Characterization of the Mechanism Underlying the Reversal of Long Term Potentiation by Low Frequency Stimulation at Hippocampal CA1 Synapses

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Reversal of long term potentiation (LTP) may function to increase the flexibility and storage capacity of neuronal circuits; however, the underlying mechanisms remain incompletely understood. We show that depotentiation induced by low frequency stimulation (LFS) (2 Hz, 10 min, 1200 pulses) was input-specific and dependent on N-methyl-d-aspartate (NMDA) receptor activation. The ability of LFS to reverse LTP was mimicked by a brief application of NMDA. This NMDA-induced depotentiation was blocked by adenosine A<sub>1</sub> receptor antagonist. However, the reversal of LTP by LFS was unaffected by metabotropic glutamate receptor antagonism. This LFS-induced depotentiation was specifically prevented by protein phosphatase (PP1) inhibitors, okadaic acid, and calyculin A but not by the PP2A or PP2B inhibitors. Furthermore, by using phosphorylation site-specific antibodies, we found that LFS-induced depotentiation is associated with a persistent dephosphorylation of the GluR1 subunit of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor at serine 831, a protein kinase C and calcium/calmodulin-dependent protein kinase II (CaMKII) substrate, but not at serine 845, a substrate of cAMP-dependent protein kinase. This effect was mimicked by bath-applied adenosine or NMDA and was specifically prevented by okadaic acid. Also, the increased phosphorylation of CaMKII at threonine 286 and the decreased PP activity seen with LTP were overcome by LFS, adenosine, or NMDA application. These results suggest that LFS erases LTP through an NMDA receptor-mediated activation of PP1 to dephosphorylate amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and CaMKII in the CA1 region of the hippocampus.

Long term potentiation (LTP)<sup>1</sup> is a long lasting form of synaptic plasticity that is thought to play important roles in learning and memory in the brain (1). Although LTP is very persistent, current work has provided evidence that various manipulations or pharmacological treatment when applied shortly after LTP induction can reverse it. For example, it has been shown that transient anoxia occurring 1–2 min after LTP induction prevented the stable expression of LTP (2). Such time-dependent reversal of LTP was also effectively induced by low frequency afferent stimulation (1–5 Hz) when delivered within 10 min of LTP induction, both in vivo (3, 4) and in vitro (5–7). In addition, antagonists that prevent cell-cell and cell-matrix interactions were also observed to reverse effectively LTP in a time-dependent manner (8, 9). This reversal of synaptic strength from the potentiated state to pre-LTP levels has been called 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 the neuronal networks (10). Although depotentiation has been consistently demonstrated in several brain regions including hippocampus (3–6, 11–14), visual cortex (15), sensorimotor cortex (16), and prefrontal cortex (17), the exact biochemical processes and molecular mechanisms responsible for this synaptic plasticity are incomplete.

We have demonstrated previously that the LFS-induced depotentiation at Schaffer collateral-CA1 synapses may attribute to an increase of extracellular adenosine acting on the A<sub>1</sub> adenosine receptors to interrupt the cAMP-PKA-dependent signaling cascade leading to the development of LTP (7). In addition, the induction of depotentiation requires the activation of PP-coupled cascades. In this study, we describe experiments in the CA1 region of rat hippocampus designed to characterize further this form of synaptic plasticity using both electrophysiological and biochemical techniques. In particular, we addressed the following questions. 1) Is the LFS-induced depotentiation input-specific and dependent on NMDA and/or mGluR activation? 2) Does the pharmacological activation of NMDA receptors mimic the LFS to elicit a time-dependent reversal of LTP? 3) What is the kind of PP participating in LFS-induced depotentiation, and what might be its substrate(s) after its activation during depotentiation? We provide evidence that a PP1-mediated dephosphorylation of GluR1 subunit of AMPA receptors at serine 831, known as a substrate of CaMKII and PKC, may be a major expression mechanism for NMDA receptor-dependent homosynaptic LFS-induced depotentiation. These results also imply that PP1 may serve as the sensor of the level of neuronal activity and in turn regulates the development of LTP at Schaffer collateral-CA1 synapses.
**EXPERIMENTAL PROCEDURES**

**Slice Preparation**—Hippocampal slices were prepared from 4 to 5-week-old male Sprague-Dawley rats as described previously (7, 18). Briefly, rats were anesthetized with halothane and decapitated soon after the disappearance of any corneal reflexes. The brain was rapidly removed and immersed in ice-cold artificial cerebral spinal fluid (ACSF) solution (composition in mm: NaCl 117, KCl 4.7, CaCl2 2.5, MgCl2 1.2, NaHCO3 25, NaHPO4 1.2, and glucose 11 at pH 7.3–7.4) bubbled with 95% O2, 5% CO2. A block of hippocampus was removed, and the middle third of the hippocampus was sectioned in the transverse plane into 400-μm thick slices with a Leica VT1000S tissue slicer (Leica, Nussloch, Germany). After their preparation, slices were placed in a holding chamber of ACSF bubbled with 95% O2, 5% CO2 and kept at room temperature for at least 1 h before recording.

