

Associative mossy fibre LTP induced by pairing presynaptic stimulation with postsynaptic hyperpolarization of CA3 neurons in rat hippocampal slice

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Abstract

Whole cell recordings of excitatory postsynaptic potentials/currents (EPSPs/EPSCs) evoked by minimal stimulation of commissural–associative (CF) and mossy fibre (MF) inputs were performed in CA3 pyramidal neurons. Paired responses (at 50 ms intervals) were recorded before, during and after hyperpolarization of the postsynaptic membrane (20–30 mV for 15–35 min). Membrane hyperpolarization produced a supralinear increase of EPSPs/EPSCs amplitude in MF-inputs. Synaptic responses remained potentiated for the rest of the recording period (up to 40 min) after resetting the membrane potential to control level ($221 \pm 60\%$, $n = 15$ and $219 \pm 61\%$, $n = 11$ for MF-EPSP and MF-EPSC, respectively). We shall refer to this effect as hyperpolarization-induced LTP (HI-LTP). In the absence of afferent stimulation, membrane hyperpolarization was unable to produce HI-LTP. In contrast to MF-EPSPs, the mean amplitude of CF-EPSPs did not increase significantly after hyperpolarization relative to controls ($138 \pm 29\%$, $n = 22$). HI-LTP was associated with modifications of classical indices of presynaptic release: paired-pulse facilitation, failures rate, coefficient of variation of EPSP amplitudes and quantal content. The induction of HI-LTP was NMDA independent but was dependent on metabotropic glutamate receptors (mGluRs) activation and calcium release from inositol 1,4,5-triphosphate (IP₃)-sensitive intracellular stores: it was prevented by mGluR antagonist, intracellular heparin and BAPTA. We conclude that while the induction of HI-LTP was postsynaptic, its expression was presynaptic.

Introduction

Persistent changes in synaptic efficacy observed during long-term potentiation (LTP) are considered to reflect major mechanisms of information storage in neural circuits. Major forms of LTP are associative in nature triggered when the postsynaptic depolarization is temporally related to presynaptic activity (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999), and postsynaptic spikes are believed to be important for long-lasting synaptic modifications (Markram *et al.*, 1997). In contrast, postsynaptic hyperpolarization was shown to prevent LTP (Kelso *et al.*, 1986; Malinow & Miller, 1986; Jaffe & Johnston, 1990) or even induce long-term depression (Stanton & Sejnowski, 1989; Fregnac *et al.*, 1994; Yang *et al.*, 1994; see however, Crepel & Jaillard, 1991).

Postsynaptic hyperpolarization has been shown recently to modify the number of response failures (N_0) and other indices of transmitter release in a subset of neocortical and hippocampal neurons (Voronin *et al.*, 1999; Berretta *et al.*, 2000; Kasyanov *et al.*, 2000). Hence, the amplitude of both minimal excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) increased more than expected from the shift in the driving force.

Here we report that hyperpolarization of the postsynaptic membrane induced reproducible long-lasting changes in synaptic efficacy in MF–CA3 but not in CF–CA3 synapses. This novel form of hyperpolarization-induced LTP (HI-LTP) was probably triggered by a rise in intracellular calcium concentration $[Ca^{2+}]_i$ in the postsynaptic cell following activation of metabotropic glutamate receptors (mGluRs) and subsequent Ca^{2+} release from intracellular stores. Changes in the paired-pulse facilitation (PPF), N_0 , coefficient of variation of EPSP amplitudes (CV) and mean quantal content indicate that HI-LTP was maintained by increased transmitter release.

Materials and methods

Slice preparation and recordings

Hippocampal slices were prepared from 7–21-day-old Wistar rats according to the method already described (Berretta *et al.*, 2000; Kasyanov *et al.*, 2000). Briefly, animals were decapitated after being anaesthetized with an intraperitoneal injection of urethane (2 g/kg). All experiments in Trieste were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609EEC) and in Moscow according to the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications #80–23). The experiments were approved by local authority veterinary service. Hippocampi were dissected free and sliced with a vibratome.

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During the experiment, slices were superfused continuously at 30–33 °C under submerged conditions, with a medium containing (in mM): NaCl, 130; KCl, 3.5; CaCl₂, 2; MgCl₂, 1.3; NaH₂PO₄, 1.25; NaHCO₃, 26; D-glucose, 11; L-glutamine, 2; bubbled with 95% O₂ and 5% CO₂. Picrotoxin (50–100 µM, Tocris Cookson Ltd, Bristol, UK) was added routinely to block GABA_A receptor-mediated responses. In most (70%) experiments α -3-(2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid (CPP, 20 µM, Tocris Cookson Ltd, Bristol, UK) was added to block N-methyl-D-aspartate (NMDA) receptors. A low concentration of tetrodotoxin (TTX, 2–10 nM, Affinity Research Products, Nottingham, UK) was added routinely to the perfusion solution to diminish polysynaptic responses and to prevent epileptiform activity (Huetter & Baughman, 1988). In control experiments, we have found that this low concentration of TTX did not significantly change the shape of action potentials. Moreover, it did not alter synaptic plasticity as in the absence of the toxin normal PPF and HI-LTP could be obtained from hippocampal slices. However, in the absence of TTX, long-lasting reliable analysis of minimal responses was difficult because of the presence of high spontaneous and sporadic seizure activity. To prevent polysynaptic activity we did not modify extracellular calcium and magnesium ion concentration as, according to our pilot experiments, this changed not only cell excitability but also release probability.

The recording pipette (resistance, 5–7 M Ω) contained (in mM): K-gluconate, 135; KCl, 5; MgCl₂, 3; K₂ATP, 2; HEPES, 10 and was corrected to 290 mosm/L with sucrose and to pH 7.3 with KOH. (*R,S*)- α -methyl-4-carboxyphenylglycine (MCPG, 0.5 mM, from Tocris Cookson, Ltd, Bristol, UK) was added in some experiments to antagonize mGluRs. In order to block inositol (1,4,5)P₃ receptors in a set of experiments ($n=4$), heparin (7 mg/mL, Sigma, Milan, Italy) was added to the intrapipette solution. Additional recordings ($n=5$ cells) were performed with patch pipettes containing the calcium chelators 1,2-bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid (BAPTA, 10 mM, purchased from Sigma, Milan, Italy).

EPSPs/EPSCs were recorded using the whole-cell configuration of the patch-clamp technique, under current- and voltage-clamp conditions, respectively. The membrane input resistance or conductance was monitored with 10 pA current pulses or 5–10 mV voltage steps, respectively. Paired (50 ms) stimuli were adjusted to evoke minimal EPSPs/EPSCs with about 50% (or more) transmission failures in response to the first pulse indicating that only a few presynaptic fibres (or even one) was activated. A high success rate to the second pulse suggested that at least one presynaptic axon was reliably activated. Pairs of stimuli were applied every 8–20 s (typically 15 s). The interpulse interval was varied across different cells to avoid low-frequency depression (Saviane *et al.*, 2002) but was constant for any given cell throughout the experiment. Bipolar tungsten wires or glass stimulating electrodes were positioned in the stratum lucidum or radiatum to activate MF or CF, respectively (Fig. 1A). When two components were present in EPSPs evoked via stimulation electrodes at presumed MF location, the component with a slower rise time (> 4.4 ms) was assumed to be mediated by CF whereas that with a faster rise time by MF (Jonas *et al.*, 1993; Henze *et al.*, 2000). Moreover, MF-inputs were identified on the basis of their capability to express LTP following tetanization with minimal stimulus strength (Johnston *et al.*, 1992; Nicoll & Malenka, 1995) and on their sensitivity to group II mGluR agonists [(2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine, DCG-IV (1 µM) or (2*S*,3*S*,4*S*)-CCG/(2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl)glycine, 10 µM; Kamiya *et al.* 1996. Altogether, recordings from 49 cells were analysed. Every cell was recorded from a separate slice preparation. Typically, responses to two inputs were recorded for each cell (using two out of three electrodes as shown in

Fig. 1A). The effects were input specific but not always expressed in all components of a composite response being present in one and absent in another (see below).

Response measurements

Responses were digitized at 5 or 10 kHz. To improve the signal-to-noise ratio, responses were measured using the standard statistical technique of principal component analysis (PCA, Jackson, 1991) modified for application to minimal postsynaptic responses (Astrelin *et al.*, 1998). Standard PCA scores were determined from a window covering the initial slope. The first component score can serve as a measure of the response magnitude (Jackson, 1991). It represents a correlation (covariance) between every single response and average waveform. This measure has been shown to correlate strongly with conventional peak amplitude measured between two windows, one set around the peak and the second ('baseline window') before the response onset (Voronin *et al.*, 1999; Berretta *et al.*, 2000; Kasyanov *et al.*, 2000). Therefore, the mean results did not depend on the measure used. However, the measure based on the first PCA component gave a better signal-to-noise ratio using most of information containing in the response waveform (Jackson, 1991) that was especially important for quantal analysis. The 'covariance amplitudes' were expressed in mV (or pA) and termed 'amplitudes' for simplicity. To obtain mV (or pA), a correction factor was introduced by dividing the peak amplitude of the average response by the mean value of the first PCA component, a procedure analogous to that commonly used for obtaining precise amplitude values from measurements over a window around a peak (Walmsley, 1993). PCA was also used to separate components (inputs) with different waveforms and latencies (see Fig. 3B–D). To separate two waveforms we used a procedure described elsewhere in detail (Astrelin *et al.*, 1998). The procedure has been tested previously with voltage- and current-clamp recordings obtained from both CA1 (Astrelin *et al.*, 1998; Voronin *et al.*, 1999) and CA3 hippocampal neurons (Berretta *et al.*, 2000; Kasyanov *et al.*, 2000). Briefly, we averaged responses, corresponding to positive values of one component and zero values of another component. For example, to identify the first component (Fig. 3C, c1) we averaged responses corresponding to the dots around zero *y*-values (see lower box in Fig. 3C). PCA has the advantage to separate responses containing pure components and in the case of mixed responses to dissect out the contribution of each component (see Astrelin *et al.*, 1998; Berretta *et al.*, 2000). This procedure is also useful to separate polysynaptic responses (identified according to their longer and more variable latencies) from monosynaptic ones. However, polysynaptic responses were very infrequent under our experimental conditions (minimal stimulation in the presence of TTX and NMDA receptor blockers).

