Evidence that heterosynaptic depolarization underlies associativity of long-term potentiation in rat hippocampus

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1. Whole-cell patch-clamp recording has been used to study the effect of heterosynaptic depolarization on pure *N*-methyl-D-aspartate (NMDA) receptor-mediated synaptic transmission in the CA1 region of rat hippocampal slices.

2. In neurones voltage clamped at −60 mV, paired-pulse stimulation of one set of Schaffer collateral–commissural fibres resulted in homosynaptic paired-pulse facilitation of the NMDA receptor-mediated excitatory postsynaptic current ($EPSC_N$). In contrast, stimulation of one set of fibres prior to stimulation of a second set of fibres (i.e. heterosynaptic paired-pulse stimulation) did not result in any heterosynaptic interactions.

3. However, under current-clamp conditions, heterosynaptic paired-pulse stimulation resulted in heterosynaptic 'paired-pulse facilitation' of the NMDA receptor-mediated excitatory postsynaptic potential ($EPSP_N$).

4. In neurones held at −50 or −40 mV, perfusion of nominally Mg^{2+}-free medium converted the response to heterosynaptic paired-pulse stimulation from 'heterosynaptic facilitation' to 'heterosynaptic depression' of $EPSP_N$.

5. When neurones were held at potentials of between −30 and +40 mV then heterosynaptic paired-pulse stimulation, in normal Mg^{2+}-containing medium, resulted in 'paired-pulse depression' of $EPSP_N$. Under voltage-clamp conditions (tested at +40 mV) no heterosynaptic interactions were seen.

6. The time course of 'heterosynaptic facilitation' at −60 mV and of 'heterosynaptic depression' at +40 mV of $EPSP_N$ was similar to the time course of $EPSC_N$.

7. We conclude, firstly, that the voltage clamp is able to prevent any voltage breakthrough associated with the synaptic activation of NMDA receptors from influencing neighbouring synapses. Secondly, when the neurone is not voltage clamped these same synapses are strongly influenced by the spreading depolarization generated by the synaptic activation of their neighbours. The time course and direction of this influence are compatible with the hypothesis that spreading synaptic depolarization, leading to a reduction of the voltage-dependent Mg^{2+} block of synaptic NMDA receptor channels, underlies the property of associativity.

Associativity is an important feature of long-term potentiation (LTP), and has contributed to its status as the primary experimental model of mammalian synaptic plasticity (Bliss & Collingridge, 1993). Associativity refers to the property which enables a 'weak' input (i.e. one which is subthreshold for the induction of LTP) to undergo LTP when activated in association with the stimulation of a separate set of afferent fibres (often referred to as a 'strong' input) onto the same neurone, or population of neurones (McNaughton, Douglas & Goddard, 1978; Levy & Steward, 1979; Barrionuevo & Brown, 1983; Gustafsson & Wigström, 1986; Kelso & Brown, 1986; Sastry, Goh & Auyeung, 1987). An explanation of the property of associativity follows from the demonstration that the induction of LTP requires the transient synaptic activation of *N*-methyl-D-aspartate (NMDA) receptors (Collingridge, Kehl & McLennan, 1983) and that these receptors are subject to a voltage-dependent block by Mg^{2+} (Ault, Evans, Francis, Oakes & Watkins 1980; Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Mayer, Westbrook &

Guthrie, 1984). Thus, a 'weak' input is thought to provide sufficient neurotransmitter (i.e. L-glutamate) to induce LTP, but does not do so because of the extent of the Mg^{2+} block of NMDA channels. The 'strong' input enables the 'weak' input to induce LTP by providing depolarization to help reduce the level of the Mg^{2+} block (Collingridge, 1985; Wigström & Gustafsson, 1985; Bliss & Collingridge, 1993). Thus, a key feature of this associativity scheme is the spread of depolarization from one synaptic input to another.

In the present study we have measured directly the influence of depolarization provided by the synaptic activation of one set of afferent fibres on the synaptic activation of NMDA receptors in a separate convergent input.

