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Calcium-dependent inactivation of the monosynaptic NMDA EPSCs in rat hippocampal neurons in culture

Igor Medina,* Xavier Leinekugel and Yehezkel Ben-Ari
INSERM, Unité 29, Hopital de PortRoyal, 123, Bd de PortRoyal, 75014 Paris, France

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Abstract

The effects of increased dendritic calcium concentration ($[Ca^{2+}]_i$) induced by single action potentials on monosynaptic glutamatergic excitatory postsynaptic currents (EPSCs) were studied in cultured rat hippocampal neurons. To investigate the respective roles of pre- and postsynaptic elements in the depolarization-induced NMDAR inactivation, we have performed simultaneous paired whole-cell recordings from monosynaptically connected pre- and postsynaptic hippocampal neurons. We report that the single firing of the postsynaptic neuron did not result in inactivation of the NMDAR-EPSC, whereas a burst of depolarizing steps transiently depressed the NMDAR-EPSCs in both pyramidal cells and interneurons. This effect was mediated by postsynaptic voltage-gated Ca^{2+} influx, as it was prevented by: (i) buffering postsynaptic $[Ca^{2+}]_i$ with 30 mM BAPTA; (ii) removing extracellular Ca^{2+}; or (iii) applying Cd^{2+}_o (100 μM), a voltage-gated calcium channel blocker. It does not involve presynaptic mechanisms as it selectively affected NMDA but not α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor-mediated EPSCs. These results suggest that inactivation of NMDAR-channels by voltage-gated Ca influx is a general property of hippocampal neurons, which may play an important role in reducing postsynaptic NMDAR Ca^{2+} influx that leads to plasticity or excitotoxicity during sustained neuronal activity.

Introduction

Because of their high Ca^{2+} permeability, the *N*-methyl-D-aspartate subtype of glutamate receptors (NMDAR) play an important role in synaptic plasticity, synapse formation and excitotoxicity (McBain & Mayer, 1994). NMDAR-channels are regulated by a large number of extracellular and intracellular modulatory sites (for review see Ascher & Johnson, 1989; Ben-Ari *et al*., 1992; McBain & Mayer, 1994), including one sensitive to intracellular calcium concentration ($[Ca^{2+}]_i$) (Mayer & Westbrook, 1985; Zorumski *et al*., 1989). A rise in $[Ca^{2+}]_i$ reduces the whole-cell currents evoked by external applications of NMDA to hippocampal neurons in culture by ≈40% (Legendre *et al*., 1993; Vyclicky, 1993; Medina *et al*., 1994), and to human embryonic kidney cells (HEK-293) expressing the NR1/NR2A or NR1/NR2B subunits of NMDAR (Medina *et al*., 1995).

A central issue that remains to be solved is the role of Ca^{2+}-induced NMDA inactivation in the modulation of NMDAR-EPSCs. Using paired-pulse protocols in autapses and monosynaptically connected neurons in cultures, Mennerick & Zorumski (1996) have shown that Ca^{2+} influx through NMDA receptors induced depression of NMDAR-EPSCs. It has been shown recently that NMDAR-mediated autaptic currents may also be modulated by calcium influx through voltage-gated Ca^{2+} channels (Rosenmund *et al*., 1995; Tong *et al*., 1995). However, because pre- and postsynaptic elements belong to the same neuron in an autapse, the activation of the presynaptic action potential-dependent glutamate release induces postsynaptic voltage-dependent Ca^{2+} influx (Mennerick & Zorumski, 1996) which obscures the role of single action potentials in the regulation of NMDAR-EPSCs.

To determine whether voltage-gated calcium channels (VDCCs) that are activated by somatic depolarization during somatic action potential firing can modulate NMDAR-EPSCs, we studied the effects of postsynaptic depolarizing steps on NMDAR-EPSCs recorded from pairs of connected neurons. Our results suggest that single action potentials only occasionally inactivate NMDA-EPSCs, whereas bursts of action potentials consistently depress the NMDAR-EPSCs. This modulation may play an important role in reducing postsynaptic NMDAR Ca^{2+} influx that leads to plasticity or excitotoxicity during sustained neuronal activity.

Methods

Primary culture of hippocampal neurons

Hippocampal neuron cultures were prepared as described (Medina *et al*., 1996). In brief, 2-day-old Wistar rats were rapidly decapitated after cervical dislocation. Hippocampi were removed from the brains and dissected free of the meninges in cooled (6 °C) oxygenated phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}. The hippocampi were then transferred into Ca^{2+}- and Mg^{2+}-free PBS, cut into small pieces and incubated with 0.3% (w/v) protease from *Aspergillus oryzae* (type XXIII, Sigma, St. Louis, MO, USA) and 0.1% (w/v) DNAse (type I, Sigma) for 20 min at 25 °C. The tissue was washed and triturated using fire-polished pipettes in Ca^{2+}- and Mg^{2+}-free PBS solution with 0.05% DNAse. After a brief centrifugation, the cell pellet was resuspended in culture medium and plated at a density of 30 000 per cm^2. Growth medium contained 8% NU Serum (Becton Dickinson, Franklin Lakes, NJ, USA) and 92% minimal essential medium (MEM, Gibco, Rockville, MD, USA)

Correspondence: Y. Ben-Ari, as above.
E-mail: ben-ari@cochin.inserm.fr

*Present address: Department of Cardiology, Children's Hospital, Boston, MA 02115, USA

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supplemented with penicillin (5 U/mL) and streptomycin (5 μg/mL). Glass coverslips were coated with poly-L-lysine (10 mg/mL, MW range 30 000–70 000, Sigma). Cells were grown for 2–3 weeks *in vitro* before use.

Electrophysiological recordings

Experiments were performed at room temperature (19–22 °C) using whole-cell patch-clamp recording from both pre- and postsynaptic neurons. The external solution contained (in mM): NaCl, 170; KCl, 1; $CaCl_2$, 2; HEPES-Na, 10; glucose, 20; glycine, 0.01; pH 7.4. Osmolarity was adjusted to 340 mOsm by adding NaCl.

Paired recordings

We selected pairs of neighbouring neurons (distance between somata of neurons 50–150 μm) in which at least one of the neurons, according to morphological criteria, appeared to be glutamatergic (Benson *et al*., 1994; see Fig. 1A). Spontaneous inhibitory and excitatory currents were consistently observed in neurons maintained in culture for more than 5 days. Direct synaptic connections between two neurons in double whole-cell patch-clamp recording were, however, observed only in neurons maintained in culture for more than 8 days. Furthermore, the probability of recording monosynaptic EPSCs and IPSCs increased significantly with time in culture. Thus, the probability of the recording of the synaptic connection between pairs of neurons kept from 8 to 12 days was ≈ 25% (16 out of 61 pairs), whereas after 14 days the synaptic connections were observed in > 90% of pairs (98 out of 108). The synapses were glutamatergic in ≈ 35% of pairs (40 out of 114).

