Postsynaptic modulation of NMDA synaptic currents in rat hippocampal microcultures by paired-pulse stimulation

Steven Mennerick* and Charles F. Zorumski†‡§

*Departments of †Psychiatry, ‡Anatomy and Neurobiology and *Program in Neuroscience, Washington University School of Medicine, St Louis, MO 63110, USA*

1. Paired-pulse synaptic stimulation of hippocampal neurons in microcultures resulted in depression of synaptic currents mediated by both NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors. However, NMDA EPSCs were more severely depressed than AMPA EPSCs.

2. Partial NMDA receptor blockade reduced paired-pulse depression of NMDA but not of AMPA synaptic currents while partial AMPA receptor blockade had no effect on paired-pulse depression of AMPA EPSCs. These results suggest that ion flux through NMDA receptors is important in paired-pulse depression of NMDA responses but has no effect on AMPA responses.

3. Low extracellular Ca^{2+} concentrations or positive postsynaptic holding potentials reduced paired-pulse depression of NMDA EPSCs to near that of AMPA responses.

4. Brief paired applications of exogenous glutamate to neurons produced Ca^{2+}-dependent depression similar to the depression of NMDA synaptic responses and synaptic stimulation depressed responses to exogenously applied NMDA.

5. Physiological concentrations of Mg^{2+} prevented expression of the postsynaptic modulation of NMDA EPSCs at −70 mV, but partial relief of Mg^{2+} block of the NMDA channel with depolarization increased paired-pulse depression of NMDA EPSCs.

Presynaptic firing frequency has important effects upon synaptic efficacy. At many synapses, frequency-dependent depression of synaptic currents occurs when the initial output of transmitter is high (Takeuchi, 1958; Betz, 1970). At most synapses where depression has been studied, depression occurs at a presynaptic locus, i.e. through a decrease in the amount of transmitter released (del Castillo & Katz, 1954). Our previous studies of paired-pulse depression of AMPA EPSCs at hippocampal microculture synapses have also suggested a presynaptic mechanism of paired-pulse depression (Mennerick & Zorumski, 1995*a*).

Although presynaptic depressants are thought to diminish AMPA and NMDA EPSCs in parallel (Perkel & Nicoll, 1994; Tong & Jahr, 1994), we find that NMDA EPSCs show a consistently larger degree of paired-pulse depression than AMPA EPSCs under many conditions. Our results suggest a postsynaptic contribution to NMDA EPSC depression and implicate Ca^{2+}-dependent receptor desensitization (Mayer & Westbrook, 1985; Zorumski, Yang & Fischbach, 1989; Clark, Clifford & Zorumski, 1990; Legendre, Rosenmund & Westbrook, 1993; Vyklicky, 1993) in the additional depression of NMDA responses. Although investigation of a synaptic role for AMPA receptor desensitization has been intense (Trussell & Fischbach, 1989; Vyklicky, Patneau & Meyer, 1991; Thio, Clark, Clifford & Zorumski, 1992; Hestrin, 1993; Trussell, Zhang & Raman, 1993), the present results represent evidence that receptor desensitization can also participate in the frequency-dependent depression of NMDA EPSCs.

METHODS

Hippocampal cultures

The microculture procedure was adapted from that of others (Segal & Furshpan, 1990) and has been detailed previously (Mennerick, Que, Benz & Zorumski, 1995). Halothane (0·5–1·0%)-anaesthetized Sprague–Dawley rats (1–3 days postnatal) were decapitated and transverse hippocampal slices, 500–800 μm thick, were prepared and treated with 1 mg ml^{-1} papain in oxygenated Leibovitz's L15 medium (Gibco). A cell suspension was obtained by gentle trituration in culture medium (Eagle's minimum essential

§ To whom correspondence should be addressed at the Department of Psychiatry, Washington University School of Medicine, 4940, Children's Place, St Louis, MO 63110, USA.

medium, supplemented with 5% horse serum, 5% fetal calf serum, 17 mM D-glucose, 400 μM glutamine, 50 u ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin). Cells (~20 000 ml^{-1}) were then plated on plastic culture dishes (35 mm diameter), treated with a layer of agarose (0·15%) and collagen droplets (0·5 mg ml^{-1} rat tail, Sigma Type I) as previously described (Mennerick *et al.* 1995). Glial proliferation was halted with 10 μM cytosine arabinoside added 3 days after plating.

Evoked autaptic and interneuronal monosynaptic responses

Whole-cell recording methods were used to study autaptic and monosynaptic responses from single-neuron and two-neuron micro-islands. For most studies pipette solutions contained (mM): 140 potassium gluconate, 4 NaCl, 0·5 $CaCl_2$, 5 EGTA, 10 Hepes, 2 MgATP, 0·2 GTP at pH 7·25. Pipettes had open tip resistances of 2·5–5 MΩ.

Autaptic currents were usually studied with an Axopatch 1D amplifier with series resistance compensation set at 80–90%. Evoked autaptic responses were elicited as previously described (Bekkers & Stevens, 1991; Mennerick *et al.* 1995) where 1·5 ms voltage steps from a negative holding potential (usually −65 mV) to a positive potential (usually +25 mV) were made to elicit evoked autaptic currents. For recordings of neuronal pairs on two-neuron micro-islands, the presynaptic cell was stimulated using an Axoclamp 2A amplifier in the discontinuous single-electrode voltage clamp mode (10–12 Hz switching frequency) while the postsynaptic cell was monitored using an Axopatch 1D amplifier. In all studies, to negate any time-dependent changes in synaptic responses, control and experimental trials were interleaved.

Solutions

The standard extracellular recording solution for all experiments contained (mM): 140 NaCl, 4 KCl, 10 Hepes and 10 glucose. Unless otherwise specified, the extracellular solution contained 3 mM $CaCl_2$, 20 μM glycine, and no added Mg^{2+}. The pH was adjusted to 7·25 with NaOH. Osmolarity of the extracellular solution was maintained at ~310 mosmol l^{-1} by addition of sucrose. For synaptic studies, extracellular solution exchanges and drug applications were achieved with a multi-barrel, gravity-fed local perfusion system. The tip of the solution exchange pipette was located 0·5–1·0 mm from the micro-island being studied. Junction potential changes at the tip of an open recording pipette indicated that complete solution exchanges could be achieved over the extent of micro-islands in 200 ms with this configuration.

For exogenous applications of glutamate, a rapid, gravity-driven flow tube apparatus was constructed. Brief applications of 4 ms were achieved with a solenoid valve (Lee Co., Essex, CT, USA) connected to a tapered flow tube (100 μm i.d.) and rapidly cleared with a second flow tube. Junction potential changes at the open tip of a patch pipette in response to solutions of differing chloride concentrations occurred with application and clearance times of ~1 ms. In whole cells, time constants of offset currents in response to 4 ms applications of 1 mM kainic acid, a non-desensitizing agonist at AMPA receptors, measured 10·1 ± 0·6 ms, suggesting that drug clearance from around cells was slower than at the tip of a patch pipette. Nevertheless, this offset value is similar to some of the slower time constants of synaptic currents in microcultures (Mennerick & Zorumski, 1995*b*). As faster application and removal of glutamate could be achieved on membrane patches, we also attempted exogenous applications onto outside-out patches to mimic synaptic responses (Lester, Clements, Westbrook & Jahr, 1990; Jonas & Sakmann, 1992). However, consistent with previous findings (Sather, Johnson, Henderson & Ascher, 1990), NMDA receptor desensitization in outside-out patches, unlike desensitization in whole cells (see Results), was resistant to removal of extracellular calcium.