Only neurons with stable resting membrane potential (within ± 4 mV) and input resistance (within $\pm 10\%$) over at least 60 trials before and after hyperpolarization and without statistically significant amplitude drift over at least 60 trials immediately before hyperpolarization (control) were analysed.

Quantal analysis

As the simplest approach (Voronin, 1993) we calculated N_0 and CV (see Faber & Korn, 1991 for reservations). N_0 was estimated using visual separation of successes and failures (Fig. 1C). We used averaging of failures and consideration of superimposed successes (Fig. 1C) to control for adequate separation. Under our conditions, the visual estimates were very similar to N_0 estimates as a double number of negative amplitudes (Voronin, 1993; Voronin *et al.*, 1999). The inverse squared of CV (CV^{-2}) was corrected for the noise variance. To determine the mean quantal content (m) we used a variant (Astrelin

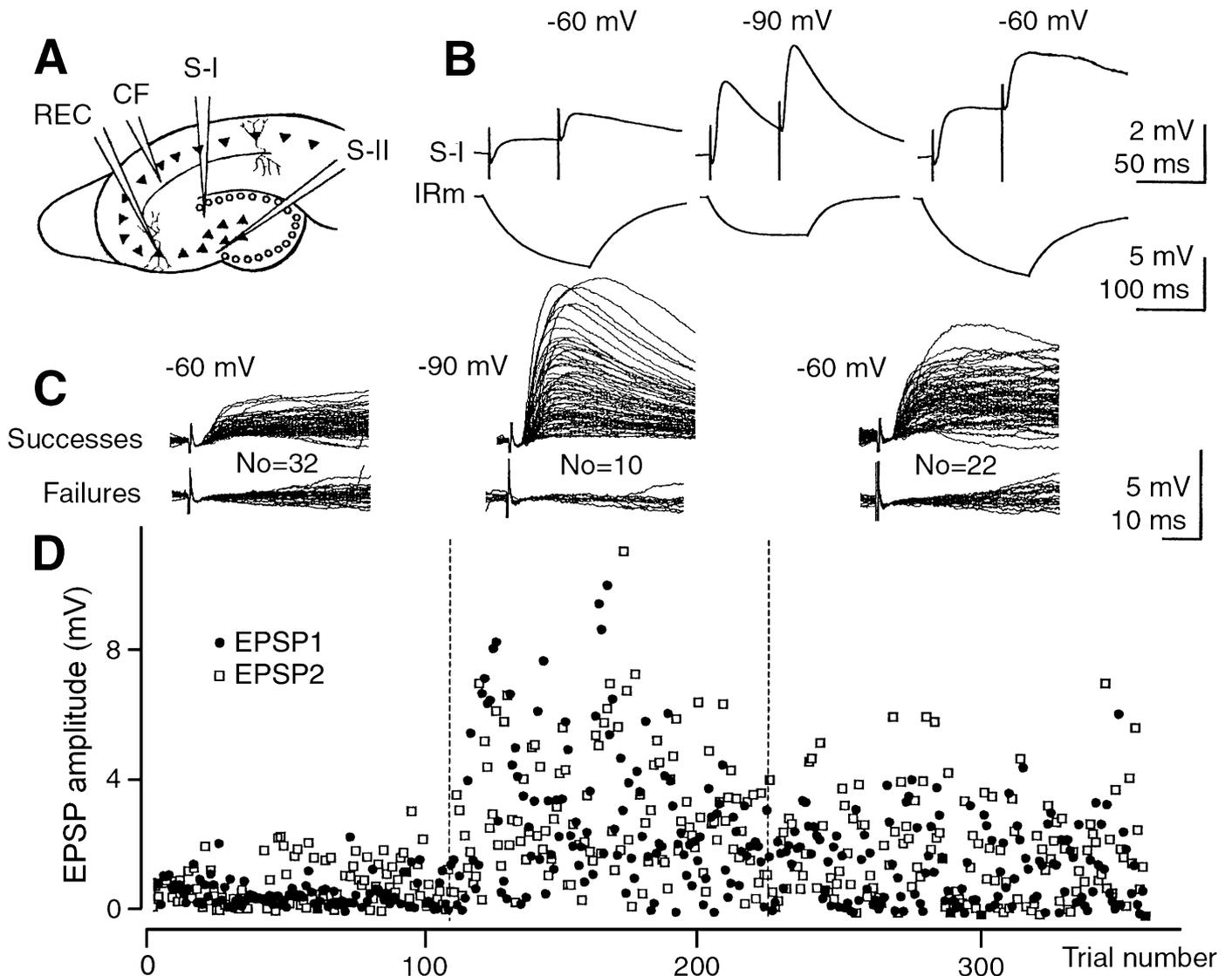


FIG. 1. Hyperpolarization-induced LTP at the mossy fibre-CA3 synapse. (A) Electrode arrangement to stimulate commissural (CF) and mossy fibres (S-I and S-II respectively). (B) Averaged EPSPs evoked via S-I stimulating electrode from resting (-60 mV) and from hyperpolarized (-90 mV) membrane potential using the paired-pulse paradigm (50 ms interval within each pair in each trial, $n = 112$ – 135 trials). Lower traces (IRm) represent the amplitude of electrotonic potentials induced by -10 pA current steps at -60 mV and -90 mV, respectively. (C) Single EPSPs from the same experiment evoked by the first pulse in the paired-pulse protocol. Successes and failures are shown separately to demonstrate a reduction in the failure number (N_0) during and after hyperpolarization. (D) Dots and squares illustrate responses to the first and second pulse in the paired-pulse paradigm (EPSP1 and EPSP2, respectively). Note a significant potentiation of both EPSPs during and after postsynaptic hyperpolarization. Paired testing pulses were at 15 s intervals between the pairs (continuous recording over 90 min).

et al., 1997) of the ‘unconstrained’ noise deconvolution technique (Redman, 1990). The algorithm searched for discrete distributions (see Fig. 8C–E, bars) with co-ordinates x_i (distance from 0) and P_i (heights). A weighted mean interval between the bars was used to define the quantal size v and the mean quantal content m ; $0.2v$ was taken as intrinsic quantal variance (Astrelin *et al.*, 1997). Because stimuli had to be applied at a low rate to avoid amplitude depression, n was typically small (150–300; mean 192 ± 3 , $n = 111$ amplitude distributions) although measurements from both responses in the paired-pulse paradigm were combined (Kullmann & Nicoll, 1992). For each case, amplitude distributions obtained from the first and second response in the paired-pulse paradigm were also analysed and compared at different periods. The basic results were the same as for the pooled data, except that control responses to the first pulse sometimes contained only a few successes. Comparison of the distributions built for the first and second pulses within each trial confirmed

constancy of v in the two responses consistent with known presynaptic nature of PPF and thus justified pooling data from two responses (Kullmann & Nicoll, 1992). Therefore, only data obtained from the pooled histograms are presented in Results. Computer simulations (Astrelin *et al.*, 1997) show that 150–200 samples can give reliable estimates of v provided that the ratio of the quantal size to the standard deviation of the baseline noise (v/σ) was > 1.3 – 1.5 . The estimated mean v/σ was 3.5 ± 0.1 ($n = 111$ distributions). Data are reported as mean \pm SEM. $P < 0.05$ was taken as ‘statistically significant’.

