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CALCIUM-MEDIATED MODULATION OF *N*-METHYL-D-ASPARTATE (NMDA) RESPONSES IN CULTURED RAT HIPPOCAMPAL NEURONES

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SUMMARY

1. Agonist-independent (inactivation) and agonist-induced (desensitization) refractory states of *N*-methyl-D-aspartate (NMDA) receptors were studied on cultured rat hippocampal neurones using whole-cell and inside-out patch-clamp techniques and a fast perfusion system.

2. Shortly after whole-cell formation, application of 100 μM NMDA in the presence of 10 μM glycine and 0·2 mM $[Ca^{2+}]_o$ induced membrane currents that desensitized by 23 %. Repeated application of NMDA at 30 s intervals resulted in a progressive increase in the degree and rate of onset of NMDA receptor desensitization.

3. Test responses to NMDA recorded in the presence of 0·2 mM $[Ca^{2+}]_o$ were reversibly inactivated by 60 % following a train of ten 1 s applications of NMDA delivered at 0·5 Hz in the presence of 2 mM $[Ca^{2+}]_o$; similar results were obtained with 2 mM $[Sr^{2+}]_o$ and 2 mM $[Ba^{2+}]_o$. In the presence of Ca^{2+} or Sr^{2+}, desensitization during the train of responses to NMDA increased by 14 and 19 % respectively, while with Ba^{2+} there was no increase in desensitization.

4. In the presence of 0·2 mM $[Ca^{2+}]_o$ at a holding potential of −60 mV, or in the presence of 2 mM $[Ca^{2+}]_o$ at a holding potential of +50 mV, a train of ten applications of NMDA failed to induce either inactivation or an increase in desensitization of test responses to NMDA. These results suggest an important role for $[Ca^{2+}]_o$ in the induction of both inactivation and desensitization of NMDA receptors.

5. Increasing the intracellular calcium concentration, $[Ca^{2+}]_i$, via repeated activation of voltage-gated Ca^{2+} channels, resulted in a reversible inactivation of test responses to NMDA by 35 % but failed to increase desensitization. In neurones dialysed with intracellular solution containing 2·5 mM Ca^{2+} NMDA receptor desensitization was similar to that in neurones dialysed with 10 nM Ca^{2+}.

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6. Block of NMDA receptor–channels by 2 mM $[Mg^{2+}]_o$ during the train application of NMDA prevented the induction of both inactivation and desensitization. In contrast 3 mM $[Mg^{2+}]_i$ was ineffective.

7. The magnitude of both inactivation and desensitization of NMDA receptors was not affected by intracellular dialysis of ATP, the non-hydrolysable ATP analogue 5′-adenylylimido-diphosphate (AMP-PNP), different Ca^{2+} chelators (EGTA or BAPTA), the Ca^{2+}-activated protease inhibitor (leupeptin), dithiothreitol, or the phosphatase inhibitors (okadaic acid and a calcineurin inhibitor).

8. Application of 2·5 mM Ca^{2+} to the cytoplasmic side of inside-out patches induced inactivation of NMDA responses similar in magnitude to the inactivation seen in whole-cell recording. Complete recovery from inactivation was, however, much faster (20 s) for inside-out patches than for whole-cell responses (usually 5–10 min).

9. These results suggest two distinct mechanisms for modulation of NMDA receptors by intracellular Ca^{2+}. Desensitization of NMDA receptors is induced when both $[Ca^{2+}]_i$ is increased and NMDA receptors activated by agonist. Inactivation of NMDA receptors is produced by increased levels of $[Ca^{2+}]_i$ and does not require NMDA receptor activation for induction.

INTRODUCTION

Desensitization of ligand-activated ion channels is a well-established experimental phenomenon which may be an important mechanism for the regulation of neuronal excitability. Desensitization of the *N*-methyl-D-aspartate (NMDA) subtype of vertebrate CNS glutamate receptor is mediated by glycine-sensitive and glycine-resistant mechanisms (Mayer, Vyklický & Clements, 1989*a*; Sather, Johnson, Henderson & Ascher, 1990; Mayer, Vyklický, Benveniste, Patneau & Williamson, 1991; Chizhmakov, Kiskin & Krishtal, 1992). The mechanism(s) underlying glycine-resistant desensitization are only partially understood. In some experiments on cultured neurones glycine-resistant desensitization was reported to evolve slowly during the course of whole-cell recording, while in others it was observed during the first application of agonist (Benveniste, Clements, Vyklický & Mayer, 1990; Sather *et al.* 1990; Sather, Dieudonne, MacDonald & Ascher, 1992). Similar results have been obtained in acutely isolated neurones (Chizhmakov *et al.* 1992).

Calcium appears to play an important role in the glycine-resistant desensitization of NMDA receptors. Mayer & Westbrook (1985) described a reversible 'fade' of responses to NMDA recorded in the presence of a low extracellular concentration of Na^+ (10 mM) with 5 mM Ca^{2+} in cultured mouse spinal cord neurones. This reversible inactivation was induced by pressure application of NMDA for 1 s or by depolarizing voltage jumps to −10 mV. Zorumski, Yang & Fischbach (1989) observed a similar decrease of responses to 1 mM glutamate that was dependent on the extracellular Ca^{2+}/Mg^{2+} ratio. In a later study Clark, Clifford & Zorumski (1990) presented data suggesting that desensitization of NMDA receptors was modulated by $[Ca^{2+}]_o$. The goal of this study was to resolve the role of Ca^{2+} in glycine-resistant NMDA receptor desensitization.

Experiments described here revealed two effects of Ca^{2+} on responses to fast application of NMDA: reversible inactivation, and a use-dependent but irreversible increase in the degree of NMDA response desensitization.

METHODS

Hippocampal cultures

Newborn Sprague–Dawley rats were killed by decapitation. The hippocampi were removed and digested for 20 min with papain (20 U/ml) activated by cysteine. After papain inactivation with trypsin inhibitor type III-O (2·5 mg/ml) the tissue was mechanically dissociated into single cells and plated at a density of 10000 cells/cm^2 onto a confluent glial cell feeder layer prepared two weeks earlier. Neuronal cultures were maintained in media composed of Eagle's minimal essential medium (MEM) supplemented with hormones and 5% horse serum (for details see Mayer, Vyklický & Westbrook, 1989*b*). Experiments were performed at room temperature (25–27 °C) on neurones maintained in culture for 3–14 days.

Solutions and drug application

Extracellular solution (ECS) contained (mM): 160 NaCl, 2·5 KCl, 10 Hepes, 10 glucose and 0·01 mg/ml Phenol Red; pH was adjusted to 7·3 with NaOH and osmolarity to 320–330 mosmol/l with sucrose. This salt solution was supplemented with Ca^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+} and phosphatidylserine as indicated in the Results section. All the test and control solutions contained 10 μM glycine, 0·5 μM TTX and 5 μM bicuculline methochloride. Control solution, which was applied between applications of NMDA, contained ECS supplemented with 2 mM Ca^{2+} and 1 mM dithiothreitol (DTT), a disulphide reducing agent, in order to prevent diminution of NMDA receptor currents caused by oxidation of disulphide bonds (Aizenman, Lipton & Loring, 1989). The recording chamber was perfused at a rate of 0·5–1 ml/min with ECS supplemented with 1 mM Mg^{2+} and 2 mM Ca^{2+}. A fast perfusion system controlled by an IBM AT computer was used to apply test and control solutions (Vyklický, Benveniste & Mayer, 1990*a*; Vyklický, Vlachova & Krusek, 1990*b*). Electrodes for whole-cell recording were filled with intracellular solution (ICS) containing (mM): 125 $CsMeSO_3$; 15 CsCl; 5 EGTA (or 5 BAPTA); 0·5 $CaCl_2$; 10 Hepes; pH was adjusted to 7·2 with CsOH and osmolarity to 305 mosmol/l with sucrose. ICS was supplemented with ethylenediaminetetraacetic acid (EDTA), Mg^{2+}, Ca^{2+}, DTT, ATP, 5′-adenylylimidodiphosphate (AMP-PNP), leupeptin, calcineurin inhibitor or okadaic acid as indicated in the Results section.

Whole-cell and inside-out patch recording

Whole-cell patch-clamp (Axoclamp 2) recordings from hippocampal neurones voltage clamped at −60 mV were obtained using 3–4 MΩ pipettes pulled from borosilicate glass. Agonist responses were stored and analysed using pCLAMP (Axon Instruments, USA). Thick-walled borosilicate glass electrodes (WPI 1B150) were coated with Sylgard (Dow-Corning, USA) and had a resistance of ≈4 MΩ. First, a cell-attached patch was formed (EPC-7 amplifier, List) using an electrode solution containing ECS supplemented with 0·2 mM Ca^{2+}, 10 μM NMDA and 10 μM glycine. If inward single-channel currents of appropriate amplitude were observed, the extracellular solution was switched to ICS containing 10 nM Ca^{2+} before forming an inside-out patch. Inside-out patches were clamped at −70 mV and single-channel activity was recorded on videotape using a VR-10 Digital Data Recorder (Instrutech Corp., USA) and Panasonic AG-2500 VCR. During analysis single-channel data were filtered at 1 kHz, digitized at 10 kHz and analysed using pCLAMP version 5.5.1 (Axon Instruments).

Data analysis

Desensitization (D) of NMDA responses was defined as

$$D = [1 - (I_{SS}/I_{PE})] \times 100, \quad (1)$$

where I_{PE} is the peak response and I_{SS} is the steady-state response to NMDA measured at the end of a 1 s application. The decay of NMDA responses, $I(t)$, in the continued presence of agonist was fitted by a single exponential function

$$I(t) = (I_{PE} - I_{SS}) \exp(-t/\tau_D) + I_{SS}, \quad (2)$$

where t is time after the start of the application of agonist and τ_D the time constant of desensitization. The desensitization rate (k_D) was defined as

$$k_D = 1/\tau_D. \quad (3)$$

The increase in the degree of desensitization of NMDA responses during the course of whole-cell recording could be fitted by a single exponential function

$$D(t) = 1 - D_0 \exp(-t/\tau), \quad (4)$$

where $D(t)$ is the degree of desensitization at time t, D_0 the initial degree of desensitization and τ the time constant of increase in the degree of desensitization.

The effect of a 20 s train of 1 s applications of 100 μM NMDA, or the activation of voltage-gated Ca^{2+} currents at 0·5 Hz, on desensitization to NMDA (ΔD) was evaluated according to the following equation

$$\Delta D = D_a(t_t) - D_b(t_t), \quad (5)$$

where t_t is the time corresponding to the first test application of NMDA after the train and D_a and D_b are defined by the function D (eqn (1)) after and before the conditioning stimulus.

Drugs were purchased from Molecular Probes (Eugene, OR, USA), Sigma (USA) and Tocris Neuramin (Bristol, UK). Results are presented as means ± S.D. One-way analysis of variance (ANOVA) and paired t tests were used to determine the significance of differences in the results.

RESULTS

In the present experiments the degree of desensitization of test responses to NMDA increased with time of recording; the initial experiments were designed to characterize the time dependence of this effect. Test responses of 100 μM NMDA were applied in the presence of a saturating concentration of glycine (10 μM) with a low $[Ca^{2+}]_o$ (0·2 mM). To prevent rapid deterioration of the neurone under study, which can occur during prolonged perfusion with low $[Ca^{2+}]_o$, 2 mM Ca^{2+} was applied between applications of NMDA, with 0·2 mM Ca^{2+} applied only 1 s before, during and for 1 s after the test application of NMDA.

