} \gg Mg^{2+} > Mn^{2+}$ for antagonism of NMDLA responses on both roots. The effects of divalent cations were reversed following 60 min wash with normal Ringer.

Divalent cations block Ca^{2+} channels (Katz & Miledi, 1967; Alvarez-Leefmans *et al.*, 1979), and can thereby prevent release of neurotransmitters (Barker *et al.*, 1975a). Since the presence of TTX in the Ringer only prevents propagated action potentials and may therefore not prevent all types of neurotransmitter release, responses to NMDLA could be generated by the release of endogenous excitory neurotransmitters. Antagonism of these responses by divalent cations may merely reflect blockade of this release. The experiment illustrated in Figure 2 was designed to test this possibility. TTX-blocked preparations were depolarized with Ringer solution containing 8 rather than 2mM K^+. This treatment should be as effective as NMDLA in promoting any indirect responses by release of endogenous excitory neurotransmitters. As illustrated in Figure 2, the response of VR to 8 mM K^+ was insensitive to the 250 μM dose of divalent ions employed. The NMDLA responses were reduced. Similar results were obtained on DR. This indicates that the mechanism of action of divalent ions in antagonizing NMDLA responses probably does not involve a reduction in depolarization – induced release of endogenous excitatory neurotransmitter(s). The experiment illustrated in Figure 2 also clearly demonstrates that $Ni^{2+} > Co^{2+} \gg Mg^{2+} > Mn^{2+}$ in antagonizing NMDLA responses.

Specificity of divalent cations in blocking NMDLA responses

Ault *et al.* (1980) have already demonstrated the specificity of divalent cations in antagonizing NMDA receptors at doses which do not affect responses to noradrenaline, substance P, carbachol, 5-hydroxytryptamine (5-HT), glutamate or γ-aminobutyric acid (GABA). In the present study, divalent cations were to be employed as specific blockers of NMDA receptors to investigate their role in synaptic transmission. It was therefore important

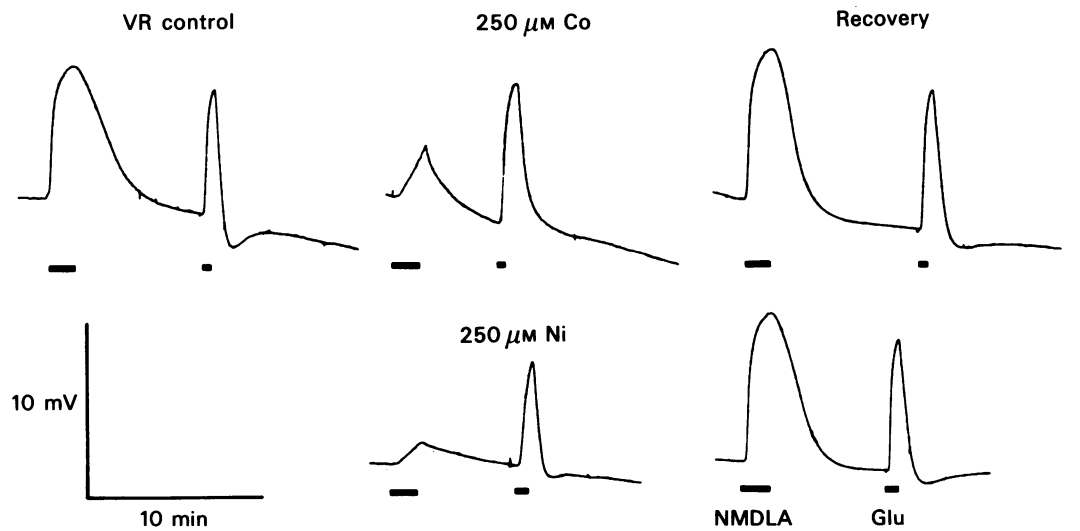

Figure 3 Sucrose gap recording from ventral root (VR) of amphibian spinal cord. Effects of 250 μM Co^{2+} and 250 μM Ni^{2+} on responses to NMDLA (0.04 mM) and L-glutamate (Glu 1 mM). NMDLA tested after 20 min exposure to divalent ions, recovery record from Co^{2+} at upper right is control record for Ni^{2+}. All traces recorded from some preparation in order shown by means of a rectilinear pen recorder. Black bars under traces indicate duration of exposure to Glu or NMDLA.

