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SLOW EXCITATORY POSTSYNAPTIC CURRENTS MEDIATED BY *N*-METHYL-D-ASPARTATE RECEPTORS ON CULTURED MOUSE CENTRAL NEURONES

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SUMMARY

1. Monosynaptic excitatory postsynaptic potentials (EPSPs) evoked between pairs of cultured neurones from either hippocampus or spinal cord were examined using the tight-seal whole-cell recording technique.

2. Using the selective *N*-methyl-D-aspartate (NMDA)-receptor antagonist, 2-amino-5-phosphonovaleric acid (APV), two components of the EPSP could be resolved in cultures from both brain regions. The APV-sensitive (slow) component had the same latency, but a much slower time-to-peak and longer duration than the APV-resistant (fast) component. Other NMDA antagonists such as ketamine also selectively blocked the slow component of the EPSP.

3. In Mg^{2+}-free medium, the dual-component EPSP had a duration lasting up to 500 ms, greatly exceeding the membrane time constant of the postsynaptic neurone, suggesting that persistent activation of NMDA receptors was responsible for the long duration of the APV-sensitive component.

4. Under voltage clamp the excitatory postsynaptic currents (EPSCs) also showed fast and slow components, both of which had a reversal potential near 0 mV in physiological saline. The synaptic current could be fitted with a sum of two exponentials with a decay time constant for the slow EPSC near 80 ms. The slow current contributed approximately 50% of the total charge transfer during the EPSC.

5. In Mg^{2+}-containing medium, the peak of the fast component was voltage insensitive, whereas the synaptic current measured at a latency of 10–50 ms was voltage dependent with a region of negative slope conductance at membrane potentials hyperpolarized to −30 mV.

6. Raising $[Ca^{2+}]_o$ from 1 to 20 mM resulted in a shift of the reversal potential of the APV-sensitive component from near 0 mV to + 10 mV, but the reversal potential of the fast component remained near 0 mV. This suggests that conductances with different ionic permeability underlie the two components of the EPSC and that the slow component is highly permeable to Ca^{2+} as well as to monovalent cations.

7. Our results demonstrate that two functionally distinct excitatory amino acid

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receptor channels are simultaneously activated by transmitter release from a single presynaptic neurone. The conductance mechanism underlying the slow component of the EPSP displays the voltage dependence and Ca^{2+} permeability expected for NMDA-receptor channels. We suggest that the available conductance generating the slow EPSP may be sufficient, even at low firing rates, to influence excitability on both a short-term and more long-lasting basis.

INTRODUCTION

An understanding of central excitatory mechanisms is of importance not only to normal function in the CNS, but also because alterations in these mechanisms may contribute to a number of neurological disease processes, including epilepsy and stroke. In the past, the role of excitatory amino acids as transmitters has been considered to be that of a mediator of 'fast' EPSPs, with a short pulse of transmitter release leading to a brief postsynaptic conductance change analogous to the action of acetylcholine at the neuromuscular junction. This contrasts with the concept of neuromodulators, exemplified by a number of peptides and catecholamines, which act more slowly to influence neuronal excitability, often via second messenger systems (Kaczmarek & Levitan, 1987). However, recent studies of excitatory amino acid receptors and their conductance mechanisms has demonstrated that one of these, the *N*-methyl-D-aspartate (NMDA)-receptor channel, has several properties well suited to a neuromodulatory role, i.e. voltage dependence (MacDonald, Porietis & Wojtowicz, 1982; Mayer & Westbrook, 1984) and a significant permeability to Ca^{2+} (Mayer & Westbrook, 1985*b*; Ascher & Nowak, 1986; MacDermott, Mayer, Westbrook, Smith & Barker, 1986; Jahr & Stevens, 1987). In addition, antagonism of NMDA receptors has been shown to block induction of long-term potentiation at the Schaffer collateral–commissural input to CA1 in the hippocampus (Collingridge, Kehl & McLennan, 1983) and to modulate 'motor programs' in the amphibian and lamprey spinal cord (Dale & Roberts, 1984; Grillner, Wallén, Dale, Brodin, Buchanan & Hill, 1987).

Despite these interesting features of the NMDA-activated conductance and their apparent functional significance, the mechanism by which these receptors are activated during synaptic transmission has been unclear. Specifically, pharmacological studies of pathways thought to use an excitatory amino acid transmitter have generally concluded that kainate or quisqualate (i.e. non-NMDA) receptors mediate fast EPSPs (see Mayer & Westbrook, 1987 for review). In addition, the synaptic conductance underlying fast EPSPs between Ia afferents and motoneurones (Finkel & Redman, 1983) or between cultured spinal cord neurones (Nelson, Pun & Westbrook, 1986) shows little voltage sensitivity, consistent with a conductance mechanism activated principally by kainate or quisqualate (Mayer & Westbrook, 1984). However, these studies with cultured spinal cord neurones were performed in medium containing 5 mM-Mg^{2+}, which markedly reduces NMDA responses near the resting membrane potential (Mayer & Westbrook, 1985*a*). In addition, the final decay of EPSPs between cultured spinal cord neurones was often contaminated by polysynaptic activity, making it difficult to resolve slow components of the monosynaptic response.

Several recent studies *in vitro* using either brain slices or isolated spinal cord

preparations have demonstrated that NMDA receptors can be activated during monosynaptic transmission (Wigström & Gustafsson, 1984; Thomson, West & Lodge, 1985; Dale & Roberts, 1985). Technical considerations limit characterization of the synaptic conductance mechanism in these preparations. Therefore, to examine in detail the properties of central excitatory synaptic mechanisms mediated by NMDA receptors, we have recorded monosynaptic EPSPs between isolated pairs of cultured mouse central neurones from both spinal cord and hippocampal regions. In more than 90% of EPSPs examined in both regions, a slow NMDA-receptor-mediated component was observed in addition to a conventional fast synaptic response. Analysis under voltage clamp revealed that the conductance underlying the slow EPSP was highly voltage dependent and showed a significant Ca^{2+} permeability, consistent with the properties of NMDA-receptor channels. We suggest that such dual-component EPSPs may be a general feature of excitatory amino acid mediated-pathways in the CNS, and thus provide an additional mechanism for influencing synaptic efficacy during normal synaptic transmission as well as under conditions of intense stimulation.

A preliminary account of some of these experiments has been presented (Forsythe & Westbrook, 1986; Mayer, Westbrook & Forsythe, 1987).

