J. Physiol. (1980), **307**, *pp.* 413–428
With 7 text-figures
Printed in Great Britain

SELECTIVE DEPRESSION OF EXCITATORY AMINO ACID INDUCED DEPOLARIZATIONS BY MAGNESIUM IONS IN ISOLATED SPINAL CORD PREPARATIONS

BY B. AULT, R. H. EVANS, A. A. FRANCIS, D. J. OAKES[1] AND J. C. WATKINS[1]

From the Department of Pharmacology and the Department of Phsysiology,[1] The Medical School, University Walk, Bristol BS8 1TD

(*Received 21 January 1980*)

SUMMARY

1. The depressant actions of Mg^{2+} and a range of other divalent ions on synaptic excitation and on responses produced by excitatory amino acids and other putative transmitters have been investigated in hemisected isolated spinal cords of frogs and neonatal rats. Some comparative studies were also made using the rat isolated superior cervical ganglion.

2. At concentrations above 10 μM, Mg^{2+} selectively antagonized *N*-methyl-D-aspartate (NMDA)-induced motoneurone depolarization as recorded from ventral roots of tetrodotoxin-blocked spinal cords. Depolarization evoked by quisqualate (unaffected by 20 mM-Mg^{2+}) was resistant to the depressant action of these ions, while depolarizations evoked by other excitant amino acids were depressed to intermediate degrees.

3. Mn^{2+}, Co^{2+} and Ni^{2+} had qualitatively similar actions to Mg^{2+}; Mn^{2+} was somewhat less potent and Co^{2+} and Ni^{2+} more potent than Mg^{2+}. The alkaline earth metal ions, Ca^{2+}, Sr^{2+} and Ba^{2+}, had very weak Mg^{2+}-like actions. Ca^{2+} and Mg^{2+} acted additively in depressing amino acid-induced responses.

4. Mg^{2+} also depressed motoneurone responses evoked by noradrenaline, substance P and carbachol in the neonatal rat isolated spinal cord. However, none of these effects were as marked as the depression of NMDA-induced responses by Mg^{2+} in this preparation. Mg^{2+} did not depress motoneurone depolarization produced by 5-HT in the rat spinal cord or the depolarizing action of GABA on primary afferent terminals of the isolated frog spinal cord.

5. At concentrations producing marked depression of NMDA-induced responses, Mg^{2+} also depressed synaptic transmission in spinal cords in the absence of an effect on ganglionic transmission. At the same concentrations, Mn^{2+}, Co^{2+} and Ni^{2+} depressed synaptic transmission in both preparations.

6. From the similarity in action between Mg^{2+} and the D-α-aminoadipate group of NMDA antagonists, it is suggested that the central depressant action of low concentrations of Mg^{2+} involves predominantly a postsynaptically mediated interference with the action of an excitatory amino acid transmitter.

0022-3751/80/2220-0748 $07.50

INTRODUCTION

Concentrations of free Mg^{2+} above that normally present in extracellular fluid have long been known to depress transmission at myoneural and synaptic junctions. The most widely accepted mechanism for this phenomenon is a competition between Mg^{2+} and Ca^{2+} in the stimulus-secretion coupling processes involved in transmitter release. This hypothesis derives mainly from studies at peripheral junctions of both vertebrates and invertebrates (see Rubin, 1970) and evidence has also been presented in support of similar phenomena at synapses within the vertebrate central nervous system (Katz & Miledi, 1963). The concentrations of Mg^{2+} most commonly used to produce these effects are high, often 20 mM or more. However, depressant effects of much lower concentrations of Mg^{2+} on central neurones have also been described. Thus, ionophoretic administration of Mg^{2+} depresses the excitability of central neurones in the cat (Kelly, Krnjević & Somjen, 1969; Rozear, de Groof & Somjen, 1971) and frog (Erulkar, Dambach & Mender, 1974). Extracellular concentrations achieved by the iontophoretic method of administration are usually regarded as being less than 1 mM (Curtis, Perrin & Watkins, 1960; Krnjević & Phillis, 1963). The marked effects of such low concentrations of Mg^{2+} might reflect a mechanism of action different from the generally accepted depression of transmitter release, for example, an antagonism of synaptic excitation at post-synaptic sites (Evans, Francis & Watkins, 1977*a*). To investigate if low concentrations of Mg^{2+} antagonize neuronal responses to putative transmitters, we have studied the effects of these ions on depolarizations induced in spinal roots by a range of transmitter agonists in isolated spinal cords of the frog or immature rat. The results suggest that depression of synaptic transmission by low concentrations of Mg^{2+} may be due to a specific interference with the action of an excitatory amino acid transmitter. Some of these results have been reported in preliminary form (Evans, Francis & Watkins, 1977*a*, *c*; Davies, Evans, Francis & Watkins, 1979).

METHODS

Recording of synaptic and agonist induced depolarizations from isolated spinal cords and ganglia

(*a*) *Frog.* Experiments were conducted on isolated hemisected spinal cords of *Rana pipiens* or *R. temporaria*, no major difference between the two species being observed. The techniques for recording ventral and dorsal root potentials following dorsal root stimulation or produced by chemical agents in the superfusion medium have been previously described (Evans, 1978*a*; Evans & Watkins, 1978*a*; Evans, Francis, Hunt, Oakes & Watkins, 1979). Briefly, hemisected spinal cords were equilibrated for 18–40 h in standard medium at 4 °C before use. The standard medium contained (mM): NaCl, 111; KCl, 2; $CaCl_2$, 2; tris, 10; glucose, 12; adjusted to pH 7·5 with HCl. For the measurement of dorsal root and ventral root responses, superfusion medium was dripped directly on to the tissue at a rate of 0·8–1·5 ml./min, the temperature being maintained at $12 \pm 0{\cdot}5°$. One dorsal root (8 or 9) was laid across a pair of stainless-steel stimulating electrodes. Potentials generated in the corresponding ventral root or an adjacent dorsal root following dorsal root stimulation (DR-VRPs and DR-DRPs, respectively) or produced by chemical agents added to the medium were recorded by Ag-AgCl electrodes from the appropriate roots. These potentials reflected changes in membrane potential of motoneuronal cell bodies and/or processes (ventral root records) or of primary afferent fibres and terminals (dorsal root records). For the measurement of root potentials generated by amino acids or other substances added to the superfusion medium, tetrodotoxin (TTX) was usually included in the medium to abolish indirect effects of the substances which were dependent on regenerative electrical activity. For this purpose, the cords were initially treated with 5 μm-TTX for 5 min, which

initiated a rapid block of both DR-VRPs, and this block was then maintained by 0·1 μM-TTX continuously present in the superfusion medium.