**Electrophysiological Experiments and Statistical Analysis**—For the electrophysiological recording, 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 at 32.0 ± 0.5 °C. Standard extracellular field recording techniques were used (7, 18). Field excitatory postynaptic potentials (fEPSPs) were recorded from the stratum radiatum of the CA1 region of the hippocampus using a glass microelectrode filled with 1 M NaCl (resistance 2–3 megohms). A bipolar stainless steel stimulating electrode was placed in stratum radiatum to activate Schaffer collateral/commissural afferents at 0.033 Hz. To stimulate independent inputs to the same cell population, two bipolar stimulating electrodes were positioned on both sides of the recording microelectrode, alternating every 15 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 spike-free response. In all experiments base-line synaptic transmission was monitored for 30 min before drug administration or delivery of low frequency afferent stimulation. The synaptic strength of synaptic transmission was quantified by measuring the slope of fEPSP. The fEPSP slopes were measured from ~20 to 70% of the rising phase using a least squares regression. LTP was induced by high frequency stimulation, at the test pulse intensity, consisting of two 1-s trains of 100 Hz, delivered with an interval of 20 s. Depotentiation was induced by application of 10 min low frequency trains of stimuli at 2 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 relative potentiation reported here were calculated as the changes in fEPSP slope measured 40 min after the end of LFS. Microelectrodes were pulled from microfiber 1.0-mm capillary tubing using a P-97 electrode puller (Sutter Instrument Co., Novato, CA). Electrical signals were collected with Axon Instruments, Foster, CA) fitted with a 1-kHz cut-off frequency, sampled at 10 kHz, and an Intel Pentium-based computer with pCLAMP software (version 7.0, Axon Instruments, Foster, CA) was used to acquire on-line and analyze off-line the data.

All drugs were applied by dissolving them in the desired final concentration in 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. The slice preparation for electrophysiological experiments. At the end of the experiments, the CA1 subregion of the hippocampal slices between the positions of the stimulating and recording electrodes was dissected out and immediately frozen on dry ice. Three to four microdissected CA1 subregions (from control, 30 min after LTP induction, 17 min after the end of LFS, 24 min after washout of adenine, or 22 min after washout of NMDA) were homogenized in the electrophysiological assay system (see below). In each experiment, an entire set of LTP or depotentiation pooled slices was taken from one animal. The microdissected subregions were lysed in ice-cold Tris-HCl buffer solution (TBS; pH 7.4) containing a mixture of protein phosphatase and protein inhibitors (50 mM Tris-HCl, 100 mM NaCl, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM microcystin-LR, 1 μM okadaic acid, 0.5% Triton X-100, 2 mM benzamidine, 60 μg/ml aprotinin, and 60 μg/ml leupeptin) to avoid dephosphorylation and degradation of proteins, and were and kept at -20°C until the day of experiment. The concentration of Me2SO in the protein of CA1 homogenate was measured with the Protein Phosphatase Assay System (Invitrogen) essentially as described by Blitzer et al. (20).

**RESULTS**

**Input Specificity of LFS-induced Depotentiation**—The first series of experiments was designed to investigate whether LFS-induced depotentiation is input-specific (i.e., homosynaptic). To address this question, two stimulating electrodes were placed on both sides of the recording microelectrode in the CA1 region to activate two groups of inputs to the same cell population. As a typical example illustrated in Fig. 1A, independence of inputs activated by the two electrodes was assessed by verifying the absence of heterosynaptic facilitation between the two inputs using paired stimuli applied at 40-ms interval. By having confirmed the independence of afferents activated, LTP was induced by a high frequency afferent stimulation (see Experimental Procedures) to both pathways at the same condition; this was followed by a 2-Hz/10-min LFS to only one pathway to reverse LTP. Under these conditions, the control (heterosynaptic) pathway that did not receive LFS exhibited a normal degree of LTP, whereas LFS resulted in a completely reversed LTP in the test (homosynaptic) pathway. At control pathway, the mean residual potentiation measured 40...
after the end of LFS was 138.5 ± 7.2% (n = 7) of base line, which was not significantly different from the LTP measured in control slices without LFS delivered (138.6 ± 9.7% of base line; n = 12) (7). In contrast, at test pathway, the mean residual potentiation measured 40 min after the end of LFS was 105.6 ± 6.5% (n = 7) of base line (Fig. 1B). These results indicated that the reversal of LTP by LFS is specific to the synapses receiving the stimulation. Therefore, LFS-induced depotentiation at Schaffer collateral-CA1 synapses is an input-specific or homosynaptic phenomenon, as is LTP (1).

LFS-induced Depotentiation Is Dependent on NMDA Receptor Activation—We next sought to investigate whether activation of NMDA receptors is necessary for LFS-induced depotentiation. The NMDA dependence of depotentiation in hippocampal CA1 region has been studied previously, but the results have been controversial (5, 11, 21, 22). To test this point further, the NMDA receptor antagonist D-APV (50 μM) was present when LFS was delivered to elicit depotentiation. As shown in the Fig. 2, in the presence of D-APV, the LFS-induced depotentiation was completely prevented. On average, the residual potentiation measured 40 min after the end of LFS was 133.2 ± 6.4% (n = 8; p < 0.05; unpaired Student’s t test) of base line. These results confirmed the previous findings of Fuji et al. (5) and O’Dell and Kandel (11) showing that LFS-induced depotentiation at this synapse requires the activation of NMDA receptors.