Results

Acute and sustained effects of postsynaptic hyperpolarization in MF-inputs

For the basic experimental series, paired-pulse minimal stimulation of mossy fibres (S-I and S-II, Fig. 1A) or commissural-associative fibres

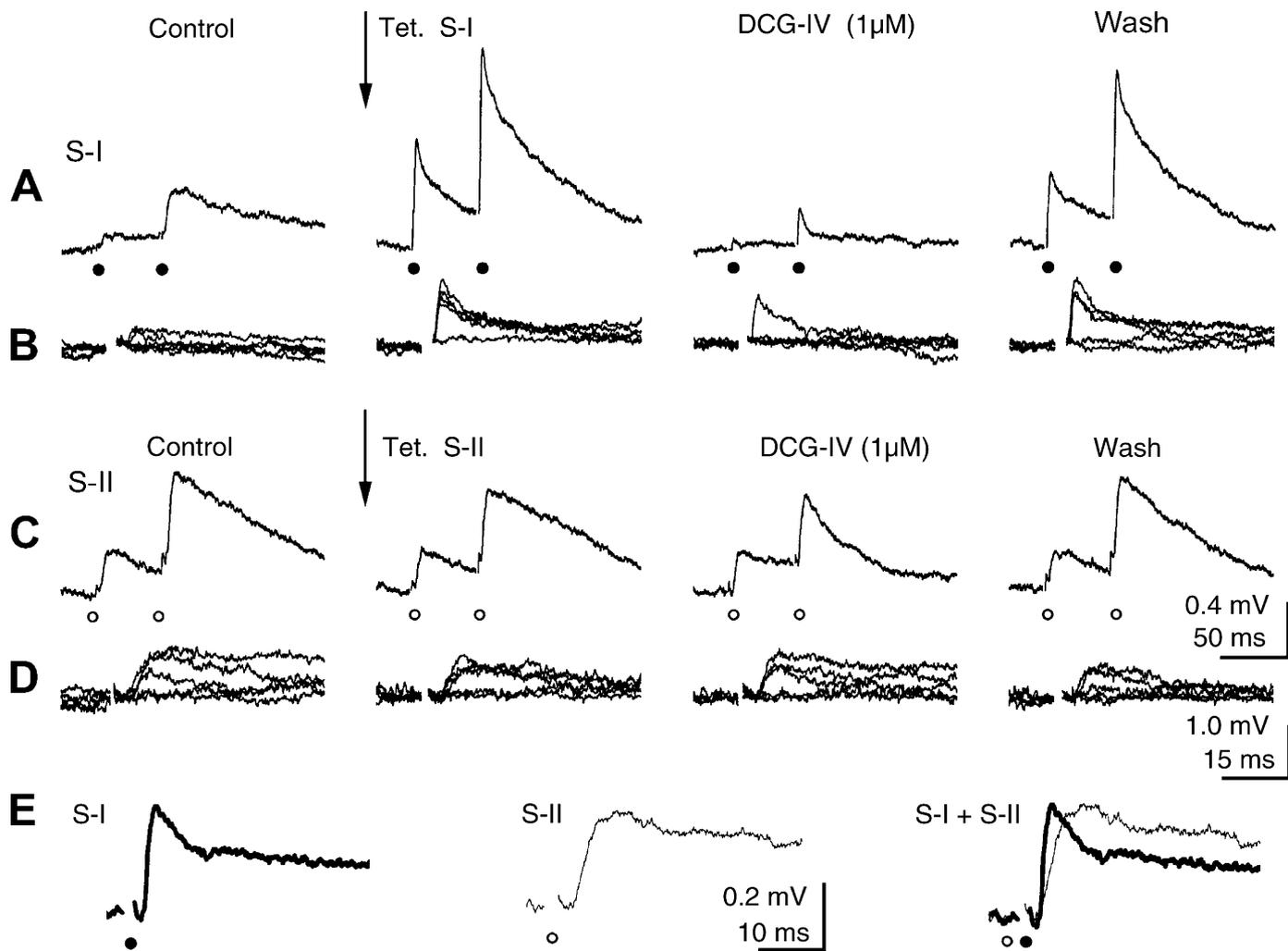


FIG. 2. Identification of EPSPs mediated by mossy fibres. Afferent tetanization (Tet.) and pharmacological analysis (DCG-IV) were used to identify EPSPs evoked in the same neuron via two stimulating electrodes positioned at two sites (S-I and S-II, see Fig. 1A). (A–D), From left to right: responses obtained in control conditions (Control); after tetanization of respective pathways (Tet. S-I and S-II); during superfusion of the mGluR agonist DCG-IV ($1 \mu\text{M}$) and after DCG-IV washout (Wash). (A and C) Average EPSPs evoked by double pulses in the paired-pulse paradigm ($n = 20$ trials with 15 s intervals between trials, 50 ms interval between the paired stimuli in each trial). (B and D) Individual responses ($n = 5$) evoked by the first pulse in the paired-pulse protocol. (E) Average of responses evoked by S-I and S-II electrodes and their superposition after scaling (S-I + S-II) to show different rise-times. Notice LTP of S-I input (A and B) after tetanization (1 s train at 100 Hz with just suprathreshold stimulus strength equal to that used to evoke minimal EPSPs) and the lack of potentiation of S-II input (C and D) when a similar train was applied via S-II electrodes. Notice also the depressant action of DCG-IV on EPSPs evoked by S-I (A and B) but not S-II electrodes (C and D). These tests identify responses evoked via S-I and S-II electrodes as mossy and commissural fibre EPSPs, respectively. In line with this, the rise time of the EPSP evoked via S-I electrodes (thick line) was faster in comparison to that evoked via S-II electrodes (thin line).

(Fig. 1A, CF) was used to evoke EPSPs in CA3 pyramidal neurons under current-clamp conditions. Cells having resting membrane potentials in the range of -55 to -68 mV were considered acceptable for analysis.

Figure 1B illustrates a strong supralinear increase in EPSP amplitude during a postsynaptic hyperpolarization of 30 mV from resting membrane potential (Berretta *et al.*, 2000). As shown, the increase in EPSP amplitude during hyperpolarization was observed in spite of the decrease in membrane input resistance (Fig. 1B, lower row). The increase in EPSP amplitude during the postsynaptic hyperpolarization was followed by a long-lasting after-effect, which persisted for the rest of the recording period (Fig. 1B–D). We shall term this after-effect HI-LTP. Both acute and long-term effects were associated with a reduction in failures rate (Fig. 1C and D). The input resistance regained

the control value after resetting the membrane potential to the initial level.

Separation of commissural-associative (CF) from mossy fibre (MF) inputs

Isolated activation of MF is problematic because of extensive collaterals of CA3 neurons (Johnston *et al.*, 1992; Henze *et al.*, 2000). As already mentioned (see Methods) we used two different approaches to separate MF from CF-inputs (Berretta *et al.*, 2000). These were based on the observations that: (i) a weak tetanus produces a persistent 'non-cooperative' potentiation of MF- but not CF-responses (Johnston *et al.*, 1992; Henze *et al.*, 2000) and (ii) activation of type II mGluRs reduces the amplitude of MF- but not CF-EPSPs, being this type of mGluR localized exclusively on MF (Kamiya *et al.*, 1996). As shown in Fig. 2,