(*b*) *Rats.* Spinal cords were obtained from 4–9 day-old rats (Otsuka & Konishi, 1974), roots L4 and L5 being used for stimulating and recording purposes as described above for the frog cord, except that the rat cords were used immediately after dissection. The composition of the medium was (mM): NaCl, 118; KCl, 2; $NaHCO_3$, 24; $CaCl_2$, 2·5; glucose, 12; gassed with 95% O_2/5% CO_2; pH 7·4. The temperature was maintained at 25 ± 0·5 °C, and the flow rate was 0·8 ml./min. Isolated superior cervical ganglia were removed from rats (150–200 g body weight) and superfused as described for hemisected rat spinal cords. The post-ganglionic nerve of the ganglion was placed in contact with the recording electrodes as for the ventral root of the spinal cord.

Uptake experiments. Frog spinal cords were prepared as for the electrophysiological experiments, then placed in fresh medium (1 ml. per hemicord) at 12 °C for 15 min. They were then transferred either to 1 ml. fresh control medium (12 °C) containing ^{35}S- or ^{14}C-labelled amino acids and [^3H] sucrose (0·1 mM), or to a similar medium containing 1 mM-Mg^{2+} in addition to all other components. Incubation was continued for 80 sec (to reproduce the time of contact of amino acids with the tissue in the electrophysiological experiments) or 15 min (to allow greater uptake) and the cords were then collected on a filter paper with light suction, washed with 1 ml. tracer-free medium (12 °C), and transferred to a counting vial containing 0·5 ml. hyamine hydroxide (1 M in methanol). The cords were digested overnight at 45 °C before adding 10 ml. scintillation fluid and counting for radioactivity content. The volume of medium that had penetrated into the cords was calculated from the ^3H counts; ^{35}S or ^{14}C counts equivalent to this volume of medium were subtracted from the total tissue content of the latter isotopes in order to correct for the maximum amounts of the amino acids estimated to be present in the extracellular medium.

Amino acids. [^{35}S]L-homocysteic acid was synthesized by the method of Cox, Headley & Watkins (1977). [U^{14}-C]L-glutamic acid and [U-^{14}C]L-aspartic acid were obtained from The Radiochemical Centre (Amersham). *N*-methyl-D-aspartic acid (NMDA) and L-homocysteic acid were synthesized as described by Watkins (1962). Quisqualic acid was a gift from Professor T. Takemoto (Tohoku University). Other chemicals and drugs were obtained from commercial sources.

RESULTS

Effects of Mg^{2+} on excitatory amino acid-induced responses in the frog spinal cord

Fig. 1 shows typical effects of 1·0 mM-Mg^{2+} in a TTX-containing medium on submaximal ventral root responses produced by NMDA and kainate (upper series) and by quisqualate, L-glutamate, L-aspartate and L-homocysteate (lower series) and indicates that whereas responses produced by NMDA and L-homocysteate were markedly reduced by Mg^{2+}, reponses to quisqualate and kainate were relatively resistant to these divalent ions. Responses to L-glutamate and L-aspartate were reduced by Mg^{2+} to an intermediate degree. These effects of Mg^{2+} on amino acid-induced responses, which were not associated with any marked change in the base line level of ventral root polarization, were stable for prolonged periods, and were reversible on return to Mg^{2+}-free media. Fig. 2 shows the effect of increasing [Mg^{2+}] on the differential antagonism of the same six amino acids and it can be seen that the major part of this antagonism develops at Mg^{2+} concentrations below 2 mM. NMDA responses were particularly sensitive, 10–20 μM-Mg^{2+} producing an evident depression.

In many of these experiments depolarizing responses evoked in a dorsal root were measured concomitantly with ventral root responses. The effects of Mg^{2+} on dorsal root responses to excitants paralleled the effects recorded from ventral roots. This correspondence between ventral and dorsal root recordings is not unexpected since

dorsal root fibres probably respond indirectly to the excitant amino acids by way of increases in extracellular K^+ arising from the action of the amino acids on cell bodies and dendrites (Evans, 1980).

As seen from Fig. 1*A*, the responses produced by NMDA were relatively prolonged, presumably due to the absence of effective uptake mechanisms for this substance in

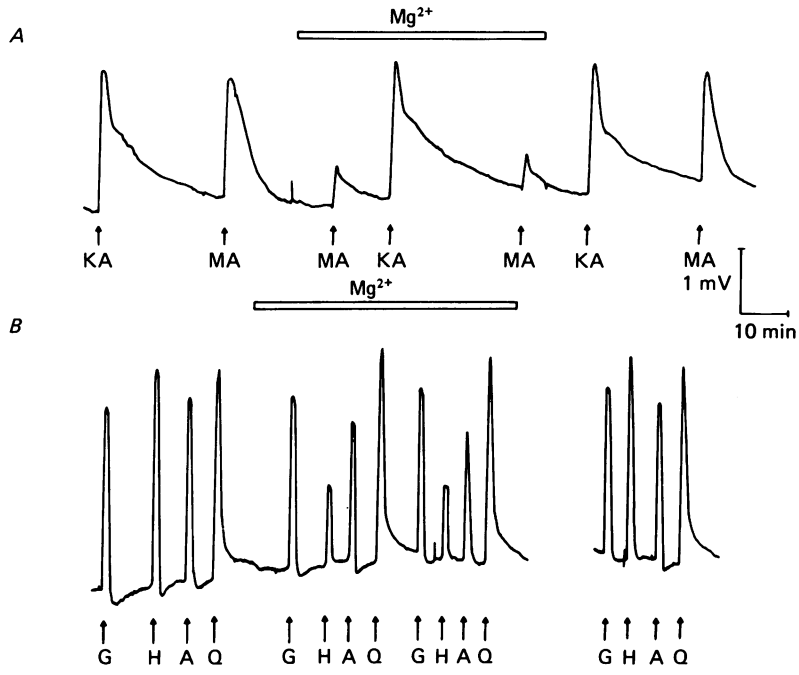

Fig. 1. Selective depression by Mg^{2+} of frog motoneurone depolarizations induced by excitant amino acids. Records show ventral root potentials induced by, upper series, 12 μM-kainate (KA) and 40 μM-NMDA(MA); lower series, 1 mM-L-glutamate (G), 100 μM-L-homocysteate (H), 1·2 mM-L-aspartate (A) and 5 μM-quisqualate (Q). Mg^{2+} (1 mM) was present in the medium during the periods represented by the bars above the records. The break in the lower record represents 20 min. Calibration: horizontal, 10 min; vertical, 1 mV.

the frog spinal cord as indicated by measurements with [^{3}H]NMDA (unpublished observations). Such a prolonged time course of action would have been inconvenient for some of the experiments carried out to investigate further the depressant action of Mg^{2+} on amino acid-induced responses. Although depressed by Mg^{2+} to a slightly lower degree than NMDA-induced depolarizations (Fig. 2), responses to L-homocysteate are rapidly terminated by uptake into the tissue (Cox *et al.* 1976; Watkins, Evans, Headley, Cox, Francis & Oakes, 1978) and this sulphonic amino acid was therefore used in preference to NMDA in some of the experiments described below.