With this protocol, for the example shown in Fig. 1 the peak response to 100 μM NMDA progressively increased over the first 5 min of recording, from 10·6 to 16·8 nA, and then remained stable. The first four applications of NMDA also produced an increase in the steady-state current, similar in proportion to the increase in peak amplitude, after which steady-state responses continually decreased during the course of whole-cell recording (Fig. 1*A* and *B*). Responses to NMDA, recorded 3–6 min after whole-cell formation, exhibited 22·6 ± 6·6 % desensitization (pooled results from thirty neurones with Mg^{2+}- and ATP-free ICS). Subsequent responses to NMDA, recorded at 30 s intervals, showed a progressive increase in the degree of desensitization, as illustrated in Fig. 1*A*. To characterize the rate of change of NMDA receptor desensitization, both the amount of desensitization and the desensitization rate were plotted as a function of time (Fig. 1*C* and *D*). Figure 1*E* shows a strong positive correlation between these parameters (correlation coefficient, $r = 0·97$). After responses to NMDA reached their maximum amplitude, the peak value was either constant in amplitude (2/5 neurones) or decreased slowly (by 15·8 ± 3·8 % over 10 min; $n = 3$).

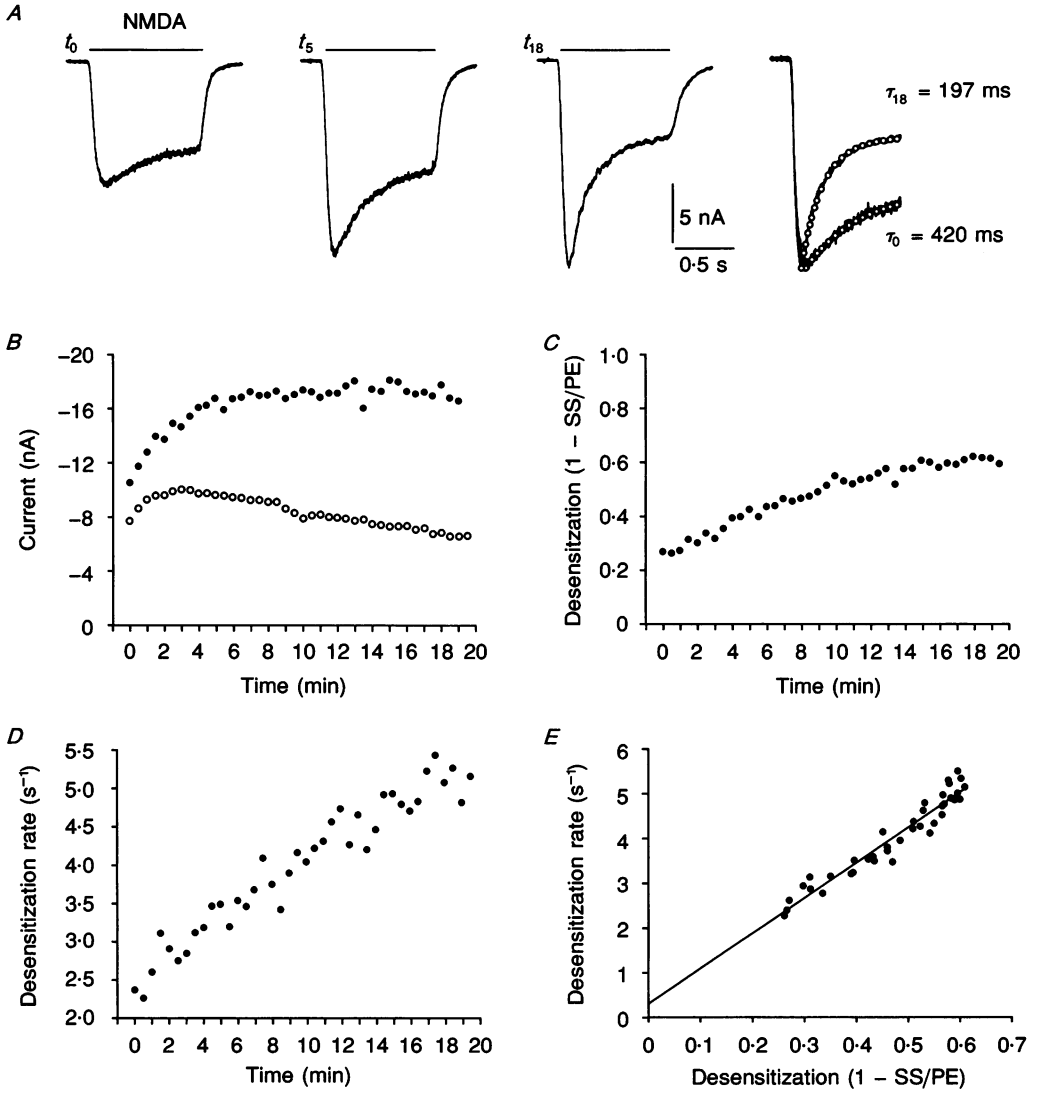

Fig. 1. Time course of changes in the kinetics of NMDA receptor responses. *A* compares the first response of a cultured hippocampal neurone to the application of 100 μM NMDA (t_0) to responses recorded 5 min (t_5) and 18 min (t_{18}) later. ECS contained 0·2 mM Ca^{2+} during NMDA applications and 2 mM Ca^{2+} in between NMDA applications. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 5 EDTA and no added Mg^{2+}. NMDA was applied every 30 s during the course of whole-cell recording. On the right, NMDA responses recorded at t_0 and t_{18} were normalized to the same peak amplitude and are shown overlaid to emphasize the increase in the desensitization rate. τ values were determined by single-exponential analysis; fits are shown by ○. For *B*, *C* and *D*, peak (PE; ●) and steady-state (SS; ○) currents, desensitization (1 − SS/PE), and the desensitization rate ($1/\tau$) are plotted *versus* time of recording. *E* illustrates the linear correlation between the degree of desensitization (*D*) and desensitization rate (k_D) ($k_D = 0{\cdot}31 + 7{\cdot}7 \times D$; $r = 0{\cdot}97$).

The behaviour illustrated in Fig. 1 was typical of the majority of experiments, although the degree of potentiation of the peak current during the first few minutes of recording was variable (mean 23·4 ± 21·1 %, $n = 30$). Such potentiation is likely to be due to a reduction of disulphide bonds by dithiothreitol (DTT) added

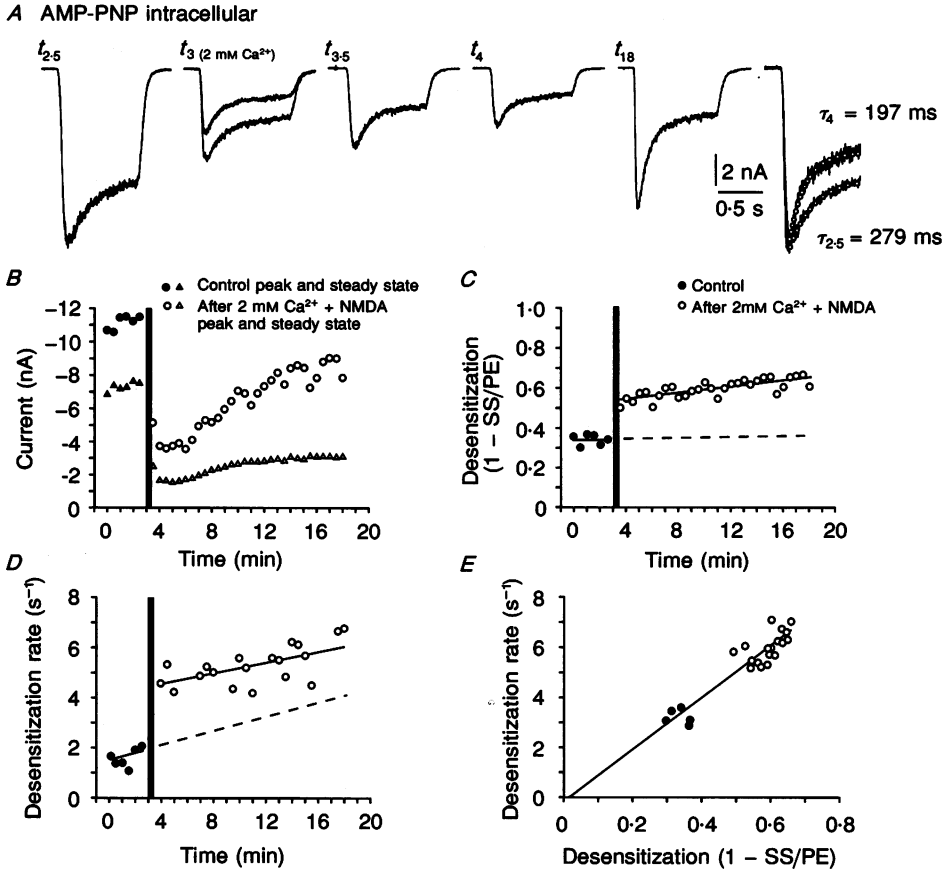

Fig. 2. Modulation of NMDA responses by sustained receptor activation in the presence of 2 mM Ca^{2+}. *A*, responses to 1 s applications of 100 μM NMDA with ECS containing 0·2 mM Ca^{2+} at times 2·5 ($t_{2·5}$), 3·5 ($t_{3·5}$), 4 (t_4) and 18 min (t_{18}) after the first application of agonist; NMDA was applied every 30 s. At 3 min a conditioning train of ten applications of 100 μM NMDA was delivered in the presence of ECS containing 2 mM Ca^{2+} (2nd and 10th responses are displayed superimposed). On the right, NMDA responses recorded at times 2·5 and 4 min were normalized to the same peak amplitude and are shown overlaid to emphasize the increase in the desensitization rate immediately after the train of NMDA applications in the presence of 2 mM Ca^{2+}. The onset of desensitization was determined by single-exponential analysis; fits are shown by ○. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+} and 1 AMP-PNP. For *B*, *C* and *D* peak (●,○) and steady-state (▲,△) current responses, the degree of desensitization, and the desensitization rate were plotted *versus* time of recording. The vertical bar indicates the time when the conditioning train of NMDA was delivered in the presence of ECS with 2 mM Ca^{2+}. *E* illustrates the linear correlation between the degree of desensitization (D) and the desensitization rate (k_D) ($k_D = -0·27 + 10·49 \times D$; $r = 0·94$). Filled symbols represent control data; open symbols data after the train of NMDA applications. The time nomenclature used in *A* applies to all subsequent figures.

to the extracellular solution (Aizenman *et al.* 1989). When a disulphide reducing agent was not used, the degree of potentiation was small and usually only the slow decline of responses to NMDA was observed. However, the finding of Sather *et al.* (1992) that in some cells there was potentiation of the peak response to 1 mM NMDA even in the absence of disulphide reducing agents, suggests that there may be other mechanisms involved in controlling NMDA receptor activity. The potentiation of NMDA responses was not examined further in the present experiments and the modulation of desensitization was analysed only after responses to NMDA reached a stable value.

