

Role for cAMP and protein phosphatase in the presynaptic expression of mouse hippocampal mossy fibre depotentiation

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Long-term potentiation (LTP) at the hippocampal mossy fibre–CA3 synapses can be reversed (depotentiated) by long trains of low-frequency stimulation (LFS). In the present study, we showed that this depotentiation is triggered by a presynaptic group II metabotropic glutamate receptor (mGluR), which reduces cytosolic cAMP level, leading to a reversal of cellular processes responsible for mossy fibre LTP expression. Furthermore, we found that both the presynaptic activity-induced elevation of Ca^{2+} and the activation of protein phosphatase (PP) activity are required for the induction of depotentiation. Thus, we conclude that mossy fibre depotentiation is expressed presynaptically through the activation of both presynaptic mGluR- and PP-coupled signalling cascades, and that the bidirectional long-term plasticity at the mossy fibre–CA3 synapses is likely to be regulated by presynaptic Ca^{2+} -dependent processes.

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Activity-dependent alteration of synaptic strength is essential for the refinement of neuronal circuitry in developing nervous systems and for the plasticity of mature brain (Siegelbaum & Kandel, 1991; Katz & Shatz, 1996). Much of our understanding of activity-dependent synaptic modification and its functional relevance comes from studies on the mammalian hippocampus. In both CA1 and CA3 regions of the hippocampus, brief high-frequency stimulation (HFS) of afferent pathways can trigger a long-lasting enhancement of synaptic strength, commonly referred to as long-term potentiation (LTP) (Bliss & Collingridge, 1993), whereas prolonged low-frequency stimulation results in a long-lasting decrease in synaptic strength, termed as long-term depression (LTD) (Mulkey & Malenka, 1992; Dudek & Bear, 1993). Although both LTP and LTD are remarkable for their stability, recent work has shown that they are initially labile and sensitive to disruption by a variety of interfering events or agents. For example, the hippocampal CA1 LTP can be reversed by afferent LFS (Barrionuevo *et al.* 1980; Fujii *et al.* 1991; Huang *et al.* 1999; Huang & Hsu, 2001), episodes of hypoxia (Arai *et al.* 1990), or pharmacological treatments that interrupt cell–cell or cell–matrix interactions (Bahr *et al.* 1997), when given shortly after LTP induction. This reversal of synaptic strength from potentiated state to pre-LTP level is referred to as depotentiation and may provide a mechanism of preventing the saturation of synaptic potentiation and increase the efficiency and the capacity of the information storage of neuronal networks (Huang & Hsu, 2001).

Since the initial discovery of depotentiation (Barrionuevo *et al.* 1980), most work on the mechanisms of this phenomenon in the mammalian brain has focused mainly on the Hebbian forms of LTP, such as hippocampal Schaffer-collateral CA1 LTP and perforant path-dentate gyrus LTP (Huang & Hsu, 2001). We have previously demonstrated that, as with Hebbian forms of LTP, non-Hebbian forms of hippocampal mossy fibre–CA3 LTP also display LFS-induced depotentiation in a slice preparation (Chen *et al.* 2001). We found that the activation of group II mGluRs and subsequent $G_{i/o}$ -protein-coupled signalling cascade is responsible for the depotentiation and neither postsynaptic depolarization nor the ionotropic glutamate receptors are required. These findings raised several immediate questions. Where is the locus of expression of LFS-induced depotentiation? Is activation of group II mGluRs alone sufficient to elicit a reversal of LTP? What are the signalling events downstream from the activation of mGluRs and $G_{i/o}$? In the present study we found that LFS-induced depotentiation is expressed presynaptically at the mossy fibre–CA3 synapses and requires two signalling events: a reduction of cAMP mediated by presynaptic group II mGluR and $G_{i/o}$, and an activity-induced elevation of presynaptic Ca^{2+} ($[Ca^{2+}]_i$) that activates PP-coupled signalling. These results underscore the crucial role of presynaptic cAMP-mediated signalling in the bidirectional plasticity of hippocampal mossy fibre–CA3 synapses and point to an active role of PPs in regulating the plasticity.

METHODS

Hippocampal slice preparation

Animal care was consistent with the guidelines set by the Laboratory Animal Center of National Cheng-Kung University. All experimental procedures were approved by the NCKU Institutional Animal Care and Use Committee. Hippocampal slices were prepared from 12- to 16-day-old or 4- to 6-week-old ICR strain mice for extracellular synaptic recordings by the procedures described previously (Huang *et al.* 1999; Chen *et al.* 2001). In brief, animals were killed by decapitation under halothane anaesthesia, and the hippocampi were removed, placed in ice cold artificial CSF (ACSF) solution and cut with a Leica VT1000S tissue slicer (Leica, Nussloch, Germany) in 400 μm thick transverse slices. After their preparation, slices were placed in a holding chamber of ACSF oxygenated with 95% O_2 -5% CO_2 and kept at room temperature for at least 1 h before recording. The composition of the ACSF solution was (mM): NaCl 117, KCl 4.7, CaCl_2 2.5, MgCl_2 1.2, NaHCO_3 25, NaH_2PO_4 1.2 and glucose 11 at pH 7.3-7.4 and equilibrated with 95% O_2 -5% CO_2 . In Ca^{2+} -free experiments, CaCl_2 was totally replaced by equimolar MgCl_2 and 100 μM EGTA.

Electrophysiological recordings

For the extracellular field potential recordings, a single slice was then transferred to a submerge-type recording chamber and held between two nylon nets. The chamber consisted of a circular well of a low volume (1-2 ml) and was continuously perfused with oxygenated ACSF at a flow rate of 2-3 ml min^{-1} at $32.0 \pm 0.5^\circ\text{C}$. Standard extracellular field recording techniques were used (Huang *et al.* 1999). Mossy fibre field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum lucidum (50-80 μm from the edge of the pyramidal cell layer) of the CA3 region of the hippocampus using a glass microelectrode filled with 1 M NaCl (resistance 2-3 $\text{M}\Omega$). A bipolar stainless steel stimulating electrode was placed in or adjacent to the dentate gyrus granule cell layers to activate mossy fibre afferents at 0.05 Hz. To stimulate independent inputs to the same cell population, two bipolar stimulating electrodes were positioned on both folia to activate two different mossy fibre afferents, alternating every 10 s. Their positions were arranged so that the same amount of current evoked two responses that did not differ from each other by > 10%. The absence of cross-pathway paired-pulse facilitation was used to ensure the two inputs were independent of each other. The stimulation strength was set to elicit response for which the amplitude was 30-40% of the maximum response. In all experiments, the baseline was recorded for at least 20 min to ensure the stability of the response. The strength of synaptic transmission was quantified by measuring the amplitude of fEPSP. The fEPSP amplitudes were calculated after subtracting the mossy fibre volley from the evoked response. The mossy fibre volley was recorded at the end of experiment after blocking synaptic transmission with 20 μM CNQX. LTP was induced by high-frequency stimulation, at the test pulse intensity, consisting of two 1 s trains of stimuli at 100 Hz, delivered with an interval of 20 s. D(-)-2-Amino-5-phosphonopentanoic acid (D-APV; 50 μM) was present for the duration of all experiments to block LTP at the CA3 to CA3 collateral synapses. Depotentiation was induced by application of 15 min low frequency trains of stimuli at 1 Hz and the stimulation intensity was the same as the test pulse intensity. The responses during the trains were not recorded, and for convenience these periods are not shown on the graph. All values of residual potentiation reported here were calculated as the changes in fEPSP amplitude measured 40 min after the end of

LFS. Microelectrodes were pulled from microfibre 1.0 mm capillary tubing on a Flaming/Brown electrode puller (Sutter Instruments, San Rafael, CA, USA). Electrical signals were collected with an Axoclamp-2B (Axon Instruments, Union City, CA, USA) filtered at 1 kHz, sampled at 10 kHz, and an Intel Pentium-based computer with pCLAMP software (version 7.0, Axon Instruments) was used to on-line acquire and off-line analyse the data.