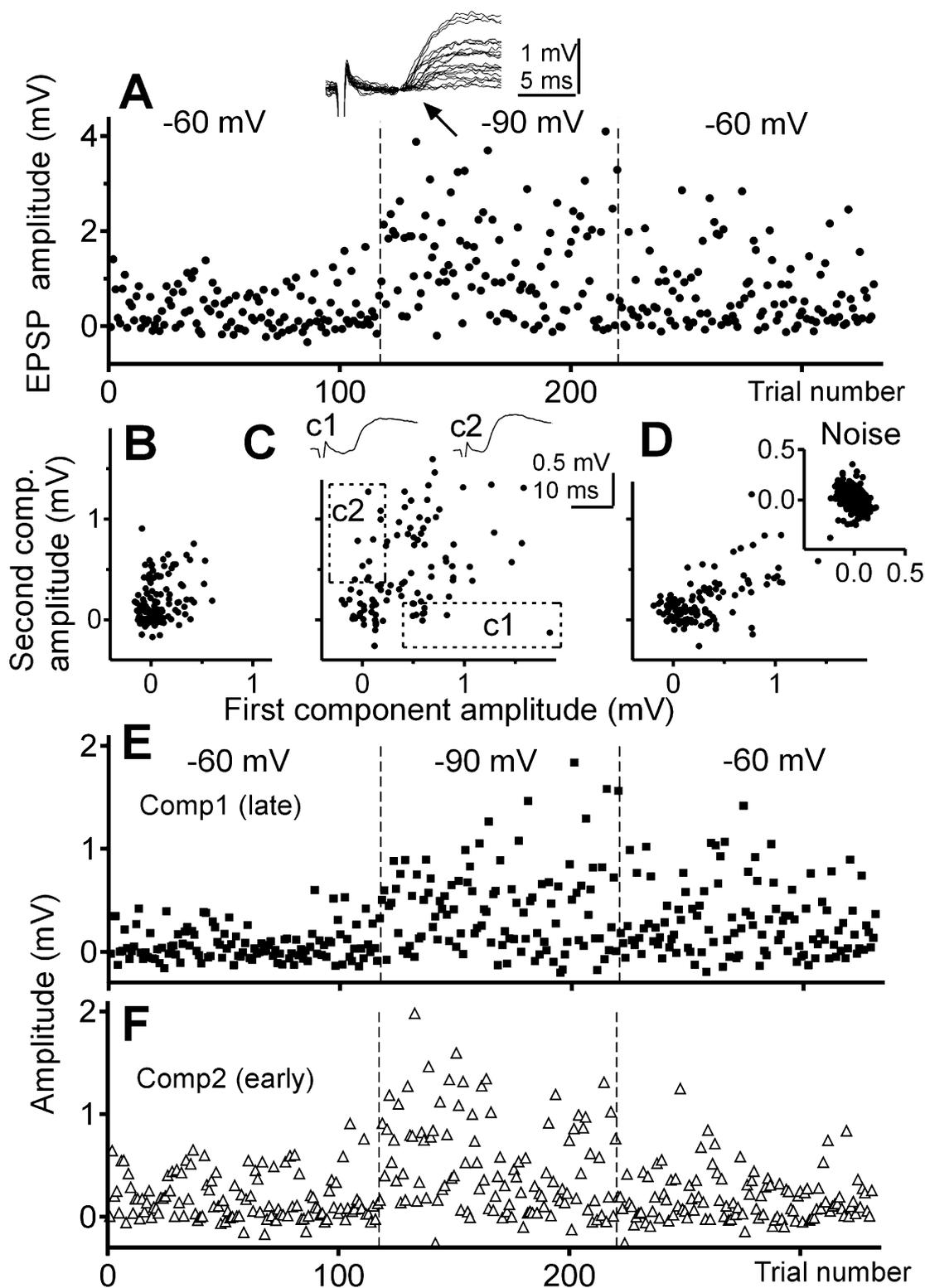


FIG. 3. Separation of two distinct EPSP components with different latencies and wave forms. (A) Time course of the amplitude of EPSPs evoked via S-I electrodes (see Fig. 1A). Inset shows superposition of 20 single traces recorded during hyperpolarization. EPSPs with a longer latency are marked by the arrow. (B–D) Component analysis of EPSPs recorded in the same experiment before, during and after postsynaptic hyperpolarization. Amplitudes ('scores') of two distinct components were obtained on the basis of the principal component analysis as described elsewhere (Astrelin *et al.*, 1998). Insets in C show average waveforms corresponding to respective dashed boxes below the insets (see Methods) to identify components with shorter (c2) and longer (c1) latency. (E and F) Amplitudes of the first (E) and second component (F) plotted against trial number. Note a persistent potentiation in E but only an acute effect of hyperpolarization in F. Testing intervals, 15 s; continuous recording over 80 min

EPSPs evoked via one of the stimulating electrode (S-I) were strongly potentiated following a weak tetanus (delivered at the same stimulus strength as used to evoke minimal EPSPs) and were suppressed by DCG-IV (1 μ M). In contrast, EPSPs evoked via the other electrode (S-II) were unaffected by analogous 'minimal' train via S-II electrodes. Therefore, both properties allowed identified the former EPSP as being evoked by MF activation. In keeping with this, the rise time of MF-EPSP was shorter than that of CF-EPSP (Fig. 2E). On the basis of these tests, out of 24 inputs activated via S-I or S-II electrodes, nine were identified as belonging to the MF and 15 to CF. The rise time of MF-EPSPs varied from 1.2–4.4 ms while that of CF from 4.6–12 ms. Therefore, EPSPs evoked via S-I or S-II electrodes having rise-times >4.4 ms were *a posteriori* considered as CF-EPSPs (see Johnston *et al.*, 1992).

It is known that minimal stimulation does not guarantee activation of a single fibre (Torii *et al.*, 1997; Astrelin *et al.*, 1998). Accordingly, we often observed that a stimulating electrode placed on the MF tract could evoke EPSPs whose latency and kinetics varied between two or more values (see inset of Fig. 3A). To make our analysis more precise we used a novel approach (Astrelin *et al.*, 1998) based on the standard statistical technique of the principal component analysis (Jackson, 1991; see Methods). This statistical approach provided a way of separating two or more components with different latencies or rise-times. Moreover, the component analysis gave quantitative measure for the contribution of each component into every single response and allowed the evaluation of changes in amplitudes separately, even in the presence of mixed responses that contained more than one component. This approach was also important for the quantal analysis because it gave better signal-to-noise ratios when compared to conventional amplitude measurements. Using this approach, we separated more than half of EPSPs evoked via S-I or S-II electrodes (Fig. 1A) into two or three distinct components having different kinetics and latencies (Fig. 3B–D). Averaging the responses corresponding to positive scores of each component with 0 scores of the other component (Fig. 3C, dashed boxes) produced two different waveforms reflecting the existence of two components (insets c1 and c2 in Fig. 3C). In the example in Fig. 3, the two components behaved differently: the first one showed HI-LTP whereas the second one did not exhibit any sizeable after-effect in spite of the significant increase in amplitude during hyperpolarization (Fig. 3E and F). This observation indicated that the after-effect is not due to general alterations in the physiological properties of the postsynaptic neuron. It also supports our previous conclusion, based on recordings from CA1 neurons, that separate components of hippocampal responses are mediated by different synapses (Astrelin *et al.*, 1998). Altogether, 20 EPSPs (or EPSP components) were classified as mediated by MF-input (MF-EPSPs) and 28 by CF-input (CF-EPSPs). Their mean rise-times were 3.2 ± 0.2 and 5.6 ± 0.5 ms, respectively ($P < 0.01$, *t*-test), again indicating that they were mediated by different synapses with distinct dendritic locations compatible with known location of MF- or CF-inputs (Johnston *et al.*, 1992). 15 MF- and 22 CF-inputs, which fulfilled the stability criteria were analysed in more details. Among MF-EPSPs, 10/15 exhibited HI-LTP of $>110\%$ relative to control.

The overall mean post-hyperpolarization amplitude was $221 \pm 60\%$ ($n = 15$, $P < 0.05$, Wilcoxon test for matched pairs here and below). This showed no apparent decrement with time (Fig. 4A and B, closed circles and black columns, respectively). The HI-LTP magnitude was correlated with EPSP amplitude increase during the hyperpolarization ($r = 0.77$, $P < 0.01$). In 12/15 inputs showing an acute effect of $>135\%$, HI-LTP was particularly strong ($400 \pm 74\%$, $P < 0.01$). None of the 3/15 EPSPs without amplitude increase during hyperpolarization expressed HI-LTP, suggesting that hyperpolarization-induced

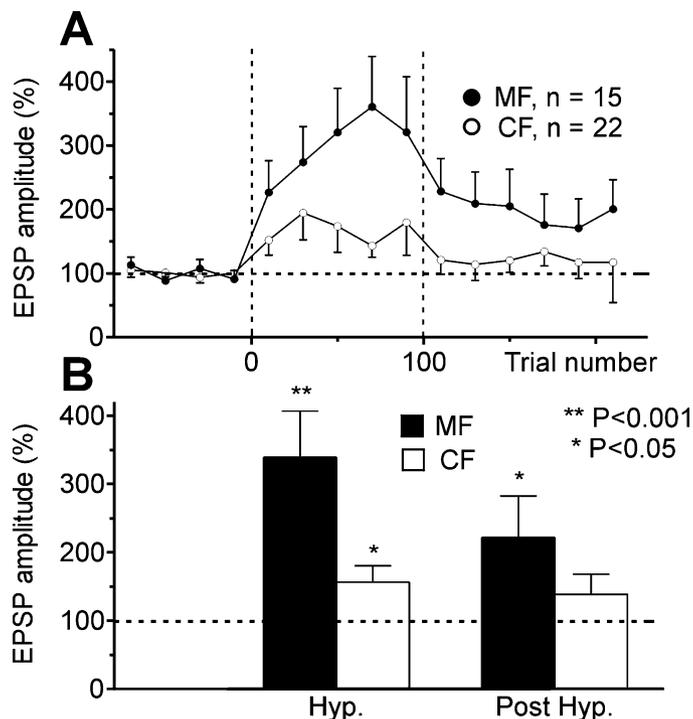


FIG. 4. Summary of the hyperpolarization effects. (A) The mean amplitudes of the first EPSP in the testing pairs (EPSP1) for all recorded inputs plotted against trial number. The data are given as percentages of the values obtained before hyperpolarization. Bars represent SEM. Data from 20 consecutive trials for each EPSP were averaged and afterwards averaged across all inputs. Intervals between trials were from 8–20 s. Typical interval was 15 s so that each dot represents the average over ≈ 5 -min recording period and the whole period shown is about 80 min. (B) Summary data representing normalized EPSP amplitudes over the whole hyperpolarization (Hyp.) and post hyperpolarization (Post Hyp.) recording periods. Some neurons were tested after hyperpolarization for more than 120 trials (shown in A). *Statistically significant differences (Wilcoxon test for matched pairs here and in other figures). Filled and open symbols (in A and B) represent data for MF-EPSPs and CF-EPSPs, respectively. The number of inputs (n) is given in A for most recording periods; for the two last points n was 12 and 8 for MF-EPSPs and 16 and 4 for CF-EPSPs.

supralinear increases in EPSP amplitude are a necessary condition for the induction of HI-LTP.

In contrast to MF-EPSPs, the mean amplitude of CF-EPSPs recorded after postsynaptic hyperpolarization was small ($138 \pm 29\%$, relative to control, $n = 22$) and the amplitude increase was not statistically significant ($P = 0.68$, Fig. 4, open symbols). Nevertheless, the after-effects significantly correlated with modification in amplitude occurring during hyperpolarization ($r = 0.62$, $n = 22$, $P < 0.01$). Among 22 CF-inputs, 10 showed an acute effect of hyperpolarization of $>135\%$ and this sample expressed a statistically significant HI-LTP ($209 \pm 56\%$, $P < 0.01$, $n = 10$).

We noticed that both the acute (supralinear) effect of postsynaptic hyperpolarization (Berretta *et al.*, 2000; Kasyanov *et al.*, 2000) and HI-LTP were also clearly seen without separation of the responses into components. Figures 1B–D and 3A present amplitude measurements from 'raw' recordings without component separation. Altogether, three pure MF-EPSPs were found. The mean post-hyperpolarization amplitude for this subset of responses was $320 \pm 162\%$. The effect was similar to the mean for the whole population of MF-inputs given above.

