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Involvement of a Postsynaptic Protein Kinase A Substrate in the Expression of Homosynaptic Long-Term Depression

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Summary

Hippocampal N-methyl-D-aspartate (NMDA) receptor–dependent long-term synaptic depression (LTD) is associated with a persistent dephosphorylation of the GluR1 subunit of AMPA receptors at a site (Ser-845) phosphorylated by cAMP-dependent protein kinase (PKA). In the present study, we show that dephosphorylation of a postsynaptic PKA substrate may be crucial for LTD expression. PKA activators inhibited both AMPA receptor dephosphorylation and LTD. Injection of a cAMP analog into postsynaptic neurons prevented LTD induction and reversed previously established homosynaptic LTD without affecting baseline synaptic transmission. Moreover, infusing a PKA inhibitor into postsynaptic cells produced synaptic depression that occluded homosynaptic LTD. These findings suggest that dephosphorylation of a PKA site on AMPA receptors may be one mechanism for NMDA receptor–dependent homosynaptic LTD expression.

Introduction

In the CA1 region of hippocampus, the activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors can induce two types of long-term synaptic change, long-term potentiation (LTP) and long-term depression (LTD). LTP typically results from strong, brief NMDA receptor activation, whereas LTD typically results from weak or prolonged NMDA receptor activation (Dudek and Bear, 1992; Cummings et al., 1996). A number of lines of evidence suggest that these forms of LTP and LTD occur by the bidirectional regulation of a common mechanism (reviewed by Bear and Abraham, 1996). An attractive hypothesis is that LTP and LTD reflect the phosphorylation and dephosphorylation, respectively, of a common set of synaptic proteins (Lisman, 1989; Bear and Malenka, 1994).

It has been suggested that LTP and LTD result, at least partly, from changes in the phosphorylation of AMPA receptors (Raymond et al., 1993; Roche et al., 1994). To aid the biochemical detection of changes following the induction of synaptic plasticity, we developed a preparation to produce NMDA receptor–dependent LTD in a majority of the synapses in the CA1 region of hippocampal slices (Lee et al., 1998 [this issue of *Neuron*]). To assist the detection of specific changes in AMPA receptors, specific phosphorylation sites were mapped on the major AMPA receptor subunit GluR1 (Roche et al., 1996; Mammen et al., 1997), and phosphorylation site–specific antibodies were generated against Ser-831, the major calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) site, and Ser-845, the major cAMP-dependent protein kinase (PKA) site. Using this new preparation and these novel reagents, we discovered that the induction of LTD causes a persistent decrease in phosphorylation of Ser-845 but not of Ser-831 (Lee et al., 1998).

The finding that LTD is associated with a selective dephosphorylation of Ser-845 (the PKA site on GluR1) was unexpected. There is considerable evidence that CaMKII activity is necessary (Malenka et al., 1989; Malinow et al., 1989) and sufficient (Petit et al., 1994; Lledo et al., 1995) for the induction of LTP, and recent work has shown that LTP may be associated with an increase in the CaMKII phosphorylation of GluR1 (Barria et al., 1997). Thus, our initial expectation was that LTD would be associated with a dephosphorylation of Ser-831, the CaMKII site on GluR1. Nonetheless, dephosphorylation of the PKA site on GluR1 is a plausible mechanism for LTD expression, since it has been shown that changes in the phosphorylation of Ser-845 can have large effects on glutamate-evoked currents mediated by AMPA receptors (Roche et al., 1996).

The aim of the present study was to test the hypothesis that the dephosphorylation of a postsynaptic PKA substrate, possibly the AMPA receptor, is one mechanism for expression of NMDA receptor–dependent homosynaptic LTD. Our strategy was (1) to test the consequences on the synaptic transmission and plasticity of manipulating postsynaptic PKA activity in hippocampal neurons and (2) to further investigate whether GluR1, which is dephosphorylated with chemLTD, may be one of the PKA substrates involved in the expression of LTD. Our results suggest that persistent dephosphorylation of a postsynaptic PKA substrate, possibly GluR1, is crucial for the expression of LTD.