Interactions of Ca^{2+} and Mg^{2+}

Mg^{2+} is known to depress neurotransmission in the frog spinal cord (Katz & Miledi, 1963; Erulkar *et al.* 1974; Evans *et al.* 1977*a*) and it has been suggested that

this depressant action is produced, at least in part, through post-synaptic interference with the action of an excitatory amino acid transmitter (Evans *et al.* 1977*a*). However, depression of transmission at the skeletal neuromuscular junction by Mg^{2+} is considered to be due to inhibition of transmitter release and is antagonized by raising the $[Ca^{2+}]$ of the medium (Del Castillo & Engbaek, 1954). Furthermore, Katz & Miledi (1963) have reported that increasing the $[Ca^{2+}]$ of the medium partially

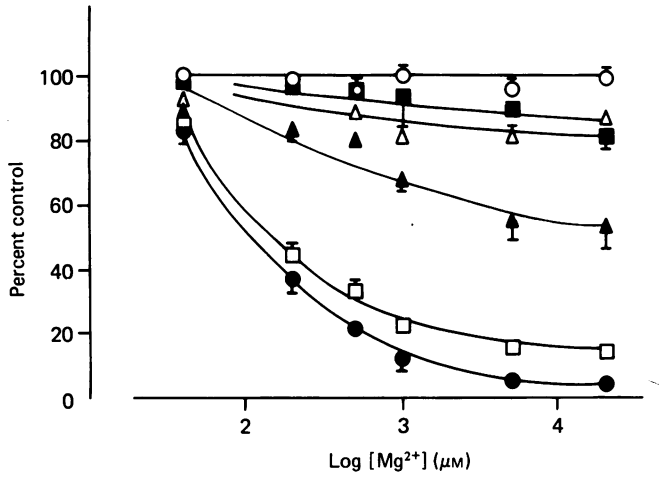

Fig. 2. Antagonism by increasing concentration of Mg^{2+} of frog ventral root depolarizations induced by a range of amino acid excitants. Amino acid-induced responses were measured in sequences of two to four amino acids, all control responses (approx. 2 mV) being matched in each preparation, by adjustment of agonist concentration, to within 10% of each other before addition of the metal ions at concentrations between 50 μM and 20 mM. Measurements in the presence of Mg^{2+} were made 10–40 min after introduction of the ions, responses to all excitants being relatively stable within this period. The bars show S.E. of mean for measurements made on three to six preparations; where not shown, they are covered by the symbols. ○, quisqualate; ■, kainate; △, L-glutamate; ▲, L-aspartate; □, L-homocysteate; ●, NMDA.

restores synaptic activity that has been blocked by Mg^{2+} in the isolated frog spinal cord. Since the antagonism of amino acid-induced responses by Mg^{2+} is not reversed by increasing $[Ca^{3+}]$ (Evans *et al.* 1977*a*) the effect of variation of $[Ca^{2+}]$ on the synaptic depression produced by Mg^{2+} might be expected to reflect the relative importance of pre- and post-synaptic mechanisms in this depression. To compare the actions of Ca^{2+} on Mg^{2+}-depressed synaptic activity and amino acid-induced depolarizations, DR-VRPs and ventral root responses to L-homocysteate, were determined first in Mg^{2+}-free/normal Ca^{2+} (2 mM) medium, then in Mg^{2+}/reduced Ca^{2+} (0·5 mM) medium (Fig. 3*A*). Lowering $[Ca^{2+}]$ caused a marked increase in the response to L-homocysteate, while the DR-VRP was slightly reduced. The addition of 0·5 mM-Mg^{2+} to the low $[Ca^{2+}]$ medium markedly reduced responses of both types. On raising the medium $[Ca^{2+}]$ from 0·5 to 5 mM (still with 0·5 mM-Mg^{2+} present) the DR-VRP was partially restored towards the pre-Mg level but the amino acid-induced responses were depressed further. Return to 0·5 mM-Ca^{2+} reversed these effects of the high-Ca^{2+} medium, the reversal of the DR-VRP responses being more

delayed than that of the amino acid-induced responses due possibly to a more prolonged retention of increased Ca^{2+} in deeper regions of the cord than in those more peripheral regions probably mediating the responses to the brief amino acid pulses. Return to the normal Mg^{2+}-free/2 mM-Ca^{2+}-containing medium restored all responses to near the control levels.

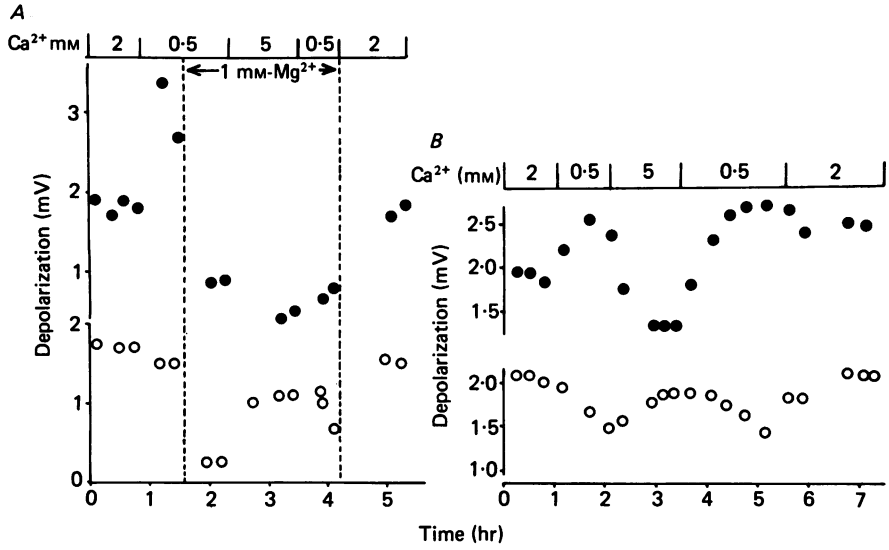

Fig. 3. Effect of variation in $[Ca^{2+}]$ on DR-VRPs (○) and L-homocysteate-induced depolarizations of motoneurones (●) recorded in ventral roots of two frog spinal cord preparations. A dorsal root of each preparation was stimulated supramaximally at 1/min. L-homocysteate (70 μM) was applied for 80 sec at the points indicated. *A*, effect of tenfold increase in $[Ca^{2+}]$ on depression produced by Mg^{2+} 1 mM. *B*, effect of tenfold increase in $[Ca^{2+}]$ in the absence of Mg^{+2}. For other details see Methods.