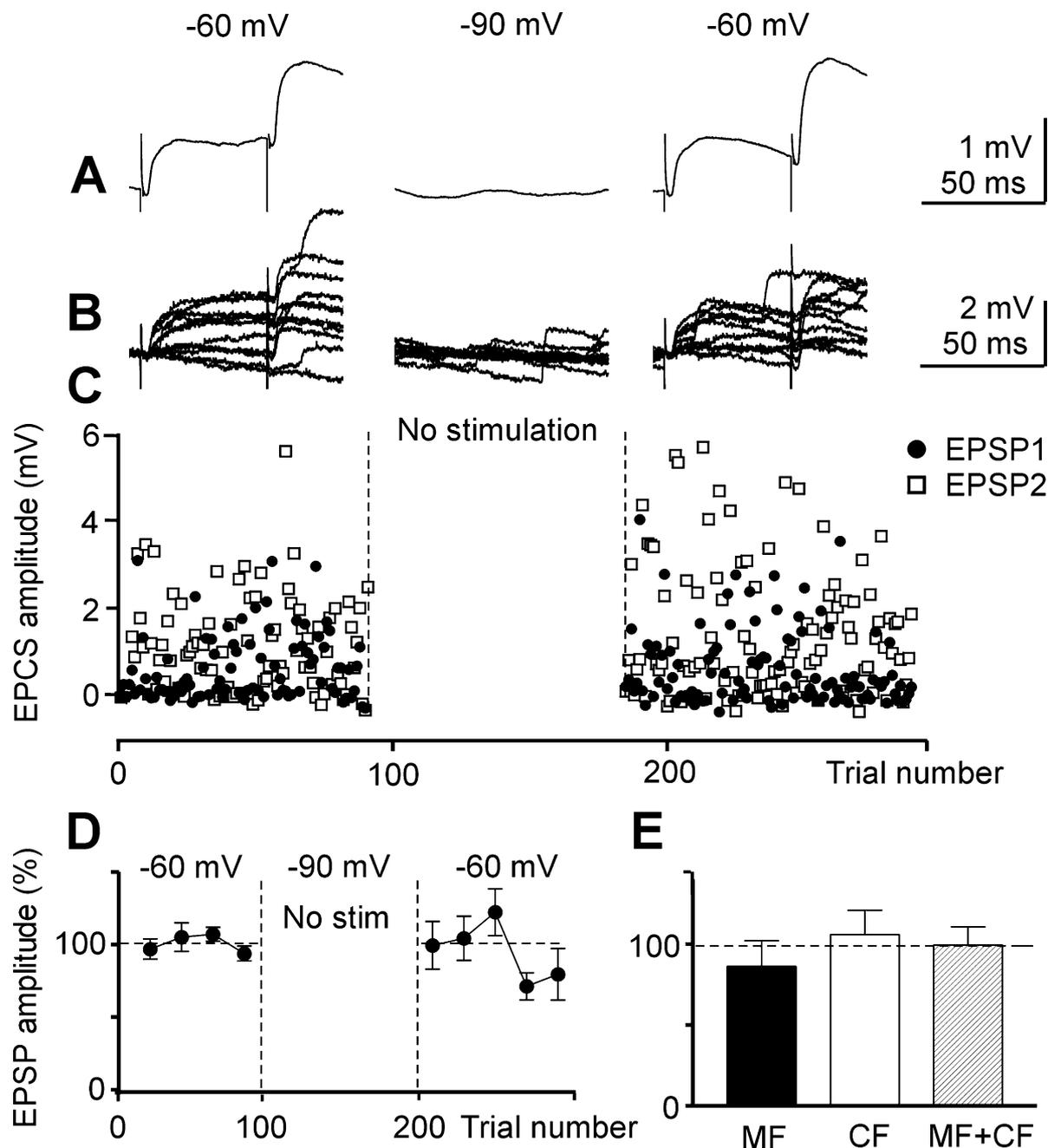


FIG. 5. In the absence of afferent stimulation, the postsynaptic hyperpolarization failed to induce HI-LTP. (A) Averaged EPSCs recorded before and after postsynaptic hyperpolarization. (B) Examples of 10 consecutive sweeps from the same periods of the same experiment. Note the presence spontaneous activity at -90 mV (middle traces). (C) EPSC amplitudes in the same experiment plotted against trial number (altogether 18 EPSPs). In the plots of C and D at -90 mV afferent inputs were not stimulated. In these plots, the inter-trial interval was 8 s (continuous recording of about 40 min). (E) Summary data showing normalized EPSP amplitudes over the whole post-hyperpolarization period for MF, CF and MF + CF-inputs (filled, open and hatched columns, respectively). In D and E, the amplitudes are given as percentages of the values obtained before hyperpolarization. There were no statistically significant changes after hyperpolarization. In E, the mean values are $86 \pm 16\%$ ($n = 6$), $105 \pm 15\%$ ($n = 12$) and $99 \pm 11\%$ ($n = 18$) for MF-EPSPs, CF-EPSPs and pooled data, respectively.

HI-LTP induction requires pairing of postsynaptic hyperpolarization with presynaptic activation

The correlation analysis described above indicates strongly that an important condition for the induction of HI-LTP is a robust (supralinear) increase in EPSP amplitude during postsynaptic hyperpolarization.

The presence of such correlation suggests that the presynaptic activation itself is necessary for HI-LTP induction. In order to see whether this was really the case, we performed a series of experiments in which we omitted the afferent stimulation during postsynaptic hyperpolarization. As shown in Fig. 5A–C, the mean amplitude of the first EPSP was similar before and after membrane hyperpolariza-

tion (0.75 ± 0.08 mV and 0.76 ± 0.08 mV, respectively). Figure 5D represents the mean course of MF- and CF-EPSPs amplitudes. In this figure, data from MF- and CF-EPSPs have been pooled together because their relative amplitudes did not differ significantly ($n = 18$). Figure 5E shows the mean amplitude of MF- and CF-EPSPs (calculated separately or together) obtained after resetting the membrane to -60 mV. Comparison of Figs 5 and 4 indicates that pairing afferent activation with postsynaptic hyperpolarization is a necessary condition for the induction of HI-LTP and that this form of LTP (Fig. 4) can not be due to instability of minimal EPSP recordings.

Mechanisms of HI-LTP induction

Induction of conventional LTP is known to depend upon postsynaptic depolarization evoked by afferent tetanus and subsequent increase in $[Ca^{2+}]_i$ in the postsynaptic cell via activation of NMDA receptors and/or voltage-dependent calcium channels (VDCC, Bliss & Collingridge, 1993). To test whether a rise of intracellular calcium was indeed responsible for HI-LTP, additional recordings were performed from five cells loaded with the calcium chelator, BAPTA (10 mM). Responses to activation of seven inputs were analysed including four MF- and three CF-EPSPs. A 30-mV membrane hyperpolarization from

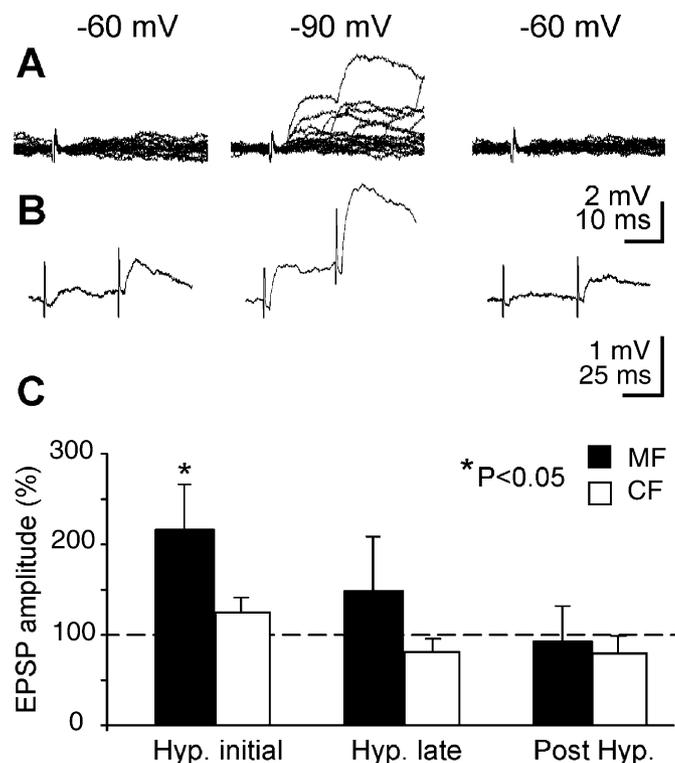


FIG. 6. Dialysis of postsynaptic neurons with the Ca^{2+} chelator BAPTA prevents the induction of HI-LTP. (A) Examples of 10 consecutive MF-EPSPs recorded before postsynaptic hyperpolarization at resting membrane potential (-60 mV), immediately after switching to -90 mV and after returning to the resting membrane potential -60 mV (left, middle and right recordings, respectively). (B) Averaged EPSPs over the same periods ($n = 50$, 20 and 100, respectively). (C) Summary plot of mean amplitudes of MF- and CF-EPSPs (filled and open columns, respectively). The mean amplitudes during (Hyp.) and after postsynaptic hyperpolarization (Post Hyp.) are given as percentages of the mean values before hyperpolarization ($n = 4$ inputs for MF- and $n = 3$ for CF-EPSPs recorded from five different cells). The period during hyperpolarization was divided into two (initial and late). *Statistically significant differences. Note large increases of MF-EPSP amplitudes but only at the initial period during hyperpolarization and the absence of their potentiation after switching off the hyperpolarizing current.

