Mechanisms of glutamate-stimulated Mg^{2+} influx and subsequent Mg^{2+} efflux in rat forebrain neurones in culture

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1. Mag-fura-2 fluorescence microscopy and whole-cell patch-clamp recordings were used to measure glutamate-induced changes in the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) and Mg^{2+} currents, respectively, in cultured forebrain neurones from fetal rats in the absence of extracellular Na^+ (Na_o^+) and Ca^{2+} (Ca_o^{2+}).

2. Increasing the extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) from 9 to 70 mM significantly enhanced the maximum $[Mg^{2+}]_i$ induced by a 5 min 100 μM glutamate plus 1 μM glycine stimulation ($[Mg^{2+}]_{i,5\ min}$) from 2·04 ± 0·07 to 2·98 ± 0·20 mM. Increasing $[Mg^{2+}]_o$ from 9 to 70 mM also significantly enhanced the initial rate of rise in $[Mg^{2+}]_i$ upon glutamate stimulation from 0·41 ± 0·02 to 0·81 ± 0·08 mM min^{-1}.

3. The glutamate-stimulated increase in $[Mg^{2+}]_i$ was not altered by prior depletion of intracellular free Na^+ (Na_i^+). For paired stimulations in single neurones, the mean $[Mg^{2+}]_{i,5\ min}$ was 1·95 ± 0·17 mM under Na^+-depleted conditions and 1·94 ± 0·16 mM under control conditions.

4. The glutamate-stimulated increase in $[Mg^{2+}]_i$ was significantly reduced when NMDA channel-permeant Cs^+ or K^+ ions were used as the Na^+ substitute instead of the presumably NMDA channel-impermeant ions *N*-methyl-D-glucamine (NMDG), Tris or sucrose. The mean $[Mg^{2+}]_{i,5\ min}$ was 0·56 ± 0·06 and 0·74 ± 0·08 mM in the presence of Cs^+ or K^+, respectively, compared with 2·13 ± 0·10, 1·93 ± 0·11 and 2·07 ± 0·22 mM in the presence of NMDG, Tris or sucrose, respectively.

5. In whole-cell recordings performed with Cs^+ as the primary intracellular cation, application of 100 μM NMDA plus 10 μM glycine induced inward currents that reversed around −55 mV in an extracellular solution containing 70 mM Mg^{2+} and 31 mM NMDG as the only cations. The currents were reversibly inhibited by DL-2-amino-5-phosphonovaleric acid (APV). In an extracellular solution containing 2 mM Mg^{2+} and 140 mM NMDG, NMDA plus glycine activated outward currents at potentials more depolarized than −90 mV.

6. In whole-cell recordings made with NMDG as the principal cation in the patch pipette, application of NMDA plus glycine in the 70 mM Mg^{2+} extracellular solution induced inward currents at voltages more negative than +15 mV. The ratio of the current measured under these conditions to the current measured in an extracellular solution containing Na^+ as the principal cation (0·073:1) was nearly constant from cell to cell.

7. Following a 5 min glutamate stimulation in the presence of 9 mM Mg^{2+}, $[Mg^{2+}]_i$ returned to basal levels at a mean rate of 58·1 ± 2·1 μM min^{-1}. Complete removal of Na_o^+ significantly inhibited the rate of recovery to 31% of control. Raising $[Mg^{2+}]_o$ to 30 mM had a similar effect, significantly inhibiting recovery to 36% of control. Raising $[Mg^{2+}]_o$ to 30 mM in combination with removal of Na_o^+ did not inhibit recovery significantly more than either manipulation alone (28% of control).

8. These results suggest that glutamate-stimulated increases in $[Mg^{2+}]_i$ that occur in the absence of Na_o^+ and Ca_o^{2+} result from Mg^{2+} entry through NMDA-activated ion channels. Furthermore, recovery from a glutamate-induced Mg^{2+} load appears to be primarily due to Mg^{2+} efflux via a mechanism whose characteristics are consistent with Na^+–Mg^{2+} exchange.

After K^+, Mg^{2+} is the most abundant cation inside mammalian cells (Romani & Scarpa, 1992). The mechanisms of Mg^{2+} homeostasis have not been studied as extensively as the cellular regulation of other cations; nonetheless, multiple mechanisms of Mg^{2+} influx and Mg^{2+} efflux have been described (Flatman, 1991; Murphy, Freudenrich & Lieberman, 1991; Romani & Scarpa, 1992; Günther, 1993). It has also been determined that most cells have a large buffering capacity for Mg^{2+}, since measurements made in a variety of cell types indicate that only 5–10% of the total intracellular Mg^{2+} exists as free Mg^{2+} (Flatman, 1991; Murphy *et al.* 1991; Günther, 1993). The majority of these studies utilized erythrocytes and cardiac myocytes, and most studies of Mg^{2+} homeostasis in neuronal tissue have been limited to the non-mammalian squid giant axon. A previous study measured changes in $[Mg^{2+}]_i$ in cultured forebrain neurones from fetal rats (Brocard, Rajdev & Reynolds, 1993) using the fluorescent dye mag-fura-2 (Raju, Murphy, Levy, Hall & London, 1989). In these neurones, glutamate stimulation triggered large increases in $[Mg^{2+}]_i$, and a major component of the glutamate-induced increase in $[Mg^{2+}]_i$ was probably due to displacement of Mg^{2+} bound within the cell by the Ca^{2+} that enters the neurone upon NMDA receptor activation (Brocard *et al.* 1993). This study also identified a second, smaller, Ca^{2+}-independent component of the glutamate-stimulated increase in $[Mg^{2+}]_i$ that reflected Mg^{2+} influx into the cell by an unknown mechanism (Brocard *et al.* 1993). Since Mg^{2+} efflux via Na^+–Mg^{2+} exchange driven by the Na^+ gradient has been described in a variety of cell types (Flatman, 1991; Murphy *et al.* 1991; Romani & Scarpa, 1992; Günther, 1993), it was proposed that the Mg^{2+} influx observed was mediated by reversal of the Na^+–Mg^{2+} exchanger, inasmuch as the Ca^{2+}-independent glutamate-stimulated increases in $[Mg^{2+}]_i$ were enhanced by removal of Na_o^+ and/or by elevating $[Mg^{2+}]_o$ (Brocard *et al.* 1993). Furthermore, the Ca^{2+}-independent glutamate-stimulated increases in $[Mg^{2+}]_i$ were blocked by the NMDA antagonist MK-801, and it was hypothesized that the K^+ efflux and subsequent membrane hyperpolarization that occurred upon NMDA receptor activation in a Na^+-free solution was necessary to activate the electrogenic Na^+–Mg^{2+} exchanger (Brocard *et al.* 1993). In the present study we have investigated further the mechanism of glutamate-stimulated increases in $[Mg^{2+}]_i$ in the absence of Na_o^+ and Ca_o^{2+} and in the presence of elevated $[Mg^{2+}]_o$, and we have investigated the mechanisms of recovery from this glutamate-induced Mg^{2+} load.

METHODS

Fluorescence microscopy

Cell culture. In the present study, all procedures using animals were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Pregnant female rats were anaesthetized by ether inhalation until consciousness was lost. The anaesthetized females were killed by decapitation, and the pups were removed and also killed by decapitation.

Primary cultures were prepared from embryonic day 17 Sprague–Dawley rat fetuses as described previously (White & Reynolds, 1995). Briefly, dissociated forebrain neurones were plated on poly-L-lysine-coated 31 mm glass coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (24 U ml^{-1}), and streptomycin (24 μg ml^{-1}). After 24 h, the medium was replaced with one containing 10% horse serum, and the coverslips were inverted to inhibit proliferation of glia. These culture conditions provide sparse, glia-poor neuronal cultures that are necessary for optimal results in the fluorescence microscopy experiments. Neurones were used after 14–18 days in culture.

Solutions. The control perfusion solution was a Hanks' balanced salt solution (HBSS) which contained (mM): 137 NaCl, 5 KCl, 20 Hepes, 5·5 glucose, 0·9 $MgSO_4$, 1·4 $CaCl_2$, 10 $NaHCO_3$, 0·6 Na_2HPO_4, and 0·6 KH_2PO_4. The pH of this solution was adjusted to 7·4 with NaOH. All other perfusion solutions used in these experiments were modifications of this control solution. The Ca^{2+}-free solutions contained no $CaCl_2$ and contained 20 μM EGTA. The Na^+-free solutions contained 5 mM $KHCO_3$ in place of $NaHCO_3$, contained K_2HPO_4 in place of Na_2HPO_4, and did not contain KCl. In these solutions NaCl was replaced with either 140 mM NMDG, Tris, CsCl, KCl, LiCl or choline chloride. The pH of the Na^+-free solutions was adjusted to 7·4 with HCl or Tris base as appropriate, except the pH of the Cl^--free NMDG solution, which was adjusted with glacial acetic acid. Solutions with concentrations of Mg^{2+} higher than 0·9 mM were supplemented with $MgCl_2$, and the concentration of the Na^+ substitute was correspondingly reduced to maintain the correct osmolality. Thus the 30, 50 and 70 mM Mg^{2+} solutions contained 90, 60 and 30 mM NMDG chloride, respectively. Bicarbonate was added to the perfusion solutions from stock solutions made fresh each day. Perfusion solutions were supplemented with sucrose as necessary to achieve the desired osmolality, and osmometer measurements confirmed that the solutions were ~300 mosmol kg^{-1}.

$[Mg^{2+}]_i$ and $[Na^+]_i$ measurements. $[Mg^{2+}]_i$ or $[Na^+]_i$ was measured in individual neurones that had been loaded with either mag-fura-2 AM or SBFI AM (Molecular Probes), respectively. To load with mag-fura-2 AM, neurones were incubated in HBSS containing 5 μM mag-fura-2 AM, 5 mg ml^{-1} bovine serum albumin, and 0·5% DMSO for 15 min at 37 °C. To load with SBFI AM, neurones were incubated in HBSS containing 5 μM SBFI AM, 0·1% w/v Pluronic F-127 (Molecular Probes), and 1% DMSO for 60 min at 37 °C. Following loading, coverslips were rinsed with HBSS, mounted in a recording chamber, and perfused with HBSS at a rate of 20 ml min^{-1}. There was an exchange of the 1 ml chamber volume every 3 s with this high flow rate. All recordings were made at room temperature (20–25 °C) using equipment previously described for fura-2 measurements (Reynolds & Aizenman, 1992). Fluorescence ratios were obtained at a rate of 0·5 Hz, and the ratio of the background-subtracted fluorescence signal was converted to $[Mg^{2+}]_i$ or $[Na^+]_i$ using the equation described by Grynkiewicz, Poenie & Tsien (1985):

$$[Mg^{2+}]_i \text{ (or } [Na^+]_i) = K^* (\text{Ratio} - R_{min})/(R_{max} - \text{Ratio}).$$

K is a constant describing the apparent affinity of the dye for Mg^{2+} or Na^+ and is related to the dissociation constant (K_d) of mag-fura-2 (1·5 mM) or SBFI (18 mM), respectively. R_{min} and R_{max} are the fluorescence ratios measured in the nominal absence and in the presence of saturating amounts of ion, respectively. K, R_{min} and

R_{max} were obtained from standard curves. The $[Mg^{2+}]_i$ standard curve was constructed using the fluorescence ratios obtained from eighteen EGTA-buffered solutions containing mag-fura-2 tetrapotassium salt (20 μM) and known concentrations of Mg^{2+} (0–50 mM). Since the spectral characteristics of SBFI in the cytosol are different from those of SBFI in solution, the $[Na^+]_i$ standard curve was constructed from fluorescence ratios obtained *in situ* upon exposing neurones loaded with SBFI AM to perfusion solutions containing known concentrations of Na^+ (0–140 mM) and 5 μM gramicidin D (Harootunian, Kao, Eckert & Tsien, 1989). However, the results of the SBFI experiments were not reported as $[Na^+]_i$ values in this study. As a consequence of the large amount of noise in the signal (see Fig. 2), when measuring very low and very high $[Na^+]_i$, some SBFI fluorescence ratios are converted to $[Na^+]_i$ values < 0 mM and > 140 mM, respectively, due to the non-linear relationship between the fluorescence ratio and $[Na^+]_i$.