Unless otherwise noted, results are reported as means ± S.E.M. and evoked traces represent the mean of 2–10 individual responses filtered at 2 kHz and digitized at 3–5 kHz. Data were analysed using commercially available software (pCLAMP, Axon Instruments) and software written in the Axobasic programming language (Axon Instruments). Modulation of synaptic responses is expressed in the text and figures using the equation:

$$\text{PPM} = ((p_2/p_1)-1) \times 100\%,$$

where PPM is the degree of paired-pulse modulation, p_2 is the test response amplitude, and p_1 is the conditioning response amplitude. Therefore, decreases in amplitude relative to control are expressed as negative percentages. Currents were quantified by measuring the peak current of pharmacologically isolated AMPA and NMDA EPSCs or excitatory autaptic currents (EACs). Alternatively, in dual-component EPSCs, the peak response was used to quantify the fast AMPA component and the mean current 60–70 ms following stimulation was used to quantify the slow NMDA component, as described previously (Hestrin, Nicoll, Perkel & Sah, 1990). For several experiments, particularly those in which small AMPA EACs were examined, a trace in the presence of postsynaptic receptor antagonists was digitally subtracted from EAC traces to eliminate any contribution of stimulus transients to measurement of the rapidly rising AMPA EAC. Drugs and chemicals were from Sigma except (−)-baclofen (gift of Ciba-Geigy); D-2-amino-5-phosphonovalerate (D-APV, Cambridge Research Biochemicals); 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2-3-dione (NBQX, generous gift of Dr John Olney, Washington University School of Medicine, St Louis, MO, USA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Research Biochemicals International).

RESULTS

As previously described, paired-pulse stimulation of single-neuron microcultures 8–16 days *in vitro* resulted in depression of test excitatory autaptic currents (EACs) compared with conditioning EACs (Mennerick & Zorumski, 1995*a*). However, when pharmacologically isolated AMPA EACs were compared with isolated NMDA EACs, NMDA EACs consistently exhibited more severe depression than AMPA EACs from the same neuron (Fig. 1*A* and *B*). In these studies we used a paired-pulse interval of 2·5 s. This allowed the slow conditioning NMDA currents to decay nearly to baseline before a test pulse was delivered and negated the problem of measuring test EAC amplitudes from the decay phase of a previous conditioning response (see below). In a sample of ten single-neuron micro-islands in which isolated AMPA and NMDA EACs were examined, AMPA EACs were depressed by −12 ± 4% and NMDA EACs by −33 ± 6% ($P < 0·001$, Student's paired t test). We have previously shown that depression of AMPA EACs is likely to be through a presynaptic mechanism (Mennerick

& Zorumski, 1995*a*) similar to depression at the vast majority of other synapses studied (Magleby, 1987 for review). Other presynaptic modulators have been reported to modulate AMPA and NMDA EPSCs in parallel (Perkel & Nicoll, 1994; Tong & Jahr, 1994). Therefore, the differential modulation of AMPA and NMDA EACs is surprising.

One possible explanation for the results in Fig. 1*A* and *B* is that transmitter release characteristics are different on paired-pulse trials in which NMDA receptors are involved. For instance, perhaps Ca^{2+} influx through NMDA channels during conditioning responses alters transmitter release during test responses. To test this possibility, we took advantage of the differential time course of AMPA and NMDA synaptic currents (Hestrin *et al.* 1990) and examined paired-pulse depression in dual-component EACs. This allowed measurement of both the fast AMPA and slow NMDA components of EACs on each paired-pulse trial. As shown in Fig. 1*C* and *D*, the difference between AMPA and NMDA paired-pulse depression persisted in dual-component EACs, arguing against the possibility that activation of NMDA receptors during conditioning responses depresses subsequent transmitter release.

We also examined the question of whether known presynaptic modulators alter AMPA and NMDA EACs in parallel. In agreement with previously reported results (Perkel & Nicoll, 1994; Tong & Jahr, 1994), we found that a low concentration of the $GABA_B$ agonist baclofen (0·35 μM) mimicked the degree of paired-pulse depression of AMPA EACs ($-11 \pm 3\%$, $n = 17$ cells) but produced a similar degree of modulation of NMDA EACs, ($-12 \pm 4\%$, $P = 0{\cdot}5$, Student's paired t test). However, in contrast to results previously reported, we found that greater presynaptic depression produced disproportionate depression of the slow, NMDA component of EACs compared with the AMPA peak current. For instance, 1 μM baclofen produced depression of $-43 \pm 6\%$ for peak EACs and $-58 \pm 6\%$ for slow EACs ($P = 0{\cdot}001$, $n = 6$ cells). The differential modulation of the two components of EACs was not due to

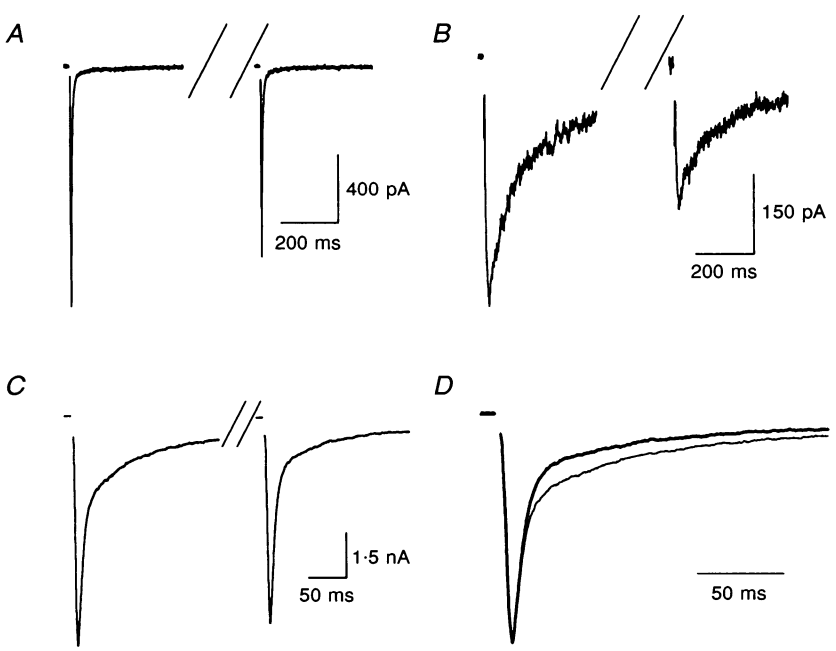

Figure 1. Paired-pulse stimulation disproportionately depresses NMDA EACs compared with AMPA EACs

A, results from an extracellular bath solution containing 3 mM Ca^{2+}, 20 μM glycine, and no added Mg^{2+}. AMPA EACs were isolated in 50 μM D-APV and stimulated with a paired-pulse interval of 2·5 s (25 s between paired-pulse trials). Test responses exhibited depression in this cell of $-21 \pm 1\%$ ($n = 4$ trials) relative to the preceding conditioning response. *B*, in the same cell, NMDA EACs were pharmacologically isolated by washing out the D-APV and adding 1 μM NBQX. The same paired-pulse stimulation produced $-42 \pm 2\%$ depression ($n = 5$ trials). Trials in the presence of different antagonists were interleaved and averaged for display. In this and subsequent figures, stimulus artifacts have been blanked for clarity unless otherwise indicated. Diagonal slashes indicate omissions in the time axis. *C*, in another cell, dual-component EACs were elicited by omitting receptor antagonists. *D*, by scaling the peak EAC of the test response (thick trace) to the conditioning response (thin trace) to account for paired-pulse depression of the AMPA component, it can be seen that the disproportionate depression of the slower NMDA EACs is also present in dual-component EACs.

an artifact of the large amplitude of EACs, since in cells treated with 80 nM NBQX and 5 μM D-APV to partially block postsynaptic receptors and limit the size of EACs to ~30% of unblocked responses, the differential modulation was still apparent (-40 ± 3 *vs.* $-52 \pm 4\%$, $P < 0{\cdot}001$, $n = 17$ cells). The differential modulation was mimicked by two other presynaptic depressants. Lowering extracellular Ca^{2+} from 3 to 0·8 mM produced depression of $-61 \pm 6\%$ for peak EACs and $-89 \pm 2\%$ for slow EACs ($P < 0{\cdot}001$, $n = 11$ cells), despite the known increase in NMDA single-channel conductance upon lowering extracellular Ca^{2+} (Jahr & Stevens, 1993). Also, 0·5 μM 2-chloroadenosine, an agonist at presynaptic adenosine receptors, produced $-60 \pm 6\%$ depression of peak EACs and $-78 \pm 5\%$ depression of slow EACs ($P < 0{\cdot}001$, $n = 9$ cells). Since the differential modulation of AMPA and NMDA EACs by presynaptic agents was apparent only with relatively high levels of presynaptic depression, we hypothesized that the additional factor contributing to NMDA depression during paired-pulse stimulation is primarily a postsynaptic factor.

