J. Physiol. (1985), **361**, *pp.* 65–90
With 12 text figures
Printed in Great Britain

THE ACTION OF *N*-METHYL-D-ASPARTIC ACID ON MOUSE SPINAL NEURONES IN CULTURE

BY MARK L. MAYER AND GARY L. WESTBROOK

From the Laboratory of Developmental Neurobiology, NICHD, Building 36, Room 2A21, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(*Received 11 July 1984*)

SUMMARY

1. Neurones from the ventral half of mouse embryo spinal cord were grown in dissociated culture and voltage clamped. The current–voltage relation of responses evoked by *N*-methyl-D-aspartic acid (NMDA), L-glutamic acid and kainic acid was recorded in media of different ionic composition.

2. On removal of Mg^{2+} from the extracellular solution, responses to NMDA and L-glutamate became less voltage sensitive, such that NMDA responses were no longer associated with a region of negative slope conductance. The antagonism of NMDA responses produced by application of Mg^{2+} to neurones bathed in nominally Mg^{2+}-free solutions shows voltage dependence and uncompetitive kinetics.

3. Voltage-jump experiments showed that the voltage-dependent action of Mg^{2+} occurred rapidly, and with complex kinetics.

4. Ni^{2+} and Cd^{2+}, two potent blockers of calcium currents in spinal cord neurones, had significantly different potencies as NMDA antagonists, Ni^{2+} being of greater potency than Mg^{2+}, and Cd^{2+} considerably weaker.

5. The voltage-dependent block of NMDA responses produced by physiological concentrations of Mg^{2+} is sufficient to explain the apparent increase in membrane resistance produced by NMDA in current-clamp experiments, and the ability of NMDA to support repetitive firing.

6. Substitution of choline for Na^+ produced a hyperpolarizing shift in the reversal potential for responses evoked by kainic acid consistent with an increase in permeability to Na^+ and K^+. In choline-substituted solutions, the reversal potential of NMDA responses was more positive than that recorded for kainic acid, and in addition NMDA responses showed enhanced desensitization.

INTRODUCTION

The conductance mechanism underlying the depolarizing action of excitatory amino acids on vertebrate neurones has not been clearly defined. Recent experiments have demonstrated the existence of several distinct excitatory amino acid receptors, which may be distinguished by the use of selective agonists and antagonists (Watkins, 1981*a*, *b*). The receptors appear to be linked to two separate conductance mechanisms (Engberg, Flatman & Lambert, 1978; MacDonald & Porietis, 1982; Mayer &

Westbrook, 1984*a*). *N*-methyl-D-aspartic acid (NMDA), a selective agonist for one type of receptor (Watkins, 1981*a*, *b*), activates a voltage-sensitive conductance (Engberg *et al.* 1978; MacDonald, Porietis & Wojtowicz, 1982; Mayer & Westbrook, 1984*a*). The inward current activated by NMDA increases as the membrane potential is depolarized, producing a 'J-shaped' agonist current–voltage plot with a negative slope conductance at membrane potentials more negative than −30 mV (MacDonald *et al.* 1982; Flatman, Schwindt, Crill & Stafstrom, 1983; Mayer & Westbrook, 1984*a*). The voltage sensitivity of the response to NMDA resembles that of regenerative sodium or calcium currents, and it has been suggested that NMDA acts by modulating voltage-dependent Na^+ or Ca^{2+} conductances (MacDonald *et al.* 1982; Dingledine, 1983; Flatman *et al.* 1983). In contrast, kainic acid and quisqualic acid, selective agonists for other amino acid receptors (Watkins, 1981*a*, *b*), produce responses with a nearly linear current–voltage relation (MacDonald & Porietis, 1982; Mayer & Westbrook, 1984*a*); thus, these receptors are linked to more conventional agonist-activated conductance mechanisms.

The reversal potential of responses evoked by selective agonists acting at NMDA or non-NMDA receptors, and measured under voltage clamp, is similar and close to 0 mV (Mayer & Westbrook, 1984*a*). Thus, it is unlikely that activation of these receptors selectively increases the membrane permeability to either Na^+ or Ca^{2+} and a mixed ionic mechanism seems probable. Studies on frog motoneurones using ion-selective micro-electrodes have shown that L-glutamate, a mixed agonist which activates both NMDA and non-NMDA receptors (Mayer & Westbrook, 1984*a*; Westbrook & Mayer, 1984), increases the membrane permeability to both Na^+ and Ca^{2+} (Buhrle & Sonnhof, 1983). In mammalian hippocampal pyramidal neurones, the reversal potential of L-glutamate shifts to a more negative value on reduction of the extracellular Na^+ concentration, and is consistent with an increase in permeability to both Na^+ and K^+ (Hablitz & Langmoen, 1982). Since intracellular injection of Cs^+ does not alter the reversal potential of excitatory amino acid responses (Mayer & Westbrook, 1984*a*; Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984), it seems probable that both NMDA and non-NMDA receptors are linked to relatively non-selective cation-permeable channels similar to cholinergic channels at the end-plate, which under physiological conditions do not discriminate well between Na^+, K^+ and Cs^+ (Huang, Catterall & Ehrenstein, 1978; Adams, Dwyer & Hille, 1980). Despite this apparent similarity in the permeability change evoked by agonists acting at NMDA and non-NMDA receptors, an additional mechanism must be present to give NMDA responses their characteristic voltage sensitivity.

In the present paper, we have used voltage-clamp recording from mouse spinal neurones in dissociated culture to investigate the mechanism of the voltage sensitivity of the NMDA response, and the ionic mechanism of responses evoked by NMDA, L-glutamate and kainate. Our results suggest that Mg^{2+} selectively bind to and block the ion channels linked to NMDA receptors, and that the affinity of Mg^{2+} depends on the membrane electric field. Preliminary reports of our findings have been published previously (Mayer & Westbrook, 1984*b*; Mayer, Westbrook & Guthrie, 1984). A paper describing voltage-dependent block by Mg^{2+} of current flow through single channels linked to NMDA receptors and activated by L-glutamate has appeared recently (Nowak *et al.* 1984).

METHODS

Details of the preparation, spinal neurones in dissociated cultures prepared from the ventral half of mouse embryo spinal cord, and the techniques for two-micro-electrode, voltage-clamp recording and data acquisition have been published previously (Mayer & Westbrook, 1983, 1984*a*). In the present study, we used micro-electrodes filled with 1 M-CsCl to reduce time- and voltage-dependent potassium currents activated on membrane potential depolarization. In a few experiments we used a giga-ohm seal, patch-clamp technique to record whole-cell currents with a single-micro-electrode, discontinuous, voltage-clamp amplifier (Axon Instruments). The patch electrodes were filled with a solution containing (mM): 140, CsCl; 2, $MgCl_2$; 1·1, EGTA (pCa 8); 10, HEPES titrated to pH 7·2 with KOH. Sucrose was added to adjust the osmolarity to 310 mosmol. In a few experiments, KCl or $KMeSO_4$ was substituted for CsCl. The results obtained using whole-cell recording were comparable to those recorded using a two-micro-electrode voltage clamp. Experiments were performed at 27–28 °C.

Composition of media

The normal recording medium used for two-electrode, voltage-clamp experiments contained (mM): 143, NaCl; 4·8, KCl; 1, $MgCl_2$; 5, $CaCl_2$; 10, glucose; 10, HEPES; 0·01 mg Phenol Red/ml and was titrated to pH 7·3. In a few experiments, the concentration of $CaCl_2$ was lowered to 2·5 mM and the concentration of $MgCl_2$ raised to 10 mM. Sucrose was added to match the osmolarity of the growth medium (325 mosmol). Tetrodotoxin (TTX) (0·6 μM) was added to block spontaneous activity and action potential generation. In one series of experiments performed in 'Mg^{2+}-free' medium, the tissue culture plates were washed 3 times with 'Mg^{2+}-free' medium before recording, to reduce, as far as possible, contamination from residual Mg^{2+} in the growth medium (0·8 mM-Mg^{2+}). Since Mg^{2+} is an effective NMDA antagonist at micromolar concentrations (Nowak *et al.* 1984), we used atomic absorption spectroscopy to measure the concentration of Mg^{2+} in the recording medium after the washing procedure. We estimate that our 'Mg^{2+}-free' recording medium used for two-electrode, voltage-clamp experiments contains 8–10 μM-Mg^{2+}; this is in part due to contamination of analar grade $CaCl_2$ with Mg^{2+} (1 μM-Mg^{2+}/mM Ca^{2+}) and such Mg^{2+} contamination may contribute to the residual voltage sensitivity of NMDA responses recorded in this medium (see Fig. 2).

Low-Na^+ medium was prepared by substituting choline chloride for NaCl; when this medium was used, plates were again carefully washed 3 times to reduce contamination from the growth medium which contains physiological levels of Na^+. Media containing divalent cations, for application to individual neurones by pressure (see below), were prepared by isosmotic addition of the required cation to the recording medium. In those experiments used to study calcium currents, tetraethylammonium chloride (10 mM) was added to the recording medium to further reduce potassium currents.

Extracellular perfusion technique

Excitatory amino acids and divalent cations were dissolved in recording medium, or in some experiments a medium in which the $MgCl_2$ concentration was different from that in the recording chamber, and applied to individual neurones by pressure ejection from extracellular micropipettes. These were fashioned on a two-stage puller, and were similar to those used for patch recording. Drug solutions were ejected using pressure pulses of 10–1000 ms duration, 1–5 lbf/in^2, applied to pipettes positioned 10–50 μm from the soma to evoke single responses of rapid rise time and brief duration. This method of drug application gave consistent levels of inward current, but the actual concentration of excitatory amino acid reaching the membrane surface was unknown. In some experiments it was convenient to evoke sustained responses, of constant amplitude, for periods of 20–60 s. To achieve this, drug pipettes were positioned close to the soma, and pressure pulses applied at 0·2–1 Hz to evoke amino acid responses which rose to a stable plateau value which was subsequently maintained by varying the duration of the pressure pulse.

The following amino acids, purchased from Sigma, were used in this study: *N*-methyl-D-aspartic acid (0·1–1 mM); kainic acid (0·2 mM); L-glutamic acid (1 mM). The divalent cations $MgCl_2$ (0·5–5 mM), $CdCl_2$ (1–5 mM), and $NiCl_2$ (0·1–0·5 mM) were of analar grade.

Conductance measurements

In this paper (and in Mayer & Westbrook, 1984*a*) the 'steady-state' chord conductance–membrane potential relation of responses to various amino acids was measured by applying a given dose of agonist repetitively and recording the ionic current at different values of membrane potential (Figs. 2 and 12). The agonist-activated chord conductance is equal to the current divided by the driving force for ionic current (Jack, Noble & Tsien, 1983). For highly non-linear responses, this method is only valid if the peak current evoked by the agonist application remains stable for a much longer period than the time constant required for the ionic current to relax to its new equilibrium value on changing the membrane potential. In our experiments, the NMDA current reaches a new equilibrium with a time constant of a few milliseconds over the voltage range −100 to −25 mV, while the agonist-activated current in response to brief pressure applications of excitatory amino acid remains stable for several hundred milliseconds (see Fig. 1, Mayer & Westbrook, 1984*b*). This method has the advantage of not requiring subtraction to separate membrane current (i.e. membrane leakage conductance, and other, voltage-sensitive conductances activated during voltage jumps) from the agonist-activated current, and in addition avoids the possibility of desensitization during prolonged agonist application. The alternative procedure of a steady agonist application coupled with voltage jumps was also used. The results of the two methods should be identical, and for the membrane potential range −100 to −10 mV this appears to be true (cf. Figs. 2, 5 and 7).