For the majority of the fluorescence microscopy experiments described below, the standard glutamate stimulation was a 5 min exposure to 100 μM glutamate plus 1 μM glycine in a Na^+- and Ca^{2+}-free solution containing either Tris or NMDG as a Na^+ substitute and containing 9 mM Mg^{2+}. In order to remove any residual Ca^{2+} or Na^+ in the perfusion chamber prior to glutamate stimulation in a Na^+- and Ca^{2+}-free solution, the coverslips were perfused with a Ca^{2+}-free solution for 2 min and then perfused with a Na^+- and Ca^{2+}-free solution for an additional 1 min immediately prior to every glutamate stimulation (see Fig. 1).

Calculations and statistics. In these studies, $[Mg^{2+}]_{i,5\ min}$ was defined as the maximum $[Mg^{2+}]_i$ achieved upon a 5 min stimulation with 100 μM glutamate plus 1 μM glycine. Plateau $[Mg^{2+}]_i$ was defined as the maximum $[Mg^{2+}]_i$ achieved upon a long ($\geqslant$ 20 min) stimulation with 100 μM glutamate plus 1 μM glycine. According to these definitions, the plateau $[Mg^{2+}]_i$ was the absolute maximum $[Mg^{2+}]_i$ possible upon stimulation, and the plateau $[Mg^{2+}]_i$ was always greater than the $[Mg^{2+}]_{i,5\ min}$, reflecting the fact that it took longer than 5 min to obtain maximal increases in $[Mg^{2+}]_i$.

The initial rate of rise in $[Mg^{2+}]_i$ was determined during the early phase of a glutamate stimulation. Since $[Mg^{2+}]_i$ increased approximately linearly during the first ~3 min of glutamate stimulation, the initial rate of rise was calculated from the slope of the line describing the increase in $[Mg^{2+}]_i$ within the first 90 s of glutamate stimulation:

$$\text{Initial rate of rise} = ([Mg^{2+}]_{i,90\ s} - [Mg^{2+}]_{i,30\ s})/1\ \text{min}.$$

$[Mg^{2+}]_{i,90\ s}$ and $[Mg^{2+}]_{i,30\ s}$ denote the $[Mg^{2+}]_i$ at 90 s and 30 s after the start of the glutamate stimulation, respectively.

Similarly, since $[Mg^{2+}]_i$ decreased approximately linearly during the first ~20 min following glutamate stimulation, the rate of recovery was determined from the slope of the line describing the decrease in $[Mg^{2+}]_i$ immediately following stimulation. Preliminary results indicated that the rate of recovery was greatest when the neurone was perfused with a Ca^{2+}-free solution (not shown). Therefore, Ca^{2+}-free solution was defined as the control solution in these recovery studies. For each neurone, a control rate of recovery was calculated from the decrease in $[Mg^{2+}]_i$ that occurred within the 6 min period of perfusion with a Ca^{2+}-free solution immediately following glutamate stimulation:

$$\text{Rate of recovery} = ([Mg^{2+}]_{i,350\ s} - [Mg^{2+}]_{i,100\ s})/250\ \text{s}.$$

$[Mg^{2+}]_{i,350\ s}$ and $[Mg^{2+}]_{i,100\ s}$ denote the $[Mg^{2+}]_i$ at 350 s and 100 s after the end of the glutamate stimulation, respectively.

For the data reported in Table 2, a treated rate of recovery was also determined for each neurone. The treated rate of recovery was determined from the decrease in $[Mg^{2+}]_i$ that occurred within the 6 min period of perfusion with an altered extracellular solution which followed the 6 min perfusion with the control (Ca^{2+}-free) solution.

In neurones exposed to an altered extracellular solution during recovery, we also calculated the rate of recovery in control solution following perfusion with the altered extracellular solution (see Fig. 9). The rate of recovery in control solution in the 6 min period following perfusion with an altered extracellular solution was not significantly different from the rate of recovery in control solution in the 6 min period preceding perfusion with an altered extracellular solution ($55{\cdot}0 \pm 2{\cdot}4$ μM min^{-1} compared with $58{\cdot}8 \pm 2{\cdot}6$ μM min^{-1}, $n = 81$, $P > 0{\cdot}05$, Student's paired t test). These results confirm that the decrease in $[Mg^{2+}]_i$ is approximately linear during the first ~15–20 min following a glutamate stimulation.

The mean initial rates of rise, the mean control rates of recovery, and the mean treated rates of recovery were compared by one-way analyses of variance with Dunnett and Bonferroni tests for *post hoc* comparisons as appropriate (Instat 2.0, Graph Pad Software, San Diego, CA, USA). The $[Mg^{2+}]_{i,5\ min}$ and the plateau $[Mg^{2+}]_i$ values were also compared by one-way analysis of variance with Dunnett and two-sided Student's unpaired t tests for *post hoc* comparisons as appropriate. In Table 2, the treated rates of recovery were compared with the control rates of recovery from corresponding neurones using the two-sided Student's paired t test. $P < 0{\cdot}05$ was considered statistically significant. All data are reported as means ± S.E.M.

Electrophysiology

Cell culture. All procedures using animals were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Primary cultures were prepared from embryonic day 16 Sprague–Dawley rat fetuses as previously described (Li-Smerin & Johnson, 1996). Briefly, pregnant rats were killed by CO_2 inhalation. Death was confirmed by the absence of a heartbeat. Fetuses were removed, decapitated and the heads chilled to about 5 °C. For each fetus, a scalpel cut through the head transecting the brainstem was made, the brain was removed, and the cerebral cortices were isolated. Dissociated cortical neurones were plated on poly-L-lysine-coated 15 mm glass coverslips in minimum essential medium (MEM) supplemented with (mM): 24 D-glucose, 18 Hepes, 1·9 L-glutamine; plus Earle's salts, 10% fetal bovine serum and 10% calf serum. Non-neuronal cell division was inhibited by addition of 5′-fluoro-2′-deoxyuridine monophosphate plus uridine for 36–48 h starting on culture day 6–8. Neurones were used after 3 to 6 weeks in culture.

Solutions. The control extracellular solution contained (mM): 140 NaCl, 2·8 KCl, 1 $CaCl_2$, and 10 Hepes; the pH was adjusted to 7·2 with NaOH. The Mg^{2+} solutions contained (mM): 31 (or 140) NMDG, 5·5 glucose, 20 Hepes, and 70 (or 2 or 0·9) $MgCl_2$: plus 20 μM EGTA. These extracellular solutions also contained 200 nM tetrodotoxin. The solution in the patch pipette contained either 120 mM CsF, 10 mM CsCl, 10 mM Hepes, and 10 mM BAPTA (pH adjusted to 7·2 with CsOH) or 130 mM NMDG, 10 mM Hepes, and 10 mM EGTA (pH adjusted with HCl).

Whole-cell recordings. The whole-cell configuration was formed using conventional patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Current was recorded with an Axopatch-1D amplifier (Axon Instruments) using pipettes pulled from thin-wall borosilicate glass capillaries with inner filaments (Clark Electromedical Instruments, Reading, UK). The pipette resistance was 4–5 MΩ. Partial compensation for series resistance was used in most experiments.

Whole-cell currents were low-pass filtered at 10 kHz, sampled at 44 kHz with a Neuro-Corder (Model DR-890, Neuro Data Instruments Corp., New York), and stored on magnetic tape for off-line analysis. Currents were also filtered at 500 Hz and recorded on chart paper with a thermal Arraycorder (WR 7700, Western Graphtec, Irvine, CA, USA). All experiments were done at room temperature (20–25 °C).

A five-barrel fast perfusion system was used to perfuse and exchange extracellular solutions during whole-cell recordings. Briefly, barrels made of square capillary tubing were arrayed in parallel and connected to reservoirs by Silastic and polyethylene tubing. After initiation of a whole-cell recording, the barrel containing the control solution was positioned ~200 μm away from the neurone under study. Exchanges of the solution perfusing the neurone were achieved by moving the array of barrels laterally with a DC linear motor. The movements of the barrels generally took 30 ms to complete. Solution exchange was 98–99% complete within 120 ms after the initiation of barrel movement (T. Blanpied & J. W. Johnson, unpublished observations).

For each measurement of a glutamate- or NMDA-activated current in a Mg^{2+} solution, the neurone was first perfused with the Mg^{2+} solution alone for 2–3 s to ensure complete washout of control solution. The neurone was then perfused with the same Mg^{2+} solution containing agonists for 2–3 s and then again perfused with the Mg^{2+} solution alone for 2–3 s. Neurones were always perfused with the control solution before the first exposure to the Mg^{2+} solution alone and after the second exposure to the Mg^{2+} solution alone.

Liquid junction potentials were measured for each of the experimental conditions. These potentials varied from −6 to +8 mV depending on the conditions, and the membrane potentials listed in Figs 5–7 have been corrected accordingly.

Calculations and statistics. The steady-state whole-cell current measured in the absence or in the presence of agonists (glutamate plus glycine or NMDA plus glycine) was determined from chart paper records. The amplitude of the whole-cell current was calculated as the difference between the response in the absence of the agonists and the response in the presence of the agonists. Statistical comparisons were performed with Systat for Windows version 5, and $P < 0.05$ was considered statistically significant (two-sided tests). All data are reported as means ± S.E.M.

RESULTS

Mechanisms of Mg^{2+} influx

The mean basal $[Mg^{2+}]_i$ (determined after 2 min of perfusion in a Ca^{2+}-free solution and 30 s of perfusion in a Na^+- and Ca^{2+}-free solution, see Methods and Fig. 1) was 0·28 ± 0·01 mM ($n = 185$). Stimulation with glutamate plus glycine caused significant increases in $[Mg^{2+}]_i$, and $[Mg^{2+}]_i$ increased approximately linearly during the first ~3 min of glutamate stimulation (Fig. 1). With longer glutamate stimulations, the rate of rise in $[Mg^{2+}]_i$ decreased over time, and the glutamate-induced increase in $[Mg^{2+}]_i$ reached a plateau after ~20 min of stimulation (Fig. 1).