Presynaptic neuron

The presynaptic neuron was recorded either in voltage-clamp or current-clamp modes using Axopatch 200A (Axon Instrument, Foster City, CA, USA) or ROK-3M (Moscow, Russia) amplifiers. Relatively high resistance (10–15 MΩ) borosilicate glass patch pipettes were used to delay the rundown of presynaptic release of glutamate. The internal solution contained (in mM): KCl, 80; K-gluconate, 100; K_4-BAPTA, 1.1; $CaCl_2$, 0.06; HEPES-K, 10; MgATP, 4.0; GTP, 0.6; creatine phosphate, 6.0; pH 7.2. The osmolarity was adjusted to 310 mOsm by adding K-gluconate.

In current-clamp mode (resting membrane potential –40 to –60 mV), action potential firing was elicited by short (1–2 ms) positive current pulses depolarizing the membrane to –10 to –20 mV. In the presence of bicuculline (20 μM) and absence of

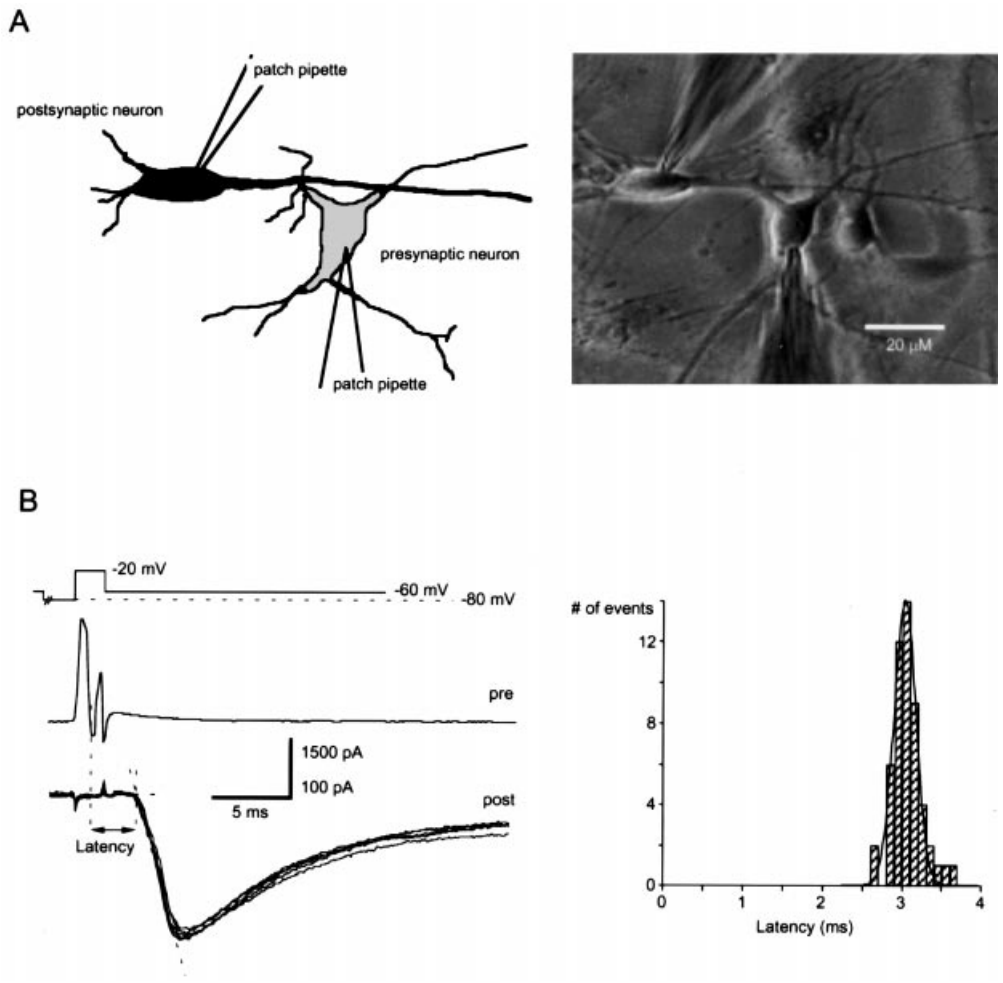

FIG. 1. Basic properties of EPSCs evoked in rat hippocampal neurons in culture. (A) Diagram and photomicrograph of synaptically connected neurons in 15-day-old cultures from hippocampi of P2 rats. (B) Latency of excitatory synaptic transmission between neurons in culture. Examples of EPSCs recording from a single pair of neurons: top trace shows the voltage pulse protocol used for exciting the presynaptic neuron in voltage-clamp mode (see Materials and methods); middle trace illustrates the current induced in the presynaptic neuron; the bottom traces show five superimposed EPSCs elicited in the postsynaptic neuron. The histogram illustrates the distribution of latencies for EPSCs elicited by 52 depolarizing pulses in this pair of neurons.

external Mg^{2+}, the presynaptic neurons were easily excited above the action potential threshold by spontaneous α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)R-EPSCs, even during the injection of hyperpolarizing current to –60 mV. Presynaptic recordings were therefore performed in voltage-clamp mode (holding potential: –60 mV) to prevent the appearance of spontaneous spikes in these conditions. To activate the presynaptic neuron, a prehyperpolarizing step (300 ms to –80 mV) was applied, immediately followed by a depolarizing step to –20 mV, of 0.5 ms longer duration (1.5–3.0 ms) than the threshold for postsynaptic responses.

Postsynaptic neuron

Postsynaptic currents were recorded using an EPC9 amplifier (Heka Electronik, Lambrecht, Germany) and pipettes with resistances of 4–6 MΩ. The internal solutions contained (in mM): CsCl, 80; Cs-gluconate, 100; K_4-BAPTA, 1.1; $CaCl_2$, 0.06; HEPES-K, 10; MgATP, 4.0; GTP, 0.6; creatine phosphate, 6.0; pH 7.2; or (when mentioned): CsCl, 40; Cs-gluconate, 100; K_4-BAPTA, 30; $CaCl_2$, 0.06; HEPES-K, 10; MgATP, 4.0; GTP, 0.6; creatine phosphate, 6.0; pH 7.2. The osmolarity was adjusted to 310 mOsm by adding Cs-gluconate. The series resistance (10–30 MΩ) compensation was at least 60%. The holding potential (V_h) was 60 mV in all experiments.

The NMDAR- and AMPAR-EPSCs were isolated pharmacologically, being recorded in the presence of CNQX (10 μM) and bicuculline (20 μM), or D-APV (100 μM) and bicuculline (20 μM), respectively. Exogenous micropipette applications of NMDA (50 μM) consisted of short (50–150 ms) pulses delivered using a Picospritzer (General Valve Corporation, Fairfield, NJ, USA), as previously described (Medina *et al.*, 1994). Activation of voltage-gated Ca^{2+} channels in the postsynaptic neuron was induced by delivering a single brief (4 ms) or trains (1 Hz for 5–10 s) of longer (500 ms) depolarizing voltage pulses from –80 to 0 mV.

