Journal of Physiology (1989), **414**, *pp.* 17–34
With 10 *text-figures*
Printed in Great Britain

REGULATION OF *N*-METHYL-D-ASPARTATE RECEPTORS REVEALED BY INTRACELLULAR DIALYSIS OF MURINE NEURONES IN CULTURE

BY J. F. MACDONALD, I. MODY* AND M. W. SALTER

From the Playfair Neurosciences Unit, Toronto Western Hospital and the Department of Physiology, University of Toronto, Toronto, Ontario, Canada M5T 2S8

(*Received 21 November 1988*)

SUMMARY

1. The whole-cell patch clamp recording technique was employed to investigate the intracellular regulation of *N*-methyl-D-aspartate (NMDA) receptors in cultured murine hippocampal neurones. Excitatory amino acids were repeatedly applied at regular intervals during intracellular dialysis with solutions of various composition.

2. Currents evoked by L-aspartate, an agonist of NMDA receptors, gradually 'washed out' to approximately 50% of their initial amplitude during dialysis with an intracellular solution containing CsCl and EGTA as a calcium buffer. In contrast, responses to kainate did not wash out. The wash-out of L-aspartate currents followed an exponential time course with a time constant of about 150 s. Wash-out did not appear to be related to desensitization of NMDA receptors.

3. Following wash-out, L-aspartate responses were blocked by Mg^{2+}, ketamine or D-2-amino-5-phosphonovalerate indicating that these responses were still mediated by NMDA receptors. Furthermore, responses to NMDA itself showed wash-out to the same extent and with a time course similar to that for L-aspartate responses.

4. Neither the time course nor the extent of the wash-out of responses to L-aspartate was affected when the Ca^{2+} concentration of the dialysate was varied from zero to $1{\cdot}5 \times 10^{-5}$ M. In addition, wash-out was unaffected by substitution of BAPTA for EGTA, indicating that wash-out was not a consequence of changes in intracellular pH related to the binding of Ca^{2+} to the buffer or to the kinetics of this binding. Therefore, the wash-out of NMDA currents could not be attributed to a gradual elevation of the concentration of intracellular Ca^{2+}.

5. The extent of the wash-out of L-aspartate currents was similar for cells held at +40 *versus* −60 mV although the rate of wash-out was slower at the depolarized potential. In addition, the reversal potential of these currents was not altered, demonstrating that a change in driving force did not account for a component of the wash-out.

6. Inclusion of an ATP regeneration solution (Forscher & Oxford, 1985) in the dialysate prevented the wash-out of L-aspartate currents. ATP alone was less effective in preventing wash-out whereas phosphocreatine and creatine phosphokinase were ineffective by themselves. Wash-out also occurred when ATP was replaced with the non-hydrolysable analogue, β,γ-methyleneATP, or with GTP. In

* Present address: Department of Neurology, Stanford University Medical Center, Stanford, CA 94305-5235, USA.

cells where wash-out of L-aspartate currents had been established, subsequent dialysis with the ATP regenerating solution partially reversed this wash-out.

7. These observations are consistent with the hypothesis that the NMDA channel may be regulated by rephosphorylation/dephosphorylation. Phosphorylation of the channel itself, or of some related protein, appears to be required to maintain the functional state of the NMDA channel.

INTRODUCTION

L-Glutamate and L-aspartate are major excitatory transmitters within the vertebrate central nervous system (CNS). Ionophoretic applications of L-glutamate to *in vivo* CNS neurones generate rapid and powerful excitations which also recover rapidly compared to other excitants such as acetylcholine (Krnjević & Phillis, 1963). This suggested that these transmitters might function for the rapid and invariant transfer of excitation between neurones. However, transmission by excitatory amino acids appears to be more complex than was first believed. For example, multiple subtypes of postsynaptic receptors have been identified and at least one of these, the *N*-methyl-D-aspartate (NMDA) receptor, has also been implicated in the establishment of long-lasting functional (Collingridge & Bliss, 1987; Cotman & Iversen, 1987; Kauer, Malenka & Nicoll, 1988; Mody, Stanton & Heinemann, 1988*a*) and morphological plasticity (Cotman & Iversen, 1987; Pearce, Cambray-Deakin & Burgoyne, 1987; Balazs, Hack & Jorgensen, 1988; Mattson, Dou & Kater, 1988) within the mammalian CNS.

Plastic changes in the CNS associated with NMDA receptors may involve modulation of the concentration of free intracellular calcium ($[Ca^{2+}]_i$). For example, activation of NMDA receptors causes an increase of $[Ca^{2+}]_i$ (Mayer, MacDermott, Westbrook, Smith & Barker, 1987; Morris, Friedlich & MacDonald, 1987; Murphy, Thayer & Miller, 1987) and this may in turn lead to a cascade of alterations of cellular metabolism. The increase could be a consequence of calcium flux directly through these channels (Mayer & Westbrook 1987*a*, *b*; Murphy *et al.* 1987). However, the actual degree of calcium permeability of NMDA channels is still controversial (Mayer & Westbrook, 1987*a*; Ascher & Nowak, 1988; Huettner, 1988; Vyklicky, Krusek & Edwards, 1988) and recent evidence indicates that activation of NMDA receptors might promote the release of calcium from intracellular stores (Mody, MacDonald & Baimbridge, 1988*a*).

Elevations of $[Ca^{2+}]_i$ are also associated with a depression of NMDA currents (Mayer *et al.* 1987) suggesting that NMDA receptors may themselves be subject to intracellular regulation. Using internally dialysed neurones we have examined the possible intracellular regulation of NMDA receptors by $[Ca^{2+}]_i$ or by dephosphorylation/rephosphorylation mechanisms which required a supply of high-energy phosphates. A brief report of some of these results has appeared elsewhere (Mody, Salter & MacDonald, 1988*b*).

METHODS

Hippocampal cultures

Fetal mice (embryonic day 18) were obtained from time-pregnant Swiss White mice (Toronto Western Hospital Vivarium) which were killed by cervical dislocations prior to removal of the fetuses. The hippocampus was microdissected from each of approximately seven fetuses and pooled

prior to dissociation. No enzymatic treatment of the tissue was employed. Instead, cells were mechanically dissociated by trituration with a series of two Pasteur pipettes (tip diameters about 500 and 150–200 μm). The dissection and dissociation were performed in cooled Hanks' solution and containers were kept on ice until the cells were plated in 35 mm collagen-coated culture dishes. Cells were plated at densities below 1×10^6 ml^{-1} and were grown in dissociated tissue culture using standard techniques (MacDonald & Wojtowicz, 1982; MacDonald, Miljkovic & Pennefather, 1987). After 2 weeks in culture hippocampal neurones formed a monolayer and were suitable for electrophysiological recordings.

Recording conditions

For recording, each culture dish was thoroughly washed with extracellular solution (in mM: 140 NaCl, 1·3 $CaCl_2$, 5·4 KCl, 25 *N*-2-hydroxyethlpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 33 glucose, pH 7·4, osmolarity of final solution 320–335 mosM, and containing tetrodotoxin (Sigma) at a concentration of 1–3 μM) and placed in a recording chamber which permitted individual neuronal somata to be observed under phase-contrast microscopy. In the majority of experiments this extracellular solution also contained 1 μM-glycine (Sigma). Glycine was added at this concentration in order to raise it to a value that would give a maximal potentiation of the NMDA response (Johnson & Ascher, 1987). This meant that variations in the amount of glycine released by the cultures themselves could not cause fluctuations in the amplitude of NMDA currents. In several experiments glycine was not added to the extracellular solution; this had no effect on the present results.