For membrane ionic currents, chord conductance can be measured either as steady-state or instantaneous functions of membrane potential (Jack *et al.* 1983). For voltage-sensitive mechanisms, the steady-state conductance measures changes in availability without providing insight into the underlying mechanism. The most widely encountered physiological mechanism producing such voltage sensitivity is a change in the probability of ion channels to undergo shut–open transitions. During the course of our experiments, it became clear that the response to NMDA is voltage sensitive because of channel block, rather than a more conventional mechanism. Thus, although we present our results as graphs showing an increase in conductance on depolarization, mechanistically our data are best interpreted as a relief of channel block with depolarization.

For neurones bathed in solution containing 1 mM-Mg^{2+} and clamped at potentials depolarized to 0 mV, the NMDA current slowly increased with time when brief pulses of agonist were used to estimate the conductance. This greatly complicated the interpretation of conductance measurements at positive membrane potentials; it should be noted that in none of our experiments did the conductance approach a maximum, even during depolarization to +70 mV. Since we did not obtain an estimate of the maximum conductance available for activation by a given dose of NMDA (i.e. the fully unblocked conductance) we are unable to present a quantitative description of the relative block produced by various divalent cations as a function of membrane potential.

Slope conductance measurements were obtained conventionally by drawing tangents to current–voltage plots of the responses evoked by amino acids (Jack *et al.* 1983). Slope conductance determines the membrane response to small variations in voltage about a given point; this is of physiological interest since the behaviour of slope conductance plots can be used to explain *apparent* changes in membrane conductance during the action of voltage-dependent agonists (see Mayer & Westbrook, 1984*a*). In this paper, slope conductance measurements are used to highlight the apparent loss of the voltage-sensitive component of the response to L-glutamate in solutions containing elevated concentrations of Mg^{2+}.

RESULTS

Antagonism of NMDA responses by Mg^{2+} is voltage dependent

Mg^{2+} have been shown to antagonize the depolarizing action of NMDA on frog motoneurones (Evans, Francis & Watkins, 1977; Ault, Evans, Francis, Oakes & Watkins, 1980). In mouse spinal neurones bathed in a 'Mg^{2+}-free' medium, and voltage clamped at membrane potentials of −60 to −70 mV, the inward current evoked by NMDA was greatly reduced on application of Mg^{2+} (Fig. 1*A*). However, when the membrane potential was depolarized, this blocking action of Mg^{2+} became

weaker (Fig. 1*B*), such that at membrane potentials positive to the NMDA reversal potential (~ 0 mV), Mg^{2+} exerted little or no blocking action on the outward current response evoked by NMDA (Fig. 1*C*).

This blocking action of Mg^{2+} is sufficient to explain the characteristic voltage sensitivity of responses evoked by NMDA. In medium containing physiological

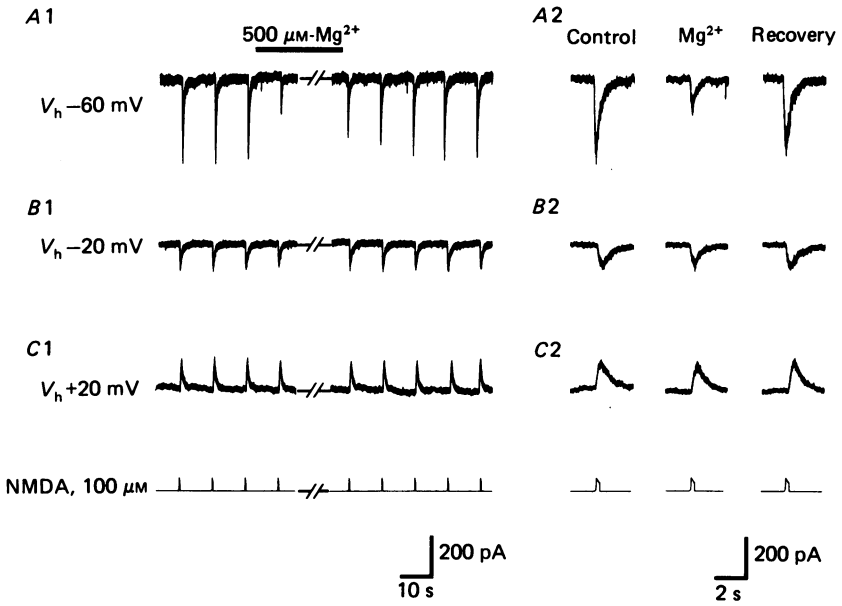

Fig. 1. Antagonism of NMDA responses by Mg^{2+} shows voltage dependence. *A*1 shows membrane currents evoked by pressure application of NMDA (100 μM, 200 ms, 2 lbf/in², 0·1 Hz, lower record); during the period indicated by a bar above the records Mg^{2+} was applied by pressure from a second micropipette (500 μM-$MgCl_2$, 300 ms, 4 lbf/in², 1·0 Hz). At a holding potential (V_h) of −60 mV the inward current evoked by NMDA falls to 38 % of control during application of Mg^{2+}; at −20 mV the NMDA response falls to 87 % of control; at +20 mV Mg^{2+} has no blocking action on the outward current response to NMDA. *A*2 shows NMDA responses on an expanded time scale recorded before, during the interrupted trace, and immediately after the records shown in *A*1. The experiment was performed using whole-cell patch-electrode recording from a spinal cord neurone bathed in 'Mg^{2+}-free' medium containing 2 mM-$CaCl_2$; voltage clamp was achieved using a single-electrode, discontinuous, voltage-clamp amplifier switching at 5 kHz. The patch electrode used for recording contained 140 mM-CsCl.

concentrations of Mg^{2+} (1 mM) current–voltage plots of NMDA responses were 'J shaped' with a negative slope conductance over the membrane potential range −30 to −80 mV (Fig. 2*A*). Ionic (chord) conductance–membrane potential plots for responses evoked by a constant dose of NMDA, derived from the driving force for ionic current (see Mayer & Westbrook, 1984*a*), showed strong outward rectification in the presence of Mg^{2+} (Fig. 2*B*) such that the mean conductance available at +20 mV was 28·7 times larger than that available at −70 mV (range 8–70, S.E. of mean 6·4, $n = 10$).

In 'Mg^{2+}-free' medium, the response to NMDA was less voltage sensitive (Fig. 2*C*),

and current–voltage plots did not show the negative slope conductance behaviour typically associated with NMDA responses (cf. MacDonald *et al.* 1982; Flatman *et al.* 1983). Thus, chord conductance plots showed much less voltage dependence (Fig. 2*D*) such that in medium containing 5 mM-Ca^{2+}, the mean conductance available at +20 mV was only 2·17 times larger than that available at −70 mV (range 1·3–3·45,

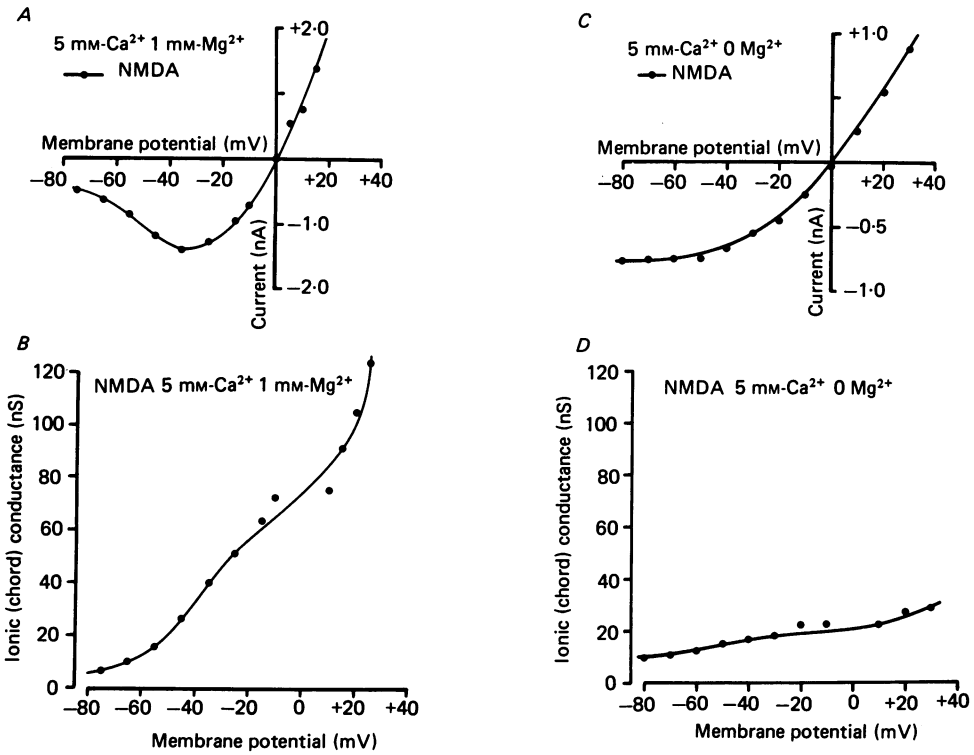

Fig. 2. Voltage sensitivity of NMDA responses requires Mg^{2+} in the extracellular medium. *A* and *C* show current–voltage plots of the peak amplitude of responses evoked by pressure application of NMDA, the dose of which was adjusted to give inward currents of 0·75 nA at −60 mV. *A* was obtained from a neurone bathed in medium containing 1 mM-$MgCl_2$; note the negative slope conductance over the voltage range −35 to −75 mV. *C* was obtained from another neurone bathed in 'Mg^{2+}-free' medium; in contrast to *A* the slope conductance is positive at all potentials. *B* and *D* show chord conductance–membrane potential relations, derived using the driving force for ionic current, for the NMDA responses shown in *A* and *C* respectively. The outward rectification ratio (G_{+20}/G_{-70}) in 1 mM-Mg^{2+} is 13·1; in 'Mg^{2+}-free' medium the rectification ratio is 2·75. Results obtained under two-electrode voltage clamp.

S.E. of mean 0·20, $n = 12$). The reversal potential of NMDA responses (~ 0 mV) was not altered by omitting Mg^{2+} from the recording solution. Thus, it is unlikely that the lack of voltage sensitivity observed in 'Mg^{2+}-free' solution reflects selective loss of a Na^+ or Ca^{2+} conductance in the absence of Mg^{2+} (cf. MacDonald *et al.* 1982; Dingledine, 1983) leaving a voltage-insensitive component of the response to NMDA, since this might be expected to result in a shift of the reversal potential to a more negative value.

The residual voltage sensitivity of the response to NMDA in 'Mg^{2+}-free' medium may reflect either the action of micromolar quantities of Mg^{2+} in the extracellular fluid (see Methods) or a weak voltage-dependent blocking action of Ca^{2+}. In experiments performed using patch electrodes for whole-cell recording, it was possible to use an extracellular Ca^{2+} concentration of 100 μM; in such solutions the response to NMDA became voltage insensitive over the potential range −80 to 0 mV, suggesting that calcium may weakly antagonize the response to NMDA.