Visualized whole-cell patch recordings of evoked excitatory postsynaptic currents (EPSCs) were conducted at room temperature (24-26 $^\circ\text{C}$) using standard methods (Edwards *et al.* 1989). Hippocampal slices (200 μm thick) were prepared from male ICR strain mice, 12- to 16-days-old, in this experiment. CA3 pyramidal neurons were visualized throughout the experiment with an upright microscope (Olympus BX50WI; Olympus, Tokyo, Japan) equipped with a water-immersion 40 \times objective using Nomarski-type differential interference contrast (DIC) optics combined with infrared videomicroscopy. Patch pipettes were pulled from borosilicate capillary tubing and heat polished. The electrode resistance was typically 4-5 $\text{M}\Omega$. The composition of intracellular solution was (mM): potassium gluconate, 110; KCl, 30; Hepes, 10; MgCl_2 , 1; EGTA, 0.5; Na_2ATP , 4; Na_3GTP , 0.3; phosphocreatine, 7; lidocaine *N*-ethyl bromide quaternary (QX-314), 5 and sucrose to bring osmolarity to 290-295 mmsol l^{-1} , pH 7.3 (adjusted with KOH). Tight-seal (> 2 $\text{G}\Omega$ before breaking into whole-cell mode) whole-cell recordings were made using a patch-clamp amplifier (Axopatch 200B; Axon Instruments). Electrical signals were low-pass filtered at 2 kHz, digitized at 4-10 kHz using a Digidata 1200B interface, and an Intel Pentium-based computer with pCLAMP software (Version 7.0; Axon Instruments) was used to on-line acquire and off-line analyse the data. Series resistance (R_s) was monitored by delivery of a -10 mV voltage step before each evoked EPSC and calculated according to $R_s = 10 \text{ mV}/I$, where I was the peak of transient current (filtered with 10 kHz) evoked by the 10 mV testing pulse when the pipette capacitance was compensated fully. Only cells demonstrating < 20 $\text{M}\Omega$ series resistance were used in these experiments. The input resistance was monitored continuously, and the recording terminated if it varied by more than 10%. Synaptic responses were evoked by low-intensity stimulation of the granule cells in the dentate gyrus of the hippocampal CA3 region via a bipolar stainless steel stimulating electrode at a frequency of 0.05 Hz. The stimulus intensity was set to the lowest values that reliably evoked a single EPSC waveform. EPSCs were included in the analysis if the rise time and decay time constants were monotonic and possessed no obvious multiple EPSCs or polysynaptic waveforms. The EPSC amplitude was determined from the response during a 1 to 2 ms window that included the peak of the waveform, and the amplitude of the baseline in a similar time window was subtracted. Bicuculline methiodide (10 μM) was routinely added to the extracellular medium to block GABAergic inhibition. Failures of synaptic responses were identified visually, averaged, and used to correct for the stimulus artifact. The group II mGluR agonists, (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-1; 20 μM) or (2S,2'R,3'R)-2-(dicarboxycyclopropyl)glycine (DCG-IV; 1 μM), were added routinely at the end of the experiments to verify that the evoked EPSCs were abolished > 80%, confirming they were mossy fibre-mediated.

Drug application

All drugs were applied by dissolving them to the desired final concentrations in the ACSF and by switching the perfusion from control ACSF to drug-containing ACSF. Appropriate stock solutions of drugs were made and diluted with ACSF just before

application. DCG-IV, forskolin and MK-801 (dizocilpine maleate) were made up to 10, 50 and 40 mM stock solution in dimethylsulfoxide (DMSO), respectively, and stored at -20°C . Aqueous dilution of these stock solutions was made daily. The concentration of DMSO in the perfusing medium was less than 0.1%, which alone had no effect on the synaptic transmission or plasticity at the hippocampal mossy fibre-CA3 synapses (Chen *et al.* 2001). The cyclosporin A perfusing solution contained 0.5% ethanol and 0.5% Tween-80. Forskolin, MK-801, okadaic acid and calyculin A were purchased from Sigma (St Louis, MO, USA); DCG-IV, CNQX, D-APV, cyclosporin A and bicuculline methiodide were obtained from Tocris Cookson Ltd (Bristol, UK); Sp-8-CPT-cAMPS was purchased from Biomol (Plymouth Meeting, PA, USA); FK-506 (tacrolimus) and 1-norkadaone were purchased from Calbiochem (La Jolla, CA, USA).

Statistical analysis

The data for each experiment were normalized relative to baseline. Data are presented as mean \pm S.E.M. The significance of the difference between the mean was calculated by a Student's paired or unpaired *t* test as appropriate. Numbers of experiments are indicated by *n*. Probability values of $P < 0.05$ were considered to represent significant differences.

RESULTS

Presynaptic expression of LFS-induced depotentiation

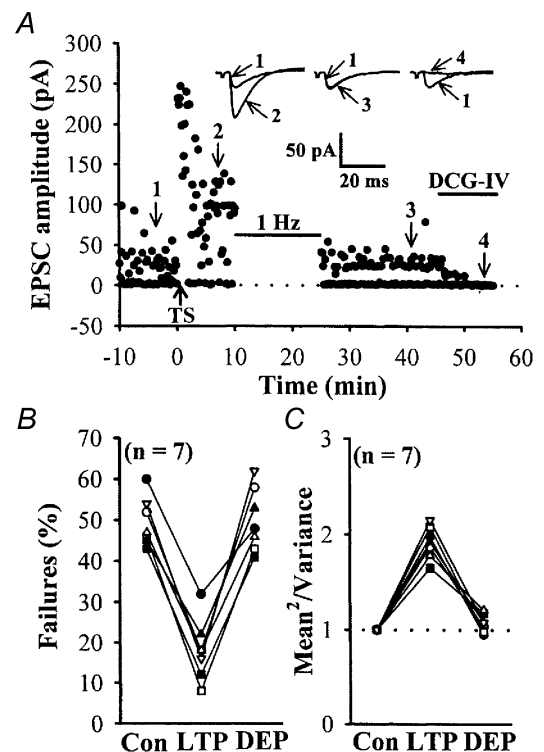
In the initial set of experiments, we examined whether the reversal of LTP by prolonged LFS (1 Hz, 15 min) is expressed presynaptically at the mossy fibre-CA3 synapses (Chen *et al.* 2001), using three different approaches. Because the hippocampal slices prepared from younger animals were easier to obtain the stable tight-seal whole-cell patch-clamp recordings and the magnitude of mossy fibre LTP of field potential recordings in slices obtained from 12- to 16-

day-old ($204 \pm 16\%$, $n = 6$; 40 min after tetanic stimulation) and 4- to 6-week-old mice ($216 \pm 13\%$, $n = 12$; 40 min after tetanic stimulation) was indistinguishable (supplementary data; $P > 0.05$; Student's unpaired *t* test), 12- to 16-day-old mice were chosen in this set of experiments. We first examined the effect of depotentiating LFS on the failure rate of single-fibre EPSCs evoked by minimal stimulation of the dentate gyrus, which reflects changes in the presynaptic transmitter release (Stevens & Wang, 1994; Raastad, 1995). At the mossy fibre-CA3 synapses, LTP is expressed presynaptically and is accompanied by a sustained decrease in the number of synaptic failures, whereas an opposite in failure rate was found for synapses undergoing LTD (Maccaferri *et al.* 1998). In agreement with the latter study, we found that LTP at the mossy fibres is accompanied by a persistent reduction of failure rate ($49.6 \pm 2.3\%$ in the control period *vs.* $18.1 \pm 2.9\%$ after LTP, $n = 7$; $P < 0.05$; Student's paired *t* test; Fig. 1A and B). In addition, we found that LFS delivered 10 min after application of the LTP induction protocol completely reversed the reduction in failure rate: the average failure rate measured at 15 min after the end of LFS was $50.1 \pm 2.9\%$, which was not significantly different from that found during the control period ($49.6 \pm 2.3\%$, $n = 7$; $P < 0.05$; Student's paired *t* test; Fig. 1A and B). At the end of experiments, $1 \mu\text{M}$ DCG-IV (selective group II mGluR agonist) was routinely added to the bath to ensure that the synaptic response obtained in our study was indeed elicited by the mossy fibre (Yokoi *et al.* 1996).