The effect of a conditioning train of NMDA applications on subsequent test responses to NMDA

The mechanism(s) underlying the time-dependent increase in desensitization of responses to NMDA have not yet been identified. Previous experiments suggested that a cytoplasmic component controlling desensitization of NMDA receptors is washed out during prolonged whole-cell recording (Sather *et al.* 1990, 1992; Benveniste *et al.* 1990; Mayer *et al.* 1991). This would predict that the rate of onset of NMDA receptor desensitization would be solely a function of time after the start of whole-cell recording. Alternatively, NMDA-induced increases in $[Ca^{2+}]_i$ (Mayer, MacDermott, Westbrook, Smith & Barker, 1987) could trigger Ca^{2+}-dependent phosphorylation or dephosphorylation processes that in turn could modulate NMDA receptors (Chen & Huang, 1991). This would predict that the development of NMDA receptor desensitization should be a function of $[Ca^{2+}]_i$.

Figure 2 shows test responses to 100 μM NMDA applied at 30 s intervals in the presence of 0·2 mM Ca^{2+} before and after a conditioning train of ten 1 s applications of 100 μM NMDA delivered in the presence of 2 mM Ca^{2+}. In order to test for a possible effect of Ca^{2+}-regulated phosphorylation on NMDA receptor activity, the non-hydrolysable ATP analogue AMP-PNP was included in the intracellular solution. The test response to NMDA, recorded < 5 s after the end of the conditioning train, showed a strong diminution of the peak current. The average inactivation, expressed as [1 − (minimum peak response after the train/peak response before the train)] × 100, was $60{\cdot}3 \pm 9{\cdot}7$ % ($n = 5$). In this cell subsequent responses to NMDA recorded at 30 s intervals were still suppressed by 69 % 1 min later, and recovered slowly, over 14 min, to 78 % of the control response (Fig. 2*A*). In other cells the response recovered completely, or became larger than the control (range 76–150 %).

The behaviour illustrated in Fig. 2*A* and *B* was typical in that the first response to NMDA, recorded 2 s after the train, was larger than the subsequent responses. It is not clear whether this was due to continued development of NMDA receptor inactivation after the end of the conditioning train or the decay of a short-lasting potentiation of responses to NMDA, but responses tested 10 s after the conditioning train did not show this effect.

Figure 2*C* illustrates the effect of a conditioning train of NMDA applications in the presence of 2 mM Ca^{2+} on the degree of desensitization of responses to NMDA, which increased from 32 % before the train to 52 % after the train (the mean increase was $13{\cdot}8 \pm 6{\cdot}1$ %, $n = 5$). Desensitization of NMDA responses during the

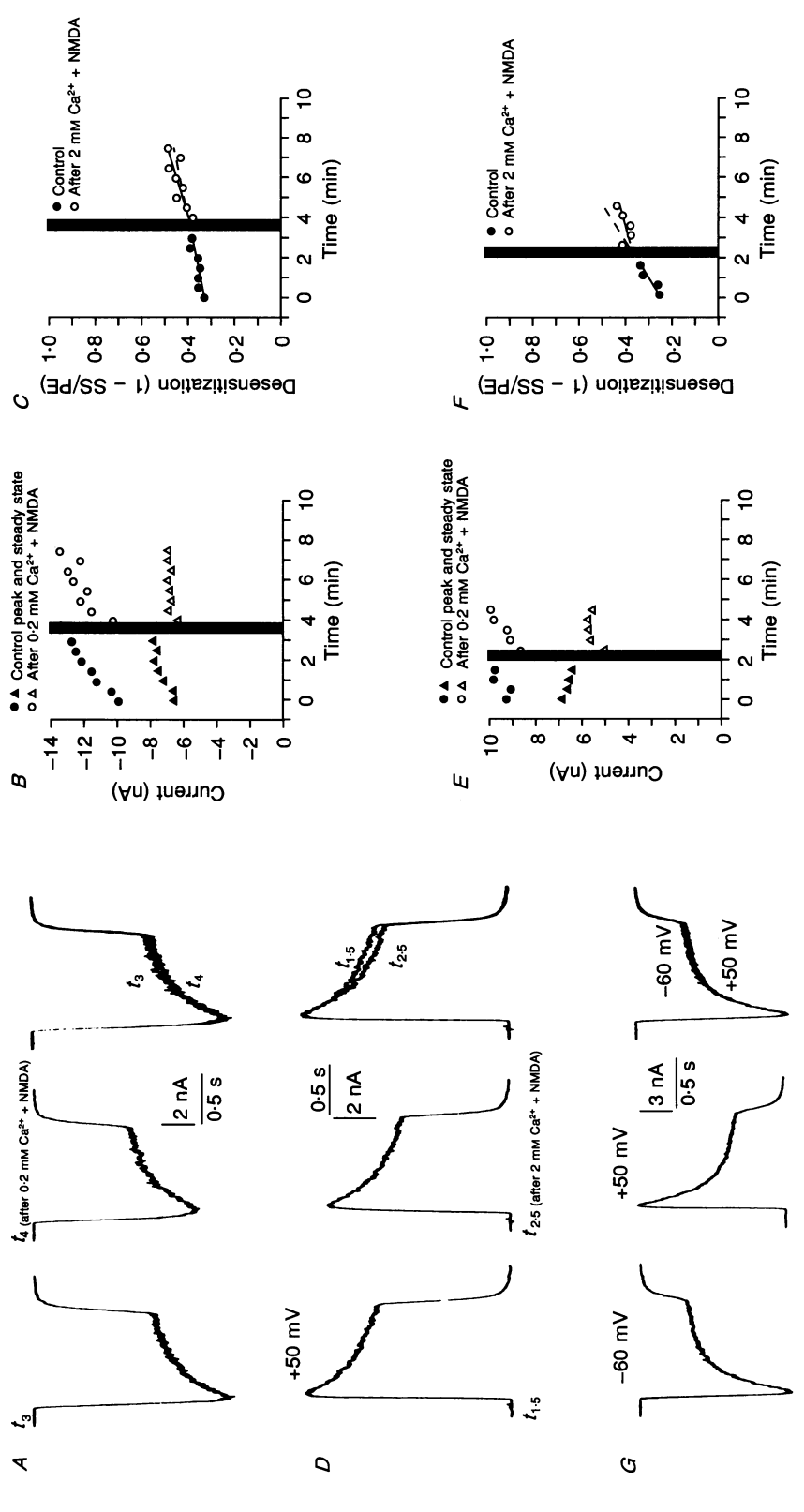

Fig. 3. For legend see facing page.

rising phase of the response to agonist is unlikely markedly to reduce the peak current because the experimentally measured increase in desensitization induced by a train of NMDA applications (usually $\approx 10\%$) was increased by only 0·4 % more if calculated from peak values extrapolated to the start of application of agonist. The increase in desensitization after the train was accompanied by a corresponding increase in the desensitization rate (Fig. 2*D*). Unlike inactivation illustrated in Fig. 2*B*, the increase in desensitization showed no recovery.

To determine the extent to which extracellular Ca^{2+} is involved in the induction of desensitization and inactivation of NMDA receptors, conditioning trains of NMDA pulses were delivered in the presence of 0·2 mM Ca^{2+}. This induced only a small increase in inactivation ($15{\cdot}9 \pm 10{\cdot}5\%$, $n = 4$) and desensitization ($4{\cdot}5 \pm 4{\cdot}4\%$, $n = 4$) as shown in Fig. 3.

These experiments did not determine whether Ca^{2+} acts at extracellular or intracellular sites to promote inactivation and to enhance desensitization of NMDA receptors. If Ca^{2+} acts at an extracellular site then both desensitization and inactivation should be inducible at positive holding potentials. To test this, the responses to a conditioning train of NMDA pulses applied in the presence of 2 mM $[Ca^{2+}]_o$ was recorded at + 50 mV (Fig. 3*D*, *E* and *F*), and revealed no effect on desensitization ($-0{\cdot}7 \pm 3{\cdot}3\%$, $n = 4$) and only a small effect on the degree of inactivation ($10{\cdot}7 \pm 18{\cdot}9\%$, $n = 4$). In order to prevent an increase in $[Ca^{2+}]_i$ due to activation of voltage-dependent Ca^{2+} channels, 1 mM Cd^{2+} was applied during the change in holding potential from −60 to + 50 mV. These experiments at + 50 mV might fail to show induction of desensitization if desensitization was intrinsically voltage dependent; this was not the case, since desensitization previously induced by a conditioning train of NMDA pulses applied in the presence of 2 mM Ca^{2+} was

Fig. 3. The induction of NMDA receptor desensitization and inactivation was suppressed in the presence of 0·2 mM Ca^{2+} or at a holding potential of + 50 mV. *A*, test responses to 100 μM NMDA recorded before and after a train of ten applications of NMDA delivered in the presence of ECS with 0·2 mM Ca^{2+}. On the right, NMDA responses recorded at 3 and 4 min after the first response to NMDA were normalized to the same peak amplitude and are shown overlaid; the holding potential was −60 mV. *B* and *C*, peak and steady-state responses, and desensitization are plotted *versus* time of recording after the first response to NMDA. The vertical bar indicates the time when the conditioning train of NMDA applications was delivered in the presence of ECS with 0·2 mM Ca^{2+}. *D*, responses to fast application of 100 μM NMDA at + 50 mV before and after a conditioning train of 100 μM NMDA applied at 0·5 Hz in the presence of ECS with 2 mM Ca^{2+}; on the right both responses are shown overlaid and normalized to the same peak amplitude. *E* and *F*, graph of peak (●,○) and steady-state (▲,△) responses, and the degree of desensitization *versus* time of recording. The vertical bar indicates the time when the conditioning train of NMDA pulses was delivered. During the change in holding potential from −60 to + 50 mV, 1 mM Cd^{2+} was applied to block Ca^{2+} influx through voltage-dependent Ca^{2+} channels. *G*, responses to 100 μM NMDA from a different neurone where desensitization was previously induced by a conditioning train of NMDA applications at a holding potential of −60 mV in the presence of 2 mM Ca^{2+}. As can be seen in comparisons of responses at −60 and + 50 mV, normalized and superimposed on the right, the desensitization of NMDA receptors was not voltage dependent. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 5 EDTA and no added Mg^{2+}.