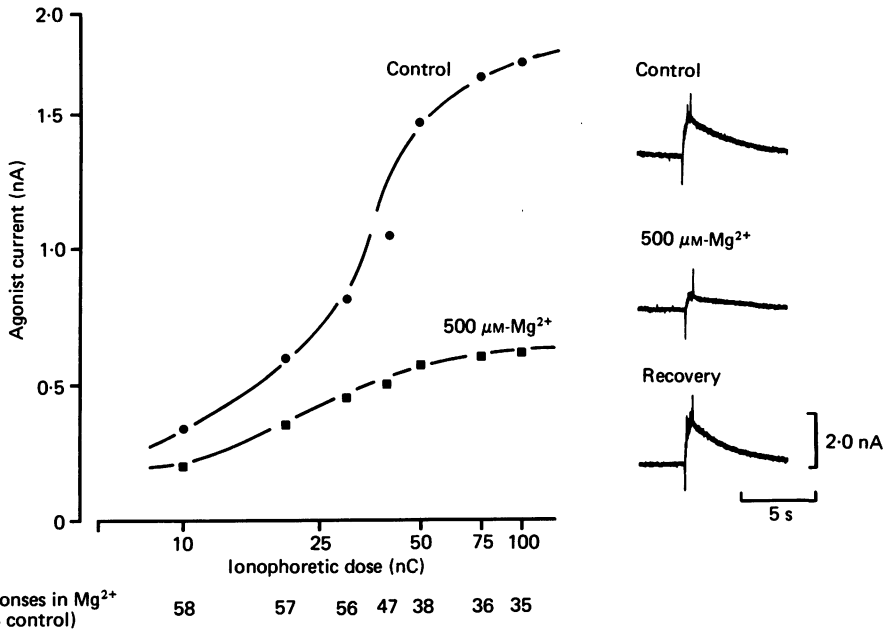

Fig. 3. Mg^{2+} antagonism shows uncompetitive kinetics. The peak amplitude of inward current responses to ionophoretic application of L-aspartic acid is plotted against the dose of L-aspartate. The neurone was clamped at a holding potential of −50 mV and bathed in 'Mg^{2+}-free' medium; $MgCl_2$ (500 μM) was dissolved in bathing medium and applied by pressure. During application of Mg^{2+} the amplitude of L-aspartate responses, expressed as a percentage of control, falls with increasing dose of agonist. The inset shows responses to 75 nC applications of L-aspartic acid recorded before, during and after application of Mg^{2+}. Results obtained under two-electrode voltage clamp.

The depolarizing action of NMDA on frog motoneurones is reduced by Mg^{2+} and a variety of competitive receptor antagonists; dose–ratio experiments suggest that these act at separate sites (Evans & Watkins, 1978). To examine the effect of Mg^{2+} on the dose–response relation of responses due to activation of NMDA receptors, we recorded inward currents evoked by ionophoretic application of L-aspartic acid, which in our experiments is a highly selective agonist for NMDA receptors on mouse spinal neurones (Mayer & Westbrook, 1984*a*). The antagonism of L-aspartate by Mg^{2+} ($\sim$ 500 μM) showed uncompetitive kinetics (Fig. 3), i.e. at a given concentration of Mg^{2+}, the antagonism increased with the dose of L-aspartic acid. The voltage-dependent action of Mg^{2+} and the evidence for uncompetitive antagonism suggest that Mg^{2+} may enter and block channels opened by NMDA, since these features of

the action of Mg^{2+} are characteristic of open channel blocking antagonists which sense the membrane electric field (cf. Ascher, Marty & Neild, 1978; Ascher, Large & Rang, 1979).

The blocking action of Mg^{2+} occurs rapidly

In the presence of certain channel-blocking antagonists acting at nicotinic sites, hyperpolarizing voltage jumps produce membrane current relaxations during the action of acetylcholine. Generally such relaxations are of two types: (1) a slow decrease of conductance producing an outward relaxation; and (2) a slow increase in conductance producing an inward relaxation (frog end-plate, Adams, 1977; *Aplysia* neurones, Ascher *et al.* 1978; rat parasympathetic ganglion neurones, Ascher *et al.* 1979). We were thus interested to see if, in the presence of NMDA and Mg^{2+}, voltage jumps produced similar current relaxations. The complex morphological and electrical structure of ventral horn neurones in culture (Guthrie & Westbrook, 1983) has unfortunate consequences for such experiments. The cable structure of the neurone limits the high-frequency response of voltage control in the dendritic tree; as a consequence, during voltage jumps capacitative currents last 1–2 ms in the dendrites and obscure other current relaxations at a time when the somatic membrane potential is well controlled (see Fig. 4 and Rall, 1969). However, if agonist application is limited to the soma, then subtraction of control current records from those recorded during NMDA responses will eliminate the slow capacitative currents and provides a useful approximation of fast conductance changes occurring at the soma.

Our experimental protocol is illustrated in Fig. 4. NMDA current relaxations were revealed by a subtraction protocol (Fig. 4*C* and *D*). In the presence of 1 mM-Mg^{2+}, hyperpolarizing voltage jumps from a holding potential of -50 mV produced outward current relaxations, i.e. a reduction of inward current, while depolarizing voltage jumps produced inward current relaxations, i.e. an increase in inward current. If Mg^{2+} acts as an open channel blocker, and the rate constant for channel opening is faster than the blocking rate constant, then a simple bimolecular pore blocking model would explain such relaxations (Ascher *et al.* 1978; Ascher *et al.* 1979). Similar observations were made in six of nine neurones after averaging and subtracting leakage currents. The relaxations recorded in the presence of NMDA and Mg^{2+} were described by single exponential functions of time constant 2–3 ms. However, extrapolation of the relaxation to the start of the voltage jump often failed to reveal instantaneous currents appropriate for the change in driving force, suggesting that part of the blocking action of Mg^{2+} occurs with very rapid kinetics beyond the resolution of our experimental technique. The small size and rapid kinetics of these responses hindered further analysis and we did not attempt to study the effect of voltage or of altering the NMDA or Mg^{2+} concentration on the time constant of the relaxation.

In 'Mg^{2+}-free' medium, no obvious relaxations were observed during NMDA responses, consistent with the weak voltage sensitivity of NMDA responses in this medium (cf. Fig. 2). During steady inward currents evoked by application of NMDA and Mg^{2+}, we did not observe changes in the variance of the current record (Fig. 4*A*). This is consistent with the rapid kinetics of the channel blocking process, and the

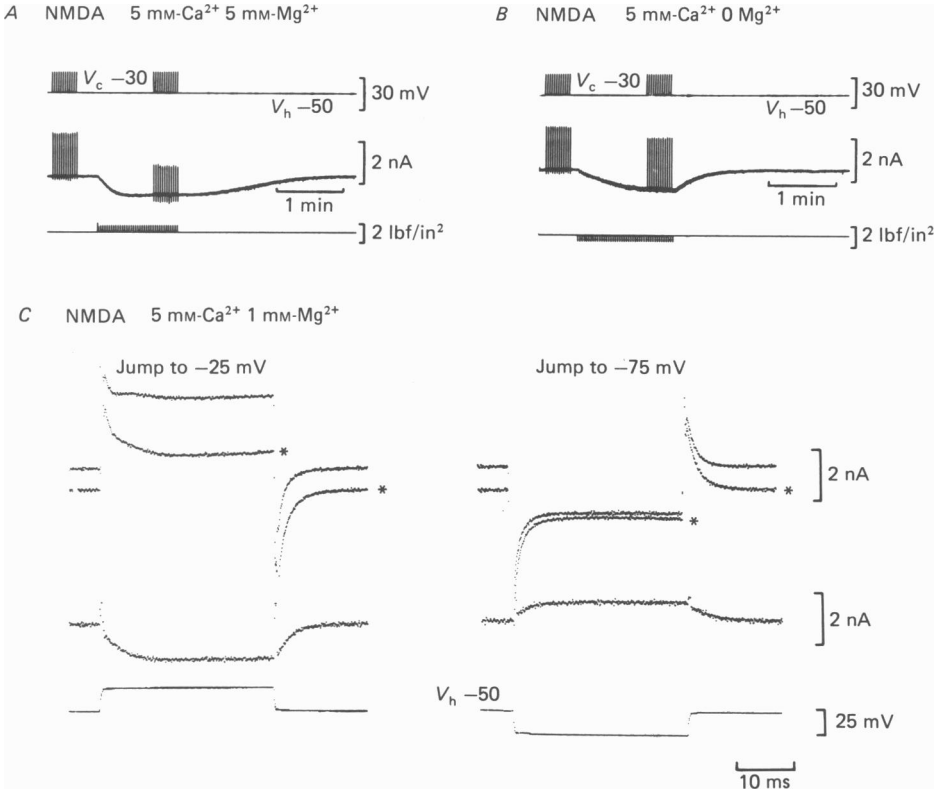

Fig. 4. The voltage-dependent blocking action of Mg^{2+} is rapid. *A* and *B* show chart records of NMDA responses recorded from a single neurone bathed in 'Mg^{2+}-free' medium containing 5 mM-Ca^{2+}. In each case the upper trace shows the membrane potential, which was clamped at a holding potential (V_h) of −50 mV and stepped to −30 mV (V_c) for 30 ms, before and during application of NMDA + Mg^{2+} (left) or NMDA alone (right). The middle trace shows the membrane current; note the increase in variance in *B* but not *A*. The lower record shows the output from a pressure transducer attached to each drug pipette. The pressure pipettes contained bathing medium with 1 mM-NMDA + 5 mM-Mg^{2+} (*A*) and 1 mM-NMDA alone (*B*); the dose from each was adjusted to give steady inward currents of ~ 1 nA. With this protocol the concentration of NMDA and Mg^{2+} at the membrane surface is unknown, but we are certain that considerable dilution occurs. The difference in time course of onset and decay of the response to NMDA in *A* and *B* in part reflects the need to eject a greater quantity of NMDA + Mg^{2+} to match the plateau current evoked by NMDA in the absence of Mg^{2+}. During voltage jumps, the membrane current and potential were digitized at 10 kHz and a computer used to average ten responses. *C* and *D* show examples of such averaged records obtained from another neurone bathed in medium containing 1 mM-Mg^{2+} using a similar protocol to apply NMDA + 1 mM-Mg^{2+}. The upper pairs of traces show the membrane current during steps to −25 mV (left) and −75 mV (right) from a holding potential of −50 mV; traces marked with an asterisk were recorded during a steady inward current of 1 nA evoked by application of NMDA. The difference current produced by subtraction of the responses recorded before and during application of NMDA is shown below the upper pair of traces. The difference-current records reveal that hyperpolarizing voltage jumps evoke outward current relaxations while depolarizing voltage jumps evoke inward relaxations. The lower record is the membrane potential recorded at the soma; note that settling occurs within ~ 500 μs. Results obtained under two-electrode voltage clamp.

limited band width of our recording system. However, in the same neurones application of NMDA alone produced an increase in variance of the current record (Fig. 4*B*). The spectral characteristics of the current noise evoked by NMDA in 'Mg^{2+}-free' medium were not determined (cf. Nowak *et al.* 1984).

Effects of other divalent cations

Ault *et al.* (1980) found that a variety of divalent cations, including Co^{2+}, Mn^{2+} and Ni^{2+}, also blocked the response of frog motoneurones to NMDA. Of these divalent cations, Co^{2+} and Ni^{2+}, which are potent calcium channel blockers (Akaike, Brown, Nishi & Tsuda, 1981), were the most effective NMDA antagonists. In our experiments, Ni^{2+} also produced a voltage-dependent block of the response to NMDA, as well as reducing the amplitude of calcium currents evoked by depolarizing voltage jumps (Fig. 5). This voltage-dependent action of Ni^{2+} is otherwise similar to that of Mg^{2+}.

In contrast, Cd^{2+}, another potent calcium channel blocker (Khrishtal, 1976), was relatively ineffective as an NMDA antagonist. Fig. 6 shows complete block of inward calcium currents evoked by depolarizing voltage jumps, by doses of Cd^{2+} that had minimal action on the inward current response to NMDA. These results suggest quite different selectivity of the divalent cation binding site in calcium channels and ion channels linked to NMDA receptors.