We next addressed the synaptic locus of LFS-induced depotentiation by examining the trial-to-trial amplitude fluctuation in EPSCs with the variance analysis. The value

Figure 1. Failure and variance analysis of LFS-induced depotentiation at the mossy fibre-CA3 synapses

A, plot of EPSC amplitude recorded at -70 mV holding potential against time, before and after high-frequency tetanic stimulation (TS) and depotentiating LFS protocol (1 Hz, 15 min), evoked at 0.05 Hz by minimal stimulation of the mossy fibres. Note that TS resulted in a decrease in the number of failures and that was completely reversed by successive application of LFS. EPSCs were blocked by $1 \mu\text{M}$ DCG-IV, confirming their mossy fibre origin. Sample traces are averages of six consecutive EPSCs recorded at the times indicated by the numbers on the graph. Upward arrow indicates application of TS. Horizontal bars indicate the period of the delivery of LFS or DCG-IV. B, summary of seven experiments comparing the failure rate before LTP, during LTP and after depotentiating LFS application. The decrease in failure rate seen with LTP was completely reversed by depotentiating LFS. C, summary of seven experiments (from the same cells shown in B) comparing the mean²/variance for minimal evoked EPSC amplitude before LTP, during LTP, and after depotentiating LFS application. The increase in mean²/variance seen with LTP was completely reversed by depotentiating LFS.



of mean²/variance varies with quantal content but is independent of changes in the postsynaptic response to a fixed amount of transmitter, and is a useful measure of changes in presynaptic function (Del Castillo & Katz, 1954; Bekkers & Stevens, 1990). Because variance analysis is best done on unitary synaptic responses (Faber & Korn, 1991), our strategy was to carry out a variance analysis of unitary single-fibre EPSCs evoked by minimal stimulation before and after depotentiating LFS application. We found, as reported previously (Xiang *et al.* 1994; Weisskopf & Nicoll, 1995), that following the induction of LTP the value of mean²/variance for unitary EPSCs was increased to $191.9 \pm 6.5\%$ of the control level ($n = 7$; $P < 0.05$; Student's paired *t* test; Fig. 1C). When LFS was delivered 10 min after LTP induction, the reversal of LTP was accompanied by a reversal in the value of mean²/variance close to pre-LTP level ($107.3 \pm 3.6\%$ of baseline, $n = 7$; $P > 0.05$; Student's paired *t* test).

While the above results are consistent with a presynaptic site for the expression of LFS-induced depotentiation, they may be attributed to a number of presynaptic mechanisms, including an alteration in the number of available vesicles, an alteration in the probability of quantal release, or changes in the numbers of release sites (Faber & Korn, 1991; Manabe *et al.* 1993). Given that the expression of mossy fibre LTP is associated with an increase in presynaptic transmitter release probability (Xiang *et al.* 1994; Weisskopf & Nicoll, 1995), we asked whether the presynaptic expression of LFS-induced depotentiation is associated with a reduction in the enhancement of release probability seen with LTP. To examine this possibility, we took advantage of the use-dependent irreversible NMDA receptor antagonist MK-801. Repeated activation of synapses in the presence of MK-801 results in a progressive decline in the amplitude of the NMDA receptor-mediated synaptic current and the rate of decay depends on the probability of transmitter release at synapses (Hessler *et al.* 1993; Rosenmund *et al.* 1993). We first examined the effect of MK-801 on the NMDA receptor-mediated EPSCs (EPSC_{NMDA}). The EPSC_{NMDA} was recorded from CA3 pyramidal neurons in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; $10 \mu\text{M}$) and at a holding potential of +50 mV to remove the voltage-dependent block of Mg²⁺ (Manabe & Nicoll, 1994). After confirming a stable baseline at a stimulation of 0.1 Hz, the stimulation was stopped and MK-801 ($40 \mu\text{M}$) was applied. Stimulation was restarted 10 min later and the EPSC_{NMDA} was recorded in the continuous presence of MK-801. Consistent with previous reports (Manabe & Nicoll, 1994; Weisskopf & Nicoll, 1995), the amplitude of EPSC_{NMDA} progressively declined with a half-decay time of 1.89 ± 0.08 min (mean \pm S.E.M., $n = 6$, Fig. 2A). We next examined the effect of the mossy fibre LTP and depotentiation on the rate of decay of successive EPSC_{NMDA} in the presence of MK-801. Figure 2B shows a typical

example of LTP of AMPA receptor-mediated EPSCs induced by tetanic stimulation at a holding potential of -70 mV. When LTP of EPSCs reach steady state (10 min after tetanic stimulation), CNQX was added and the holding potential was switched to +50 mV to record the NMDA receptor-mediated synaptic response. After establishing a stable baseline, stimulation was stopped and MK-801 was applied. When stimulation was restarted after a 10 min period, EPSC_{NMDA} progressively declined in amplitude (Fig. 2B). The half-decay time after induction of LTP was 1.35 ± 0.05 min (mean \pm S.E.M., $n = 6$), which was significantly different from that of the control values ($P < 0.05$; Student's unpaired *t* test; Fig. 2D). Under identical experimental conditions, we measured the half-decay time of depotentiation after LFS application 10 min after LTP induction. We found that LFS induced a depotentiation of previously established LTP and resulted in a slower decline of EPSC_{NMDA} (half-decay time 1.86 ± 0.07 min, $n = 6$), which was significantly different from that found normally after induction of LTP ($P < 0.05$; Student's unpaired *t* test; Fig. 2D), and was identical to the control values ($P > 0.05$; Student's unpaired *t* test). Taken together, these results strongly support the notion that a reversal of LTP-associated increase in release probability is the presynaptic mechanism underlying the LFS-induced depotentiation at the hippocampal mossy fibre-CA3 synapses.