METHODS

Cell culture

Primary dissociated hippocampal cultures were prepared from 16–17 day embryos (C57Bl/6 mice), and plated at low density (25000–50000 cells/35 mm dish) onto confluent layers of astrocytes. Embryos of this stage were chosen in an attempt to optimize survival of post-mitotic principal neurones (i.e. pyramidal cells) which have earlier birthdates than interneurones, including GABAergic neurones (see e.g. Banker & Cowan, 1977). The astrocyte feeder layers were prepared from the hippocampi of newborn mice. Following treatment with 0·125% trypsin for 15 min, the tissue was triturated and plated at $2{\cdot}5 \times 10^5$ cells into 35 mm tissue culture dishes coated with Vitrogen (Collagen Corp., Palo Alto, CA, U.S.A.) and poly-L-lysine (30000–70000 MW, 10^{-5} M in 0·15 M-borate, pH 8·4, Sigma). The resulting feeder layers were grown in minimal essential medium (MEM, formula no. 82-0234 with added bicarbonate, Gibco) with 10% fetal bovine serum until confluent (12–14 days); feeder layers prepared in this way were devoid of neurones and consisted primarily of flat cells which were positively stained by antibodies to glial fibrillary acidic protein. When hippocampal neurones were added, the medium was switched to the following composition: 95% MEM, 5% horse serum (Hyclone) and an added nutrient supplement containing insulin, transferrin, putrescine, selenium, corticosterone, progesterone and triiodothyronine. The lot of horse serum used in these experiments contributed a final concentration of 7·5 μM-L-glutamate and 38·5 μM-glycine to the growth medium, based on measurements provided by the supplier. Hippocampal cultures were treated 1 day after plating of neurones with antimitotics to suppress overgrowth of background cells; half-changes of medium were done weekly thereafter. The small isolated islands of two to five neurones in these cultures were ideal for physiological studies since ongoing spontaneous synaptic activity and evoked polysynaptic activity was minimal. Spinal cord cultures were prepared from 13-day embryos using techniques similar to those described previously (Mayer & Westbrook, 1984).

Electrophysiology

Experiments were performed at room temperature (24–25 °C) on the stage of an inverted microscope. Most experiments were done in a static bath; however, in a few cases bath perfusion at 0·5–1 ml/min was used. Cultures were washed extensively to remove growth medium before each experiment and placed in a recording solution containing (mM): NaCl, 135; KCl, 3·0; glucose, 10; HEPES, 10; $CaCl_2$, 1 or 2; with no added Mg^{2+}. The pH was adjusted to 7·3 with NaOH and the osmolarity to 325 mosM with sucrose. Picrotoxin (10–100 μM) was added to block inhibitory synaptic activity.

Patch electrodes were fabricated with Corning no. 7740 borosilicate glass (WPI no. TW150F) using a two-stage vertical puller and fire-polished. Electrodes were filled with (mM): potassium or caesium methyl sulphate, 140; HEPES, 10 and EGTA, 1·1; they had DC resistances of 3–5 MΩ. Drugs were diluted in the recording solution and delivered by pressure ejection (3–20 kPa) from 'puffer' electrodes which were identical to those used for whole-cell recording except that the tips were not fire-polished. The puffer pipettes were positioned within 50–100 μm of the postsynaptic neurone and the drug delivered with a pulse train (usually 400 ms, 2 Hz) for 0·5–2 min; this allowed sustained application, but did not result in removal by rapid perfusion of any conditioning substances in the recording solution (see Johnson & Ascher, 1987). However, in experiments where the reversal potential was measured in high $[Ca^{2+}]_o$, rapid local perfusion (35 kPa) was used to deliver the high-Ca^{2+} solution; in these cases 10 μM-glycine (and 1 μM-strychnine) was added to the solutions to circumvent problems with wash-out of conditioning substances. Drugs applied were DL-2-amino-5-phosphonovaleric acid (DL-APV, Sigma & Cambridge Research Biochemicals) ketamine HCl (Parke-Davis), $MgCl_2$, $CaCl_2$ and kynurenic acid (Sigma).

Experimental procedure and data analysis

Tight-seal whole-cell recordings were made from two separate neurones within the same field of view (700 μm diameter) in current clamp. Action potentials were stimulated with brief intracellular current pulses (1–5 ms) to search for excitatory monosynaptic connections in either direction. An EPSP was judged to be monosynaptic if the synaptic latency was no more than 5 ms and showed no detectable intertrial variation. Current clamp experiments were performed using patch electrodes containing potassium methyl sulphate with the membrane potential near its resting value (about −65 mV in physiological saline). The presynaptic neurone was stimulated at a continuous rate (0·1–0·5 Hz) and the evoked response was digitized and stored on a LSI 11/23 microcomputer either as single trials or averages of ten to twenty records. Voltage clamp recordings were made with a discontinuous one-electrode voltage clamp (switch frequency 8–15 kHz) using a patch electrode containing caesium methyl sulphate for the postsynaptic neurone. Currents were digitized at 5–10 kHz and filtered at 2 kHz (8-pole Bessel). The membrane time constant (τ_m) of the postsynaptic neurone was determined either by analysis of the final decay of the fast component of the EPSP in the presence of APV, or from the voltage response to brief (1–2 ms, 50 pA) hyperpolarizing current pulses (see e.g. Jack & Redman, 1971). Shape indices of the EPSPs were obtained by measuring the time-to-peak and half-duration of the fast component normalized to τ_m (Jack, Miller, Porter & Redman, 1971).

RESULTS

Monosynaptic EPSPs were analysed from fifty-eight pairs of spinal cord neurones (SC–SC EPSPs) and ninety pairs of hippocampal neurones (HPC–HPC EPSPs). Current clamp results from the two preparations were similar; voltage clamp studies were primarily performed on HPC–HPC EPSPs.

Dual-component EPSPs

The EPSPs evoked between cultured neurones usually consist of an early monosynaptic component followed at variable latencies by both polysynaptic inhibitory and excitatory synaptic responses (Ransom, Christian, Bullock, & Nelson, 1977). Earlier pharmacological studies demonstrated that the peak amplitude of fast EPSPs between cultured spinal cord neurones is relatively unaffected by NMDA-receptor antagonists such as APV, but is antagonized by non-selective excitatory amino acid antagonists including *cis*-2,3-piperidine dicarboxylic acid and γ-D-glutamylglycine (Nelson *et al.* 1986). Similar observations have been made by Rothman & Samaie (1985) in hippocampal cultures. In order to examine the late components of these EPSPs we have made tight-seal whole-cell recordings from