Results

Chem-LTD Is Inhibited by Postsynaptic Activation of PKA

A brief application of NMDA (20 μM, 3 min) to hippocampal slices results in a long-term synaptic depression in CA1 (termed chemLTD), and this correlates with a persistent dephosphorylation of GluR1 on Ser-845 (Lee et al., 1998). Since Ser-845 is specifically phosphorylated by PKA, we examined whether the dephosphorylation of a postsynaptic PKA substrate is involved in chemLTD expression. To test this hypothesis, we injected a PKA activator in postsynaptic cells to see if the manipulation would prevent LTD expression.

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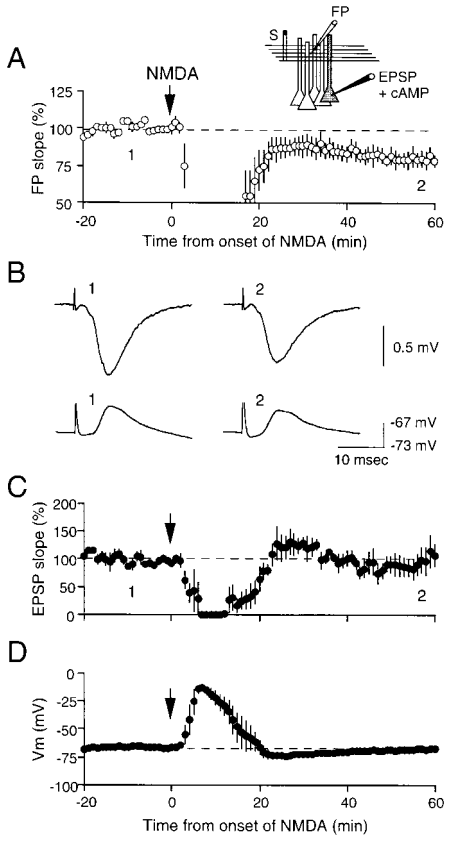

Figure 1. Chem-LTD Is Blocked by Postsynaptic Injection of a cAMP Analog

(A) The inset shows the preparation in which simultaneous intracellular and extracellular recordings were made in CA1. The intracellular recording electrode was filled with 10 mM Sp-cAMPS, a nonhydrolizable cAMP analog. The graph shows the average effect of NMDA application (20 μM, 3 min) on the FP slope (79% ± 5% of baseline at 60 min post-NMDA, n = 4).

(B) Simultaneously recorded FPs and EPSPs (averages of four consecutive traces) from a representative case, taken at the time points indicated in (A).

(C) In contrast to the LTD observed in the FPs, there was no lasting depression of the EPSPs in the cells filled with Sp-cAMPS (101% ± 19% of baseline).

(D) Average changes in the membrane potential during the course of the experiment.

Intracellular recordings were obtained with sharp microelectrodes filled with 10 mM 3′,5′-monophosphothioate (Sp-cAMPS), a nonhydrolizable analog of cAMP (Yusta et al., 1988). Diffusion of the drug into the cell was confirmed by measuring changes in spike adaptation to depolarizing step currents (+0.1 or +0.2 nA, 1 s). Within 10 min of impalement, the number of action potentials evoked with a current step doubled (from 6 ± 2 spikes per pulse to 13 ± 2 spikes per pulse), reflecting a decrease in spike adaptation. We allowed 20–40 min for Sp-cAMPS to diffuse into the cell before attempting to induce chemLTD. As shown in Figure 1, there was a complete block of chemLTD expression in the Sp-cAMPS-loaded cells (101% ± 19% of baseline excitatory postsynaptic potential (EPSP) slope, measured 60 min post-NMDA, n = 4 cells) without affecting chemLTD measured in the field potential (79% ± 5% of baseline field potential (FP) slope, 60 min post-NMDA). Control recordings from neurons not loaded with Sp-cAMPS confirmed that expression of chemLTD in the FP normally is paralleled by depression of the intracellular EPSP slope (Lee et al., 1998). Thus, activation of postsynaptic PKA can block chemLTD in CA1 neurons.