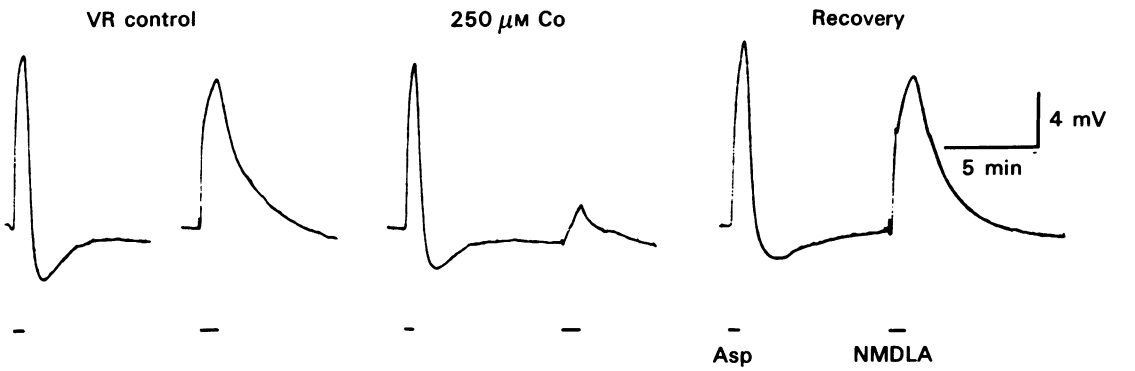

Figure 4 Sucrose gap recording from ventral root (VR) of amphibian spinal cord. Effects of 250 μM Co^{2+} on responses to NMDLA (0.04 mM) and aspartate (Asp, 1 mM). Left hand traces: control responses. Centre traces: responses to Asp tested after 20 min exposure to Ringer containing Co^{2+}, NMDLA response recorded after 35 min in Co^{2+} Ringer. Right hand traces: recovery records following 75 min wash with normal Ringer. Black bars indicate duration of exposure to Asp or NMDLA.

to show that the 250 μM doses of divalent cations to be used had minimal effects on receptors for other putative amino acid neurotransmitters such as glutamate (Glu). A typical experiment is illustrated in Figure 3, although 20 min incubation in 250 μM Co^{2+} significantly reduced the amplitude of the NMDLA response, the Glu response was slightly enhanced (cf. Padjen & Smith, 1981). This apparent enhancement could result from the suppression of the sodium pump-mediated Glu after hyperpolarization (Padjen & Smith, 1980b). Among the divalent cations tested, only Ni^{2+} at 250 μM produced noticeable reduction of the Glu response (Figure 3). Since this ion was extremely effective in antagonizing the response to NMDLA, Ni^{2+} could still be considered as relatively specific for NMDA receptors. In another series of experiments such as that illustrated in Figure 4, it was noted that NMDLA responses were much more sensitive to divalent cations than responses to aspartate.

Effects of divalent cations on synaptic transmission in bullfrog sympathetic ganglia

If antagonism of NMDLA responses by various divalent cations results from an action of these ions on a Ca^{2+} channel underlying the electrogenesis of the response, then these ions should exhibit a similar rank order of potency for blockade of both NMDA responses and Ca^{2+} channels. In an attempt to assess the relative potencies of Ni^{2+}, Mn^{2+}, Co^{2+}, and Mg^{2+} as calcium channel blockers, their effects on synaptic transmission in bullfrog sympathetic ganglia was examined. This process ultimately depends on presynaptic Ca^{2+} influx to release acetylcholine (Blackman *et al.*, 1963).

Ni^{2+}, Co^{2+}, Mn^{2+} and Mg^{2+} at 250 μM had little or no effect on ganglionic transmission. At 1 mM these cations reduced the amplitude of the orthodromic B-fibre action potential to 13.3 ± 4.4% (Mn^{2+}) ($n = 6$), 68.2 ± 7.8%, (Co^{2+}) ($n = 6$), 76 ± 11.2% (Ni^{2+}) ($n = 5$) and 96.7 ± 4.7% (Mg^{2+}) ($n = 6$), (percentages of control responses following a 20 min incubation). In any given preparation, the order of potency was $Mn^{2+} > Co^{2+} > Mg^{2+}$ but the potency of Ni^{2+} varied from being more effective than Co^{2+} to less effective than Mg^{2+}. In some preparations the response observed in the presence of 1 mM Mg^{2+} was larger than control. This may have resulted from a 'membrane stabilization' effect of the divalent ion and consequent superior recording of responses. A typical experiment is illustrated in Figure 5a. Thus, if the cation sensitivity of synaptic transmission in ganglia is accepted as a suitable model for the cation sensitivity of Ca^{2+} channels, it would seem that there is a clear cut difference in sensitivity of NDMLA responses and Ca^{2+} channels, i.e. NMDA receptors are antagonized by $Ni^{2+} > Co^{2+} > Mg^{2+} > Mn^{2+}$ at 250 μM whereas Ca^{2+} channels are antagonized by $Mn^{2+} > Co^{2+} > Ni^{2+} > Mg^{2+}$ at 1 mM.