Electrodes

Patch electrodes were used for recording in the whole-cell configuration. The electrodes were constructed from borosilicate thin-walled glass (o.d. 1·5 mm, TW150F-4, WPI) which contained a filament to facilitate filing. A Narashige PP88 vertical puller was used to form electrodes of an approximately uniform tip diameter (1·5–2 μm) and shape. Each electrode was then fire-polished and a concerted effort was made to keep the electrode tip diameter and geometry as consistent as possible. Once filled with one of the intracellular solutions electrodes had resistances of 3–4 MΩ. Filled electrodes were dipped in Sigmacote (Sigma) which eliminated the meniscus between the electrode and the surface of the extracellular solution and thus improved visibility and the capacitive properties of the recording electrode. Omitting Sigmacote had no effect on the time-dependent changes presently reported.

Solutions for intracellular dialysis

The intracellular solution contained (in mM): 140 CsCl or KCl, 10 HEPES, 11 ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) and 35 mM CsOH or KOH used to dissolve the EGTA; in some experiments 1 mM-$CaCl_2$ 1–2 mM-$MgCl_2$ or 2 mM-tetraethylammonium chloride (TEA) were added. All intracellular solutions had a pH of 7·3. In many experiments an ATP regenerating system (Forscher & Oxford, 1985) containing (in mM): 2 TEA, 2 $MgCl_2$, 4 Tris-ATP, 20 phosphocreatine and 50 U ml^{-1} creatine phosphokinase (all from Sigma) was included. In these experiments the osmotic balance of the solution was maintained by decreasing CsCl or KCl to 120 mM. To avoid degradation of labile components in the solutions they were either made freshly just prior to the beginning of the experiment or were aliquoted into smaller volumes and frozen until they were used. After 4–6 h the solution was then discarded. In some experiments ATP was replaced with an equimolar amount of β,γ-methyleneATP (Sigma), an analogue which is resistant to enzymatic hydrolysis (Yount, 1975) or with 500 μM-GTP (Sigma).

In one series of experiments the calcium-buffering capacity of the intracellular solution was systematically varied by changing the Ca^{2+}: EGTA ratio. In these cases the free concentrations of calcium in the recording solutions were calculated using a computer program (Stockbridge, 1987). The following pK values for the stability constants were used: $HEGTA^{3-}$, 9·19; H_2EGTA^{2-}, 8·66, H_3EGTA^-, 2·57; H_4EGTA, 1·96; $MgEGTA^{2-}$, 5·21; $MgHEGTA^-$, 7·62; $CaEGTA^{2-}$, 10·97; $CaHEGTA^-$, 3·79. Substantial shifts in the pH of the intracellular solution occurred when the EGTA and calcium concentrations were varied. Therefore, just prior to use the pH of each solution was adjusted, with HCl or CsOH as appropriate. In other experiments, the pH-insensitive calcium buffer 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA, Sigma) (Tsien, 1980) was substituted for EGTA.

In experiments designed to determine if the wash-out of NMDA currents could be reversed we

filled recording electrodes twice, each time with a different solution. For example, an electrode was filled first with a solution lacking the ATP regenerating solution. Because of the filament in the electrode this solution was immediately transported to the electrode tip by capillary flow. The electrode was then flushed with a solution containing the ATP regenerating solution which removed the bulk of the first solution. The first solution, however, remained in the shank and tip of the electrode and a period of approximately 10–15 min was required for the second solution to diffuse to the tip. The approximate time required for this diffusion was determined by including Trypan Blue in the second solution. When electrodes were double-filled in this fashion recording was made within 2 min after flushing the electrode.

Single-electrode voltage clamp

Recordings were made at room temperature (21–23 °C) using a single-electrode voltage clamp switching between hold and current injection at frequencies of 20–28 kHz (Axoclamp 2A) and therefore series resistance compensation was not required. Care was taken to ensure adequate capacitive settling of the clamp during the response to excitatory amino acids. In a few experiments a non-switching patch clamp with series resistance compensation (Axopatch 1A) was employed but this had no effect on the outcome of the experiments.

The recording of current and voltage as well as the application of excitatory amino acids was controlled via an aquisition program (EXPS; NIH, Laboratory of Neurophysiology) running on a LSI-11 23 computer. Data were recorded continuously on a Gould Brush Pen recorder and also were routinely sampled (2·5 ms/point) and stored on hard disk for analysis.

Cells included in this study were recorded for extended periods ranging from 20 min to 5 h and did not demonstrate swelling or other gross morphological changes during the recording period. Input resistance ranged from 80 to 400 MΩ and little or no change in leakage resistance was detected in these cells even after several hours of recording. Cells which failed to meet these criteria were not included.

Application of amino acids and drugs

Excitatory amino acids (L-aspartate, 100–250 μM; kainate, 50–100 μM; or NMDA, 250–500 μM; all as sodium salts from Sigma) were dissolved in the extracellular solution and applied by pressure (Picospritzer II, 10–150 kPa) from glass micropipettes with tips located approximately 20–50 μm from the soma under study. Exchanging the entire volume of the bath several times at any stage of the experiments had no influence on the size of the recorded currents. Using this technique and pressure applications of 10–50 ms we could routinely evoke highly reproducible L-aspartate currents that peaked within 50–200 ms and recovered fully by 1–2 s. Cells and pressure pipettes which demonstrated slower L-aspartate kinetics were not used for the present study. These brief applications of amino acid did not result in a detectable accumulation of amino acid because the injected volume was small (several picolitres) relative to bath volume (about 1 ml). Active uptake of L-aspartate by the cells of the culture may have also limited any possible accumulation. Excitatory amino acids were applied at low frequencies (either 0·034 or 0·025 Hz) to minimize desensitization.

Each dish could be continuously perfused with the extracellular solution. However, this was not usually done because the non-laminar flow of solution greatly reduced the reproducibility of amino acid responses, thus in most cases recordings were made in a static bath. To apply ketamine (5–20 μM; Park Davis), D-2-amino-5-phosphonovaleric acid (APV) (50 μM; Cambridge) and Mg^{2+} (500 μM), which were dissolved in the extracellular solution, the entire volume of the culture dish was exchanged several times. In the absence of drugs, such exchange had no influence on the size of the recorded currents. In some experiments a rapid laminar flow system was used to change the extracellular solution.

Data analysis

To compare responses between different cells the peak amplitude of inward current at any given time was normalized with respect to amplitude of the first response. Population results are expressed as mean ± S.E.M. Smooth curves were calculated by a non-linear least-squares analysis using the Simplex method.

RESULTS

In these and previous experiments we employed L-aspartate to activate NMDA receptors. When similar application protocols to those employed here were previously used (MacDonald *et al.* 1987) it was possible to record highly reproducible currents. In some experiments, however, a very slow decline of the L-aspartate currents with a time constant of about 40 min could be observed for which deterioration the