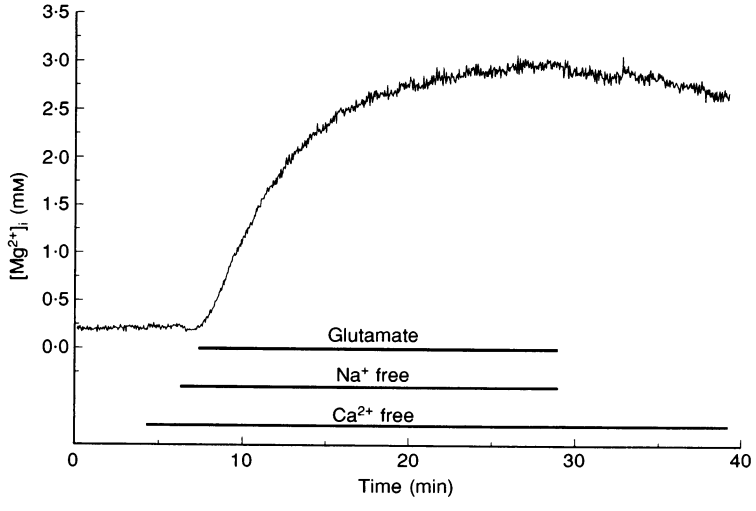

Figure 1. Glutamate-stimulated increases in $[Mg^{2+}]_i$ reached a plateau with longer stimulations
$[Mg^{2+}]_i$ was measured using the fluorescent dye mag-fura-2. The neurone was stimulated with 100 μM glutamate + 1 μM glycine for ~20 min in a Na^+- and Ca^{2+}-free solution containing 9 mM Mg^{2+}. In order to remove any residual Ca^{2+} or Na^+ in the perfusion chamber prior to glutamate stimulation in a Na^+- and Ca^{2+}-free solution, all coverslips were perfused with a Ca^{2+}-free solution for 2 min and then perfused with a Na^+- and Ca^{2+}-free solution for an additional 1 min immediately prior to glutamate stimulation. This trace is representative of results obtained in 14 additional neurones. Qualitatively similar results were obtained in 13 additional neurones stimulated with glutamate in a Na^+- and Ca^{2+}-free solution containing 70 mM Mg^{2+}.

Table 1. Elevated $[Mg^{2+}]_o$ increases the initial rate of rise in $[Mg^{2+}]_i$, the $[Mg^{2+}]_{i,5\ min}$ and the plateau $[Mg^{2+}]_i$ that occur upon glutamate stimulation

$[Mg^{2+}]_o$ (mM)	Initial rate of rise in $[Mg^{2+}]_i$ (mM min^{-1})	$[Mg^{2+}]_{i,5\ min}$ (mM)	Plateau $[Mg^{2+}]_{i,20\ min}$ (mM)
9	0·41 ± 0·02	2·04 ± 0·07	2·99 ± 0·26 †
30	0·52 ± 0·09	2·08 ± 0·16	n.d.
50	0·58 ± 0·06	2·47 ± 0·10	n.d.
70	0·81 ± 0·08 *	2·98 ± 0·20 *	4·77 ± 0·21 * †

Glutamate stumulation was either a 5 min or a 20 min exposure to 100 μM glutamate plus 1 μM glycine in a Na^+- and Ca^{2+}-free solution. Initial rates of rise were determined from the increase in $[Mg^{2+}]_i$ within the first 90 s of glutamate stimulation. There were significant differences in the initial rates ($P < 0·05$, one-way ANOVA). $[Mg^{2+}]_{i,5\ min}$ was defined as the maximum $[Mg^{2+}]_i$ achieved upon a 5 min glutamate stimulation. Plateau $[Mg^{2+}]_i$ was defined as the maximum $[Mg^{2+}]_i$ achieved upon a long (≥ 20 min) glutamate stimulation. There were significant differences in the $[Mg^{2+}]_{i,5\ min}$ values ($P < 0·05$, one-way ANOVA). All values represent the mean ± S.E.M. from 10–107 neurones. * Results significantly different from the corresponding results obtained with $[Mg^{2+}]_o$ of 9 mM ($P < 0·05$, Dunnett or Student's unpaired t test). † Plateau $[Mg^{2+}]_i$ significantly different from corresponding $[Mg^{2+}]_{i,5\ min}$ ($P < 0·05$, Student's unpaired t test). n.d., not determined.

Glutamate-stimulated increases in $[Mg^{2+}]_i$ are enhanced by raising $[Mg^{2+}]_o$

Increasing $[Mg^{2+}]_o$ significantly enhanced both the $[Mg^{2+}]_{i,5\ min}$ and the plateau $[Mg^{2+}]_i$ induced by either a 5 min or a 20 min glutamate stimulation, respectively (Table 1). Increasing $[Mg^{2+}]_o$ from 9 to 70 mM significantly enhanced the mean $[Mg^{2+}]_{i,5\ min}$ induced by glutamate stimulation by 146% (Table 1), but had no effect on $[Mg^{2+}]_i$ in the absence of glutamate (not shown). Similarly, increasing $[Mg^{2+}]_o$ from 9 to 70 mM significantly enhanced the mean plateau $[Mg^{2+}]_i$ induced by a 20 min glutamate stimulation by 160% (Table 1). The initial rate of rise in $[Mg^{2+}]_i$ (determined within the first 90 s of glutamate stimulation) was also significantly enhanced by increasing $[Mg^{2+}]_o$. Raising $[Mg^{2+}]_o$ from 9 to 70 mM increased the initial rate of rise in $[Mg^{2+}]_i$ by 198% (Table 1). These results support the hypothesis that the increase in $[Mg^{2+}]_i$ upon glutamate stimulation in a Na^+- and Ca^{2+}-free solution is due to Mg^{2+} influx.

Measurements of glutamate-induced changes in $[Na^+]_i$

In a previous study, the increases in $[Mg^{2+}]_i$ that occurred upon glutamate stimulation in a Ca^{2+}-free solution were reduced when Na^+ was included in the extracellular solution or when $[Mg^{2+}]_o$ was reduced from 9 to 0·9 mM (Brocard *et al.* 1993). These results suggested that part of the increase in $[Mg^{2+}]_i$ upon glutamate stimulation in a Ca^{2+}-free solution may have been due to reverse operation of the Na^+–Mg^{2+} exchanger. Therefore, in the present study, we used the fluorescent dye SBFI (Minta & Tsien, 1989) to monitor $[Na^+]_i$ upon glutamate stimulation in a Na^+- and Ca^{2+}-free solution in an attempt to detect decreases in $[Na^+]_i$ concomitant with increases in $[Mg^{2+}]_i$.

In the control (Na^+-containing) perfusion solution, glutamate increased $[Na^+]_i$ in a concentration-dependent manner (Fig. 2*A*). Following a 30 s glutamate exposure, $[Na^+]_i$ slowly returned to basal levels within ~5–15 min, and the length of time required for recovery depended on the concentration of glutamate used. Since the SBFI fluorescence ratios were not converted to $[Na^+]_i$ values in Fig. 2*A*, an *in situ* calibration using the pore-forming antibiotic gramicidin D is shown for comparison (inset). This calibration also illustrates the usefulness of the dye for detecting changes in $[Na^+]_i$ in the 1–100 mM range.

Exposing neurones to a Na^+-free solution in the absence of glutamate decreased $[Na^+]_i$ (Fig. 2*B*). Na^+-free solutions containing Tris, NMDG or choline as a Na^+ substitute had similar effects on $[Na^+]_i$, but a Na^+-free solution containing Li^+ as a Na^+ substitute quenched the fluorescence of SBFI (not shown) and was not used in further experiments. After ~15 min of perfusion with a Na^+-free solution, the SBFI fluorescence ratio reached a stable minimum lower than that which was routinely observed upon exposure to a 1 mM Na^+ calibration solution. This effect was rapidly reversible, and $[Na^+]_i$ returned to or exceeded baseline values within ~2–3 min of reperfusion with the control Na^+-containing solution.

Glutamate-stimulated increases in $[Mg^{2+}]_i$ are not due to reverse Na^+–Mg^{2+} exchange

If part of the increase in $[Mg^{2+}]_i$ that occurs upon glutamate stimulation in a Na^+- and Ca^{2+}-free solution were due to reversal of the Na^+–Mg^{2+} exchanger, then it should be possible to reduce the glutamate-stimulated increase in $[Mg^{2+}]_i$ by depleting $[Na^+]_i$ prior to stimulation (via a 15 min pretreatment in a Na^+-free solution). We compared

the glutamate-stimulated increases in $[Mg^{2+}]_i$ after both a 1 min and a 15 min exposure to a Na^+-free solution (Fig. 3). The mean $[Mg^{2+}]_{i,5\ min}$ achieved after a 1 min pretreatment in a Na^+-free solution was 1·94 ± 0·16 mM. In the same neurones, the mean $[Mg^{2+}]_{i,5\ min}$ achieved after a 15 min pretreatment in a Na^+-free solution was 1·95 ± 0·17 mM ($n = 10$, $P > 0·05$, Student's paired t test). Since the $[Mg^{2+}]_{i,5\ min}$ was not altered by prior $[Na^+]_i$ depletion, these results suggest that Mg^{2+} influx does not occur via reverse Na^+–Mg^{2+} exchange.

Glutamate-stimulated Mg^{2+} influx may occur via the ion channel of the NMDA receptor

Since previous studies indicated that the increase in $[Mg^{2+}]_i$ that occurs upon glutamate stimulation in a Na^+- and Ca^{2+}-free solution was blocked by MK-801 (Brocard *et al.* 1993), a channel blocker of NMDA-activated ion channels, we explored the possibility that Mg^{2+} might enter neurones via the ion channel of the NMDA receptor in the absence of other permeant cations. If glutamate-stimulated Mg^{2+}