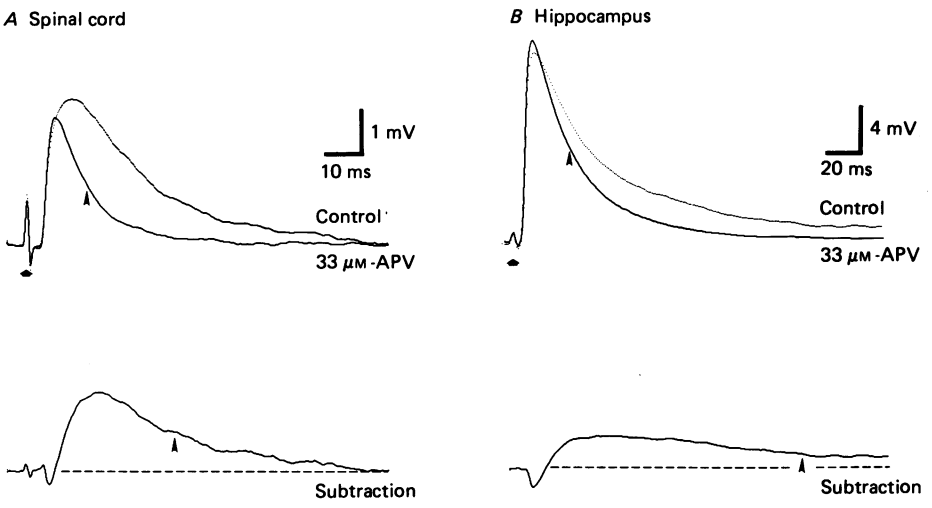

Fig. 1. Activation of NMDA receptors contributes a slow component to monosynaptic EPSPs in spinal cord and hippocampal cultures. An SC–SC EPSP (*A*) and HPC–HPC EPSP (*B*) are shown in the top two traces before (dotted line) and during (continuous line) the application of 33 μM-DL-APV from a puffer pipette positioned within 100 μm of the postsynaptic neurone. The broad arrow indicates the artifact from stimulation of the presynaptic neurone. Digital subtraction of the two records (lower trace) reveals a slowly rising and long-duration response which represents the NMDA-receptor-mediated component of the EPSP. The difference in duration of the two components of the EPSP is apparent in comparing the half-decay time (narrow arrow) of the APV-resistant component with the half-decay time of the subtracted record. The solution in the patch electrode contained potassium methyl sulphate; extracellular solution contained 1 mM-Ca^{2+}, picrotoxin and no added Mg^{2+}. Membrane potential of the postsynaptic neurone was −59 mV in *A* and −68 mV in *B*. Each trace is average of ten to twenty records.

neurones in low-density cultures to reduce polysynaptic activity, and performed most experiments in Mg^{2+}-free recording solutions to increase the availability of the conductance activated by NMDA receptors.

Under these conditions the monosynaptic EPSPs between spinal cord neurones had a rapid rise, but an extremely long decay phase, often lasting 500 ms. The results for EPSPs in hippocampal cultures were remarkably similar, as shown in the top traces of Fig. 1. In the presence of the NMDA-receptor antagonist APV, two distinct components of the EPSP were distinguishable. The early portion of the EPSP was not reduced by APV whereas there was a marked reduction in the late component of the response. This could be seen as a substantial reduction in the half-duration of the EPSP (see arrows, Fig. 1). The NMDA-receptor-mediated (slow) component of the EPSP, as revealed by digital subtraction, did not differ in latency from the APV-resistant (fast) component, but had a much slower time-to-peak as well as a longer duration. The slow component was also blocked by other NMDA-receptor antagonists including 25 or 50 μM-ketamine (Anis, Berry, Burton & Lodge, 1983; $n = 6$), and reduced by 100 μM-Mg^{2+} ($n = 11$, and see Fig. 2). Kynurenic acid (500 μM, $n = 3$) blocked both components of the synaptic responses consistent with its action as a non-selective excitatory amino acid-receptor antagonist (Perkins &

Stone, 1982; Ganong, Lanthorn & Cotman, 1983). An unexpected finding in our experiments was a small, but consistent *increase* in the amplitude of the fast component EPSP during application of NMDA antagonists (seen as a downward deflection in the subtracted records in Fig. 1). This was not accompanied by an obvious change in the somatic input resistance of the postsynaptic neurone (not shown), and it is possible that this indicates that there are NMDA receptors on the presynaptic terminals.

TABLE 1. Properties of dual-receptor EPSPs

	SC–SC	HPC–HPC
EPSPs with two components	34/36	29/34
Time-to-peak of slow EPSP (ms)	36·7 ± 3·4	73·4 ± 8·6
Half-width of slow EPSP (ms)	90·1 ± 9·3	159·0 ± 20·2
Amplitude slow/fast component	0·60 ± 0·22	0·32 ± 0·07

Data from voltage recording of monosynaptic EPSPs in spinal cord (SC–SC) and hippocampal cultures (HPC–HPC). The presence of dual fast and slow components of the EPSPs were assessed using only those cell pairs tested with the NMDA antagonist, APV (33 μM). The half-width of the slow EPSP was measured at 50% of peak amplitude. The amplitude of the fast component was measured at the peak of the APV-resistant EPSP; amplitude of the slow component was measured at the peak of the APV-sensitive component. Any EPSPs with no slow component were excluded from the calculations of the slow/fast amplitude ratio. The slow/fast amplitude ratio was not significantly different between spinal cord and hippocampus (two-sample t test, $P = 0{\cdot}35$). Data expressed as mean ± S.E.M.

The relative amplitudes of the fast and slow components was variable between cell pairs, but the amplitude ratio of slow/fast EPSP was quite similar for SC–SC and HPC–HPC EPSPs. On a few occasions (six of seventy, see Table 1), no slow component could be detected; however, unlike results in the isolated amphibian spinal cord in which one-third of the responses lacked a fast component (Dale & Roberts, 1985), we have seen only one example of 148 EPSPs examined which appeared to be exclusively mediated by NMDA receptors. A summary of the characteristics of the dual-receptor components of the EPSPs is shown in Table 1.

Time course of the slow EPSP exceeds the membrane time constant

The long duration of the slow component of the EPSP could conceivably result from a synaptic location on remote dendrites. One test of this hypothesis is to compare the time course of the EPSP with the electrotonic properties of the postsynaptic neurone. As for the Ia motoneurone EPSP in the cat (Finkel & Redman, 1983), the conductance mechanism underlying the fast EPSP between cultured spinal cord neurones is much briefer than the membrane time constant of the postsynaptic neurone (Nelson *et al.* 1986). This is also the case for the fast EPSC in hippocampal cultures (see below). As a result, the terminal decay of the Ia EPSP has been used to determine the value of τ_m for cat motoneurones (Jack & Redman, 1971). For the dual-receptor EPSPs examined in these experiments, the duration of the synaptic response could be as much as 20 times longer than τ_m, as measured in response to a brief current pulse injected into the postsynaptic neurone. However, in the presence of an NMDA-receptor antagonist, the final decay of the fast component

of the EPSP was well fitted by a single exponential with a time constant nearly identical to τ_m (Fig. 2). For eighteen EPSPs, the final decay time constant of the fast SC–SC EPSP was 27·1 ± 3·0 ms and τ_m was 24·6 ± 2·6 (mean ± S.E.M., $n = 9$); for fast HPC–HPC EPSPs the final decay time was 45·4 ± 5·5 ms and τ_m was 44·8 ± 6·2 ms ($n = 9$). The longer τ_m seen for hippocampal neurones probably reflects an age-