Homosynaptic LTD Is Inhibited by Postsynaptic Activation of PKA

Chem-LTD was developed as a preparation to facilitate investigation of the mechanism of homosynaptic LTD that is typically induced using low-frequency synaptic stimulation (LFS; Dudek and Bear, 1992). The chemLTD experiments implicate a postsynaptic PKA substrate in the mechanism of NMDA receptor–mediated synaptic depression. Therefore, we investigated the effects of PKA activators on LFS-induced homosynaptic LTD. To confirm the specific involvement of postsynaptic PKA in homosynaptic LTD, cells were filled with Sp-cAMPS (10 mM), and LFS was delivered to the Schaffer collaterals.

Intracellular recordings from control cells confirmed that LFS produces robust LTD of the EPSP slope (54% ± 7% of baseline 60 min post-LFS onset, n = 7 cells) and that this correlates with LTD of the simultaneously recorded FP (79% ± 3% of baseline; Figure 2). In contrast, intracellular recordings from cells loaded with Sp-cAMPS (confirmed by changes in spike adaptation) showed little LTD 60 min after LFS (86% ± 9% of baseline, $p > 0.7$, paired t test, n = 6) even though LTD of the FP still was observed (75% ± 2% of baseline; Figure 2). Statistical comparison confirms that the change in EPSP slope 60 min after LFS in control cells was significantly different than that in Sp-cAMPS-loaded cells ($p < 0.03$, t test). These data show that selective activation of postsynaptic PKA can inhibit the establishment of stable homosynaptic LTD.

Similar results were obtained with bath application of PKA activators on LFS-induced homosynaptic LTD. These data are summarized in Table 1. Both bath-applied forskolin (an activator of adenylyl cyclase) and 8-bromo-cAMP significantly reduced the magnitude of homosynaptic LTD (see below).

Inhibition of Postsynaptic PKA Causes Synaptic Depression that Occludes Homosynaptic LTD

One interpretation of our data is that the postsynaptic PKA activators prevent stable expression of LTD by keeping a PKA substrate in a phosphorylated state. According to this model, PKA inhibitors should have two effects. First, the inhibitor by itself should cause dephosphorylation of a PKA substrate and hence a depression of synaptic transmission. Second, synaptic transmission thus depressed should no longer undergo LTD following LFS. We tested these predictions by recording intracellularly with electrodes filled with 10 μM PKI (6–22 amide), a highly selective inhibitor of PKA (Kemp et al., 1988; Glass et al., 1989).

Consistent with our first prediction, the intracellular EPSP slope started to decrease in size 20–30 min after the impalement of cells with PKI-filled electrodes (Figure

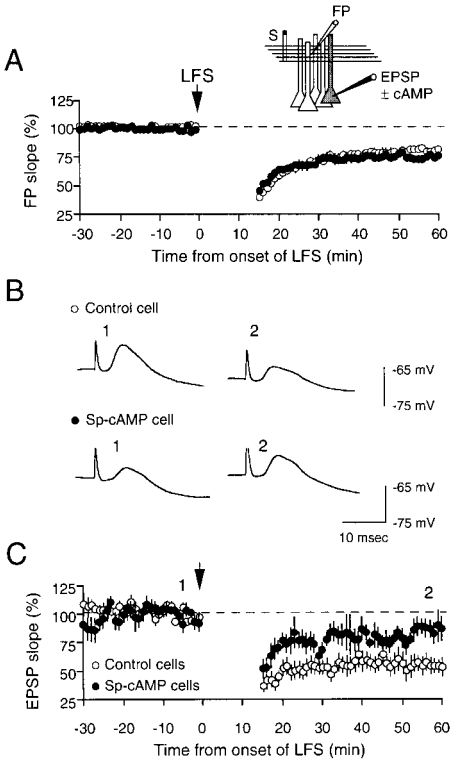

Figure 2. Homosynaptic LTD Is Blocked by Postsynaptic Injection of a cAMP Analog

The insets show the preparation in which simultaneous intracellular and extracellular recordings were made in CA1. Results from control experiments are shown in open symbols, and results from experiments in which the intracellular recording electrodes were filled with 10 mM Sp-cAMPS are shown in closed symbols.

(A) The graphs show the average effect of LFS on the FP slope (controls: 79% ± 3% of baseline at 60 min post-LFS, n = 7; Sp-cAMPS group: 75% ± 2% of baseline, n = 6).