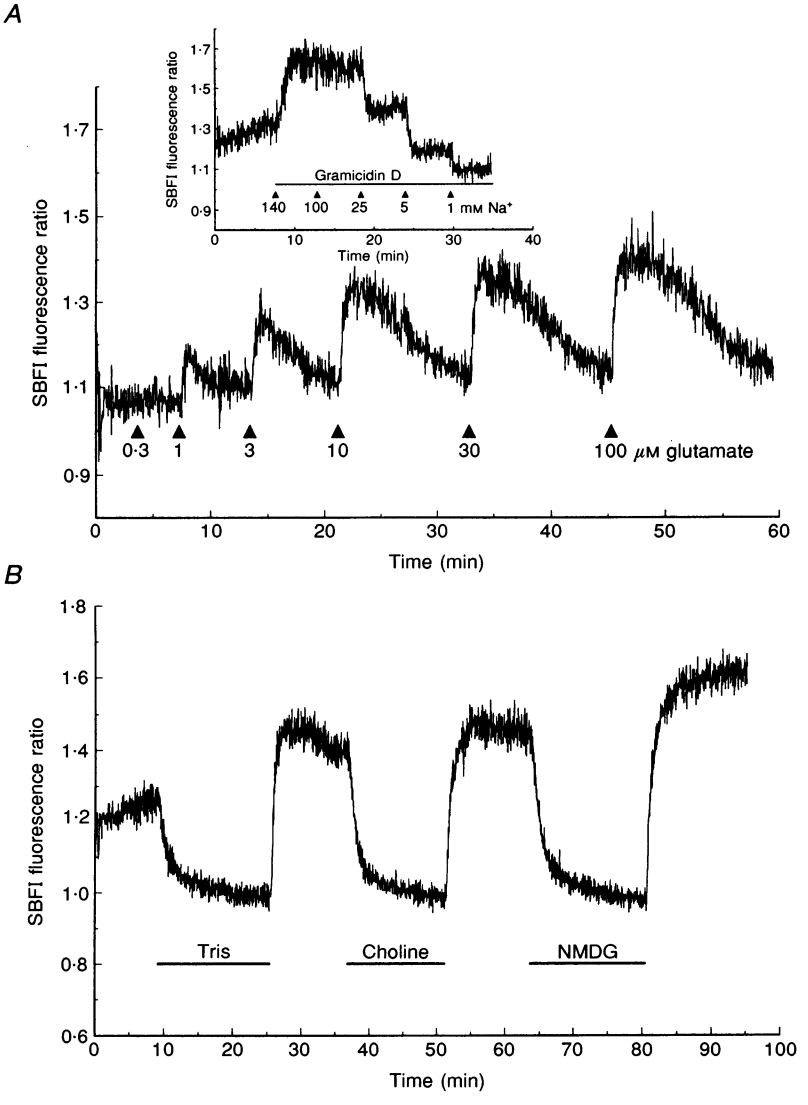

Figure 2. The differential effects of glutamate and Na^+-free solutions on $[Na^+]_i$

A, glutamate dose dependently increased $[Na^+]_i$ in neurones. $[Na^+]_i$ was measured using the fluorescent dye SBFI. Each arrowhead denotes the beginning of a 30 s exposure to glutamate (at the indicated concentration) in combination with 1 μM glycine in the control (Na^+-containing) perfusion solution. These data are representative of results obtained in 3 additional neurones. Inset, the pore-forming antibiotic gramicidin D was used for *in situ* calibration of SBFI (Harootunian *et al.* 1989). The perfusion solution was changed from the control (Na^+-containing) solution to one containing gramicidin D, and each arrowhead denotes the introduction of a different calibration solution in the presence of gramicidin D. These data are representative of results obtained in 10 additional neurones. *B*, Na^+-free solutions caused a profound but rapidly reversible depletion of $[Na^+]_i$. The neurone was alternately perfused with the control (Na^+-containing) solution and solutions in which all Na^+ had been replaced with Tris, choline or NMDG. Similar results were obtained in up to 8 additional neurones.

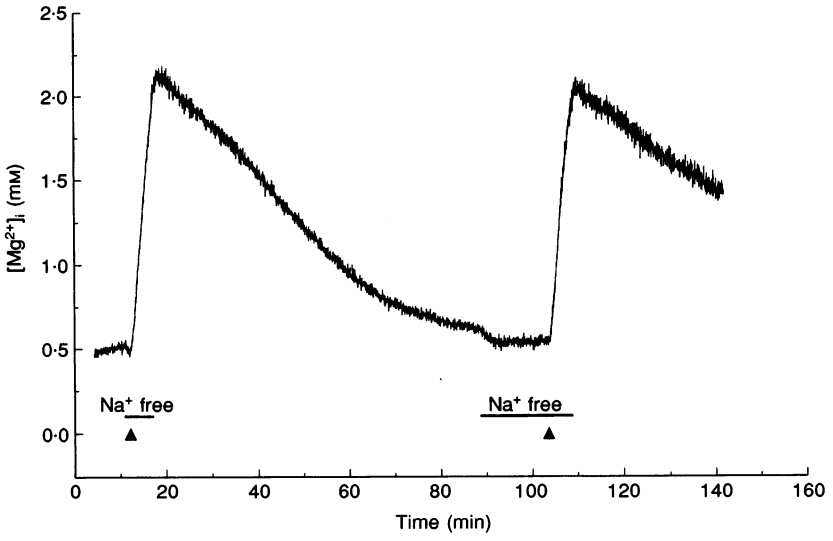

Figure 3. Glutamate-stimulated increases in $[Mg^{2+}]_i$ were not altered by prior depletion of $[Na^+]_i$

At the arrowheads the neurone was stimulated with 100 μM glutamate + 1 μM glycine for 5 min in a Na^+- and Ca^{2+}-free solution containing 9 mM Mg^{2+} after either a 1 min or a 15 min pretreatment in a Na^+- and Ca^{2+}-free solution. This trace is representative of results obtained in 9 additional neurones.

influx occurs via this channel, then addition of NMDA channel-permeant monovalent cations to the extracellular solution may inhibit Mg^{2+} influx by competing with Mg^{2+} for entry through the channel. Therefore, we hypothesized that the glutamate-stimulated increase in $[Mg^{2+}]_i$ that occurs upon stimulation in a Na^+- and Ca^{2+}-free solution would be reduced if the Na^+ substitute could permeate the channel of the NMDA receptor. When Cs^+ or K^+ was used as a Na^+ substitute instead of the presumably impermeant NMDG, Tris or sucrose, glutamate-stimulated increases in $[Mg^{2+}]_i$ were significantly reduced ($P < 0{\cdot}05$, one-way ANOVA with Bonferroni tests for *post hoc* comparisons, Fig. 4). The mean $[Mg^{2+}]_{i,5\ min}$ was $0{\cdot}56 \pm 0{\cdot}06$ mM ($n = 13$) or $0{\cdot}74 \pm 0{\cdot}08$ mM ($n = 15$) when the Na^+

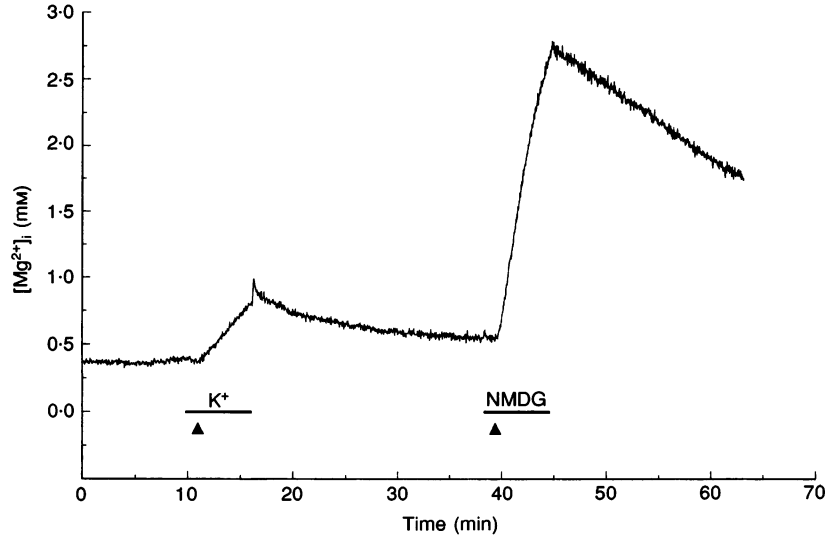

Figure 4. Glutamate-stimulated increases in $[Mg^{2+}]_i$ were significantly reduced in the presence of a Na^+ substitute which permeates the channel of the NMDA receptor

At the arrowheads the neurone was stimulated with 100 μM glutamate + 1 μM glycine for 5 min in a Na^+- and Ca^{2+}-free solution containing 9 mM Mg^{2+} after a 1 min pretreatment in the same Na^+- and Ca^{2+}-free solution. For the first stimulation, the Na^+- and Ca^{2+}-free solution contained K^+ as a Na^+ substitute, and for the second stimulation, the Na^+- and Ca^{2+}-free solution contained NMDG as a Na^+ substitute. These data are representative of results obtained in 14 additional neurones, and similar results were obtained in 13 additional neurones when Cs^+ was used as the Na^+ substitute.

substitute was Cs^+ or K^+, respectively, and these means were not significantly different from each other. In contrast, in the presence of NMDG, Tris or sucrose, the mean $[Mg^{2+}]_{i,5\ min}$ was $2{\cdot}13 \pm 0{\cdot}10$ ($n = 42$), $1{\cdot}93 \pm 0{\cdot}11$ ($n = 35$) or $2{\cdot}07 \pm 0{\cdot}22$ mM ($n = 10$), respectively. These means were not significantly different from each other either. While K^+ or Cs^+ may have reduced Mg^{2+} influx by competing with Mg^{2+} for entry through NMDA channels, it is also possible that K^+ or Cs^+ influx depolarized neurones and that the reduction in the glutamate-stimulated increase in $[Mg^{2+}]_i$ was due to a decrease in the driving force for Mg^{2+} influx.

Electrophysiological studies of Mg^{2+} permeation

To test directly the hypothesis that Mg^{2+} enters neurones via the channel of the NMDA receptor, we performed whole-cell patch-clamp experiments. We first tested whether the endogenous transmitter glutamate could activate inward whole-cell currents in an extracellular solution containing Mg^{2+} and NMDG as the only cations (besides H^+). In whole-cell patch-clamp experiments performed with Cs^+ in the patch pipette, glutamate (100 μM) plus glycine (1 μM) activated an inward current at negative membrane potentials in an extracellular solution containing 70 mM Mg^{2+} and 31 mM NMDG as the only cations (not shown). We then examined the NMDA-activated current carried by Mg^{2+} and compared the currents recorded in the Mg^{2+} and the Na^+-containing control solutions.