METHODS

Experiments were performed on transverse hippocampal slices (400 μm thick) prepared from halothane-anaesthetized (3·5%) female albino rats of between 3 and 5 weeks of age. Slices were maintained submerged in a low volume bath (1·8 ml) and perfused at 6–8 ml min^{-1} with medium which comprised (mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1·2; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10; bubbled with a 95% O_2–5% CO_2 mixture; at room temperature (22–28 °C). Whole-cell recordings were obtained from CA1 neurones using the 'blind patch' technique (Blanton, Loturco & Kreigstein, 1989) with patch electrodes filled with (mM): $CsMeSO_4$, 130; QX-314 (2(triethylamino)-*N*-(2,6-dimethylphenyl) acetamide, 5; NaCl, 1; $MgCl_2$, 1; EGTA, 0·05; Hepes, 5 (pH 7·2–7·4). In a few experiments the electrodes were filled with (mM): $KMeSO_4$, 122·5; KCl, 17·5; NaCl, 9; EGTA, 0·2; Hepes, 10; pH 7·4). After a whole-cell recording was obtained, picrotoxin (50 μM) was added to the perfusate to block the γ-aminobutyric acid $(GABA)_A$ receptor-

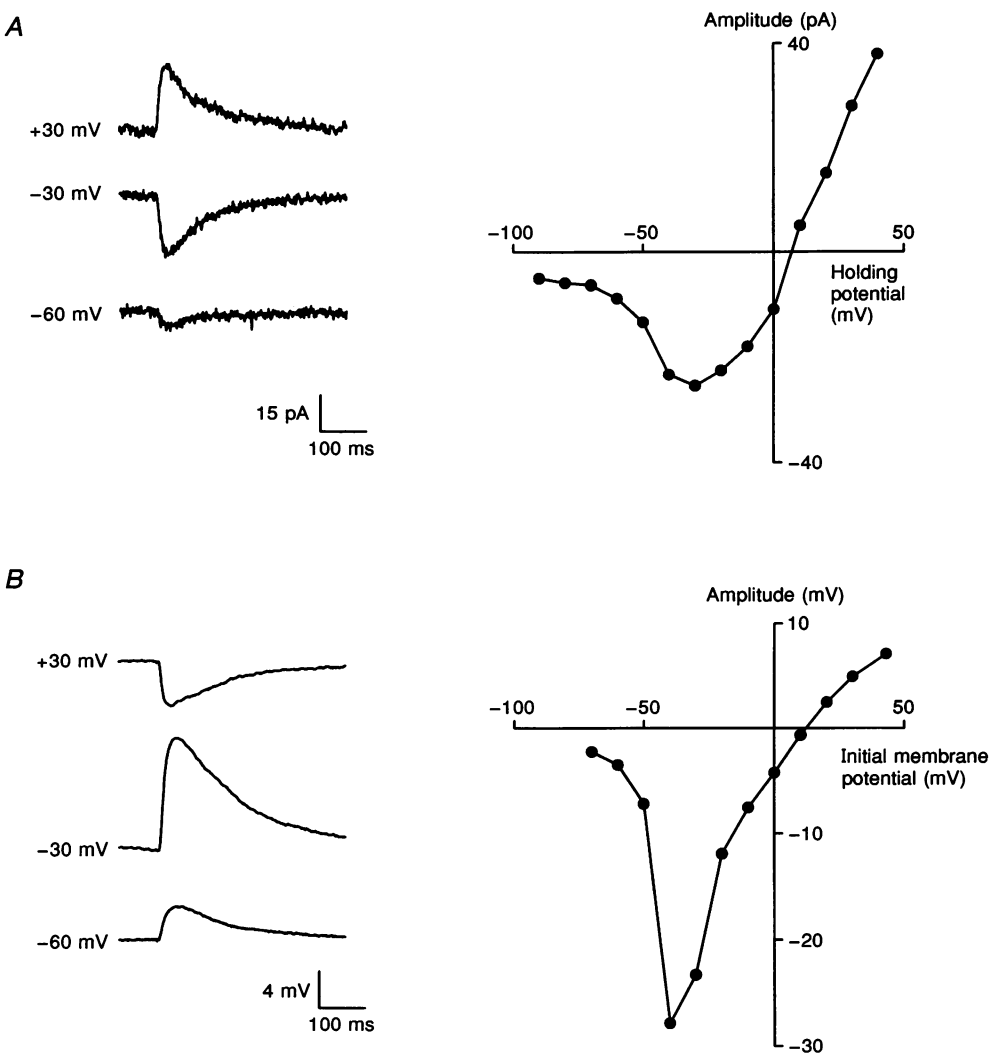

Figure 1. Voltage dependence of the pharmacologically isolated NMDA receptor-mediated synaptic response

A, the relationship between the peak amplitude of the synaptic current and the voltage-clamped membrane potential. *B*, the equivalent plot obtained under current-clamp conditions, for another neurone. In this graph the membrane potential refers to the potential immediately prior to stimulation. Similar $EPSP_N$ voltage plots, with maxima at −40 or −30 mV, were obtained in 4 neurones.