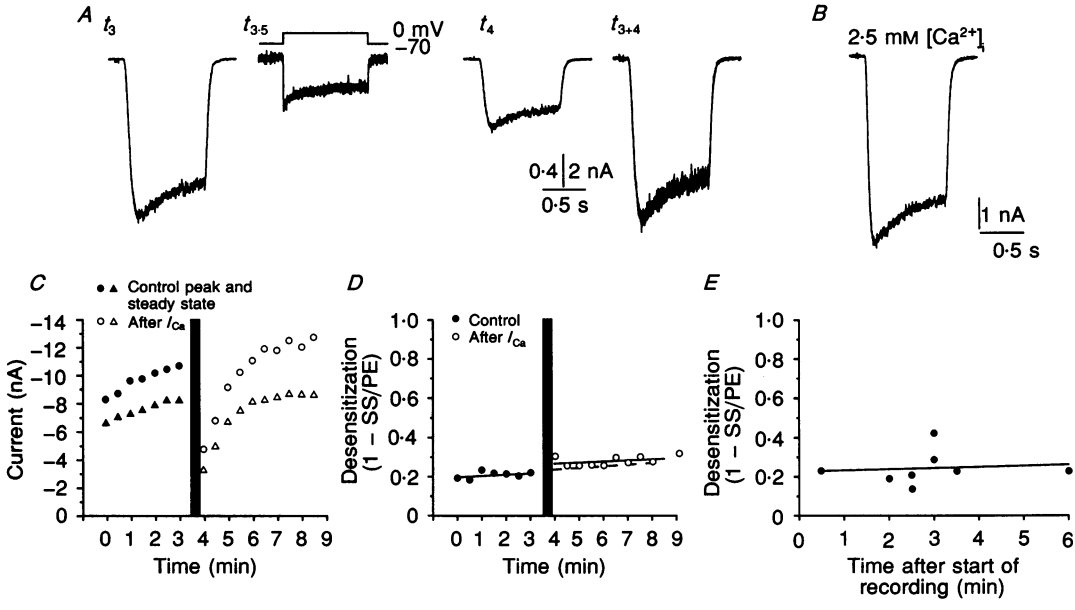

Fig. 4. The effect of increased $[Ca^{2+}]_i$ on NMDA receptor responses. *A*, NMDA responses recorded in the presence of ECS with 0·2 mM Ca^{2+} before and after activation of Ca^{2+} currents by a train of ten 1 s depolarizing steps to 0 mV from a holding potential of −70 mV in the presence of ECS with 2 mM Ca^{2+}. The first Ca^{2+} current response is illustrated ($t_{3\cdot5}$), with the voltage step shown above the current trace. Note that the calibration bar of 0·4 nA relates to the Ca^{2+} current and 2 nA to the NMDA currents. On the right, NMDA responses recorded at 3 (t_3) and 4 min (t_4) after the first response to NMDA were normalized to the same peak amplitude and are shown overlaid. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 3 Mg^{2+}, 1·5 ATP and 100 μM cAMP (cAMP was used to reduce run-down of Ca^{2+} currents). *B*, the first response to 100 μM NMDA recorded from a different neurone with ECS containing 0·2 mM Ca^{2+} and ICS containing 2·5 mM Ca^{2+} (5 mM EGTA with 7·5 mM Ca^{2+}) and no added Mg^{2+}, 6 min after whole-cell formation. *C* and *D* show graphs of peak (●,○) and steady-state (▲,△) responses illustrated in *A*, and the degree of desensitization *versus* time of recording. The vertical bar indicates the time when the conditioning train of ten voltage steps was applied. The train of Ca^{2+} currents induced 55·5 % inactivation of NMDA currents but only increased desensitization of NMDA receptors by 3·7 %. *E*, the degree of NMDA receptor desensitization to application of 100 μM NMDA is plotted as a function of time after the start of whole-cell recording with ICS containing 2·5 mM Ca^{2+}; each data point represents a different neurone. The lack of correlation ($D = 0{\cdot}225 + 0{\cdot}005 \times t$) between the degree of desensitization (D) and the time (t) after the start of whole-cell recording suggests that desensitization is essentially independent of time.

essentially voltage independent (see Fig. 3*G*). Together, the above results suggest that a rise in $[Ca^{2+}]_i$ is important for the induction of inactivation and desensitization, but did not determine whether activation of NMDA receptors plays a role independent of a rise in $[Ca^{2+}]_i$.

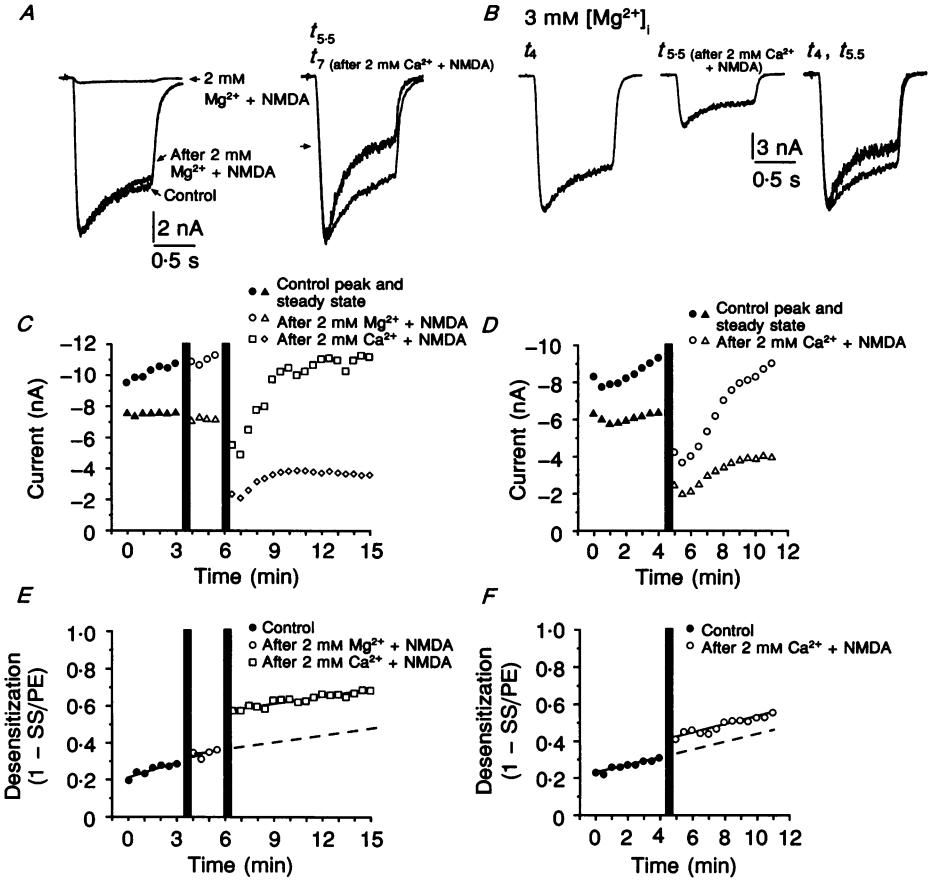

Fig. 5. The effect of Mg^{2+} on induction of inactivation and desensitization of NMDA responses. *A*, on the left, superimposed responses to 100 μM NMDA before (control) and immediately after a conditioning train of ten NMDA applications delivered in the presence of ECS with 2 mM Ca^{2+} and 2 mM Mg^{2+}. On the right, superimposed test responses before and after a conditioning train of ten NMDA applications delivered in the presence of ECS with 2 mM Ca^{2+} but without added Mg^{2+}. The response to NMDA recorded at time 7 min was normalized with respect to the peak response before the train; the actual value of the peak response is indicated by an arrow. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 5 EDTA and no added Mg^{2+}. Holding potential was −60 mV. *B*, responses to NMDA recorded with ICS containing (mM): 5 EGTA, 0·5 Ca^{2+} and 3 Mg^{2+}, before and after a conditioning train of NMDA applications delivered in the presence of ECS with 2 mM Ca^{2+}. On the right, NMDA responses recorded at 4 and 5·5 min were normalized to the same peak amplitude and are shown overlaid. *C* and *D* show peak (●,○,□) and steady-state (▲,△,◇) responses to NMDA illustrated in *A* and *B*, respectively, plotted *versus* time of recording. In *C*, the vertical bar at 3·5 min indicates the time when the conditioning train of NMDA was delivered in the presence of ECS with 2 mM Ca^{2+} and 2 mM Mg^{2+}; the vertical bar at 6 min in *C* and at 4·5 min in *D* indicates when the conditioning train of ten NMDA pulses was delivered in the presence of ECS with 2 mM Ca^{2+} and no added Mg^{2+}. *E* and *F*, desensitization of NMDA responses illustrated in *A* and *B*, respectively, *versus* time of recording. The vertical bars have the same meaning as in *C* and *D*.

In the next series of experiments a train of ten 1 s voltage steps from −70 to 0 mV was used to activate voltage-gated Ca^{2+} channels in order to increase $[Ca^{2+}]_i$ independent of NMDA receptor activation. The peak amplitude of NMDA responses was initially suppressed following activation of Ca^{2+} currents, but showed recovery (Fig. 4 *A* and *C*), indicating that a rise in $[Ca^{2+}]_i$ can reversibly inactivate NMDA receptors. However, the average inactivation ($35{\cdot}3 \pm 15{\cdot}5$ %, $n = 5$) was smaller than inactivation induced by a conditioning train of NMDA pulses applied in the presence of 2 mM Ca^{2+} (Fig. 2), perhaps due to a relatively smaller Ca^{2+} load or a different localization of NMDA receptors and voltage-gated Ca^{2+} channels. In contrast to its effect on inactivation, a train of voltage-dependent Ca^{2+} currents did not affect NMDA receptor desensitization, which changed only $1{\cdot}5 \pm 1{\cdot}9$ % ($n = 5$) as shown in Fig. 4 *A* and *D*. However, in the same cells it was possible subsequently to increase the degree of desensitization with a conditioning train of NMDA pulses applied in the presence of 2 mM Ca^{2+}. Furthermore, when using intracellular solution containing 2·5 mM Ca^{2+}, the degree of NMDA receptor desensitization in response to the initial application of NMDA was similar ($24{\cdot}1 \pm 8{\cdot}3$ %, $n = 8$) to that for neurones studied using a solution containing 10 nM $[Ca^{2+}]_i$ ($22{\cdot}6 \pm 6{\cdot}6$ %, $n = 30$), and essentially independent of time after whole-cell formation ($D = 0{\cdot}225 + 0{\cdot}005 \times t$) as shown in Fig. 4 *B* and *E*. Together, these results suggest that, while raising $[Ca^{2+}]_i$ by any means can induce reversible inactivation of NMDA receptors, both an increase in $[Ca^{2+}]_i$ and concurrent activation of NMDA receptors is required to increase desensitization.

The effect of Mg^{2+}, Sr^{2+} and Ba^{2+} on inactivation and desensitization of responses to NMDA

Another series of experiments was performed to evaluate whether inactivation and desensitization of NMDA receptors could be modulated by Mg^{2+}, a voltage-dependent blocker of NMDA receptor ion channels. A conditioning train of NMDA pulses applied in the presence of both 2 mM $[Mg^{2+}]_o$ and 2 mM $[Ca^{2+}]_o$ neither increased desensitization ($-0{\cdot}3 \pm 3{\cdot}0$ %, $n = 4$) nor induced inactivation ($-1{\cdot}2 \pm 2{\cdot}9$ %) of NMDA receptor responses (see Fig. 5 *A*, *C* and *E*). These results further support the hypothesis that desensitization and inactivation of NMDA receptors are induced by an increase in $[Ca^{2+}]_i$ and do not occur if the Ca^{2+} flux through NMDA channels is blocked by Mg^{2+}. Subsequent experiments were designed to explore the effect of intracellular Mg^{2+} on inactivation and desensitization induced by a conditioning train of NMDA applications. The addition of 3 mM Mg^{2+} to the intracellular solution did not prevent induction of desensitization ($10{\cdot}0 \pm 3{\cdot}1$ %, $n = 4$) nor inactivation ($58{\cdot}4 \pm 6{\cdot}2$ %) by a conditioning train of NMDA pulses applied in the presence of 2 mM Ca^{2+} (Fig. 5 *B*, *D* and *F*).