Mg^{2+} and the response to L-glutamate

In medium containing 1 mM-Mg^{2+}, the response to L-glutamate is voltage dependent, and current–voltage plots are of a characteristic shape (Mayer & Westbrook, 1984*a*): over the potential range -70 to -30 mV, the slope conductance is much smaller than expected when compared to the agonist-activated chord conductance, derived using the driving force for ionic current (see Table 1). At more positive potentials, the slope conductance increases steeply with further depolarization, and the chord and slope conductance values converge. This behaviour reflects the mixed-agonist action of L-glutamate at both NMDA and non-NMDA receptors (Mayer & Westbrook, 1984*a*). In 'Mg^{2+}-free' medium, current–voltage plots of responses to L-glutamate were more linear, and the slope conductance increase produced by depolarization was less marked and similar to that produced by L-glutamate in the presence of the NMDA receptor antagonist (±)-2-amino-5-phosphonovaleric acid (2-APV, see Table 1).

In medium containing 10 mM-Mg^{2+}, the ratio of the slope/chord conductance of the response to L-glutamate measured at -60 mV was similar to that recorded in the presence of 2-APV or in 'Mg^{2+}-free' medium (Table 1) suggesting that in 10 mM-Mg^{2+} responses to L-glutamate have become voltage insensitive; however, at more positive membrane potentials the slope conductance increased to large positive values (Table 1). This reflects relief of voltage-dependent block by Mg^{2+} of NMDA channels opened by L-glutamate. At -60 mV, in 10 mM-Mg^{2+}, essentially all current flow through NMDA channels is blocked; thus, the slope conductance measurement of the response to L-glutamate reflects activation of some other amino acid receptor such as that for kainate or quisqualate (Mayer & Westbrook, 1984*a*). In contrast, at -60 mV in 1 mM-Mg^{2+}, the probability of NMDA channels being blocked by Mg^{2+} is lower, and slope conductance measurements of the response to L-glutamate reflect

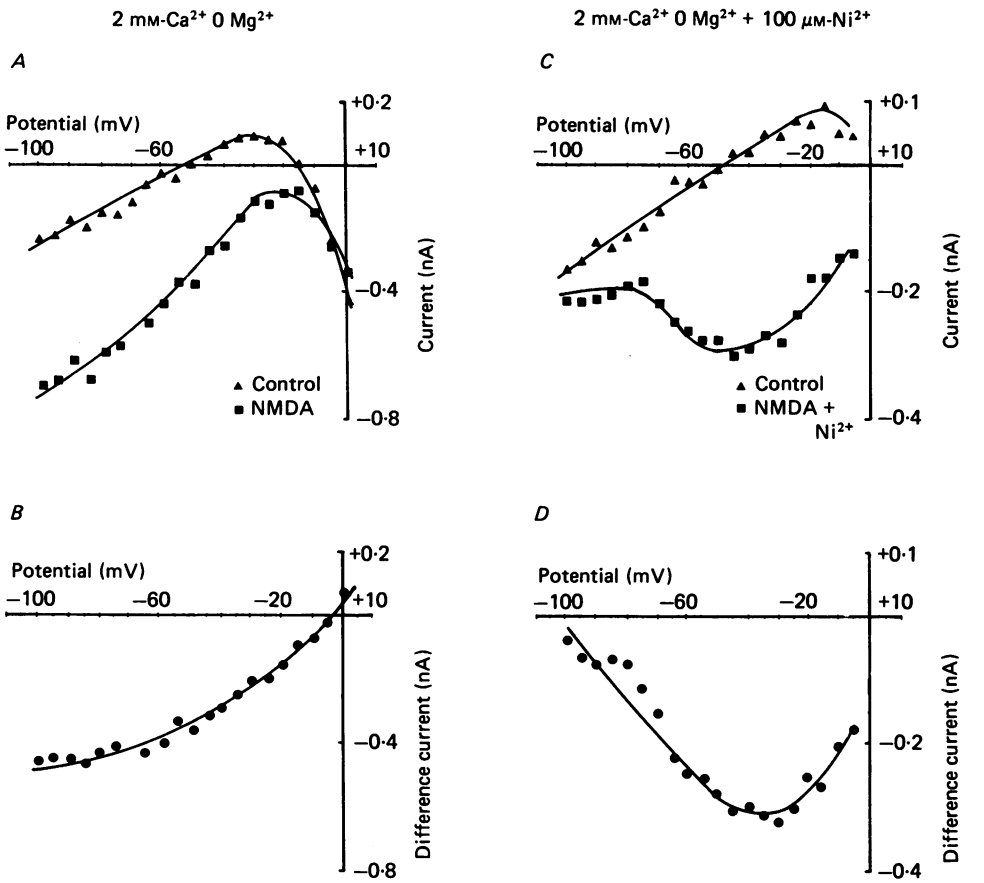

Fig. 5. Ni^{2+} shows a voltage-dependent effect on currents evoked by NMDA. *A* and *C* show current–voltage plots obtained from Cs^+-loaded spinal cord neurones at rest and during application from pressure pipettes of *A*, 500 μM-NMDA and *C*, 500 μM-NMDA plus 100 μM-Ni^{2+}. The 'Mg^{2+}-free' bathing medium contained 2 mM-Ca^{2+} and 0·6 μM-TTX; experiments were performed using CsCl-filled patch pipettes for whole-cell recording under discontinuous voltage clamp. Current–voltage plots were obtained using 30 ms voltage jumps from a holding potential of −50 mV. Each data point represents the average of four consecutive digitized samples at 100 μs per point, taken 5 ms after the beginning of the voltage jump. Subtraction of records obtained during the response to NMDA from control records was used to reveal the current–voltage relation of the NMDA-activated current (*B* and *D*). In the absence of Ni^{2+}, the response to NMDA shows a small degree of outward rectification over the potential range 0 to −100 mV (*B*). The control records *A* and *C* show a region of negative slope conductance between −30 and 0 mV due to the activation of calcium currents during depolarizing voltage jumps; NMDA did not appear to alter the amplitude of these (*A*), but application of Ni^{2+} together with NMDA blocked the activation of calcium currents (*C*). In addition, in the presence of Ni^{2+}, the response to NMDA has a negative slope conductance over the range −30 to −100 mV (*D*) that is not present in *A*.

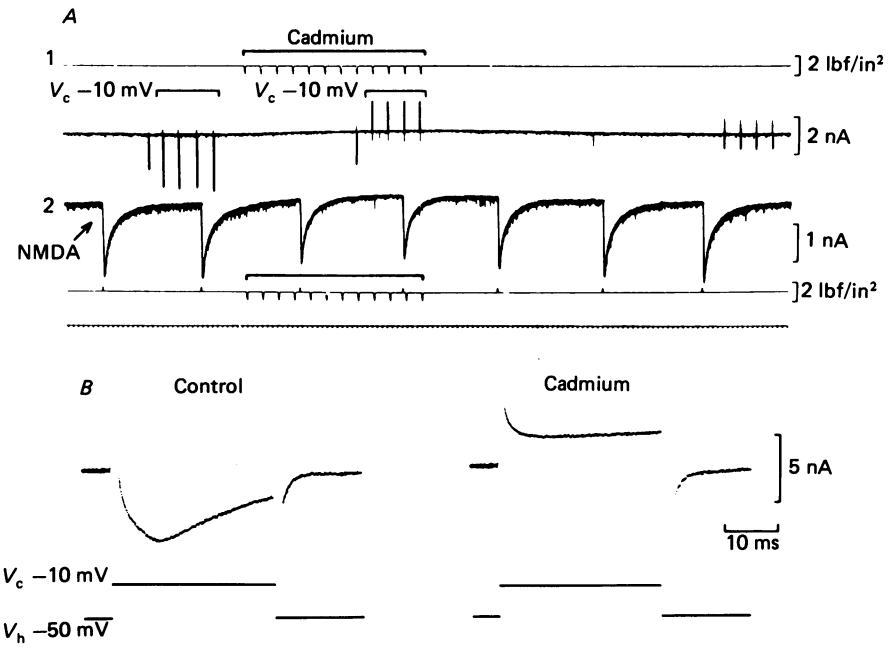

Fig. 6. NMDA responses are resistant to antagonism by doses of cadmium which block voltage-sensitive calcium currents. All traces were obtained from one neurone bathed in 'Mg^{2+}-free' medium containing 10 mM-tetraethylammonium chloride. The experiment was performed at a holding potential of −50 mV using a two-electrode voltage clamp. The upper set of chart records labelled *A*1 show (top) the output from the pressure transducers used to monitor cadmium and NMDA application, and (bottom) the membrane current. Deflexions in the current record are responses to a series of 30 ms voltage jumps applied before, during and after cadmium application. The first downward deflexion is the response to a 40 mV *hyperpolarizing* voltage jump used to measure the leak conductance; the next four responses are produced by 40 mV *depolarizing* voltage jumps which activate inward calcium currents, or during application of cadmium outward leak currents. These responses were digitized at 100 μs per point and averaged. *B* shows the averaged records obtained before and during cadmium application. The lower set of chart records, labelled *A*2, show at higher gain the membrane current (top), the pressure transducer output (middle) and 1 s timing marks (bottom). A second application of cadmium, using the same protocol as that used in *A*1 to block calcium currents (1 mM-Cd^{2+}, 200 ms pulses at 1·5 lbf/in²), produced only a 15% attenuation of the response to NMDA.

current flow through ion channels linked to both NMDA and other amino acid receptors. Since we were unable to obtain an estimate of the maximal conductance available for a given dose of L-glutamate these experiments were performed by evoking inward currents of similar size at −60 mV. As a consequence, larger doses of L-glutamate were required in 10 mM-Mg^{2+} resulting in a higher occupancy of NMDA receptors; current flow through these receptors occurred only on sufficient depolarization to relieve the blocking action of Mg^{2+}.

Effect of NMDA on the membrane current–voltage relation

The depolarizing action of NMDA is usually associated with an apparent increase in membrane resistance (Engberg *et al.* 1978). This is now shown to be a consequence of the voltage-dependent blocking action of Mg^{2+}. In the presence of 1 mM-Mg^{2+},

NMDA reduced the membrane slope conductance, measured under voltage clamp using 30 ms voltage jumps (Fig. 7*B*), and produced a region of non-linearity in the current–voltage relation such that it was impossible to accurately extrapolate a reversal potential for the NMDA-evoked current. In fact, over the potential range −60 to −80 mV, extrapolation gave a false reversal potential close to that expected

TABLE 1. Chord and slope conductance measurements derived from responses to L-glutamate in media containing different concentrations of Mg^{2+}. Each measurement is the mean obtained from five to sixteen neurones as shown on the left. The chord conductance at −60 mV (G_{c-60}), derived using the driving force for ionic current, is presented to show that in each solution similar amplitude responses were obtained to allow meaningful comparison of the slope conductance at −60 mV (G_{s-60}) and +10 mV (G_{s+10}). Slope conductance measurements were obtained from tangents to current–voltage plots recorded over the range −70 to +20 mV. Note that despite a large increase in slope conductance on depolarization to +10 mV, the response recorded at −60 mV in 10 mM-Mg^{2+} has a similar slope conductance to that obtained in the presence of 2-APV or in 'Mg^{2+}-free' medium

	Divalent cations (mM)		L-glutamate chord and slope conductance measurements (nS)		
n			G_{c-60}	G_{s-60}	G_{s+10}
16	5 Ca	1 Mg	20·7	5·6	74·7
5	5 Ca	0 Mg	22·2	15·0	50·8
9	5 Ca	1 Mg + 2-APV	21·0	13·4	38·1
5	2·5 Ca	10 Mg	23·7	15·0	120

for the potassium equilibrium potential, E_K. Because the blocking action of Mg^{2+} occurs rapidly (see Fig. 4), the membrane current–voltage relation approached steady state within 5–10 ms. The result of this is to increase the effective membrane resistance for even brief depolarizing events such as excitatory post-synaptic potentials (e.p.s.p.s). However, in 'Mg^{2+}-free' medium, NMDA behaves as a classical excitatory agonist and evoked increases in membrane slope conductance (Fig. 7*D*) such that extrapolation gave an estimate of the reversal potential for the response to NMDA similar to that recorded directly (Mayer & Westbrook, 1984*a*).