Effects of divalent cations on synaptic responses in frog spinal cord

Magnesium As illustrated in Figure 2, Mg^{2+} produced marked and specific depression of NMDLA responses at 250 μM. Since this treatment would not be expected to block neurotransmitter release, any effect of this dose of Mg^{2+} on synaptic transmission in spinal cord should be largely due to postsynaptic effects at NMDA receptors. After 20 min incubation in Ringer solution containing 250 μM Mg^{2+}, the slow (polysynaptic) component of DR-VRP and DR-DRP was depressed. VR-DRP was relatively unaffected or even slightly enhanced. The results of all

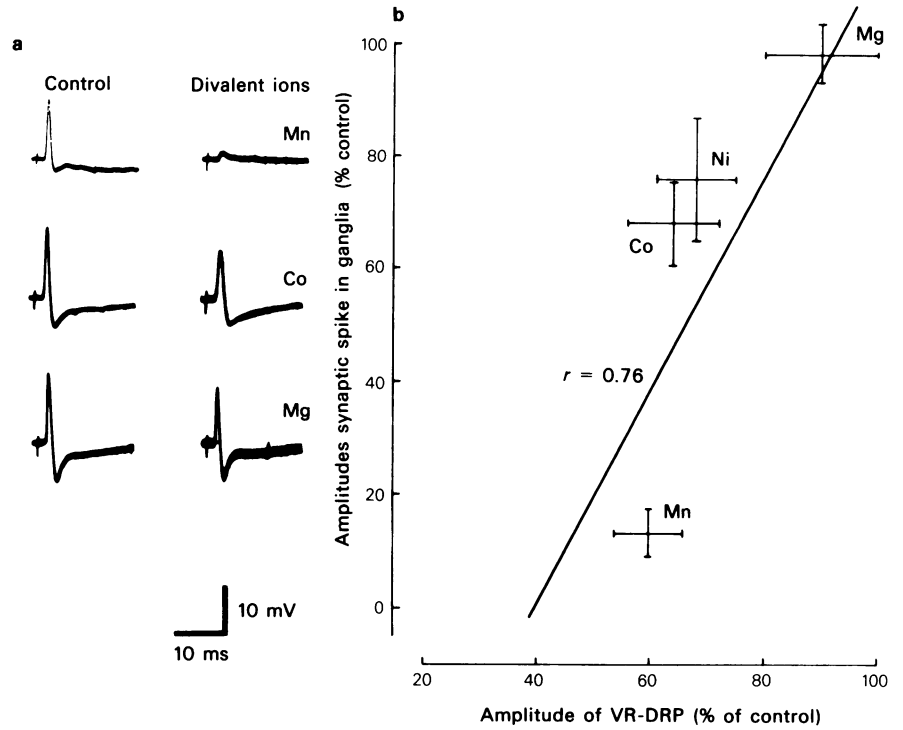

Figure 5 (a) Sucrose gap recording from bullfrog sympathetic ganglion. Control: orthodromic (synaptic) B fibre population action potentials recorded following stimulation of the sympathetic chain with supramaximal 0.5 ms pulses. Divalent ions: orthodromic B fibre population action potentials recorded following 20 min incubation in Ringer containing Mn^{2+}, Co^{2+} or Mg^{2+} (1 mM). All traces are photographs of oscilloscope recordings from the same preparation. (b) Graphical comparison of the effects of divalent cations (250 μM) on the ventral root-dorsal root potential (VR-DRP) of amphibian spinal cord with their effects (at 1 mM) on the synaptic B fibre spike in sympathetic ganglia. Amplitudes of the two responses as percentage of control in the presence of Co^{2+}, Ni^{2+}, Mn^{2+} or Mg^{2+} are plotted against each other. $n > 5$ for both VR-DRP and ganglionic spike. Error bars indicate s.e.mean response size under each experimental condition. Line of best fit and correlation coefficient (r) computer calculated.

experiments are summarized in Table 1 and data from a typical experiment are illustrated by means of the histogram in Figure 6a.

Cobalt Figure 2 also illustrates that 250 μM Co^{2+} was more potent than Mg^{2+} in antagonizing NMDLA responses but is also more unlikely to affect neurotransmitter release (Figure 5). Thus the effects of 250 μM Co^{2+} upon synaptic transmission in spinal cord may be ascribed both to antagonism of NMDA receptors and reduction of neurotransmitter release. Although 250 μM Co^{2+} (20 min incubation) tended to depress preferentially DR-VRP and DR-DRP rather than VR-DRP, it was somewhat less selective than Mg^{2+} (Table 1). In some experiments however, such as that illustrated in Figure 6a and 7, a relatively selective depression of DR-VRP and DR-DRP was observed. In an attempt to minimize effects on

Table 1 Effects of divalent cations (250 μM) on synaptic transmission in frog spinal cord