We explored whether the extra paired-pulse depression in NMDA EPSCs could be distinguished from the depression of AMPA responses on the basis of differential recovery

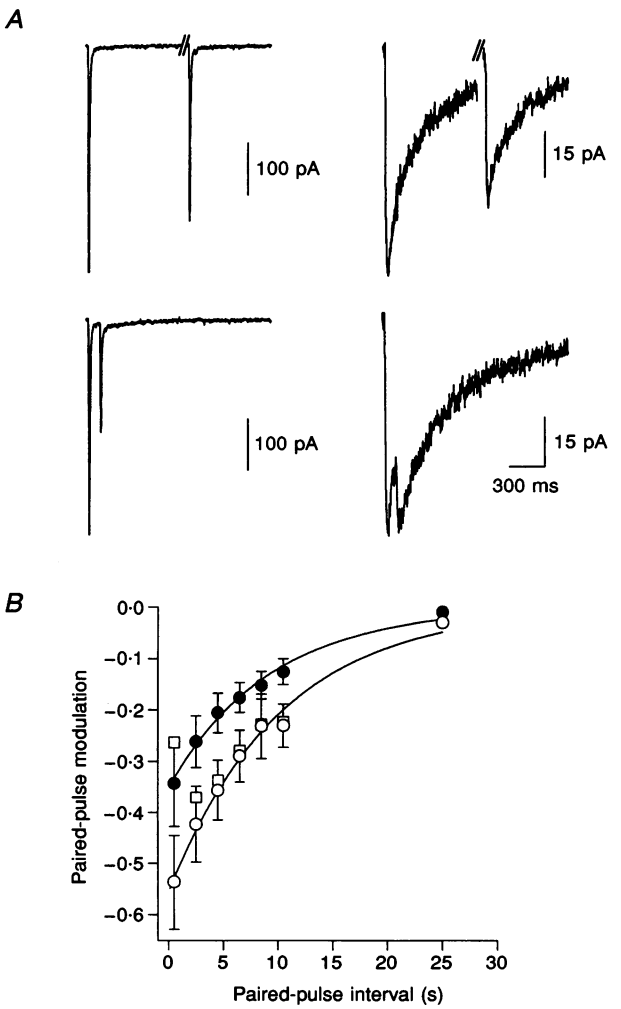

Figure 2. Recovery from AMPA and NMDA depression occurs over seconds

A, quantification of NMDA depression at brief paired-pulse intervals is complicated by slow decays of conditioning EPSCs. The left panels show AMPA EPSCs and the right panels show NMDA EPSCs from a postsynaptic cell. AMPA EPSCs were pharmacologically isolated with 50 μM D-APV, and NMDA EPSCs were isolated with 10 μM CNQX in the bath solution. The top panels show the paired-pulse depression elicited with a paired-pulse interval of 2·5 s. The bottom panels show depression elicited with a 100 ms paired-pulse interval. *B*, the symbols represent the average depression observed at various paired-pulse intervals, beginning with a 500 ms interval, in dual-component EACs from 6 single-cell micro-islands. □, the amount of NMDA depression when test EACs were measured from the initial baseline holding current level preceding conditioning stimulation. The lines represent a single exponential fit by least squares minimization to the data represented by circles. For the AMPA component (●) the time constant of recovery was 9·3 s; for the NMDA component (○) the time constant of recovery was 10·3 s.

time courses. It was difficult to quantify NMDA paired-pulse modulation at very brief paired-pulse intervals because of the dramatic difference in the decay time course of AMPA and NMDA EPSCs. The difficulty has been discussed in detail with regard to inhibitory synaptic currents (Roepstorff & Lambert, 1994) and is illustrated by the example EPSCs shown in Fig. 2*A*. The traces show pharmacologically isolated AMPA (left panels) and NMDA (right panels) EPSCs from a postsynaptic neuron on a two-neuron micro-island. The top panels show that at the paired-pulse interval of 2·5 s, both components are depressed, with the NMDA component exhibiting slightly

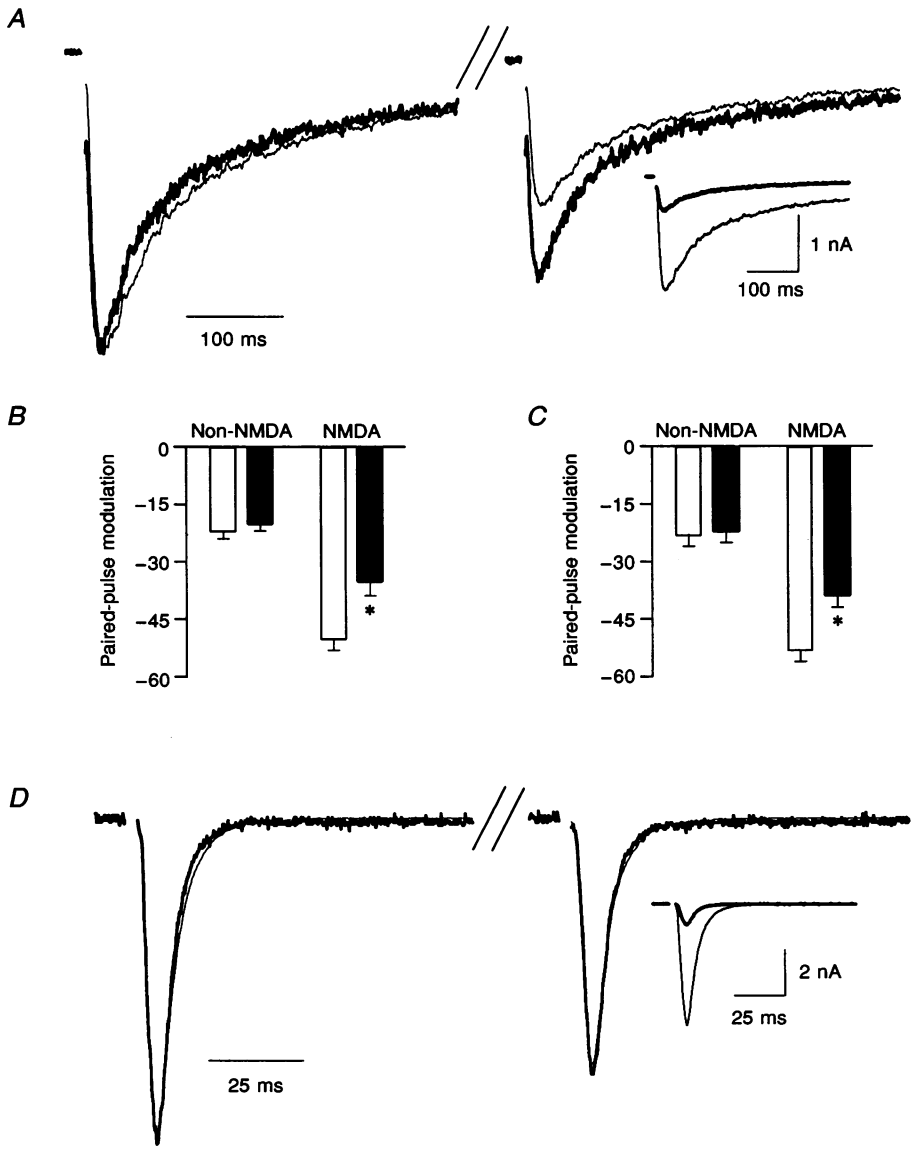

Figure 3. Partial postsynaptic blockade reduces NMDA paired-pulse depression but not AMPA paired-pulse depression