the resting potential (ranging from -60 mV to -68 mV) induced a significant initial increase in the amplitude of MF-EPSPs as exemplified in Fig. 6A and B ($n = 4$ MF-EPSPs recorded from four different cells) while CF-EPSPs showed no significant changes (Fig. 6C, white columns). The mean initial increase of MF-EPSP amplitudes during hyperpolarization was equal to $216 \pm 50\%$ relative to control ($P < 0.05$; Fig. 6C). However, amplitudes of 3/4 recorded MF-EPSPs declined with repeated trials in spite of continuous hyperpolarization. The time course and magnitude of the decline differed across the cells and reached approximately steady level during the last 40 trials. The mean data calculated over the late regions of postsynaptic hyperpolarization was not significantly different from control ($149 \pm 60\%$, $n = 4$ MF-EPSPs, $P > 0.2$; Fig. 6C, Hyp. late). No HI-LTP was observed after repolarizing the membrane to the resting level (Fig. 7C, Post Hyp). These experiments indicate clearly that an increase in intracellular calcium is essential for this form of synaptic plasticity. A rise of intracellular calcium may originate from extracellular sources through activation of NMDA receptors and/or VDCCs. Both NMDA receptors and VDCC can be activated due to supralinear increase in EPSP amplitude during postsynaptic hyperpolarization (>3 fold, on average, Fig. 4B). However, we can exclude the contribution

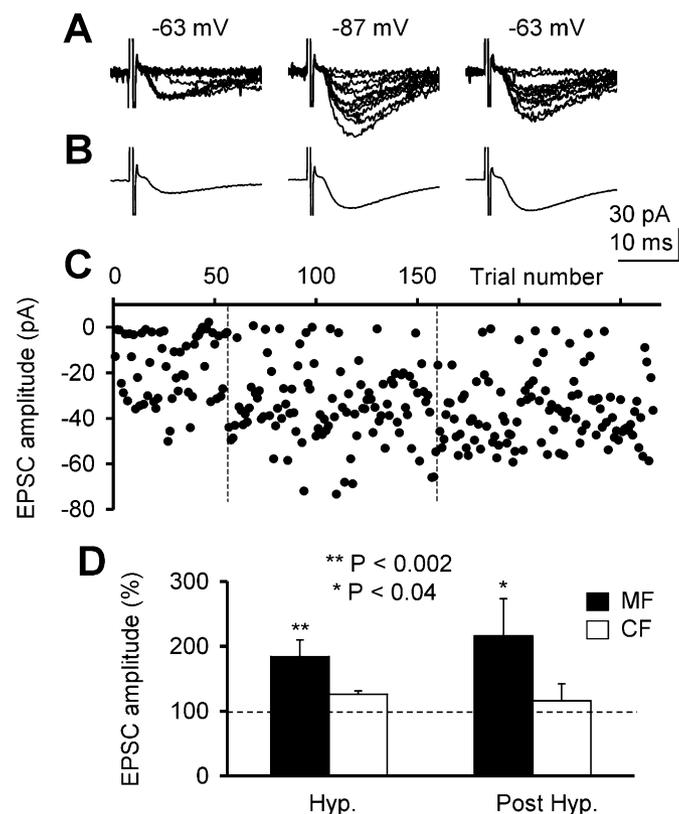


FIG. 7. Postsynaptic hyperpolarization induces a persistent potentiation of the MF-inputs under voltage-clamp conditions. (A) Examples of 12 consecutive single MF-EPSCs before, during, and after postsynaptic hyperpolarization. (B) Averaged EPSCs over the same periods ($n = 55$, 102 and 108, respectively). (C) EPSC amplitudes in the same experiment plotted against trial number (8 s intertrial interval, continuous recording of 36 min). (D) Summary plot of changes in amplitudes of MF- and CF-EPSCs (filled and open columns, respectively). The mean amplitudes during (Hyp.) and after hyperpolarization (Post Hyp.) are given as percentages of the mean values before hyperpolarization. *Statistically significant differences ($n = 11$ inputs for MF- and $n = 3$ for CF-EPSCs).

of NMDA receptors as the majority of recordings were performed in the presence of NMDA receptor antagonists (see Methods). We can also exclude activation of VDCC as HI-LTP was still observed when EPSCs were recorded in voltage-clamp conditions that should have suppressed synaptic activation of VDCC (Markram & Sakmann, 1994). Thus, 8/11 MF-EPSCs expressed a HI-LTP of $>110\%$ (Fig. 7A–C). On average, for all recorded MF-EPSCs the magnitude of HI-LTP was similar to that found under current-clamp conditions: $219 \pm 61\%$ (Fig. 7D, filled bars). The magnitude of the after-effect was correlated strongly with the amplitude increase observed during membrane hyperpolarization ($r=0.88$, $P=0.001$, $n=11$). In six inputs with acute hyperpolarization effects of $>135\%$, an increase in EPSC amplitude of $325 \pm 93\%$ was observed similar to the results obtained in current-clamp conditions.

An alternative source for $[Ca^{2+}]_i$ increases are intracellular stores. Calcium release from intracellular stores can be triggered by activation of IP_3 receptors via group I mGluRs that have been shown to play an important role in several forms of synaptic plasticity (Bortolotto *et al.*, 1999; Henze *et al.*, 2000). To see whether mGluRs are involved in HI-LTP, we used MCPG (0.5 mM), a competitive antagonist of mGluRs. Addition of MCPG to the extracellular solution did not significantly change the mean amplitude of either MF- or CF-EPSPs. Their mean amplitudes were $106 \pm 16\%$ ($n=13$) and $110 \pm 20\%$ ($n=8$) relative to control, respectively. In the presence of MCPG, postsynaptic hyperpolarization produced a significant ‘supralinear’ increase in MF-EPSP amplitude ($184 \pm 29\%$, $n=10$, $P<0.02$, Fig. 8A and B, Hyp., black column), but almost no change in the mean amplitude of CF-EPSPs (Fig. 8B, Hyp., white column). However, after switching the hyperpolarization off, a response depression rather than HI-LTP was found in MF-inputs (Fig. 8A and B, Post Hyp., black column). In order to block the transduction cascade activated by mGluR, we used recording pipettes filled with heparin, which inhibits the binding of IP_3 to its receptor (Taylor & Broad, 1998). In these conditions, the mean hyperpolarizing effect in MF-inputs was supralinear, virtually the same as in the experiments with MCPG ($182 \pm 12\%$, $n=4$) but the after-effect was blocked ($97 \pm 7\%$, $n=4$, Fig. 8C and D). These results should be taken with caution as heparin, like other compounds commonly used to antagonize IP_3 receptors (Xestospongine C or 2-APB), have a doubtful specificity of action (Taylor & Broad, 1998; Bootman *et al.*, 2002; Solovyova *et al.*, 2002).

Mechanisms of HI-LTP expression

To explore the expression mechanisms of HI-LTP, we tested the contribution of changes in passive membrane properties. The resting membrane potential level could hardly contribute because the mean HI-LTP magnitude was similar in current- and voltage-clamp conditions. Furthermore, in current-clamp mode, small changes in membrane potential level (<1 – 2 mV) occasionally occurring after switching off the hyperpolarization could not account for EPSP amplitude increase. The postsynaptic hyperpolarization was often associated with a significant reduction in membrane input resistance (Maccaferri *et al.*, 1993; see Fig. 1B and Berretta *et al.*, 2000), the mean decrease being $32 \pm 11\%$ below control ($P<0.002$). However, after resetting the membrane to its initial value (-60 mV), these changes were negligible ($-3 \pm 20\%$ for 13 MF-EPSPs) and did not correlate with HI-LTP magnitude ($r=-0.09$). Altogether, these data indicate that changes in passive membrane properties do not contribute to HI-LTP maintenance.

To test whether HI-LTP maintenance was associated with presynaptic changes, we determined several traditional measures of presynaptic transmitter release.

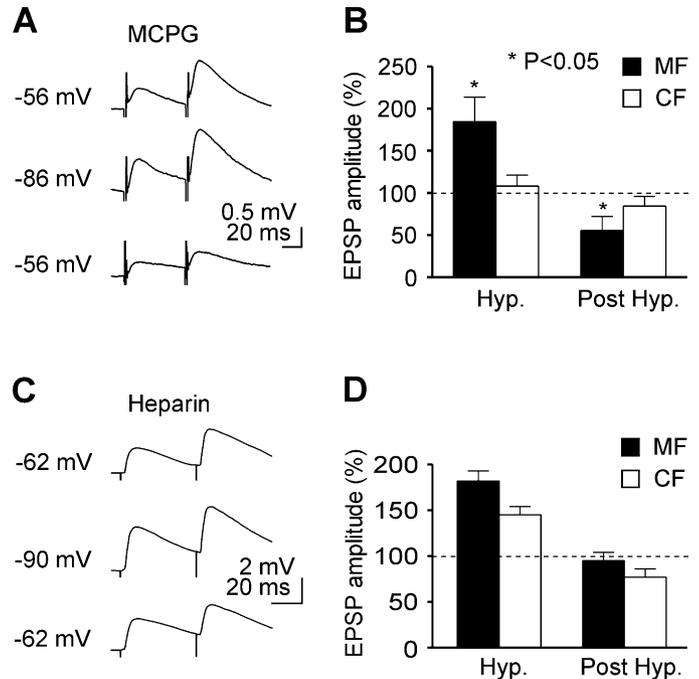


FIG. 8. MCPG and heparin prevent the induction of HI-LTP. (A) Averaged EPSPs induced by paired stimulation of the MF tract before, during, and after postsynaptic hyperpolarization in the presence of MCPG (0.5 mM; $n=60$, 100 and 66, respectively; 15 s intertrial interval; recording of about 70 min). (B) Summary data for all experiments with MCPG. The columns show mean amplitudes of the first EPSP in the paired-pulse paradigm normalized to the initial control before MCPG application (dashed line). Hyp. and Post Hyp., during and after postsynaptic hyperpolarization, respectively. Black and white columns represent data for the MF- and CF-EPSPs, $n=5$ and 4, respectively. Number of CF-inputs: eight and four for MCPG application and other periods, respectively. (C) MF-EPSPs, recorded with a patch pipette containing heparin, before, during, and after postsynaptic hyperpolarization ($n=21$, 89 and 47, respectively). (D) Summary data for all experiments with heparin obtained with recordings of MF- ($n=4$ inputs) and CF-EPSPs ($n=3$). The data are presented as in B. Note large increases of MF-EPSP amplitudes during hyperpolarization in both experimental series and the absence of their potentiation after switching off the hyperpolarizing current.