Perfusion of the neurons

During all experiments, the recording chamber was continuously perfused with an external solution applied through one channel of the two-barrel fast perfusion system. The inner diameter of the channels was ≈300 μm. When application of calcium-free or 100 μM Cd^{2+} solution was required, the first channel was moved and perfusion through the second channel was started 2 s before the application of the depolarizing pulses to the postsynaptic neuron. Effective perfusion was confirmed by the disappearance of the EPSCs in response to stimulation of the presynaptic neuron. At the end of the postsynaptic depolarizing train, the calcium-free or 100 μM Cd^{2+} solution was stopped, and the first channel was replaced to allow the rapid exchange of external solution.

Data analysis

Data were filtered at 3 kHz and digitized at 15 kHz using pCLAMP 6 software (Axon Instruments) and a Labmaster DMA board (DIPSI, France).

The latency of the EPSCs was defined as the time between the presynaptic spike (maximum of the presynaptic inward current) and the onset of the EPSC (cf. Katz & Miledi, 1965). The onset of the EPSC was taken as the intersection between the baseline and a line joining the points of the rising EPSC phase at 20 and 80% of peak amplitude.

The degree of inactivation of EPSCs was estimated using the equation $(I_0I)/I_0$, where I_0 is the amplitude of the EPSC or NMDA test current before application of the depolarizing pulses, and I is the amplitude of the EPSC or NMDA test current 4 s after the train of postsynaptic depolarizing pulses.

All results are presented as mean values ± SEM. Data were compared statistically using Student's *t*-test. Statistical significance was taken to be at the 5% level.

Results

Basic properties of evoked mono-synaptic EPSCs

The traces presented in Fig. 1B illustrate the basic properties of EPSCs evoked by stimulation of the presynaptic neuron, and recorded in voltage-clamp mode in Mg^{2+}-free solution containing bicuculline (20 μM). A short (2 ms) depolarizing step in the presynaptic neuron evoked an EPSC with a latency varying from 2.6 to 3.7 ms (Fig. 1B, left traces). The distribution of the latencies was fitted by a Gaussian curve with a constant single peak at 3.0 ± 0.03 ms ($n = 52$) (Fig. 1B, right). Similar EPSCs were observed in 38 out of 52 pairs of neurons with excitatory connections. In 14 experiments, the depolarizing step induced bursts of two to 10 EPSCs, whose latencies varied between 2 and 50 ms (not shown). Shorter or smaller depolarizing steps resulted in the all-or-none disappearance of the bursts of EPSCs in these pairs. These experiments were not included in the analysis. Therefore, activation of the presynaptic neuron induced, in most of the pairs, monosynaptic EPSCs.

In keeping with earlier reports (Keller *et al.*, 1991; Hestrin, 1992; Khazipov *et al.*, 1995), the evoked EPSCs had two clearly distinguishable fast and slow components blocked by CNQX (10 μM) and D-APV (100 μM), respectively, suggesting that the EPSCs are mediated by AMPA and NMDA receptors (see Fig. 5B below). The amplitude of the fast AMPA component varied from 30 to 3000 pA (mean = 615 ± 204 pA, $n = 18$, $V_h = -60$ mV), and that of the slow NMDA component from 5 pA to 1 nA (mean = 198 ± 66 pA, $n = 22$, $V_h = -60$ mV). The 10–90% rise time of NMDAR-EPSCs was 8.0 ± 1.0 ms ($n = 13$), the decay time was well fitted by two exponentials with time constants of 59.5 ± 5.9 ms ($n = 11$) and 317 ± 31 ms ($n = 13$). In contrast to the kinetics of NMDAR-EPSCs, the kinetic parameters of evoked AMPAR-EPSCs were consistently slower than values reported previously for AMPAR-EPSCs (Hestrin, 1992): the mean 10–90% rise time was 2.6 ± 0.3 ms ($n = 13$), the decay time constant was 7.4 ± 0.5 ms. The slower kinetics of evoked AMPA-EPSCs in our experiments were not related to the postsynaptic recording conditions or particular properties of AMPA receptors, because the kinetics of spontaneous AMPAR-EPSCs recorded from the same postsynaptic neurons were about twice as fast: 10–90% rise time was 1.8 ± 0.2 ms (significant difference, $P < 0.01$, paired *t*-test, $n = 13$) and decay time constant 4.8 ± 0.3 ms (significant difference, $P < 0.01$, paired *t*-test, $n = 13$). These differences were observed independently of the presynaptic recording mode: in five experiments, switching from voltage-clamp (VC) to current-clamp (CC) mode had no effect on the 10–90% rise time [2.5 ± 0.5 ms (VC) and 2.3 ± 0.5 ms (CC), $P = 0.21$, paired *t*-test] or on the decay time constant [7.2 ± 0.7 ms (VC) and 6.9 ± 0.6 ms (CC), $P = 0.17$, paired *t*-test]. Although answering this question is beyond the scope of the present study, the slower kinetic parameters of the evoked AMPAR-EPSCs may be due to altered presynaptic transmitter release during presynaptic whole-cell recording.

Activation of postsynaptic voltage-gated Ca^{2+} channels inactivates NMDAR-EPSCs

Figure 2A and B illustrates pharmacologically isolated monosynaptic NMDAR-mediated currents (NMDAR-EPSCs) recorded using a weak Ca^{2+} buffer in the postsynaptic patch pipette (1.1 mM BAPTA + 0.06 mM Ca^{2+}; free $[Ca^{2+}]_i = 10^{-8}$ M). The stimulation of the presynaptic neuron by depolarizing pulses with intervals of 10–