(B) Representative EPSPs (averages of four consecutive traces) from a control cell and a cell filled with Sp-cAMPS, taken at the time points indicated in (C).

(C) Intracellularly recorded LTD of the EPSP slopes in control cells (54% ± 7% of baseline) and in cells filled with Sp-cAMPS (86% ± 9% of baseline). There was no significant LTD in Sp-cAMPS cells ($p > 0.7$, paired t test).

3B). This change in the EPSP was accompanied by a slight depolarization (−73 ± 1 mV to −71 ± 3 mV in the 20 min period; paired t test, $p > 0.1$, n = 5 cells) and an increase in the number of spikes elicited with a step depolarizing current injection (+0.1 or 0.2 nA for 1 s; 2 ± 1 spikes/pulse to 7 ± 2 spikes/pulse). The increase in the number of spikes could be due to the inhibition of PKA phosphorylation of voltage gated Na^+ channels, which has been reported to decrease spike threshold (Costa and Catteral, 1984; Gershon et al., 1992; Li et al., 1992).

The EPSP stabilized 30–40 min after impalement with the PKI-filled electrode at a value of 53% ± 5% of the original magnitude ($p < 0.005$, paired t test, n = 5 cells; Figure 3B). The depression of the EPSP does not reflect a general reduction in synaptic transmission in the slice, since there was no significant change in the extracellular FP. Furthermore, the EPSP depression in PKI-loaded cells is not likely to be due to the "rundown" of responses during the long baseline recordings, since control cells did not show a similar decrease in the EPSP when held for the identical 40–60 min baseline period (n = 4 cells; Figure 3A).

Following stabilization of the EPSP, we tested our second prediction by delivering LFS. Although LFS produced clear LTD of the FP (78% ± 3% of baseline 1 hr post-LFS onset; Figure $3B_1$), there was no further decrease in the EPSP in the PKI-loaded neurons (106% ± 15% of pre-LFS baseline, measured 1 hr after LFS onset, $p > 0.7$, paired t test, n = 5 cells; Figure $3B_3$). The absence of homosynaptic LTD in PKI-filled neurons is not accounted for by the wash-out of essential intracellular factors during the long recording period, since control cells recorded for a comparable duration still exhibited normal LTD (57% ± 11% of pre-LFS baseline, measured 1 hr after LFS onset, $p < 0.02$, paired t test, n = 4 cells; Figure $3A_3$). The simplest interpretation of these data is that inhibition of postsynaptic PKA directly causes a depression of synaptic transmission that occludes homosynaptic LTD.

Activation of Postsynaptic PKA Reverses Previously Established Homosynaptic LTD

Our experiments have shown that a postsynaptic PKA substrate is critically involved in the mechanism for LTD. However, they have not enabled us to determine if the involvement is in LTD induction or LTD expression. Our working hypothesis is that the postsynaptic expression of synaptic strength is determined by the phosphorylation state of PKA substrates, which can be altered by inducing LTD. If this is the case, the activation of postsynaptic PKA would be expected to cause synaptic potentiation directly. However, we did not observe any consistent effect on baseline synaptic transmission of loading neurons with a cAMP analog (e.g., Figure 1C). One possible explanation of this lack of an effect is that the phosphorylation of the PKA sites on the putative substrate is saturated under "basal" conditions. Thus, prior dephosphorylation of these sites might be required to observe a potentiating effect of a PKA activator. Our data suggest that the induction of LTD dephosphorylates the PKA sites on the AMPA receptor. Therefore, we predicted that the postsynaptic injection of cAMP would selectively potentiate synapses that had previously undergone LTD.

To test this prediction and to clarify the role of the PKA pathway in the expression of LTD, we performed the experiment shown in Figure 4. In the first phase of the experiment, we monitored the FPs evoked by stimulating two independent inputs converging onto the same population of neurons (Figure $4A_1$). We then induced LTD at the synapses of one input (S1) by giving two episodes of LFS (1 Hz, 15 min). After allowing 30 min for the LTD to stabilize, we began the second phase of the experiment, which entailed impaling individual CA1 pyramidal cells with an electrode filled with Sp-cAMPS (Figure $4A_2$). In agreement with our prediction, we observed a gradual potentiation of the EPSP evoked by stimulation of the previously depressed input (S1: 157% ± 15% of the initial 10 min baseline, measured