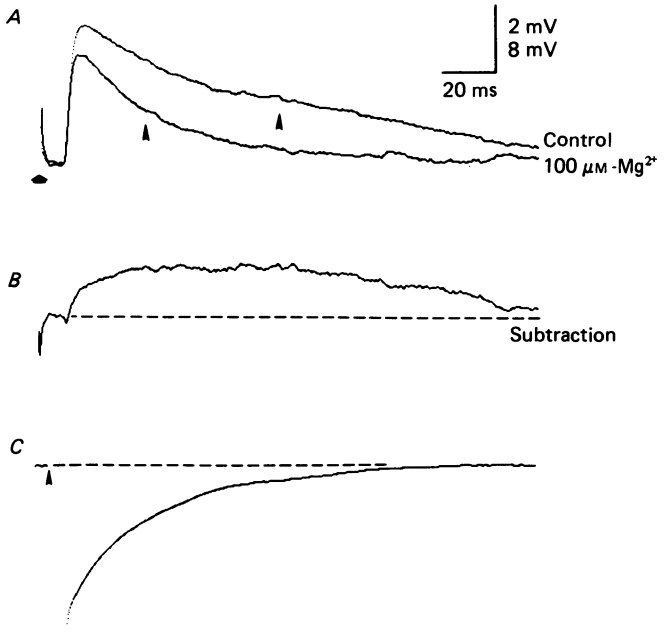

Fig. 2. The duration of the slow EPSP exceeds the membrane time constant. *A*, SC–SC EPSP is shown before and during the application of 100 μM-Mg^{2+} which blocks the NMDA-receptor channels. The half-decay times are indicated by the arrows. The subtracted record demonstrating the time course of the slow EPSP is shown in *B*. The voltage response of the postsynaptic neurone to a hyperpolarizing current pulse (0·1 nA, 3 ms) is shown in *C*. The duration of the slow EPSP is much longer than the voltage decay following the current pulse, suggesting that persistent activation of NMDA receptors underlies the long-duration response. However, the terminal decay of the fast EPSP (shown here in the presence of 100 μM-Mg^{2+}) was the same (time constant = 24·1 ms) as the membrane time constant calculated from the response in *C* (26·6 ms). Experimental conditions were the same as Fig. 1. Amplitude scale: *A* and *B*, 2 mV; *C*, 8 mV.

dependent increase in membrane resistivity since they were studied at 1–2·5 weeks in culture *versus* 3 weeks for spinal cord cultures. Thus a dendritic location of NMDA receptors is insufficient to account for the long duration of the slow EPSP and suggests rather that persistent activation of NMDA receptors is responsible for the long duration.

Spillover of transmitter onto extrasynaptic regions is one mechanism which could account for activation of NMDA receptors during synaptic transmission, as has been reported for the action of glycine at inhibitory synapses onto goldfish Mauthner cells (Faber, Funch & Korn, 1985). If this were true, the increased number of active boutons generating large amplitude EPSPs might show interactions between release

sites, i.e. transmitter release from one bouton could lead to activation of receptors in the postsynaptic region of adjacent boutons. In this situation, the contribution of NMDA-receptor current should correlate with the amplitude of the EPSP (i.e. this predicts a larger slow/fast EPSP amplitude ratio with increasing EPSP size).

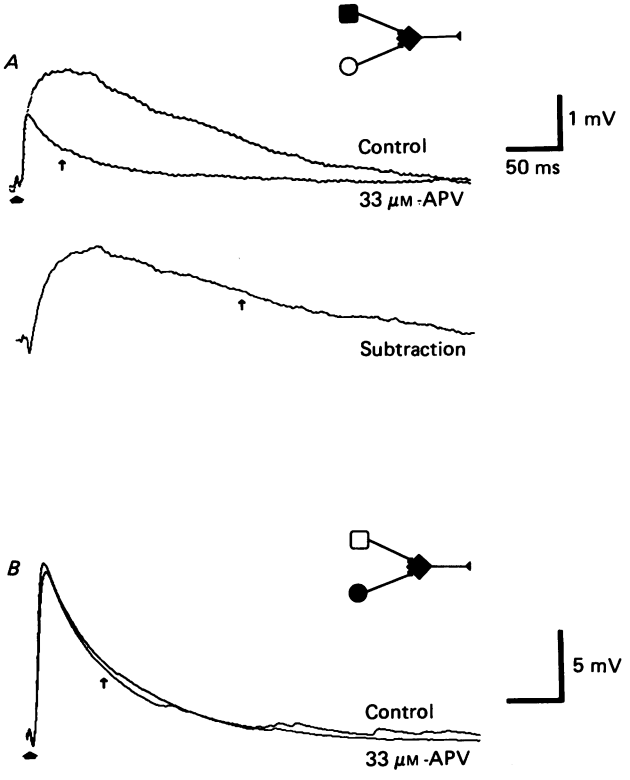

Fig. 3. Two different presynaptic hippocampal neurones evoked monosynaptic EPSPs in the same hippocampal neurone. In *A*, a small EPSP had a large NMDA-receptor-mediated component as revealed by application of DL-APV. In *B*, a second presynaptic neurone evoked a much larger EPSP with no apparent slow component. This suggests that NMDA receptors are not homogeneously distributed, but rather the slow EPSP results from activation of NMDA receptors located at specific synaptic sites. Membrane potential −65 mV.

However, the reverse was actually the case. The ratio of slow/fast for the large EPSPs (> 14 mV) was significantly smaller ($0{\cdot}19 \pm 0{\cdot}03$, $n = 15$) compared to EPSPs with amplitudes less than 5 mV ($0{\cdot}52 \pm 0{\cdot}1$, $n = 18$, two-sample t test, $P < 0{\cdot}01$). This is particularly well illustrated by the two HPC–HPC EPSPs in Fig. 3. In this case, monosynaptic EPSPs from two different presynaptic neurones were recorded in the same postsynaptic neurone. Stimulation of one neurone evoked a large EPSP with no slow component whereas stimulation of another neurone evoked a small EPSP with a large NMDA-receptor-mediated component. Although analysis of transmission at single release sites will be necessary to determine the exact organization of postsynaptic excitatory amino acid receptors, our analysis suggests

that the NMDA receptors responsible for the slow EPSPs are indeed associated with discrete synaptic locations rather than distributed homogeneously in the extra-synaptic membrane. This is further supported by the presence of large numbers of NMDA-receptor binding sites in preparations of postsynaptic densities from rat cortex (Fagg & Matus, 1984).