The above experiment indicates that increasing $[Ca^{2+}]$ partially counteracts the depressant effects of Mg^{2+} on DR-VRPs, but acts additively with Mg^{2+} in depressing amino-induced responses yet further. The question arises as to whether the effect of Ca^{2+} in reversing Mg^{2+}-induced depression of DR-VRPs is due to a Ca-Mg competition for membrane Ca^{2+} sites important for transmission processes (particularly transmitter release), or whether the Ca^{2+} effects are independent of Mg^{2+}. To investigate this question, the experiment of Fig. 3*B* was performed in which the addition of Mg^{2+} was omitted. It can be seen that the effect of variation in $[Ca^{2+}]$ on the synaptic response was similar to that observed in the presence of Mg^{2+} (Fig. 3*A*). It can therefore be concluded that the increase in the DR-VRP caused by high Ca^{2+} is not due to displacement of Mg^{2+} from membrane sites. Hence the partial reversal by Ca^{2+} of the action of Mg^{2+} in reducing the DR-VRP cannot be taken as evidence that the Mg^{2+} effect was presynaptically mediated.

Effects of other divalent metal ions

Some other divalent metals were tested for possible Mg^{2+}-like actions on motoneurone responses to different amino acid excitants. In this respect, Mn^{2+}, Co^{2+} and

Ni^{2+} were found to have similar properties to Mg^{2+}. The effect is illustrated by Fig. 4 which shows that depolarizations induced by kainate were relatively unaffected by these ions at a concentration (0·25 mM) which produced clear depression of NMDA-induced responses.

Table 1 shows the effects of 0·5 mM-Mg^{2+}, Co^{2+} and Mn^{2+} on the responses produced by six excitants and indicates that the relative susceptibilities of the agonists

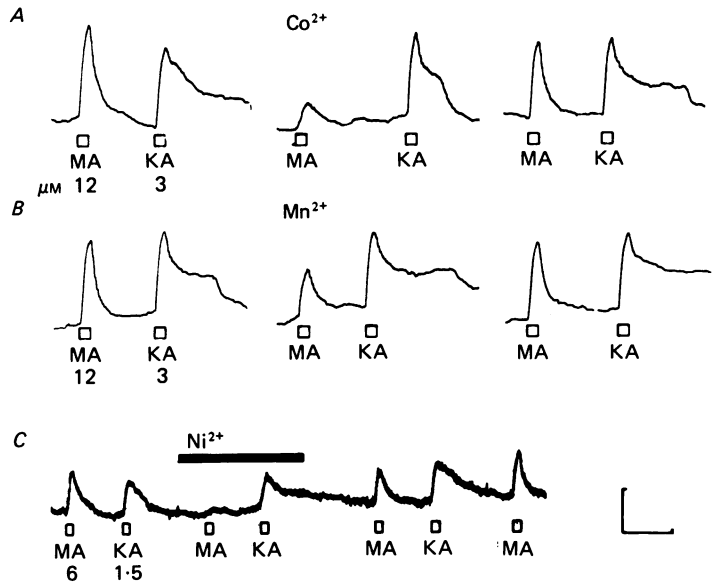

Fig. 4. Ventral root recordings of the effect of Co^{2+}, Mn^{2+} and Ni^{2+} (0·25 mM) on depolarizing responses produced by 2 min applications of N-methyl-D-aspartate (MA) or kainate (KA) in three frog TTX-blocked spinal cord preparations. In *A* and *B* the metal ions were introduced 10 min before the start of the centre traces and the right hand traces show recovery 25 min after removal of the metal ions from the bathing medium. In *C* the trace is continuous and Ni^{2+} was introduced during the period indicated by the bar above the trace. Vertical calibration 1 mV in *A* and *B*, 0·5 mV in *C*. Horizontal calibration 10 min.

to the actions of the ions were similar in each case. However, Mn^{2+} was less potent than either Mg^{2+} or Co^{2+}. A more quantitative measure of the relative potencies of the divalent metal ions was obtained by comparing dose ratios for depression of NMDA-induced responses. For 1 mM concentrations of Mn^{2+}, Mg^{2+}, Co^{2+} and Ni^{2+} the respective dose ratios observed were: (mean ± S.E. of mean, number of preparations in parentheses) 3·8 ± 0·5 (3), 5·7 ± 0·4 (7), 13·3 (2) and > 20 (3). In these experiments it was observed that the effects of Co^{2+} and Ni^{2+} (particularly) increased progressively with time of contact of the ions with the tissue, the values given above referring to a contact time for all the ions of approximately 30 min. By contrast, responses measured in the presence of Mg^{2+} or Mn^{2+} were relatively stable after about 10 min.

The results illustrated by Fig. 3 suggest that Ca^{2+} has an effect on excitant amino acid-induced responses similar to, but weaker than, Mg^{2+}. This effect of Ca^{2+} con-

trasts with the effect of these ions in antagonizing the presynaptic blocking action of Mg^{2+} at the frog neuromuscular junction (del Castillo & Engbaek, 1954). At the squid giant synapse (Katz & Miledi, 1969) and frog neuromuscular junction (Miledi, 1966), Sr^{2+} and Ba^{2+} resemble Ca^{2+} in their effects whereas Mg^{2+} and the transition metal ions antagonize the action of Ca^{2+}. The actions of Ca^{2+}, Sr^{2} and Ba^{2+} were therefore compared with that of Mg^{2+} on the frog spinal cord. Addition of Ca^{2+}, Sr^{2} or Ba^{2+}

TABLE 1. Effects of divalent metal ions (0·5 mM) on responses of frog spinal motoneurones to excitatory amino acids

	Percent control responses		
	Mg^{2+}	Co^{2+}	Mn^{2+}
NMDA	22 ± 1	17 ± 3	57 ± 1
L-homocysteate	34 ± 1	40 ± 5	64 ± 3
L-aspartate	82 ± 2	64 ± 11	81 ± 5
L-glutamate	89 ± 2	93 ± 5	91 ± 3
Kainate	96 ± 3	96 ± 7	95 ± 1
Quisqualate	95 ± 1	105 ± 2	98 ± 1

Amino acid-induced ventral root depolarizations (approx. 2 mV) were measured, in the presence of TTX (10^{-7} M) as described in the legend to Fig. 2. Measurements in the presence of the metal ions (0·5 mM) were made during a period of 10–40 min after their introduction. The values given are the amino acid-induced depolarizations in the presence of the divalent metal ions expressed as a percentage, ± S.E. of mean (three to six preparations), of the depolarizations obtained before treatment with these ions. Mg^{2+} and Mn^{2+} produced a stable depression of agonist responses with 10 min of their application. However, the depression of responses produced by Co^{2+} increased progressively with time and the values given for this ion are the average of two responses measured at an interval of 30 min in each preparation.