Excitability changes during NMDA responses

Depolarizing responses produced by NMDA in the presence of 1 mM-Mg^{2+} evoked repetitive firing, and an increase in the frequency of synaptic barrage, probably as a result of spread of NMDA to adjacent neurones; smaller doses of NMDA caused an apparent increase in the input resistance (Fig. 8*A*). In the absence of Mg^{2+}, the depolarizing response to NMDA occurred with a decrease in input resistance, and presumably as a result of this the excitability of the neurone was lower, such that NMDA did not evoke repetitive firing or an increase in synaptic barrage due to excitation of adjacent neurones (Fig. 8*B*). This action of NMDA in the absence of Mg^{2+} was similar to that of kainic acid, which we have found much less able to support repetitive firing than (in the presence of Mg^{2+}) L-glutamate or NMDA.

Reversal potential of amino acid responses in low Na^+

In a previous series of experiments performed in medium containing 143 mM-Na^+ and 4·8 mM-K^+, the reversal potential of kainate and NMDA was similar and close to 0 mV (Mayer & Westbrook, 1984*a*). On reducing $[Na^+]_o$, substituting choline Cl

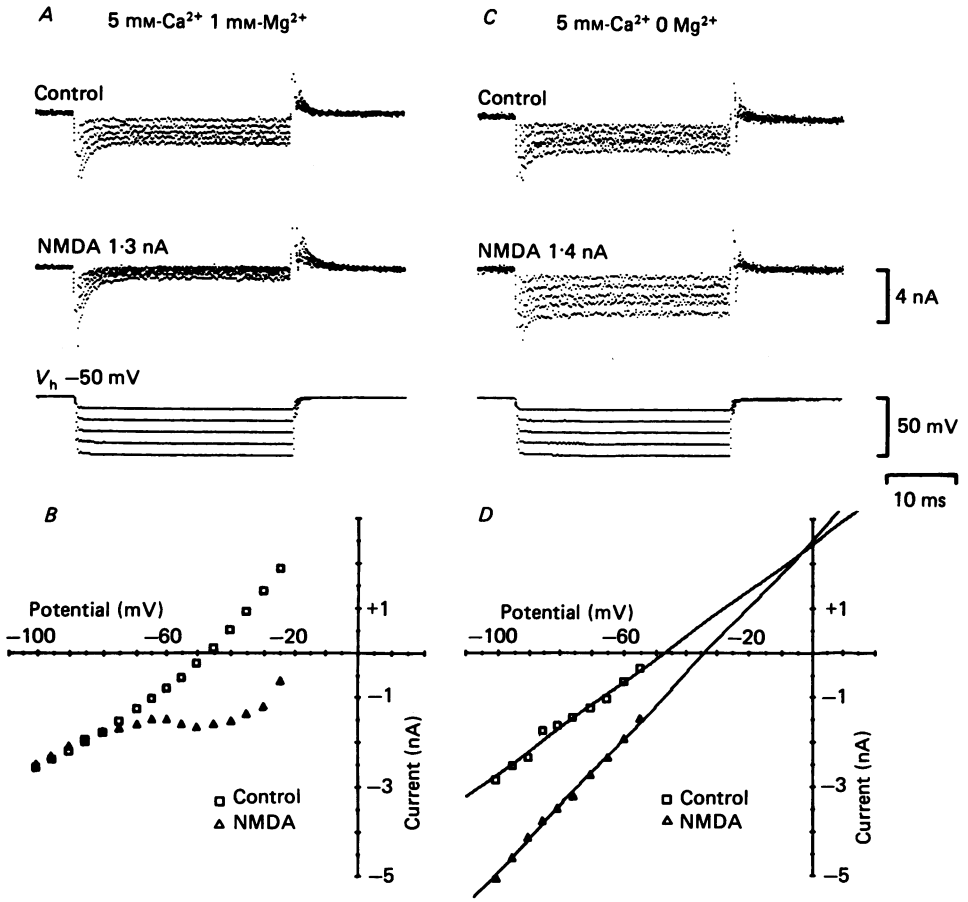

Fig. 7. Membrane resistance changes evoked by NMDA. *A* and *B* show the effect of NMDA on a spinal cord neurone bathed in medium containing 1 mM-$MgCl_2$. The traces in *A* show the membrane current, digitized at 5 kHz, during a series of hyperpolarizing voltage jumps before (top record) and during application of sufficient NMDA to evoke a steady inward current of 1·3 nA at −50 mV (middle record); the lower record shows the membrane potential (two-electrode clamp) during 30 ms hyperpolarizing voltage jumps incrementing by −10 mV. NMDA evokes an apparent increase in the membrane resistance. The current–voltage plot in *B* was obtained from a more extensive series of voltage jumps; note the non-linear relation induced by NMDA and the lack of cross-over of the current–voltage plots at membrane potentials negative to −80 mV. *C* and *D* show the effect of NMDA on another neurone bathed in 'Mg^{2+}-free' solution, and recorded using the protocol described above. NMDA reduces the membrane resistance; extrapolation of the resting membrane current–voltage relation (fitted by linear regression) with that recorded during application of sufficient NMDA to evoke 1·4 nA of inward current at −50 mV gives intersection at −5 mV, the reversal potential of the NMDA-evoked current.

for NaCl, the reversal potential for responses evoked by kainate was consistently shifted to a more negative potential (Fig. 9). In nine neurones bathed in medium containing 10 mM-Na^+, the mean kainate reversal potential was −16·3 mV (range −7 to −27 mV, S.E. of mean 2·2). In this low-Na^+ medium, the time course and voltage

sensitivity of kainate responses was similar to that recorded in control experiments (cf. Fig. 9 and Fig. 2 in Mayer & Westbrook, 1984*a*). In contrast, NMDA responses were greatly prolonged in medium containing 10 mM-Na^+ (Fig. 9), and tended to 'fade' or desensitize during experiments such that in most neurones it was impossible to obtain reproducible responses. Despite this, in four neurones a reversal potential was obtained for NMDA responses in 10 mM-Na^+ and was 5 to 27 mV (mean 12·5) more positive than the kainate reversal potential recorded in the same neurones (see Fig. 9).

L-glutamate appears to activate both NMDA and either kainate or quisqualate receptors (Mayer & Westbrook, 1984*a*). In medium containing 10 mM-Na^+ and 1 mM-Mg^{2+}, L-glutamate responses became complex. At the most negative membrane potentials examined, the ion channels linked to NMDA receptors are strongly blocked by Mg^{2+}, and L-glutamate responses were of similar time course to those recorded in control experiments (Fig. 10). As the membrane potential was shifted positive to about −50 mV, the blocking action of Mg^{2+} became weaker, and L-glutamate responses developed a biphasic time and voltage dependence (Fig. 10). The initial fast component of the response resembled that evoked by kainic acid in 10 mM-Na^+; the slower component of the response had a more depolarized reversal potential and resembled responses evoked by NMDA (cf. Figs. 9 and 10).

Inactivation of NMDA responses

'Fade' of NMDA responses in medium containing 10 mM-Na^+ greatly complicated measurement of the agonist current–voltage relation. This phenomenon is similar to classical desensitization at nicotinic receptors (Katz & Thesleff, 1957) in that it was reversibly increased on application of a high dose of NMDA (Fig. 11). However, a similar reversible inactivation occurred during a depolarizing voltage-jump protocol which was sufficient to activate voltage-sensitive calcium currents; responses to kainic acid did not show this behaviour (Fig. 11). This inactivation of NMDA responses during depolarization was variable and did not occur in all neurones. We gained the impression that it was most severe in media which would tend to impair intracellular calcium regulation, i.e. low Na^+, 'Mg^{2+}-free' solutions, but further experiments are required to confirm this.

In 'Mg^{2+}-free' solutions containing 143 mM-Na^+, responses to NMDA occasionally displayed voltage-dependent inactivation similar to, though less pronounced than that recorded in 10 mM-Na^+. As a result the agonist current–voltage plot had an inflexion between −40 and +20 mV that was absent in neurones not showing such marked inactivation of NMDA responses (cf. Figs. 2 and 12). Calculation of the NMDA chord conductance–potential relation for responses showing such voltage-dependent inactivation in medium containing 143 mM-Na^+, revealed conductance plots (Fig. 12) with inactivation over the voltage range usually associated with maximal entry of calcium through voltage-sensitive channels, approximately −30 to +30 mV. This behaviour also occurred, to a much smaller extent, for the responses shown in Fig. 2*C* and *D*. Responses to kainic acid did not show voltage-dependent inactivation in any of the recording solutions used in the present experiments.

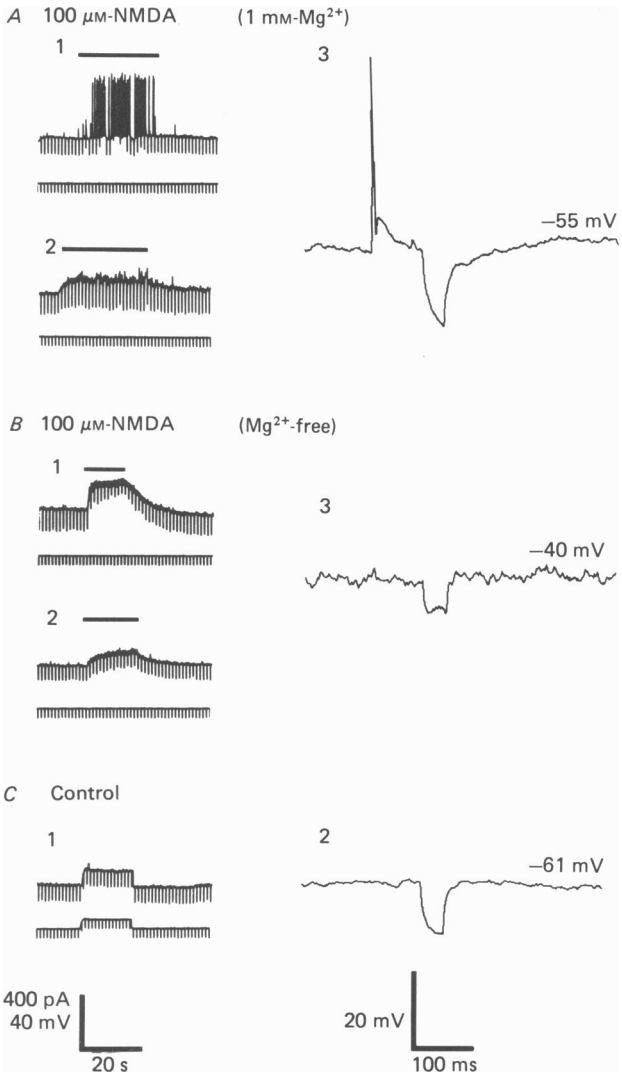

Fig. 8. Excitability changes during NMDA receptor activation reflect the voltage-dependent action of Mg^{2+}. *A*, *B* and *C* show current-clamp records from one spinal cord neurone obtained using a $KMeSO_4$-filled patch electrode. The pairs of traces at left show chart records of membrane potential (upper trace) and current (lower trace). Digitized voltage records (sampling rate 0·5 kHz) obtained during the peak NMDA responses (*A*1, *B*1) are shown at right. Current pulses (50 ms, 60 pA, 1 Hz) were used to monitor membrane resistance. The bathing solution contained 2 mM-Ca^{2+} and '0' Mg^{2+}. In order to reduce spontaneous activity, 25 nM-TTX was added to the bath; as a result the background activity of most neurones consisted of occasional synaptic events, but action potentials could still be easily evoked with intracellular current pulses. Perfusion pipettes contained 100 μM-NMDA with 1 mM-Mg^{2+} (*A*1–3), or 100 μM-NMDA in 'Mg^{2+}-free' medium (*B*1–3) and were positioned in similar positions close to the soma. The dose of NMDA or NMDA plus Mg^{2+} was adjusted by varying the duration of pressure pulses (see Methods); the period of drug application is indicated by bars above the chart records. During a small dose of NMDA plus Mg^{2+} (*A*2), adjusted to give a 10 mV depolarization, there was an increase in synaptic activity and an apparent membrane resistance increase; a larger dose (*A*1) led to a prolonged burst of action potentials, the amplitude of which