A, pharmacologically isolated NMDA EACs were stimulated as in Fig. 1 (control, thin traces). On alternating paired-pulse trials, 30 μM Mg^{2+} was added to the extracellular bath solution. The thick trace represents trials in 30 μM Mg^{2+} with the conditioning response peak amplitude scaled to the peak amplitude of control responses to show the reduction in NMDA paired-pulse depression. The inset shows the reduction in NMDA conditioning EACs with 30 μM Mg^{2+}. *B* and *C*, in dual-component EACs, the fast peak response showed no change in paired-pulse modulation with the addition of 30 μM Mg^{2+} (*B*, filled bars) or with the addition of the competitive antagonist D-APV (8–10 μM; *C*, filled bars). However, both the non-competitive and the competitive antagonists reduced the amount of NMDA paired-pulse depression. * $P < 0{\cdot}001$, antagonist *versus* control conditions, $n = 11$ cells for *B* and $n = 10$ cells for *C*. *D*, the AMPA receptor antagonist NBQX (90 nM; thick trace) depressed AMPA EACs by −83% (inset, thick traces, NBQX) but had no effect upon AMPA EAC paired-pulse depression as shown by the responses scaled with respect to conditioning responses. Control, thin traces.

greater depression than the AMPA component. The bottom panels show that while the AMPA component is clearly more depressed at a paired-pulse interval of 100 ms than at the 2·5 s interval, quantification of the NMDA EPSC depression is complicated by the decaying conditioning EPSC. If one measures the test NMDA EPSC from the original baseline holding current (preceding the conditioning EPSC), the test NMDA EPSC is only slightly depressed; however, if the test EPSC is measured from the decaying conditioning EPSC, the test NMDA EPSC shows greater depression at the 100 ms interval than at 2·5 s and greater depression than the AMPA component. The decaying conditioning response may be considered the appropriate baseline for measuring the test EPSC if receptors bound by transmitter during the conditioning response do not occlude the ability of transmitter released by test stimulation to bind and activate receptors. In other words, if receptors at synapses activated by the conditioning response are well below maximum occupancy for transmitter or if test EPSCs occur primarily at synaptic sites not activated during the conditioning response, the decaying conditioning EPSC can be considered the more appropriate baseline for test EPSC measurement. However, to avoid making assumptions about the measurement of NMDA EPSCs at brief paired-pulse intervals, we focused on intervals > 500 ms, to allow for the decay of conditioning EPSCs.

Figure 2*B* shows the average recovery time course of six dual-component EACs. The depression of the NMDA component was greater than the AMPA component at all paired-pulse intervals when test EPSCs were measured from decaying conditioning EPSCs. Furthermore, when measured in this manner, recovery from depression in the two components occurred in parallel (Fig. 2*B*). The AMPA component recovery was well fitted by a single exponential with a time constant of 9·3 s, while the NMDA component recovered with a time constant of 10·3 s. Therefore, it was not possible to confidently distinguish the extra component of NMDA EPSC paired-pulse depression on the basis of recovery time course.

The mechanisms underlying paired-pulse depression may be thought of as leaving the synapse refractory to subsequent stimulation. With the frequency-dependent presynaptic depression that occurs at many synapses, partial presynaptic blockade (e.g. lowering extracellular Ca^{2+}) can diminish the amount of depression (Betz, 1970). This has led to the hypothesis that conditioning presynaptic stimulation leaves the presynaptic terminal refractory to subsequent stimulation, perhaps through depletion of quantal stores (del Castillo & Katz, 1954). Likewise, if a refractory postsynaptic mechanism unique to NMDA receptors is responsible for the additional depression seen in NMDA EACs, then perhaps partial postsynaptic blockade of NMDA receptors should diminish

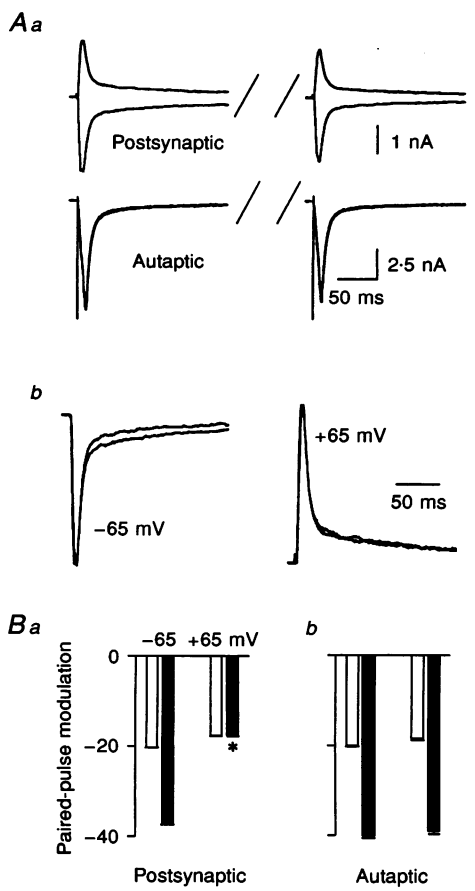

Figure 4. A positive membrane potential reduces NMDA synaptic depression

A a, the traces exemplify the experimental protocol. Dual recordings were made from two-neuron micro-islands. One cell was designated presynaptic (bottom traces). The other cell was designated postsynaptic (top traces). Postsynaptic holding potential was maintained at −65 mV (inward postsynaptic currents) or at +65 mV (outward postsynaptic currents) on alternating trials. The presynaptic cell was maintained at −65 mV throughout (except during stimulation). Part of the stimulus artifacts in the autaptic traces have been left intact for clearer identification of the presynaptic cell. *A b*, the peak postsynaptic test response from the cell in *A* was scaled to the peak conditioning response. At −65 mV (left panel) the slow NMDA component was depressed more than the fast AMPA component. The same scaling procedure for the currents at +65 mV (right panel) demonstrates that at the positive potential, both components were depressed to a similar extent. *Ba*, paired-pulse depression of AMPA and NMDA EPSCs is shown for 11 postsynaptic cells examined as in *A*. Open bars non-NMDA, filled bars NMDA. A two-way repeated measures analysis of variance revealed a significant interaction between the type of postsynaptic response (AMPA or NMDA) *versus* postsynaptic holding potential ($P < 0·001$). Bonferroni corrected *t* tests showed a significant difference between AMPA and NMDA responses at −65 mV but not at +65 mV. There was no significant difference between AMPA paired-pulse modulation at the two holding potentials. The data in the right panel (+65 mV data) are from Mennerick & Zorumski (1995*a*). *B b*, changes in the holding potential of the postsynaptic cell caused no change in the paired-pulse depression of NMDA autaptic responses recorded from presynaptic neurons, which were maintained at −65 mV.

the amount of paired-pulse depression of NMDA EACs. In support of this idea, partial blockade of NMDA receptors with the competitive antagonist D-APV (8–10 μM) or with the non-competitive antagonist Mg^{2+} (30 μM) decreased NMDA paired-pulse depression (Fig. 3*A–C*). In contrast, neither D-APV nor Mg^{2+} caused a change in the paired-pulse depression of AMPA EACs (Fig. 3*B* and *C*). Furthermore, partial blockade of AMPA receptors with the competitive AMPA receptor antagonist NBQX (90 nM) produced no consistent change in paired-pulse depression of isolated AMPA EACs (Fig. 3*D*, $P > 0{\cdot}3$, $n = 8$ cells) despite a $-75 \pm 4\%$ depression of the AMPA conditioning response. These results are consistent with the idea that ion flow through postsynaptic NMDA receptors is involved in the more severe paired-pulse depression of NMDA EACs.