mediated component of synaptic inhibition and 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX; 1–2 μM) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) was added to eliminate the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor-mediated component of the excitatory post-synaptic current (EPSC). The Cs^+-based electrode solution eliminated the $GABA_B$ receptor-mediated component of synaptic inhibition, by blocking the associated K^+ channels. In experiments using the K^+-based electrode solution the $GABA_B$ antagonist 3-*N*[1-(*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*P*-benzyl-phosphinic acid (CGP 55845A; 1 μM) was also added to the perfusate to eliminate this synaptic component. Synaptic responses were evoked by stimulating Schaffer collateral–commissural fibres, using bipolar stimulating electrodes. The two electrodes were positioned, on either side of the recording electrode, in stratum radiatum at approximately the same distance from stratum pyramidale. Each pathway was stimulated at 10 or 15 s intervals. Homo- or heterosynaptic paired-pulse stimulation was delivered using an interstimulus interval of between 10 and 1500 ms. Averages of up to eight synaptic records were used to calculate facilitation and depression ratios and for purposes of illustration.

AP5 and CNQX were gifts from Professor J. C. Watkins (Bristol University), NBQX a gift from Dr T. Honoré (Nova Nordisk, Málov) and CGP 55845A was a gift from Dr M. F. Pozza (Ciba). Picrotoxin and QX-314 were purchased from Sigma and Alomone laboratories, respectively. Data are presented as means ± 1 S.E.M.

RESULTS

Experiments were performed, using Cs^+-based electrode solutions, on twenty neurones, which had resting membrane potentials, on breakthrough, of −56 ± 1 mV and input resistances (at −60 mV) of 295 ± 29 MΩ. A further three neurones were recorded using a K^+-based electrode solution. The EPSC, recorded in the presence of the pharmacological cocktail, was entirely NMDA receptor-mediated; it displayed a typical non-linear dependence on holding potential (Fig. 1*A*; Hestrin, Nicoll, Perkel & Sah, 1990; Konnerth, Keller, Ballanyi & Yaari, 1990; Randall, Schofield & Collingridge, 1990). A steeper relationship between response amplitude and membrane potential was observed under current-clamp conditions (Fig. 1*B*). The synaptic response was eliminated by the specific NMDA antagonist (*R*)-2-amino-5-phosphonopentanoate (AP5; 50 μM) (data not shown).

For each neurone the independence of the two inputs was established, under voltage-clamp conditions at −60 mV, by delivering paired stimuli to one input (homosynaptic paired-pulse stimulation) and by stimulating one input shortly before the second (heterosynaptic paired-pulse stimulation). As illustrated in Fig. 2*A*, homosynaptic stimulation resulted