Sr^{2+} and Ba^{2+} are known to permeate NMDA channels (Mayer & Westbrook, 1987; Ascher & Nowak, 1988). To test whether these divalent ions could modulate NMDA receptors in a similar way to Ca^{2+}, a conditioning train of 100 μM NMDA pulses was delivered in the presence of either Sr^{2+} or Ba^{2+}; no other divalent ion was present. The effect of 2 mM Sr^{2+} on subsequent test responses to NMDA recorded in the presence of 0·2 mM Ca^{2+} is illustrated in Fig. 6 *A*, *C* and *E*. Sr^{2+} produced effects similar to those of Ca^{2+} and induced both inactivation ($61{\cdot}6 \pm 3{\cdot}8$ %, $n = 4$) and desensitization ($18{\cdot}8 \pm 7{\cdot}9$ %). When compared with the action of Ca^{2+}, recovery

from Sr^{2+}-induced inactivation was faster in all neurones studied and test responses recorded immediately after the conditioning train always increased in amplitude, with no evidence for a short-lasting potentiation as observed with Ca^{2+} (Fig. 6 *A* and *C*). A conditioning train of NMDA applied in the presence of 2 mM Ba^{2+} also

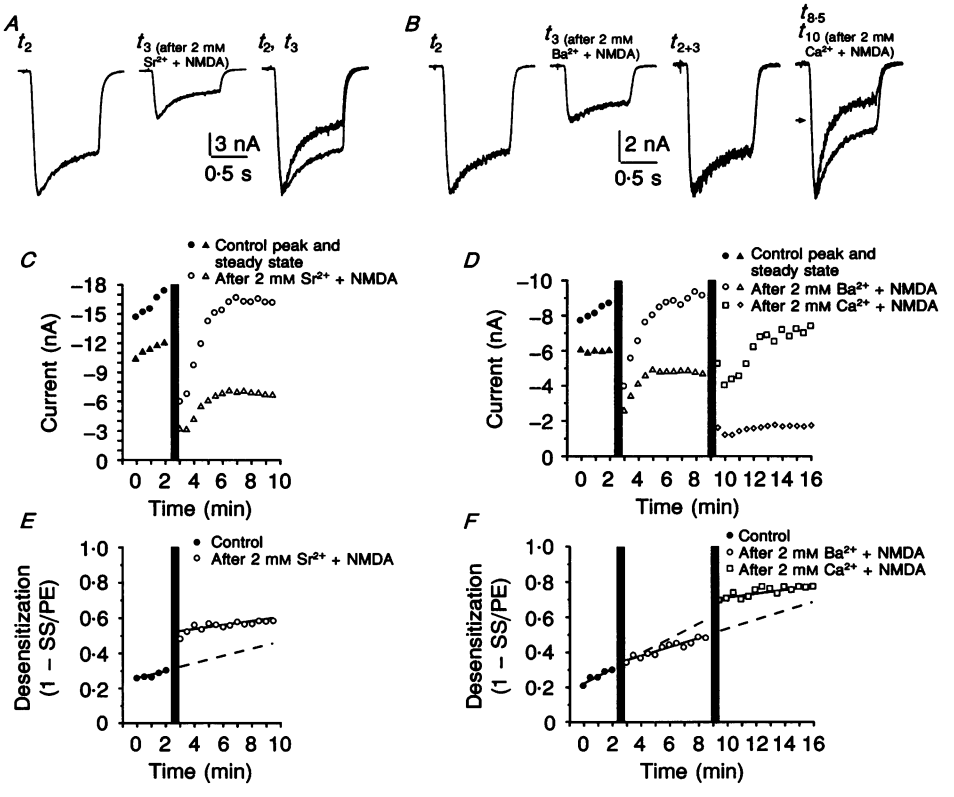

Fig. 6. Sr^{2+} and Ba^{2+} have different effects on desensitization of NMDA responses. *A*, test responses to 100 μM NMDA before and immediately after a conditioning train of ten NMDA applications delivered in the presence of ECS with 2 mM Sr^{2+} and no added Ca^{2+}. On the right, NMDA responses recorded at 2 and 3 min were normalized to the same peak amplitude and are shown overlaid. *B*, responses to NMDA recorded in the presence of ECS with 0·2 mM Ca^{2+} before and immediately after a conditioning train of NMDA pulses delivered in the presence of ECS with 2 mM Ba^{2+} and no added Ca^{2+}. Responses recorded at 2 and 3 min were normalized to the same peak amplitude and are shown overlaid; note that Ba^{2+} did not increase desensitization of NMDA receptors. On the right, superimposed NMDA responses recorded in the presence of ECS with 0·2 mM Ca^{2+} before and after delivery of a conditioning train of ten NMDA pulses in the presence of ECS with 2 mM Ca^{2+} but without added Ba^{2+}. The response to NMDA recorded at 10 min was normalized with respect to the peak response before the train; the actual value of the peak is indicated by an arrow. ICS contained 5 mM EGTA and 0·5 mM Ca^{2+} for both *A* and *B*. *C* and *D*, peak (●,○,□) and steady-state (▲,△,◇) responses to NMDA illustrated in *A* and *B*, respectively, plotted versus time of recording. In *C* the vertical bar at 3·5 min indicates the time when the conditioning train of NMDA pulses was applied in the presence of 2 mM Sr^{2+}. In *D*, vertical bars at 2·5 and 9 min indicate conditioning trains of NMDA in the presence of 2 mM Ba^{2+} and 2 mM Ca^{2+}, respectively. *E* and *F*, desensitization of NMDA responses illustrated in *A* and *B*, respectively, versus time of recording. The vertical bars have the same meaning as in *C* and *D*.

induced inactivation of NMDA receptors (Fig. 6*D*; 51·5 ± 12·1 %, $n = 5$) similar to the effect of Sr^{2+} and Ca^{2+}, but as shown in Fig. 6*F* Ba^{2+} did not enhance NMDA receptor desensitization (1·8 ± 2·8 %). The lack of effect of Ba^{2+} on NMDA receptor desensitization was not due to an irreversible inactivation of the mechanism

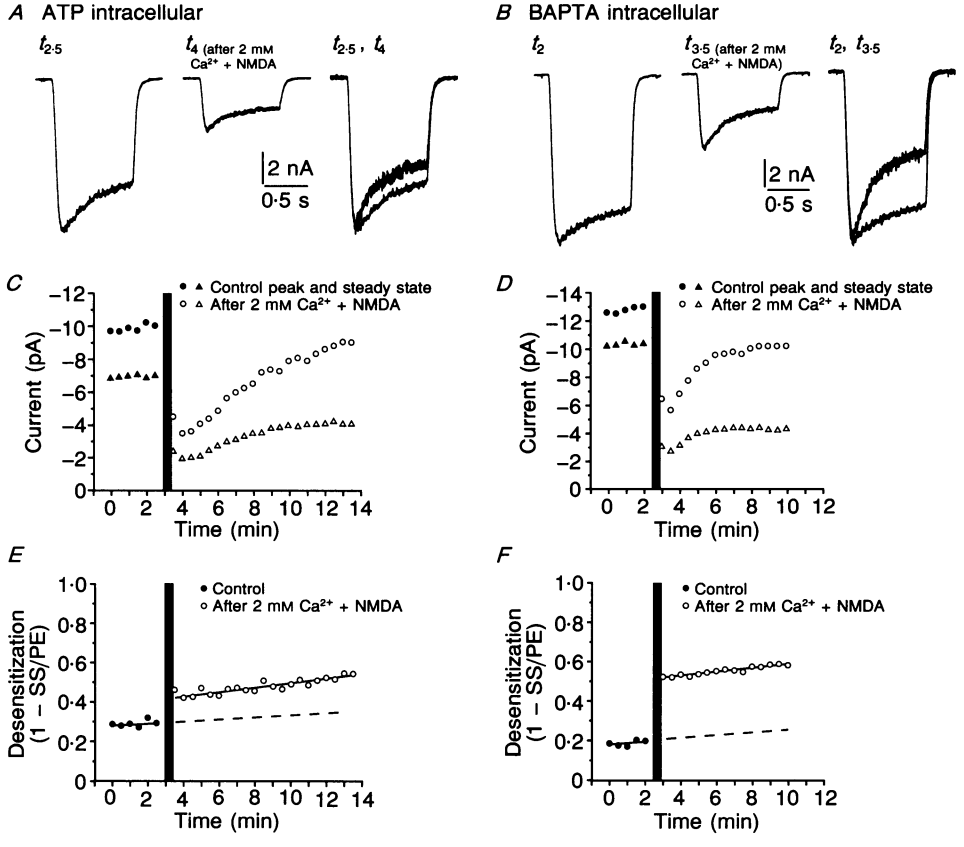

Fig. 7. The effect of intracellular ATP and BAPTA on induction of inactivation and desensitization of NMDA responses. Responses to 100 μM NMDA before and after a conditioning train of ten NMDA applications delivered in the presence of ECS with 2 mM Ca^{2+}. On the right, both NMDA responses are shown overlaid and normalized to the same peak amplitude. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 1·5 ATP, 3 Mg^{2+} (*A*) or 5 BAPTA, 0·5 Ca^{2+}, 5 EDTA and no added Mg^{2+} (*B*). *C* and *D* show peak (●,○) and steady-state (▲,△) responses to NMDA illustrated in *A* and *B*, respectively, plotted versus time of recording. The vertical bar indicates when the train of NMDA pulses was applied in the presence of 2 mM Ca^{2+}. *E* and *F*, desensitization of NMDA responses illustrated in *A* and *B*, respectively, *versus* time of recording. The vertical bars have the same meaning as in *C* and *D*.

controlling desensitization, because in the same cells it was possible subsequently to induce desensitization with a conditioning train of NMDA pulses applied in the presence of 2 mM Ca^{2+} (Fig. 6*B* and *F*). The fact that Ba^{2+} or the activation of voltage-gated Ca^{2+} channels (see Fig. 4) induced inactivation of NMDA responses but did not increase desensitization suggests that the desensitization and inactivation are independent processes.

Inactivation and desensitization are not influenced by intracellular ATP and BAPTA

Wash-out of NMDA receptor activity (MacDonald, Mody & Salter, 1989) has been reported to be greatly retarded when high-energy phosphates are used in