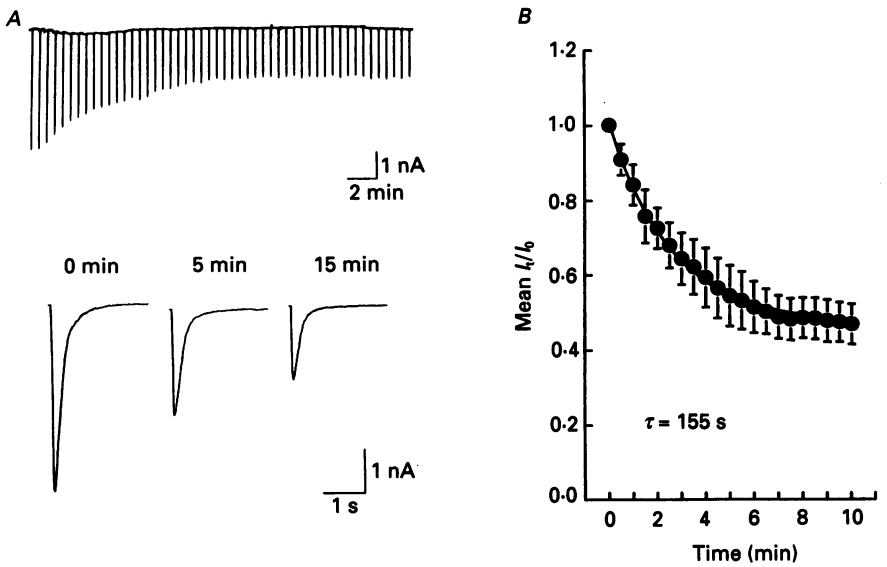

Fig. 1. Wash-out of inward currents evoked by L-aspartate when the intracellular solution does not contain high-energy phosphates. *A*, responses to pressure applications of L-aspartate (250 μM, 20 ms, 130 kPa, every 30 s) are shown in the continuous pen recorder trace of membrane current in the upper record. The holding potential in this and subsequent figures was −60 mV except where otherwise indicated. In the lower record individual responses taken at the times indicated above the traces are shown with an expanded time scale. Note the gradual and progressive decrease in the peak amplitude of the responses during the first 10 min of recording. Thereafter, the responses were stable at approximately 40% of the initial amplitude. *B*, in the graph the mean of the ratio, I_t/I_0, the peak amplitude of the L-aspartate-induced current at time t relative to the initial amplitude at $t = 0$, is plotted for six neurones (error bars show S.E.M.). The continuous line shows the single exponential best fitted to the means; the time constant (τ) is 155 s. In all cases the intracellular solution contained (in mM): 140 CsCl, 35 CsOH, 10 HEPES, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$ and 2 TEA.

recordings could not be held responsible (J. F. MacDonald, unpublished observations). The whole-cell patch electrodes employed in those experiments had tip diameters well below 1 μm and had resistances in the 7–10 MΩ range and as such were unlikely to have been very effective at dialysing the inside of cells (Pusch & Neher, 1988). In the present experiments the tip diameter of the electrodes was over 1 μm and they had resistances of 3–4 MΩ. Using these large-tipped electrodes it became apparent that L-aspartate currents rapidly declined over the first 10–15 min of recording, finally stabilizing at about 50% of the initial value (see Fig. 1). In the

same cells voltage-dependent calcium currents also washed-out completely in about the same time required for the partial wash-out of L-aspartate currents (Fig. 2 and Mody *et al.* 1988*b*). The wash-out of the L-aspartate currents was consistent from cell to cell provided the quality of the recordings was high. Furthermore, when KCl was substituted for CsCl in the recording electrodes a similar degree of wash-out of L-aspartate currents was observed ($66 \pm 9\%$ of initial level 10 min after the first response; $n = 5$).

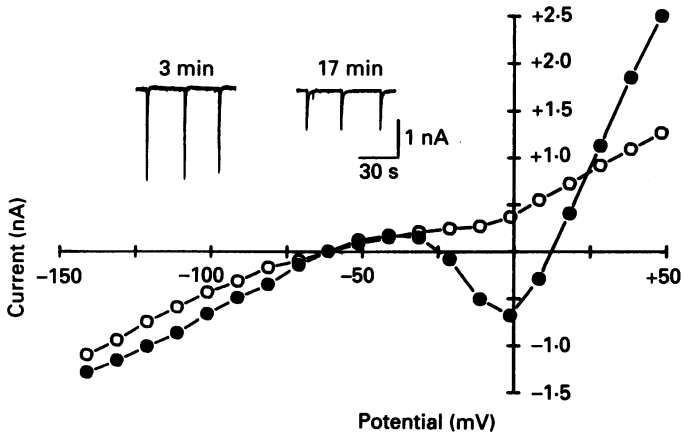

Fig. 2. Whole-cell current-voltage relationships of voltage-activated Ca^{2+} current before and after wash-out. One minute (●) and 12 min (○) after the start of the recording, a series of voltage steps, 100 ms in duration, was made from the holding potential of −60 mV. The peak inward current, subtracted from the baseline, is plotted *versus* the potential during the step. This was a voltage-dependent calcium current as it was appropriately sensitive to changes in extracellular Ca^{2+} and was blocked by low concentrations of Cd^{2+} (100 μM). The leak conductance (calculated from the step to −70 mV) decreased from 14·5 to 9·7 nS. Note also the marked decline in the voltage-dependent Ca^{2+} current. The insets are current traces showing three responses to L-aspartate immediately after each series of voltage steps. The intracellular solution contained (in μM): 140 CsCl, 35 CsOH, 10 HEPES, 11 EGTA and 2 $CaCl_2$.

A gradual increase in leak conductance might have been responsible for the decline in size of the L-aspartate responses. This was not the case, however, as neither the current required to hold the cell at −60 mV (Fig. 1*A*) nor the slope conductance (Fig. 2) increased during the course of the wash-out. In fact, Fig. 2 illustrates that the slope conductance generally decreased with time even though voltage-activated calcium currents washed out.

In order to quantitate the time course of wash-out, possible sources of variation were reduced by making recordings from cells of similar size (15–20 μm) derived from a relatively small number of dissections. In addition, the time between break-through of the patch and the first amino acid application was minimized, never exceeding 2 min. In total, eighty recordings were made in this way; all of the cells were held at −60 mV and L-aspartate applications were made every 30 s. The amplitude of the initial inward L-aspartate currents ranged from 1 to 10 nA but most currents were greater than 2 nA.

Figure 1*B* illustrates that the responses to L-aspartate declined to $47 \pm 5\%$ of their initial amplitude after 10 min of recording. The final current amplitude was

maintained throughout the remainder of the recording period which in some cases was as long as 2 h. Figure 1*B* also shows that the rate of wash-out was well fitted by a single exponential with a time constant of 155 s. To determine whether the kinetics (time to peak, rate of decay) of the responses was altered, individual responses were compared before and after wash-out (Fig. 1*A*). By examining a large number of cells, it was found that there was no consistent change in the kinetics of the individual currents.

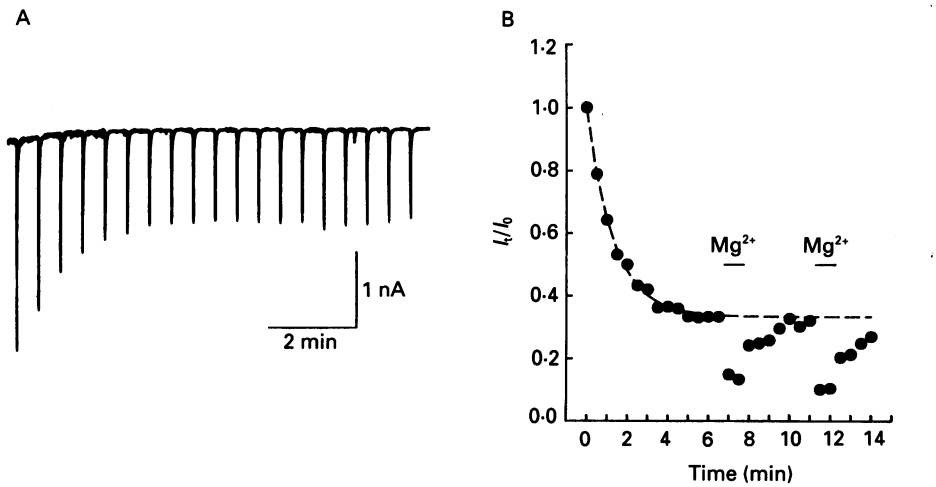

Fig. 3. Wash-out of NMDA responses. *A*, the current trace shows that the amplitude of responses to pressure applications of NMDA (200 μM, 20 ms, 130 kPa) gradually declined and then stabilized at 46% of the initial level. The intracellular solution contained (in μM): 140 CsCl, 35 CsOH, 10 HEPES, 11 EGTA, 0 $CaCl_2$, 2 $MgCl_2$ and 2 TEA. *B*, I_t/I_0 is plotted for the responses of another neurone to applications of L-aspartate (250 μM). In this case the culture dish was continuously perfused with extracellular solution. The perfusate contained $MgCl_2$ (1 mM) during the periods shown by the lines above the data points. During the perfusion with this solution the responses to L-aspartate were depressed by 33% and there was complete recovery upon return to the Mg^{2+}-free solution. The dashed line shows the exponential which provided the best fit for the decay of the responses (τ = 80 s). The intracellular solution was the same as in *A*.