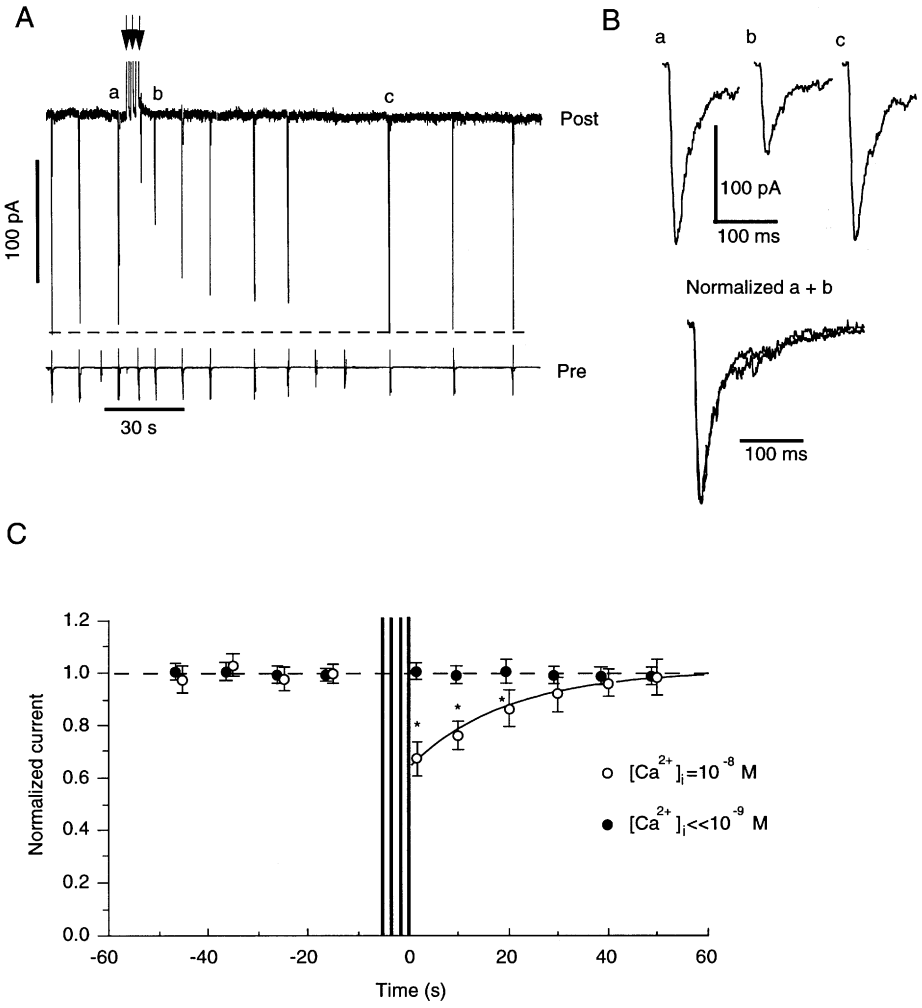

FIG. 2. Postsynaptic depolarizing pulses transiently inactivate evoked NMDAR-mediated postsynaptic currents. (A) Effect of postsynaptic depolarization on NMDAR-EPSCs (top trace) recorded in the presence of 10 μM glycine, 10 μM CNQX and 20 μM bicuculline. The bottom trace illustrates the currents induced by brief depolarizing pulses applied to the presynaptic neuron. Note the decrease of EPSCs amplitude after the train of depolarizing pulses applied to the postsynaptic cell (marked by arrows). (B) Examples of the same EPSCs as in (A) on a faster time base. Note that the decrease of NMDAR-EPSCs amplitude was not associated with a change in decay time course. (C) Summary of inactivation of NMDAR-EPSCs induced by 5–10 s depolarizing trains in the presence of weak (1.1 mM BAPTA + 0.06 mM Ca^{2+}; free $Ca^{2+} = 10^{-8}$ M) (○) and strong (30 mM BAPTA) (●) intracellular calcium buffers in the postsynaptic neuron. The times of application of depolarizing pulses to the postsynaptic neuron are shown by vertical lines. Zero time corresponds to the end of depolarizing steps. Data are means from 18 (○) and five (●) experiments. The amplitudes have been normalized to the mean control amplitude. Asterisks indicate the values significantly different ($P < 0.05$) from those measured 10 s before postsynaptic depolarization.

20 s evoked NMDAR-EPSCs of stable amplitude in the postsynaptic neuron. A train of depolarizing voltage pulses in the postsynaptic neuron induced a transient decrease in the amplitude of the NMDAR-EPSCs (33 and 51% of control, respectively, 2 and 9 s after the end of the train). This inactivation was associated with a change in the amplitudes but not the kinetics of the NMDAR-EPSCs, as the normalized EPSCs before and 9 s after the depolarizing train application were identical (Fig. 2B).

On average, 5–10 s trains of depolarizing pulses decreased the amplitude of NMDAR-EPSCs by 33 ± 6% (measured 2 s after depolarization, $n = 18$) in the absence of Mg^{2+}_o (Fig. 2C), and by 37 ± 4%, $n = 6$ in the presence of 0.5 mM Mg^{2+}_o (not shown). The time constant of the recovery from inactivation varied from 5 to 30 s, and on average was 17 ± 2 s ($n = 18$) in Mg^{2+}-free external solution and 12 ± 2 s, $n = 4$ in the presence of 0.5 mM Mg^{2+}_o.

To determine whether Ca^{2+} increase induced by a single action potential could depress NMDAR-EPSCs, we tested the effect of single postsynaptic short (4 ms) voltage pulses (Fig. 3A). In three out of eight experiments, this depolarization caused a transient depression of the NMDAR-EPSCs by 21 ± 2% ($n = 3$) 10 s later. Because in the remaining five experiments a single 4 ms depolarizing step did not modulate NMDAR-EPSCs, on average the amplitude of the EPSCs (92 ± 5%, $n = 8$) was not significantly different from the value measured before depolarization (100 ± 2%, paired t-test, $P = 0.33$). Depolarization of the same postsynaptic neurons by three consecutive pulses (each lasting 100 ms at intervals of 300 ms) did reduce the EPSCs by 30 ± 3% 10 s after depolarization, $n = 8$ (Fig. 3B). There is therefore some variability in short depolarization-induced NMDAR inactivation.

In experiments performed in the absence of bicuculline, nine cells could be identified as interneurons ($n = 6$) or pyramidal cells ($n = 3$) on the basis of two criteria: (i) their firing pattern (spiking accommodation during depolarizing pulses); or (ii) when the two cells of a pair were reciprocally connected, their transmitter [γ-aminobutyric acid (GABA) or glutamate] could be identified from the corresponding evoked postsynaptic currents. No significant difference in depolarization- (by trains of depolarization) induced NMDAR inactivation was observed between pyramidal cells and interneurons: 37 ± 7%, $n = 6$ in interneurons and 47 ± 11%, $n = 3$ in pyramidal cells, $P = 0.46$, suggesting that the inactivation of NMDAR-EPSCs is a general feature of hippocampal neurons.