Pathway	*% of control response**		
	Mg^{2+}	Co^{2+}	Mn^{2+}
VR-DRP	90.5 ± 10.2 ($n = 7$)	64.0 ± 8.6 ($n = 6$)	60.1 ± 6.3 ($n = 7$)
DR-DRP	47.3 ± 5.4 ($n = 7$)	29.5 ± 4.4 ($n = 6$)	36.5 ± 3.7 ($n = 7$)
DR-VRP	38.1 ± 4.9 ($n = 7$)	25.3 ± 4.3 ($n = 6$)	32.4 ± 4.4 ($n = 7$)

$$\text{*\% of control response} = \frac{\text{Area under curve of response in presence of divalent cation}}{\text{Area under curve of control response}} \times \frac{100}{1}$$

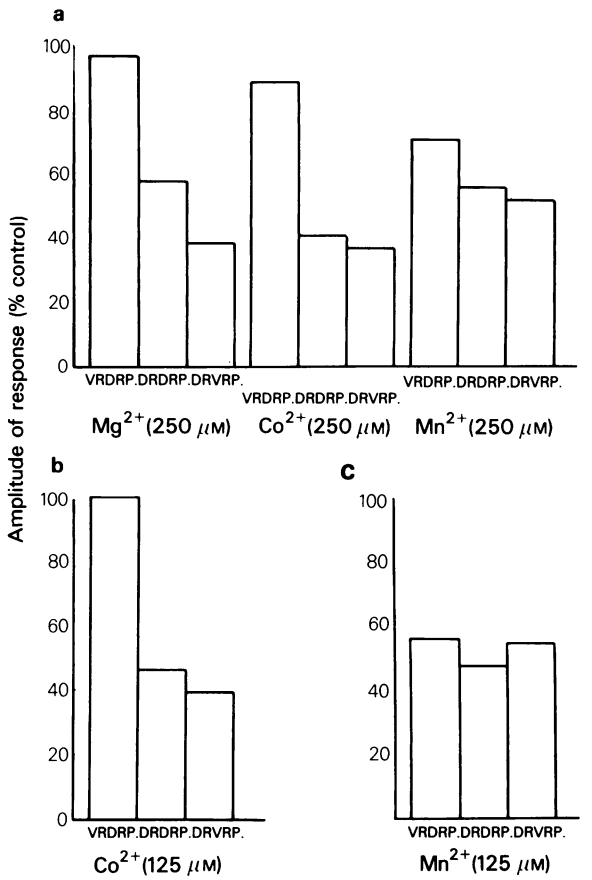

Figure 6 Histograms illustrating the depression of synaptic responses in amphibian spinal cord by divalent cations. (a) Effects of Mg^{2+}, Co^{2+}, Mn^{2+} (250 μM) on VR-DRP, DR-DRP and DR-VRP. Percentage changes of responses obtained by measuring area under traces on original data records such as that illustrated in Figure 7. All data obtained from same preparation. Note that Co^{2+} and Mg^{2+} preferentially depress DR-VRP and DR-DRP whereas Mn^{2+} tends to reduce all responses. (b) Histograms of data from another preparation illustrating effect of 125 μM Co^{2+}. Note selective depression of DR-VRP and DR-VRP. (c) Histograms of data from a third preparation illustrating effect of 125 μM Mn^{2+}. Note non-selective depressions of all responses.

neurotransmitter release, some experiments, such as that presented in Figure 6b, were performed with 125 μM Co^{2+}. With this dose it was often possible to produce selective depression of DR-DRP and DR-VRP with little or no depression of VR-DRP.

Manganese Unlike Co^{2+} and Mg^{2+}, 250 μM Mn^{2+} was relatively ineffective in blocking NMDA receptors (Figure 2) so that its effects on synaptic transmission in spinal cord may be mainly ascribed to inhibition of neurotransmitter release. Although after 20 min incubation in 250 μM Mn^{2+}, VR-DRP seemed, on average, to be slightly less depressed than DR-DRP or DR-VRP (Table 1) this was not the case for all preparations. In the results illustrated in Figure 6a no such selective depression of VR-DRP could be observed. Mn^{2+} often produced non-selective depression of all three synaptic responses, marked depression of DR-DRP was always associated with marked depression of VR-DRP. With lower doses of Mn^{2+} (125 μM), (Figure 6c), it was still not possible to demonstrate selective depression of any single pathway.

Nickel As illustrated in Figure 2, 250 μM Ni^{2+} was very effective in blocking NMDA receptors but was somewhat erratic in blocking ganglionic transmission. Although Ni^{2+} (250 μM) consistently reduced VR-DRP to an average of 67.2 ± 7.1% of control ($n = 5$), (Figure 8), its effects on DR-DRP and DR-VRP were varied, both depressions and enhancements of these responses were observed. The effects of all divalent cations were reversed following 60 min wash with normal Ringer solution.