It is possible, although unlikely, that dialysis might have accentuated or uncovered the activation of non-NMDA receptors by L-aspartate as it is not an absolutely specific agonist for NMDA receptors. Therefore, we tested the more selective agonist, NMDA, itself. Figure 3*A* shows currents evoked by NMDA; the rate of wash-out is about the same as that of the wash-out of L-aspartate currents. On average, currents evoked by NMDA declined to $54 \pm 5\%$ ($n = 5$) of the initial level after 10 min of recording. The NMDA and L-aspartate currents remaining at the end of wash-out were reduced by specific blockers of NMDA receptors/channels, such as Mg^{2+} (Fig. 3*B*), ketamine and APV (not shown). These blockers were as effective against L-aspartate responses, following wash-out, as they were against non-washed-out responses.

L-Aspartate currents evoked by applications at intervals of less than 15 s declined as a consequence of desensitization (see Fig. 1 of MacDonald *et al.* 1987). However,

more rapid components of desensitization may have occurred during the exposure to a single application of agonist (Grantyn & Lux, 1988). Therefore, wash-out of NMDA currents could represent the conversion of the channels to a lower conductance state by promoting desensitization of the receptors. We could not readily determine if the wash-out enhanced the rapid component of desensitization. On the other hand, if the slow component of NMDA desensitization was enhanced and if this enhancement also required repeated exposure to the agonist, we would predict that the time course and degree of wash-out would also depend directly upon the number of applications of L-aspartate. In several cells following the break-through, L-aspartate was applied

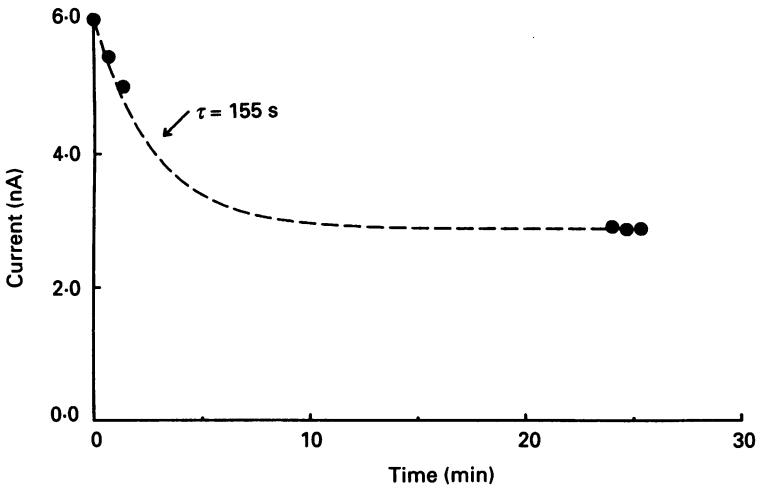

Fig. 4. Wash-out of responses to L-aspartate does not require continuously repeated applications. For this cell L-aspartate was applied three times at intervals of 40 s and then three additional applications were made 22 min later. At this time the responses had decreased to a stable amplitude which was 48% of the initial response. The intracellular solution was the same as in Fig. 1 and the dashed line shows the theoretical decay calculated using results from Fig. 1*B*.

just two or three times to record the initial current amplitude. Subsequent amino acid applications were suspended for at least 20 min. The time course and extent of wash-out was the same even though the cell had been exposed to only a small number of applications of L-aspartate (Fig. 4). Furthermore, with repeated applications at intervals of less than 15 s the decline of L-aspartate currents was identical to that observed in the absence of wash-out.

To determine whether wash-out was specific for NMDA-mediated currents, we used applications of kainate to activate a non-NMDA type of excitatory amino acid receptor (the kainate receptor). In contrast to L-aspartate or NMDA currents, those evoked by kainate did not wash-out (Fig. 5). These non-NMDA currents were stable even in the same cells which demonstrated wash-out of L-aspartate currents ($n = 4$).

To investigate the possibility that the wash-out was due to a gradual increase in $[Ca^{2+}]_i$, we compared the time course of wash-out using dialysis solutions which contained 11 mM-EGTA with 0, 1 or 11 mM added calcium. This would vary the $[Ca^{2+}]$ within the recording solution from nominally zero to $1{\cdot}5 \times 10^{-5}$ M. In addition,

a solution lacking both EGTA and calcium was used. Byerly and Moody (1984) have reported that by changing the ratio of Ca^{2+} to buffer during perfusion of large invertebrate neurones they were unable to maintain $[Ca^{2+}]_i$ at levels other than 10^{-7} (11 EGTA–0 Ca^{2+}) and 10^{-5} (11 EGTA–5 Ca^{2+}). If this were the case in our cultured hippocampal neurones then we may not have adequately controlled $[Ca^{2+}]_i$ at intermediate levels. Nevertheless, we would predict that if increased $[Ca^{2+}]_i$ was responsible for the wash-out of NMDA currents, then changing the buffering capacity of the intracellular solution over a wide range ought to alter the time constant of wash-out of the currents. Figures 6*A*, *B* and *D* demonstrate that the time constant of wash-out differed little even when the $[Ca^{2+}]$ was varied a million-fold.

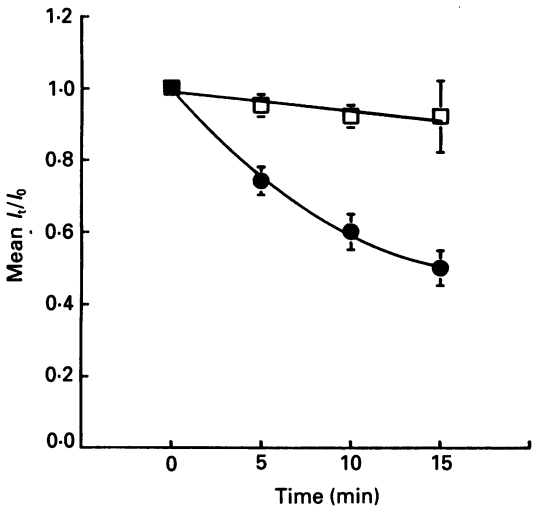

Fig. 5. Responses to kainate do not wash out. During recordings made with an intracellular solution containing (in mM): 140 CsCl, 35 CsOH, 10 HEPES, 11 EGTA and 1 $CaCl_2$ the mean amplitude of response to L-aspartate (●; $n = 10$) declined to $50 \pm 5\%$ of the initial level after within 15 min of recording. By contrast, after the same duration, responses to kainate (□; $n = 7$) were $92 \pm 10\%$ of control.

The wash-out actually appears to be slightly less complete when calcium concentrations were at their greatest. In addition, to minimize entry of Ca^{2+} from the extracellular medium, experiments were done in the presence of Cd^{2+} (100 μM). In these cases, wash-out L-aspartate currents persisted with the responses having decreased to $58 \pm 2\%$ at 10 min ($n = 4$).

While these results are consistent with the idea that the wash-out does not result from a slow rise of $[Ca^{2+}]_i$ it has been recognized that EGTA often fails to effectively buffer transient increases in $[Ca^{2+}]_i$ occurring near the cell membrane. Thus, it remains possible that rapid, transient increases in $[Ca^{2+}]_i$ following activation of NMDA receptors are responsible for the wash-out. Therefore, in six experiments we have used BAPTA as a calcium buffer because it binds Ca^{2+} more rapidly than does EGTA (Tsien, 1980). When BAPTA was substituted for EGTA, and calcium was not added to the dialysis solution, neither the time course nor the completeness of wash-out was altered (Fig. 6*C*).