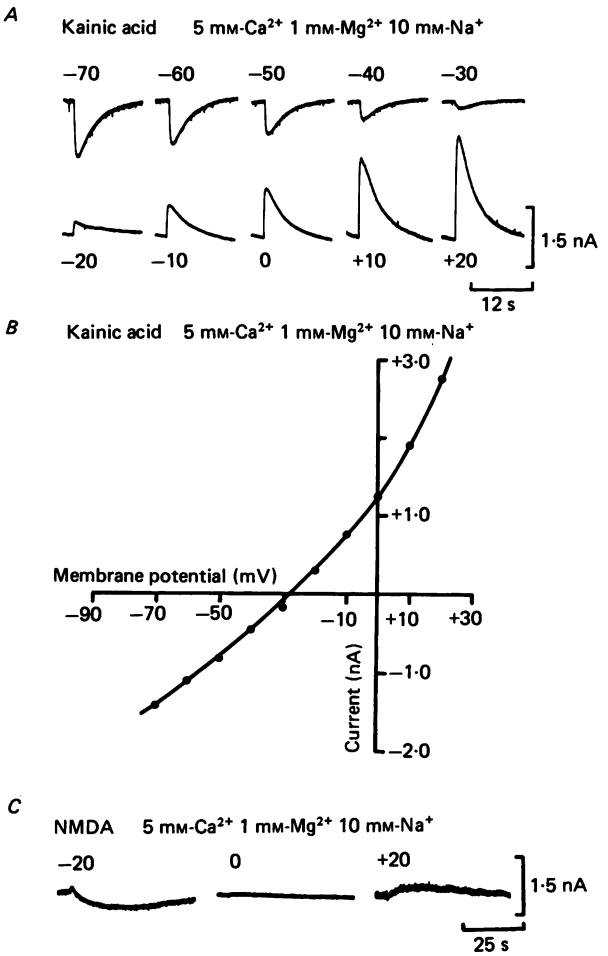

Fig. 9. Amino acid responses recorded in choline-substituted medium containing 10 mM-Na^+, 5 mM-Ca^{2+} and 1 mM-Mg^{2+} (two-electrode voltage clamp). *A* shows chart records of membrane currents evoked by pressure application of kainic acid over the potential range −70 to +20 mV; note that from −20 to +20 mV kainic acid evokes outward current responses. *B* shows a current–voltage plot of these responses; the interpolated reversal potential is −27 mV. *C* shows responses evoked by NMDA and recorded in the same neurone; note the extreme prolongation of the responses compared to those recorded in control experiments (see Fig. 3 in Mayer & Westbrook, 1984*a*), and reversal of the NMDA response at ~ 0 mV. The small size and long time course of the response to NMDA, coupled with rundown, prevented study of the current–voltage relation over a wider range of membrane potentials.

is attenuated by the frequency response of the chart recorder and the slow sampling rate in *A*3. Both small (*B*2) and large (*B*1, *B*3) doses of NMDA in 'Mg^{2+}-free' medium evoked depolarization with membrane resistance decreases; longer applications led to a plateau depolarization at −35 mV which took several minutes to recover. Even during the larger depolarization there was no increase in synaptic activity, or generation of action potentials, but a striking increase in voltage noise is apparent in *B*3 compared to control (*C*2). A 10 mV depolarization with current injection (*C*1) did not reproduce the changes in excitability seen in *A*, nor the increase in voltage noise seen in *B*.

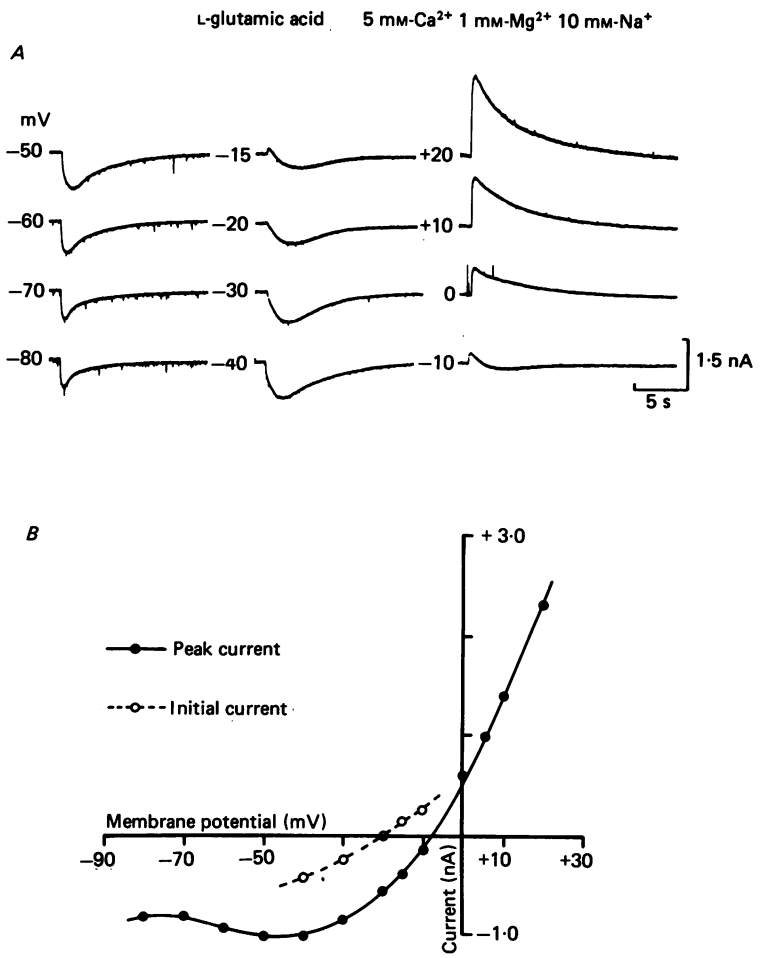

Fig. 10. Biphasic responses evoked by L-glutamate in medium containing 10 mM-Na^+ and 1 mM-Mg^{2+}. *A* shows chart records of currents evoked by pressure application of L-glutamate as the membrane potential was varied from −80 to +20 mV (two-electrode voltage clamp). Note that at hyperpolarized potentials, the responses are brief compared to those recorded at more positive membrane potentials. At −15 and −10 mV the response is biphasic: the early rapidly developing outward current is followed by a slowly developing inward current; at −30 and −40 mV both phases are inward, but it is still possible to distinguish two components and estimate the amplitude of the initial response, which reverses at −20 mV. Small deflexions in the records, especially noticeable in the traces recorded at −80 to −50 mV, are spontaneous synaptic currents which occur in low-Na^+ solution despite the presence of 0·6 μM-TTX. *B* shows a current–voltage plot of the data illustrated in *A*. The peak current has a highly non-linear voltage dependence, and reverses at −8 mV. The initial current, which can only be distinguished over the range −40 to −10 mV, appears to have a more linear voltage dependence.

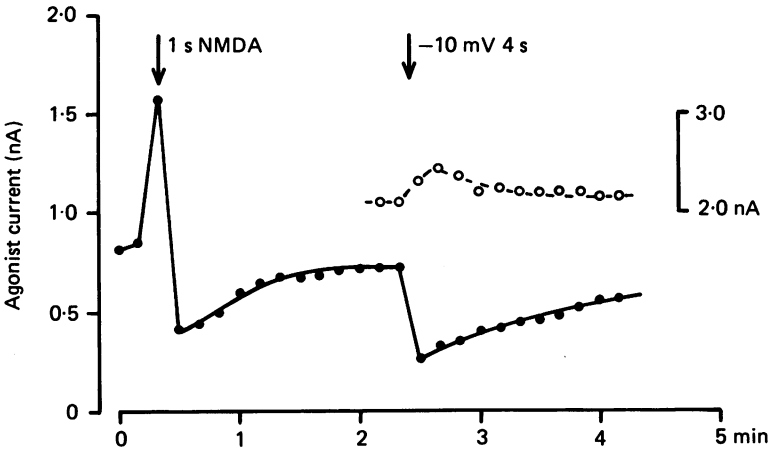

Fig. 11. Time course of changes in amino acid responses following a large dose of agonist or depolarizing voltage jumps. Responses were evoked by pressure application of NMDA (1 mM, 100 ms, 4 lbf/in^2, ●) or kainic acid (0·2 mM, 100 ms, 4 lbf/in^2, ○) in a spinal cord neurone voltage clamped (two-electrode system) at a holding potential of −50 mV, and bathed in 'Mg^{2+}-free' medium containing 10 mM-Na^+ and 5 mM-Ca^{2+}. The peak amplitude of amino-acid-evoked current response (ordinate) is plotted against time. Application of a large dose of NMDA (achieved by increasing the duration of the pressure pulse to 1 s) reduced the amplitude of subsequent responses. A similar reduction occurred following a depolarizing voltage jump to −10 mV for 4 s. During this experiment the amplitude of the NMDA responses also gradually declined with time, producing a sloping base line. In contrast, responses evoked by kainic acid were stable and did not show inactivation following a depolarizing voltage jump, or after application of a larger dose of kainic acid.

DISCUSSION

Mg^{2+} block of NMDA responses

There has been little agreement about the nature of the permeability change evoked by excitatory amino acids in vertebrate neurones. The results presented in this paper, and those obtained by Nowak *et al.* (1984) and ourselves (Mayer & Westbrook, 1984*a*), help to explain the paradoxical voltage-dependent action of certain excitatory amino acids. Agonists acting at NMDA receptors produce highly voltage-sensitive responses; previously it was suggested that this resulted from modulation of an undefined voltage-dependent conductance carrying Na^+ or Ca^{2+} current (MacDonald & Porietis, 1982; Flatman *et al.* 1983; Dingledine, 1983). Our results and those of Nowak *et al.* (1984) suggest that voltage dependence of the NMDA response is a consequence of Mg^{2+} block, and not the behaviour of a conductance mechanism linked to a classical intramembrane gating particle. The responses evoked by other amino acids, such as L-glutamate and D- and L-homocysteate, are more complex because these agonists activate both NMDA receptors and other amino acid receptors linked to a voltage-insensitive mechanism (Mayer & Westbrook, 1984*a*). Kainic and quisqualic acids are selective agonists for these other receptors, and produce responses with a more linear current–voltage plot.