(2 mM) failed to alter significantly the antagonism of NMDA-induced responses produced by 1 mM-Mg^{2+} in two frog spinal cord preparations bathed in Ca^{2+}-free medium. In another type of experiment, three preparations were bathed in low Ca^{2+} (0·5 mM) medium and it was found that an increase of 2–3 mM in [Ca^{2+}] produced a depression of L-homocysteate-induced responses equivalent to that produced by addition of 0·2 mM-Mg^{2+} to this medium. In two analogous experiments addition of Sr^{2+} or Ba^{2+} were found to be of the same or lower potency, compared with Ca^{2+}, in depressing L-homocysteate-induced responses.

Thus it appears that although all the alkaline earth metal ions have qualitatively similar effects, Mg^{2+} is uniquely potent amongst this group in selectively depressing amino acid-induced motoneuronal depolarizations.

Effects of Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} on transmission in the rat superior cervical ganglion and the immature rat spinal cord

D-α-aminoadipate, α,ϵ-diaminopimelate and other related compounds selectively antagonize responses of spinal neurones to excitatory amino acids, the pattern of this antagonism being similar to that reported here for divalent metal ions. These compounds also depress spinal synaptic transmission and it has been suggested that this action involves the blockade of an excitatory amino acid transmitter (Biscoe, Davies, Dray, Evans, Martin & Watkins, 1978; Evans & Watkins, 1978; Evans, Francis

& Watkins, 1978; Evans *et al.* 1979). Thus the possibility must be considered that the depressant action of divalent metal ions on central transmission is also due, at least in part, to interference with the post-synaptic actions of an excitatory amino acid transmitter. If this were the predominant mechanism involved, then transmission in autonomic ganglia should be relatively unaffected by the low concentrations of these ions necessary to depress amino acid-induced and synaptic excitation in spinal cord preparations. Ganglionic transmission is unlikely to involve amino acids as transmitters, and is not blocked by D-α-aminoadipate and related compounds (Evans & Watkins, 1978*a*). If, however, the effects of the ions on spinal cord synaptic activity are due to non-specific membrane effects, as proposed by Kato, Kelly, Krnjević & Somjen (1968) for cerebral cortical neurones, or to presynaptic actions as occurs at the frog neuromuscular junction (Del Castillo & Engbaek, 1954; Meiri & Rahaminoff, 1972; Weakley, 1973; Kita & Van der Kloot, 1973) or the squid stellate ganglion (Katz & Miledi, 1969), then ganglionic transmission might also be expected to be antagonized by the same low concentrations of the ions as those that depress spinal synaptic activity.

Fig. 5 shows a comparison between the effects of Mg^{2+}, Mn^{2+} and Co^{2+} (2 mM) on transmission in an isolated superior cervical ganglion and transmission in the isolated hemisected spinal cord of a neonatal rat. All three metal ions were effective in depressing spinal cord transmission but, unlike the other two, Mg^{2+} was relatively ineffective in depressing ganglionic transmission at this concentration. Similar effects were observed in two other preparations of spinal cords and ganglia which were treated with 0·5 and 1 mM metal ions. Mn^{2+}, Co^{2+} and Ni^{2+} produced evident depression of ganglionic transmission at 0·5 mM whereas Mg^{2+} had no significant action on ganglionic transmission at 0·5 or 1 mM. In one ganglion preparation, superfused with atropine (1 μM), depolarizations induced by carbachol were unaffected by these metal ions (2 mM).

Thus it would appear that, if the effects of these ions on ganglionic transmission can be taken as an indication of their central presynaptic actions, the central actions of Mn^{2+}, Co^{2+} and Ni^{2+} are likely to involve both pre- and post-synaptic effects, whereas the central depressant action of Mg^{2+}, at levels of 2 mM or less, is largely the result of post-synaptic effects.

Effects of Mg^{2+} on responses to other types of putative transmitter

D-α-aminoadipate and other similar pharmacological antagonists of NMDA are quite selective in their action; thus, depolarizing responses induced by non-amino acid transmitter agonists, recorded in ventral roots of the neonatal rat spinal cord, are resistant to these agents (Evans & Watkins, 1978*a*). In the present investigation the specificity of the antagonism produced by Mg^{2+} was tested on the neonatal rat preparation. Submaximal depolarizing responses induced by 5-HT (B. Ault & R. H. Evans, unpublished observations) were insensitive to Mg^{2+} (1 mM). However, similar responses induced by substance P (Konishi & Otsuka, 1974), noradrenaline (Ault & Evans, 1978) or carbachol (Evans, 1978*a*) were all depressed by Mg^{2+} (1 mM), noradrenaline-induced responses being the most sensitive. These effects of Mg^{2+} were greater than those estimated in our preliminary study (Evans & Watkins, 1978*a*)