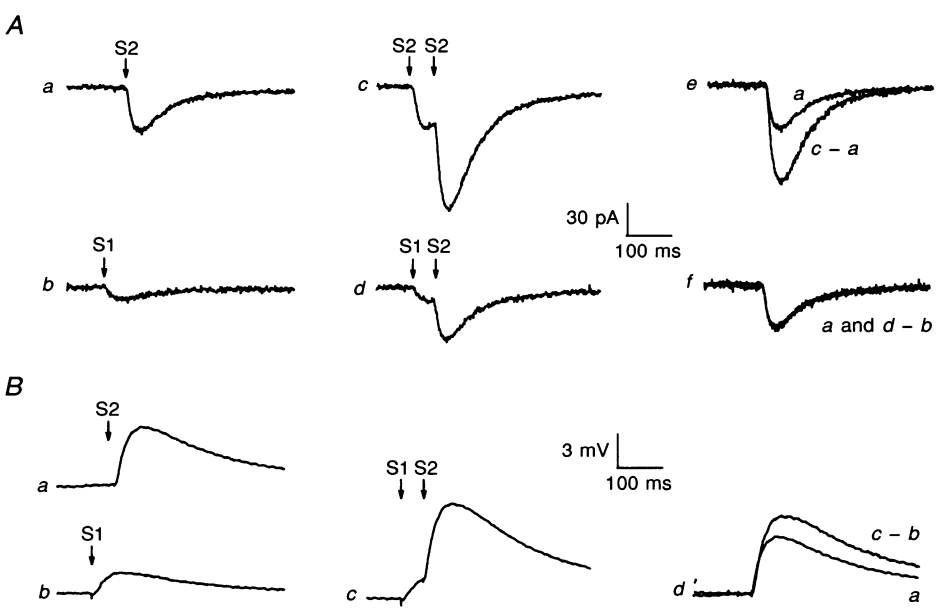

Figure 2. Heterosynaptic paired-pulse facilitation of $EPSP_N$

A, voltage-clamp experiments. *a* and *b*, $EPSC_N$s evoked at a holding potential of −60 mV, by stimulation of two sets of fibres (S2 and S1, respectively). *c*, paired-pulse facilitation of $EPSC_N$ elicited by delivering two identical shocks to S2 with an interstimulus interval of 50 ms. *e*, the extent of this facilitation illustrated by the superimposition of the response to a single stimulus (*a*) and the facilitated response (*c* − *a*). *d*, the response to heterosynaptic paired-pulse stimulation. The lack of heterosynaptic paired-pulse facilitation can be seen in *f* by the superimposition of the response to S2 obtained 50 ms following stimulation of S1 (*d* − *b*) and the response to a single stimulus of S2 (*a*). *B*, current-clamp experiments. *a* and *b*, the $EPSP_N$s, recorded at an initial holding potential of −60 mV, which correspond to the $EPSC_N$s illustrated in *A*. *c*, the response to heterosynaptic stimuli. The extent of paired-pulse heterosynaptic facilitation can be seen in *d* by the superimposition of the facilitated response (*c* − *b*) with the response to a single stimulus (*a*). Subtractions were made as described previously (Clark *et al.* 1994).

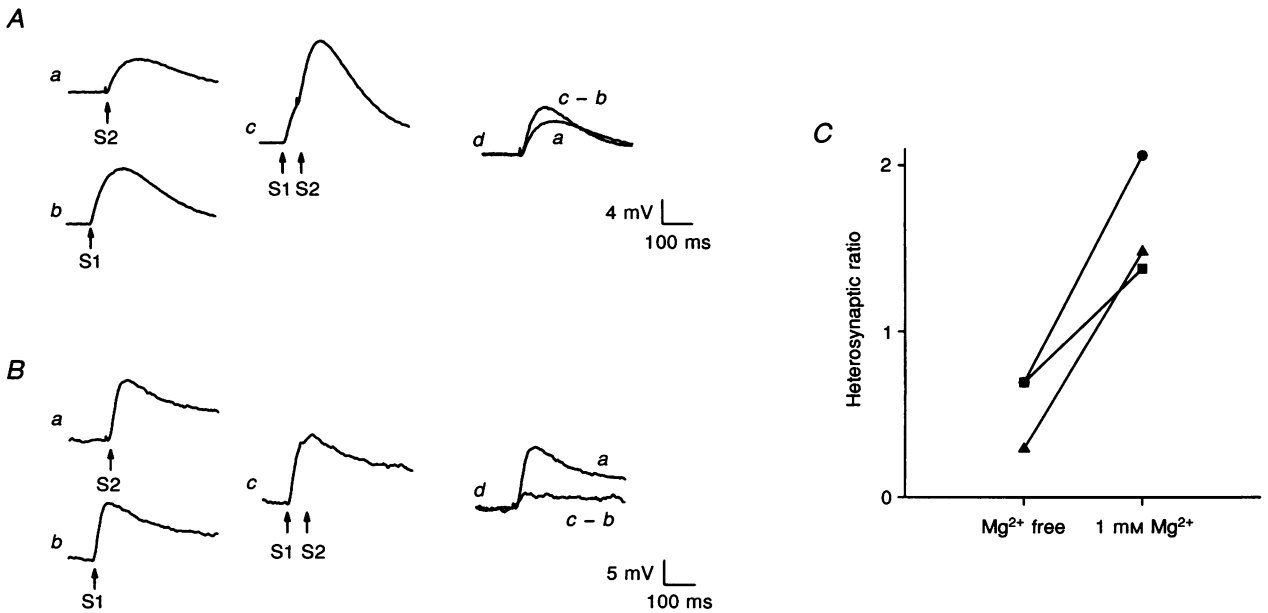

Figure 3. The effect of Mg^{2+} on heterosynaptic interactions

A, $EPSP_N$s evoked at −40 mV in the presence of 1 mM Mg^{2+}. Data are plotted as in Fig. 2*B* and show 'heterosynaptic facilitation'. *B*, the corresponding experiment performed following perfusion with nominally Mg^{2+}-free medium. The stimulus intensities were reduced to match roughly the amplitudes of $EPSP_N$s. Note that removal of Mg^{2+} results in 'heterosynaptic depression'. *C*, the heterosynaptic ratio *versus* Mg^{2+} concentration for the 3 neurones where the same protocol was used. The lines connect points from the same cell. The facilitation ratio was obtained by dividing the peak amplitude of the facilitated response ($c - b$) by the peak amplitude of the initial response (a).