Statistical examination of data

If VR-DRP is generated by pathways which do not involve NMDA receptors (Padjen & Smith, 1980a; cf. Homma, 1981) it is likely that any depression of this response by divalent cations results from an action of these substances on transmitter release. If this is the case, then the ability of Mn^{2+}, Co^{2+}, Ni^{2+} and Mg^{2+} to block ganglionic transmission should be correlated with their ability to block VR-DRP. For both responses the effectiveness of the cations was $Mn^{2+} > Co^{2+} > Ni^{2+} > Mg^{2+}$. This is illustrated graphically in Figure 5b where the relative potencies of divalent ions in antagonizing the two responses are plotted against each other. Since these phenomena were linearly correlated ($r = 0.76$) it is likely that similar processes were involved in blockade of both ganglionic transmission and VR-DRP. Antagonism of DR-VRP and DR-DRP were not at all well correlated with antagonism of ganglionic transmission. The r values were 0.37 and 0.27 respectively, indicating that different processes were involved in the antagonism in each case. On the other hand, antagonism of DR-VRP was very well correlated with antagonism of DR-DRP ($r = 0.98$). This is consistent with the possibility that essentially identical processes were involved in the antagonism in each case. Antagonism of VR-DRP correlated very poorly with antagonism of DR-DRP ($r = 0.15$) and DR-VRP ($r = 0.04$) again indicating that different processes were involved in the antagonism of the responses in these pathways.

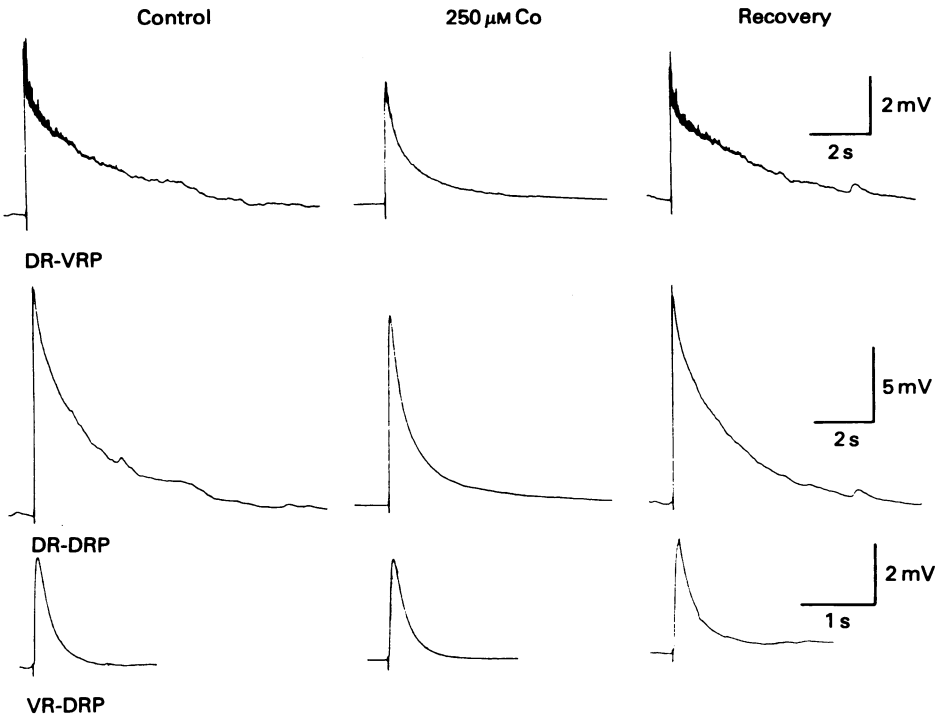

Figure 7 Sucrose gap recording from isolated hemisected amphibian spinal cord. Effect of Co^{2+} on synaptic potentials. Left hand traces: controls: Centre traces: DR-VRP, DR-DRP and VR-DRP recorded after 20 min superfusion with Ringer containing 250 μM Co^{2+}. Note preferential depression of DR-VRP and DR-DRP compared to VR-DRP. Right hand traces: Recovery of responses following 75 min washout of Co^{2+}. Traces from rectilinear pen holder.

Discussion

The rank order of potency for divalent cations in blocking Ca^{2+} channels (ganglionic transmission) was $Mn^{2+} \gg Co^{2+} > Ni^{2+} \gg Mg^{2+}$. On the other hand, their rank order of potency in antagonizing NMDLA responses was $Ni^{2+} > Co^{2+} \gg Mg^{2+} > Mn^{2+}$. Divalent cations were effective against these responses in concentrations below 100 μM, whereas, concentrations exceeding 1 mM were required for blockade of Ca^{2+} channels. NMDLA responses and Ca^{2+} channels thus displayed very different pharmacological sensitivities to divalent cations. This indicates that the blockade of NMDLA responses is not due to an effect of these substances on a Ca^{2+} ionophore which may underlie the generation of the response. This conclusion is based on the assumption that all types of Ca^{2+} channels exhibit similar sensitivity to divalent cations; if this were not the case, NMDLA-activated Ca^{2+} channels would have to exhibit rather remarkable properties in that (i) they would have to be at least ten times more sensitive to divalent ions than voltage-sensitive Ca^{2+} channels and (ii) they would have to be relatively insensitive to Mn^{2+}.