To establish further that the LFS-induced depotentiation is mediated through the activation of NMDA receptors, it is essential to demonstrate that LFS-induced depotentiation should be mimicked by the direct application of NMDA. For this purpose, the effect of NMDA on the development of LTP was investigated. The base-line responses to stimulation of two independent inputs to a common population of CA1 neurons were monitored in alteration. A high frequency tetanic stimulation was given only to the test pathway to induce homosynaptic LTP, and then NMDA was bath-applied to the slice at different times after LTP induction. In the initial experiments, NMDA was applied immediately after the induction of LTP. A concentration of 10 μM was used because previous work (23–25) showed that a brief application of this concentration did not produce a long-lasting effect of the evoked synaptic response. A brief (5 min) bath application of NMDA (10 μM) to hippocampal slices resulted in a robust depression of fEPSP, as described (23–25). After washout of NMDA, the fEPSP of control pathway was recovery completely (98.7 ± 5.3% of base line, n = 8; p > 0.05; paired Student’s t test). However, at the test pathway, the fEPSP recovered to close to the pretetanus base line after washout of NMDA. On the average, the fEPSP slope measured 40 min after washout of NMDA was 109.8 ± 7.1% (n = 8; p > 0.05; paired Student’s t test) of base line (Fig. 3A). As shown in Fig. 3B, the reversal of LTP similar to the above results was also obtained with administration of NMDA beginning 3 min after the LTP induction. The slope of fEPSP measured 40 min after NMDA washout was 114.6 ± 6.7% (n = 7; p > 0.05; paired Student’s t test) of base line. In contrast, when NMDA was applied 10 min after LTP induction, the synaptic responses consistently recovered to the potentiated level, i.e. the slope of fEPSP measured 40 min after NMDA washout was 152.4 ± 8.9% (n = 8; p < 0.05; paired Student’s t test) of base line (Fig. 3C). These results confirmed and extended the previous findings of Lee et al. (24) showing that direct application of NMDA can reveal a time-dependent reversal of LTP in the CA1 region of rat hippocampal slices.

Relationship of NMDA Receptor Activation and Adenosine-mediated Signaling Pathways in Triggering Depotentiation—The above results promote the idea that activation of NMDA receptors may initiate a process that interrupts the biochemical mechanisms leading to the stabilization of LTP. We next investigated what was the biochemical step downstream of NMDA receptor activation that generates LFS-induced depotentiation. It was therefore of interest to examine the relationship between the NMDA receptor activation and adenosine-mediated signaling pathways in the development of depotentiation. We began by confirming that LFS-induced depotentiation can be mimicked by the direct extracellular application of adenosine. In agreement with our previous findings (7), bath application of adenosine (0.2 mM for 3 min) beginning 3 min after LTP induction completely reversed LTP. On average, the slope of fEPSP measured 40 min after washout of adenosine was 108.8 ± 6.7% (n = 6; p > 0.05; unpaired Student’s t test) of base line (Fig. 4A). We next determined whether adenosine was downstream signaling...
The end of LFS (n depotentiation was not affected when NMDA receptor antagonist D-APV (50 μM) was applied during the time-dependent induction of depotentiation, if application started 1 min after LTP induction and continued until the end of adenosine application. On average, the EPSP slope measured 40 min after washout of adenosine was 106.3 ± 6.8% (n = 5; p > 0.05; unpaired Student’s t test) of base line (Fig. 4C). Therefore, adenosine seems to be the downstream signal for NMDA receptor activation to trigger depotentiation.

mGluR Antagonists Did Not Affect LFS-induced Depotentiation—Previous studies (21, 28, 29) have revealed that metabotropic glutamate receptor (mGluR) activation is primarily necessary for the induction of depotentiation because its induction can be blocked by the mGluR antagonist MCPG (30). However, this conclusion has been questioned, because a recent report (30), conducted independently by the laboratories of Bear and Malenka, failed to find any effect of MCPG on CA1 depotentiation. The reason for this discrepancy remains unclear. In an attempt to gain further insight into the role of mGluRs in triggering depotentiation, we next carried out a series of experiments in which we analyzed the effects of mGluR antagonists on the development of LFS-induced depotentiation. Fig. 5 summarizes our pharmacological examination of LFS-induced depotentiation. Initially, to assess the role of group I mGluR in the LFS-induced depotentiation, the selective group I mGluR antagonist AIDA was applied during the delivery of LFS. Under this condition, we did not see any effect of AIDA (500 μM) on the LFS-induced depotentiation; the level of depotentiation did not differ from the control values. The residual potentiation measured 40 min after the end of LFS was 106.6 ± 6.3% (n = 8; p > 0.05; unpaired Student’s t test) of base line (Fig. 5A). In additional experiments we tested the effect of group II and group III mGluR antagonists on the LFS-induced depotentiation. As illustrated in Fig. 5, B and C, neither the non-selective group II mGluR antagonist MCPG (500 μM) nor the selective group III mGluR antagonist α-methyl-l-2-amino-4-phosphonobutyrate (MAP4, 500 μM) affects the induction of depotentiation, if application started 1 min after LTP induction and continued until the end of LFS. The residual potentiation measured 40 min after the end of LFS was 107.2 ± 6.7% (n = 9; p > 0.05; unpaired Student’s t test) and 108.2 ± 6.7% (n = 7; p > 0.05; unpaired Student’s t test) of base line, respectively. These results confirmed and extended the previous findings (30) showing that activation of mGluRs is not an absolute requirement for the LFS-induced depotentiation in the hippocampal CA1 region.