Since NMDA receptors are much more permeable to Ca^{2+} than most AMPA receptors, Ca^{2+} is a good candidate for mediating postsynaptic depression of NMDA EACs. The desensitization of whole-cell responses to prolonged applications of exogenous NMDA receptor agonists in the presence of saturating concentrations of glycine has been shown previously to be Ca^{2+} dependent (Mayer & Westbrook, 1985; Zorumski, Yang & Fischbach, 1989; Clark *et al.* 1990; Legendre *et al.* 1993; Vyklicky, 1993). However, since this form of desensitization is relatively slow, it is not clear that this mechanism can contribute to depression of synaptic responses, where glutamate is present only briefly (Clements, Lester, Tong, Jahr & Westbrook, 1992). Nevertheless, the slow dissociation of glutamate from NMDA receptors makes it conceivable that this mechanism contributes to paired-pulse depression of NMDA synaptic responses (Lester & Jahr, 1992).

Several reports suggest that the Ca^{2+}-dependent desensitization of NMDA receptors is reduced by positive membrane potentials (Clark *et al.* 1990; Legendre *et al.* 1993; Vyklicky, 1993), presumably by preventing intracellular Ca^{2+} accumulation (Legendre *et al.* 1993). Therefore, we varied postsynaptic membrane potential and examined the effects upon paired-pulse depression of dual-component EPSCs. For this study we examined two-neuron microislands and monitored both the autaptic current of the presynaptic cell and the conventional monosynaptic response in the second neuron (Fig. 4*A*). Using this protocol, we were able to manipulate the membrane potential of the postsynaptic cell while using the autaptic response of the presynaptic cell as an additional internal control. Figure 4 shows that reversing the polarity of the postsynaptic membrane potential reduced the paired-pulse depression of NMDA EPSCs without significantly changing AMPA EPSC paired-pulse depression (Fig. 4*Ba*). In contrast, presynaptic EACs, measured in the same paired-pulse trials as the postsynaptic EPSCs, showed no change in the degree of NMDA paired-pulse depression with changes in the membrane potential of the postsynaptic cell

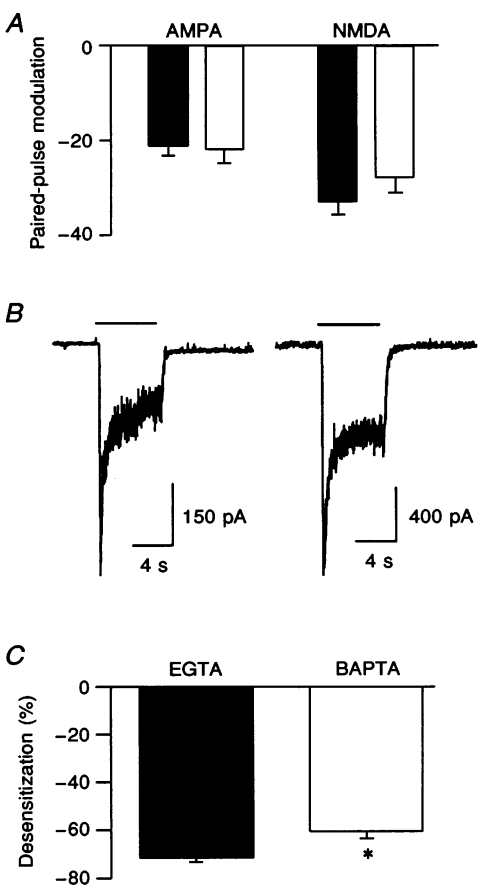

Figure 5. Intracellular BAPTA has subtle effects upon NMDA depression and desensitization

A, the effect of 20 mM intracellular BAPTA (open bars) upon depression of AMPA and NMDA EPSCs and EACs. Presynaptic cells were loaded with the standard pipette solution containing 5 mM EGTA (filled bars); postsynaptic cells were loaded with the same solution but with EGTA replaced with 20 mM BAPTA. Osmolarity of solutions was matched using sucrose. Dual-component autaptic and interneuronal responses were measured in a manner similar to that in Fig. 4*Aa*. A 2 × 2 ANOVA test with repeated measures for receptor type was used to analyse the effect of BAPTA upon AMPA and NMDA responses. Significance levels are given in the text (see Results). *B*, traces demonstrate the effect of the pipette solutions used in *A* on whole cell responses to exogenously applied NMDA. The left trace is an example from an EGTA-loaded cell, the right trace from a BAPTA-loaded cell. Bars above the traces represent the duration of application of 1 mM NMDA. *C*, summary of results from 5 cells filled with EGTA and 5 cells filled with BAPTA. * $P < 0{\cdot}01$, Student's independent two-tailed t test. Percentage desensitization was calculated by subtracting 1 from the ratio of the steady state to the peak NMDA-induced current.

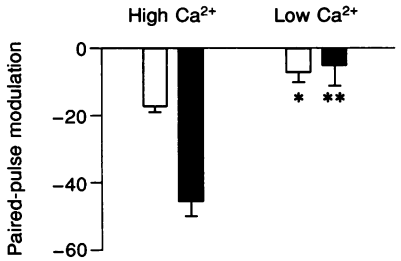

Figure 6. Lowering extracellular Ca^{2+} disproportionately reduces NMDA paired-pulse depression
The effect of lowering extracellular Ca^{2+} from 3 mM (high Ca^{2+}) to 0·8 mM (low Ca^{2+}) upon paired-pulse depression of AMPA (open bars) and NMDA components of EACs (filled bars) is shown. A two-way repeated measures analysis of variance showed a significant interaction between type of response (AMPA *vs.* NMDA) and the high *vs.* low Ca^{2+} conditions; ** $P < 0{\cdot}001$, $n = 11$ cells. Bonferroni corrected t tests also revealed a significant effect of Ca^{2+} upon AMPA EAC paired-pulse depression, * $P = 0{\cdot}001$, as described previously (Mennerick & Zorumski, 1995*a*).

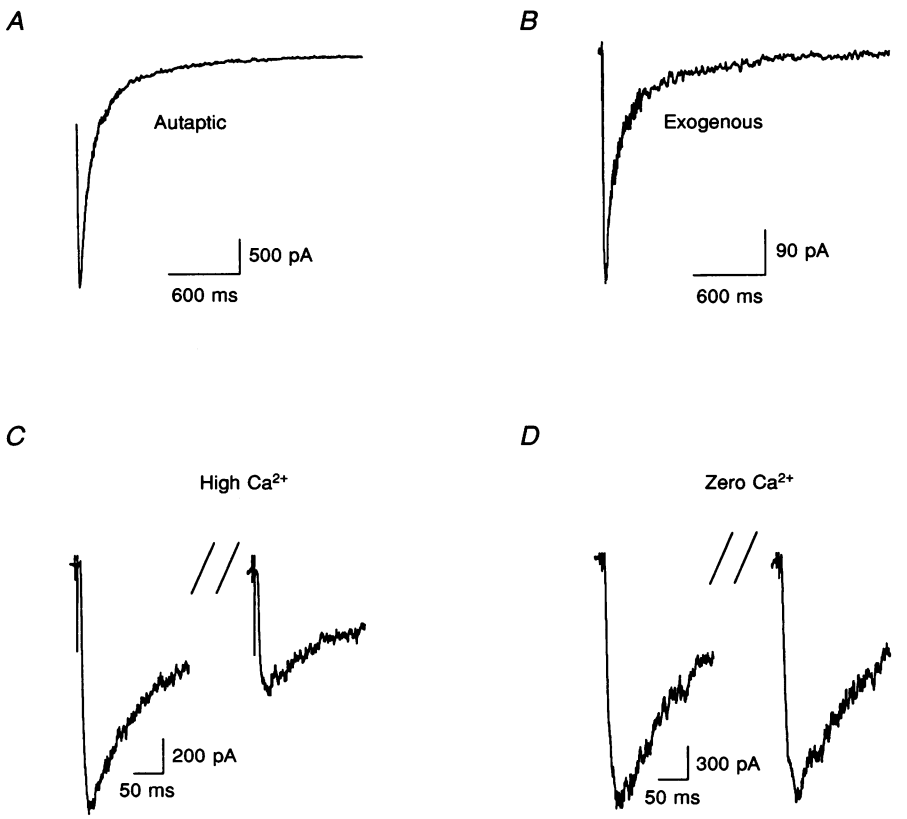

Figure 7. Rapid exogenous applications of 1 mM glutamate to whole cells cause Ca^{2+}-dependent depression of test responses
A and *B*, comparison of the kinetics of autaptic currents from a single-neuron micro-island (*A*) with exogenous application of glutamate for 4 ms in another cell (*B*). *C*, a representative example of currents in response to paired applications of exogenous glutamate in 3 mM Ca^{2+}. The traces in *D* show an example of paired glutamate applications in another cell in which the bath solution contained no added Ca^{2+} and 1 mM EGTA. NBQX (1 μM) was present for all experiments.