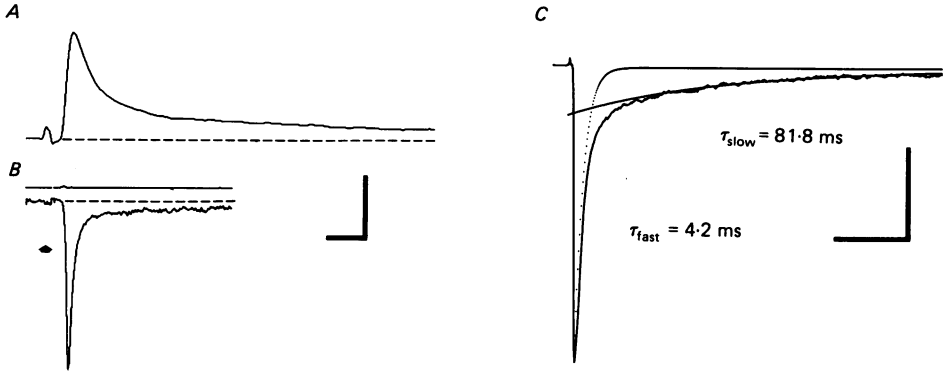

Fig. 4. The EPSC under voltage clamp has two kinetically distinct components. *A*, SC–SC EPSP evoked at a membrane potential of −76 mV in a bath containing 1 mM-Ca^{2+} and no added Mg^{2+} shows a rapid peak amplitude followed by a slow response lasting > 200 ms. The EPSC for the same cell pair is shown in the lower trace in *B* at a holding potential of −76 mV. Arrow indicates presynaptic stimulation. Note that in addition to the rapid peak of inward current there is a long-lasting inward current which underlies the slow decay of the EPSP in *A*. The EPSC returned to the initial current shown as the dotted line, but in this example the length of the data sample was limited by the high sampling rate used to adequately resolve the fast current. *C*, an excitatory synaptic current for a HPC–HPC EPSP is shown in *C* at a holding potential of −46 mV. The time course of the synaptic current could be well fitted with the sum of two exponentials. Curve fitting was performed by a linear regression analysis of the logarithm of the current. The time constant of the fast component was 4·2 ms (dotted line) and 81·8 ms for the slow component (continuous line). Note that the slow component of the synaptic current does not show the slow time-to-peak characteristic of the slow EPSP (see Fig. 1 and Table 1). Calibration for *A* and *B* is 20 ms and 5 mV/0·37 nA; for *C* calibration is 40 ms, 0·3 nA.

Likewise, using shape indices determined from the APV-resistant component of the response, EPSPs with slow components were not confined to distal locations. Proximal inputs were defined as a time-to-peak/τ_m < 0·2 and distal inputs as a time-to-peak/τ_m > 0·4 (see Jack *et al.* 1971). The ratio of slow/fast peak amplitude for EPSPs with the most proximal synaptic locations (0·38±0·1, $n = 16$) was not significantly different from the distal inputs (0·28±0·05, $n = 14$, $P = 0·36$, paired t test).

Voltage dependence of the slow EPSC

The fast excitatory synaptic current for the SC–SC synapse decays with a time constant of less than 1 ms (Nelson *et al.* 1986) whereas the time course of the NMDA-receptor-mediated EPSP is sufficiently slow for the conductance mechanisms for the two components to be easily distinguishable under voltage clamp. Figure 4*A* shows a SC–SC EPSP in Mg^{2+}-free solution which had a slow component of duration

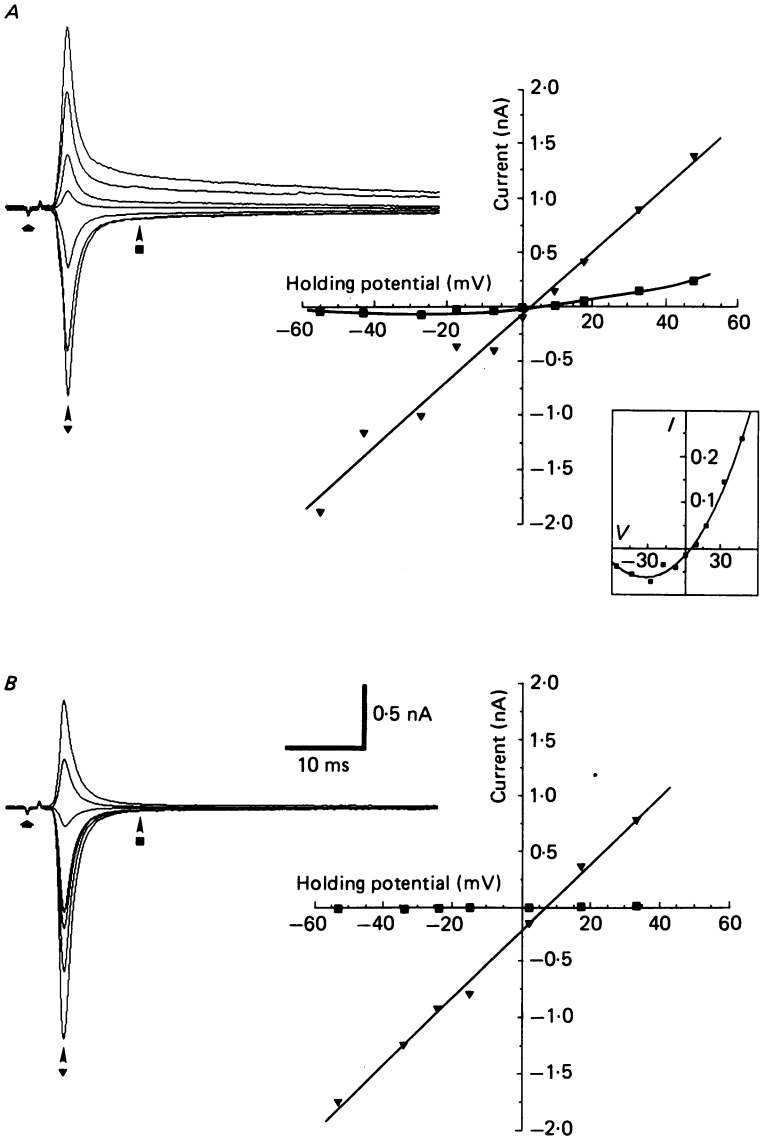

Fig. 5. For legend see facing page.

> 200 ms, and the underlying excitatory synaptic current clamped at the resting potential of −76 mV (Fig. 4*B*.). The EPSC consists of an initial peak and rapid decay followed by a smaller-amplitude, slowly decaying inward current that corresponds to the slow component of the EPSP. Similar results were obtained for twenty EPSCs.

The synaptic current could be well fitted with a sum of two exponentials (Fig. 4*C*). At holding potentials near the resting potential, the values for the decay time constants for thirteen EPSCs were 3·9 ± 0·1 ms for the fast current and 85·5 ± 4·5 ms for the slow current. Although the peak amplitude of the slow current was much

smaller than the fast current, the current carried by the slow EPSC (measured as the integral APV-sensitive current at −60 mV in Mg^{2+}-free medium) comprised nearly 50% of the available conductance (0·49 ± 0·01, $n = 5$). Unlike the slow time-to-peak of the APV-sensitive EPSP, the slow EPSC appeared to reach a peak within a few ms (Fig. 4*C*; see Discussion). We could not examine the onset of the slow current in isolation since highly selective antagonists for non-NMDA receptors are not yet available.

The average decay time constant for the fast EPSC in the present experiments is longer than previously reported for fast EPSCs located on the soma or proximal dendrites of cultured spinal cord neurones (Nelson *et al.* 1986); this difference is most probably due to space clamp limitations rather than to differences in the kinetics of ion channels mediating the fast current. Since our primary objective in the present experiments was analysis of the slow current, which is much less subject to spatial distortion (e.g. Johnston & Brown, 1983), EPSCs were not excluded from analysis on the basis of synaptic location.