NMDA-activated current carried by Mg^{2+}

NMDA responses recorded in the presence of internal Cs^+. NMDA (100 μM) plus glycine (10 μM) also activated an inward current at negative membrane potentials in an extracellular solution containing 70 mM Mg^{2+} and 31 mM NMDG as the only cations (Fig. 5). At membrane potentials near −70 mV, NMDA activated an inward current in the control (140 mM Na^+) solution as well as in the 70 mM Mg^{2+} solution but activated an outward current in the 2 mM Mg^{2+} solution (Fig. 5). The amplitude of the current recorded in

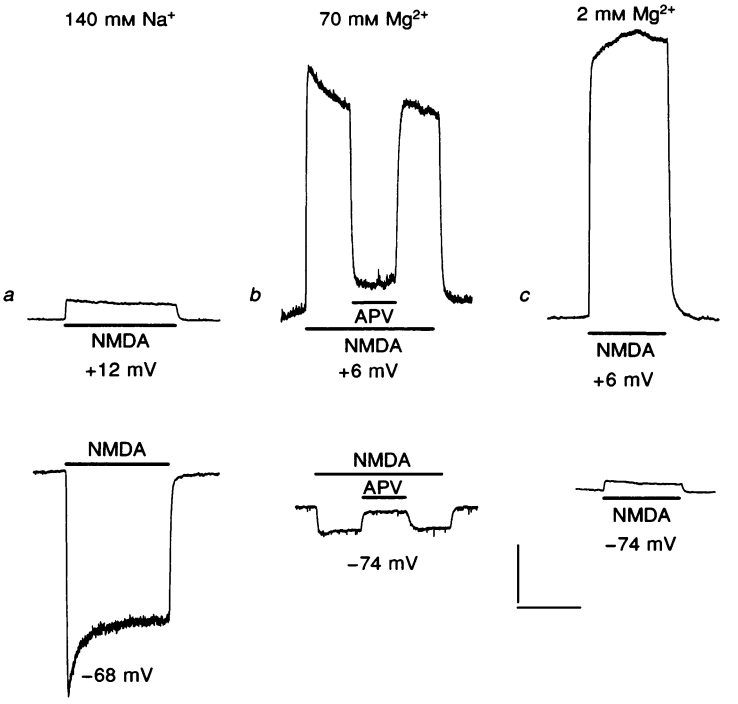

Figure 5. NMDA activated an inward current at negative membrane potentials in an extracellular solution containing 70 mM Mg^{2+} and 31 mM NMDG as the only cations

All records were obtained from the same neurone. The extracellular solution contained either 140 mM Na^+, 2·8 mM K^+ and 1 mM Ca^{2+} (left column), 70 mM Mg^{2+} and 31 mM NMDG (middle column), or 2 mM Mg^{2+} and 140 mM NMDG (right column) as the only cations. The currents were recorded at either positive (upper row) or negative (lower row) membrane potentials, and the indicated potentials represent the membrane potentials after correction for liquid junction potentials. The horizontal bars in the left and right columns indicate application of 100 μM NMDA plus 10 μM glycine. In the middle column, the longer horizontal bars indicate application of 100 μM NMDA plus 10 μM glycine, and the shorter horizontal bars indicate application of 200 μM DL-2-amino-5-phosphonovaleric acid (APV) in combination with NMDA plus glycine. The amplitude (vertical) scale bar is 1000 pA for the left column, 150 pA for the middle column and 500 pA for the right column. The time (horizontal) scale bar is 2 s for all records. All the currents were recorded with 130 mM Cs^+ in the patch pipette. Similar results were obtained in 12 additional neurones.

the 70 mM Mg^{2+} solution was always much smaller than that of the current recorded in the Na^+-containing control solution at the same membrane potential in the same neurone ($n = 13$). In the 70 mM Mg^{2+} solution, inward current was observed at membrane potentials more negative than −55 mV (the approximate reversal potential with Cs^+ in the patch pipette). This NMDA-activated inward current was reversibly inhibited by DL-2-amino-5-phosphonovaleric acid (APV) (Fig. 5). APV (200 μM) blocked 95 ± 3% of the current in the 70 mM Mg^{2+} solution at a membrane potential of −74 mV ($n = 5$). At positive membrane potentials, NMDA plus glycine activated an outward current in the control solution and in both of the Mg^{2+} solutions (Fig. 5). The outward current also was reversibly inhibited by APV.

These results suggest that the inward current recorded in the 70 mM Mg^{2+} solution at negative membrane potentials was carried primarily by Mg^{2+}. If NMDG had carried the inward current recorded in the 70 mM Mg^{2+} solution, we would have also measured inward currents at negative membrane potentials in the solution containing 2 mM Mg^{2+} and 140 mM NMDG.

NMDA responses recorded in the absence of internal Cs^+. The results of the electrophysiological studies presented thus far show that Mg^{2+} can permeate NMDA-activated ion channels in the absence of other permeant cations and generate an inward current at negative membrane potentials. However, these experiments did not permit us to quantitate Mg^{2+} currents because, even at hyperpolarized voltages, a small outward flux of internal Cs^+ is expected to contribute to the net current measured. In order to permit isolation and measurement of inward current, we conducted experiments in which internal Cs^+ was replaced with NMDG.

NMDA (100 μM) plus glycine (10 μM) activated inward currents at negative membrane potentials in both the control and the 70 mM Mg^{2+} solutions when internal Cs^+ was replaced with NMDG (Fig. 6*A*). The input resistances in the 70 mM Mg^{2+} solution and in the control 140 mM Na^+ solution were not significantly different (120 ± 14% of control, $n = 9$, $P > 0.05$, Student's unpaired t test). This suggests that membrane integrity is perserved in the high Mg^{2+} solution and that Mg^{2+} influx is not due to membrane

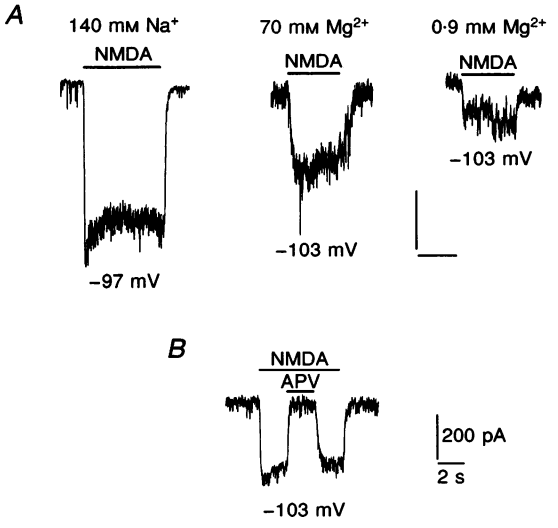

Figure 6. In the absence of internal Cs^+, NMDA activated an inward current at negative membrane potentials in extracellular solutions containing Mg^{2+} and NMDG as the only cations

All currents were recorded with 130 mM NMDG in the patch pipette. The currents were recorded at membrane potentials near −100 mV, and the indicated potentials represent the membrane potentials after correction for liquid junction potentials. *A*, all records were obtained from the same neurone. The extracellular solution contained either 140 mM Na^+, 2·8 mM K^+ and 1 mM Ca^{2+} (left column), 70 mM Mg^{2+} and 31 mM NMDG (middle column), or 0·9 mM Mg^{2+} and 140 mM NMDG (right column) as the only cations. The horizontal bars indicate application of 100 μM NMDA plus 10 μM glycine. The amplitude (vertical) scale bar is 200 pA for the left column and 25 pA for the middle and right columns. The time (horizontal) scale bar is 2 s for all records. Similar results were obtained in 2 additional neurones. *B*, the inward current activated by NMDA plus glycine in an extracellular solution containing 70 mM Mg^{2+} and 31 mM NMDG was reversibly blocked by APV. The longer horizontal bar indicates application of 100 μM NMDA plus 10 μM glycine, and the shorter horizontal bar indicates application of 200 μM APV in combination with NMDA plus glycine. Similar results were obtained in 1 additional neurone, and the records in *A* and *B* were obtained from different neurones.

leakage. NMDA plus glycine also activated an inward current in the 0·9 mM Mg^{2+} solution when the patch pipette contained NMDG instead of Cs^+ (Fig. 6*A*). The amplitude of the inward current recorded in the 70 mM Mg^{2+} solution was much greater than the amplitude of the inward current recorded in the 0·9 mM Mg^{2+} solution. These results again suggest that the inward current is carried by Mg^{2+} and not by NMDG. The NMDA-activated inward current recorded in the 70 mM Mg^{2+} solution was reversibly blocked by APV (Fig. 6*B*). APV (200 μM) completely inhibited the current recorded under these conditions ($n = 2$). NMDA-activated currents in the 70 mM Mg^{2+} solution typically reversed around +15 mV, suggesting that we may not have fully washed out permeant intracellular ions. However, any outward currents at the voltages used for Figs 6 and 7 should have been minimal.

The inward currents shown in Fig. 6 are activated by NMDA and inhibited by APV, suggesting that they are mediated by flux through the channel of the NMDA receptor. To test this hypothesis further, we compared the magnitudes of the currents recorded in the control solution with those recorded in the Mg^{2+} solutions. For these comparisons, we used NMDG in the patch pipette. If the current measured under each condition is carried by the same population of receptors, then the ratio of the current measured in the Mg^{2+} solution to the current measured in the control solution should be constant from cell to cell. In accord with this prediction, a plot of the NMDA-activated currents recorded in the 70 mM Mg^{2+} solution as a function of the control currents measured in the same neuron revealed a close correlation (Fig. 7*A*). A linear regression fit to the data points yielded $r^2 = 0{\cdot}98$, and the correlation was statistically significant ($P < 0{\cdot}001$, Pearson correlation test). Since we were not able to fully compensate for series resistance, the current amplitudes for the largest recorded control currents may have been underestimated due to series resistance error. Therefore, the slope of the linear regression shown in Fig. 7*A* may be slightly underestimated. Nevertheless, the amplitude of the current recorded in the 70 mM Mg^{2+} solution was proportional to the amplitude of the current recorded in the control solution, indicating that Mg^{2+} and Na^+ both permeated the same population of NMDA-activated ion channels.

The amplitudes of the currents recorded at membrane potentials near −60 mV in each Mg^{2+} solution expressed as a fraction of the control current recorded in the same neurone are shown in Fig. 7*B*. In the presence of 70, 2 and 0·9 mM Mg^{2+}, the fractional current recorded in the Mg^{2+} solution

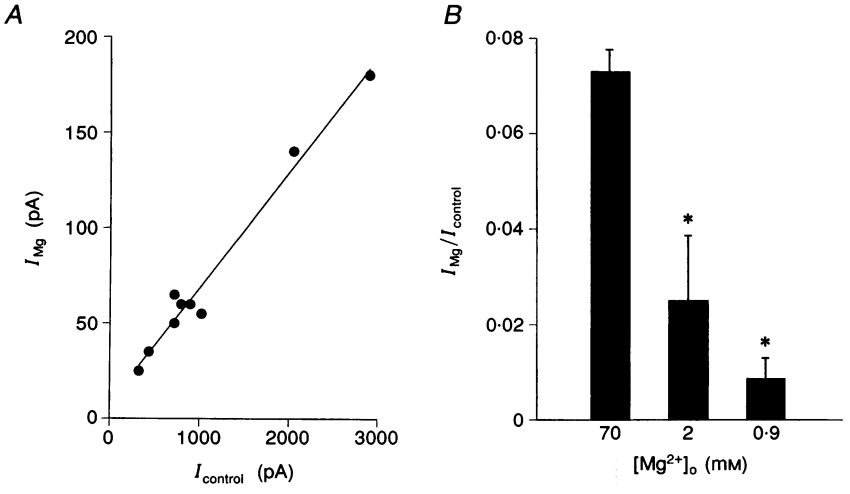

Figure 7. The NMDA-activated currents recorded in the Mg^{2+} solutions were proportional to the NMDA-activated currents recorded in the control solution

All currents were recorded with 130 mM NMDG in the patch pipette. *A*, the current recorded at a membrane potential of −63 mV in an extracellular solution containing 70 mM Mg^{2+}(I_{Mg}) was plotted against the current recorded in the control solution ($I_{control}$) at −57 mV in the same neurone. (The membrane potentials were corrected for liquid junction potentials.) Each symbol represents currents recorded from one neurone ($n = 9$). Linear regression fit to the data points yielded $r^2 = 0{\cdot}98$, and the correlation was statistically significant ($P < 0{\cdot}001$, Pearson correlation test). Due to the potential series resistance error for larger currents, the two largest values of $I_{control}$ may have been underestimated (see text). *B*, the currents measured in the Mg^{2+} solutions at a membrane potential of −63 mV were divided by the currents measured in the control solution at −57 mV in the same neurone. (The membrane potentials were corrected for liquid junction potentials.) Each point represents the mean ± S.E.M. from 9, 5 and 3 neurones, respectively. * The fractional currents calculated for the 2 mM Mg^{2+} and the 0·9 mM Mg^{2+} data points were significantly different from the fractional current calculated for the 70 mM Mg^{2+} data point ($P < 0{\cdot}05$, one-way ANOVA with Tukey's tests for *post hoc* comparisons).

was $0{\cdot}073 \pm 0{\cdot}005$ ($n = 9$), $0{\cdot}025 \pm 0{\cdot}014$ ($n = 5$), and $0{\cdot}0087 \pm 0{\cdot}0044$ ($n = 3$) (Fig. 7*B*). The fractional current was significantly reduced when $[Mg^{2+}]_o$ was reduced from 70 mM to 2 or 0·9 mM ($P < 0{\cdot}05$, one-way ANOVA with Tukey's tests for *post hoc* comparisons).