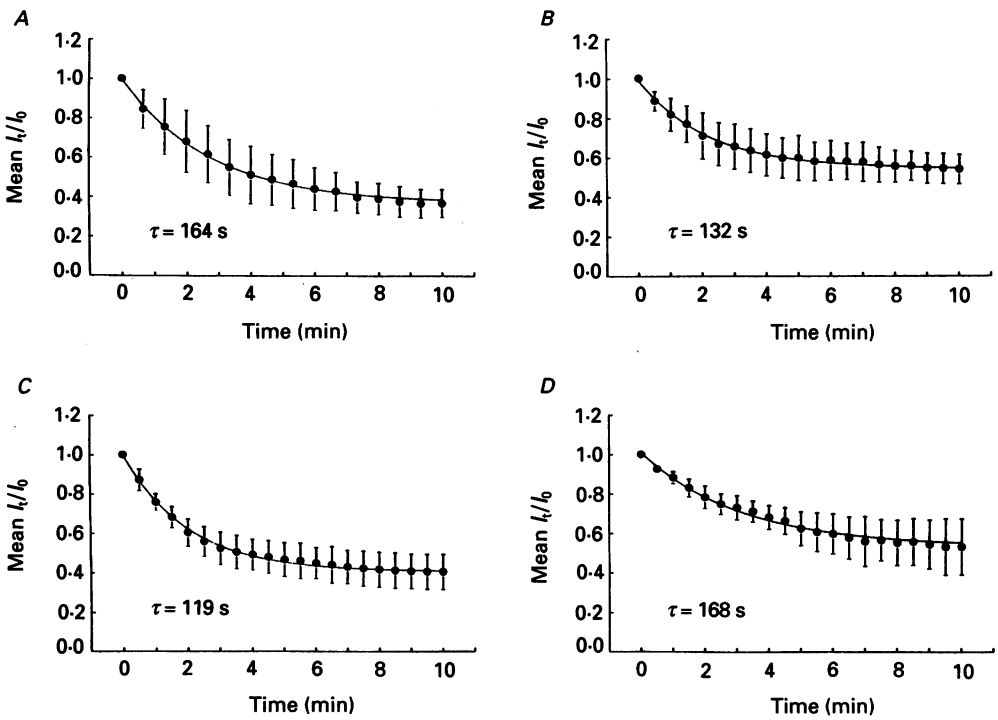

Fig. 6. Wash-out of responses to L-aspartate under four conditions of calcium buffering. The graphs show I_t/I_0 when the intracellular solution contained (in mM): 11 EGTA–0 Ca^{2+} (*A*, $n = 6$); 11 EGTA–11 Ca^{2+} (*B*, $n = 5$); 11 BAPTA–0 Ca^{2+} (*C*, $n = 6$) and 0 EGTA–0 Ca^{2+} (*D*, $n = 7$). In each case the other constituents of the intracellular solution were (in mM): 140 CsCl, 35 CsOH, and 10 HEPES. For each graph the continuous line is the single exponential with the best fit and τ is indicated. Note that I_t/I_0 was not significantly affected by the level of calcium buffering (one-way analysis of variance, $F = 2{\cdot}02$, $P > 0{\cdot}05$).

The holding potential used in most of these experiments (i.e. −60 mV) may have permitted activation of some low-threshold calcium current which also could have contributed to an accumulation of intracellular calcium. This holding potential was chosen because it was difficult to obtain long-term and steady recordings in cells held at more hyperpolarized holding potentials. However, in a few cases where we were successful at holding the cell at −90 mV the wash-out of the L-aspartate currents was not appreciably different than at −60 mV.

Depolarization of the cell to positive potentials evoked a slowly declining outward current tail which is associated with a selective but transient depression of L-aspartate but not kainate currents (MacDonald *et al.* 1987). Despite the complication presented by this transient depression we also examined whether or not wash-out would occur at positive holding potentials. Cells were clamped at −60 mV and then slowly stepped to +40 mV. Under these circumstances it is difficult to estimate the initial value of the L-aspartate current because of the imposed depression of these currents during the slow relaxation. The L-aspartate currents increased in amplitude during the decline of this tail current (Fig. 7). Nevertheless, outward L-aspartate currents then washed-out. When the later responses were fitted with an exponential

it was possible to extrapolate back to an estimate of the initial current amplitudes which suggested that the completeness of wash-out was similar at −60 or +40 mV. On the other hand, the rate of wash-out was slower when holding at +40 mV (τ = 465 s at +40 mV *vs.* 155 s at −60 mV).

Fig. 7. Wash-out of outward L-aspartate currents at a holding potential of +40 mV. Within 30 s of the start of recording the membrane potential (lower record) was changed from −60 to +40 mV. This evoked a small inward current followed by a slowly decaying outward current (upper record). Repeated applications of L-aspartate (250 μM, 20 ms, 140 kPa) were begun when the current had stabilized, approximately 90 s after the beginning of the recording. The subsequent increase and then decrease in the amplitude of the L-aspartate responses was best fitted by a double exponential with the form $I_t = I_0$ $(0{\cdot}52e^{-0{\cdot}0022t} - 0{\cdot}66e^{-0{\cdot}016t} + 0{\cdot}48)$. Thus, there appeared to be an initial depression of the response with a time constant of 63 s followed by a slower wash-out (τ = 465 s). In addition, the amplitude at the end of the wash-out was 48% of the extrapolated original level when the initial depression is taken into account. The intracellular solution was the same as for Fig. 3.

The wash-out of L-aspartate currents might have resulted from a gradual shift of the reversal potential to more hyperpolarized values resulting in a reduction in the driving force on the permeant ions. The reversal potential for NMDA-activated currents in cultured hippocampal neurones is about 0 mV during whole-cell intracellular dialysis with solutions similar to those used here (Mayer & Westbrook, 1987*a*, *b*). Once the wash-out of the L-aspartate currents had been achieved the reversal potential remained near to 0 mV regardless of the concentration of EGTA or calcium in the recording solution and regardless of whether or not BAPTA was employed (Fig. 8).

In several types of cells the inclusion of an ATP regenerating system in the intracellular dialysis solution has been reported to greatly retard the wash-out of calcium currents (Forscher & Oxford, 1985; Chad & Eckert, 1986). For this reason we examined responses to L-aspartate during intracellular dialysis with this solution. It was found that there was no appreciable decline of L-aspartate currents for extended periods of recording (Fig. 9). In some cells, stable L-aspartate currents could be recorded for periods of an excess of 2 h. To prevent wash-out of L-aspartate it was critical to use solutions which had just been prepared or thawed. Solutions which had been stored for a number of hours were ineffective at preventing wash-out presumably due to instability of the high-energy phosphates in the ATP regenerating system. When only ATP (with Mg^{2+}) was included in the intracellular solution, wash-out of L-aspartate currents was reduced but not prevented with the current having declined to 77±5% (n = 11) after 10 min. In addition, inclusion of only