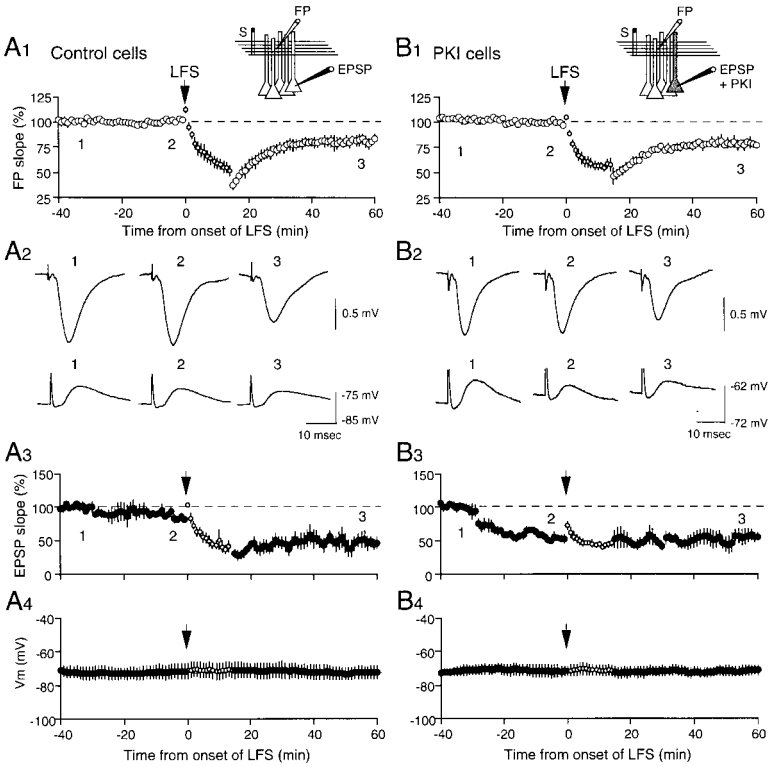

Figure 3. Inhibition of Postsynaptic PKA Causes Depression of the EPSP that Occludes Homosynaptic LTD

The insets show the preparation in which simultaneous intracellular and extracellular recordings were made in CA1. Results from control experiments are shown in (A), and results from experiments in which the intracellular recording electrodes were filled with 10 μM PKI (6–22 amide) are shown in (B).

(A_1 and B_1) The graphs show the average effect of LFS on the FP slope (A_1: 84% ± 4% of baseline at 60 min post-LFS, n = 4; B_1: 78% ± 3% of baseline, n = 5).

(A_2 and B_2) Simultaneously recorded FPs and EPSPs (averages of four consecutive traces) from representative cases, taken at the time points indicated in A_1 and B_1.

(A_3) Intracellularly recorded LTD of the EPSP slopes in control cells (57% ± 11% of pre-LFS baseline).

(B_3) In cells filled with PKI, there was a gradual decline in the EPSP that stabilized at a value of 53% ± 5% of the initial value. Subsequent LFS produced no further synaptic depression in the PKI-loaded cells (106% ± 15% of pre-LFS baseline measured 1 hr after LFS onset).

(A_4 and B_4) Average resting membrane potential before, during, and after LFS in control and PKI-filled neurons.

60 min after impalement, n = 7 cells, paired t test, $p < 0.007$), while the EPSP evoked by stimulation of the control path did not show any change (S2: 100% ± 11% of 10 min baseline, measured 60 min after impalement, paired t test, $p > 0.3$). The potentiation of the response to S1 was restricted to the intracellularly recorded neuron; there was no corresponding change in the extracellularly recorded FP (S1 FP: 114% ± 8% of the initial 10 min baseline, measured 60 min after impalement, paired t test, $p > 0.1$; S2 FP: 109% ± 7% of the initial baseline, paired t test, $p > 0.1$). Moreover, the potentiation was a specific consequence of Sp-cAMPS in the intracellular electrode, since no significant change in the S1 EPSP was observed in identical control experiments using electrodes that did not contain the drug (S1 EPSP slope: 85% ± 11% of the initial 10 min baseline, 1 hr post impalement, n = 4; S2 EPSP slope: 76% ± 15% of baseline, paired t test, $p > 0.2$).