If it is accepted that divalent cations do not act on a Ca^{2+} ionophore associated with electrogenesis of the NMDLA response, it must be assumed that they interfere with either receptor-agonist binding or receptor-ionophore coupling. The high potency of divalent cations and the demonstration of inhibition of [^{3}H]-NMDA binding to rodent brain membrane fractions by Mg^{2+} (IC_{50} = 450 μM) (Snodgrass, 1979) suggests that divalent ions interfere with receptor-agonist binding. On the other hand, it has been suggested that the divalent cations exhibit a different mechanism of action from 'organic' NMDA antagonists (Evans & Watkins, 1978; Evans *et al.*, 1979) which presumably compete with agonists for receptor binding. If both receptor and ionophore blockade are excluded it may be suggested that the divalent cations act on receptor-ionophore coupling. Such an effect has recently been proposed for divalent cations and 5-HT receptors in sympathetic ganglia (Nash & Wallis, 1981).

Regardless of their exact mechanism of action, the

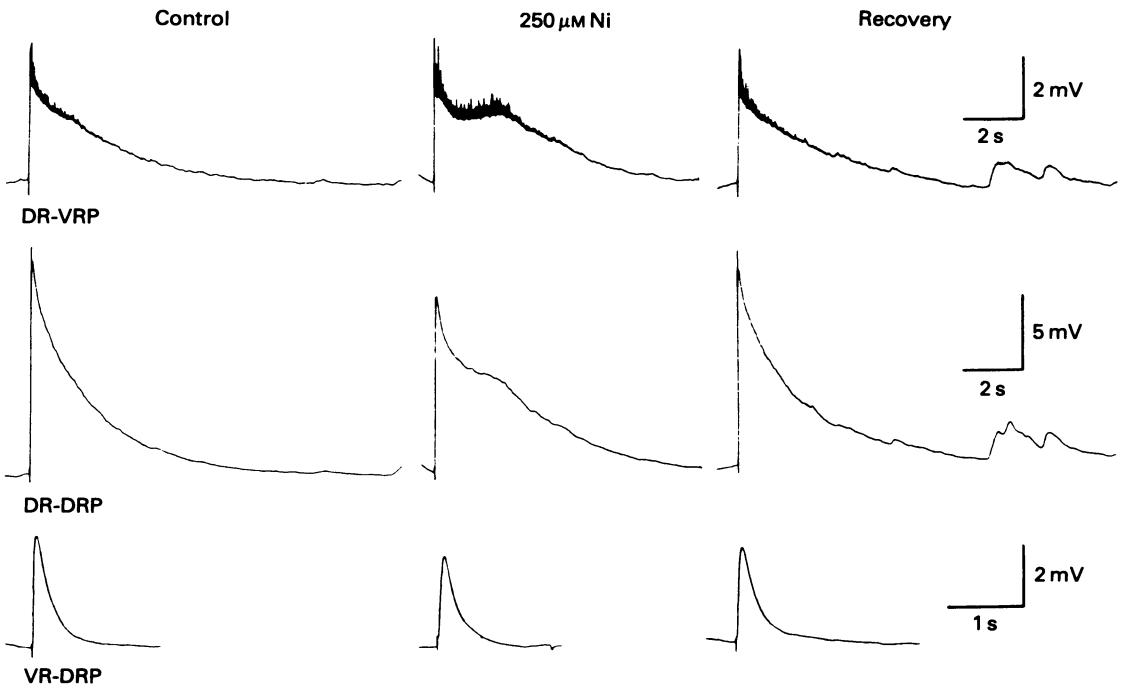

Figure 8 Sucrose gap recording from isolated hemisected amphibian spinal cord. Effect of Ni^{2+} on synaptic responses. Left hand traces: controls. Centre traces: DR-VRP, DR-DRP and VR-DRP recorded after 20 min superfusion with Ringer containing 250 μM Ni^{2+}. Note apparent enhancement of DR-VRP. Right hand traces: recovery of responses following 105 min washout of Ni^{2+}. Traces from rectilinear pen recorder.