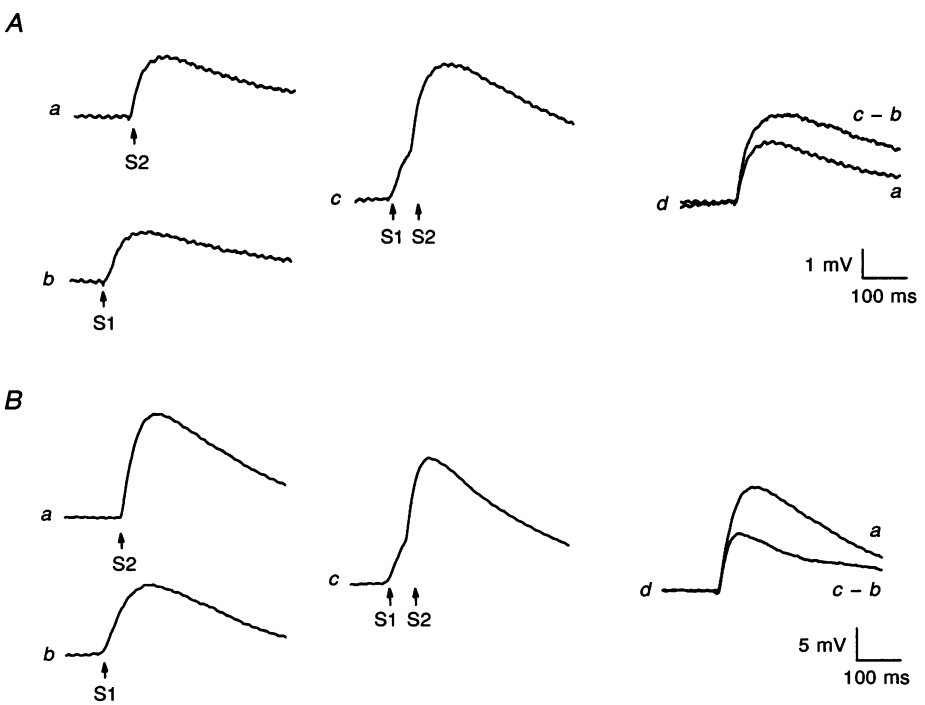

Figure 4. The effect of membrane potential on heterosynaptic interactions

A, $EPSP_N$s evoked at −50 mV. Data are plotted as in Fig. 2*B* and show 'heterosynaptic facilitation'. *B*, the corresponding experiment performed in the same neurone at −20 mV; it shows 'heterosynaptic depression'.

in paired-pulse facilitation of the NMDA receptor-mediated EPSC ($EPSC_N$). In contrast, heterosynaptic stimulation resulted in no paired-pulse facilitation, thereby demonstrating the complete independence of the two sets of afferent fibres.

In contrast, when the same heterosynaptic paired-pulse stimulation protocol was repeated under current-clamp conditions (with a prestimulus holding potential of −60 mV), there was a marked increase in the size of the NMDA receptor-mediated excitatory postsynaptic potential ($EPSP_N$), evoked by the second stimulus, in every neurone examined (Fig. 2*B*; $n = 11$). This interaction, which reflects changes in EPSP size but not the underlying conductance, is termed here 'heterosynaptic facilitation'.

The present results could be explained by a spread of depolarization from one input leading to a reduction in the Mg^{2+} block of NMDA receptor channels at another and thereby enhancing the size of the $EPSP_N$ elicited. We investigated this possibility by examining the effects of Mg^{2+} and membrane potential on heterosynaptic facilitation.

In three neurones the effects of heterosynaptic stimulation were compared in the standard Mg^{2+}-containing medium and in medium to which no Mg^{2+} had been added. Experiments were performed at −50 or −40 mV since this is a potential range which is in the region of negative slope conductance in the presence of Mg^{2+} but not when nominally Mg^{2+}-free medium is used; more negative potentials are still in a region of negative slope conductance presumably due to residual Mg^{2+} block (Hestrin *et al.* 1990; Konnerth *et al.* 1990; Clark, Randall & Collingridge, 1994). In each case, 'heterosynaptic facilitation' was converted to 'heterosynaptic depression' after perfusion with nominally Mg^{2+}-free medium for sufficient time (approximately 30 min) to reduce or eliminate the voltage-dependent block of NMDA responses (Fig. 3).