Further analysis of these experiments suggests that the EPSP potentiation in the Sp-cAMPS-filled neurons reflects the complete reversal of the previously established LTD (Figure 4B). Because homosynaptic LTD was saturated in pathway S1, initially the intracellular EPSP evoked by stimulation of this pathway was significantly smaller than that evoked by stimulation of the control path (EPSP slope: S1 = 1.1 ± 0.3 mV/msec; S2 = 2.0 ± 0.5 mV/msec, measured 0 min after impalement, paired t test, $p < 0.05$). Over time, however, the EPSP slope in the depressed path potentiated until it was of magnitude similar to the control path EPSP (EPSP slope: S1 = 1.9 ± 0.5 mV/msec; S2 = 2.0 ± 0.3 mV/msec, measured 60 min after impalement with Sp-cAMPS-filled electrode, paired t test, $p > 0.8$). The results of this experiment strongly suggest that a postsynaptic PKA substrate controls the expression of homosynaptic LTD.

Correlation of Chem-LTD and GluR1 Dephosphorylation: Effects of Bath-Applied PKA Activators

The electrophysiological analysis strongly suggests that the expression mechanism of both homosynaptic LTD and chemLTD involves the persistent dephosphorylation of a postsynaptic PKA substrate. This analysis was motivated by our previous finding that chemLTD is associated with the dephosphorylation of Ser-845 on the GluR1 subunit of the AMPA receptor, a PKA substrate. We therefore next asked if treatment of hippocampal slices with PKA activators prevents the NMDA-stimulated dephosphorylation of GluR1 and chemLTD.

The effects of bath application of the PKA activator forskolin on synaptic transmission and chemLTD are summarized in Figures $5A_1$–$5A_3$. As has been reported previously (Chavez-Noriega and Stevens, 1992, 1994), bath application of forskolin (50 μM) resulted in an increase in synaptic transmission that stabilized after 20–30 min (120% ± 7% of baseline, measured 50 min after washing on forskolin, n = 6; Figure $5A_1$). This effect has been attributed to an increase in presynaptic glutamate release, as it is correlated with an increase in mEPSP frequency without much effect on their amplitude (Chavez-Noriega and Stevens, 1994). In accordance with this interpretation, a decrease in the paired pulse facilitation (PPF) ratio accompanied the forskolin-induced synaptic enhancement (PPF ratio: 89% of baseline after 50 min in forskolin, n = 2; data not shown). However, as shown in Figures $5A_1$–$5A_3$, forskolin also