present work confirms that divalent cations are relatively specific antagonists of responses evoked at NMDA receptors (Ault *et al.*, 1980; Watkins & Evans, 1981). Since they also block neurotransmitter release (Alvarez-Leefmans *et al.*, 1979) no previous attempts have been made to use divalent cations to identify specific neuronal pathways involving NMDA receptors. In the present study it was possible to examine three different synaptic responses in amphibian spinal cord, the usual orthodromic DR-VRP, as well as DR-DRP and an antidromic VR-DRP. Low doses of Mg^{2+} and Co^{2+} were used to investigate the role of NMDA receptors in specific pathways because they were relatively ineffective in blocking neurotransmitter release but highly effective in blocking NMDA receptors. These divalent cations produced a similar pattern of antagonism of responses in spinal cord to DL α-aminoadipate (Padjen & Smith, 1980a). Both classes of antagonists blocked or reduced the slow (polysynaptic) component of DR-VRP and DR-DRP with minimal depression of VR-DRP. These results suggest an involvement of NMDA receptors in DR-DRP and DR-VRP but not in VR-DRP. The general lack of effect of 250 μM Mg^{2+} and 125 μM Co^{2+} on VR-DRP serves as a control to indicate that the blockade of DR-DRP and DR-VRP was not due to impairment of neurotransmitter release. Mn^{2+} produced non-specific depression of all responses. This lack of selective depression with Mn^{2+}, which is a poor NMDA receptor blocker, adds further support to the hypothesis that NMDA receptors may be involved in only DR-DRP and DR-VRP and not VR-DRP. The relative abilities of the divalent cations Ni^{2+}, Mn^{2+}, Co^{2+} and Mg^{2+} to depress VR-DRP was correlated with their relative abilities to block ganglionic transmission. This suggests that antagonism of both ganglionic transmission and VR-DRP could be attributed to blockade of neurotransmitter release and could further imply a lack of involvement of NMDA receptors in the VR-DRP pathway. The higher potency of the divalent ions in the case of VR-DRP may be because this pathway is polysynaptic whereas ganglionic transmission is monosynaptic. In a recent paper, Homma (1981) demonstrated antagonism of VR-DRP in frog spinal cord with 1 mM DL-α-aminoadipate. This would suggest an involvement of NMDA receptors in this pathway. This result is inconsistent with both the present results and with the previous finding that 3 mM DL-α-aminoadipic acid

had little effect on this response (Padjen & Smith, 1980a). There is at present no obvious explanation for this difference in data. Since there was little correlation between the ability of various divalent ions to block VR-DRP and their ability to block DR-DRP and DR-VRP it would seem that different mechanisms were involved in each case. Blockade of DR-DRP and DR-VRP was well correlated, suggesting similar processes were involved in the blockade.

Ni^{2+} was the most potent antagonist of NMDLA in the present experiments, DR-VRP and DR-DRP were relatively insensitive to this ion and in some experiments responses were slightly enhanced. It was noted that the action of Ni^{2+} on ganglion transmission was highly variable. This variability could result from poor penetration of Ni^{2+} into ganglia. Poor penetration to subsynaptic sites could also explain the inability of this ion to block DR-DRP and DR-VRP.

In general, the present data support the idea of an involvement of NMDA receptors in the polysynaptic components of DR-DRP and DR-VRP (cf. Evans *et al.*, 1981). Recent evidence suggests that, the putative neurotransmitter, aspartic acid may not act on NMDA receptors (Lambert, Flatman & Engberg, 1981); glutamate is thought to act primarily on quisqualate receptors (Watkins & Evans, 1981). Furthermore, the present results indicate that neither aspartate nor glutamate responses are especially sensitive to divalent cations. This may indicate that neither aspartate nor glutamate are the transmitters involved in DR-DRP and DR-VRP, further investigation is therefore necessary to clarify this situation and perhaps to 'identify the endogenous ligand' for NMDA receptors (Anis, Burton & Lodge, 1982).

Supported by the University of Alberta Medical Research Fund and the Alberta Heritage Foundation for Medical Research. I would like to thank Dr Tessa Gordon for reading the manuscript, Dr Ante Padjen for useful discussion, Mrs Kochu Rani Dombro for skilled technical assistance, and Miss Rosalind Millar for typing the manuscript.

References

ALVAREZ-LEEFMANS, F.J., DE SANTIS, A. & MILEDI, R. (1979). Effects of some divalent cations on synaptic transmission in frog spinal motornueones. *J. Physiol.*, **294,** 387–406.

ANIS, N.A., BURTON, N.R. & LODGE, D. (1982). Ketamine antagonises N-methylaspartate and synaptic excitation of spinal neurones. *Br. J. Pharmac.*, **75,** 52P.

AULT, B., EVANS, R.H., FRANCIS, A.A., OAKES, D.J. & WATKINS, J.C. (1980). Selective depression of excitatory amino acid induced depolarizations by magnesium ions in isolated spinal cord preparations. *J. Physiol.*, **307,** 413–428.

BARKER, J.L., NICOLL, R.A. & PADJEN, A. (1975a). Studies on convulsants in the isolated frog spinal cord. I. Antagonism of amino acid responses. *J. Physiol.*, **245,** 521–536.

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

BLACKMAN, J.G., GINSBORG, B.L. & RAY, C. (1963). On the quantal release of the transmitter at a sympathetic synapse. *J. Physiol.*, **167,** 402–415.