Mechanisms underlying LFS-induced depotentiation

Having confirmed that LFS-induced depotentiation is expressed at the presynaptic site, we next examined the subcellular signalling mechanism that underlies this form of plasticity. We have shown recently that LFS-induced depotentiation can be mimicked by bath-applied group II mGluR agonist and was specifically inhibited by group II mGluR antagonist (Chen *et al.* 2001). Thus, the activation of presynaptic group II mGluR and its downstream signalling pathways are likely candidates of biochemical cascades responsible for the induction of depotentiation. To further test this hypothesis, we first examined whether the activation of group II mGluRs alone was sufficient to induce depotentiation by applying the group II mGluR agonist DCG-IV ($3 \mu\text{M}$). Two independent pathways converging into the same postsynaptic population of CA3 neurons were stimulated. After establishing a stable baseline for the two pathways, LTP was induced by a tetanic stimulation to both pathways simultaneously. This was followed by a brief (5 min) bath application of DCG-IV to reverse LTP. In the control pathway, synaptic transmission was monitored throughout the application of DCG-IV, whereas the afferent stimulation in the test pathway was terminated as soon as DCG-IV was applied and the stimulation was restarted until 10 min after washout of DCG-IV. Consistent with our previous report (Chen *et al.* 2001), in the control pathway, application of

DCG-IV at 10 min after LTP induction successfully reversed LTP in the same manner as LFS – the fEPSP amplitude measured 40 min after washout of DCG-IV was $108 \pm 13\%$ ($n = 7$) of the control baseline (Fig. 3A). However, absence of stimulation during DCG-IV application in the test pathway completely prevented the depotentiation effect of DCG-IV. The average fEPSP amplitude measured 40 min after washout of DCG-IV was $208 \pm 16\%$ ($n = 7$) of baseline, which was significantly different from that measured in the control pathway ($P < 0.05$; Student's paired t test). Thus, activation of group II mGluRs alone could not effectively induce depotentiation and an additional

factor associated with the presynaptic stimulation is required for the induction of depotentiation.

Why is presynaptic stimulation required for the induction of depotentiation? An obvious consequence of presynaptic stimulation is Ca^{2+} entry into presynaptic terminals. To address the potential role of presynaptic $[\text{Ca}^{2+}]_i$ in the depotentiation induction, we examined the effect of EGTA-AM, a membrane-permeant Ca^{2+} chelator. When EGTA-AM ($200 \mu\text{M}$) was bath-applied for 60 min, the fEPSP amplitude gradually decreased to $68.5 \pm 5.6\%$ ($n = 4$) of baseline and remained depressed at a steady level (Fig. 3B). Furthermore, this manipulation significantly

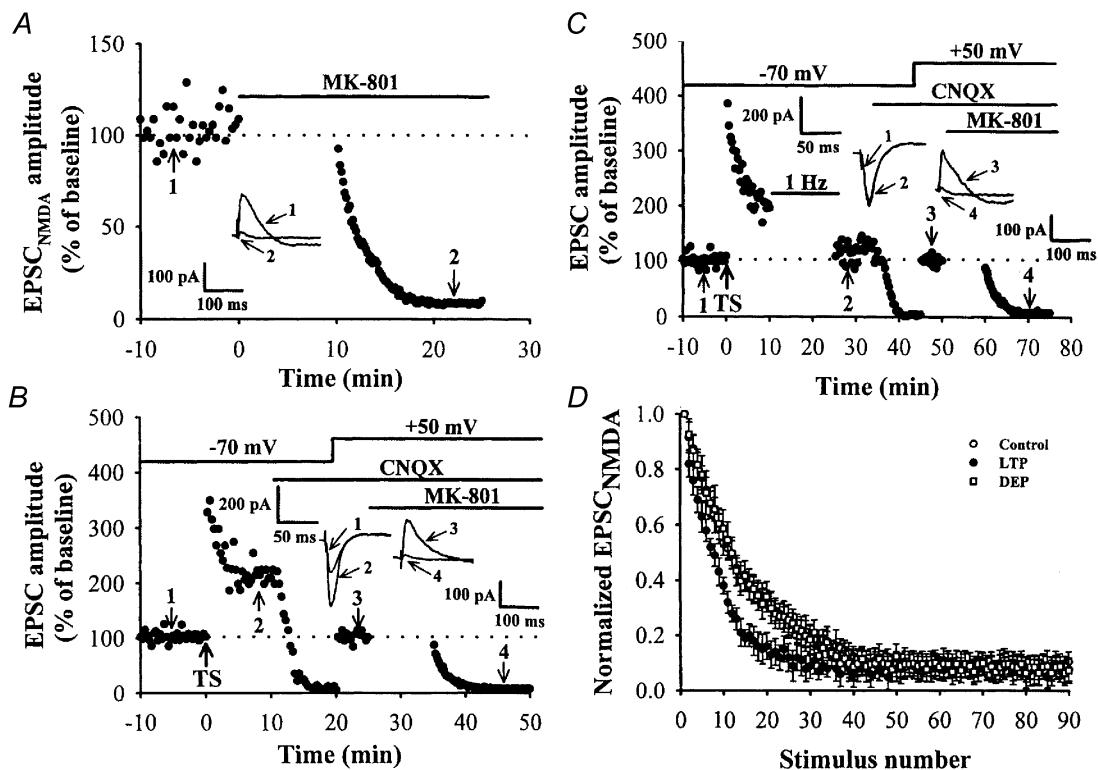


Figure 2. LFS-induced depotentiation is associated with a change in the glutamate release probability assessed from the decay time course of NMDA receptor-mediated EPSC amplitude in the presence of MK-801

A, a typical example showing the time course of blockade of NMDA receptor-mediated EPSC ($\text{EPSC}_{\text{NMDA}}$) by MK-801 ($40 \mu\text{M}$). Stimulation was interrupted for 10 min while MK-801 was washed in. B, the effect of LTP on the decay time course of $\text{EPSC}_{\text{NMDA}}$ amplitude in the presence of MK-801. After establishing a stable LTP of EPSC by TS at a -70 mV holding potential, CNQX ($10 \mu\text{M}$) was applied and the membrane was shifted to a positive holding potential of $+50 \text{ mV}$ to record the NMDA receptor-mediated synaptic response, and the stimulation frequency was changed from 0.05 to 0.1 Hz. After confirming a stable baseline, stimulation was stopped and MK-801 was applied. C, the effect of LFS-induced depotentiation on the decay time course of $\text{EPSC}_{\text{NMDA}}$ amplitude in the presence of MK-801. LFS protocol was delivered 10 min after LTP induction. In this case, subsequent LFS was able to reverse the synaptic potentiation caused by TS. After reversing the synaptic strength from the potentiated state to pre-LTP level, CNQX ($10 \mu\text{M}$) was applied and the membrane was shifted to a positive holding potential of $+50 \text{ mV}$ to record the $\text{EPSC}_{\text{NMDA}}$, and the stimulation frequency was changed from 0.05 to 0.1 Hz. MK-801 was then applied to measure the glutamate release probability. D, the time course of block of $\text{EPSC}_{\text{NMDA}}$ by MK-801 in control (○; $n = 6$), LTP (●; $n = 6$) and LFS-induced depotentiation experiments (□; $n = 6$). The subsequent decay of responses, normalized to the first response after MK-801 application, was monitored. The decay of the response was fitted by a double exponential function and the time necessary to reach the 50% response level (half-decay) was determined. Note that the responses expressing LTP decayed more rapidly than the control response and the application of LFS significantly reversed this facilitatory effect.

reduced the depotentiation normally induced by DCG-IV ($3 \mu\text{M}$) application. Because EGTA-AM takes about 20 min to produce its maximal effect on synaptic response, DCG-IV was applied at 20 min after EGTA-AM application to induce depotentiation. As shown in Fig. 3C, the magnitude of DCG-IV-induced depotentiation in slices treated with EGTA-AM was significantly smaller than that in the control (control: $92 \pm 18\%$, $n = 5$; EGTA-AM: $20.8 \pm 8.3\%$, $n = 5$; $P < 0.05$; Student's unpaired t test). Thus, in addition to group II mGluR activation, an activity-mediated increase in the presynaptic $[\text{Ca}^{2+}]_i$ is also required to trigger mossy fibre depotentiation.