If heterosynaptic depolarization is indeed acting to reduce the Mg^{2+} block then the second $EPSP_N$ should be smaller, rather than larger, if the membrane potential at which it is evoked falls outside of the region of negative slope conductance of the NMDA receptor-mediated synaptic response (due to the reduction in driving force). We

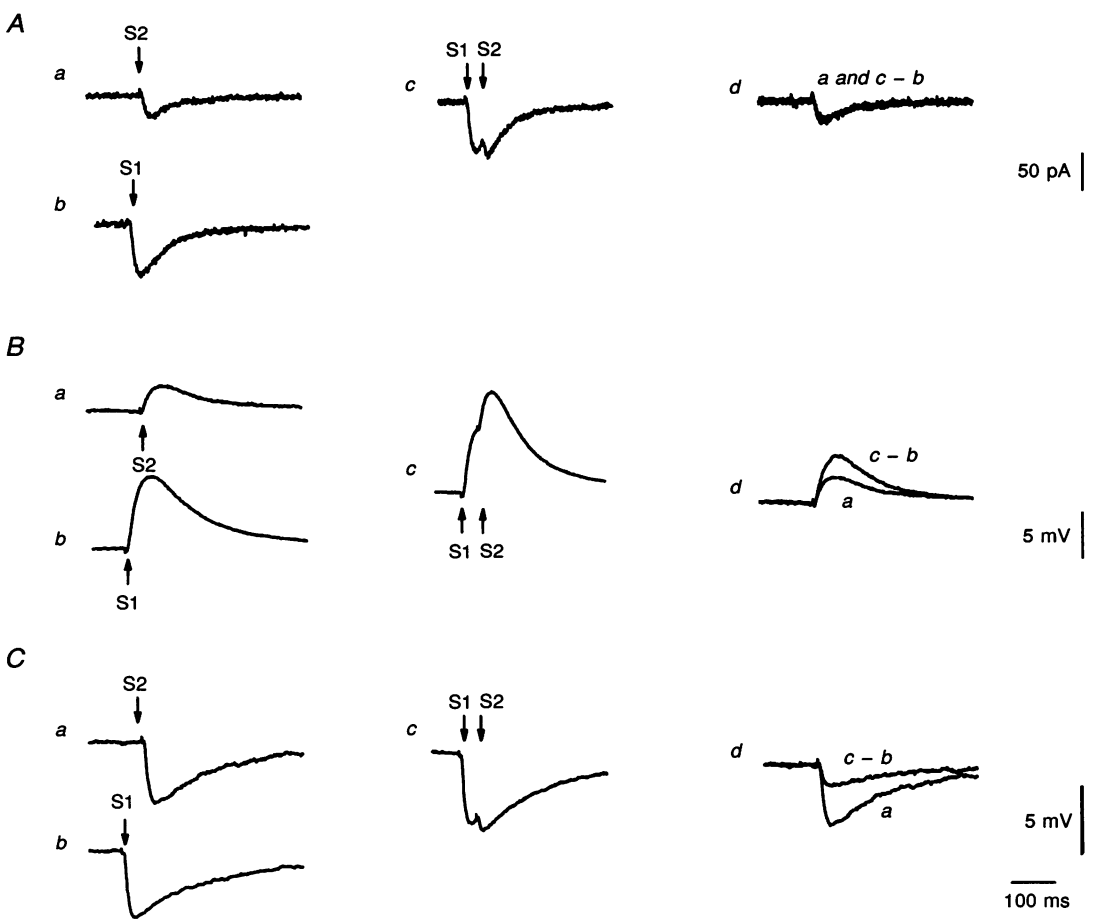

Figure 5. Heterosynaptic paired-pulse depression

A and *B* plot the heterosynaptic responses at −60 mV, under voltage-clamp and current-clamp conditions, respectively. The protocol was similar to that illustrated in Fig. 2. *C* shows the equivalent current-clamp experiment conducted using an initial holding potential of +40 mV. Note the marked heterosynaptic paired-pulse depression of the reversed $EPSP_N$.

therefore compared the effects of heterosynaptic stimulation at negative potentials either side of the peak of the $EPSP_N$–voltage relationship (Fig. 1*B*). In four neurones heterosynaptic paired-pulse facilitation was always observed at potentials of between −60 and −40 mV whereas heterosynaptic paired-pulse depression was always seen at potentials of between −30 and 0 mV (Figs 4 and 6).

In a further four neurones, experiments were also performed beyond the reversal potential of the synaptic response. In each neurone tested at +40 mV there was heterosynaptic paired-pulse depression of $EPSP_N$ (Figs 5 and 6). In contrast, under voltage-clamp conditions there were no heterosynaptic interactions (Fig. 5).