DAVIES, J., EVANS, R.H., FRANCIS, A.A. & WATKINS, J.C. (1979). Excitatory amino acid receptors and synaptic excitation in mammalian central nervous system. In *Advances in Pharmacology and Therapeutics,* Vol. 2, Neurotransmitters. ed. Simon, P. pp. 161–170. Oxford: Pergamon.

EVANS, R.H. (1980). Evidence supporting the indirect depolarization of primary afferent terminals in the frog by excitatory amino acids. *J. Physiol.*, **298,** 25–35.

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.*, **67,** 591–603.

EVANS, R.H., SMITH, D.A.S. & WATKINS, J.C. (1981). Differential role of excitant amino acid receptors in spinal transmission. *J. Physiol.*, **320,** 55P.

EVANS, R.H. & WATKINS, J.C. (1978). Dual sites for antagonism of excitatory amino acids on central neurones. *J. Physiol.*, **277,** 57P.

HEINEMANN, U. & PUMAIN, R. (1981). Effects of tetrodotoxin on changes in extracellular free calcium induced by repetitive electrical stimulation and iontophoretic application of excitatory amino acids in the sensorimotor cortex of cats. *Neurosci. Letts,* **21,** 87–91.

HOMMA, S., (1981). Depression of ventral root-dorsal root potential by DL-α-aminoadipate in frog spinal cord. *Brain. Res.*, **208,** 240–243.

KATZ, B. & MILEDI, R. (1967). The timing of calcium action during neuromuscular transmission. *J. Physiol.*, **189,** 535–544.

KRNJEVIĆ, K. (1974). Chemical nature of synaptic transmission in vertebrates. *Physiol. Rev.*, **54,** 418–540.

LAMBERT, J.D.C., FLATMAN, J.A. & ENGBERG, I. (1981). Actions of excitatory amino acids on membrane conductance and potential in motoneurones. In *Glutamate as a Neurotransmitter.* ed. DiChiara, G. & Gessa, G.L. pp. 205–216. New York: Raven Press.

MacDONALD, J.F. & WOJTOWICZ, J.M., (1980). Two conductance mechanisms activated by applications of L-glutamic, L-aspartic, DL-homocysteic, N-methyl-D-aspartic and DL-kainic acids to cultured mammalian central neurones. *Can. J. Physiol. Pharmac.*, **58,** 1393–1397.

MacDONALD, J.F. & WOJTOWICZ, J.M. (1981). Excitatory amino acids evoke a voltage-dependent decrease in the

conductance of cultured murine neurones. *Soc. Neurosci. Abstracts.*, **7**, 336.

NASH, H.L. & WALLIS, D.I. (1981). Effects of divalent cations on responses of a sympathetic ganglion to 5-hydroxytryptamine and 1,1-dimethyl-4-phenyl piperazinium. *Br. J. Pharmac.*, **73**, 759–772.

NISHI, S. & KOKETSU, K. (1968). Analysis of slow inhibitory post synaptic potential in bullfrog sympathetic ganglion. *J. Neurophysiol.*, **31**, 717–728.

PADJEN, A.L. & SMITH, P.A. (1980a). Specific effects of α-D,L-aminoadipic acid on synaptic transmission in frog spinal cord. *Can. J. Physiol. Pharmac.*, **58**, 692–698.

PADJEN, A.L. & SMITH, P.A. (1980b). Glutamate evoked after hyperpolarization (GLU-AHP) in frog motorneurones (MN): Evidence for electrogenic Na pump. *Proc. XXVII International Congress of Physiol Sci.*, **14**, 627.

PADJEN, A.L. & SMITH, P.A. (1981). Possible role of divalent cations in amino acid responses of frog spinal cord. In *Amino Acid Neurotransmitters*. ed. De Feudis, F.V. & Mandel, P. pp. 271–279. New York: Raven Press.

PUIL, E. (1981). S-Glutamate: its interactions with spinal neurons. *Brain. Res. Rev.*, **3**, 229–322.

SHAPOVALOV, A.I., SHIRIAEV, B.I. & VELUMAIN, A.A. (1978). Mechanisms of post synaptic excitation in amphibian motoneurones. *J. Physiol.*, **279**, 437–455.

SNODGRASS, S.R. (1979). In vitro binding studies with ^{3}H-N-methyl aspartate. *Soc. Neurosci. Abstracts*, **5**, 572.

SONNHOF, U. & BÜHRLE, C.H. (1981). An analysis of glutamate induced ion fluxes across the membrane of spinal motorneurons of the frog. In *Glutamate as a Neurotransmitter*. ed. Dichiara, G. & Gess, G.L. pp. 195–204. New York: Raven Press.

WATKINS, J.C. & EVANS, P.R. (1981). Excitatory amino acid transmitters. *A. Rev. Pharmac. Tox.*, **21**, 165–204.

(*Received May 7, 1982.*)