In the next series of experiments, we investigated the mechanism of the depolarization-induced NMDAR inactivation. To test the hypothesis that this effect is due to Ca^{2+} influx via voltage-dependent Ca^{2+} channels, we used three different approaches: first, a high concentration of the Ca^{2+} chelator BAPTA was included in the postsynaptic intracellular solution; second, external calcium was removed during the depolarization of the postsynaptic neuron; and third, the voltage-gated calcium channel blocker Cd^{2+} was applied during the depolarization of the postsynaptic neuron. When the postsynaptic intracellular solution contained 10 mM BAPTA, the effects of the depolarizing trains were highly variable; inactivation ranged from 8 to 34%, $n = 3$. We therefore used a higher concentration of BAPTA (30 mM) and found a complete block of

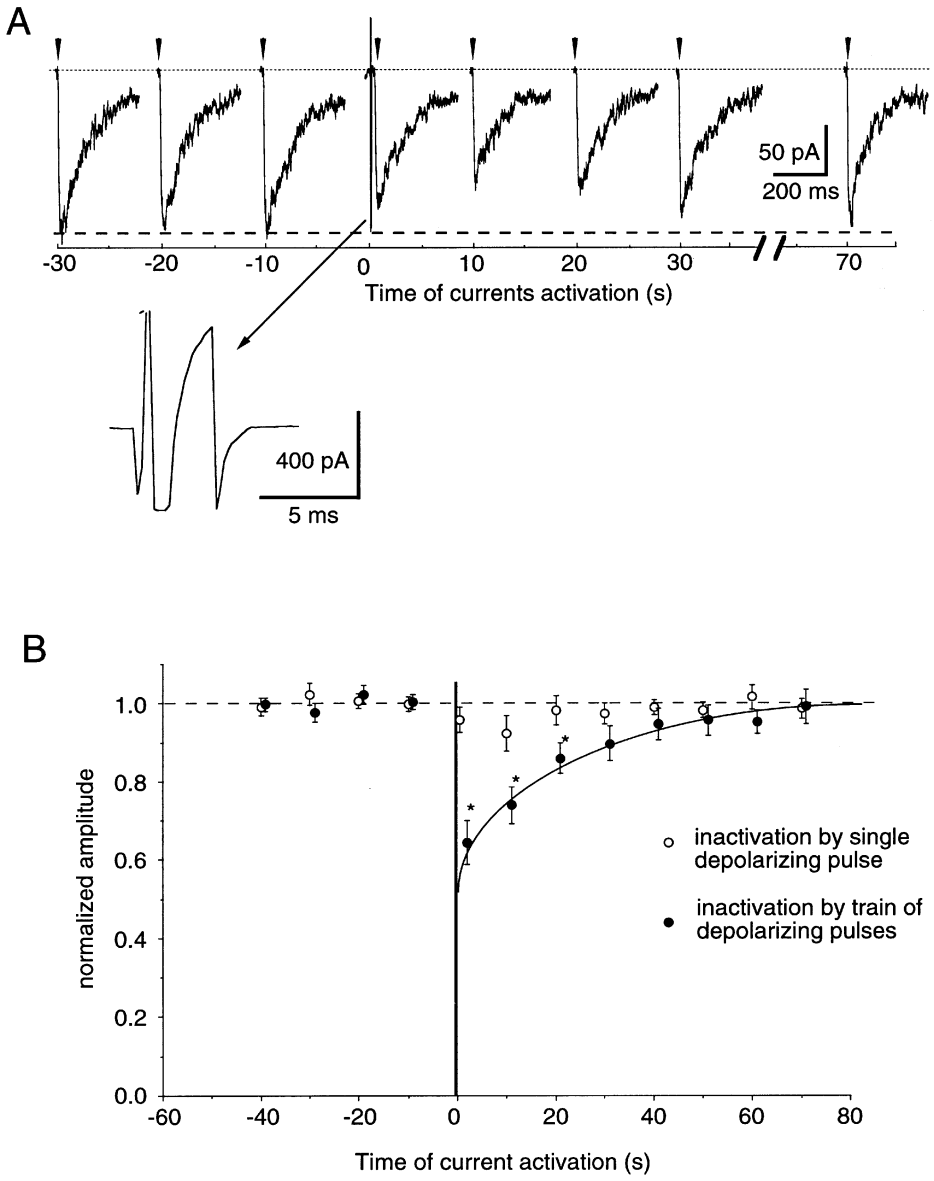

FIG. 3. A single depolarizing step is sufficient to inactivate evoked NMDAR-EPSCs in individual but not all experiments. (A) NMDAR-EPSCs traces were evoked every 10 s. The thick calibration bars are for the amplitude and duration of individual current traces. The position of arrows above EPSCs shows the time of EPSC activation (timescale below traces). Zero time corresponds to the end of the depolarizing step. The insert below the traces shows a fast timescale current induced by 4 ms depolarizing step from –80 to 0 mV. Note the 25% decrease of the EPSC evoked 10 s after single depolarization. (B) Averaged amplitude of NMDAR-EPSCs inactivated by single depolarizing pulse, as shown in A (○) and EPSCs inactivated by a train of three depolarizing pulses in the same experiments (●). Zero time corresponds to the end of the depolarizing steps. The horizontal dashed line was drawn through the mean value of EPSCs before inactivation. Data are means ± SEM from eight experiments. The amplitudes have been normalized to the mean control amplitude. Asterisks indicate the values significantly different ($P < 0.05$) from those measured 10 s before postsynaptic depolarization.

the inactivation by depolarization of the postsynaptic neuron ($\Delta I/I_0 = 1 \pm 2\%$, $n = 5$; Fig. 2C). As illustrated in Fig. 4A, a 5-s depolarizing train applied to the postsynaptic neuron – in the presence of Ca^{2+}-free external solution containing 1 mM EGTA – failed to modulate the NMDAR-EPSC evoked 10 s later (left trace), whereas a subsequent similar train of depolarizing pulses applied to the same neuron in the presence of 2 mM $[Ca^{2+}]_o$ induced the inactivation of EPSCs (right trace). On average, from five experiments, the depolarizing train in the absence of external calcium reduced NMDAR-EPSCs by $-1 \pm 1\%$ ($\Delta I/I_0$) after 10 s, as compared to $28 \pm 7\%$ when the depolarizing train is realized in the presence of external calcium (Fig. 4B). As illustrated in Fig. 4C, direct blockade of voltage-dependent calcium channels by bath application of 100 μM Cd^{2+} during the postsynaptic depolarizing train also significantly decreased the inactivation rate of NMDAR-EPSCs.

Altogether, these data suggest that the depolarization-induced transient decrease of NMDAR-EPSCs was due to the increase of postsynaptic calcium, presumably through voltage-gated calcium channels. In addition to the inactivation of NMDA currents by Ca^{2+}, two other mechanisms could explain the reduction of the NMDAR-EPSCs: (i) retrograde inhibition of presynaptic terminals by a diffusible Ca^{2+}-dependent messenger (Llano *et al.*, 1991; Glitsch *et al.*, 1996); or (ii) persistent depolarization of the postsynaptic membrane. If so, depolarizing pulses should similarly modulate non-NMDAR-EPSCs. The subsequent experiments therefore aimed to determine whether the depolarizing pulses also modulate AMPAR-mediated evoked EPSCs.