Involvement of Protein Phosphatase 1 in LFS-induced Depotentiation—Because hippocampal CA1 LTP is generally thought to be due, at least in part, to the activation of several postsynaptic protein kinases including Ca²⁺/calmodulin-dependent protein kinase II (CaMK II), protein kinase C (PKC), mitogen-activated protein (MAP) kinase, protein kinase A (PKA), and Src family protein tyrosine kinases (1, 31, 32), it is therefore reasonable to suggest that LFS may elicit depoten-
tiation by interfering with the protein phosphorylation processes required for LTP, perhaps by activating PP-coupled cascades. This proposal was supported by the observation that pretreatment of the hippocampal slices with serine/threonine-PP inhibitors, okadaic acid, or calyculin A hippocampal slices prevents the LFS-induced depotentiation at Schaffer collateral-CA1 synapses (7, 11, 22). The findings of PP inhibitors to effectively prevent the induction of depotentiation raise an important question: which subtype of PPs is essential for the depotentiation induction? To address this question, we have examined the effects of multiple subtypes of PP inhibitors on the LFS-induced depotentiation.

If PP1 is critical for LFS-induced depotentiation, blockade of PP1 activity should inhibit the induction of depotentiation by LFS. To test this idea, we examined the effect of potent PP1 inhibitors, okadaic acid and calyculin A, on the development of depotentiation. Consistent with previous reports (7, 11), following a 2–4-h preincubation in okadaic acid (1 μM), the LFS-induced depotentiation was markedly inhibited. On average, the residual potentiation measured 40 min after the end of LFS was 132.7 ± 4.9% (n = 12; p < 0.05) when compared with control depotentiation slices; unpaired Student’s t test) of base line. Moreover, calyculin A (1 μM) also prevented induction of depotentiation. The mean residual potentiation measured 40 min after the end of LFS was 143.7 ± 5.1% (n = 12; p < 0.05) when compared with control depotentiation slices; unpaired Student’s t test) of base line (Table I). In contrast, preincubation of slices in 1-norkadone (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. The mean residual potentiation measured 40 min after the end of LFS was 104.6 ± 3.7% (n = 4; p > 0.05) when compared with control depotentiation slices; unpaired Student’s t test) of base line. Moreover, all of these treatments alone failed to affect the LTP induction. The fEPSP slope measured 40 min after LTP induction was 147.6 ± 5.3% (n = 8; p > 0.05 when compared with control LTP slices; unpaired Student’s t test), 143.9 ± 6.2% (n = 8; p > 0.05 when compared with control LTP slices; unpaired Student’s t test), and 142.6 ± 3.7% (n = 4; p > 0.05 when compared with control LTP slices; unpaired Student’s t test) of base line, respectively. Because both okadaic acid and calyculin A, at a dose of 1 μM, inhibit not only the PP1 (IC50 = 10–15 and 2 nM, respectively) but also PP2A (IC50 = 0.5–1 and 0.5–1 nM, respectively) (33, 34), it is possible that the blockade of depotentiation induction by these two pharmacological agents observed here is due to their inhibition of PP2A activity. To explore this possibility, we performed experiments in which the dose of okadaic acid was reduced to 3 nM, a dose that has been shown to inhibit selectively PP2A without affecting PP1 activity. In 3 nM okadaic acid pretreatment slices, robust LFS-induced depotentiation was observed. The mean residual potentiation measured 40 min after the end of LFS was 105.8 ± 6.5% (n = 6) of base line, which is not significantly different from control slices. Similar results were also obtained by preincubation of the slices with another PP2A inhibitor, fostriecin (10 nM; IC50 = 1.5–3.2 nM for PP2A and IC50 = 131 μM for PP1) (35, 36). On average, the residual potentiation measured 40 min after the end of LFS was 112.1 ± 6.1% (n = 6; p > 0.05 when compared with control depotentiation slices; unpaired Student’s t test) of base line (Table I). Moreover, neither the okadaic acid nor fostriecin treatment alone affects the LTP induction. The fEPSP slope measured 40 min after LTP induction was 139.3 ± 6.7% (n = 6; p > 0.05 when compared with control LTP slices; unpaired Student’s t test) and 139.7 ± 5.8% (n = 6; p > 0.05 when compared with control LTP slices; unpaired Student’s t test) of base line, respectively. These results indicate that the activation of PP1 but not PP2A plays an essential role in the development of LFS-induced depotentiation.