The results presented thus far indicate that Mg^{2+} ions can permeate the ion channel of the NMDA receptor. They also suggest that the glutamate-stimulated increase in $[Mg^{2+}]_i$ that occurs in the absence of Na_o^+ and Ca_o^{2+} is due to this influx pathway.

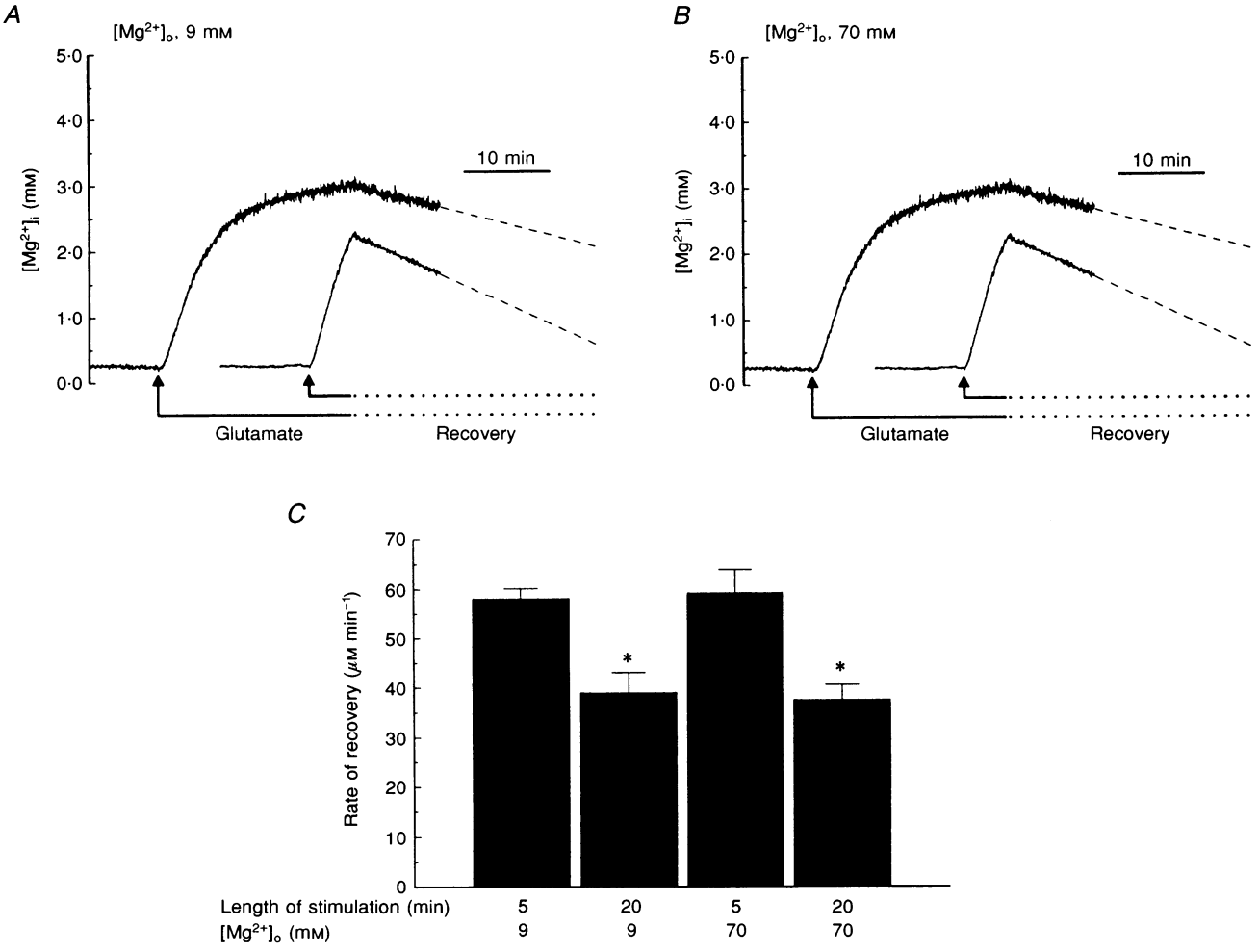

Figure 8. Recovery from a glutamate-induced Mg^{2+} load was significantly inhibited after longer stimulations

A, arrows denote the beginning of either a 20 min or 5 min stimulation with 100 μM glutamate + 1 μM glycine in a Na^+- and Ca^{2+}-free solution containing 9 mM Mg^{2+}. The 20 min and 5 min glutamate stimulations were performed in two different neurones but are depicted together for comparison. In addition, the times of glutamate removal are aligned to illustrate the differences in the rates of recovery. These traces are representative of results obtained in 14 and 120 additional neurones, respectively. Dashed lines represent extrapolations of the decrease in $[Mg^{2+}]_i$ based on the initial control rate of recovery determined as described in the Methods. *B*, arrows denote the beginning of either a 20 min or 5 min stimulation with 100 μM glutamate + 1 μM glycine in a Na^+- and Ca^{2+}-free solution containing 70 mM Mg^{2+}. The 20 min and 5 min glutamate stimulations were performed in two different neurones but are depicted together for comparison. In addition, the times of glutamate removal are aligned to illustrate the differences in the rates of recovery. These traces are representative of results obtained in 11 and 18 additional neurones, respectively. Dashed lines represent extrapolations of the decrease in $[Mg^{2+}]_i$ based on the initial control rate of recovery determined as described in the Methods. *C*, a summary of the data described in *A* and *B*. Each point represents the mean ± S.E.M. from 12 or more neurones. * Rate of recovery following a 20 min stimulation was significantly different from the rate of recovery following a 5 min stimulation in the same $[Mg^{2+}]_o$ ($P < 0{\cdot}05$, one-way ANOVA with Student's unpaired *t* tests for *post hoc* comparisons).

Mechanisms of Mg^{2+} efflux

We continued our study of glutamate-induced changes in $[Mg^{2+}]_i$ by investigating the mechanisms by which $[Mg^{2+}]_i$ returns to basal levels following glutamate stimulation. We were interested in determining whether the mechanisms of Mg^{2+} efflux in cultured neurones from fetal rats were similar to those described in other cell types. Using mag-fura-2 microfluorimetry, we measured the rate of decrease in $[Mg^{2+}]_i$ following removal of glutamate.

Rate of recovery is slower following longer glutamate stimulations

Representative traces illustrating recovery from glutamate-induced increases in $[Mg^{2+}]_i$ are shown in Fig. 8*A* and *B*. Neurones were stimulated with glutamate plus glycine for either 5 or 20 min in the presence of 9 mM Mg^{2+} (Fig. 8*A*) or 70 mM Mg^{2+} (Fig. 8*B*). The mean rate of recovery following a 5 min stimulation in the presence of 9 mM Mg^{2+} was not significantly different from the mean rate of recovery following a 5 min stimulation in the presence of 70 mM Mg^{2+} ($58{\cdot}1 \pm 2{\cdot}1$ μM min^{-1} compared with $59{\cdot}3 \pm 4{\cdot}6$ μM min^{-1}; $n = 121$ and 19, respectively; Fig. 8*C*). Similarly, the mean rate of recovery following a 20 min stimulation in the presence of 9 mM Mg^{2+} was not significantly different from the mean rate of recovery following a 20 min stimulation in the presence of 70 mM Mg^{2+} ($38{\cdot}9 \pm 4{\cdot}1$ μM min^{-1} compared with $37{\cdot}5 \pm 3{\cdot}1$ μM min^{-1}; $n = 15$ and 12, respectively; Fig. 8*C*). These results indicate that the rate of recovery was independent of the $[Mg^{2+}]_o$ during stimulation. However, the mean rate of recovery following a 20 min stimulation was significantly slower than the mean rate of recovery following a 5 min stimulation in the presence of either 9 mM Mg^{2+} (67% of 5 min control; Fig. 8*C*) or 70 mM Mg^{2+} (63% of 5 min control; Fig. 8*C*). These results suggest that longer stimulations somehow compromise the ability of neurones to extrude or sequester Mg^{2+}.

Changes in the perfusion solution alter the rate of recovery

In an attempt to determine the types of ion transporters present in neurones which contribute to Mg^{2+} efflux, we tested the effects of changing the ion composition of the perfusion solution on the rate of recovery from a glutamate-induced Mg^{2+} load. Immediately following glutamate stimulation, neurones were first perfused with control recovery solution for ~6 min, then perfused with the altered extracellular solution for ~6 min, and finally returned to the control perfusion solution (see Fig. 9). Complete removal of Na_o^+ significantly inhibited the rate of recovery following a standard 5 min glutamate stimulation (31% of control, Table 2 and Fig. 9). Increasing $[Mg^{2+}]_o$ from 0·9 mM to 9 or 30 mM also significantly inhibited recovery (55 and 36% of control, respectively, Table 2). However, the inhibition caused by 30 mM Mg^{2+} was not significantly different from the inhibition caused by removal of Na_o^+. The inhibition of recovery caused by the combination of 30 mM Mg^{2+} plus 0 mM Na^+ was not significantly greater than the inhibition caused by either manipulation alone. Recovery in a Na^+-free

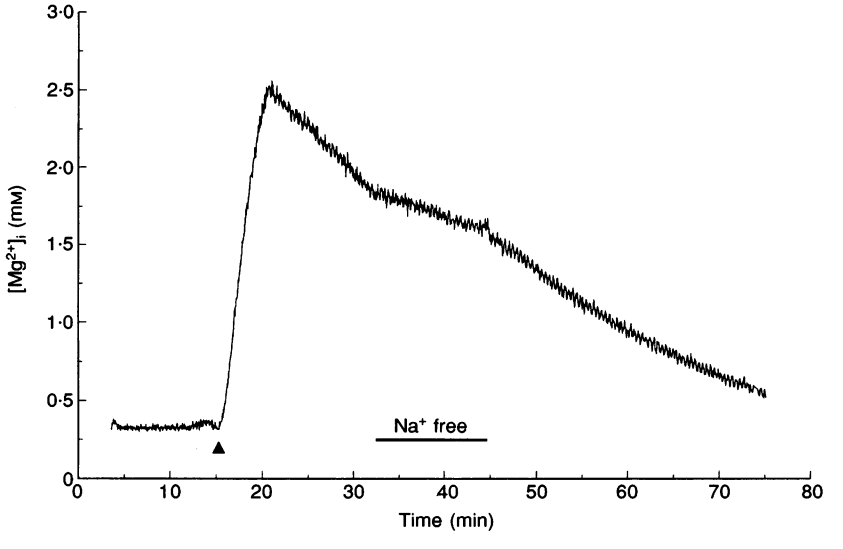

Figure 9. Removal of $[Na^+]_o$ significantly inhibited recovery from a glutamate-induced Mg^{2+} load

At the arrowhead, the neurone was stimulated with 100 μM glutamate + 1 μM glycine for 5 min in a Na^+- and Ca^{2+}-free solution containing 9 mM Mg^{2+}. After glutamate stimulation, the neurone was initially perfused with the control solution (Ca^{2+} free but Na^+ containing, see Methods) and then perfused with a Na^+- and Ca^{2+}-free solution which contained Tris as a Na^+ substitute. This trace is representative of results obtained in 6 additional neurones, and similar results were obtained in 4 additional neurones when NMDG was used as a Na^+ substitute.