Depolarization of the postsynaptic neuron does not inactivate AMPAR-EPSCs

Figure 5A illustrates the results of an experiment in which AMPA and NMDA components of EPSCs were measured simultaneously. In control conditions (20 s intervals between EPSCs), EPSCs had a characteristic slow NMDAR-mediated component (trace a, left, trace a–c, right). In control conditions, the slow component had a relative amplitude of 58% of the early AMPAR component. As shown in Fig. 5B, depolarizing pulses did not affect the amplitude of the AMPAR-EPSCs ($106 \pm 13\%$, $n = 9$), while they transiently decreased by up to 35% the relative amplitude of the slow component of the EPSCs (traces b, left and b,c, right in Fig. 5A). The NMDAR component of EPSCs recovered to control values within 20–40 s ($n = 5$, not shown). Therefore, the depolarization-induced NMDAR

inactivation most probably involves Ca-dependent postsynaptic but not presynaptic mechanisms.

Parallel time course of inactivation of exogenous and synaptic NMDAR responses

Further evidence in favour of a postsynaptic involvement of calcium-induced inactivation of NMDA receptors was obtained by monitoring simultaneously the effect of a depolarizing train on NMDAR-EPSCs and currents induced by local application of exogenous NMDA to the postsynaptic neuron (Fig. 6A). As illustrated in Fig. 6A, depolarizing pulses reduced similarly both the NMDAR-EPSC and NMDAR-induced currents by 39 and 28%, respectively (measured 2–4 s after depolarization). On average, maximal reductions were $38 \pm 4\%$ and $26 \pm 4\%$ ($n = 6$, not significant: $P = 0.11$, Fig. 6B), and time constants of recovery from inactivation were also similar: $\tau = 31 \pm 5$ s and $\tau = 28 \pm 8$ s ($n = 6$) for EPSCs and application-induced currents, respectively.

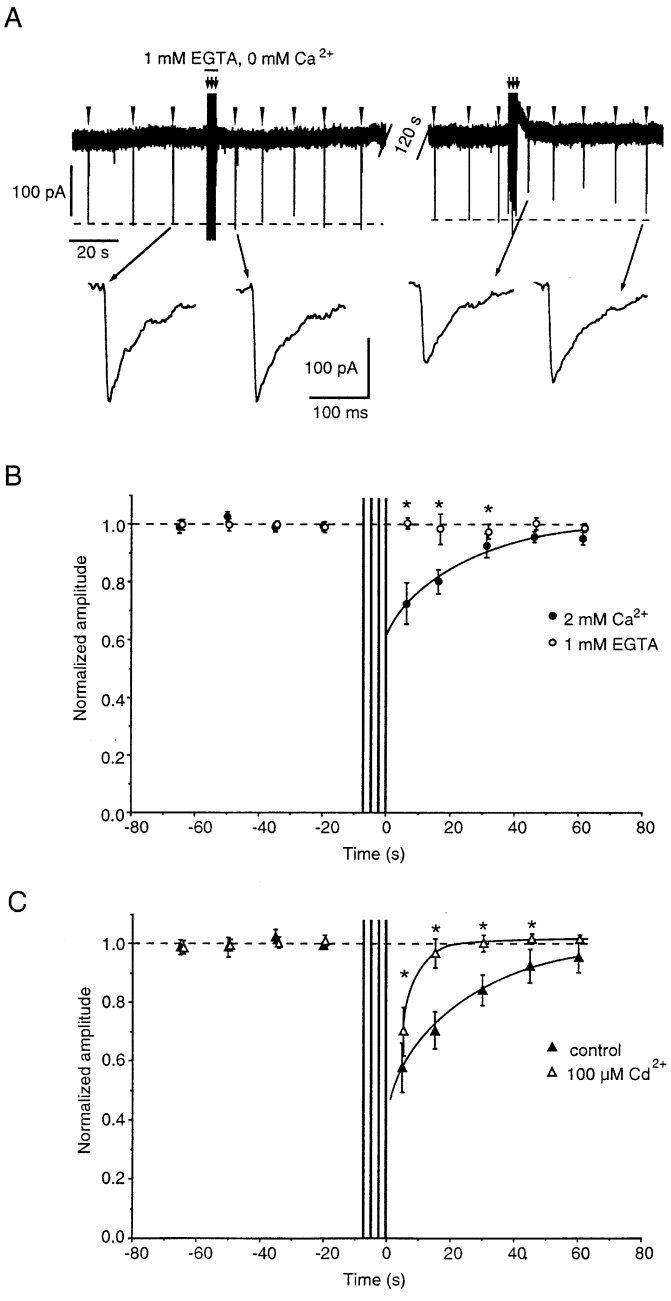

FIG. 4. NMDAR-EPSC inactivation is suppressed by reducing $[Ca^{2+}]_o$ and by applying Cd^{2+} during postsynaptic depolarization. (A) Traces show depolarization-induced inactivation of NMDAR-EPSCs in the absence (left) and presence (right) of 2 mM Ca^{2+} in the external solution during application of depolarizing pulses. Application of the Ca^{2+}-free external solution containing 1 mM EGTA started 1–2 s before the depolarizing train, indicated by a bar above the trace. In the illustrated experiment, the postsynaptic pipette contained K-gluconate in place of Cs-gluconate. Depolarizing pulses in the presence of 2 mM $[Ca^{2+}]_o$ activated the outward potassium current, as determined by measuring the reversal potential using voltage ramp application (not shown) and decreased the amplitude of the NMDAR-EPSCs. The disappearance of this outward current was used as a test for the efficacy of the Ca^{2+} removal during the depolarizing train shown in the left panel. To prevent the possible artifacts of NMDA-EPSCs changes related to the activation of outward current, the majority of experiments, including most of the experiments in this serial (three out of five), were performed using Cs^+ in the postsynaptic pipette which blocked potassium currents. (B) The amplitude of the NMDAR-EPSCs recorded after depolarization in the absence (1 mM EGTA) (○) and presence of 2 mM $[Ca^{2+}]_o$ (●). The means ± SEM of five experiments are presented. The amplitudes have been normalized to the mean control amplitude. Asterisks mark the statistically significant difference between amplitudes of EPSCs measured in the absence (1 mM EGTA) and presence of 2 mM $[Ca^{2+}]_o$ (paired t-test, $P < 0.05$). (C) The application of 100 µM $[Cd^{2+}]_o$ during depolarization of postsynaptic neuron reduces the degree of inactivation of NMDAR-EPSCs. The means ± SEM of five experiments are shown. Statistically significant differences are marked by asterisks (paired t-test, $P < 0.05$).

Discussion

The present results suggest that the rise of $[Ca^{2+}]_i$ induced by depolarizing pulses inactivates NMDAR-EPSCs. This effect is Ca^{2+} dependent and affects selectively NMDAR-EPSCs, AMPAR-EPSCs remaining unchanged.