To examine the possible contribution of PP2B (calcineurin) to depotentiation, slices were preincubated for 2–4 h in cyclopasin A (250 μM). We found that cyclosporin A possessed no significant effect on the LFS-induced depotentiation; the degree of depotentiation did not differ from the control slices. The mean residual potentiation measured 40 min after the end of LFS was 109.4 ± 6.3% (n = 8; p > 0.05 when compared with control depotentiation slices; unpaired Student’s t test) of base line, 109.2 ± 6.4% (n = 8; p > 0.05 when compared with control LTP slices; unpaired Student’s t test) of base line, respectively. Moreover, all of these treatments alone failed to affect the LTP induction. The fEPSP slope measured 40 min after LTP induction was 147.6 ± 5.3% (n = 8; p > 0.05 when compared with control LTP slices; unpaired Student’s t test), 143.9 ± 6.2% (n = 8; p > 0.05 when compared with control LTP slices; unpaired Student’s t test), and 142.6 ± 3.7% (n = 4; p > 0.05 when compared with control LTP slices; unpaired Student’s t test) of base line, respectively. Because both okadaic acid and calyculin A, at a dose of 1 μM, inhibit not only the PP1 (IC50 = 10–15 and 2 nM, respectively) but also PP2A (IC50 = 0.5–1 and 0.5–1 nM, respectively) (33, 34), it is possible that the blockade of depotentiation induction by these two pharmacological agents observed here is due to their inhibition of PP2A activity. To explore this possibility, we performed experiments in which the dose of okadaic acid was reduced to 3 nM, a dose that has been shown to inhibit selectively PP2A without affecting PP1 activity. In 3 nM okadaic acid pretreatment slices, robust LFS-induced depotentiation was observed. The mean residual potentiation measured 40 min after the end of LFS was 105.8 ± 6.5% (n = 6) of base line, which is not significantly different from control slices. Similar results were also obtained by preincubation of the slices with another PP2A inhibitor, fostriecin (10 nM; IC50 = 1.5–3.2 nM for PP2A and IC50 = 131 μM for PP1) (35, 36). On average, the residual potentiation measured 40 min after the end of LFS was 112.1 ± 6.1% (n = 6; p > 0.05 when compared with control depotentiation slices; unpaired Student’s t test) of base line (Table I). Moreover, neither the okadaic acid nor fostriecin treatment alone affects the LTP induction. The fEPSP slope measured 40 min after LTP induction was 139.3 ± 6.7% (n = 6; p > 0.05 when compared with control LTP slices; unpaired Student’s t test) and 139.7 ± 5.8% (n = 6; p > 0.05 when compared with control LTP slices; unpaired Student’s t test) of base line, respectively. These results indicate that the activation of PP1 but not PP2A plays an essential role in the development of LFS-induced depotentiation.
significant inhibition of PP activity from 7.6 ± 1.2 (n = 6) to 3.7 ± 1.1 nmol/mg/min (n = 6; p < 0.05; unpaired Student’s t test). This inhibitory effect on PP activity seen with LTP was completely reversed by depotentiating stimulation application, including LFS applied at 3 min after LTP induction (7.9 ± 1.6 nmol/mg/min measured at 17 min after the end of LFS, n = 6; p > 0.05 when compared with control; unpaired Student’s t test), extracellular application of adenosine (0.2 mM, 3 min) at 3 min after LTP induction (8.2 ± 1.5 nmol/mg/min measured at 24 min after washout of adenosine, n = 6; p > 0.05 when compared with control; unpaired Student’s t test), or a brief (5 min) bath application of NMDA (10 μM) beginning 3 min after LTP induction (7.2 ± 1.3 nmol/mg/min measured at 22 min after washout of NMDA, n = 6; p > 0.05 when compared with control slices; unpaired Student’s t test).

Correlation of LFS-induced Depotentiation and Dephosphorylation of AMPA Receptor GluR1 Subunit—How might PP1 activation be involved in the development of LFS-induced depotentiation? One possibility would be that activating PP1 dephosphorylates the phosphoproteins, which contribute to the stable expression of LTP, and then LTP fails. If this is the case, what substrates are involved in PP1-dependent processes, which lead to depotentiation expression. Because many ion channels, neurotransmitter receptors, protein kinases, and transcription factors are phosphorylated after the induction of LTP (1), the possible candidates are numerous, and it is likely that multiple substrates are involved. Over the last few years, evidence has accumulated that an increase in AMPA receptor GluR1 subunit phosphorylation is a key process underlying LTP expression in the CA1 region of hippocampus (37–39). This led us to investigate whether PP1-mediated dephosphorylation of GluR1 subunit has an important role in LFS-induced depotentiation. Recent studies (38–40) have demonstrated that GluR1 subunit is regulated by protein phosphorylation at two sites on its intracellular C-terminal domain. Serine 831 is phosphorylated by CaMKII and PKC, whereas serine 845 is phosphorylated by PKA. Phosphorylation of either of these serine residues potentiates AMPA receptor function (37–40). To determine changes in phosphorylation of GluR1 subunit at these residues after the synaptic induction of LTP and depotentiation, we performed the quantitative immunoblotting using two phosphorylation site-specific antibodies that recognize the serine 831 and serine 845 sites of GluR1 subunit, respectively. As shown in Fig. 7A, high frequency stimulation that induced LTP also increased GluR1 phosphorylation at serine 831 (116.4 ± 3.7% of control slices, n = 7; p < 0.05; unpaired Student’s t test). However, no significant changes in serine 845 phosphorylation were observed 30 min after LTP induction (103.8 ± 4.2% of control slices, n = 7; p > 0.05; unpaired Student’s t test). In LFS-induced depotentiation experiments, we found that there was significant dephosphorylation of GluR1 at serine 831 (88.3 ± 3.6% of LTP slices, n = 7; p < 0.05; unpaired Student’s t test) but no significant change at serine 845 (103.2 ± 3.7% of LTP slices, n = 7; p > 0.05; unpaired Student’s t test) (Fig. 7B). In addition, preincubation of slices with okadaic acid (1 μM) for 2–4 h blocked the LFS-induced dephosphorylation of GluR1 at serine 831 (100.2 ± 2.5% of LTP slices, n = 4; p < 0.05; unpaired Student’s t test; Fig. 8A). However, preincubation of slices with cyclosporin A (250 μM) for 2–4 h had no significant effect on LFS-induced dephosphorylation of GluR1 at serine 831 (80.2 ± 5.7% of LTP slices, n = 4; p > 0.05; unpaired Student’s t test; Fig. 8B). Moreover, neither okadaic acid nor cyclosporin A pretreatment significantly affects the GluR1 phosphorylation at serine 831 during LTP expression. Also, a brief bath application of adenosine (0.2 mM, 3 min) or NMDA (10 μM, 5 min) beginning 3 min after LTP induction mimicked LFS to elicit a significant dephosphorylation of GluR1 at serine 831 (adenosine-induced depotentiation slice group, 81.1 ± 2.4% of LTP slices measured at 22 min after washout of adenosine; n = 4; p < 0.05; unpaired Student’s t test) (Fig. 8, C and D). Together, these results suggest that the reversal of LTP induced by LFS is associated with a PP1-mediated dephosphorylation of GluR1 at a CaMKII/PKC phosphorylation site, serine 831.