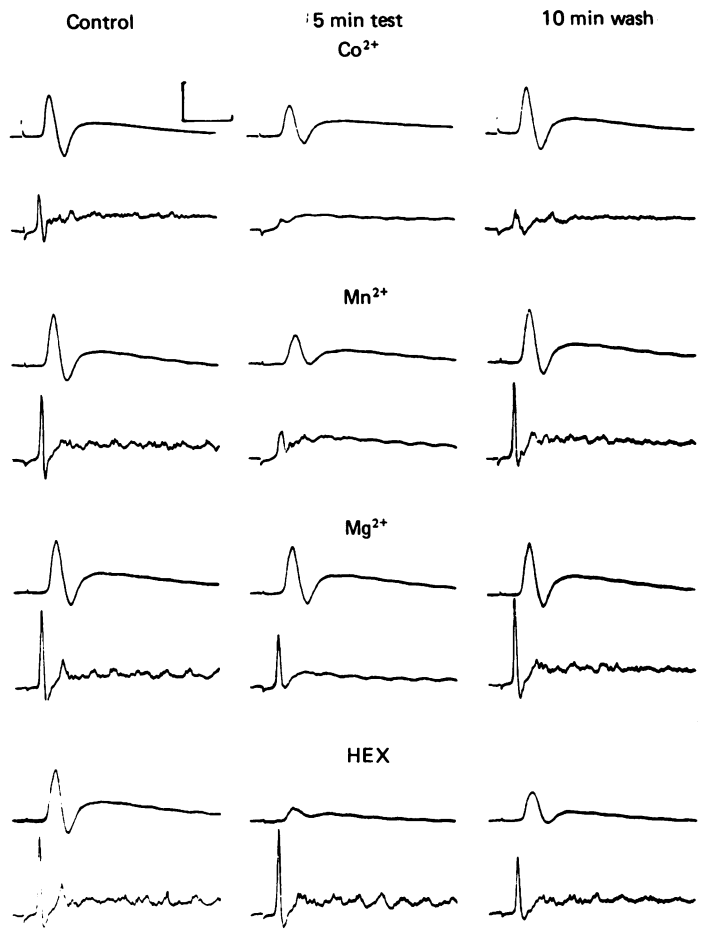

Fig. 5. Effect of Co^{2+}, Mn^{2+} and Mg^{2+} (all at 2 mM) and hexamethonium (HEX, 0·5 mM) on ganglionic and spinal transmission. A rat superior cervical ganglion and a neonatal rat spinal cord preparation were bathed in the same medium and simultaneous records of the post-ganglionic response to preganglionic nerve stimulation (upper traces), and DR-VRPs (lower traces) were made. The preparations were supramaximally stimulated at 1/min. Test substances were introduced 5 min before the centre recordings were made and recovery is shown in the right hand trace 10 min after removal of test substances from the bathing medium. Intervals of 60 min were allowed between applications of test substances. Vertical calibration, ganglion recordings 2·7 mV, spinal cord recordings 2 mV. Horizontal calibration, 20 msec. For other details see Methods.

probably because of failure to ensure that the responses to these substances were sufficiently submaximal in our earlier experiments. However, in conformity with the conclusions of this preliminary study, by far the most marked effects of Mg^{2+} were observed in the case of NMDA-induced responses. The relative effects of Mg^{2+} on the responses to these various agonists are illustrated in Fig. 6.

The selectivity of Mg^{2+} for excitant amino acid-induced responses was further tested on depolarizations induced by L-homocysteate or GABA and recorded in

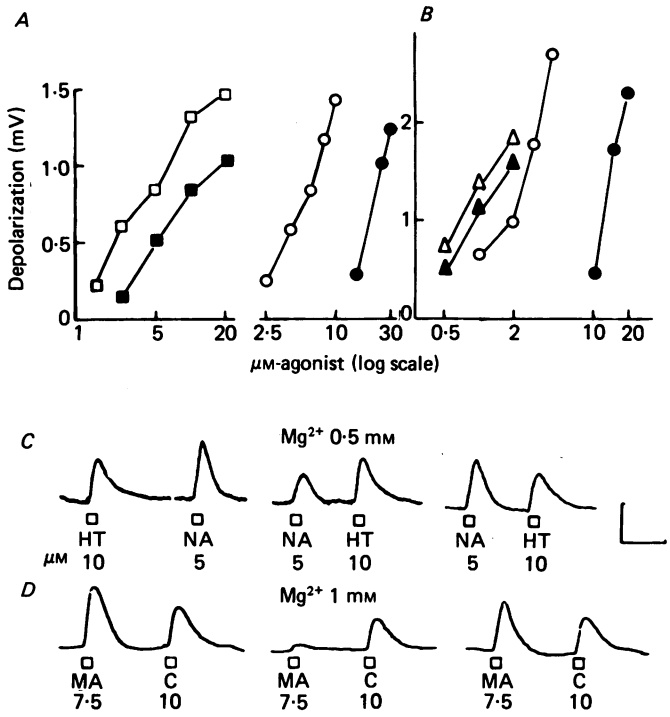

Fig. 6. Ventral root recordings from neonatal rat TTX-blocked spinal cord preparations. *A*, effect of 1 mM-Mg^{2+} (filled symbols) on depolarizing responses induced by noradrenaline (□) and N-methyl-D-aspartate (○). The coefficient of variation was 20 %, as assessed from the variance of responses at the 5 μM dose level. *B*, depolarizing responses induced by substance P (△). Other details as for *A*. *C*, depolarizing responses induced by 5-hydroxytryptamine (HT) and noradrenaline (NA). Mg^{2+} was introduced 10 min before the start of the centre trace. Recovery is shown in the right hand trace which commenced 25 min after removal of Mg^{2+} from the bathing medium. *D*, depolarizing responses induced by N-methyl-D-aspartate (MA) and carbachol (C). Other details as for *C*. Vertical calibration, 0·5 mV in *C*, 1 mV in *D*. Horizontal calibration, 10 min. All agonists were applied for 2 min.

dorsal roots of frog isolated spinal cords. In contrast to the responses produced by the excitatory amino acid, responses induced by GABA were unaffected by 1 mM-Mg (Fig. 7).

Lack of effect of Mg^{2+} on amino acid uptake

Changes in the monovalent cation concentration of the superfusion medium have marked effects on the rates of uptake of amino acids (Watkins *et al.* 1978) which can be correlated with the effects of the same ionic changes on root potentials generated by the amino acids in the frog spinal cord (Evans, Francis & Watkins, 1977*b*). To investigate whether Mg^{2+} also has effects on rates of amino acid uptake such as might contribute to the differential depression of amino acid-induced responses by these divalent ions, the uptake of [$U^{14}C$] L-glutamate, [$U^{14}C$] L-aspartate and [^{35}S]L-homocysteate into frog spinal cord were compared in the presence and absence of

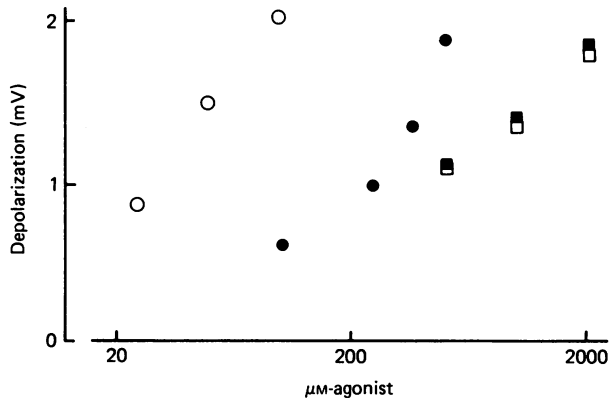

Fig. 7. Effect of 1 mM-Mg^{2+} (filled symbols 20 min after introduction of Mg^{2+}) on dorsal root depolarizations induced by 80 sec applications of GABA (□) or L-homocysteate (○) in a frog spinal cord preparation blocked with TTX. For other details see Methods.

TABLE 2. Uptake of L-[$U^{14}C$]glutamate, L-[$U^{14}C$]aspartate and L-[^{35}S]homocysteate in hemisected frog spinal cord

		Uptake (n-mole/g wet wt.)	
	Time	Control	+Mg^{2+} (1 mM)
L-[$U^{14}C$]glutamate	80 sec	77 ± 8·6 (5)	78 ± 7·2 (5)
	15 min	509 ± 26 (5)	508 ± 44 (5)
L-[$U^{14}C$]aspartate	80 sec	58 ± 5 (6)	53 ± 5 (6)
	15 min	574 ± 29 (6)	507 ± 35 (6)
L-[^{35}S]homocysteate	15 min	68 ± 10 (4)	74 ± 4 (4)

Hemisected frog spinal cords were incubated for either 80 sec or 15 min in medium containing 0·5 mM-L-[$U^{14}C$]glutamate, 0·5 mM-L-[$U^{14}C$]aspartate or 70 μM-L-[^{35}S]homocysteate in the presence or absence of 1 mM-Mg^{2+}. Other details are given in the Methods section. The amounts of the amino acids taken up show no significant differences ($P > 0·1$, Student's t test) between control values and values measured in the presence of Mg^{2+}.