First, we measured PPF, which is a well-known presynaptic phenomenon (see Voronin, 1993). The PPF ratio for MF-EPSPs significantly decreased during hyperpolarization, and the decrease persisted after switching off the hyperpolarizing current (Fig. 9A, PPF). For better comparison with acute effects, Fig. 9 relates only with inputs expressing facilitatory effects during hyperpolarization (12/15 MF-EPSP). PPF changed significantly after resetting the membrane potential to its initial value and changes correlated with the magnitude of HI-LTP ($r=-0.74$, $P<0.02$, $n=12$). For all MF-EPSPs ($n=15$) the correlation was $r=-0.60$ ($P<0.05$).

Then we calculated the logarithm of failure rates $[-\ln(N_0/N)] = \ln(N/N_0)$. This value (as well as CV^{-2}) represents the mean quantal content (m) for the simplest (Poisson) model of transmitter release (Redman, 1990; Voronin, 1993) and its changes indicate modifications of presynaptic release. Figure 9A shows a significant increase in $\ln(N/N_0)$ after hyperpolarization. It was correlated with HI-LTP magnitude for the illustrated sample ($r=0.85$, $P<0.01$, $n=12$) and for all MF-EPSP recordings ($r=0.87$, $P<0.001$, $n=15$).

CV^{-2} also tended to increase both during and after postsynaptic hyperpolarization (Fig. 9A) although the latter increase did not reach the significance level. However, the changes in CV^{-2} significantly

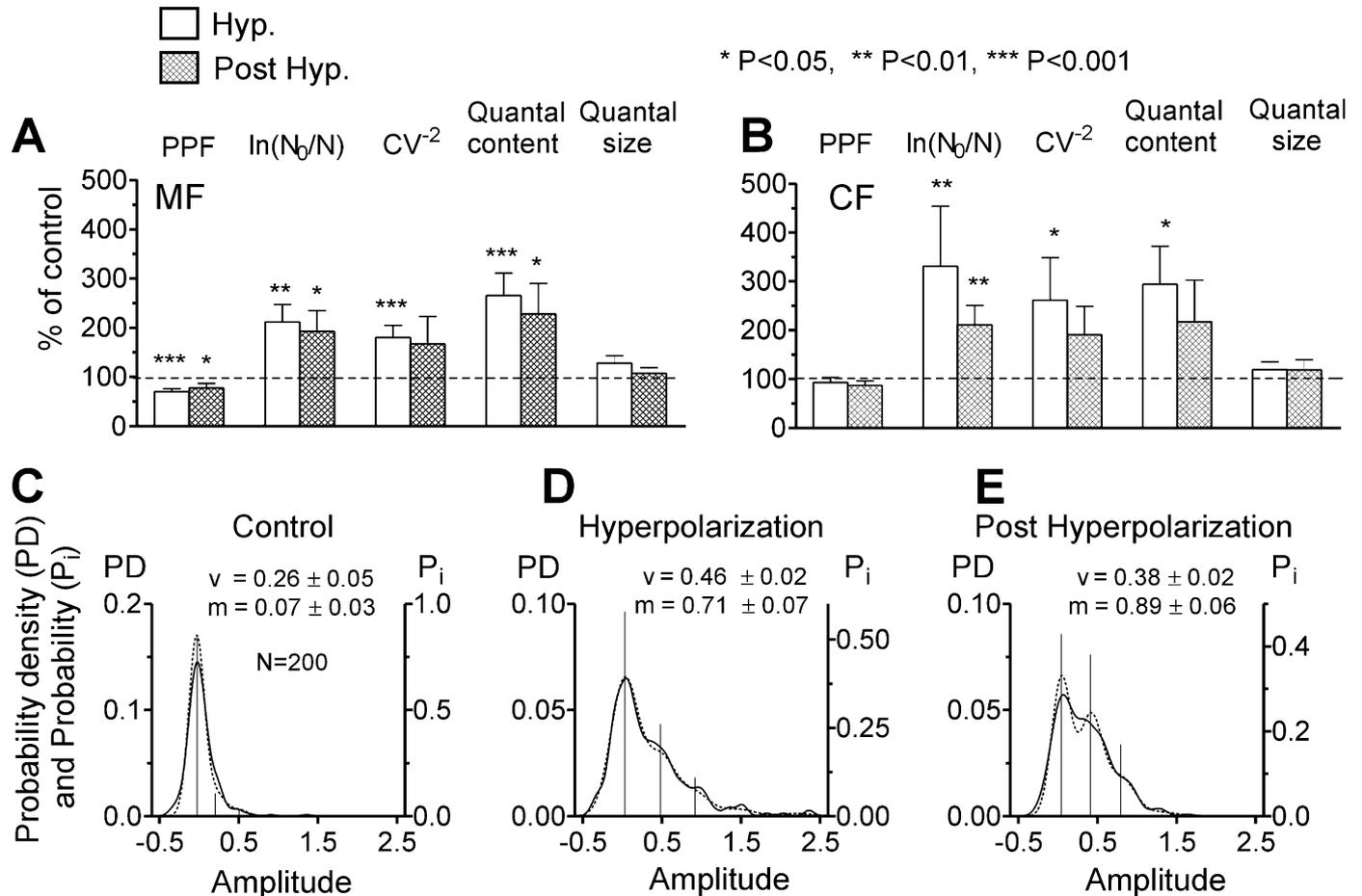


FIG. 9. Changes in indices of transmitter release suggest presynaptic mechanisms of HI-LTP expression. (A) Summary changes in the paired-pulse facilitation (PPF) ratio, logarithm of the failure rate [$\ln(N_0/N)$], inverse squared coefficient of variation of EPSP amplitude (CV^{-2}), quantal content and quantal size for MF-EPSPs during and after postsynaptic hyperpolarization (open and double hatched columns, respectively). (B) As above but for CF-EPSPs. For calculations of the quantal parameters based on the deconvolution techniques (see C–E) measurements from both responses in the paired-pulse paradigm were combined according to Kullmann & Nicoll (1992). Error bars represent SEM; *Statistically significant differences from controls (at about -60 mV). For better comparison of the acute effect during hyperpolarization with after-effect, only results from inputs that expressed a significant ($>135\%$) acute facilitatory effect are plotted ($n = 12$ for MF-inputs and $n = 10$ for CF-inputs). (C–E) Noise deconvolution analysis of MF-EPSPs from a single experiment. Smoothed experimental (continuous curves) and predicted (dashed curves) amplitude distributions and deconvolved components (bars) found by the noise deconvolution procedure (Astrelin *et al.*, 1997) and used to estimate quantal size (v) and mean quantal content (m) in control conditions (C), during (D) and after hyperpolarization (E). Probability densities (left scales, PD) are for distributions; probabilities (right scales, P_i) are for bars.

($P < 0.01$) correlated with MF-EPSP amplitude changes: $r = 0.85$ ($n = 12$) and $r = 0.86$ ($n = 15$).

Finally, we determined m more directly using a noise deconvolution procedure (Astrelin *et al.*, 1997). The example in Fig. 9C–E shows approximately equal (quantal) distances between the bars representing the deconvolution solution. The mean distance that determines quantal size (v) increased significantly only during hyperpolarization (Fig. 9D) as compared to control (Fig. 9C). In contrast, the mean quantal content, m , increased more than 10 times both during and after the hyperpolarization. The mean data for 12 MF-EPSPs are given in Fig. 9A. The increase in m was correlated with changes in MF-EPSPs amplitude after hyperpolarization, i.e. with HI-LTP magnitude ($r = 0.84$, $P < 0.01$). In contrast, the smaller increase in v observed during and after hyperpolarization (Fig. 9A) was not significantly correlated with HI-LTP magnitude ($r = 0.05$, $P > 0.1$).

CF-EPSPs showed no significant after-effect (see Fig. 4B) and no significant changes in PPF, CV^{-2} , quantal content or quantal size. Nevertheless, in those cases with supralinear amplitude increase during hyperpolarization (Fig. 9B; $n = 10$; CF-EPSPs), PPF, CV^{-2}

and m tended to change in a direction compatible with presynaptic involvement and the increase in $\ln(N/N_0)$ was statistically significant. The modifications in m correlated strongly with amplitude changes: $r = 0.82$ for the sample of Fig. 9B ($n = 10$ CF-EPSPs with ‘supralinear’ amplitude increase during hyperpolarization) and $r = 0.85$ for all recorded CF-EPSP ($n = 22$, $P < 0.01$).