NMDAR-channels were shown to be transiently inactivated by intracellular Ca^{2+} increases that can be mediated by various pathways. Previous studies of responses to exogenous NMDA have shown that an increase in intracellular Ca^{2+} via VDCCs (Legendre *et al.*, 1993), intracellular stores (ryanodine stores, Kyrozis *et al.*, 1996), NMDA-R channels (Mennerick & Zorumski, 1996; Zorumski *et al.*, 1989) and AMPA/KA-R Ca^{2+}-permeable channels (Medina *et al.*, 1994; Kyrozis *et al.*, 1995) were all able to transiently inactivate NMDAR activity. Several mechanisms have been suggested to underlie the calcium-induced inactivation of NMDAR: (i) calcineurin-dependent dephosphorylation (Lieberman & Mody, 1994; Tong & Jahr, 1994); (ii) interaction with the cytoskeleton (Rosenmund & Westbrook, 1993); (iii) inactivation by a putative Ca^{2+}-dependent diffusible factor (Vyclicky, 1993; Medina *et al.*, 1996); (iv) interaction of calmodulin with the NR1 subunit through two different low- and high-affinity binding sites (Ehlers *et al.*, 1996). Synaptic spines are compartmentalized, and it is not clear how close the source of Ca^{2+} must be from the NMDAR to produce inactivation of their activity. Therefore, it is a central issue to determine which of the various sources of intracellular Ca^{2+} modulate NMDAR-EPSCs.

Previous studies reported that Ca^{2+} influx through NMDAR efficiently inhibited NMDAR-EPSCs, suggesting that highly localized Ca^{2+} influx in the direct vicinity of NMDAR was efficient to produce their inactivation (Mennerick & Zorumski, 1996; Zorumski *et al.*, 1989). Similarly, the inactivation of the NMDAR-EPSCs has been reported in response to activation of the calcium influx through VGCCs (Rosenmund *et al.*, 1995; Tong *et al.*, 1995). However, the autapses used in these studies have a number of technical limitations and do not allow one to determine if a single somatic spike can reduce

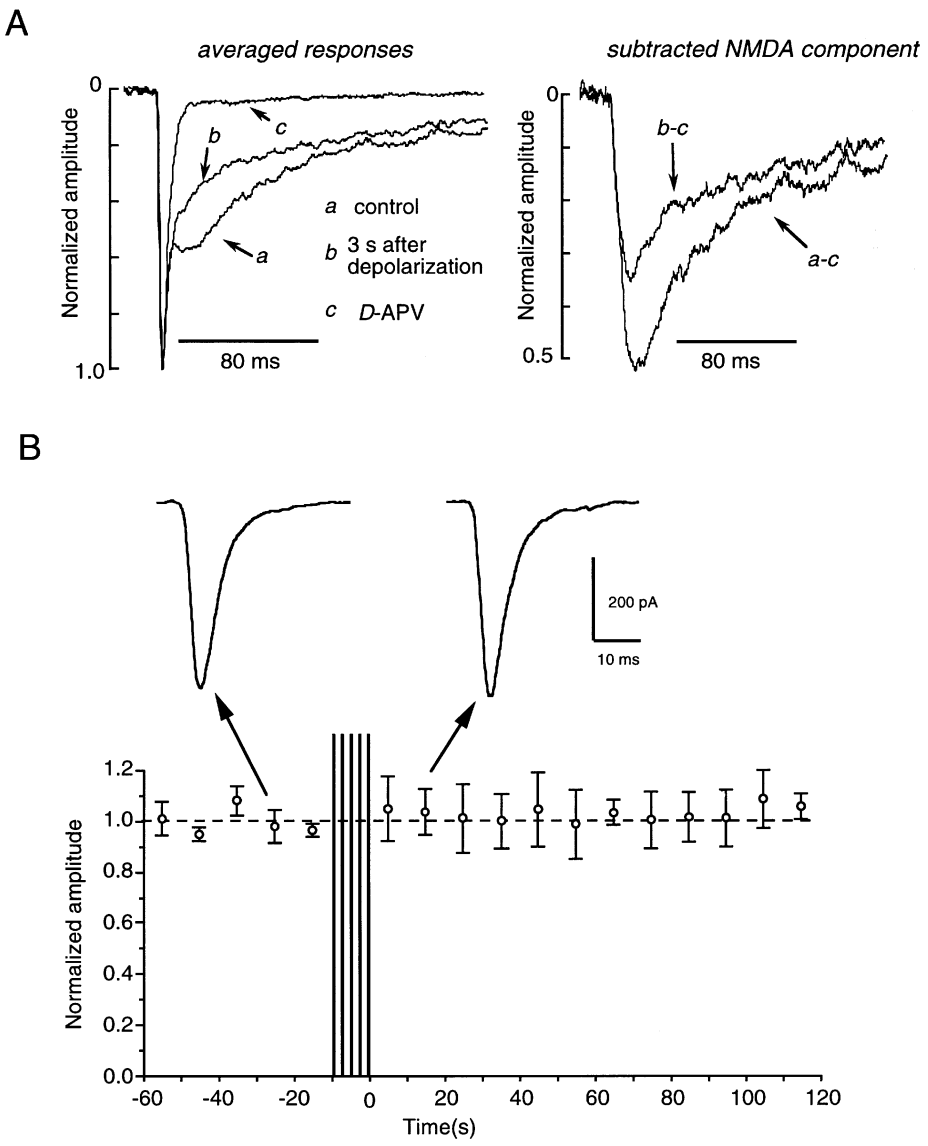

FIG. 5. Postsynaptic depolarizing pulses inactivate NMDAR- but not AMPAR-EPSCs. (A) EPSCs recorded in the presence of 10 μM glycine and 20 μM bicuculline. Five trains of depolarizing pulses were applied at intervals of 2–3 min. Trace a (control) represents the averaged mixed AMPAR/NMDAR response evoked before application of postsynaptic depolarizing pulses. Trace b is the averaged response evoked 3 s after depolarizing trains. To demonstrate the changes in the NMDAR-mediated component of EPSCs, at the end of the experiment the AMPAR responses were recorded in the presence of 100 μM APV (trace c), and then the NMDAR-mediated current was obtained by subtraction of the traces, as illustrated at the right and indicated by corresponding letters. (B) Normalized amplitude of AMPAR-mediated EPSCs before and after a depolarizing train. The means from five experiments are illustrated. The amplitudes have been normalized to the mean control amplitude. The traces above illustrate the averaged responses corresponding to the points indicated by arrows.

the amplitude of the NMDAR-EPSCs. By using double-patch recordings from two synaptically connected neurons in the present study, we have shown that, while the single firing of the postsynaptic neuron does not significantly modulate the amplitude of the NMDAR-EPSCs, bursts of action potentials effectively reduce this amplitude. This effect was most probably due to a postsynaptic increase in $[Ca^{2+}]_i$ as the following conditions prevented the depolarization-induced inactivation of NMDAR-EPSCs: (i) strong buffering of postsynaptic intracellular calcium by 30 mM BAPTA; (ii) removal of calcium from the external solution; and (iii) voltage-dependent calcium channels blockade by Cd^{2+}. Recent studies showed the existence of VGCCs in postsynaptic spines (Yuste & Denk, 1995; Segal, 1995), suggesting that calcium influx through dendritic calcium channels could directly inactivate synaptic NMDA receptors.