1 mM-Mg^{2+}. The concentrations of amino acids used for these uptake experiments were those that produced approximately equal depolarizations in the absence of Mg^{2+}. Table 2 shows that Mg^{2+} had no significant effect on the uptake of any of these amino acids measured over an 80 sec period (approximating to the period of contact of the amino acid with the spinal cord in electrophysiological experiments) or for 15 min. This indicates that the depolarizing responses produced by these amino acids are not related to the uptake processes, particularly in the case of L-homocysteate, the responses to which are strongly antagonized by Mg^{2+}. Furthermore, the lesser effect of Mg^{2+} on depolarizations produced by L-glutamate and L-aspartate, compared with L-homocysteate, cannot be ascribed to any masked enhancement of the responses to these latter two amino acids due to an inhibition by Mg^{2+} of their uptake systems.

DISCUSSION

The results illustrate a selective blocking action of Mg^{2+} on depolarization of motoneurones induced by certain excitatory amino acids. This selectivity is exemplified by the marked susceptibility of NMDA-induced responses and the relative resistance of quisqualate and kainate-induced responses to these metal ions. Other divalent ions produced similar effects, the descending order of potency being Ni^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+}. Among alkaline earth metal ions, Ca^{2+} had a very weak Mg^{2+}-like action in the absence of the latter ions and enhanced the action of Mg^{2+} when both ions were added together; Sr^{2+} and Ba^{2+} had actions which were similar to or weaker than that of Ca^{2+}. The lack of antagonism of the effects of Mg^{2+} by Ca^{2+} rules out a competition between these ions as a mechanism underlying the action of Mg^{2+} such as that proposed for the blockade of transmitter release by these ions at peripheral junctions (Del Castillo & Engbaek, 1954).

The pattern of antagonism shown by the divalent metal ions is similar to that described for D-α-aminoadipate and structurally similar compounds, the descending order of agonist susceptibility to both types of antagonism being NMDA, L-homocysteate, L-aspartate, L-glutamate, kainate and quisqualate (Evans *et al.* 1978, 1979). These observations indicate the existence of at least two types of receptors (or receptor/ionophore systems) for excitatory amino acids, one type (NMDA receptors) being sensitive to the action of both the organic and inorganic antagonists and other receptors being insensitive to the actions of either type of antagonist. On this basis, many amino acids, including L-glutamate and L-aspartate would be presumed to have mixed actions on different receptors.

Preliminary measurements of the antagonism produced by mixtures of D-α-aminoadipate and related antagonists with Mg^{2+} indicate that separate sites are involved in the actions of the organic and inorganic antagonists (Evans & Watkins, 1978*b*). The organic compounds probably compete with the agonist at the amino acid receptor site, while the metal ions act at a different site, possibly the ionophore involved in the depolarizing response (Davies *et al.* 1979).

An important difference observed between Mg^{2+} and the organic NMDA antagonists, undetected in our preliminary experiments (Evans & Watkins, 1978*a*), was the ability of Mg^{2+} to depress depolarizing responses of motoneurones of the immature rat evoked by carbachol, noradrenaline and substance P. It has also been reported that Mg^{2+} depresses acetylcholine- as well as amino acid-induced excitation in the cat central nervous system (Kato *et al.* 1968; Davies & Watkins, 1977). In the present work, however, the effects of Mg^{2+} on the responses to these other agonists were far less pronounced than the depression of NMDA-induced responses by the divalent ions. Although the receptors for these various transmitter agonists appear to be quite distinct (Evans, 1978*b*; Evans & Watkins, 1978*a*; Ault & Evans, 1978) it is possible that the ionophores involved bear some structural similarities to one another, rendering them all sensitive to Mg^{2+} to a certain extent. However, the fact that the depolarizing responses produced in rat motoneurones by 5-hydroxytryptamine or by GABA in frog primary afferent fibres were unaffected by Mg^{2+} indicates that such structural features are not an essential part of all transmitter-activated ionophores in the mammalian and amphibian spinal cords.

The organic NMDA antagonists show a clear parallelism in their order of potency for depression of NMDA-induced responses and synaptic transmission in spinal cords. This has led to the suggestion that the organic antagonists mediate their effects by the blockade of the action of an excitatory amino acid transmitter (Evans *et al.* 1979). The same mechanism could underlie the depression of DR-VRPs by low concentrations of Mg^{2+}. An alternative explanation that this effect was predominantly due to a depression of transmitter release similar to that observed at peripheral junctions (Del Castillo & Engbaek, 1954), would seem less likely. Firstly, the threshold concentration of Mg^{2+} necessary to depress DR-VRPs, around 10–50 μM (Davies *et al.* 1979), is considerably lower than that generally considered necessary for the depression of transmitter release. Secondly, although DR-VRPs that had been depressed by Mg^{2+} could be increased towards control values by increasing $[Ca^{2+}]$, this effect can not be ascribed to a Ca^{2+}/Mg^{2+} antagonism similar to that believed to underlie the depression of transmitter release at peripheral junctions, since DR-VRPs measured in the absence of Mg^{2+} were similarly enhanced by raised $[Ca^{2+}]$. These effects were probably due to an increase in transmitter release resulting from the raised extracellular concentration of Ca^{2+}, presumably leading to an increased intra-terminal influx of the ions during synaptic activity.

On the other hand, depression of transmitter release would seem likely to have contributed significantly to the depression of synaptic activity produced by Co^{2+}, Mn^{2+} and Ni^{2+}. Unlike the organic NMDA antagonists, the divalent metal ions did not exhibit a parallelism in their depressant effects on NMDA-induced and synaptically-evoked responses. In particular, Mn^{2+} was less potent than Mg^{2+} in antagonizing NMDA-induced responses, but was more potent than Mg^{2+} in depressing synaptic excitation. Mn^{2+}, Co^{2+} and Ni^{2+} are all considerably more potent than Mg^{2+} in depressing transmitter release at peripheral junctions (Meiri & Rahaminoff, 1972; Weakley, 1973; Kita & Van der Kloot, 1973). In the present work, these three transition metal ions had pronounced depressant actions on transmission in sympathetic ganglia, whereas Mg^{2+} had no such action at concentrations able to produce marked antagonism of NMDA-induced responses and synaptic excitation in spinal cords. Thus, it is suggested that the central depressant action of Mg^{2+} at low concentrations is largely the result of an interference with post-synaptic actions of a transmitter acting at NMDA receptors, while the central depressant effects of Mn^{2+}, Co^{2+} and Ni^{2+} involve this same post-synaptic mechanism acting synergistically with a presynaptic depression of transmitter release. Such post-synaptic actions would demand that caution be exercised in the use of these ions as inhibitors of transmitter release in studies of synaptic transmission in the vertebrate central nervous system (Barker, Nicoll & Padjen, 1975).