In summary, these data are compatible with an increased presynaptic release of glutamate from MF-inputs during and after postsynaptic hyperpolarization. The results suggest presynaptic mechanisms for HI-LTP maintenance with increased number of quanta released by each presynaptic volley without significant changes in the efficacy of a single quantum.

Discussion

HI-LTP constitutes an unusual form of long-lasting increase in synaptic strength as it was induced by pairing afferent stimulation with hyperpolarization rather than depolarization. Usually, hyperpolarizing the postsynaptic membrane prevents the induction of LTP by limiting

the depolarization of the cell during the tetanus (Kelso *et al.*, 1986; Malinow & Miller, 1986). Therefore, our observation seems to contradict at least one of the basic properties of LTP, namely its associative nature. However, this discrepancy is only apparent as HI-LTP can be still considered associative as its induction required presynaptic activation of excitatory inputs together with postsynaptic hyperpolarization.

HI-LTP was observed only in inputs with large increase in EPSP amplitudes during postsynaptic hyperpolarization. As discussed already (Voronin *et al.*, 1999; Berretta *et al.*, 2000; Kasyanov *et al.*, 2000) the 'supralinear' increase is very likely due to a strong enhancement of transmitter release. This hypothesis is substantiated by changes in respective indices of transmitter release including PPF. In previous work (Voronin *et al.*, 1999; Berretta *et al.*, 2000; Kasyanov *et al.*, 2000), the supralinear increase in EPSP amplitudes during postsynaptic hyperpolarization has been attributed to the positive intrasynaptic electrical (ephaptic) feedback in large synapses (Byzov & Shura-Bura, 1986; Voronin *et al.*, 1995). Although other possibilities have been discussed (including changes in local ion gradients, Voronin *et al.*, 1999; Berretta *et al.*, 2000; Kasyanov *et al.*, 2000) Byzov's hypothesis has been considered the most likely. For example, this assumption explains why, in the same neurons, postsynaptic hyperpolarization produces 'classical' linear voltage-dependence of multifibre (composite) MF responses (Barrionuevo *et al.*, 1986; Hestrin *et al.*, 1990; Jonas *et al.*, 1993) but supralinear increases of minimal MF-EPSCs (see Kasyanov *et al.*, 2000 for details). The different effects on minimal vs. composite responses account for the finding that postsynaptic hyperpolarization may prevent conventional LTP (Kelso *et al.*, 1986; Malinow & Miller, 1986) but not LTP-like changes of minimal responses as described here.

We would like to stress here, that, independently of the underlying mechanisms the effect of postsynaptic hyperpolarization is to enhance glutamate release from a large proportion of MF synapses.

Mechanisms of HI-LTP induction

The present data suggest that an increase in intracellular calcium in postsynaptic cells is crucial for the induction of HI-LTP. This was substantiated by the experiments with BAPTA showing no HI-LTP in cells recorded with this calcium chelator. It should be stressed that with BAPTA the 'supralinear' increase in amplitude of MF-EPSPs was not maintained for all the period of membrane hyperpolarization but started declining after 40–50 trials. The nature of this phenomenon is unclear and needs further study. It could be that, in the absence of LTP-like mechanisms that would support the pool of the available transmitter, low-frequency depression would prevail. Thus, under conditions of increased transmitter release such as during hyperpolarization of the postsynaptic membrane (Berretta *et al.*, 2000) an enhanced depletion of the readily available vesicles would occur leading to synaptic depression (see Saviane *et al.*, 2002).

The increase in calcium may originate from extracellular or intracellular sources following activation of NMDA receptors, VDCCs or release from intracellular stores. BAPTA experiments do not allow discrimination between the pathways by which calcium rises. However the contribution of NMDA receptors can be excluded because the majority of the experiments were performed in the presence of NMDA receptor antagonists. The contribution of VDCCs is unlikely because HI-LTP was still present in MF-responses recorded under voltage-clamp conditions that should have suppressed calcium influx via VDCCs. Another theoretical possibility is a rise of $[Ca^{2+}]_i$ due to calcium tail current elicited when the hyperpolarization is turned off. Although at present it is difficult to completely exclude the contribu-

tion of this current, this possibility seems unlikely on the following basis: (i) HI-LTP started developing during postsynaptic hyperpolarization (see Fig. 4A), a condition that should have suppressed calcium influx through VDCCs (Magee *et al.*, 1996). In cases without after-effect, the slow delayed increase was absent (compare Fig. 3E and F; see also Fig. 8C). (ii) Different potentiating effects were observed in MF and CF responses recorded from the same neuron. This allows the exclusion of a general postsynaptic alteration. (iii) HI-LTP did not develop in those cases in which the postsynaptic hyperpolarization was not paired with presynaptic activation. In these cases, a similar tail current should have occurred. Alternatively, the source of calcium may be intracellular. One possibility is calcium release from intracellular stores, triggered by IP_3 receptors following activation of postsynaptic mGluRs which are known to contribute to several forms of synaptic plasticity (Bortolotto *et al.*, 1999). This hypothesis seems likely as HI-LTP was prevented by heparin and MCPG, known to inhibit the binding of IP_3 to its receptor and to antagonize mGluRs, respectively. Therefore, the increased release of glutamate, following membrane hyperpolarization would have activated mGluRs localized on the subsynaptic membrane (Chrupka & Gähwiler, 1991; Shigemoto *et al.*, 1997) leading to an elevation of intracellular calcium following release from intracellular IP_3 -sensitive calcium stores. In line with this hypothesis it has been demonstrated recently that a moderate activation of MF is able to activate group I mGluRs and to evoke calcium release from IP_3 -sensitive internal stores in CA3 pyramidal cells – an effect that is blocked by heparin but not by dantrolene (Kapur *et al.*, 2001). Calcium rise would trigger the conventional cascade of events postulated for other postsynaptically induced forms of LTP in CA1 (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999) and in CA3 area (Urban & Barrionuevo, 1996; Yeckel *et al.*, 1999; Henze *et al.*, 2000).

Mechanisms of HI-LTP maintenance

As to the maintenance mechanisms, changes in classical indices of transmitter release suggest a predominantly presynaptic location. In principle, changes in failures rate, CV^{-2} and m could result from the appearance of new subsynaptic receptors in previously silent synapses (Malenka & Nicoll, 1999). However, we consider this explanation unlikely, at least for the MF-inputs for which there is a common consensus on the involvement of presynaptic mechanisms in the maintenance of conventional (tetanus-induced) LTP (see Henze *et al.*, 2000). Our more specific reasons are the following: (i) Conversion of silent synapses into active ones presumes activation of NMDA receptors and most of our experiments were performed in the presence of NMDA receptor antagonists. (ii) When completely silent synapses are expressed, EPSP components with different latency and/or kinetics should appear (Voronin *et al.*, 1999). Such novel components were found neither during nor after hyperpolarization of CA3 neurons. (iii) Under our experimental conditions, a postsynaptic site of maintenance would require selective 'all-or-none' modifications of subsynaptic receptors at some sites without essential changes at other sites as no significant changes in quantal efficacy were found. (iv) The explanation based on expression of postsynaptically silent synapses is difficult to reconcile with reduction in PPF (see also Gasparini *et al.*, 2000). The concept of insertion of glutamate receptors of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid type (AMPA receptor) as a major mechanism for early LTP maintenance has been challenged recently. Evidence from our (Gasparini *et al.*, 2000) and other groups (Choi *et al.*, 2000; Renger *et al.*, 2001) suggests that apparently silent synapses actually contain AMPA receptors and are silent because of very low glutamate release (i.e. they are 'presynaptically', not 'postsynaptically' silent). In line with this view is the observation that in

CA1 pyramidal neurons the ratio of amplitudes of spontaneous AMPA- to NMDA-mediated synaptic events is equal to 1 and this does not change over the first postnatal week, indicating that newly formed glutamatergic synapses express both AMPA and NMDA receptors (Groc *et al.*, 2002). Moreover, it has been shown recently, that in CA1 hippocampal neurons from adult rats, the amplitudes of AMPA and NMDA receptor-mediated synaptic currents equally increased after LTP induction when measured simultaneously under the same experimental conditions (Bayazitov *et al.*, 2002; see also Niu *et al.*, 1998; Bayazitov & Kleschevnikov, 2000).

Summarizing, HI-LTP can provide a novel mechanism for lasting synaptic modifications that could expand the known repertoire of possible ways of information storage and consolidation of memory traces in large synapses, which could appear during development or as a result of potentiation.

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Abbreviations

BAPTA, 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid; CF, commissural-associative fibres; CPP, α -3-(2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid; CV, coefficient of variation; DCG-IV, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; HI-LTP, hyperpolarization-induced LTP; IP3, inositol 1,4,5-triphosphate; LTP, long-term potentiation; m, mean quantal content; MCPG, (R,S)- α -methyl-4-carboxyphenylglycine; MF, mossy fibres; mGluR, metabotropic glutamate receptor; N0: number of transmission failures; NMDA, N-methyl-D-aspartate; PCA, principal component analysis; PPF, paired-pulse facilitation; r, coefficient of linear correlation; v, quantal size; VDCC, voltage-dependent calcium channels.

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