The following observations suggest that the depolarization-induced inactivation of NMDAR-EPSCs is mediated by a postsynaptic mechanism: (i) depolarizing pulses depressed the responses to exogenous NMDA applications; (ii) when both fast CNQX-sensitive (AMPA) and slow APV-sensitive (NMDA) components of EPSCs were recorded altogether, the depolarizing pulses selectively modulated the NMDAR, the AMPAR component of EPSCs remaining unaffected. This suggests that the release of glutamate by presynaptic terminals was not affected by the postsynaptic depolarization. This observation also eliminates the possibility that changes in EPSCs amplitude could be due to insufficient voltage-clamp in dendrites, because in such a case, similar changes in NMDAR-and AMPAR-EPSC amplitude should be observed. Therefore, the inactivation of NMDAR-EPSCs does not involve a retrograde messenger released upon activation of postsynaptic voltage-dependent Ca^{2+} channels as recently suggested for GABAergic synaptic transmission in the cerebellum (Glitsch *et al.*, 1996).

In our experiments, potentiation of AMPAR-EPSCs by intracellular calcium (as previously reported: Wyllie *et al.*, 1994) was observed in two out of nine experiments by 12 and 25% (9 s after the depolarizing train) that did not recover to the control value within 2 min (not shown). But because in the remaining seven experiments AMPAR-EPSCs were not modulated, the average amplitude of the AMPAR-EPSCs after postsynaptic depolarization was not significantly different from the control value (Fig. 5). Therefore, our results also support the notion that NMDA receptors are the only subtype of glutamate-activated channels inactivated by intracellular calcium (Legendre *et al.*, 1993; Vyclicky, 1993; Medina *et al.*, 1994; Kyrozis *et al.*, 1995).

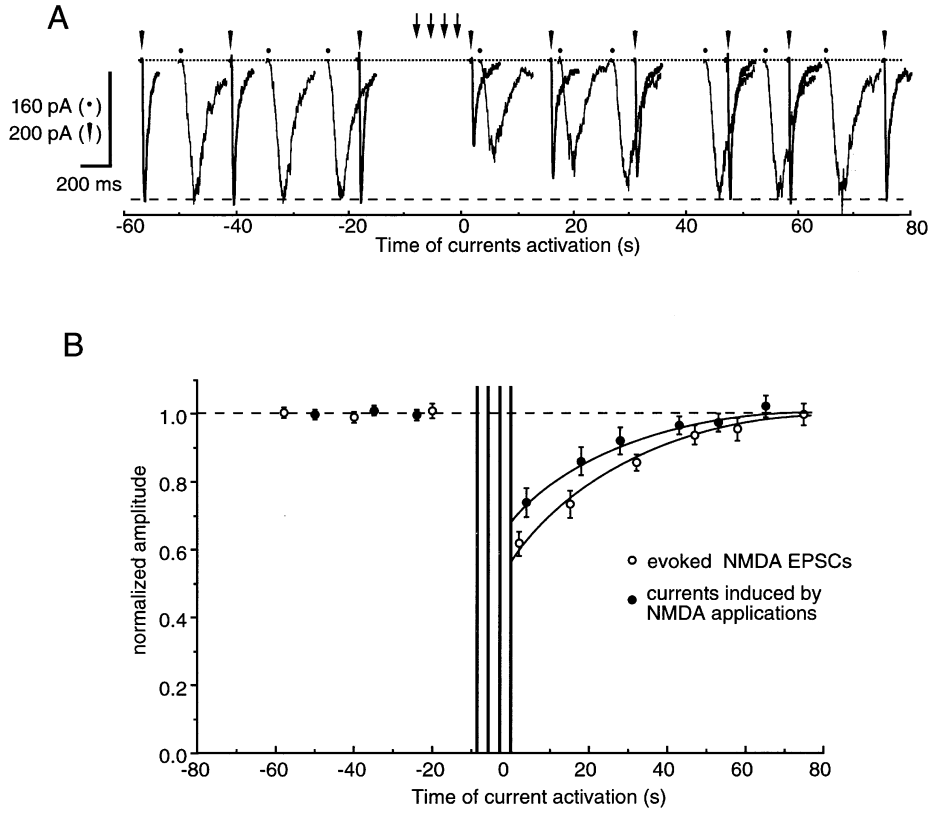

FIG. 6. NMDAR-EPSCs and NMDA-induced currents show similar inactivation by postsynaptic depolarization. (A) Examples of evoked NMDAR-EPSCs (traces marked by triangles) and currents evoked by brief (50 ms) applications of 100 μM NMDA (traces marked by closed circles). The positions of triangles and circles correspond to the times of EPSC stimulation and NMDA application current activations, respectively (timescale below traces). The thick calibration bars at the left are for the amplitude and duration of individual current traces. The arrows above the traces show times of the postsynaptic neuron depolarization. (B) Normalized amplitudes of NMDAR-EPSCs (○) and currents induced by NMDA (●). Vertical lines show the times of depolarization of the postsynaptic neuron. Averaged data from six experiments are presented.

It is generally accepted that Ca^{2+} is a major intracellular messenger involved in various processes, e.g. synaptic plasticity, neuronal development and excitotoxicity. The transient rise of Ca^{2+} in neurons has consequences that are much longer lasting than the rise of $[Ca^{2+}]_i$: long-term potentiation (LTP) or long-term depression (LTD) of synaptic efficacy (Bear, 1995), and sustained enhancement of postsynaptic AMPAR responses (Wyllie *et al.*, 1994). In contrast to these processes, the effect of Ca^{2+} on NMDAR-EPSCs is transient. Physiologically, the Ca^{2+}-dependent reduction of NMDAR activity may decrease Ca^{2+} influx and therefore regulate the balance between LTP and LTD, or protect cells from excitotoxicity during neuronal hyperexcitation. Future studies are required to evaluate more precisely the role of calcium originating from different types of intracellular stores, VGCCs and AMPAR-channels in the modulation of the NMDAR component of EPSCs.

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; CC, current-clamp; CNQX, 6-cyano-7-nitro-quinoxaline-2,3 dione; D-APV, D-2-amino-5-phosphonopentanoic acid; EGTA, ethylene glycol-bis(beta-aminoethylether)-N,N,N′,N′-tetraacetic acid; EPSCs, excitatory postsynaptic currents; GABA, γ-aminobutyric acid; LTD, long-term depression; LTP, long-term potentiation; MEM, minimum essential medium; NMDAR, *N*-methyl-D-aspartate receptor; PBS, phosphate-buffered saline; VC, voltage-clamp; VDCCs, voltage-gated calcium channels.

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