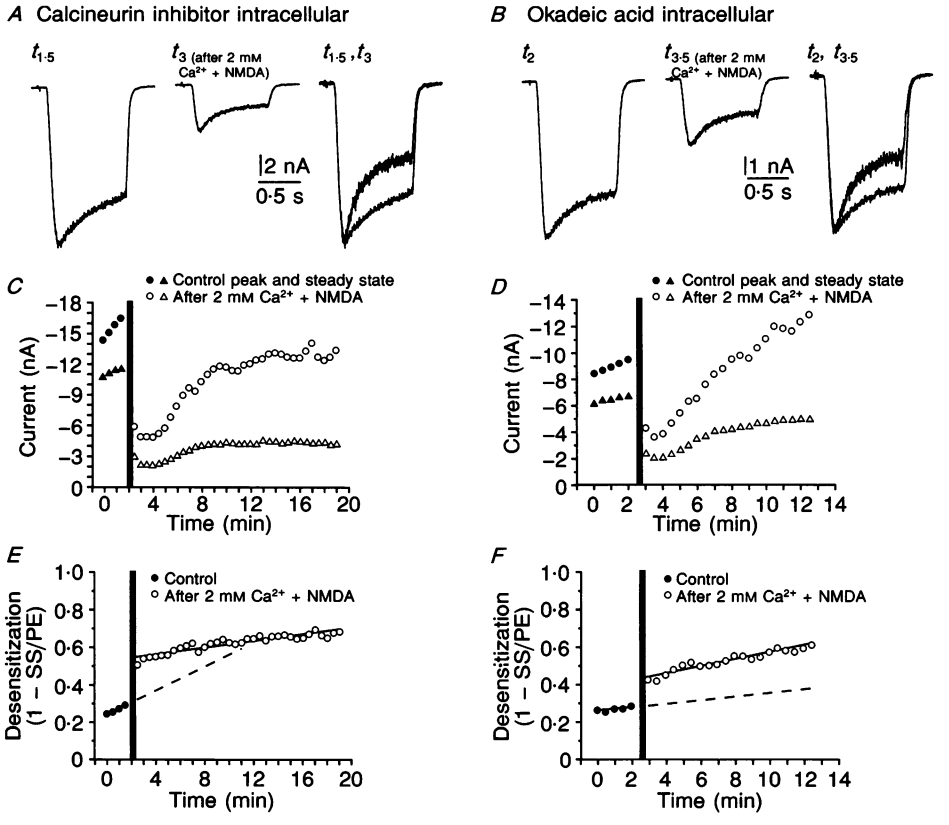

Fig. 8. Inactivation and desensitization of NMDA responses is not affected by protein phosphatase inhibitors. Responses to 100 μM NMDA before and after a conditioning train of ten NMDA applications that was delivered in the presence of ECS with 2 mM Ca^{2+}. On the right, both NMDA responses are shown overlaid and normalized to the same peak amplitude. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 5 EDTA, no added Mg^{2+} and 250 μM calcineurin inhibitor (*A*) or 5 EGTA, 0·5 Ca^{2+}, 5 EDTA, no added Mg^{2+} and 1 μM okadaic acid (*B*). *C* and *D* show peak (●,○) and steady-state (▲,△) responses to NMDA illustrated in *A* and *B*, respectively, plotted *versus* time of recording. The vertical bar indicates when the conditioning train of NMDA pulses was applied in the presence of 2 mM Ca^{2+}. *E* and *F*, desensitization of NMDA responses illustrated in *A* and *B*, respectively, *versus* time of recording. The vertical bars have the same meaning as in *C* and *D*.

intracellular solutions. To investigate the possibility that ATP could modulate the induction and maintenance of desensitization of NMDA receptors 1·5 mM ATP and 3 mM Mg^{2+} were included in the intracellular solution. As shown in Fig. 7*A*, *C* and *E*, the increase in desensitization ($10\cdot9 \pm 4\cdot1$ %, $n = 5$) and magnitude of inactivation ($54\cdot4 \pm 6\cdot2$ %, $n = 5$) induced by a conditioning train of NMDA was not significantly influenced by intracellular MgATP. Intracellular MgATP also

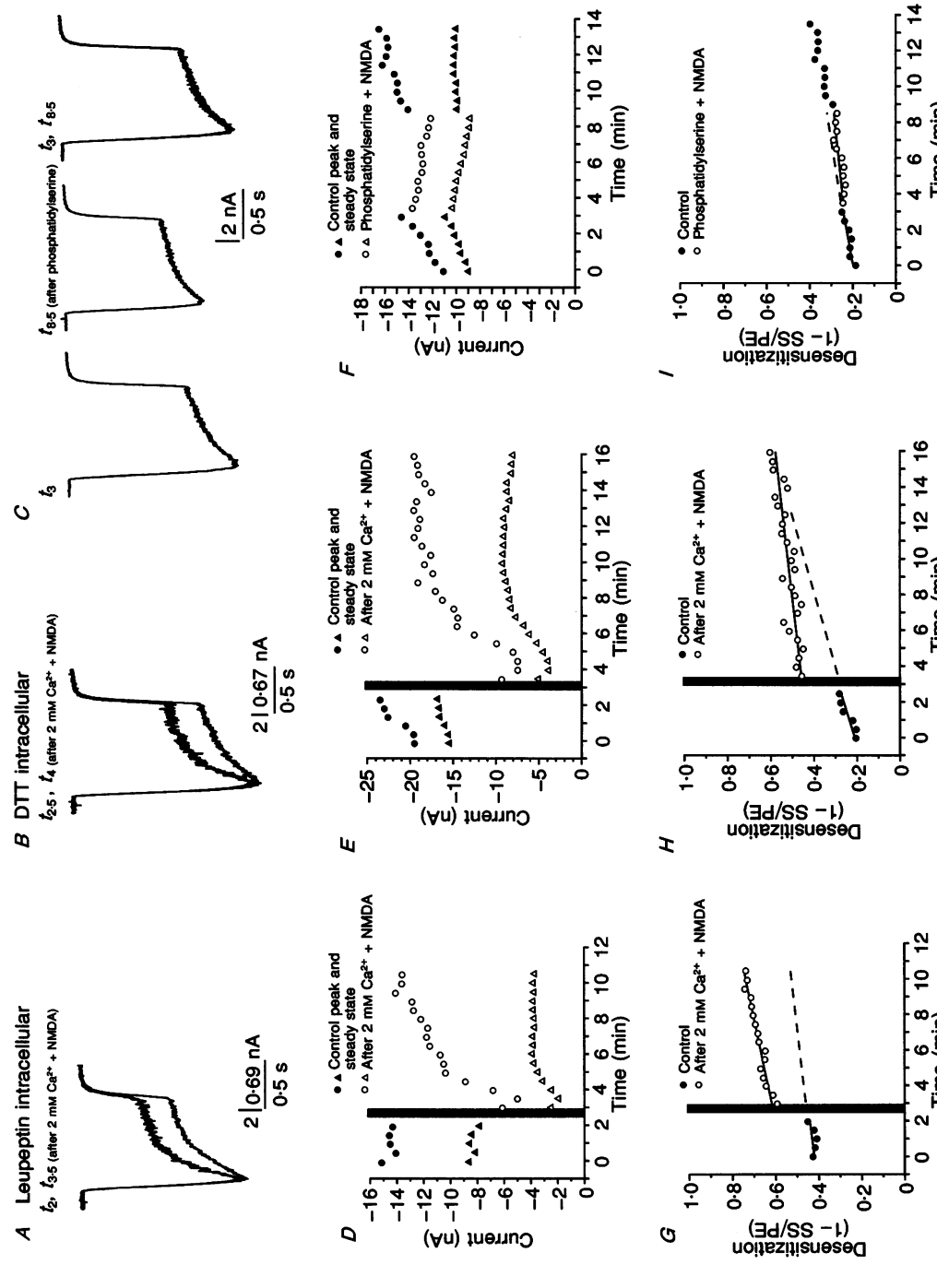

Fig. 9. For legend see facing page.

failed to reverse the progressive increase in desensitization of NMDA receptor responses which occurs during whole-cell recording (Fig. 7*E*). Similar results were obtained in five neurones.

These results are consistent with the hypothesis that $[Ca^{2+}]_i$ participates in the induction of NMDA receptor inactivation and desensitization. Alternatively, the observed NMDA receptor modulation might be caused by H^+, due to a change in the intracellular pH caused by binding of Ca^{2+} to EGTA. To exclude this possibility, BAPTA, which binds Ca^{2+} without releasing protons (Tsien, 1980), was used in place of EGTA. In the presence of BAPTA (5 mM) neither the degree of inactivation ($45{\cdot}2 \pm 14{\cdot}4$ %, $n = 4$) nor desensitization ($23{\cdot}7 \pm 7{\cdot}9$ %) of NMDA receptors induced by a conditioning train of agonist was significantly reduced relative to that observed in the presence of EGTA.

Ca^{2+}-induced modulation of NMDA receptors is neither prevented by protein phosphatases, peptidase inhibitors and DTT nor mimicked by phosphatidylserine

The next series of experiments focused on the potential mechanism by which Ca^{2+}-dependent inactivation and desensitization of NMDA receptors could be induced, including Ca^{2+}-mediated dephosphorylation, proteolysis, changes in the lipid environment around the receptor–channel complex, and on oxidation of functionally important sites on the NMDA receptor.

Protein phosphorylation and dephosphorylation is an important regulatory mechanism for the control of ion channel activity (Huganir & Greengard, 1990). To test whether inhibition of Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin; for review see Klee, Draetta & Hubbard, 1988) could interfere with the modulation of NMDA responses induced by a conditioning train of NMDA applied in the presence of 2 mM $[Ca^{2+}]_o$, a calcineurin inhibitor (250 μM; peptide 3; Hashimoto, Perrino & Soderling, 1990) was included in the intracellular solution. Peptide 3 did not prevent the induction of desensitization ($21{\cdot}8 \pm 9{\cdot}0$ %) or inactivation ($64{\cdot}8 \pm 8{\cdot}1$ %, $n = 5$) of NMDA receptor responses (Fig. 8*A*, *C* and *E*). To test whether protein phosphatase 1 and protein phosphatase 2A are involved in

Fig. 9. Ca^{2+}-induced modulation of NMDA receptors is not prevented by peptidase inhibitors or DTT or mimicked by phosphatidylserine. Responses to 100 μM NMDA before and after a conditioning train of ten NMDA applications in the presence of ECS with 2 mM Ca^{2+} are shown overlaid and normalized to the same peak amplitude. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 5 EDTA, no added Mg^{2+} and 250 μM leupeptin (*A*) or 5 EGTA, 0·5 Ca^{2+}, 5 EDTA, no added Mg^{2+} and 1 DTT (*B*). Note that the 2 nA calibration bar in *A* and *B* relates to the NMDA response before the conditioning train and the other calibration to the records after the train. *C*, NMDA responses recorded in the presence of ECS with 0·2 mM Ca^{2+} and after 5·5 min perfusion with ECS containing 0·2 mM Ca^{2+} and 30 μM phosphatidylserine. On the right, both NMDA responses are shown overlaid and normalized to the same peak amplitude. ICS contained (mM): 5 EGTA, 0·5 Ca^{2+}, 5 EDTA and no added Mg^{2+}. *D*, *E* and *F* show peak (●,○) and steady-state (▲,△) responses to NMDA illustrated in *A*, *B* and *C*, respectively, *versus* time of recording. The vertical bars in *D* and *E* indicate when the train of NMDA applications was applied in the presence of 2 mM Ca^{2+}. Open symbols in *F* indicate peak and steady-state responses recorded in the presence of phosphatidylserine. *G*, *H* and *I* show plot of desensitization of NMDA responses illustrated in *A*, *B* and *C*, respectively, *versus* time of recording. The vertical bars and open symbols have the same meaning as in *D*, *E* and *F*.

the modulation of NMDA responses okadaic acid was added to the intracellular solution. Okadaic acid at a concentration of 1–6 μM, which should completely inhibit activity of both proteases (Cohen, Holmes & Tsukitani, 1990), did not prevent the increase in desensitization of NMDA receptors (15·8 ± 4·2 %) or the induction of inactivation of responses to NMDA (52·1 ± 12·6 %, $n = 6$) induced by a conditioning train of agonist applications (Fig. 8*B*, *D* and *F*).

It is now recognized that Ca^{2+}-activated neutral proteases such as calpain may play a regulatory rather than degradative role in cellular protein metabolism (Croall & Demartino, 1991). These proteases are also activated by NMDA receptor stimulation (Siman & Noszek, 1988; Seubert, Larson, Oliver, Jung, Baudry & Lynch, 1988). To test whether the induction of NMDA receptor desensitization by a conditioning train of agonist applications is mediated by activation of such proteases, leupeptin, an inhibitor of calpain activity, was added to the intracellular solution (Wang, 1990). Leupeptin (250 μM) had no significant effect on inactivation or desensitization induced by a conditioning train of agonist applications (Fig. 9*A*, *D* and *G*); the average inactivation was 55·2 ± 6·9 % and the increase in desensitization was 11·8 ± 3·4 % ($n = 4$).