To examine the voltage dependence of the two components of the synaptic current, fast inward currents were measured at their peak, and slow currents were measured at a latency of > 5 times the decay time constant of the fast EPSC. This limited a contribution of the fast EPSC to the slow current measurement to much less than 1%. The postsynaptic membrane potential was varied between −60 and +50 mV and Mg^{2+} (100 μM) was applied by local perfusion ($n = 3$). As shown for a HPC–HPC EPSC in Fig. 5, the peak amplitude of the fast current was quite symmetrical about the reversal potential of 0 mV, whereas the slow synaptic current was much larger at membrane potentials positive to the reversal potential. The current–voltage (*I–V*) relationship of the fast current was linear with a slope conductance of 29·9 nS, while the slow current was highly voltage dependent with a region of negative slope at membrane potentials hyperpolarized to −30 mV. The slow current *I–V* plot is shown at higher gain in the inset of Fig. 5. The reversal potential for both components was near 0 mV with $[Ca^{2+}]_o = 1$ mM.

As expected from the effects of NMDA-receptor antagonists on the slow component

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Fig. 5. The slow EPSC is voltage dependent in the presence of Mg^{2+}. *A*, monosynaptic EPSC between two hippocampal neurones at a series of holding potentials from −60 mV to +47 mV is shown at left. The postsynaptic neurone was perfused with a solution containing 100 μM-Mg^{2+} and 1 mM-Ca^{2+}. The records have been leak subtracted; stimulation artifact from the current pulse injected into the presynaptic neurone is shown with the broad arrow. The early peak current is symmetrical around the reversal potential of 0 mV, whereas there is a much larger slow current at positive membrane potentials than at hyperpolarized potentials. The peak current (▼) and the current at a latency of 10 ms (■) are plotted at right. The peak current is voltage insensitive but the slow current (shown at higher gain in inset) shows a region of negative slope conductance at holding potentials hyperpolarized to −30 mV. *B*, perfusion with DL-APV (33 μM, in the absence of Mg^{2+}) completely eliminated the slow component of the EPSC, but had no effect on the fast component. Same EPSC as in *A*. Note the absence of evoked current 10 ms after the onset in *B* compared to the slow current at the same latency in *A*. The current–voltage plot at right in *B* demonstrates that the slope conductance of the fast current is unaffected by antagonism of NMDA receptors. Two whole-cell recordings with the patch electrode filled with caesium methyl sulphate for the postsynaptic neurone, and filled with potassium methyl sulphate for the presynaptic neurone.

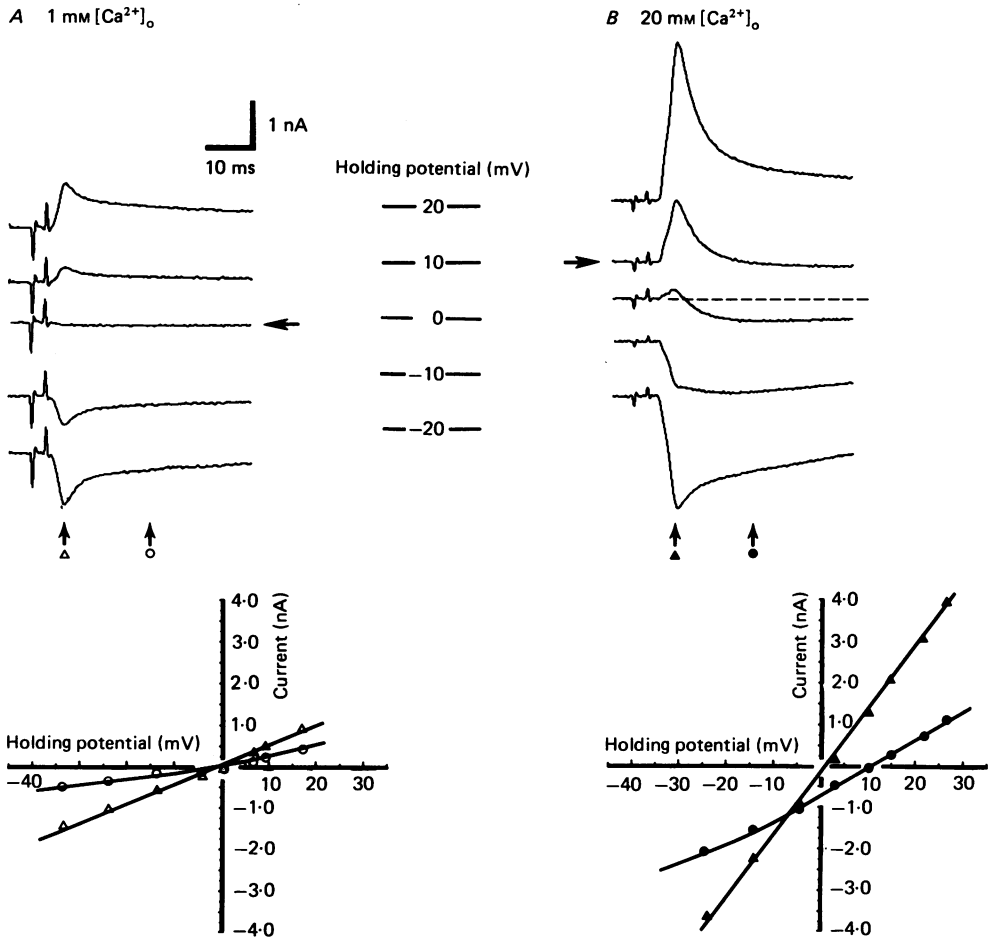

Fig. 6. The conductance underlying the slow EPSC is permeable to Ca^{2+}. The HPC–HPC EPSC is shown for a series of holding potentials near the reversal potential in the presence of 1 mM Ca^{2+} (*A*) and during local perfusion of 20 mM-Ca^{2+} (*B*). Current–voltage plots of the peak current (triangles) and the slow current at a latency of 20 ms (circles) are plotted below the individual current traces. In 1 mM-Ca^{2+} the reversal potential of both the peak current and the slow current is near 0 mV (see horizonal arrow in *A*). However, during perfusion with 20 mM-Ca^{2+}, the reversal potential of the slow current shifts to +10 mV (horizontal arrow in *B*) while the reversal potential of the peak current does not shift. Thus the peak current is outward at a holding potential of +10 mV and the monosynaptic current is biphasic at a holding potential of 0 mV. The increase in slope conductance seen in *B* is due to the increase in transmitter output in the high-Ca^{2+} solution. Extracellular solution contained 135 mM-Na^{+}, no added Mg^{2+} with picrotoxin; 160 mM-caesium methyl sulphate solution was used in postsynaptic patch electrode.

of the EPSP (see Fig. 1), only the slow current was blocked by APV. In Fig. 5*B*, APV completely blocked the synaptic current measured at a latency of 10 ms without affecting the peak amplitude of the EPSC. The slope conductance of the fast EPSC in the presence of APV was 30·8 nS, essentially unchanged from the control value. The decay time constant of the fast EPSC in this example was 1·1 ms at a holding potential of −54 mV.