(Fig. 4*Bb*). The ratio of the NMDA to AMPA components of conditioning EPSCs was not altered by changing the holding potential (0·18 ± 0·04 at −65 mV *vs.* 0·22 ± 0·05 at +65 mV; $P > 0·1$, $n = 13$), indicating that any calcium influx during the depolarization of the neuron to +65 mV did not cause significant NMDA channel desensitization.

If intracellular Ca^{2+} participates in paired-pulse depression of NMDA EPSCs, one might expect that high concentrations of intracellular Ca^{2+} chelators such as BAPTA should reduce NMDA depression. Differences exist in the literature regarding the effectiveness of intracellular BAPTA in reducing Ca^{2+}-dependent NMDA receptor desensitization. In several reports BAPTA has been ineffective in preventing desensitization of NMDA responses to exogenously applied agonists (Clark *et al.* 1990; Vyklicky, 1993). In other recent reports, BAPTA has been effective in reducing the decline in NMDA responses (Legendre *et al.* 1993). In our experiments, the effect of BAPTA upon NMDA paired-pulse depression was equivocal (Fig. 5*A*). Depression of dual-component EACs and EPSCs was measured in nineteen pairs of neurons in which the presynaptic cell was loaded for 5–8 min with 5 mM EGTA and in which the postsynaptic cell was loaded with 20 mM BAPTA via the patch pipette. Using a repeated measures ANOVA test, a statistically significant interaction was detected between the buffer condition and the type (NMDA *vs.* AMPA) of postsynaptic response ($P = 0·01$). Means for the depression of the AMPA component were similar (−22 ± 3% for BAPTA-loaded and −21 ± 2% for EGTA-loaded cells; Fig. 5*A*). The NMDA components showed depression of −28 ± 3% for BAPTA-loaded and −33 ± 3% for EGTA-loaded cells (Fig. 5*A*). These means suggest that the significant interaction between the buffer condition and the type of response is due to a smaller NMDA depression in the BAPTA-loaded cells. However, *post hoc* comparisons (Bonferroni corrected *t* tests) revealed no significant difference between the NMDA depression in EGTA-loaded and BAPTA-loaded cells ($P > 0·1$). Additionally, the difference between AMPA and NMDA depression in BAPTA-loaded cells was still significantly different for both buffer conditions ($P = 0·001$ for BAPTA, $P = 0·000001$ for EGTA).

Given the weak effect of BAPTA upon synaptic depression, we examined the effect of BAPTA upon the desensitization of NMDA receptors exogenously applied to agonist under the conditions of our synaptic experiments (3 mM Ca^{2+}, 0 mM Mg^{2+} and 20 μM glycine in the extracellular solution). Cells were challenged with a 6 s exposure to 1 mM NMDA. During NMDA exposure, currents were desensitized in a manner similar to that previously described (Clark *et al.* 1990; Fig. 5*B*). Figure 5*B* and *C* shows that while there was a statistically significant reduction in the degree of desensitization to exogenously applied NMDA in BAPTA-loaded cells compared with EGTA-loaded cells, the effect was rather subtle and possibly explains the inability to detect a significant reduction in NMDA EPSC depression in BAPTA-loaded cells.

Lowering the extracellular Ca^{2+} concentration from 3 to 0·8 mM reduced NMDA EAC paired-pulse depression to levels similar to those of AMPA EAC paired-pulse depression (Fig. 6). However, the interpretation of this experiment is complicated by the fact that lowering the extracellular Ca^{2+} concentration has both postsynaptic effects on the Ca^{2+}-permeable NMDA ion channels and upon presynaptic transmitter release. Furthermore, the ability to lower extracellular Ca^{2+} levels is impeded by the need to maintain a basal extracellular Ca^{2+} concentration (>0·3 mM) to support transmitter release for the measurement of EPSCs.

Therefore, to determine whether synaptic release of glutamate is likely to evoke Ca^{2+}-dependent desensitization

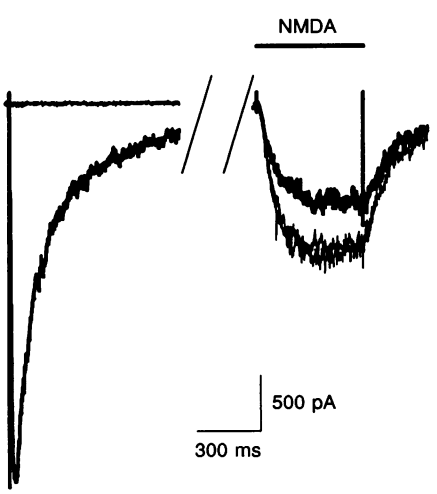

Figure 8. Synaptic activation of NMDA receptors depresses responses to exogenous NMDA

Applications of 500 ms duration of 10 μM NMDA were delivered either without (thin traces) or with (thick trace) prior synaptic activation. The thin traces show one trial before the synaptic stimulation trial and one trial following the synaptic stimulation trial. As with synaptic paired-pulse experiments, the interval between synaptic stimulation and NMDA application was 2·5 s. The interval between trials was 25 s. Autaptic stimulus-associated transients have been truncated.

of NMDA receptors, we examined currents in response to brief (4 ms) applications of 1 mM glutamate to whole cells in the presence of 1 μM NBQX to block the AMPA component of glutamate currents. Short pulses of 1 mM glutamate in 3 mM extracellular Ca^{2+} followed by rapid washing elicited currents with kinetics similar to those of synaptic responses (Lester *et al.* 1990; Fig. 7*A* and *B*). Paired-pulse applications of glutamate in the same extracellular conditions caused depression of test responses (2·5 s paired-pulse interval, Fig. 7*C*). In six cells, the average depression was $-38 \pm 4\%$. In eight cells with no added Ca^{2+} and with 1 mM EGTA in the extracellular bath, depression was reduced to $-8 \pm 3\%$ (Fig. 7*D*, $P < 0{\cdot}001$, Student's independent two-tailed t test). In contrast to NMDA receptor-mediated currents, AMPA receptor-mediated currents in 3 mM Ca^{2+} (studied with 4 ms pulses of glutamate in the presence of 50 μM D-APV and in the absence of NBQX) decayed with a time constant of $7{\cdot}0 \pm 0{\cdot}8$ ms and displayed little or no paired-pulse depression at an interval of 2·5 s ($-3 \pm 1\%$, $n = 8$ cells, data not shown).

These data show that brief pulses of glutamate are capable of eliciting Ca^{2+}-dependent desensitization that is present seconds after agonist exposure. However, to explore whether single synaptic stimuli are capable of causing postsynaptic receptor desensitization, we elicited NMDA currents with 10 μM NMDA with or without autaptic stimulation 2·5 s prior to agonist application. As shown in Fig. 8, currents in response to exogenous NMDA were depressed when preceded by a conditioning synaptic stimulation ($-18 \pm 5\%$, $n = 10$ cells, $P < 0{\cdot}01$). The depression of responses to exogenous NMDA was not caused by time-dependent rundown of receptor function, since responses recovered to $98 \pm 4\%$ of control with the following unconditioned application of NMDA (Fig. 8). These data provide a direct demonstration that single synaptic stimuli depress postsynaptic NMDA receptor sensitivity.