We have previously shown that LFS-induced depotentiation at the mossy fibre-CA3 synapse is not input specific (homosynaptic) but occurs heterosynaptically (Chen *et al.* 2001), perhaps through a heterosynaptic activation of group II mGluRs by spillover of glutamate from neighbouring synapses. Having observed that an activity-mediated change in presynaptic $[\text{Ca}^{2+}]_i$ is required to trigger homosynaptic depotentiation, we next examined whether heterosynaptic activity-dependent depotentiation requires activity in the heterosynaptic inputs. To address this issue, two stimulating electrodes were placed on both edges of the stratum granulosum of dentate gyrus to activate two group inputs to the same cell population. The independence of inputs activated by two stimulatory electrodes was assessed by verifying the absence of heterosynaptic facilitation between the two inputs using paired stimuli applied at intervals of 30 ms. As previously

reported (Chen *et al.* 2001), induction of depotentiation was found to affect the LTP previously induced on a separate input (Fig. 4A). At the control pathway, the mean residual potentiation measured 40 min after the end of LFS was $145 \pm 15\%$ of baseline ($n = 4$), which was not significantly different from the residual potentiation at the test pathway ($123 \pm 14\%$ of baseline; $P > 0.05$; Student's paired t test; Fig. 4B). We next examined the effect of heterosynaptic activity on heterosynaptic depotentiation induction. A single experiment is shown in Fig. 4C, in which the presynaptic stimulation of the control pathway was stopped during LFS application in the test pathway. Stopping stimulation completely prevented the generation of heterosynaptic depotentiation by LFS. This phenomenon was observed in all six slices tested in this study. At the control pathway, the mean residual potentiation measured 40 min after the end of LFS was $197 \pm 18\%$ of baseline ($n = 6$), which was significantly different from that measured at test pathway ($129 \pm 15\%$ of baseline; $P < 0.05$; Student's paired t test; Fig. 4D). These results indicated that presynaptic activity is essential for both homosynaptic and heterosynaptic depotentiation induction at the mossy fibre-CA3 synapses.

Protein phosphorylation mediated by the protein kinase A (PKA) pathway has been identified as an important element in mossy fibre LTP expression (Huang *et al.* 1994; Weisskopf *et al.* 1994). How does the increase in presynaptic $[\text{Ca}^{2+}]_i$ interfere with PKA-mediated phosphorylation, resulting in depotentiation? Since Ca^{2+} is known to activate

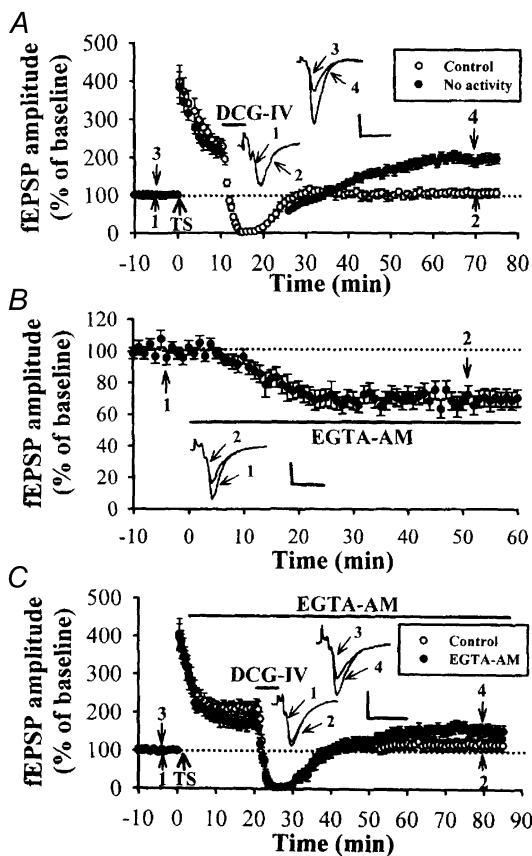


Figure 3. DCG-IV-induced depotentiation requires presynaptic activity

A, summary graph of seven experiments. Two stimulatory electrodes were used to activate two independent groups of afferents (confirmed by no heterosynaptic facilitation). After establishing a stable baseline, LTP was induced on two afferent pathways by coactivation, followed by application of DCG-IV ($3 \mu\text{M}$) for 5 min. In the test pathway, the stimulation was stopped during the application of DCG-IV and did not restart stimulation until 10 min after the washout of DCG-IV. Depotentiation was induced only in the control stimulation pathway ($n = 7$). B, summary of experiments ($n = 4$) showing effects of EGTA-AM ($200 \mu\text{M}$) on synaptic response. C, summary of experiments ($n = 5$) in which EGTA-AM ($200 \mu\text{M}$) was applied before application of DCG-IV ($3 \mu\text{M}$). The magnitude of DCG-IV-induced depotentiation in slices treated with EGTA-AM was smaller than that in the control condition ($n = 8$, $P < 0.05$). The superimposed fEPSP in the inset of each graph illustrates respective recordings from example experiments taken at the time indicated by numbers. Upward arrow indicates application of TS. Horizontal bars indicate the period of the delivery of DCG-IV or EGTA-AM. The horizontal dashed lines indicate the average value of the normalized amplitude during the control period. Calibration: 0.2 mV, 10 msec.

protein phosphatases (PPs), it is reasonable to inquire whether LFS may elicit depotentiation by activating PP-coupled cascades. This possibility was further tested by examining the effects of multiple subtypes of PP inhibitors on the LFS- or DCG-IV-induced depotentiation.

Several serine/threonine PP, including PP1, PP2A and PP2B, are present in principal neurons of the hippocampus (Winder & Sweatt, 2001). If PP1 is critical for depotentiation, blockade of PP1 activity should inhibit the induction of LFS- and DCG-IV-induced depotentiation. Thus, we examined the effect of potent PP1 inhibitors, okadaic acid and calyculin A, on the development of depotentiation. We found that, following a 2–4 h preincubation in okadaic acid (1 μM), both the LFS- and DCG-IV-induced depotentiations were inhibited. The residual potentiation measured 40 min after the end of LFS or washout of DCG-IV was $183 \pm 11\%$ ($n = 10$; $P < 0.05$; Student's unpaired t test) and $193 \pm 12\%$ ($n = 5$; $P < 0.05$; Student's unpaired t test) of the control baseline, respectively

(Table 1). Similarly, another PP1 inhibitor, calyculin A (1 μM), also prevented the induction of depotentiation by LFS or DCG-IV (Table 1). In contrast, preincubation of slices in 1-norkadaone (1 μM), a compound with physical and chemical properties similar to those of okadaic acid but lacking any phosphatase inhibitory activity had no effect on the ability to generate depotentiation (Table 1). Moreover, treatment with each of these drugs alone failed to affect the induction of LTP (Table 1).