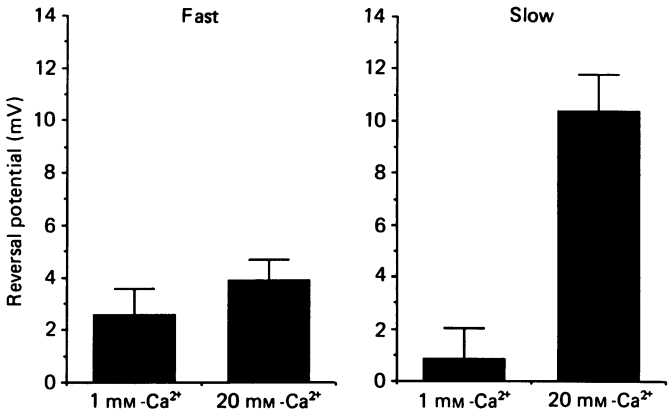

Fig. 7. Comparison of reversal potentials of the fast and slow EPSCs in medium containing 135 mM-Na^+ and either 1 or 20 mM-Ca^{2+}. Reversal potentials at the peak of the synaptic current (histogram at left) demonstrate that there was no significant change in the reversal potential of the fast EPSC during perfusion with high Ca^{2+}. However, the reversal potential of the slow EPSC did show a significant shift to a more positive potential in 20 mM-Ca^{2+}.

The slow excitatory current is partly carried by calcium ions

The Mg^{2+} sensitivity and voltage dependence of the slow synaptic current demonstrate that synaptically activated NMDA-receptor channels exhibit one of the properties expected on the basis of voltage clamp and single-channel studies of NMDA-activated channels (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Mayer, Westbrook & Guthrie, 1984; Mayer & Westbrook, 1985*a*). NMDA-receptor channels also have a high permeability to Ca^{2+}, sufficient to result in a transient increase in cytoplasmic Ca^{2+} when NMDA is applied to cultured spinal cord neurones under voltage clamp (MacDermott *et al.* 1986). Based on shifts in the reversal potential of currents evoked by excitatory amino acids, the relative permeability ratio (calculated using the extended constant field voltage equation) for the NMDA-activated conductance was $P_{Ca}/P_{Na} = 10{\cdot}6$, compared to $P_{Ca}/P_{Na} = 0{\cdot}15$ for responses to kainic or quisqualic acids (Mayer & Westbrook, 1988). To examine the Ca^{2+} permeability of the slow synaptic current, reversal potentials for the fast and slow monosynaptic EPSC were first measured in a solution containing 1 mM-Ca^{2+} and 135 mM-Na^+, and then during perfusion with a high-Ca^{2+} solution (Fig. 6).

For twelve neurones the reversal potentials were $+2{\cdot}5 \pm 1{\cdot}0$ mV for the fast current and $+0{\cdot}8 \pm 1{\cdot}2$ mV for the slow current in the low-Ca^{2+} solution. However, when the postsynaptic neurone was locally perfused with a solution containing 20 mM-Ca^{2+} and 135 mM-Na^+, the reversal potential of the slow current shifted to a more positive voltage (horizontal arrows, Fig. 6*A* and *B*). The shift in the reversal potential was significantly greater for the slow component ($\Delta V = +9{\cdot}6 \pm 1{\cdot}4$ mV) than for the fast component ($\Delta V = +1{\cdot}3 \pm 0{\cdot}8$, $P < 0{\cdot}01$, paired two-sample t test, $n = 8$), consistent with a significant Ca^{2+} permeability of the slow current mediated by activation of NMDA receptors (Fig. 7). The extended constant field voltage equation using a P_{Ca}/P_{Na} ratio of 10·6 for NMDA-receptor channels would predict a shift of +15 mV on raising $[Ca^{2+}]_o$ from 1 to 20 mM. It is likely that the smaller shift

of +9·6 mV is due to dilution of the high-Ca^{2+} solution inevitable with the local perfusion method used in these experiments.

DISCUSSION

Our experiments demonstrate that NMDA-receptor activation is responsible for a slow component of monosynaptic EPSPs evoked by action potentials in single presynaptic neurones, and that the synaptic currents underlying the fast and slow components of the EPSP are kinetically distinct. The slow synaptic current is voltage dependent, due to block by extracellular Mg^{2+}, and has a high permeability to Ca^{2+}; thus the behaviour of these synaptically activated channels closely matches those seen in voltage and patch clamp studies of NMDA-receptor channels. The similarity of our results in neurones of the spinal cord and hippocampus suggests that slow EPSPs mediated by NMDA receptors may be a feature of many central synapses that use an acidic amino acid as an excitatory neurotransmitter.

Is the dual-receptor EPSP a general phenomenon?

That NMDA receptors are activated during synaptic transmission is not a new observation. Evans, Smith & Watkins (1981) demonstrated that APV blocked a slow component of the ventral root potential evoked by low-frequency stimulation of dorsal root fibres in the isolated rat spinal cord; this was interpreted as indicating a role for NMDA receptors in 'polysynaptic' activation. However, the lack of effect of APV on many fast EPSPs (see Mayer & Westbrook, 1987 for review), combined with the appearance of an APV-sensitive component of synaptic responses during tetanic stimulation, has led to the working hypothesis that NMDA receptors are quiescent during normal synaptic transmission and only become activated under conditions of intense synaptic bombardment (e.g. see Herron, Lester, Coan & Collingridge, 1986). Despite this, as a number of pathways have been carefully examined, taking into consideration Mg^{2+} sensitivity of the NMDA response, a contribution by NMDA receptors to synaptic potentials mediated by low-frequency stimulation has now been demonstrated in cerebral cortex (Thomson *et al.* 1985), hippocampus (Wigström & Gustafsson, 1984) and spinal cord (Dale & Roberts, 1985). Physiological activation of primary afferent inputs also involves NMDA receptors in the ventrobasal thalamus (Salt, 1986); and in spike-triggered averages of monosynaptic EPSPs evoked in frog motoneurones by Ia afferents (E. Frank, personal communication).

Studies of excitatory synaptic currents on cultured chick spinal neurones by O'Brien & Fischbach (1986) and the results presented here suggest that release of the excitatory transmitter leads simultaneously to the activation of both NMDA receptors and another excitatory amino acid receptor. It is interesting in this regard that all endogenous excitatory amino acids examined to date, including L-glutamate, L-aspartate, L-homocysteate and the dipeptide *N*-acetylaspartylglutamate, appear to act preferentially at NMDA receptors (Olverman, Jones & Watkins, 1984; Westbrook, Mayer, Namboodiri & Neale, 1986), although at higher concentrations these agonists also activate other excitatory amino acid receptors. Thus a pure 'fast' EPSP (as in Fig. 3*B*) requires segregation of kainate or quisqualate receptors at the

synaptic site whereas a pure 'slow' EPSP (see e.g. Fig. 11, Dale & Roberts, 1985) could occur either on the basis of NMDA-receptor segregation or simply could be due to preferential activation of the receptor with the higher affinity for the excitatory transmitter.