Our conclusion of a predominantly postsynaptic effect of low concentrations of Mg^{2+} does not conflict with the observations of Katz & Miledi (1963) and Erulkar (1974) that spontaneous junctional potentials can be recorded intracellularly in the presence of high external $[Mg^{2+}]$. The potentials recorded under these conditions could represent the action of spontaneously released transmitter(s) on Mg^{2+}-insensitive receptors such as those activated by quisqualate and, less specifically, by kainate and L-glutamate in the present work. The existence of these divalent metal ion-resistant, presumably post-synaptic receptors for excitatory amino acids may also be

relevant to the persistence of short-latency motoneuronal e.p.s.p.s evoked by dorsal root stimulation in the frog spinal cord bathed in low-Ca^{2+} medium containing Co^{2+} or Mg^{2+}. Such e.p.s.p.s have recently been postulated to be due to electrical coupling between primary afferents and motoneurone dendrites (Shapovalov, Shiriaev & Velumian, 1978; Alvarez-Leefmans, De Santis & Miledi, 1979), though chemical transmission could not be totally excluded. Specific blocking agents for the Mg^{2+}-resistant type(s) of excitatory amino acid receptors would clearly be of value in the further investigation of such phenomena.

REFERENCES

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

AULT, B. & EVANS, R. H. (1978). The action of catecholamines on the isolated hemisected spinal cord of the immature rat. *J. Physiol.* **278**, 41*P*.

BARKER, J. L., NICOLL, R. A. & PADJEN, A. (1975). Studies on convulsants in the isolated frog spinal cord. 1. Antagonism of amino acid responses. *J. Physiol.* **245**, 521–536.

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

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

CURTIS, D. R., PERRIN, D. D. & WATKINS, J. C. (1960). The excitation of spinal neurones by the ionophoretic application of agents which chelate calcium. *J. Neurochem.* **6**, 1–20.

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

DAVIES, J. & WATKINS, J. C. (1977). Effects of magnesium ions on the responses of spinal neurones to excitatory amino acids and acetylcholine. *Brain Res.* **130**, 364–368.

DEL CASTILLO, J. & ENGBAEK, L. (1954). The nature of the neuromuscular block produced by magnesium. *J. Physiol.* **124**, 370–384.

ERULKAR, S. D., DAMBACH, G. E. & MENDER, D. (1974). The effect of magnesium at motoneurones of the isolated spinal cord of the frog. *Brain Res.* **66**, 413–424.

EVANS, R. H. (1978*a*). The effects of amino acids and antagonists on the isolated hemisected spinal cord of the immature rat. *Br. J. Pharmac.* **62**, 171–176.

EVANS, R. H. (1978*b*). Cholinoceptive properties of motoneurones of the immature rat spinal cord maintained *in vitro*. *Neuropharmacology* **17**, 277–281.

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., FRANCIS, A. A. & WATKINS, J. C. (1977*a*). Selective antagonism by Mg^{2+} of amino acid-induced depolarization of spinal neurones. *Experientia* **33**, 489–491.

EVANS, R. H., FRANCIS, A. A. & WATKINS, J. C. (1977*b*). Effects of monovalent cations on the responses of motoneurones to different groups of amino acid excitants in frog and rat spinal cord. *Experientia* **33**, 246–248.

EVANS, R. H., FRANCIS, A. A. & WATKINS, J. C. (1977*c*). Selective antagonism by Mg^{2+} of amino acid-induced depolarization of frog and rat spinal neurones. *J. Physiol.* **265**, 42–43*P*.

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

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

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

KATO, G., KELLY, J. S., KRNJEVIĆ, K. & SOMJEN, G. (1968). Anaesthetic action of magnesium ions. *Can. anaes. Soc. J.* **15**, 539–544.

KATZ, B. & MILEDI, R. (1963). A study of spontaneous miniature potentials in spinal motoneurones. *J. Physiol.* **168**, 389–422.

KATZ, B. & MILEDI, R. (1969). Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol.* **203**, 459–487.

KELLY, J. S., KRNJEVIĆ, K. & SOMJEN, G. (1969). Divalent cations and electrical properties of cortical cells. *J. Neurobiol.* **1**, 197–208.

KITA, H. & VAN DER KLOOT, W. (1973). Action of Co and Ni at the frog neuromuscular junction. *Nature, New Biol.* **245**, 52–53.

KONISHI, S. & OTSUKA, M. (1974). Excitatory action of hypothalamic substance P on spinal motoneurones of newborn rats. *Nature, Lond.* **252**, 734–735.

KRNJEVIĆ, K. & PHILLIS, J. W. (1963). Iontophoretic studies of neurones in the mammalian cerebral cortex. *J. Physiol.* **165**, 274–304.

MEIRI, U. & RAHAMINOFF, R. (1972). Neuromuscular transmission: inhibition by manganese ions. *Science, N.Y.* **176**, 308–309.

MILEDI, R. (1966). Strontium as a substitute for calcium in the process of transmitter release at the neuromuscular junction. *Nature, Lond.* **212**, 1233–1234.

OTSUKA, M. & KONISHI, S. (1974). Electrophysiology of mammalian spinal cord *in vitro*. *Nature, Lond.*, **252**, 733–734.

ROZEAR, M., DE GROOF, R. & SOMJEN, G. (1971). Effects of microiontophoretic administration of divalent metal ions on neurons of the central nervous system of cats. *J. Pharmac. exp. Ther.* **176**, 109–118.

RUBIN, R. P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. *Pharmac. Rev.* **22**, 389–428.

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

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

WATKINS, J. C., EVANS, R. H., HEADLEY, P. M., COX, D. W. G., FRANCIS, A. A. & OAKES, D. J. (1978). Role of uptake in excitation of central neurones by glutamate-related amino acids: possible value in transmitter identification. In *Iontophoresis and Transmitter Mechanisms in the Mammalian Central Nervous System*, ed. RYALL, R. W. & KELLY, J. S. pp. 397–399. Amsterdam: Elsevier/North Holland Biomedical Press.

WEAKLEY, J. N. (1973). The action of cobalt ions on neuromuscular transmission in the frog. *J. Physiol.* **234**, 597–612.