Given that postsynaptic receptor blockade by Mg^{2+} reduces NMDA EPSC paired-pulse depression (Fig. 3*A* and *B*), it is of interest to determine whether NMDA

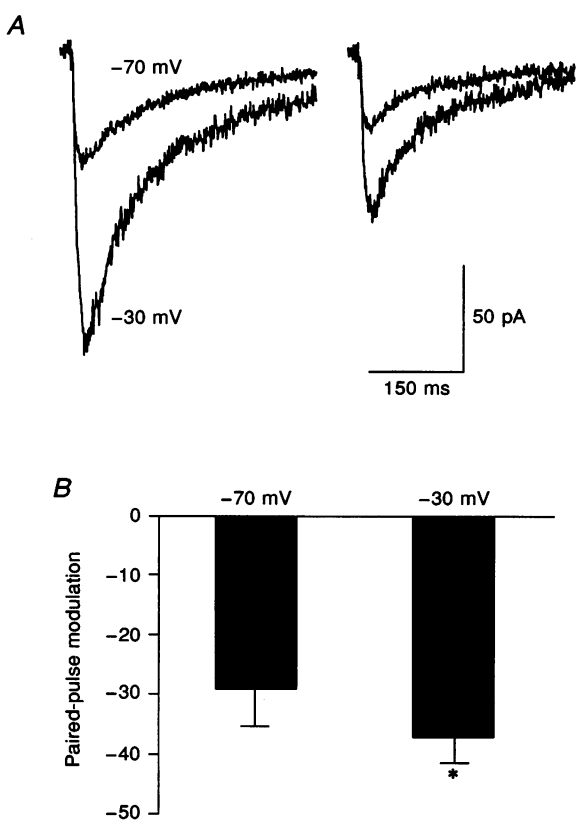

Figure 9. NMDA EPSC paired-pulse depression is increased with partial relief of Mg^{2+} block
NMDA EPSCs (interneuronal) were examined in dual recordings from 6 two-neuron micro-islands in the presence of 2 mM Ca^{2+}, 1 mM Mg^{2+} and 1 μM NBQX. The membrane potential of the postsynaptic cell was alternated between −70 mV and −30 mV. *A*, the traces show an example of the mean responses from one postsynaptic cell. The paired-pulse depression in this cell at −70 mV was −33% *vs.* −46% at −30 mV. *B*, mean depression from the sample of 6 postsynaptic neurons. * $P = 0{\cdot}01$, Student's paired t test.

receptor desensitization can modulate NMDA EPSCs in the presence of physiological concentrations of extracellular Mg^{2+}. The large amplitude of microculture EPSCs allowed measurement of NMDA EPSCs even at hyperpolarized potentials in the presence of extracellular Mg^{2+} (Fig. 9*A*). In a bath solution of 2 mM Ca^{2+} and 1 mM Mg^{2+} and at −70 mV, there was no significant difference between the paired-pulse depression of the AMPA and NMDA components of EACs (−23 ± 4% AMPA *vs.* −29 ± 7% NMDA; $n = 8$ cells, $P > 0{\cdot}2$), indicating that at hyperpolarized potentials, Mg^{2+} block of the NMDA channel precludes expression of the postsynaptic modulation of NMDA responses. When pairs of neurons were examined so that postsynaptic membrane potential could be manipulated, depolarizing the postsynaptic membrane potential from −70 to −30 mV increased isolated conditioning NMDA EPSCs by 252 ± 42% (from −17 ± 4 to −57 ± 13 pA) and significantly increased the degree of NMDA paired-pulse depression from −29 ± 6 to −37 ± 4% ($n = 6$; Fig. 9*B*), indicating that with partial relief of Mg^{2+} block, single synaptic stimuli can desensitize NMDA receptors.

DISCUSSION

Our results demonstrate a postsynaptic component of paired-pulse synaptic depression of NMDA receptors in culture. Culture systems have proved to be a valuable model of various aspects of synaptic function. However, the extent to which many results generalize from reductionist models like microcultures to more intact preparations remains to be explored. Although in mass cultures (Thio *et al.* 1992) and in microcultures of hippocampal cells (Mennerick & Zorumski, 1995*a*), the majority of synapses exhibit frequency-dependent depression, the only synapse to exhibit paired-pulse depression reliably under physiological conditions in the intact hippocampus appears to be the medial perforant pathway synapses onto dentate granule cells (McNaughton, 1980). Therefore, this synapse may be an *in situ* candidate for the exploration of the form of postsynaptic modulation of NMDA receptors examined in the present study.

The possible role of the rapid and profound desensitization of AMPA receptors in dictating the time course of fast EPSCs at glutamatergic synapses and in modulating synaptic efficacy has been intensively investigated (Trussell & Fischbach, 1989; Vyklicky *et al.* 1991; Thio *et al.* 1992; Trussell *et al.* 1993). Although recently it has been demonstrated that trains of synaptic stimuli can cause Ca^{2+}-dependent desensitization of NMDA receptors (Rosenmund, Feltz & Westbrook, 1995; Tong, Shepherd & Jahr, 1995), little attention has been given to modulation of NMDA receptors by single synaptic stimuli. The slow rate of NMDA receptor desensitization in response to prolonged agonist application (Lester & Jahr, 1992) coupled with the brief lifetime of transmitter in the synaptic cleft (Clements *et al.* 1992) would seem to preclude an important role for NMDA receptor desensitization in synaptic modulation. However, because glutamate dissociates slowly from NMDA receptors, NMDA synaptic currents are prolonged relative to AMPA currents (Lester *et al.* 1990), and receptors may have an increased likelihood of entering a desensitized state before agonist dissociation (Lester & Jahr, 1992).

Two groups have recently reported results consistent with those presented here (Rosenmund *et al.* 1995; Tong *et al.* 1995). The present results add to these initial reports in several respects. Firstly, both previous reports relied exclusively upon autaptic EPSCs to demonstrate synaptically induced NMDA receptor desensitization. Under these conditions it is difficult to rule out the fact that calcium influx through voltage-gated calcium channels during presynaptic stimulation participates in NMDA receptor desensitization (Legendre *et al.* 1993). The results of Fig. 4 demonstrate that at negative membrane potentials, conventional monosynaptic currents exhibit differential modulation of AMPA and NMDA components nearly identical with those of EACs. These results, combined with results demonstrating that several manipulations of postsynaptic receptors modulate the degree of NMDA paired-pulse depression (Figs. 3 and 4), implicate ion flux through postsynaptic receptor channels as the primary cause of synaptically induced NMDA receptor desensitization. Secondly, both previous reports used trains of stimuli to examine synaptically elicited NMDA receptor depression. Our results demonstrate that NMDA receptor desensitization can be elicited by single conditioning EPSCs in the absence of extracellular Mg^{2+}. Even in the presence of physiological concentrations of extracellular Mg^{2+}, our results suggest that with partial relief of Mg^{2+} block of NMDA channels, as is achieved during long-term potentiation induction, single synaptic stimuli can elicit NMDA receptor desensitization.

With higher degrees of presynaptic depression induced pharmacologically, NMDA synaptic currents were more severely depressed than AMPA synaptic currents. This result appears to conflict with those previously reported (Tong & Jahr, 1994), although non-parallel modulation similar to that seen here has been reported with adenosine antagonists in hippocampal slices (Perkel & Nicoll, 1994). Further work should clarify the reasons for the discrepancy. One possibility is that our results are explained in part by the large quantal content of autaptic EACs under the conditions of our experiments. We have previously shown that prolonged glutamate presence can contribute to the decay time course of AMPA EACs under conditions of high quantal content (Mennerick & Zorumski, 1995*b*). If these effects are due to spill-over of transmitter from adjacent release sites to regions of extrasynaptic receptors (Hartzell *et al.* 1975; Faber & Korn, 1988), it is possible that slowly rising NMDA peak EACs can be augmented by these spill-over effects under conditions of high transmitter output

(Faber & Korn, 1988). Significantly lowering transmitter output would be expected to reduce the effects of spill-over upon synaptic currents in a non-linear fashion (Hartzell *et al.* 1975) and could explain the non-parallel modulation of AMPA and NMDA responses by presynaptic depressants. The results of Figs 3–9 suggest that at the levels of presynaptic depression achieved with paired-stimulated, calcium-dependent desensitization of NMDA receptors is the primary mediator of the non-parallel modulation. However, we cannot exclude the possibility of non-parallel modulation by the presynaptic mechanism in some cells, particularly those which show the most paired-pulse depression of AMPA responses.