These data are consistent with the hypothesis that spread of synaptic depolarization on stimulation of the first input 'facilitates' the response to the second input by voltage-dependent relief of the Mg^{2+} block; in which case, the time course of heterosynaptic interactions should follow the time course of the synaptic response evoked on stimulation of the first input. We therefore investigated the time course of heterosynaptic interactions at −60 and +40 mV by delivering two stimuli with interstimulus intervals of between 10 and 1500 ms. At −60 mV, 'heterosynaptic facilitation' lasted for at least 250 ms, but was not detected at intervals of 500 ms or greater. The maximum 'facilitation' occurred at between 10 and 25 ms. Over the entire interstimulus interval range there was no 'heterosynaptic facilitation' of $EPSC_N$ (Fig. 7*A* and *B*). The time course of 'heterosynaptic facilitation' was compared with the time courses of $EPSC_N$ and $EPSP_N$ for six neurones. The 'facilitation' profile of $EPSP_N$ was similar to the time course of $EPSC_N$, whereas the time course of $EPSP_N$ was somewhat slower (Fig. 7*A*). At +40 mV, 'heterosynaptic depression' of $EPSP_N$ had a time course similar to that of the $EPSC_N$ (evoked by the first input) recorded at the same potential. Thus, it lasted longer and was just detectable at an interpulse interval of 500 ms (Fig. 7*C*). Over the entire interpulse stimulus interval range there was no 'heterosynaptic depression' recorded under voltage clamp (Fig. 7*C*).

In keeping with practically all patch-clamp studies investigating LTP or related properties, the above experiments were performed using a Cs^+-based electrode solution to block voltage-dependent conductances and thereby improve the space clamp. To determine whether heterosynaptic interactions could be recorded under more physiological conditions, heterosynaptic paired-pulse stimuli were delivered to three neurones recorded with a K^+-based electrode solution. In each case, heterosynaptic paired-pulse stimulation, delivered at −60 mV with an interstimulus interval of 50 ms, resulted in no interactions under voltage clamp but led to heterosynaptic facilitation under current clamp (data not shown). These data are therefore consistent with the findings with the Cs^+-based electrode solution.

DISCUSSION

The present results demonstrate the influence of heterosynaptic depolarization on the synaptic activation of the NMDA receptor system. They are entirely consistent with the hypothesis that depolarization, by reducing the level of Mg^{2+} block of NMDA channels, increases the synaptic activation of the NMDA receptor system and that this underlies the property of associativity (Collingridge, 1985; Wigström & Gustafsson, 1985; Bliss & Collingridge, 1993). Since the effect is caused by depolarization (provided by S1) and hence a change in the driving force of the synaptic response (S2) we refer to the phenomenon as 'heterosynaptic facilitation' to distinguish it from classical facilitation.

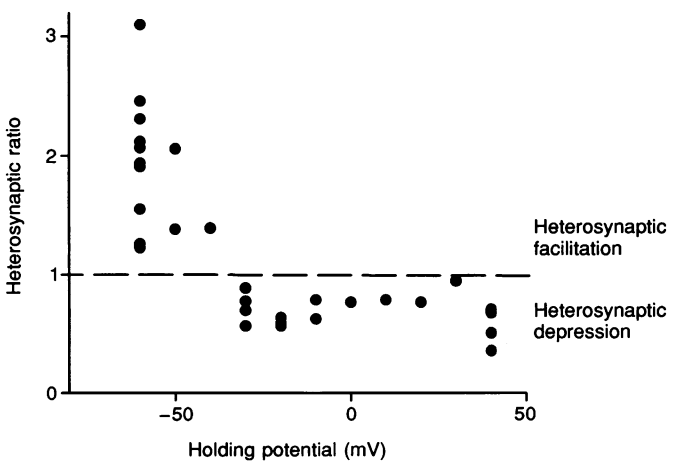

Figure 6. Voltage dependence of heterosynaptic interactions

The graph plots the degree of 'heterosynaptic facilitation' or 'heterosynaptic depression' (i.e. ratio of less than 1) *versus* membrane potential for 17 neurones.

The time course of 'heterosynaptic facilitation' is similar to that by which a 'strong' input or injection of depolarizing current can enable a 'weak' input to elicit LTP (Gustafsson, Wigström, Abraham & Huang, 1987). The latter was attributed to the time course of the NMDA receptor-mediated EPSP *per se*. The similarity of the profile of 'heterosynaptic facilitation' and the time course of $EPSC_N$ are consistent with this explanation, since the time course of the local depolarization change is likely to follow closely the conductance change, in view of its slow kinetics.

In the present study we have used $EPSP_N$s to both provide and test for the effect of the associative depolarization. Of course, in the normal functioning of the hippocampus the level of depolarization will be determined by the net effect of excitatory and inhibitory synaptic and intrinsic influences. In this wider context other timings are important. For example, a strong input arriving, say, 200 ms prior to a 'weak' stimulus may provide an 'associative' influence by depressing the strength of synaptic inhibition coincident with the 'weak' stimulus (Larson, Wong & Lynch, 1986; Diamond, Dunwiddie & Rose, 1988; Davies, Starkey, Pozza & Collingridge, 1991).