Time course of the slow EPSP

The most striking features of the NMDA-receptor-mediated component of the EPSP are the slow time-to-peak and long duration. Our experiments are consistent with those of Wigström & Gustafsson (1984) and Dale & Roberts (1985) in demonstrating a slow rise of the NMDA-receptor-mediated EPSP. However, Thomson *et al.* (1985) and O'Brien & Fischbach (1986) concluded that an early component of excitatory synaptic responses was sensitive to NMDA-receptor antagonists. These differences could reflect uncertainties in eliminating polysynaptic activity or asynchronous monosynaptic contributions to the peak response, difficulties which are avoided using simultaneous pre- and postsynaptic intracellular recordings.

Our results demonstrate that factors other than the location of the synaptic input must be responsible for the long duration of the NMDA-receptor-mediated response. This is supported by calculations of electrotonic decay in equivalent cylinder neuronal models (Rall, 1977). For example, an equivalent cylinder model predicts only a 3- to 5-fold increase in the current decay time constant measured at the soma in response to a brief conductance change at the end of an equivalent cylinder for a typical central neurone of electrotonic length = 1 (see Johnston & Brown, 1983; Fig. 10, Nelson *et al.* 1986). Alternative possibilities for the long duration of the APV-sensitive EPSP are: (1) open states of NMDA channels with very long lifetimes, (2) transmitter molecules persist in the synaptic cleft, or are continuously released at low levels, and diffuse onto nearby extrasynaptic regions resulting in repeated receptor activation until diffusion and/or reuptake terminates the response, or (3) loose coupling of receptor activation to channel opening, reflecting second-messenger activation.

There is some evidence for the first possible mechanism. Mean lifetimes for NMDA-receptor channels in the absence of Mg^{2+} have been reported as 4·7 ms (Nowak *et al.* 1984) and 5·2–7·1 ms (Cull-Candy & Usowicz, 1987). However, Jahr & Stevens (1987) noted both short and long gating modes of NMDA-activated channels with mean lifetimes of 1–3 and 10–15 ms, with some bursts lasting hundreds of milliseconds. A long bursting mode could result in a long-duration EPSP, but does not by itself explain the slow time-to-peak. Although channel activation kinetics could possibly be responsible for the slow rise of the EPSP the peak of the slow synaptic current had no apparent delay, suggesting that the time-to-peak of the slow EPSP was due to the time necessary to charge the resistive and capacitative elements of the postsynaptic membrane. In fact, the time-to-peak of the slow EPSPs was longer than the postsynaptic membrane time constant, consistent with this possibility.

Alternatively, it seems plausible that persistent activation of NMDA receptors is the factor responsible for the long duration of the EPSP. The mixed agonist action of L-glutamate, with a higher affinity for NMDA receptors, suggests that a low concentration of agonist would be sufficient to generate the slow EPSP. This is

further supported by the sensitivity of the slow EPSP to low concentrations of APV. With local perfusion of 33 μM-DL-APV, the effective D-APV concentration at the neuronal surface in our experiments was probably $< 10\ \mu$M. Since APV is a competitive NMDA-receptor antagonist (Harrison & Simmonds, 1985; Westbrook & Mayer, 1987), complete block by these low concentrations of APV suggests that the subsynaptic L-glutamate concentration during the slow EPSP could easily be $< 10\ \mu$M, based on the relative affinities of L-glutamate and APV for the NMDA receptor (Olverman *et al.* 1984). Such low occupancy of NMDA receptors under normal conditions suggests that the synaptic response could be considerably amplified during intense stimulation. This contrasts with the neuromuscular junction where the high acetylcholine concentration may activate 50–75% of the available receptors at the peak of the end-plate current (Hartzell, Kuffler & Yoshikami, 1975).

The slow EPSP in normal synaptic transmission

An important question is to what extent the NMDA-activated conductance participates in synaptic transmission under physiological levels of stimulation, and whether the channel is totally blocked by Mg^{2+} at resting membrane potentials. Technical factors almost certainly have contributed to the difficulty in detecting the slow EPSP in our prior experiments. For example, the slow time course would limit the apparent contribution of NMDA-receptor channels if only the peak amplitude of the EPSP is measured. The activation of large, slow conductances mediating inhibitory synaptic potentials and spike after-potentials may also obscure and/or shunt the slow EPSP. However, this would not prevent the influx of ions (including Ca^{2+}) through synaptically activated NMDA channels. In addition, it seems likely that the somatic shunt associated with microelectrode recording in cultured neurones, which can decrease the input resistance of spinal cord neurones from 200 to 20 MΩ, made it more difficult to see the slow EPSP. In fact, using whole-cell recording, slow synaptic currents can be observed in 1 mM-Mg^{2+} (Forsythe & Nelson, 1988).

A number of factors for which quantitative data are not available need to be considered in assessing the contribution of the slow EPSP in normal synaptic transmission. For example, since the depolarization on a dendritic spine will be much larger than that recorded at the soma (Rall, 1974) it seems quite plausible that NMDA-receptor channels may overcome a voltage-dependent block, even in millimolar Mg^{2+}. Other factors to consider are the concentrations of the excitatory transmitter and Mg^{2+} in the synaptic cleft, as well as the effects of endogenous modulators of NMDA receptors such as glycine (Johnson & Ascher, 1987) and zinc (Peters, Koh & Choi, 1987; Westbrook & Mayer, 1987). Despite these unknowns, Gustafsson, Wigström, Abraham & Huang (1987) clearly show that a single presynaptic volley activates NMDA receptors for at least 100 ms, even in the presence of 4 mM-Mg^{2+}, and that this is sufficient to induce long-term potentiation when coupled with a postsynaptic depolarization.

Properties of synaptically activated NMDA-receptor channels

The voltage dependence of NMDA-receptor channels appears to have a well-defined physiological role in short-term modulation of excitability. A second

messenger role for Ca^{2+} influx through NMDA channels is more circumstantial (Mayer, MacDermott, Westbrook, Smith & Barker, 1987). Our results demonstrate that, although the membrane potential depolarization during the slow EPSC may be small, the charge transfer (and thus entry of Ca^{2+}) may be surprisingly large when integrated over several hundred milliseconds. Furthermore, the relative Ca^{2+} permeability of synaptically activated NMDA channels is in reasonable agreement with voltage clamp studies of agonist-activated currents (Mayer & Westbrook, 1988); also, it is much higher than the Ca^{2+} permeability associated with other excitatory synaptic currents such as the frog end-plate current (e.g. Adams, Dwyer & Hille, 1980). Thus it is reasonable to consider this transmitter-gated Ca^{2+} influx as potentially important to a number of cell functions. For example, Ca^{2+} influx via a slow synaptic response may affect modulation of Ca^{2+}-dependent conductances during rhythmic bursting.

Two unresolved issues central to an understanding of these dual-receptor synapses are the location of NMDA receptors, since the exact site of Ca^{2+} entry may be critical as it appears to be in presynaptic membrane (e.g. Chad & Eckert, 1984), and whether regulation of NMDA receptors by endogenous modulators or via switching between multiple conductance levels (Jahr & Stevens, 1987; Cull-Candy & Usowicz, 1987) can influence the output of single synapses as well as that of neuronal networks.

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