Recent experiments have shown that inactivation of K^+ channels is strongly enhanced by cysteine oxidation. This effect could be restored by the antioxidant dithiotreitol (Ruppersberg, Stocker, Pongs, Heinemann, Frank & Koenen, 1991). The use of an intracellular solution containing 1 mM DTT did not reverse the initial desensitization of NMDA receptors present during the first application of NMDA and did not significantly modulate the induction of desensitization (13·6 ± 8·4 %) or inactivation (61·3 ± 8·4 %, $n = 5$) of NMDA responses induced by a conditioning train of agonist applications (Fig. 9*B*, *E* and *H*).

Phosphatidylserine has been proposed to act extracellularly to modulate the state of postsynaptic excitatory amino acid receptors (for review see Baudry, 1991). In a further series of experiments phosphatidylserine was tested for an effect on inactivation and desensitization of responses to NMDA. The extracellular application of 30 μM phosphatidylserine reduced responses to NMDA recorded in the presence of 0·2 mM Ca^{2+} only slightly (88·9 ± 2·9 %) (Fig. 9*C*, *F* and *I*). This effect was quickly reversible. There was no change in the degree of desensitization of NMDA receptors induced by application of phosphatidylserine for several minutes (−2·5 ± 3·7 %, $n = 4$).

Inactivation of NMDA currents in inside-out patches

With whole-cell recording the rate of recovery of responses to NMDA after inactivation by a conditioning train of agonist is very slow, usually requiring 5–15 min for complete recovery. The time needed for recovery was much longer than expected from estimates of the kinetics of diffusion of Na^+ (time constant ≈ 5 s) from a recording electrode into a chromaffin cell (Marty & Neher, 1983). To clarify whether the difference is due to a maintained high $[Ca^{2+}]_i$ or due to a slow recovery of the NMDA receptor–channel complex from Ca^{2+}-induced modulation, experiments were performed using the inside-out patch configuration. Intracellular solution containing either low (10 nM) or high (2·5 mM) Ca^{2+} was applied on the cytoplasmic side of an inside-out patch containing NMDA receptor–channels. In order to avoid exposure of the patch to a high concentration of Ca^{2+} during inside-

out patch formation, the neurone under study was perfused with intracellular solution containing 10 nM Ca^{2+} during this procedure. NMDA receptors were activated in the presence of 0·2 mM $[Ca^{2+}]_o$ with 10 μM NMDA. NMDA-activated channels were identified as inward currents with a reversal potential close to 0 mV

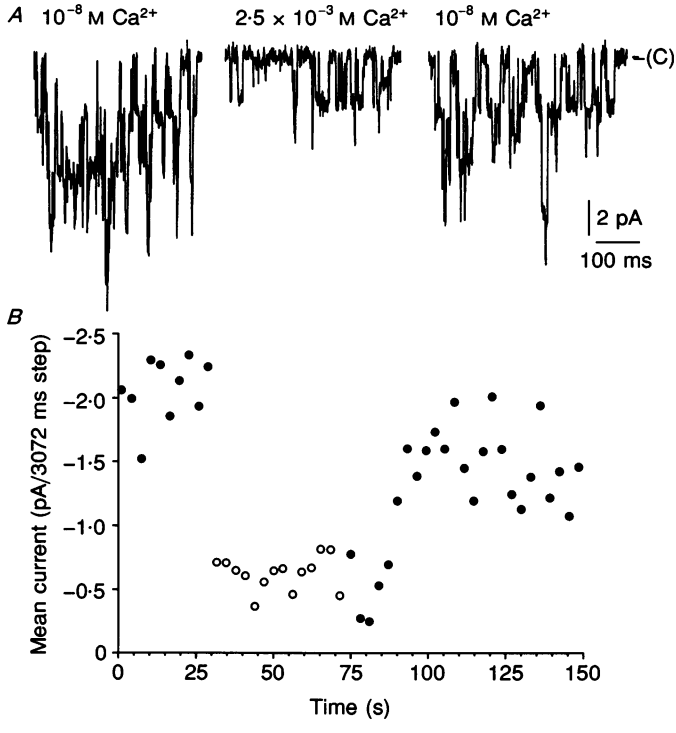

Fig. 10. Ca^{2+}-sensitive inactivation of NMDA receptors, recorded from an inside-out patch. *A* shows NMDA receptor single-channel activity induced in the continuous presence of 10 μM NMDA in ECS containing 0·2 mM Ca^{2+} applied to the extracellular side of the inside-out patch; the holding potential was −70 mV. During and after formation of the inside-out patch, ICS contained 10 nM Ca^{2+} (5 mM EGTA with 0·5 mM Ca^{2+}). There were at least five active NMDA channels with the same reversal potential (+ 2 mV) and principal single-channel conductance (53 pS). With ICS containing 2·5 mM Ca^{2+} (5 mM EGTA with 7·5 mM Ca^{2+}) the activity of NMDA channels was strongly reduced, but single-channel conductance was unaffected. This effect was largely reversible upon returning to ICS with 10 nM Ca^{2+}. (C) indicates the current level where all channels are closed. *B*, graph showing the mean NMDA activity, calculated as mean current per 3072 ms epoch, *versus* the time following the start of inside-out recording. The solution facing the inside of the patch was changed from ICS containing 10 nM Ca^{2+} to ICS containing 2·5 mM Ca^{2+} at 30 s and at 73·4 s it was changed back to control ICS. ● indicates mean NMDA currents with ICS containing 10 nM Ca^{2+}, and ○ mean NMDA currents with ICS containing 2·5 mM Ca^{2+}.

and a conductance close to 50 pS. As shown in Fig. 10, the mean NMDA current (estimated from a fit of the amplitude histogram based on all data points in 3072 ms long segments), was suppressed by 70 % when the concentration of free Ca^{2+} on the cytoplasmic side of the patch was changed from 10 nM to 2·5 mM. The onset of suppression was fast (< 3 s), but it took about 21 s for recovery of NMDA

receptor–channel activity after reperfusion of the patch with a low (10 nM) Ca^{2+} concentration. Such recovery was much faster than for whole-cell recording experiments. Similar results were obtained on three additional patches, but it was not possible to determine exactly the time course of recovery because the level of single channel activity was too low.

DISCUSSION

The results described in this paper illustrate two distinct forms of Ca^{2+}-mediated regulation of NMDA receptors: (1) inactivation, which is reversible, is induced in the absence of NMDA receptor activation and most probably depends on an increase in $[Ca^{2+}]_i$ for both induction and maintenance, and (2) glycine-resistant desensitization, which requires both NMDA receptor activation and an increase in $[Ca^{2+}]_i$ for induction, and is irreversible. The distinction between desensitization and inactivation is based on Katz & Thesleff's (1957) definition of desensitization, as an agonist-induced state in which the receptor becomes refractory to agonist application; inactivation of NMDA receptors by a rise in $[Ca^{2+}]_i$ occurs in the absence of agonist. The results of this study expand our knowledge of the regulatory role of Ca^{2+} in modulation of NMDA receptors as previously suggested by Mayer & Westbrook (1985); Mayer *et al.* (1987); Zorumski *et al.* (1989); Mayer *et al.* (1989*a*); Clark *et al.* (1990); Lerma, Zukin & Bennett (1990); Vyklický *et al.* (1990*a*) and Legendre, Rosenmund & Westbrook (1993). In addition to the effects described in this paper, Ca^{2+} is known to be highly permeant through NMDA receptor–channels and may interfere with monovalent cation permeability and the potency of zinc as an NMDA receptor antagonist (MacDermott, Mayer, Westbrook, Smith & Barker, 1986; Mayer *et al.* 1987; Mayer & Westbrook, 1987; Ascher & Nowak, 1988; Mayer *et al.* 1989*b*). Ca^{2+} also potentiates NMDA responses in low extracellular glycine (Gu & Huang, 1991; L. Vyklický & V. Vlachova, unpublished observations). Although most studies on Ca^{2+} modulation of NMDA receptors show an inhibitory effect, recordings from the CA3 region of hippocampal slices suggest that there may also be a developmentally regulated potentiation of NMDA receptor responses by Ca^{2+} (Brady, Smith & Swann, 1991).

Ca^{2+}-induced NMDA receptor inactivation

The term inactivation, as used in this paper, refers to a reversible suppression of responses to NMDA induced by repeated application of agonist in the presence of 2 mM $[Ca^{2+}]_o$, or by activation of voltage-gated Ca^{2+} channels. Inactivation appears to be similar to the modulation of NMDA receptors referred to in previous papers as desensitization, fade or inactivation, in studies on cultured spinal cord neurones (Mayer & Westbrook, 1985; Mayer *et al.* 1987), poly(A) + RNA-injected oocytes (Lerma *et al.* 1990) and cultured hippocampal neurones (Mayer *et al.* 1989*a*; Vyklický *et al.* 1990*a*; Legendre *et al.* 1993).

Inactivation was induced by NMDA receptor stimulation in the presence of a physiological $[Ca^{2+}]_o$ (2 mM), but not when $[Ca^{2+}]_o$ was decreased to 0·2 mM, which suggests an important role for Ca^{2+} in this form of NMDA receptor modulation. Furthermore, the induction of NMDA receptor inactivation was voltage dependent and also was greatly reduced when the application of NMDA was delivered in the presence of Mg^{2+}, a voltage-dependent NMDA receptor–channel blocker. These

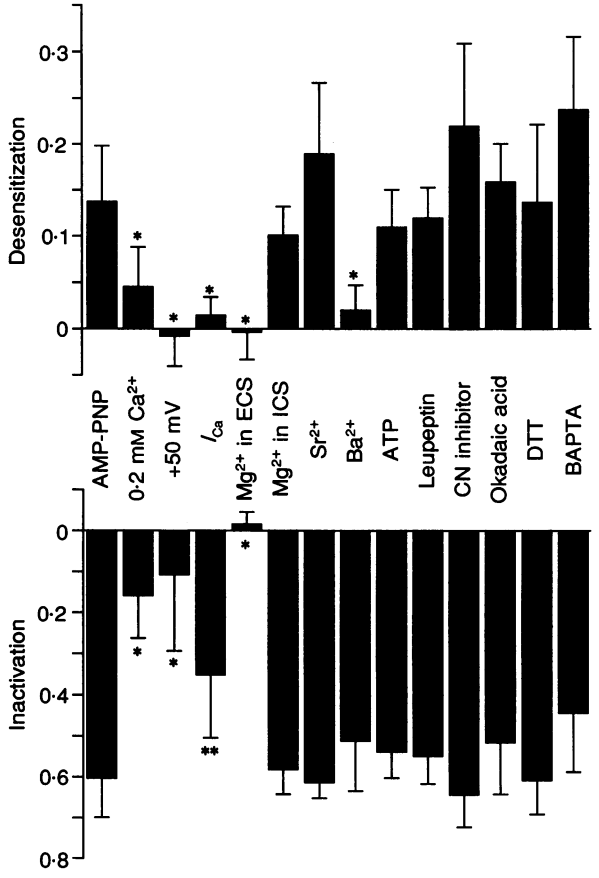

Fig. 11. Histogram comparing the effect of a conditioning train of ten NMDA applications or activation of voltage-dependent Ca^{2+} currents (I_{Ca}) on inactivation and desensitization of NMDA responses. ECS contained (mM): 0·2 Ca^{2+}, 2 Mg^{2+} (Mg^{2+} ECS), 2 Sr^{2+} (Sr^{2+}), or 2 Ba^{2+} (Ba^{2+}). ECS containing 2 mM Ca^{2+} was used in experiments at + 50 mV, during activation of I_{Ca}, and when ICS was supplemented with 1 mM AMP-PNP (AMP-PNP), 3 mM Mg^{2+} (Mg^{2+} ICS), 1·5 mM ATP + 3 mM Mg^{2+} (ATP), 250 μM leupeptin, 250 μM calcineurin inhibitor (CN inhibitor), 1–6 μM okadaic acid, 1 mM DTT or when 5 mM BAPTA was substituted for EGTA. Values are plotted as means ± S.D. and one-way ANOVA was used to evaluate the significance of differences between (1) control (train of ten NMDA applications in the presence of ECS with 2 mM Ca^{2+} and ICS containing 1 mM AMP-PNP) and test responses (a train of NMDA applications or voltage steps in the presence of test ECS or ICS as appropriate) and (2) paired t tests between the degree of desensitization or inactivation before and after the train. * indicates a significant ($P < 0·05$) reduction of inactivation or desensitization relative to control that was not significantly ($P > 0·05$) different from inactivation or desensitization of NMDA responses before the train. ** indicates a significant reduction of inactivation relative to control that was significantly ($P < 0·05$) different from inactivation of NMDA responses before the train.