To examine the possible contribution of PP2B (calcineurin) to depotentiation, slices were preincubated for 2–4 h in cyclosporin A (250 μM). We found that cyclosporin A also prevented the generation of both LFS- and DCG-IV-induced depotentiation. The mean residual potentiation measured 40 min after the end of LFS or washout of DCG-IV was $187 \pm 21\%$ ($n = 10$; $P < 0.05$; Student's unpaired t test) and $180 \pm 15\%$ ($n = 6$; $P < 0.05$; Student's unpaired t test) of the control baseline, respectively (Table 1). Similarly, another inhibitor of PP2B, FK-506 (10 μM), also

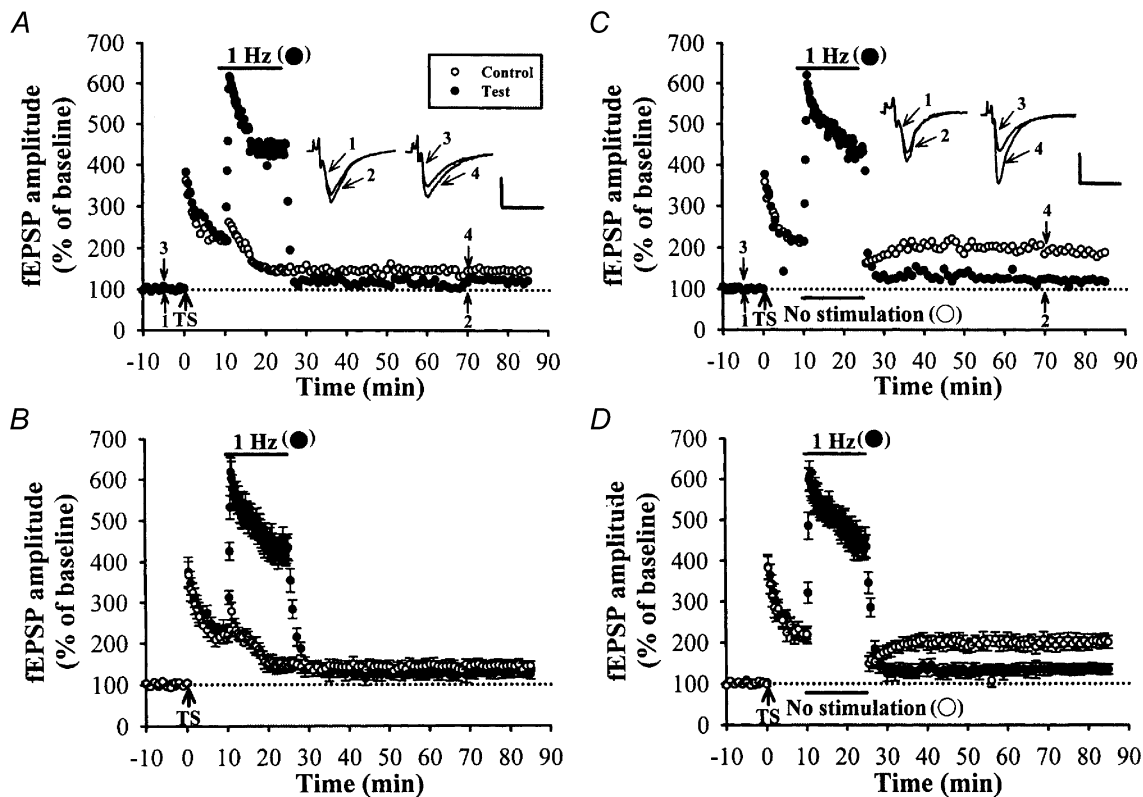


Figure 4. Heterosynaptic reversal of LTP by LFS requires activity in the heterosynaptic inputs

A, a typical example showing LTP was first induced on two independent afferents (no heterosynaptic facilitation) by coactivation, followed by LFS (1 Hz, 15 min) applied to only one afferent (test pathway). This results in a homosynaptic reversal of LTP at the test pathway and also in a heterosynaptic reversal of LTP induced previously at the control pathway. B, summary of data from four experiments performed as in A. C, an example experiment identical to those shown in A, with the exception that the presynaptic stimulation of control pathway was stopped during LFS application in the test pathway. Stopping stimulation completely prevented the generation of heterosynaptic reversal of LTP. D, summary data from six experiments performed as in C. The superimposed fEPSP in the inset of each graph illustrates respective recordings from example experiments taken at the time indicated by numbers. Upward arrow indicates application of TS. Horizontal bars indicate the period of the delivery of LFS. The horizontal dashed lines indicate the average value of the normalized amplitude during the control period. Calibration: 0.2 mV, 10 msec

Table 1. Effects of protein phosphatase inhibitor treatments on LTP or LFS- or DCG-IV-induced depotentiation at the hippocampal mossy fibre-CA3 synapses

Drug treatment	LTP 40 min after TS (%)	Residual LTP 40 min after LFS following TS (%)	Residual LTP 40 min after washout of DCG-IV (%)
Control	216 ± 13 (12) *	127 ± 15 (10)	108 ± 11 (8)
Okadaic acid (1 μM for 2–4 h)	208 ± 15 (8) *	183 ± 11 (10) *	193 ± 12 (5) *
Calyculin A (1 μM for 2–4 h)	212 ± 18 (6) *	178 ± 14 (10) *	186 ± 13 (6) *
Norkadaone (1 μM for 2–4 h)	207 ± 15 (3) *	109 ± 10 (3)	113 ± 11 (3)
Cyclosporin A (250 μM for 2–4 h)	221 ± 18 (8) *	187 ± 21 (10) *	180 ± 15 (6) *
FK-506 (10 μM for 2–4 h)	215 ± 17 (8) *	190 ± 19 (8) *	197 ± 14 (6) *

LFS or DCG-IV (3 μM) was applied 10 min after LTP induction. The data represent the percentage of baseline for fEPSP amplitude. Number of experiments is in parentheses. * Significantly different from baseline (Student's unpaired *t* test, *P* < 0.05).

prevented the induction of depotentiation by LFS DCG-IV. Moreover, treatment with either of these drugs alone failed to affect the induction of LTP. Because the concentrations of okadaic acid and calyculin A used in this study inhibit not only PP1 but also PP2A, we suggest that the activation of both PP1 and/or PP2A and PP2B is indeed an absolute requirement for LFS- and DCG-IV-induced depotentiation at the mossy fibre-CA3 synapses.

Depotentiation elicited by LFS and DCG-IV require a decrease in presynaptic cAMP level

In the last set of experiments we examined how group II mGluRs activation might reverse the previously established LTP. As for mechanisms responsible for the group II mGluR-mediated presynaptic modulation at these synapses, two possibilities have been reported. First, it reduces the action potential-induced presynaptic Ca²⁺ influx and inhibits the release machinery (Kamiya & Ozawa, 1999). Second, it inhibits the production of cAMP via the activation of a G_{i/o} and subsequent inhibition of adenylyl cyclase (Pin & Duvoisin, 1995). The decrease in cAMP could, in turn, reduce the PKA activity, which is known to be an important regulator of mossy fibre synaptic transmission and plasticity (Huang *et al.* 1994; Weisskopf *et al.* 1994). If group II mGluR-mediated decrease in presynaptic Ca²⁺ influx is a key trigger for LFS-induced depotentiation, a manipulation that decreases Ca²⁺ influx should mimic LFS to elicit depotentiation. To test this possibility, we tested the effect of decreasing Ca²⁺ influx by removal of extracellular Ca²⁺ on the development of LTP. We found that application of Ca²⁺-free medium for 10 min beginning 10 min after LTP induction caused a rapid suppression of evoked synaptic response. After returning to the normal extracellular Ca²⁺ concentration medium, the synaptic responses consistently returned to a potentiated level – the amplitude of fEPSP measured 40 min after washout of Ca²⁺-free medium was 196 ± 16% (*n* = 6) of the control value. These results suggest that group II mGluR-mediated depotentiation is not due to a reduction of presynaptic Ca²⁺ influx and also confirm the previous

finding (Regehr & Tank, 1991) that the maintenance of mossy fibre LTP is independent of sustained presynaptic Ca²⁺ influx.