### Table I

| Drug treatment | LTP 40 min after TS | The residual LTP 40 min after LFS following TS |
|---------------|---------------------|---------------------------------------------|
| Control       | 141.2 ± 5.7% (n = 18)* | 108.7 ± 6.4% (n = 16) |
| Okadaic acid (1 μM for 2–4 h) | 147.6 ± 5.3% (n = 8)* | 132.7 ± 4.9% (n = 12)* |
| Calyculin A (1 μM for 2–4 h) | 143.9 ± 6.2% (n = 8)* | 143.7 ± 5.1% (n = 12)* |
| Norkadaone (1 μM for 2–4 h) | 142.6 ± 4.8% (n = 4)* | 104.6 ± 3.7% (n = 4) |
| Okadaic acid (3 mM for 2–4 h) | 139.3 ± 6.7% (n = 6)* | 105.8 ± 6.5% (n = 6) |
| Paftricin (10 mM for 2–4 h) | 139.7 ± 5.8% (n = 6)* | 112.1 ± 6.2% (n = 6) |
| Cyclosporin A (250 μM for 2–4 h) | 136.7 ± 5.4% (n = 9)* | 109.4 ± 6.3% (n = 8) |
| Cypermethrin (250 μM for 2–4 h) | 142.8 ± 6.5% (n = 8)* | 111.4 ± 6.3% (n = 9) |

*p Significantly different from baseline (unpaired Student t test, p < 0.05).
CaMKII activity in hippocampal slices (41) accompanied with autophosphorylation of the kinase and phosphorylation of presynaptic and postsynaptic proteins (42). This, along with the results from studies showing that autophosphorylation of CaMKII could be reversed by PP-1 activation in synaptic membranes (43), led us to investigate whether dephosphorylation of the phosphorylated state of CaMKII (threonine 286-phosphorylated CaMKII) has an important role in the development of LFS-induced depotentiation. We determined the phosphorylation state of CaMKII by immunoblotting analysis with an antibody that specifically recognizes phosphorylated threonine 286 of CaMKII. Protein phosphatase 1 (PP1) is involved in LFS-induced dephosphorylation of CaMKII at threonine 286 (81.1 ± 2.1% of LTP slices, n = 4; p > 0.05; unpaired Student’s t test; Fig. 9C). Furthermore, neither okadaic acid nor cyclosporin A pretreatment significantly affects the CaMKII phosphorylation at threonine 286 during LTP expression. A brief bath application of adenosine (0.2 mM, 3 min) or NMDA (10 μM, 5 min) at 3 min after LTP induction mimicked LFS to elicit a significant dephosphorylation of CaMKII at threonine 286 (adenosine-induced depotentiation slice group, 75.6 ± 4.3% of control slices, n = 5; p < 0.05; unpaired Student’s t test; Fig. 9C). These results suggest that dephosphorylation of phosphorylated state CaMKII may also be one potential mechanism participating in the induction of hippocampal CA1 depotentiation.

DISCUSSION

In the present study, using both electrophysiological and biochemical approaches, we have made some progress in understanding the fundamental characteristics and cellular substrates responsible for LFS-induced depotentiation at Schaffer

![Image](https://example.com/image1.png)

**FIG. 7.** LFS-induced depotentiation is accompanied by dephosphorylation of a CaMKII site on GluR1 subunit. A, CA1 homogenates were probed using antibody against phosphorylated serine 831 (PKC/CaMKII site) and phosphorylated serine 845 (PKA site) on GluR1 (top gel) and an antibody recognizing total GluR1 (bottom gel). Bar plots representing the phosphorylation changes in serine 831 and serine 845 30 min after LTP induction were determined by densitometry. Note that LTP is accompanied by a significant increase in GluR1 phosphorylation at serine 831, whereas phosphorylation of serine 845 was not significantly increased 30 min after LTP induction. B, LFS-induced depotentiation (DEP) results in dephosphorylation of serine 831 site of GluR1. There was no significant change in phosphorylation of serine 845 following LFS. Data are presented as means ± S.E. The number of experiments is indicated by n. Asterisk, p < 0.05 (unpaired Student’s t test) as compared with the control group.