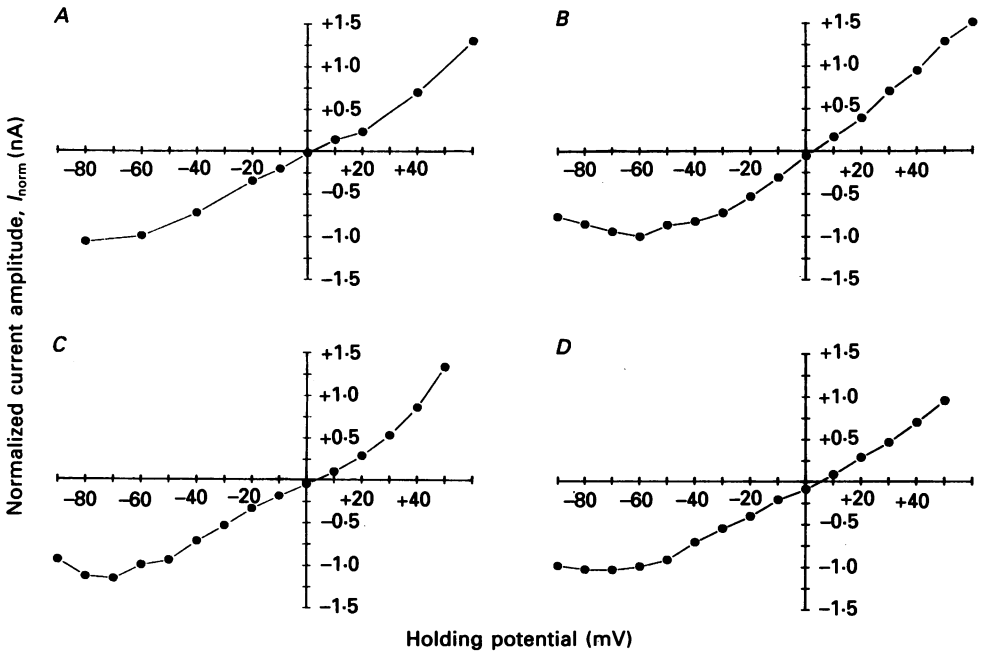

Fig. 8. Voltage dependence of L-aspartate currents under four conditions of Ca^{2+} buffering. Responses to L-aspartate were elicited at various holding potentials 15–20 min after the start of recording (i.e. after wash-out had occurred). To facilitate comparison among different cells, the response amplitude at each potential was then normalized to that when the holding potential was −60 mV. Thus, in each graph the normalized amplitude (I_{norm}) for a single cell is plotted *versus* the holding potential. The intracellular solution contained (in mM): 11 EGTA–0 Ca^{2+} (*A*); 11 EGTA–11 Ca^{2+} (*B*); 11 BAPTA–0 Ca^{2+} (*C*) and 0 EGTA–0 Ca^{2+} (*D*) in addition to 140 CsCl, 35 CsOH and 10 HEPES. In each case the reversal potential for the L-aspartate response was between 0 and +5 mV.

phosphocreatine and creatine phosphokinase was ineffective in preventing wash-out ($n = 5$).

Three cells were studied in which the ATP in the regenerating solution was replaced with the non-hydrolysable analogue β,γ-methyleneATP. In each case responses to L-aspartate washed-out and the currents were $53 \pm 1\%$ of the initial amplitude 10 min after the start of recording. This finding indicates that hydrolysis of ATP rather than its mere presence is critical for preventing the wash-out. In addition, it was found that GTP failed to prevent wash-out; responses to L-aspartate decreased to $56 \pm 4\%$ ($n = 4$) at 10 min.

Wash-out of calcium currents is at least partially reversible in some cells (Chad & Eckert, 1986). In order to investigate the reversibility of the wash-out of L-aspartate currents, we double-filled electrodes as described in the Methods section. This allowed initial dialysis of the neurone by a solution devoid of the ATP regenerating system, followed after a delay by the diffusion of a second solution containing the ATP regenerating system. Under these conditions L-aspartate currents initially declined as anticipated, but unlike any of our previous recordings, the currents then increased and reached a maximum amplitude approximately 15–20 min after the first response (Fig. 10).

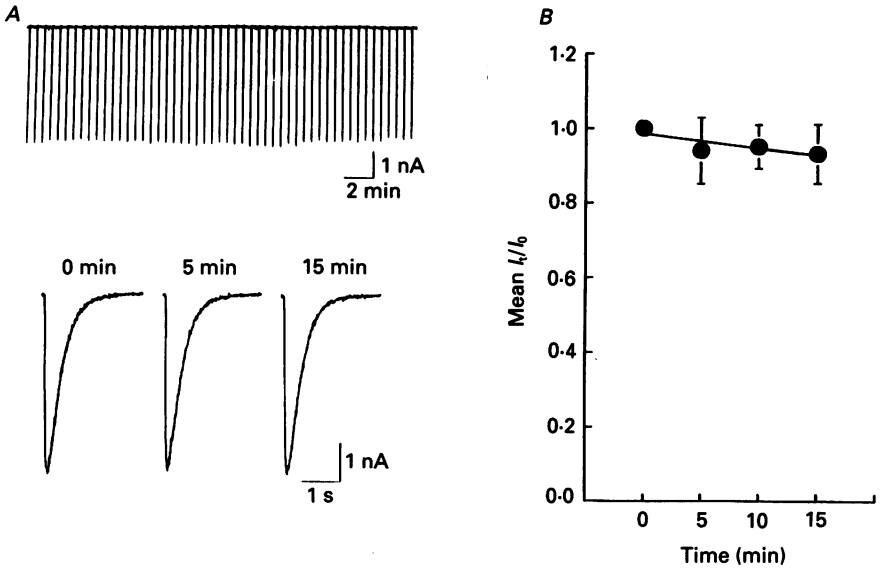

Fig. 9. Wash-out of L-aspartate responses is prevented by inclusion of an ATP regenerating system in the intracellular solution. *A*, continuous current recording (upper trace) shows that inward currents by application of L-aspartate (250 μM, 20 ms, 140 kPa) were stable during the recording. Individual responses, taken at the times indicated, are illustrated below. *B*, I_t/I_0 is plotted for eight cells.

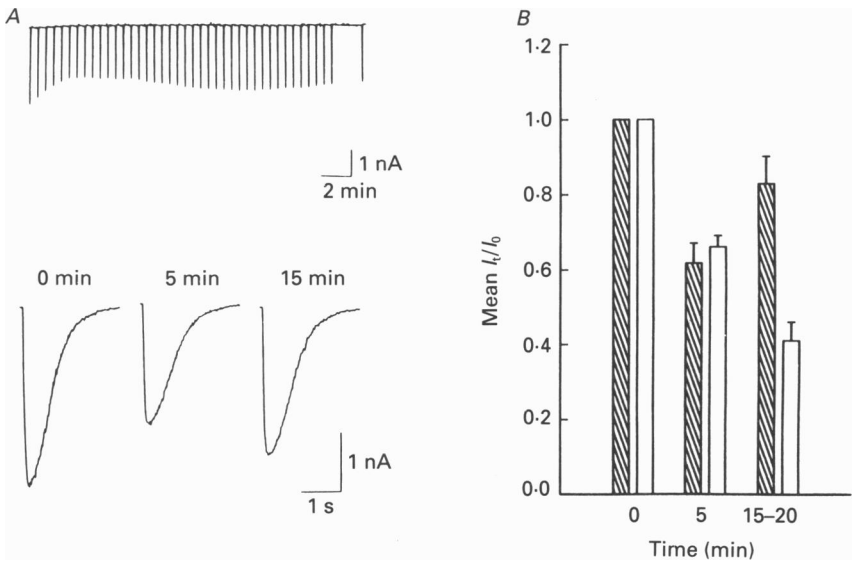

Fig. 10. Reversal of wash-out. *A*, continuous current recording (upper trace) of responses to L-aspartate when the electrode was double-filled as described in the Methods. Individual responses, at the times indicated, are shown below, *B*, histograms illustrating that the response amplitude increased for cells recorded with electrodes that had been double-filled (hatched bars, $n = 5$) *versus* cells recorded with only the control solution (open bars $n = 6$). Note that for each cell the response amplitudes have been normalized with respect to the first response at $t = 0$.

It was not practical to determine the current–voltage relationship, and hence the reversal potential, at the beginning of the recording (in the absence of ATP regenerating solution) because the wash-out would have been occurring progressively during the taking of measurements. However, after the L-aspartate responses had stabilized at the end of the wash-out, the reversal potential was found to be similar to that of responses from cells where the ATP regenerating system was used (Mody *et al.* 1988*b*).

A gradual decrease in the affinity of the agonist for the NMDA receptor might have been responsible for the wash-out of the L-aspartate currents. This would have resulted in a shift of the dose–response curve to the right along the concentration axis. It was not feasible to make such measurements during the wash-out itself. However, as a compromise we evoked a submaximum response before wash-out had occurred and then we examined the dose–response relationship after wash-out had been achieved. This was done by varying the duration of the pressure applications of L-aspartate. It is clear that the maximum response following wash-out was considerably smaller than the submaximum current activated before wash-out. This was not the case when ATP regenerating solution was employed and the initial response lay on the same dose–response curve constructed after 20 min of recording (not shown).