We next examined the second possibility that a group II mGluR-mediated decrease in cAMP in mossy fibre nerve terminals is essential for triggering depotentiation. If a decrease in presynaptic cAMP levels is indeed required for the induction of depotentiation, then preventing this decrease should block the induction of depotentiation. To examine this idea, we continuously applied an adenylyl cyclase activator, forskolin, or a non-hydrolysable and membrane-permeant cAMP analogue, Sp-8-CPT-cAMPS, to clamp presynaptic cAMP concentration. Application of forskolin (50 μM) alone caused a persistent increase in the amplitude of fEPSPs (398 ± 47% of the control, *n* = 6; *P* < 0.05; Student's paired *t* test; Fig. 5A) and completely blocked the DCG-IV- (3 μM; 368 ± 36% of the control, *n* = 6; *P* > 0.05 when compared with the response during the application of forskolin alone; Student's unpaired *t* test; Fig. 5B) or LFS-induced long-term synaptic depression (396 ± 26% of the control, *n* = 6; *P* > 0.05 when compared with the response during the application of forskolin alone; Student's unpaired *t* test; Fig. 5C). Similarly, bath application of Sp-8-CPT-cAMPS (100 μM) alone also caused the expected increase in the synaptic response (173 ± 14% of the control, *n* = 6; *P* < 0.05; Student's paired *t* test; Fig. 5D) and completely blocked the DCG-IV- (3 μM; 174 ± 15% of the control, *n* = 5; *P* > 0.05 when compared with the response during the application of Sp-8-CPT-cAMPS alone; Student's unpaired *t* test; Fig. 5E) and LFS-induced long-term synaptic depression (179 ± 15% of the control baseline, *n* = 5; *P* > 0.05 when compared with the response during the application of Sp-8-CPT-cAMPS alone; Student's unpaired *t* test; Fig. 5F). These results provide further support for our hypothesis that LFS-induced depotentiation is mediated via a group II mGluR-coupled decrease in the presynaptic cAMP level of the mossy fibre boutons.

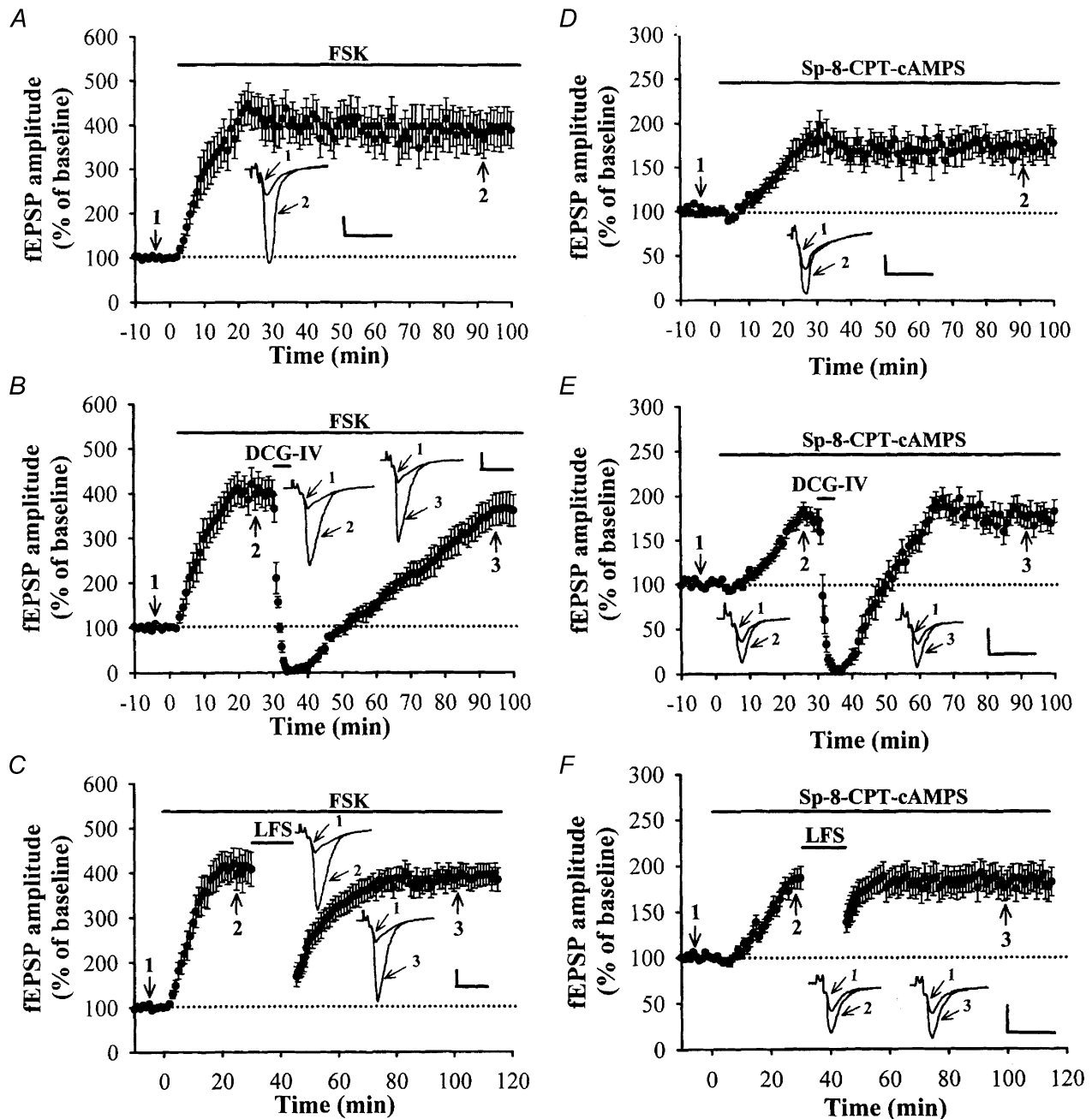


Figure 5. A decrease in cAMP level is important for the induction of mossy fibre depotentiation.

A, summary of experiments ($n = 6$) in which forskolin (50 μ M) was applied alone caused a persistent increase in the synaptic response. *B*, summary of experiments ($n = 5$) in which forskolin (50 μ M) was applied prior the application of DCG-IV (3 μ M) for 5 min. Forskolin completely blocked the DCG-IV-induced long-term synaptic depression. *C*, summary of experiments ($n = 5$) in which forskolin was applied prior the delivery of LFS (1 Hz, 15 min). Forskolin completely blocked LFS-induced long-term synaptic depression. *D*, summary of experiments ($n = 6$) in which Sp-8-CPT-cAMPS (100 μ M) was applied alone caused a persistent increase in the synaptic response. *E*, summary of experiments ($n = 5$) in which Sp-8-CPT-cAMPS (100 μ M) was applied prior the application of DCG-IV (3 μ M) for 5 min. Sp-8-CPT-cAMPS completely blocked the DCG-IV-induced long-term synaptic depression. *F*, summary of experiments ($n = 5$) in which Sp-8-CPT-cAMPS (100 μ M) was applied prior the application of LFS. Sp-8-CPT-cAMPS completely blocked LFS-induced long-term synaptic depression. The superimposed fEPSP in the inset of each graph illustrates recordings from example experiments taken at the time indicated by numbers. Upward arrow indicates application of TS. Horizontal bars indicate the period of the delivery of LFS or pharmacological agents as indicated. The horizontal dashed lines indicate the average value of the normalized amplitude during the control period. Calibration: 0.2 mV, 10 msec.

DISCUSSION

The major new findings in the present study are as follows. First, the non-Hebbian forms of mossy fibre LTP could be reversed by long trains of LFS protocol and this LFS-induced depotentiation is expressed, at least in part, by a reversal of the enhancement of release probability associated with LTP. Second, the activation of presynaptic group II mGluRs is necessary, but not sufficient for the LFS-induced depotentiation. It also requires an activity-dependent elevation of presynaptic $[Ca^{2+}]_i$, and the activation of PP-coupled signalling. Third, a group II mGluR-mediated decrease in adenylyl cyclase activity, which reduces the cytosolic cAMP level is involved in the induction of depotentiation by LFS protocol.