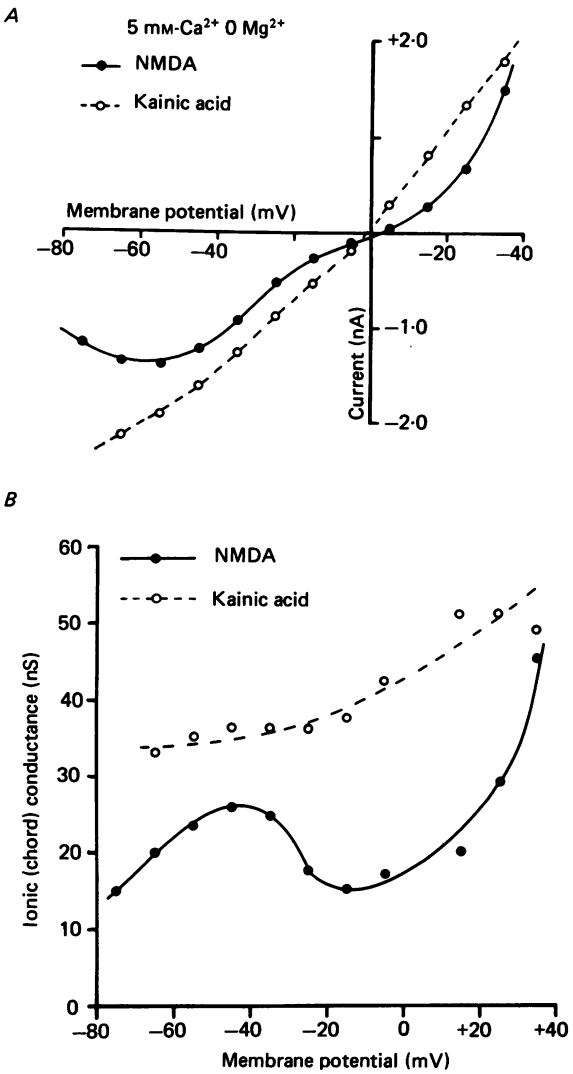

Fig. 12. Voltage-dependent inactivation of NMDA responses in a spinal cord neurone bathed in medium containing 5 mM-$CaCl_2$ and 143 mM-Na^+. *A* shows a current–voltage plot of the peak amplitude of responses evoked by pressure application of NMDA and kainic acid to a voltage-clamped spinal cord neurone (two-electrode system). The kainic acid response is relatively linear with respect to membrane potential; in contrast, the NMDA response has a negative slope conductance over the potential range −65 to −75 mV (presumably due to contamination of the 'Mg^{2+}-free' medium with Mg^{2+}), and shows an inflexion due to a decrease in slope conductance over the potential range −15 to +15 mV. *B* shows the data illustrated in *A* following conversion to agonist-activated chord conductance, using the driving force for ionic current. The response to kainic acid shows a small degree of outward rectification. In contrast, the NMDA conductance–potential relation has a pronounced N shape, with a threshold for inactivation of approximately −40 mV.

Our results do not contain sufficient quantitative detail to allow estimation of any of the rate constants determining binding and dissociation of Mg^{2+} at the blocking site. During voltage jumps, the inward current relaxation produced by NMDA in the presence of 1 mM-Mg^{2+} has at least two kinetic components. This is in accord with the single-channel studies of Nowak *et al.* (1984): in 100 μM-Mg^{2+} at -60 mV the mean open time of channels opened by 2–10 μM-L-glutamate is 0·7 ms, and the mean burst duration 3·2 ms; these time constants are expected to become faster with 1 mM-Mg^{2+}. Over the potential range -80 to -10 mV, in the presence of 1 mM-Mg^{2+}, the conductance linked to NMDA receptors increases e-fold on depolarization by 21–28 mV ($n = 4$). By assuming that the conductance available for the action of NMDA is proportional to the fraction of unblocked channels, it is possible to calculate what fraction of the membrane electric field influences the binding of Mg^{2+} to its site of action within the ion channel from the relation (see Woodhull, 1973)

$$\Delta G = \exp\left[\frac{z\delta FE}{RT}\right]$$

where ΔG represents an e-fold increase in the conductance activated by NMDA on depolarizing the membrane potential by E mV; z is the valency of the blocking ion; δ the fraction of the membrane potential acting at the blocking site; F, R and T are the Faraday, the gas constant and the absolute temperature respectively. In four experiments, we obtained δ values of 0·46–0·62 (mean 0·53). However, at the most positive potentials examined in our experiments (+60 to +70 mV), the conductance activated by NMDA is still increasing with depolarization and shows no sign of approaching an asymptote (see Fig. 2). At these positive potentials, NMDA currents show a tendency to gradually increase with time. Such creep in the response amplitude may represent agonist-triggered unblocking of a stable closed blocked state as has been described for cholinergic responses in crustacean muscle fibres by Lingle (1983). This slow increase in the response to NMDA greatly complicates measurement of the maximal conductance available for the action of NMDA. Single-channel recording experiments suggest that the kinetic description of the block of NMDA channels by Mg^{2+} is indeed more complicated that that predicted by a fast open channel block mechanism (Nowak *et al.* 1984). Further experiments are required to see if NMDA channels can enter a closed blocked state, and to determine the relative rate constants of the transitions from closed blocked to open blocked, and from open blocked to unblocked channels.

Previous experiments had shown that high concentrations of Mg^{2+} (10 mM) can completely block the voltage-sensitive component of responses to L-glutamate (MacDonald & Wojtowicz, 1982). This may seem paradoxical if Mg^{2+} block of ion channels is the cause of such voltage sensitivity. However, the blocking model described by Woodhull (1973) predicts a depolarizing shift in the titration curve for dissociation of the blocking ion from its binding site on increasing the concentration of the blocking ion, such that at high concentrations of Mg^{2+}, NMDA channels will become unblocked only at membrane potentials depolarized to the agonist reversal potential. In a series of experiments performed in medium containing 10 mM-Mg^{2+}, we were unable to record inward NMDA currents larger than 0·4–0·5 nA; however, at membrane potentials positive to the reversal potential it was easy to evoke large

(3–4 nA) outward currents with NMDA. In addition, the current–voltage relation of responses to L-glutamate evoked in 10 mM-Mg^{2+} and recorded over the potential range of −20 to −80 mV was considerably more linear than that recorded in the presence of 1 mM-Mg^{2+} in control experiments, reflecting nearly complete block by 10 mM-Mg^{2+} of inward current flow through ion channels linked to NMDA receptors. However, at more positive membrane potentials the L-glutamate slope conductance increases to large positive values, as the channels linked to NMDA receptors become unblocked.

Although our results suggest that Mg^{2+} enter NMDA ion channels, the permeability of the channel to Mg^{2+} is probably quite low. The intracellular activity of Mg^{2+} in excitable cells is of the order of several millimolar (Hess & Weingart, 1981), but such concentrations of Mg^{2+} do not strongly block outward current flow through NMDA ion channels (Nowak *et al.* 1984). Thus, as in other systems, the blocking site is only accessible from the outer face of the membrane (Coronado & Miller, 1979, 1980). The Mg^{2+} binding site has affinity for other divalent cations, Ni^{2+} also producing a voltage-dependent block of NMDA responses. However, concentrations of Cd^{2+} which completely block calcium currents activated by depolarizing voltage jumps to 0 mV have relatively little effect on responses to NMDA.

Neuronal excitability

In the presence of physiological concentrations of Mg^{2+} (1 mM) the current–voltage relation of responses evoked by NMDA has a negative slope conductance at potentials more negative than −30 mV. At low doses of NMDA, this negative slope conductance behaviour effectively increases the membrane resistance over the potential range −80 to −30 mV; as a consequence, other depolarizing inputs such as e.p.s.p.s are potentiated (see Engberg, Flatman & Lambert, 1979), while outward currents which tend to slow or inhibit repetitive firing become less effective. This effect of NMDA on the excitability of neurones is reminiscent of the action of muscarine on the M-current (Adams, Brown & Constanti, 1982). The negative slope behaviour of the NMDA current–voltage relation is much better able to support repetitive firing than other excitatory amino acids such as kainate and quisqualate (or NMDA in the absence of Mg^{2+}), which behave classically and produce depolarization accompanied by an increase in membrane conductance.

Larger doses of NMDA can produce a region of negative slope conductance in the *membrane* current–voltage relation and, as a consequence, the membrane potential exhibits bistable behaviour when the dose of NMDA is large enough to produce a region of net inward current (see Jack *et al.* 1983). This dose-dependent action of NMDA on neuronal excitability has been elegantly demonstrated for cat neocortical neurones *in vitro* (Flatman *et al.* 1983). The tendency of NMDA receptor stimulation to evoke epileptiform-like discharges will be most pronounced in those neurones in which depolarization activates other persistent inward currents. An example would be hippocampal neurones which respond to NMDA (Dingledine, 1983) and possess a non-inactivating Ca^{2+} conductance (Brown & Griffith, 1983). When the membrane potential is not voltage clamped, potentiation by NMDA of regenerative calcium spikes could simply result from activation of two mechanisms which both produce non-linear membrane current–voltage relations. A specific interaction between the conventional voltage-sensitive conductance mechanism and the response triggered

by agonists acting at NMDA receptors seems improbable (cf. Dingledine, 1983; Flatman *et al.* 1983).

The action of NMDA on neuronal excitability is further complicated by the activation of voltage-dependent outward currents during NMDA-evoked depolarization. In particular, calcium entry during bursts of action potentials may trigger large hyperpolarizing responses which will further enhance the production of epileptiform- and pace-maker-like activity; for discussion of such a model in molluscan neurones, see Gorman, Hermann & Thomas (1981).

Desensitization of NMDA responses

Recent studies suggest that in the vertebrate C.N.S. responses to a number of excitatory amino acids, including NMDA, exhibit desensitization (Fagni, Baudry & Lynch, 1983). At the vertebrate neuromuscular junction, desensitization is a well-documented phenomenon, the molecular mechanisms of which are not yet established. It is of interest to note that a number of studies have described an apparent link between calcium and desensitization at the end-plate (Manthey, 1966; Nastuk & Parsons, 1970; Scubon-Mulieri & Parsons, 1977). Our results suggest that procedures which either enhance membrane calcium fluxes (i.e. 'Mg^{2+}-free' solutions), or overload intracellular Ca^{2+} homoeostatic mechanisms (i.e. low-Na^+ solutions which reduce sodium–calcium exchange) tend to enhance desensitization to NMDA. Responses to kainic acid did not show desensitization.

Scubon-Mulieri & Parsons (1977) suggest that at the end-plate, the intrinsic rate of desensitization to acetylcholine may be a function of the intracellular free ionized Ca^{2+} concentration. Our observation that depolarization into a membrane potential region which produces activation of inward Ca^{2+} currents enhances desensitization to NMDA, suggests that certain molecular features of the desensitization process may be common to both cholinergic and NMDA conductance mechanisms.

The ionic mechanism of amino acid responses

Relatively little is known about the ionic mechanism of excitatory amino acid responses in vertebrate neurones. The major area of uncertainty lies in assigning values to the relative permeabilities to Na^+ (P_{Na}), Ca^{2+} (P_{Ca}) and K^+ (P_K). Although Buhrle & Sonnhof (1983) suggest that on frog motoneurones L-glutamate does not increase P_K, the directly measured reversal potential of 0 mV for all excitatory amino acids suggests a mixed ionic mechanism similar to that operating at the vertebrate end-plate (Adams *et al.* 1980). Studies on the ionic mechanism of L-glutamate at the *Drosophila* neuromuscular junction also convincingly demonstrate a mixed ionic mechanism with significant permeability to both monovalent and divalent cations, although in this tissue the increase in permeability to Mg^{2+} is much larger than P_{Ca} (Jan & Jan, 1976). In locust muscle, L-glutamate increases the membrane permeability to Na^+, K^+ and Ca^{2+} (Anwyl, 1977). Studies on frog motoneurones, also using L-glutamate, suggest an agonist-triggered increase in P_{Ca} (Buhrle & Sonhoff, 1983). Recent studies have demonstrated Na^+ dependence of the reversal potential of L-glutamate responses in hippocampal neurones (Hablitz & Langmoen, 1982), but the mixed-agonist action of this amino acid complicates interpretation of this result since it is not known if L-glutamate was acting at NMDA or other amino acid receptors.