![Image](https://example.com/image2.png)

**FIG. 8.** Protein phosphatase 1 (PP1) is involved in LFS-induced dephosphorylation of GluR1 at serine 831. A, immunoblot of slices pretreated with okadaic acid (Oka; 1 μM for 2–4 h) using an antibody recognizing phosphorylated GluR1 at serine 831. Okadaic acid pretreatment prevented the dephosphorylation of GluR1 at serine 831 associated with LFS-induced depotentiation (DEP). B, cyclosporin A (CsA; 250 μM for 2–4 h) pretreatment did not affect the LFS-induced dephosphorylation of GluR1 at serine 831. C, extracellular application of adenosine (0.2 mM, 3 min) elicited a significant dephosphorylation of GluR1 at serine 831 in the same way as LFS. D, A brief (5 min) bath application of NMDA (10 μM) mimicked LFS to dephosphorylate GluR1 at serine 831. **DEP**, LFS-induced depotentiation slices. **Ade-DEP**, adenosine-induced depotentiation slices. **NMDA-DEP**, NMDA-induced dephosphorylation slices. Data are presented as means ± S.E. Number of experiments is indicated by n. Asterisk, p < 0.05 (unpaired Student’s t test) as compared with the control or LTP group.
FIG. 9. The increased phosphorylation of CaMKII at threonine 286 following LTP induction was overcome by subsequent LFS application. A, CA1 homogenates were probed with an antibody specific for threonine 286-phosphorylated CaMKII (top gel) and an antibody recognizing total CaMKII (bottom gel). In high frequency stimulation-receiving slices, LTP is concomitant with an increase in the amount of threonine 286-phosphorylated CaMKII determined by densitometry. The increase in CaMKII phosphorylation at threonine 286 is completely reversed by subsequent depotentiation. The increase in CaMKII phosphorylation at threonine 286 is associated with LFS-induced depotentiation (DEP). C, cyclosporin A (CsA; 250 μM for 2–4 h) pretreatment did not affect the LFS-induced dephosphorylation of CaMKII at threonine 286. D, extracellular application of adenosine (0.2 mM, 3 min) elicited a significant dephosphorylation of CaMKII at threonine 286 in the same way as LFS. E, a brief (5 min) bath application of NMDA (10 μM) mimicked LFS to dephosphorylate CaMKII at threonine 286. DEP, LFS-induced depotentiation slices. Ade-DEP, adenosine-induced depotentiation slices. NMDA-DEP, NMDA-induced depotentiation slices. Data are presented as means ± S.E. Number of experiments is indicated by n. Asterisk, p < 0.05 (unpaired Student’s t test) as compared with the control or LTP group.

collateral-CA1 synapses. The major findings in this study are as follows. 1) LFS-induced depotentiation is input-specific and dependent on NMDA receptor activation. 2) The time-dependent reversal of LTP by LFS was mimicked by a brief application of NMDA. 3) NMDA-induced depotentiation was blocked by A1 adenosine receptor antagonist DPCPX. 4) The ability of LFS to reverse LTP was not affected by mGlurR antagonist. 5) LFS-induced depotentiation was selectively prevented by PP1 inhibitors, okadaic acid and calyculin A. 6) The expression of depotentiation was accompanied by a persistent dephosphorylation of the GluR1 subunit of AMPA receptor at serine 831, a PKC/CaMKII substrate, but not at serine 845, a PKA substrate. 7) The increased phosphorylation of CaMKII at threonine 286 and decreased PP activity seen with LTP were overcome by LFS, adenosine, or NMDA application. These findings and their interactions are discussed below.

Input specificity is a characteristic feature of many types of activity-dependent synaptic plasticity. For example, LTP is input-specific, because the potentiation is restricted to the tetanized inputs and not in untetanized inputs, even though they terminated on the same postsynaptic neurons (1, 44). The issue of whether LFS-induced depotentiation is an input-specific phenomenon remains controversial at the present time. The notion that potentiation is input-specific was supported by the results of experiments, established in rat hippocampal CA1 region in vitro, in which two independent inputs are potentiated simultaneously, followed by depotentiating stimulation delivered to one input and demonstration of the absence of heterosynaptic effects on the second potentiated input pathway (22, 27, 45). In contrast, Muller et al. (46) have demonstrated that repeated induction of long term depression on one input was associated with a heterosynaptic reversal of the LTP previously induced in a separate pathway. Thus, they proposed that depotentiation may also occur heterosynaptically. The reason for this discrepancy is not clear but could be attributed partially to the use of different stimulation paradigms for depotentiation (5 Hz, 300 stimuli versus 1 Hz, 900 stimuli), resulting in activation of different biochemical processes that may vary in their mode of action and produce different types of depressive effects. The results of the present study demonstrated that the reversal of LTP by LFS was specific to the synapses receiving the stimulation, indicating that the pattern of stimulation used in this study to elicit depotentiation was input-specific.