The locus of expression of LFS-induced depotentiation

In addressing the locus of expression of synaptic plasticity, a change in paired-pulse facilitation (PPF) is frequently used as an indication of presynaptic expression. Our previous result showed that LFS-induced depotentiation at the mossy fibre-CA3 synapses is accompanied by a marked reduction of PPF attenuation during LTP, suggesting that the expression of LFS-induced depotentiation is presynaptic (Chen *et al.* 2001). However, there is now increasing evidence that the magnitude of PPF is also regulated by postsynaptic mechanisms (Wang & Kelly, 1996). In the present work, further results obtained by examining the failure and variance of synaptic response, and the rate decay of the EPSC_{NMDA} in the presence of MK-801 have further confirmed a presynaptic mechanism for LFS-induced depotentiation, and demonstrated that the expression of LFS-induced depotentiation involves a reduction in the enhancement of release probability seen with LTP. These results, however, cannot exclude the possibility that the apparent decrease in the release probability involves a reduction in the number of functional synapses during the expression of depotentiation. Recent evidence has shown the existence of silent synapses and silent release sites, which become functional during LTP (Kullmann, 1994; Liao *et al.* 1995). LFS-mediated reduction in quantal release could thus result from a downregulation of the number of functional synapses or release sites, a change reciprocal to that which occurs in LTP.

Signalling events downstream from the activation of mGluRs and G_{i/o}

Metabotropic glutamate receptors are a class of G-protein coupled receptors and produce a variety of effects depending upon the subtype of receptor activated (Anwyl, 1999). It was shown previously that mGluRs are involved in the induction of LFS-induced depotentiation in the dentate gyrus (Kulla *et al.* 1999) and CA1 region of the hippocampus (Bashir & Collingridge, 1994; but see Selig *et al.* 1995). On the other hand, experiments utilizing mutant mice and pharmacological antagonists suggest that mGluRs

also play a pivotal role in mediating the induction and expression of mossy fibre LTP and LTD (Conquet *et al.* 1994; Kobayashi *et al.* 1996; Yokoi *et al.*, 1996; but see Hsia *et al.* 1995). We have shown previously that LFS-induced depotentiation was prevented by the non-selective group II mGluR antagonist MCPG and the potent group II mGluR antagonist LY 341495, and was mimicked by bath-applied potent group II mGluR agonist DCG-IV, and have suggested that activation of group II mGluRs may contribute to the induction of mossy fibre depotentiation (Chen *et al.* 2001). Here, we have extended these findings by showing that a decrease in presynaptic cAMP levels contributes to this process. We found that both DCG-IV and LFS-induced long-term synaptic depression were prevented by the pharmacological elevation of cAMP levels. Our data also exclude the possibility that group II mGluR-mediated decrease in presynaptic Ca^{2+} influx is the trigger for LFS-induced depotentiation, because the maintenance of mossy fibre LTP is independent of a sustained increase of the presynaptic Ca^{2+} influx (Regehr & Tank, 1991).

Activation of group II mGluRs alone is insufficient for depotentiation

A surprising finding was that triggering mossy fibre depotentiation required both synaptic activity and group II mGluR activation. Because suppression of a rise in the presynaptic $[Ca^{2+}]_i$ by a membrane-permeant Ca^{2+} chelator, EGTA-AM, reduced the magnitude of DCG-IV-induced depotentiation, an elevation of presynaptic $[Ca^{2+}]_i$ due to synaptic activation is an additional presynaptic factor required for the induction of depotentiation. Similar results were also previously obtained in mossy fibre LTD (Tzounopoulos *et al.* 1998; Kobayashi *et al.* 1999). These findings are of interest because mossy fibre LTP induced by high-frequency stimulation is also thought to require the increase in presynaptic $[Ca^{2+}]_i$ (Castillo *et al.* 1994). How can the same signal, the increase in presynaptic $[Ca^{2+}]_i$, cause opposite effects on the synaptic strength? A potential explanation of this may be that the differential increase in the magnitude of presynaptic $[Ca^{2+}]_i$ may determine the direction of synaptic strength via the activation of distinct enzymes with different affinities for Ca^{2+} (Lisman, 1989). In fact, previous study has demonstrated that, in the mossy fibre terminals, 100 Hz tetanic stimulation may cause a larger elevation of $[Ca^{2+}]_i$ than 1 Hz LFS did (Regehr & Tank, 1991). Thus, the magnitude of Ca^{2+} increase in the presynaptic terminal is an important factor to determine the direction of plasticity at the hippocampal mossy fibre synapses. In addition, we have previously reported that LFS-induced depotentiation at this synapse is not input specific, perhaps through spillover mechanisms (Chen *et al.* 2001). In the present study, we have extended this finding by observing that heterosynaptic activity-dependent depotentiation also requires activity in the heterosynaptic input.

Protein phosphatases are involved in the induction of depotentiation

What is the biological step downstream of Ca^{2+} entry responsible for the induction of depotentiation? The most likely candidates are PP-coupled signalling processes. Because mossy fibre LTP is known to be mediated through the activation of PKA, it is reasonable to speculate that the reversal of LTP by LFS protocol is due to preferential activation of PP to override and shut off the PKA-dependent signalling cascades. This idea is supported by the findings that both the LFS- and DCG-IV-induced depotentiation were prevented by PP inhibitors (Table 1). Interestingly, we found that both of the PP1/PP2A inhibitors okadaic acid and calyculin A and PP2B inhibitors cyclosporin A and FK-506 prevented the generation of LFS- and DCG-IV-induced depotentiation, suggesting that these subtypes of serine/threonine PP contribute to the generation of mossy fibre depotentiation. Since intracellular Ca^{2+} does not directly influence PP1, there should be another Ca^{2+} -dependent mediator or signalling cascade to translate the Ca^{2+} signal into an increase in PP1 activity (Lisman, 1989). The most likely candidate for this process is PP2B, a Ca^{2+} /calmodulin-dependent PP that can indirectly increase the PP1 activity by dephosphorylating the inhibitory protein (I1) of PP1 (Shenolikar & Nairn, 1991). Thus, it is possible that Ca^{2+} influx induced by LFS activates calmodulin and PP2B, which in turn dephosphorylates and hence inactivates PP1 regulatory protein I1. This removes the braking effect of I1 on PP1, allowing PP1 to become active, and therefore dephosphorylates the presynaptic phosphoproteins participating in the stable expression of LTP. An open question that deviated from this observation is that of which target phosphoproteins are involved in PP-coupled processes and lead to the expression of depotentiation. A plausible candidate is RIM1 α , an active zone protein that binds to synaptic vesicle protein Rab3A and is also a PKA substrate (Wang *et al.* 1997, 2000). This prediction is based on the current findings of Castillo *et al.* (2002), who demonstrated the genetic evidence that mossy fibre LTP in the hippocampus is abolished in mice lacking RIM1 α .

In conclusion, our study suggests that mossy fibre depotentiation is expressed presynaptically through the activation of both presynaptic mGluRs and PPs. Our current findings may add to a growing body of literature suggesting that the modulation of the presynaptic $[\text{Ca}^{2+}]_i$ and cAMP-mediated signalling pathways plays an important role in the bidirectional modulation of synaptic strength at the mossy fibre synapses by activity (Tzounopoulos *et al.* 1998; Henze *et al.* 2000).

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Supplementary material

The online version of this paper can be found at:

<http://www.jphysiol.org/cgi/content/full/543/3/767>

and contains material entitled:

The induction of mossy fibre LTP

The data demonstrate that the magnitude of mossy fibre LTP of field potential recordings in slices obtained from 12- to 16-day-old and 4- to 6-week-old mice was indistinguishable.