Table 2. Changes in the composition of the perfusion solution decrease the rate of recovery from a glutamate-induced Mg^{2+} load

Extracellular solution composition	Control rate (μM min^{-1})	Treated rate (μM min^{-1})	Percentage of control
0 mM Na^+	53·9 ± 4·6	16·6 ± 3·4*	31
9 mM Mg^{2+}	55·5 ± 5·4	30·4 ± 2·9*	55
30 mM Mg^{2+}	45·2 ± 4·8	16·2 ± 2·6*	36
1·4 mM Ca^{2+}	62·8 ± 5·7	50·6 ± 6·1*	81
0 mM Na^+ + 9 mM Mg^{2+}	64·4 ± 6·6	16·8 ± 5·0*	26
0 mM Na^+ + 30 mM Mg^{2+}	66·8 ± 8·4	18·6 ± 4·1*	28
0 mM Na^+ + 1·4 mM Ca^{2+}	76·8 ± 7·2	3·9 ± 3·1*†	5
0 mM Na^+ + 0 mM Cl^-	67·2 ± 12·8	18·3 ± 3·6*	27
0 mM HCO_3^-	53·8 ± 4·3	50·9 ± 3·5	95

Rates of recovery were determined after a 5 min stimulation with 100 μM glutamate plus 1 μM glycine in a Na^+- and Ca^{2+}-free solution. Control rates were determined from the decrease in $[Mg^{2+}]_i$ within the 6 min period immediately following glutamate stimulation. For recovery experiments, the control perfusion solution contained 148·2 mM Na^+, 0 mM Ca^{2+}, 0·9 mM Mg^{2+}, 10 mM HCO_3^- and 142 mM Cl^- (see description of Ca^{2+}-free solution in Methods). There were no significant differences in the control rates ($P > 0·05$, one-way ANOVA). Treated rates were determined from the decrease in $[Mg^{2+}]_i$ within the 6 min perfusion with an altered extracellular solution that followed the 6 min perfusion with the control solution. There were significant differences in the treated rates ($P < 0·05$, one-way ANOVA). All values represent the mean ± S.E.M. from 8–13 neurones. * Treated rate was significantly different from the control rate ($P < 0·05$, Student's paired t test). † Treated rate was significantly different from treated rate for 0 mM Na^+ alone ($P < 0·05$, Bonferroni test).

solution was not altered by removal of extracellular Cl^- (Table 2). Removal of bicarbonate had no effect on the rate of recovery (Table 2). Addition of 1·4 mM Ca^{2+}caused a small but statistically significant decrease in the recovery rate (81% of control, Table 2), and the combination of 1·4 mM Ca^{2+} plus 0 mM Na^+ was significantly more effective at inhibiting recovery than 0 mM Na^+ alone. These results indicate that Na^+-, Mg^{2+}- and Ca^{2+}-sensitive efflux mechanisms contribute to recovery from a glutamate-induced Mg^{2+} load.

DISCUSSION

The principal findings of this study are as follows: (1) the channel of the NMDA receptor is permeable to Mg^{2+} in the absence of Na_o^+ and Ca_o^{2+}; (2) Mg^{2+} influx through NMDA-activated ion channels can cause significant increases in $[Mg^{2+}]_i$; and (3) the primary mechanism of recovery from a glutamate-induced Mg^{2+} load in neurones is via a process of Mg^{2+} efflux consistent with Na^+–Mg^{2+} exchange, in that it was dependent on $[Na^+]_o$ and was inhibited by elevated $[Mg^{2+}]_o$.

While the extracellular solutions used for glutamate stimulation in these studies are not physiological (Na^+ and Ca^{2+} free), these conditions allowed us to study Mg^{2+} homeostasis in the absence of potentially confounding changes in $[Ca^{2+}]_i$. Though the increases in $[Ca^{2+}]_i$ that occur upon glutamate stimulation in a Ca^{2+}-containing solution generally are not large enough to interfere with detection of $[Mg^{2+}]_i$ changes using mag-fura-2 (Brocard *et al.* 1993; Rajdev & Reynolds, 1993), it is possible that Ca^{2+} influx may alter the way in which a cell handles Mg^{2+}. There is evidence that Ca^{2+} and Mg^{2+} compete for intracellular binding sites (McLaughlin, Mulrine, Gresalfi, Vaio & McLaughlin, 1981; Murphy, Freudenrich, Levy, London & Lieberman, 1989; Brocard *et al.* 1993), supporting the hypothesis that some Ca^{2+} and Mg^{2+} homeostatic processes may overlap.

Mechanisms of Mg^{2+} influx

We have previously shown that the glutamate-stimulated increases in $[Mg^{2+}]_i$ that occur in the absence of Na_o^+ and Ca_o^{2+} are dependent on $[Mg^{2+}]_o$ (Brocard *et al.* 1993). In the present study, increasing $[Mg^{2+}]_o$ enhanced both the $[Mg^{2+}]_{1,5\ min}$ induced by a glutamate stimulation and enhanced the initial rate of rise in $[Mg^{2+}]_i$ upon glutamate stimulation. It was previously hypothesized that the Mg^{2+} influx that occurs upon glutamate stimulation in the absence of Na_o^+ and Ca_o^{2+} might occur via reverse Na^+–Mg^{2+} exchange (Brocard *et al.* 1993). However, the results of the present study disprove the Na^+–Mg^{2+} exchange hypothesis and are consistent with the more parsimonious explanation that Mg^{2+} influx occurs via the ion channel of the NMDA receptor. We used whole-cell patch-clamp experiments to confirm this hypothesis directly, and the results of the present study indicate that NMDA-activated ion channels are permeable to Mg^{2+} in the absence of other permeant

cations in the extracellular solution. Consistent with the results obtained in our electrophysiological studies, NMDA (in a Na^+- and Ca^{2+}-free solution) also increased $[Mg^{2+}]_i$ in a concentration-dependent manner (not shown).

The following calculation demonstrates that the Mg^{2+} currents measured in our patch-clamp experiments were of sufficient magnitude to explain the increases in $[Mg^{2+}]_i$ we observed in our fluorescence microscopy experiments. For the purposes of this calculation we assumed a constant current of 50 pA (see Fig. 7*A*) carried entirely by Mg^{2+} and assumed that there was no Mg^{2+} buffering during stimulation. Under these conditions, the $[Mg^{2+}]_i$ expected in a spherical neurone 20 μm in diameter after a 5 min stimulation in a Na^+- and Ca^{2+}-free solution containing 70 mM Mg^{2+} would be ~19 mM. However, in the present study, the $[Mg^{2+}]_{i,5\ min}$ measured under similar conditions was only ~3 mM (Table 1), suggesting that the majority of the Mg^{2+} entering the neurone is rapidly buffered (i.e. bound, sequestered or extruded) and does not contribute to the final $[Mg^{2+}]_i$ observed. These results are similar to those of studies which indicate that $[Ca^{2+}]_i$ does not accurately reflect total Ca^{2+} entry upon glutamate stimulation due to extensive intracellular buffering (Hartley, Kurth, Bjerkness, Weiss & Choi, 1993; Eimerl & Schramm, 1994; White & Reynolds, 1995) and is consistent with reports that Mg^{2+} and Ca^{2+} share intracellular binding sites (McLaughlin *et al.* 1981; Murphy *et al.* 1989; Brocard *et al.* 1993).

Similarly, one can calculate the initial rate of rise in $[Mg^{2+}]_i$ one might expect based on the results of our electrophysiological studies. Using the same assumptions as above, one would predict an initial rate of rise of ~4 mM min^{-1}, which is ~5-fold greater than the observed rate (Table 1). These calculations provide further evidence that a large percentage of the Mg^{2+} entering the cell during stimulation is rapidly buffered. Alternatively, it is possible that the amplitude of the Mg^{2+} current may not be constant over the entire time course of a 5 min glutamate stimulation due to receptor desensitization. Mg^{2+} influx then would not be as great as we assumed above, and this also could explain the discrepancies between the predicted and the observed $[Mg^{2+}]_{i,5\ min}$ values and initial rates of rise. The differences between the predicted and the observed values are probably not due to a reduction of Mg^{2+} influx in the mag-fura-2 experiments compared with the patch-clamp experiments due to competition with the low concentrations of K^+ (5·6 mM) present in the extracellular solution used in the mag-fura-2 experiments, since preliminary results indicate that glutamate-stimulated increases in $[Mg^{2+}]_i$ in a Na^+-, Ca^{2+}- and K^+-free solution are not significantly different from those observed in a Na^+- and Ca^{2+}-free solution (not shown). It should be noted that the membrane potential in the fluorescence experiments is unknown and that this may hamper direct comparison of the results from the two types of studies.

While previous studies have shown that Mg^{2+} blocks NMDA-activated inward currents, these studies were done using Na^+-containing solutions (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Mayer, Westbrook & Guthrie, 1984; Mayer & Westbrook, 1987; Ascher & Nowak, 1988). The Mg^{2+} blockade described in these previous reports may in part reflect very slow permeation of Mg^{2+} in the presence of Na_o^+ and Ca_o^{2+}. Interestingly, Mayer & Westbrook (1987) reported that Mg^{2+} may permeate NMDA receptor channels in the presence of Na_o^+ when the membrane potential is extremely hyperpolarized (−150 to −200 mV), and mutant NMDA receptors that are Mg^{2+} permeable have been constructed (Burnashev *et al.* 1992).