DISCUSSION

Our findings demonstrate that NMDA currents recorded from cultured hippocampal neurones wash out during intracellular dialysis with a conventional recording solution. In contrast, currents evoked by activating kainate receptors are not subject to wash-out and remain stable in amplitude for extended periods of recording. Thus, in addition to the various pharmacological agents that are known to distinguish these subtypes of excitatory amino acid channels by acting from the extracellular side of the membrane (i.e. Mg^{2+}, Zn^{2+} and glycine), there must also be different factors which regulate channel activity on the cytoplasmic side of the membrane.

A portion of the NMDA current was found to be dependent upon a supply of high-energy phosphates that was provided by the ATP regenerating solution. Furthermore, dialysis of this solution could at least partially reverse wash-out of NMDA currents. The maintenance of phosphorylation reactions seems to be critical in preventing this wash-out. For example, the non-hydrolysable ATP analogue, β,γ-methyleneATP, failed to prevent the wash-out, the ATP regenerating system rapidly lost its ability to prevent wash-out if it was permitted to degrade, and wash-out persisted when GTP was substituted for ATP. Also, ATP or creatine phosphokinase/creatine phosphate on its own were unable to prevent wash-out, although fresh solutions of ATP were able to significantly retard the wash-out. Taken together these findings suggest that the wash-out of NMDA currents occurs as a consequence of the gradual loss of the substrates of phosphorylation from the interior of the cell.

One component of the NMDA current proved highly resistant to wash-out. A possible explanation is that the agonists we employed were activating non-NMDA receptors. This was unlikely because NMDA possesses a high specificity for its own receptor and previous experiments in cultured neurones have clearly demonstrated

that applications of NMDA and L-aspartate, similar to those employed here, activate exclusively NMDA receptors (Mayer & Westbrook, 1987*b*; Watkins & Olverman, 1987). Furthermore, the pharmacological properties of these currents were not dramatically altered by wash-out. For example, regardless of whether or not the ATP regenerating solution was used, NMDA currents were readily blocked by the competitive NMDA antagonist APV as well as by voltage-dependent NMDA channel blockers such as Mg^{2+} and ketamine.

The partial wash-out of NMDA currents may suggest that there are several subtypes of NMDA receptors/channels, one of which depends upon a supply of high-energy phosphates. The possibility of subtypes of NMDA channels is also suggested by the observation that cerebellar neurones can possess different complements of subconductance states of this channel (Cull-Candy, Howe & Ogden, 1988). In addition, a number of recent studies of NMDA binding sites in the CNS have in fact proposed a subdivision of the NMDA receptor (Monaghan, Cotman, Olverman & Watkins, 1988) and it is possible that a more rigorous examination of the pharmacological properties of these two components would reveal more subtle differences than we have been able to detect. Our demonstration that the dose–response relationship for NMDA was unlikely to have been shifted in a parallel fashion implies that the affinity of the NMDA receptor was not dramatically altered by wash-out.

Recently, Stelzer, Kay & Wong (1988) have reported that the chloride currents coupled to $GABA_A$ receptors of acutely isolated hippocampal neurones wash out almost entirely during whole-cell dialysis. Wash-out of $GABA_A$ currents in cultured hippocampal cells was also to about 50% of the initial value. Similar to NMDA currents this wash-out of $GABA_A$ currents could also be prevented by including a supply of high-energy phosphates in the dialysis solution. In apparent contrast to our present observations, they found that currents evoked by L-glutamate did not wash out in isolated CA1 neurones. However, L-glutamate is not a particularly specific NMDA agonist (Watkins & Olverman, 1987) and it is possible that the enzymatic treatment that was used to isolate these cells may have degraded their NMDA receptors (Akaike, Kaneda, Hori & Krishtal, 1988; Allen, Brady, Swann, Hori & Carpenter, 1988).

The wash-out of calcium currents in invertebrate neurones consists of a reversible and an irreversible component (for example see Chad & Eckert, 1986). The irreversible component was suppressed by the inclusion of leupeptin, an inhibitor of calcium-activated proteolysis, in the dialysate. However, a remaining component of the wash-out could be reversed by including ATP and the catalytic subunit of cylic AMP-dependent protein kinase in the dialysis solution. Chad & Eckert (1986) postulated that the calcium channels were gradually dephosphorylated during the dialysis unless an appropriate source of high-energy phosphates was provided to continuously rephosphorylate the channels. Further support for this hypothesis was provided by the recent demonstration that some calcium channel activity requires channel phosphorylation (Armstrong & Eckert, 1987).

In opposition to the theory that calcium channels are directly regulated by dephosphorylation/rephosphorylations, others have suggested that wash-out reflects calcium-dependent inactivation of calcium channels arising as a consequence of a

gradual rise in $[Ca^{2+}]_i$. For example, Byerly & Moody (1984) have found that even when invertebrate neurones were dialysed with exceptionally high concentrations of EGTA (> 100 mM) it was possible for the actual value of $[Ca^{2+}]_i$ to differ significantly from the free concentration in the dialysate. The failure of EGTA to accurately control $[Ca^{2+}]_i$ was thought to be related to a loss during dialysis of cellular components necessary to maintain intracellular sequestration or extrusion of calcium. High-energy phosphates might be one of these cellular components. Thus, the ATP regenerating solution might prevent wash-out simply by maintaining low $[Ca^{2+}]_i$.

Unlike these experiments we saw no obvious irreversible component of the wash-out of NMDA currents as they declined to 50% of their initial value and then remained stable for periods of several hours. Our results also suggest that activation of calcium-dependent proteases was unlikely to contribute to the wash-out of NMDA currents. Furthermore, inclusion of leupeptin in the dialysate did not alter the wash-out of NMDA current (J. F. MacDonald, M. W. Salter & I. Mody, unpublished results). This discrepancy between the run-down of calcium currents in muscle cells and large invertebrate neurones and the wash-out of NMDA currents is probably due to the much smaller size of the cultured mammalian neurones and to our inclusion in the majority of experiments of substantial concentrations of calcium buffers which may have improved our ability to successfully buffer $[Ca^{2+}]_i$.

Our experiments suggest that wash-out of NMDA currents was not the consequence of an accumulation of intracellular calcium. Specifically, changes neither in the free concentration of calcium nor in the concentration of calcium buffer within the dialysate had an appreciable effect on the time course of wash-out of NMDA currents. Even if none of the solutions we employed successfully controlled $[Ca^{2+}]_i$ it seems highly unlikely that the rate at which calcium rose to a new stable value would be uninfluenced by such large changes in the calcium concentration and buffering capacity of the dialysate.

The state of the NMDA channel would therefore appear to be modulated by rephosphorylation/dephosphorylation perhaps of the channel itself or of some closely related protein. However, wash-out of the NMDA currents is not complete and NMDA single-channel activity can be routinely recorded from outside-out patches in the absence of an ATP regenerating solution (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984). Thus, some NMDA channel activity must not be dependent upon phosphorylation. It is known that NMDA channel activity recorded from patches demonstrates a number of subconductance states as well as a main conductance state of about 50 pS (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Ascher & Nowak, 1988). The probability of the channel opening to any particular conductance state or the time it remains in a conductance state might then be determined by phosphorylation of the channel.

There are many possible enzymes including protein kinase C that could be responsible for the phosphorylation and we are currently examining several possibilities. Implication of NMDA receptor activation (Kauer *et al.* 1988) and translocation of protein kinase C during long-term potentiation in the hippocampus (Akers, Lovinger, Colly, Linden & Routtenberg, 1986; Lovinger, Wong, Murakami & Routtenberg, 1987) and the recruitment of NMDA receptors into synaptic

transmission following kindling (Mody & Heinemann, 1987) raises the possibility that an increased phosphorylation of NMDA channels might be responsible for some aspects of synaptic plasticity.