Several groups have shown that NMDA receptor desensitization in the presence of saturating concentrations of glycine is dependent upon extracellular Ca^{2+} (Clark *et al.* 1990; Legendre *et al.* 1993; Vyklicky, 1993). Our data suggest that this form of receptor desensitization can be elicited even by very brief applications of exogenous glutamate and is largely responsible for the augmented depression of NMDA receptors caused by paired synaptic stimulation of hippocampal cells in culture. This form of synaptic modulation might be expected to influence the induction of long-term potentiation and long-term depression, which are typically elicited by repeated synaptic activation of NMDA receptors at $\geqslant 1$ Hz.

Bekkers, J. M. & Stevens, C. F. (1991). Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. *Proceedings of the National Academy of Sciences of the USA* **88**, 7834–7838.

Betz, W. J. (1970). Depression of transmitter release at the neuromuscular junction of the frog. *Journal of Physiology* **206**, 629–644.

Clark, G. D., Clifford, D. B. & Zorumski, C. F. (1990). The effect of agonist concentration, membrane voltage and calcium on *N*-methyl-D-aspartate receptor desensitization. *Neuroscience* **39**, 787–797.

Clements, J. D., Lester, R. A., Tong, G., Jahr, C. E. & Westbrook, G. L. (1992). The time course of glutamate in the synaptic cleft. *Science* **258**, 1498–1501.

del Castillo, J. & Katz, B. (1954). Statistical factors involved in neuromuscular facilitation and depression. *Journal of Physiology* **124**, 574–585.

Faber, D. S. & Korn, H. (1988). Synergism at central synapses due to lateral diffusion of transmitter. *Proceedings of the National Academy of Sciences of the USA* **85**, 8708–8712.

Hartzell, H. C., Kuffler, S. W. & Yoshikami, D. (1975). Post-synaptic potentiation: interaction between quanta of acetylcholine at the skeletal neuromuscular synapse. *Journal of Physiology* **251**, 427–463.

Hestrin, S. (1993). Different glutamate receptor channels mediate fast excitatory synaptic currents in inhibitory and excitatory cortical neurons. *Neuron* **11**, 1083–1091.

Hestrin, S., Nicoll, R. A., Perkel, D. J. & Sah, P. (1990). Analysis of excitatory synaptic action in pyramidal cells using whole-cell recording from rat hippocampal slices. *Journal of Physiology* **422**, 203–225.

Jahr, C. E. & Stevens, C. F. (1993). Calcium permeability of the *N*-methyl-D-aspartate receptor channel in hippocampal neurons in culture. *Proceedings of the National Academy of Sciences of the USA* **90**, 11573–11577.

Jonas, P. & Sakmann, B. (1992). Glutamate receptor channels in isolated patches from CA1 and CA3 pyramidal cells of rat hippocampal slices. *Journal of Physiology* **455**, 143–171.

Legendre, P., Rosenmund, C. & Westbrook, G. L. (1993). Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. *Journal of Neuroscience* **13**, 674–684.

Lester, R. A., Clements, J. D., Westbrook, G. L. & Jahr, C. E. (1990). Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents. *Nature* **346**, 565–567.

Lester, R. A. & Jahr, C. E. (1992). NMDA channel behavior depends on agonist affinity. *Journal of Neuroscience* **12**, 635–643.

McNaughton, B. L. (1980). Evidence for two physiologically distinct perforant pathways to the fascia dentata. *Brain Research* **199**, 1–19.

Magleby, K. L. (1987). Short-term changes in synaptic efficacy. In *Synaptic Function*, ed. Edelman, G. M., Gall, W. E. & Cowan, W. M., pp. 13–31. John Wiley and Sons, New York.

Mayer, M. L. & Westbrook, G. L. (1985). The action of *N*-methyl-D-aspartic acid on mouse spinal neurones in culture. *Journal of Physiology* **361**, 65–90.

Mennerick, S., Que, J., Benz, A. & Zorumski, C. F. (1995). Passive and synaptic properties of neurons grown in microcultures and in mass cultures. *Journal of Neurophysiology* **73**, 320–332.

Mennerick, S. & Zorumski, C. F. (1995*a*). Paired-pulse modulation of fast excitatory synaptic currents in microcultures of rat hippocampal neurons. *Journal of Physiology* **488**, 85–101.

Mennerick, S. & Zorumski, C. F. (1995*b*). Presynaptic influence upon the time course of fast excitatory synaptic currents in cultured hippocampal neurons. *Journal of Neuroscience* **15**, 3178–3192.

Perkel, D. J. & Nicoll, R. A. (1994). Evidence for all-or-none regulation of neurotransmitter release: implications for long-term potentiation. *Journal of Physiology* **471**, 481–500.

Roepstorff, A. & Lambert, J. D. (1994). Factors contributing to the decay of the stimulus-evoked IPSC in rat hippocampal CA1 neurons. *Journal of Neurophysiology* **72**, 2911–2926.

Rosenmund, C., Feltz, A. & Westbrook, G. L. (1995). Calcium-dependent inactivation of synaptic NMDA receptors in hippocampal neurons. *Journal of Neurophysiology* **73**, 427–430.

Sather, W., Johnson, J. W., Henderson, G. & Ascher, P. (1990). Glycine-insensitive desensitization of NMDA responses in cultured mouse embryonic neurones. *Neuron* **4**, 725–731.

Segal, M. M. & Furshpan, E. J. (1990). Epileptiform activity in microcultures containing small numbers of hippocampal neurons. *Journal of Neurophysiology* **64**, 1390–1399.

Takeuchi, A. (1958). The long-lasting depression in neuromuscular transmission of the frog. *Japanese Journal of Physiology* **8**, 102–113.

Thio, L. L., Clark, G. D., Clifford, D. B. & Zorumski, C. F. (1992). Wheat germ agglutinin enhances EPSCs in cultured postnatal rat hippocampal neurons by blocking ionotropic quisqualate receptor desensitization. *Journal of Neurophysiology* **68**, 1930–1938.

Tong, G. & Jahr, C. E. (1994). Multivesicular release from excitatory synapses of cultured hippocampal neurons. *Neuron* **12**, 51–59.

Tong, G., Shepherd, D. & Jahr, C. E. (1995). Synaptic desensitization of NMDA receptors by calcineurin. *Science* **267**, 1510–1512.

Trussell, L. O. & Fischbach, G. D. (1989). Glutamate receptor desensitization and its role in synaptic transmission. *Neuron* **3**, 209–218.

TRUSSELL, L. O., ZHANG, S. & RAMAN, I. M. (1993). Desensitization of AMPA receptors upon multiquantal neurotransmitter release. *Neuron* **10**, 1185–1196.

VYKLICKY, L. JR (1993). Calcium-mediated modulation of *N*-methyl-D-aspartate (NMDA) responses in cultured rat hippocampal neurones. *Journal of Physiology* **470**, 575–600.

VYKLICKY, L. JR, PATNEAU, D. K. & MAYER, M. L. (1991). Modulation of excitatory synaptic transmission by drugs that reduce desensitization at AMPA/kainate receptors. *Neuron* **7**, 971–984.

ZORUMSKI, C. F., YANG, J. & FISCHBACH, G. D. (1989). Calcium-dependent, slow desensitization distinguishes different types of glutamate receptors. *Cellular and Molecular Neurobiology* **9**, 95–104.

Acknowledgements

This work was supported by NIH Research Scientist Development Award MH-00964, grants MH-45493, AG-11355, and a Fellowship from the Bantly Foundation. The authors thank Ann Benz and Jian Que for technical assistance and Kel Yamada, Jim Huettner, Bob Wilkinson, Steve Rothman, Yuki Izumi, and Liu Lin Thio for discussion. The authors also thank Tamara Hershey for statistical advice.

Received 11 April 1995; accepted 19 July 1995.