In whole-cell patch-clamp experiments using Cs^+ in the patch pipette, we have also measured NMDA-activated inward currents at negative membrane potentials in the presence of an extracellular solution which contained 91 mM $MgCl_2$ and 0 mM NMDG (not shown). These currents were comparable in size to those recorded in the 70 mM Mg^{2+}–31 mM NMDG solution (Fig. 5). In contrast, Burnashev *et al.* (1992) reported no measurable NMDA-induced currents in a 110 mM $MgCl_2$ solution in human embryonic kidney cells transfected with the NMDA receptor subunits NR1 and NR2A or NR1 and NR2C. The electrophysiological results obtained in the 91 mM Mg^{2+} solution, in combination with the fluorescence microscopy data obtained using sucrose as a Na^+ substitute, indicate that the Mg^{2+} currents we have observed are not dependent on the use of NMDG as the Na^+ substitute. While NMDG has been reported to be a low-affinity NMDA channel blocker (Villarroel, Burnashev & Sakmann, 1995), NMDG did not significantly block glutamate-stimulated increases in $[Mg^{2+}]_i$ or Mg^{2+} currents in this study.

The data presented here suggest that the interaction between Mg^{2+} ions and the channel of the NMDA receptor in the (Na^+- and Ca^{2+}-free) Mg^{2+} solutions is fundamentally different from their interaction in the (Na^+- and Ca^{2+}-containing) control solution. In the 70 mM Mg^{2+} solution, the NMDA-activated current was on average 0·073 times as large as the current measured in the control solution (see Fig. 7*B*). We can use these data to estimate single-channel current in the 70 mM Mg^{2+} solution. In the control solution, NMDA-activated single-channel current is typically ~2·8 pA at −55 mV (Nowak *et al.* 1984). Assuming that the open probability is the same in both the control and in the Mg^{2+} solutions, single-channel current in the 70 mM Mg^{2+} solution would be ~0·2 pA. This corresponds to a flux of Mg^{2+} of ~$6{\cdot}4 \times 10^5$ ions s^{-1}. To achieve this rate of flux, each Mg^{2+} ion could have a maximum residence time in the channel of only 1·6 μs. However, in normal NaCl-based solutions, the unbinding rate of Mg^{2+} from the NMDA-activated channel is ~1100–1800 s^{-1} (Ascher & Nowak, 1988; Jahr & Stevens, 1990), corresponding to a mean

residence time of 560–910 μs. Clearly, the typical residence time of Mg^{2+} in the channel must be much lower in the 70 mM Mg^{2+} solution than in the NaCl-based control solutions used in the previous studies. While the assumption that the open probability is identical in the control and the Mg^{2+} solutions may be in error, it is unlikely that the error could be large enough to explain the discrepancy in Mg^{2+} residence times calculated here. One possible explanation is that the influx of a Mg^{2+} ion bound in the NMDA-activated channel can be 'enhanced' by the binding of a second Mg^{2+} ion. A similar idea has been proposed to explain the high flux of Ca^{2+} through voltage-activated Ca^{2+} channels (Hess & Tsien, 1984). An alternative explanation for the very low maximum residence time calculated here is that there is a small subpopulation of Mg^{2+}-permeable NMDA receptors that carry the Mg^{2+} current. However, the data shown in Fig. 7*A* argue against this explanation.

Measurements of $[Na^+]_i$ in neurones

The depletion of $[Na^+]_i$ upon exposure to a Na^+-free solution containing NMDG has previously been reported in studies of parietal and chief cells from rabbit gastric glands (Negulescu, Harootunian, Tsien & Machen, 1990). The results of the present study confirm these findings in neurones and demonstrate similar effects with choline and Tris. The depletion of $[Na^+]_i$ that occurs upon exposure to a Na^+-free solution has been proposed to be due to the continuous activity of the Na^+–K^+-ATPase as well as passive efflux (Negulescu *et al.* 1990).

Similarly, the glutamate-induced increases in $[Na^+]_i$ observed in the present study are consistent with previous results obtained in cerebellar granule cells (Kiedrowski, Brooker, Costa & Wroblewski, 1994*a*; Kiedrowski, Wroblewski & Costa, 1994*b*). The relatively large increases in $[Na^+]_i$ that occur upon brief stimulations with non-toxic concentrations of glutamate and the slow recovery of $[Na^+]_i$ following stimulation may have implications for Mg^{2+} homeostasis *in vivo*. Glutamate-induced increases in $[Na^+]_i$ could inhibit subsequent Mg^{2+} efflux via Na^+–Mg^{2+} exchange and consequently decrease recovery of $[Mg^{2+}]_i$ following glutamate receptor activation *in vivo*.

Mechanisms of Mg^{2+} efflux

Na^+–Mg^{2+} antiport driven by the Na^+ gradient has been described in a variety of cell types (for reviews see Flatman, 1991; Murphy *et al.* 1991; Romani & Scarpa, 1992; Günther, 1993). Since a specific inhibitor of the Na^+–Mg^{2+} antiporter is not yet available, the most effective way to manipulate Na^+–Mg^{2+} exchange is to alter Na^+ and/or Mg^{2+} gradients. The results from the current study are consistent with the hypothesis that a Na^+–Mg^{2+} exchanger operates in cultured forebrain neurones from fetal rats. Moreover, Mg^{2+} efflux via Na^+–Mg^{2+} exchange appears to be an important mechanism of recovery from a glutamate-induced Mg^{2+} load in these cells, since removing Na^+_o or elevating $[Mg^{2+}]_o$ decreased the rate of recovery to approximately one-third of the control rate. In contrast, there do not appear to be any additional Na^+-independent, Mg^{2+}-gradient-sensitive Mg^{2+} carriers or channels involved in Mg^{2+} efflux, since increasing $[Mg^{2+}]_o$ in the absence of Na^+_o did not inhibit recovery significantly more than Na^+ removal alone.

The rate of recovery from a glutamate-induced Mg^{2+} load was decreased in the presence of $[Ca^{2+}]_o$, suggesting the existence of a Ca^{2+}-inhibited Mg^{2+} extrusion pathway which has not been described previously. The results of the present study do not support the existence of the Na^+-independent Mg^{2+} efflux pathway sensitive to inhibition by Cl^- previously described in erythrocytes (Günther & Vormann, 1989, 1990), since removing Cl^- from the extracellular solution did not enhance the rate of recovery in a Na^+-free solution. The residual recovery that could not be inhibited by manipulation of the ion composition of the perfusion solution may involve either intracellular sequestration or energy-dependent Mg^{2+} efflux pumps that are less sensitive to $[Mg^{2+}]_o$. Mg^{2+}-ATPases involved in Mg^{2+} influx have been cloned from *Salmonella typhimurium*, and one of these proteins is homologous to a rat sarcoplasmic reticulum Ca^{2+}-ATPase (Smith & Maguire, 1993). It is possible that similar proteins in neurones may remove Mg^{2+} from the cytoplasm via extrusion across the plasma membrane or uptake into the endoplasmic reticulum.

The results of the current study also suggest that longer glutamate stimulations somehow compromise the ability of neurones to extrude or sequester Mg^{2+}. One might expect that the rate of recovery of $[Mg^{2+}]_i$ would be greater following longer stimulations, since the longer exposures to a Na^+-free solution associated with the longer glutamate stimulations should deplete $[Na^+]_i$ to a greater extent and enhance the rate of Na^+–Mg^{2+} exchange when the neurones are returned to a Na^+-containing solution following stimulation. However, our results do not confirm this hypothesis. Instead, the decrease in the rate of recovery following longer stimulations may be related to the greater total Mg^{2+} load imposed by longer stimulations. Similarly, the rate of recovery does not correlate with the magnitude of the glutamate-induced increase in $[Mg^{2+}]_i$ prior to the start of recovery. While the increases in $[Mg^{2+}]_i$ produced by either a 5 min stimulation in 70 mM Mg^{2+} or a 20 min stimulation in 9 mM Mg^{2+} were equivalent (Table 1), the rates of recovery following these stimulations were significantly different (Fig. 8), again suggesting that the rate of recovery is dependent on the total Mg^{2+} load. As discussed above, the increase in $[Mg^{2+}]_i$ that occurs upon glutamate stimulation in a Na^+- and Ca^{2+}-free solution may not accurately reflect the total amount of Mg^{2+} that enters a cell, yet it is not obvious how large amounts of Mg^{2+} which are bound or sequestered inside the cell during longer stimulations might inhibit recovery, since the primary mechanism of recovery in these neurones appears to be

Na^+–Mg^{2+} exchange. However, we did not determine directly whether the recovery mechanisms following a 20 min stimulation are the same as those in effect following a 5 min stimulation. One possible explanation of the data is that the large amount of Mg^{2+} that is presumably sequestered or buffered during the long glutamate stimulations is slowly released into the cytoplasm during recovery to cause an apparent decrease in the rate of recovery of $[Mg^{2+}]_i$.

Role of Mg^{2+} homeostasis in excitotoxicity

While glutamate is the predominant excitatory neurotransmitter in mammalian brain (Di Chiara & Gessa, 1981), glutamate can also be toxic to neurones (Olney, 1969). It has been shown previously that glutamate exposures which trigger excitotoxicity in cultured neurones (100 μM for 5 min; Choi, Maulucci-Gedde & Kriegstein, 1987) can also produce large increases in $[Mg^{2+}]_i$ in these cells (Brocard *et al.* 1993), raising the interesting possibility that elevated $[Mg^{2+}]_i$ may play a role in excitotoxic cell death. While part of the tissue damage that occurs upon traumatic brain injury or ischaemia is thought to be due to the release of excitatory amino acids (Simon, Swan, Griffiths & Meldrum, 1984; Faden, Demediuk, Panter & Vink, 1989), several whole animal studies indicate that $[Mg^{2+}]_i$ and total brain Mg^{2+} levels are significantly reduced following a traumatic or ischaemic brain injury (Vink, McIntosh, Demediuk & Faden, 1987; Vink, McIntosh, Demediuk, Weiner & Faden, 1988; Vande Linde *et al.* 1991). This apparent discrepancy may be resolved by considering the different time points examined in these two types of studies. The increase in $[Mg^{2+}]_i$ observed in the fluorescence microscopy experiments occurred simultaneous to an excitotoxic glutamate application, while the decreases in $[Mg^{2+}]_i$ and total cellular Mg^{2+} content observed in the whole animal studies occurred several hours following injury. Thus initial glutamate-stimulated increases in $[Mg^{2+}]_i$ may reflect Mg^{2+} mobilization which precipitates eventual depletion of cellular Mg^{2+} (i.e. the Ca^{2+}-dependent displacement of intracellularly bound Mg^{2+} that occurs upon glutamate receptor activation may lead to extrusion of Mg^{2+} from the cell via Na^+–Mg^{2+} exchange). It is possible that the subsequent decreases in total cellular Mg^{2+} content and $[Mg^{2+}]_i$ may ultimately be what is detrimental to neurones. If loss of intracellular Mg^{2+} subsequent to glutamate receptor activation turns out to be an important component of excitotoxic cell death, then compounds which inhibit Mg^{2+} efflux by blocking Na^+–Mg^{2+} exchange might be useful clinically. The experimental protocol presented here (recovery from a glutamate-stimulated increase in $[Mg^{2+}]_i$) could easily be used to identify compounds which modulate Na^+–Mg^{2+} exchange.

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