Our results suggest that the response to kainic acid is due to an increase in permeability to sodium and potassium. For NMDA the situation is more complex. The results with low-Na^+ solutions suggest a difference in the ionic selectivity of channels linked to NMDA and kainate receptors, but considerable caution is required in the interpretation of our results. It is possible that the ratio P_{Ca}/P_{Na} is greater for NMDA than for kainate, and that this difference is exaggerated in low-Na^+ solutions, hence the difference in reversal potential for the action of NMDA and kainate. It is, however, important to distinguish between ion fluxes directly triggered by agonists, and those secondary to agonist-triggered calcium flux, since intracellular Ca^{2+} can activate potassium (Meech & Standen, 1975), chloride (Owen, Segal & Barker, 1984) and non-specific cation conductances (Colquhoun, Neher, Reuter & Stevens, 1981; Yellen, 1982). The Ca^{2+}-activated chloride current produces slow inward tail currents following depolarizing voltage jumps in chloride-loaded spinal neurones (Owen *et al.* 1984); these tail currents are prolonged by NMDA (M. L. Mayer & G. L. Westbrook, unpublished observations). Such prolongation may result from an additional load on intracellular calcium sequestration mechanisms imposed by the agonist (NMDA)-triggered calcium flux, rather than from direct modulation by amino acids, for example L-aspartic acid (MacDonald & Schneiderman, 1984), of a voltage-dependent slow inward current. Similarly prolongation of NMDA responses in low-Na^+ solution may result from reduction of sodium–calcium exchange, and in chloride-loaded spinal neurones consequent activation by Ca^{2+} of an inward chloride current with a reversal potential close to 0 mV.

Little is known about the regulation of calcium ion activity in spinal cord neurones in culture. If sodium–calcium exchange plays a major role, and prolonged exposure to sodium-substituted solutions allows the intracellular calcium activity to rise above physiological levels, it is possible this is the mechanism of enhanced desensitization of responses to NMDA. The depolarizing response of spinal cord neurones to L-aspartic acid is markedly depressed in choline-substituted, sodium-free solution (MacDonald, 1984). It is unclear if this results from enhanced desensitization, or a direct action of choline on the agonist-triggered ion channel; changes in driving force do *not* appear sufficient to explain the reduction in current.

We thank Dr P. G. Nelson for his encouragement and support of this work, Dr P. B. Guthrie and the Laboratory of Preclinical Studies, NIAAA for computer programs, Sandy Fitzgerald for preparing and maintaining the cultures, and Linda Bowers for photographic assistance.

REFERENCES

ADAMS, D. J., DWYER, T. M. & HILLE, B. (1980). The permeability of end-plate channels to monovalent and divalent metal cations. *Journal of General Physiology* **75**, 493–510.

ADAMS, P. R. (1977). Voltage-jump analysis of procaine action at frog end-plate. *Journal of Physiology* **268**, 291–318.

ADAMS, P. R., BROWN, D. A. & CONSTANTI, A. C. (1982). Pharmacological inhibition of the M-current. *Journal of Physiology* **332**, 223–262.

AKAIKE, N., BROWN, A. M., NISHI, K. & TSUDA, Y. (1981). Actions of verapamil, diltiazam and other divalent cations on the calcium-current of *Helix* neurones. *British Journal of Pharmacology* **74**, 87–95.

ANWYL, R. (1977). The effect of foreign cations, pH and pharmacological agents on the ionic permeability of an excitatory glutamate synapse. *Journal of Physiology* **273**, 389–404.

ASCHER, P., LARGE, W. A. & RANG, H. P. (1979). Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *Journal of Physiology* **295**, 139–170.

ASCHER, P., MARTY, A. & NEILD, T. O. (1978). The mode of action of antagonists of the excitatory response to acetylcholine in *Aplysia* neurones. *Journal of Physiology* **278**, 207–235.

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. *Journal of Physiology* **307**, 413–428.

BROWN, D. A. & GRIFFITH, W. H. (1983). Persistent slow inward calcium current in voltage-clamped hippocampal neurones of the guinea-pig. *Journal of Physiology* **337**, 303–320.

BUHRLE, CH. PH. & SONNHOF, U. (1983). The ionic mechanism of the excitatory action of glutamate upon the membrane of motoneurones of the frog. *Pflügers Archiv* **396**, 154–162.

COLQUHOUN, D., NEHER, E., REUTER, H. & STEVENS, C. F. (1981). Inward current channels activated by intracellular Ca in cultured cardiac cells. *Nature* **294**, 752–754.

CORONADO, R. & MILLER, C. (1979). Voltage-dependent caesium blockade of a cation channel from fragmented sarcoplasmic reticulum. *Nature* **280**, 807–810.

CORONADO, R. & MILLER, C. (1980). Decamethonium and hexamethonium block K^+ channels of sarcoplasmic reticulum. *Nature* **288**, 495–497.

DINGLEDINE, R. (1983). *N*-methyl aspartate activates voltage-dependent calcium conductance in rat hippocampal pyramidal cells. *Journal of Physiology* **343**, 385–405.

ENGBERG, I., FLATMAN, J. A. & LAMBERT, J. D. C. (1978). The action of *N*-methyl-D-aspartic and kainic acids on motoneurones with emphasis on conductance changes. *British Journal of Pharmacology* **64**, 384–385*P*.

ENGBERG, I., FLATMAN, J. A. & LAMBERT, J. D. C. (1979). The actions of excitatory amino acids on motoneurones in the feline spinal cord. *Journal of Physiology* **228**, 227–261.

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

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

FAGNI, L., BAUDRY, M. & LYNCH, G. (1983). Classification and properties of acidic amino acid receptors in hippocampus. I. Electrophysiological studies of an apparent desensitisation and interactions with drugs which block transmission. *Journal of Neuroscience* **3**, 1538–1546.

FLATMAN, J. A., SCHWINDT, P. C., CRILL, W. E. & STAFSTROM, C. E. (1983). Multiple actions of *N*-methyl-D-aspartate on cat neocortical neurones in vitro. *Brain Research* **266**, 169–173.

GORMAN, A. L. F., HERMANN, A. & THOMAS, M. V. (1981). Intracellular calcium and the control of neuronal pacemaker activity. *Federation Proceedings* **40**, 2233–2239.

GUTHRIE, P. B. & WESTBROOK, G. L. (1983). Electrotonic analysis of mouse ventral horn neurons in culture: A.C. analysis. *Society for Neuroscience Abstracts* **9**, part 1, P233.

HABLITZ, J. J. & LANGMOEN, I. A. (1982). Excitation of hippocampal pyramidal cells by glutamate in the guinea-pig and rat. *Journal of Physiology* **325**, 317–331.

HESS, P. & WEINGART, R. (1981). Free magnesium in cardiac and skeletal muscle measured with ion-selective micro-electrodes. *Journal of Physiology* **318**, 14*P*.

HUANG, L. M., CATTERALL, W. A. & EHRENSTEIN, G. (1978). Selectivity of cations and non-electrolytes for acetylcholine-activated channels in cultured muscle cells. *Journal of General Physiology* **71**, 397–410.

JACK, J. J. B., NOBLE, D. & TSIEN, R. W. (1983). *Electric Current Flow in Excitable Cells*, 2nd edn., pp. 225–260. Oxford: Clarendon Press.

JAN, L. Y. & JAN, Y. N. (1976). L-glutamate as an excitatory transmitter at the *Drosophila* larval neuromuscular junction. *Journal of Physiology* **262**, 215–236.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitisation' produced by acetylcholine at the motor end-plate. *Journal of Physiology* **138**, 63–80.

KHRISHTAL, O. A. (1976). Blocking actions of cadmium ions on calcium channels in the membrane of nerve cells. *Dockladÿ Akademiinauk SSSR* **231**, 1003–1005.

LINGLE, C. (1983). Blockade of cholinergic channels by chlorisondamine on a crustacean muscle. *Journal of Physiology* **339**, 395–417.

MACDONALD, J. F. (1984). Substitution of extracellular sodium ions blocks voltage-dependent decrease of input conductance evoked by L-aspartic acid. *Canadian Journal of Physiology and Pharmacology* **62**, 109–115.

MACDONALD, J. F. & PORIETIS, A. V. (1982). DL-Quisqualic and L-aspartic acids activate separate excitatory conductances in cultured spinal cord neurones. *Brain Research* **245**, 175–178.

MACDONALD, J. F., PORIETIS, A. V. & WOJTOWICZ, J. M. (1982). L-aspartic acid induces a region of negative slope conductance in the current voltage relationship of cultured spinal cord neurones. *Brain Research* **237**, 248–253.

MACDONALD, J. F. & SCHNEIDERMAN, J. H. (1984). L-aspartic acid potentiates 'slow' inward current in cultured spinal cord neurones. *Brain Research* **296**, 350–355.

MACDONALD, J. F. & WOJTOWICZ, J. M. (1982). The effects of glutamate and its analogues upon the membrane conductances of central murine neurones in culture. *Canadian Journal of Physiology and Pharmacology* **60**, 282–296.

MANTHEY, A. A. (1966). The effect of calcium on the desensitisation of membrane receptors at the neuromuscular junction. *Journal of General Physiology* **49**, 963–976.

MAYER, M. L. & WESTBROOK, G. L. (1983). A voltage-clamp analysis of inward (anomalous) rectification in mouse spinal sensory ganglion neurones. *Journal of Physiology* **340**, 19–45.

MAYER, M. L. & WESTBROOK, G. L. (1984*a*). Mixed-agonist action of excitatory amino acids on mouse spinal cord neurones under voltage clamp. *Journal of Physiology* **354**, 29–53.

MAYER, M. L. & WESTBROOK, G. L. (1984*b*). Channel block by magnesium ions may underlie voltage sensitivity of *n*-methyl-D-aspartic acid action on mouse spinal neurones in culture. *Journal of Physiology* **349**, 14*P*.

MAYER, M. L., WESTBROOK, G. L. & GUTHRIE, P. B. (1984). Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurones. *Nature* **309**, 261–263.

MEECH, R. W. & STANDEN, N. B. (1975). Potassium activation of *Helix aspersa* neurones under voltage clamp: a component mediated by calcium influx. *Journal of Physiology* **249**, 211–239.

NASTUK, W. L. & PARSONS, R. L. (1970). Factors in the inactivation of postjunctional membrane receptors of frog skeletal muscle. *Journal of General Physiology* **56**, 218–249.

NOWAK, L., BREGESTOVSKI, P., ASCHER, P., HERBET, A. & PROCHIANTZ, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* **307**, 462–465.

OWEN, D. G., SEGAL, M. & BARKER, J. L. (1984). A Ca-mediated Cl^- conductance is present in cultured mouse spinal neurones. *Nature* **311**, 567–570.

RALL, W. (1969). Time constants and electrotonic length of membrane cylinders and neurons. *Biophysical Journal* **9**, 1483–1508.

SCUBON-MULIERI, B. & PARSONS, R. L. (1977). Desensitisation and recovery at the frog neuromuscular junction. *Journal of General Physiology* **69**, 431–447.

WATKINS, J. C. (1981*a*). Pharmacology of excitatory amino acid receptors. In *Glutamate: Transmitter in the Central Nervous System*, ed. ROBERTS, P. J., STORM-MATHISEN, J. & JOHNSTON, G. A. R., pp. 1–24. New York: John Wiley and Sons.

WATKINS, J. C. (1981*b*). Pharmacology of excitatory amino acid transmitters. In *Amino Acid Neurotransmitters*, ed. DE FEUDIS, F. V. & MANDEL, P., pp. 205–212. New York: Raven Press.

WESTBROOK, G. L. & MAYER, M. L. (1984). Glutamate currents in mammalian spinal neurones: resolution of a paradox. *Brain Research* **301**, 375–379.

WOODHULL, A. M. (1973). Ionic blockage of sodium channels in nerve. *Journal of General Physiology* **61**, 687–708.

YELLEN, G. (1982). Single Ca^{2+}-activated non selective cation channels in neuroblastoma. *Nature* **296**, 357–359.