Recent studies (5, 11, 47) have demonstrated that the triggering of depotentiation relied on synaptic activation of NMDA receptors. Consistent with these findings, our data showed that D-APV specifically prevented the LFS-induced depotentiation (Fig. 2). Furthermore, we have found that a direct extracellular application of NMDA itself was also sufficient to produce a time-dependent reversal of LTP (Fig. 3), as LFS did. Combined, the above findings favor the assumption that the reversal process of LTP is an NMDA receptor-dependent phenomenon. Other reports have recently shown that the activation of NMDA receptors is not required for some depotentiating stimulation to reverse LTP. Two such examples are revealed by Bashir and Collingridge (21) and Staubli and Chun (22), who found that D-APV was unable to prevent the reversal of LTP produced by LFS at 2 Hz (900 pulses) or 9 Hz (500 stimuli) in the same CA1 region of rat hippocampal slices. Thus, there appears to be at least two mechanistically distinct forms of depotentiation, which can coexist at the same synapses, one is triggered by activation of NMDA receptors and another is independent of NMDA receptors. These discrepancy observations also point to the possibility that the use of different patterns of depotentiating stimulation (e.g., with variable strength, duration and frequency) may result in the activation of different cellular processes that may vary in their mode of
action to exert their depressive effects.

The dependence of depotentiation induction on NMDA receptor activation implies that increases in intracellular Ca^{2+} via influx through NMDA receptor are required for this phenomenon. However, what are the biochemical steps downstream of Ca^{2+} entry through NMDA receptors to undergo the induction of depotentiation? The most likely candidate is PP cascades, because PP activation is known to play an essential role in the development of LFS-induced depotentiation (7, 11, 22, 48). We have reported previously (7) that the selective PP1/2A inhibitors, okadaic acid and calyculin A, prevent LFS-induced depotentiation. In the present study, we have extended these findings by observing that the reversal of LTP by LFS was not blocked by the selective inhibitors of PP2A, indicating that PP1 but not PP2A contributes to the processes of depotentiation. Because PP1 is not directly influenced by intracellular Ca^{2+}, there should be another Ca^{2+}-dependent mediator or signaling cascade to translate the Ca^{2+} signal into an increase in PP1 activity (49). There are two potential candidates that could be responsible for this process. One is PP2B (calcineurin), a Ca^{2+}/calmodulin-dependent PP, that can indirectly increase the PP1 activity by a mechanism to dephosphorylate the inhibitory protein (I1) of PP1. I1, in its phosphorylated but not dephosphorylated form, acts as a protein inhibitor of PP1 (50). It is possible that under depotentiating stimulation, Ca^{2+} can enter through NMDA receptors and bind to calmodulin, resulting in the activation of PP2B. PP2B dephosphorylates PP1 regulatory protein I1, which no longer inhibits PP1. The PP1 then dephosphorylates the phosphoproteins, which contribute to the stable expression of LTP. This prediction is based on the current findings of Zhuo et al. (48) who demonstrated the genetic evidence that LFS-induced depotentiation was abolished completely in calcineurin Aa-deficient mice. However, in the present study, we could not inhibit depotentiation by applying the selective PP2B inhibitors, cyclosporin A and cyclosporin A, suggesting that the protocol used in the present study to elicit LFS-induced depotentiation was not attributable to the activation of PP2B-mediated signaling processes. A similar result was also observed by Lu et al. (51), who demonstrated that PP2B inhibitors, FK506 and cyclosporin A, inhibited induction of LTP but had no effect on depotentiation in the CA1 region of rat hippocampal slices.

The second candidate system that could increase the PP1 activity is adenosine-mediated signaling cascades. Because the activation of NMDA receptors is one potential mechanism that can result in an increase in extracellular adenosine in hippocampal slices (25), and the increase of extracellular adenosine acting on the A1 adenosine receptors to interrupt the cAMP/PKA-dependent signaling cascades leading to the suppression of PP1 activity.

Which target phosphoproteins are involved in PP1-dependent processes, which lead to depotentiation expression? The prime candidates are CaMKII and the specific subunit of AMPA receptors, because it is known that LTP is accompanied by an increase in the CaMKII-mediated phosphorylation of GluR1 subunit of AMPA receptors (37, 53), and both of CaMKII and GluR1 can be dephosphorylated by PP1 (50, 53). By using phosphorylation site-specific antibodies, we have demonstrated that LFS-induced depotentiation is associated with dephosphorylation of serine 831, but not serine 845, of the GluR1 subunit (Fig. 6), and this decrease in GluR1 phosphorylation was blocked by pretreatment of the slices with PP1 inhibitor but not by PP2B inhibitor. These results are in agreement with a recent report (39) demonstrating that long term depression induction in potentiated synapses, the major CaMKII site on GluR1, is dephosphorylated. We also found that LFS-induced depotentiation is accompanied by a dephosphorylation of CaMKII at threonine 286, a site that appears to be constitutively phosphorylated after triggering of LTP (37). Because the increase in CaMKII activity is parallel with its autophosphorylation at threonine 286 and an increase of autophosphorylated CaMKII is required for LTP, manipulations that could dephosphorylate CaMKII may lead to inhibiting its activity and then reversing LTP to pre-LTP level. From these results, it seems reasonable to speculate that the persistent dephosphorylation of CaMKII and its substrate, GluR1 subunit, is a plausible mechanism for the expression of depotentiation.

At present the precise functional relevance of depotentiation is not entirely clear. To the extent that LTP represents the cellular correlate of memory, the processes involved in the depression of synaptic potentiation may provide a mechanism of memory loss (“forgetting”). Considering that stimulation protocols necessary for depotentiation observed in recent studies are common firing patterns observed in endogenous hippocampal θ rhythm during exploration, it is possible that the loss of memory might result from an active process triggered by physiological patterns associated with particular behavioral circumstances (10).

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