results, together with the fact that the NMDA channel is highly permeable to Ca^{2+} (MacDermott *et al.* 1986; Mayer & Westbrook, 1987), suggest that influx of Ca^{2+} through NMDA receptor–channels can mediate NMDA receptor inactivation (for summary see Fig. 11). But the fact that a similar reversible suppression of NMDA

receptors was induced in the absence of NMDA by activation of voltage-gated Ca^{2+} currents, suggests that Ca^{2+} alone is sufficient to induce NMDA receptor inactivation and furthermore that it is mediated by an increase in $[Ca^{2+}]_i$. An intracellular site of action for Ca^{2+} is further supported by results from inside-out patch experiments in which NMDA receptor single-channel activity was reversibly suppressed by application of Ca^{2+} to the intracellular side of the patch.

In cultured chick spinal cord neurones, $[Ca^{2+}]_o$ induces a reversible 'desensitization' of NMDA-type glutamate receptors, while kainate/quisqualate receptors are not affected (Zorumski *et al.* 1989). Clark *et al.* (1990) found that responses to pressure ejection of NMDA in the presence of 10 mM $[Ca^{2+}]_o$ were reversibly attenuated by 55 %. Similar to the inactivation of NMDA responses described in this article, the attenuation was dependent on both the $[Ca^{2+}]_o$ and membrane potential. However, responses to NMDA were not altered by manipulations intended to affect $[Ca^{2+}]_i$, which led the authors to suggest an extracellular site of action of calcium.

This conclusion was based on three sets of observations. First, the degree of NMDA receptor desensitization was not altered by an increase in the intracellular Ca^{2+}-buffering capacity using 5–10 mM BAPTA. However, similar experiments in which the effect of $[Ca^{2+}]_i$ on GABA responses was studied showed that 10 mM BAPTA was insufficient to prevent the Ca^{2+}-mediated modulation and that 30 mM BAPTA was required to control $[Ca^{2+}]_i$ (Llano, Leresche & Martry, 1991). Second, treatment of neurones with the Ca^{2+} ionophore A23187 or caffeine before whole-cell formation did not promote NMDA desensitization. The interpretation of these results is complicated by the fact that neither the change in $[Ca^{2+}]_i$ induced by these manipulations nor the effect of subsequent whole-cell recording on $[Ca^{2+}]_i$ was known. Third, activation of voltage-gated Ca^{2+} channels before or during application of NMDA failed to promote desensitization. However, the present results suggest that activation of voltage-gated Ca^{2+} channels, combined with application of NMDA in the presence of 5 mM Ca^{2+} is unlikely to exert a modulatory effect above that induced by Ca^{2+} influx through NMDA channels alone. It is thus likely that the similarities between the reversible 'desensitization' observed by Clark *et al.* (1990) and the inactivation described here reflect a common process which is mediated by an increase in $[Ca^{2+}]_i$.

The results of this study suggest that Ca^{2+} inactivates NMDA receptors directly because the degree of inactivation in whole-cell recordings was not affected by drugs interfering with protein phosphorylation/dephosphorylation, and inactivation could be induced in inside-out patches where all diffusible intracellular components are likely to be washed out. An alternative explanation is that Ca^{2+} affects a component of the plasma membrane or a protein closely associated with the NMDA receptor. Responses to NMDA could not be fully inactivated even by a high concentration of Ca^{2+} (2·5 mM) in inside-out patch experiments or by prolonged intracellular dialysis with solutions containing 2·5 mM Ca^{2+} (see Figs 4 and 10). This suggests that either 2·5 mM Ca^{2+} is not a saturating concentration for inducing inactivation or that Ca^{2+} induces only partial inhibition of NMDA receptors. An alternative explanation is that the cells studied may express multiple subtypes of NMDA receptors or modulatory proteins associated with NMDA receptors and that these have different sensitivities to Ca^{2+}. Further experiments are required to resolve this issue.

The recovery from inactivation of NMDA receptor responses recorded in the whole-cell mode was much slower than in inside-out patches. It seems unlikely that the long recovery times with whole-cell recording reflect a persistently elevated $[Ca^{2+}]_i$, because direct measurements with Arsenazo III have shown that elevations of $[Ca^{2+}]_i$ produced by similar stimulation protocols recover much more quickly (Mayer *et al.* 1987).

Although there are pitfalls in comparing the induction of NMDA receptor inactivation by Sr^{2+} and Ba^{2+} with Ca^{2+}-mediated inactivation, because of the differing permeabilities of these ions through NMDA receptor–channels (Mayer & Westbrook, 1987) and their different affinity for EGTA (Martell & Smith, 1974), the results summarized in Fig. 11 show that both Sr^{2+} and Ba^{2+} reversibly inactivate the NMDA receptor to the same degree as Ca^{2+}. These results are in agreement with those of Legendre *et al.* (1993), but conflict with the results of Clark *et al.* (1990), who found that 'desensitization' was induced by Ca^{2+} and Sr^{2+} but not by Ba^{2+}. The fact that the degree of Ca^{2+}-induced inactivation did not alter when $[Mg^{2+}]_i$ was raised to 3 mM suggests that Mg^{2+} is neither able to induce NMDA receptor inactivation nor to antagonize the Ca^{2+} effect. The Ca^{2+}-induced inactivation of NMDA receptors is clearly different from the voltage-dependent block of NMDA-activated ion channels by intracellular Mg^{2+} and Ca^{2+} (Johnson & Ascher, 1990). At positive potentials intracellular Mg^{2+}, and to a lesser degree Ca^{2+}, can reduce NMDA receptor single-channel conductance by fast ion channel block. This is in contrast to Ca^{2+}-mediated inactivation, for which no obvious change in the amplitude of NMDA receptor single-channel currents was observed at −70 mV. Intracellular Mg^{2+} did not prevent inactivation of NMDA receptors by Ca^{2+}, which further suggests that Ca^{2+} and Mg^{2+} modulate the NMDA receptor–channel complex via different sites.

Desensitization of NMDA receptors

There are at least two types of NMDA receptor desensitization, glycine sensitive and glycine resistant (for review see Mayer *et al.* 1991). Glycine-sensitive desensitization is characterized by a progressive increase in NMDA receptor desensitization as the extracellular concentration of glycine decreases (Mayer *et al.* 1989*a*; Benveniste *et al.* 1990; Vyklický *et al.* 1990*a*; Lerma *et al.* 1990; Traynelis & Cull-Candy, 1990). Benveniste *et al.* (1990) modelled this form of desensitization with a negative allosteric interaction between the binding of NMDA and glycine, such that when the receptor has bound either NMDA or glycine, its affinity for the other ligand decreases. However, an additional desensitization state had to be proposed to explain the occurrence of glycine-resistant desensitization. Desensitization which is insensitive to the concentration of glycine is usually present to a variable extent at the beginning of whole-cell recording and progressively increases thereafter, especially when long applications of NMDA receptor agonists are made in the presence of 1–5 mM $[Ca^{2+}]_o$ (Sather *et al.* 1990, 1992; Mayer *et al.* 1991; Chizhmakov *et al.* 1992). Recently, glycine-resistant desensitization was described using a cyclic model of the NMDA receptor with two desensitized states (Sather *et al.* 1992).

A rise in intracellular Ca^{2+} seems to participate in the induction of glycine-resistant NMDA receptor desensitization because induction was dependent on

$[Ca^{2+}]_o$, holding potential and $[Mg^{2+}]_o$. However, an increase in the $[Ca^{2+}]_i$ without concomitant NMDA receptor activation did not increase desensitization, suggesting that this process requires both an increase in $[Ca^{2+}]_i$ *and* the binding of agonist (see Fig. 4).

Once established, the degree of NMDA receptor desensitization is voltage independent and insensitive to $[Ca^{2+}]_i$ suggesting that Ca^{2+} is only important for the induction of NMDA receptor desensitization, and not for its maintenance (compare Figs 2, 3 and 4). It was possible to induce desensitization following substitution of Sr^{2+} for Ca^{2+} but, in contrast to its effects on inactivation, Ba^{2+} failed to induce NMDA receptor desensitization (see Fig. 6).

Protein phosphorylation is a regulatory mechanism participating in the control of neurotransmitter receptor desensitization (for review see Huganir & Greengard, 1990). Activation of NMDA receptors in cultured hippocampal cells leads to Ca^{2+}-dependent phosphorylation of proteins related or identical to microtubule-associated protein 2 kinase (Bading & Greenberg, 1991). A similar mechanism, protein kinase C-mediated phosphorylation, has been shown to modulate glutamate responses (Chen & Huang, 1991). It is tempting to speculate that NMDA receptor desensitization would also be regulated by phosphorylation; but the fact that the induction of desensitization was not affected by intracellular perfusion with drugs inhibiting protein phosphorylation (AMP-PNP, low $[Mg^{2+}]_i$) provides no support for such a mechanism. NMDA-mediated dephosphorylation has been shown for the cytoskeletal protein MAP2 (Halpain & Greengard, 1990), and a similar mechanism could lead to modulation of NMDA receptors with a resulting change in desensitization. However, three phosphatase inhibitors (okadaic acid, calcineurin inhibitor peptide 3, low Mg^{2+}) had no effect on Ca^{2+}-induced desensitization of NMDA receptors, suggesting mechanisms other than dephosphorylation. However, the occurrence of unidentified phosphatases with different pharmacological sensitivities cannot be completely ruled out. The fact that the induction of NMDA receptor desensitization was use dependent suggests the possibility that the site regulating desensitization is hidden and available for modulation only when the NMDA receptor is activated.

It seems unlikely that the inhibitors used in this study failed to reach their site of action before a conditioning train of NMDA was applied (usually 5–7 min after whole-cell formation). Estimates of the rate at which molecules diffuse from patch pipettes to neurones in slices of medulla show that fluorescence-labelled serum albumin (molecular weight 67 000) fills neurones in 3–5 min (Chen & Huang, 1991). These results suggest that the inhibitors, including the calcineurin inhibitor peptide 3, which had the largest molecular weight (2929) used in this study, should reach a final concentration well before the increase in $[Ca^{2+}]_i$ induced by the conditioning application of NMDA.

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