Irrespective of the source of the 'associative' depolarization, the associative 'sensor' is the NMDA receptor-mediated synaptic conductance. Under voltage-clamp conditions, there was no 'heterosynaptic facilitation' which meant, firstly, that the two inputs were completely independent and, secondly, that there was no transfer of depolarization, which would have occurred had the clamp not been working adequately. This shows that a somatic voltage clamp can control the membrane potential at synapses sufficiently to prevent detectable voltage changes associated with $EPSC_N$. In turn, this means that the somatically recorded $EPSC_N$ (Collingridge, Herron & Lester, 1988; Hestrin *et al.* 1990; Konnerth *et al.* 1990; Randall *et al.* 1990) provides an accurate reflection of the time course of $EPSC_N$ at its synaptic origin.

In summary, the use of voltage-clamp techniques has enabled the influence of depolarization from one set of synapses on the level of activation at another to be determined. The results concord with the hypothesis that the influence of heterosynaptic depolarization on the synaptic activation of the NMDA receptor system underlies the property of associativity.

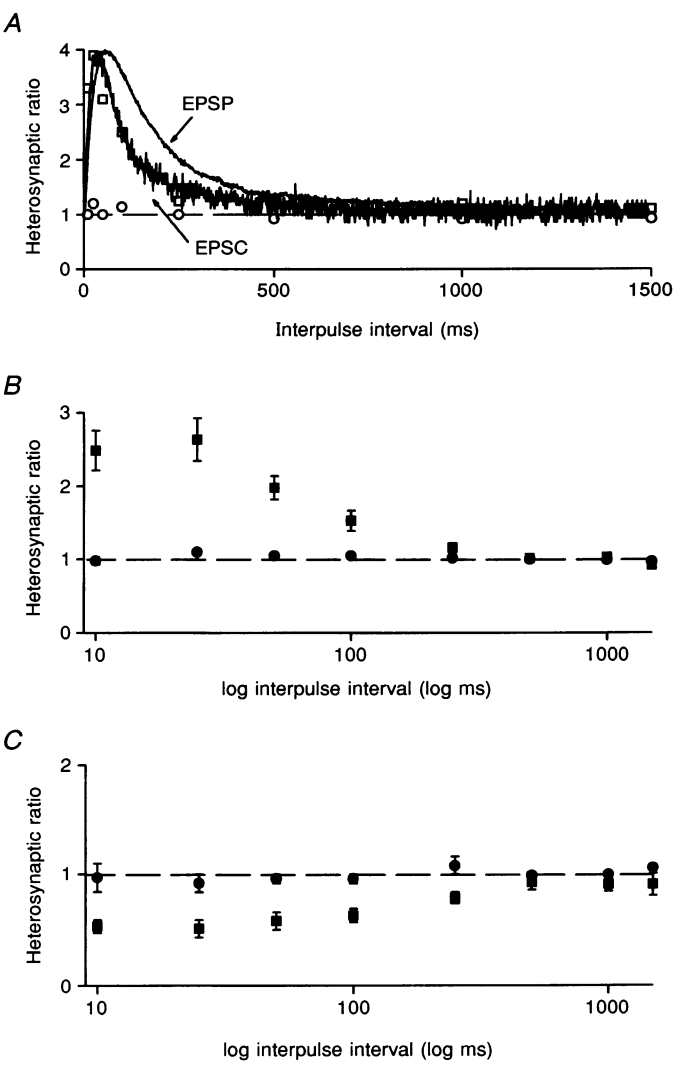

Figure 7. The time course of heterosynaptic paired-pulse facilitation

A, the heterosynaptic facilitation ratio *versus* time, under current-clamp (□) and voltage-clamp (○) conditions for a single neurone. Superimposed upon the graph is an $EPSP_N$ and an $EPSC_N$ (inverted) plotted on the same time scale; in both cases the peak amplitude has been matched to the maximum facilitation ratio value. Note the similarity between the heterosynaptic facilitation profile of the $EPSP_N$ and the time course of the $EPSC_N$. *B*, heterosynaptic ratio of $EPSP_N$ (■) and $EPSC_N$ (●) *versus* log interpulse interval for 11 neurones, obtained at a membrane potential of −60 mV. *C*, plots of the equivalent data for 4 neurones recorded at +40 mV.

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Acknowledgements

We are grateful to Professor J. C. Watkins, Dr M. F. Pozza and Dr T. Honoré for gifts of compounds. We thank Professor R. G. M. Morris for providing laboratory facilities and the MRC for financial support. K.A.C. was a recipient of a Wellcome Trust Prize Studentship.

Received 13 September 1994; accepted 21 July 1995.