We thank Ms M. Deverill for expert technical assistance. M. C. Bartlett participated in some of the experiments. This work was supported by a Medical Research Council of Canada grant to J.F.M. I.M. is a Fellow of the MRC of Canada and of the I. W. Killam Foundation; M.W.S. is an E. B. Eastburn Fellow of the Hamilton Foundation.

REFERENCES

AKAIKE, N., KANEDA, M., HORI, N. & KRISHTAL, O. A. (1988). Blockade of *N*-methyl-D-aspartate response in enzyme-treated rat hippocampal neurons. *Neuroscience Letters* **87**, 75–79.

AKERS, R. F., LOVINGER, D. M., COLLEY, P. A., LINDEN, D. J. & ROUTTENBERG, A. (1986). Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science* **231**, 587–589.

ALLEN, C. N., BRADY, R., SWANN, J., HORI, N. & CARPENTER, D. O. (1988). *N*-methyl-D-aspartate (NMDA) receptors are inactivated by trypsin. *Brain Research* **458**, 147–150.

ARMSTRONG, D. & ECKERT, R. (1987). Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization, *Proceedings of the National Academy of Sciences of the USA* **84**, 2518–2522.

ASCHER, P. & NOWAK, L. (1988). The role of divalent cations in the NMDA responses of mouse central neurones in culture. *Journal of Physiology* **399**, 247–266.

BALAZS, R., HACK, N. & JORGENSEN, O. S. (1988). Stimulation of the *N*-methyl-D-aspartate receptor has a trophic effect of differentiating cerebellar granule cells. *Neuroscience Letters* **87**, 80–86.

BYERLY, L. & MOODY, W. J. (1984). Intracellular calcium ions and calcium currents in perfused neurones of the snail, *Lymnaea stagnalis*. *Journal of Physiology* **352**, 637–652.

CHAD, J. E. & ECKERT, R. (1986). An enzymatic mechanism for calcium current inactivation in dialysed *Helix* neurones. *Journal of Physiology* **378**, 31–51.

COLLINGRIDGE, G. L. & BLISS, T. V. P. (1987). NMDA receptors – their role in long-term potentiation. *Trends in Neurosciences* **10**, 288–293.

COTMAN, C. W. & IVERSEN, L. L. (1987). Excitatory amino acids in the brain – focus on NMDA receptors. *Trends in Neurosciences* **10**, 263–265.

CULL-CANDY, S. G., HOWE, J. G. & OGDEN, D. C. (1988). Noise and single channels activated by excitatory acids in rat cerebellar granule neurones. *Journal of Physiology* **400**, 189–222.

CULL-CANDY, S. G. & USOWICZ, M. M. (1987). Multiple conductance channels activated by excitatory amino acids in cerebellar neurons. *Nature* **325**, 525–528.

FORSCHER, P. & OXFORD, G. S. (1985). Modulation of calcium channels by norepinephrine in internally dialyzed avian sensory neurons. *Journal of General Physiology* **85**, 743–763.

GRANTYN, R. & LUX, H. D. (1988). Similarity and mutual exclusion of NMDA- and proton-activated transient Na^+-currents in rat tectal neurons. *Neuroscience Letters* **89**, 198–203.

HUETTNER, J. E. (1988). Calcium current through channels gated by NMDA receptors. *Society for Neuroscience Abstracts* **14**, 5.

JAHR, C. E. & STEVENS, C. F. (1987). Glutamate activates multiple single channel conductances in hippocampal neurons. *Nature* **325**, 522–525.

JOHNSON, J. W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* **325**, 529–531.

KAUER, J. A., MALENKA, R. C. & NICOLL, R. A. (1988). NMDA application potentiates synaptic transmission in the hippocampus. *Nature* **334**, 250–252.

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

LOVINGER, D. M., WONG, K. L., MURAKAMI, K. & ROUTTENBERG, A. (1987). Protein kinase C inhibitors eliminate hippocampal long-term potentiation. *Brain Research* **436**, 177–183.

MACDONALD, J. F., MILJKOVIC, Z. & PENNEFATHER, P. (1987). Use-dependent block of excitatory amino-acid currents in cultured neurons by ketamine. *Journal of Neurophysiology* **58**, 251–266.

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

Mattson, M. P., Dou, P. & Kater, S. B. (1988). Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *Journal of Neuroscience* **8**, 2087–2100.

Mayer, M. L, MacDermott, A. B., Westbrook, G. L., Smith, S. J. & Barker, J. L. (1987). Agonist- and voltage-gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured using Arsenazo III. *Journal of Neuroscience* **7**, 3230–3244.

Mayer, M. L. & Westbrook, G. L. (1987*a*). Permeation and block of NMDA acid receptor channels by divalent cations in mouse cultured central neurones. *Journal of Physiology* **394**, 501–527.

Mayer, M. L. & Westbrook, G. L. (1987*b*). The physiology of excitatory amino acids in the vertebrate nervous system. *Progress in Neurobiology* **28**, 197–276.

Mody, I. & Heinemann, U. (1987). NMDA receptors of dentate gyrus granule cells participate in synaptic transmission following kindling. *Nature* **326**, 701–704.

Mody, I., MacDonald, J. F. & Baimbridge, K. G. (1988*a*). Release of intracellular calcium following activation of excitatory amino acid receptors in cultured hippocampal neurons. *Society for Neuroscience Abstracts* **14**, 94.

Mody, I., Salter, M. W. & MacDonald, J. F. (1988*b*). Requirement of NMDA receptor/channels for intracellular high energy phosphates and the extent of intraneuronal calcium buffering. *Neuroscience Letters* **93**, 73–78.

Mody, I., Stanton, P. K. & Heinemann, U. (1988*c*). Activation of *N*-methyl-D-aspartate receptors parallels changes in cellular and synaptic properties of dentate gyrus granule cells after kindling. *Journal of Neurophysiology* **59**, 1033–1054.

Monaghan, D. T., Cotman, C. W., Olverman, H. J. & Watkins, J. C. (1988). Two classes of NMDA recognition sites: Differential distribution and regulation by glycine. In *Frontiers in Excitatory Amino Acids Research*, vol. 46, eds. Calvalheiro, E. A., Lehmann, J. & Turski, L., pp. 543–550. New York: Alan R. Liss, Inc.

Morris, M. E., Friedlich, J. J. & MacDonald, J. F. (1987). Intracellular calcium in mammalian brain cells: fluorescence measurements with Quin-2. *Experimental Brain Research* **65**, 520–526.

Murphy, S. N., Thayer, S. A. & Miller, R. J. (1987). The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons *in vitro*. *Journal of Neuroscience* **7**, 4146–4158.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* **307**, 462–465.

Pearce, I. A., Cambray-Deakin, M. A. & Burgoyne, R. D. (1987). Glutamate acting on NMDA receptors stimulates neurite outgrowth from cerebellar granule cells. *FEBS Letters* **223**, 143–147.

Pusch, M. & Neher, E. (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Archiv* **411**, 204–211.

Stelzer, A., Kay, A. R. & Wong, R. K. S. (1988). $GABA_A$-receptor function in hippocampal cells is maintained by phosphorylation factors. *Science* **241**, 339–341.

Stockbridge, N. (1987). EGTA. *Computers in Biological Medicine* **17**, 299–304.

Tsien, R. Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. *Biochemistry* **19**, 2396–2404.

Vyklicky Jr, L., Krusek, J. & Edwards, C. (1988). Differences in the pore sizes of the *N*-methyl-D-aspartate and kainate cations channels. *Neuroscience Letters* **89**, 313–318.

Watkins, J. C. & Olverman, H. J. (1987). Agonists and antagonists for excitatory amino acid receptors. *Trends in Neurosciences* **10**, 265–272.

Yount, R. G. (1975). ATP analogs. *Advances in Enzymology* **43**, 1–56.