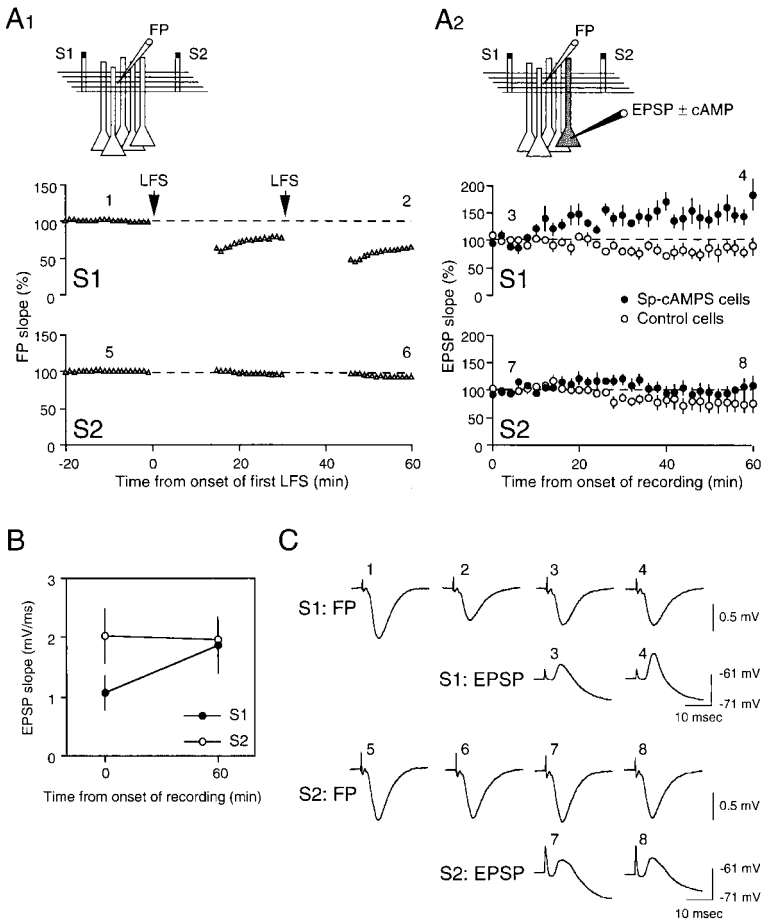

Figure 4. Activation of Postsynaptic PKA Reverses Previously Established Homosynaptic LTD

Responses to stimulation of two independent inputs to a common population of CA1 neurons were monitored. In the first phase of the experiment (A_1), only FPs were recorded (inset). Two episodes of LFS (1 Hz; 2 × 900 pulses) were given to one pathway (S1) to saturate homosynaptic LTD. Once the LTD had stabilized (20–60 min after the second LFS), the second phase of the experiment was begun. In this phase (A_2), individual CA1 pyramidal cells were impaled with microelectrodes containing 10 mM Sp-cAMPS (as shown in the inset), and changes in the intracellular EPSPs evoked by stimulation of S1 and S2 were monitored (closed symbols). While the intracellular response to stimulation of S2 remained stable, the response to stimulation of S1 gradually increased to a potentiated value (157% ± 15% of the initial 10 min baseline, measured 60 min after impalement, n = 7 cells, paired t test, $p < 0.01$). In experiments where the Sp-cAMPS was omitted from the recording electrode (open symbols), the EPSP slopes in response to S1 and S2 did not change significantly over this time ($p > 0.1$).

(B) Analysis of the absolute EPSP slope measurements at the time of impalement (t = 0) and 1 hr after recording with electrodes filled with Sp-cAMPS (t = 60). At the time of impalement, the initial slopes of EPSPs evoked from the previously depressed pathway (S1: 1.1 ± 0.3 mV/msec) were significantly smaller than those evoked from the control pathway (S2: 2.0 ± 0.5 mV/msec, paired t test, $p < 0.05$). However, over the 1 hr recording period, the slope of the EPSP evoked by stimulation of S1 (1.9 ± 0.5 mV/msec) grew until it was no different from that evoked from S2 (2.0 ± 0.3 mV/msec).

(C) FPs and EPSPs (averages of four consecutive sweeps) from a representative case, taken at the time points indicated in (A).

completely blocked chemLTD (95% ± 3% of renormalized baseline 60 min post-NMDA, n = 6; control: 82% ± 3% of baseline, n = 7, t test, $p < 0.01$). Treating slices with 8-bromo-cAMP (100 μM) also significantly reduced chemLTD (Table 1). These findings corroborate the earlier result using intracellular Sp-cAMPS (Figure 1) and show that bath-applied PKA activators can inhibit chemLTD. We therefore next asked if the same treatments would block the dephosphorylation of GluR1, which is stimulated by NMDA.

In the absence of NMDA treatment, 50 μM forskolin increased phosphorylation of Ser-845, with little effect on Ser-831 phosphorylation, as previously described (Mammen et al., 1997; Lee et al., 1998; Table 2). Interestingly, however, pretreatment of the hippocampal slices with forskolin also completely blocked the NMDA-induced decrease in Ser-845 phosphorylation (Figure $5A_4$). In addition, 8-Bromo-cAMP (100 μM), a nonhydrolizable membrane permeable analog of cAMP (Hei et al., 1991; Sandberg et al., 1991), did not significantly increase the phosphorylation of Ser-845 but did significantly reduce (n = 7, $p < 0.05$) the NMDA-induced dephosphorylation of GluR1 (Table 2). These experiments indicate that activating PKA in hippocampal slices can antagonize the NMDA-induced dephosphorylation of GluR1. Thus, the effects of PKA activators on chemLTD correlate with their effects on GluR1 dephosphorylation.

Correlation of Chem-LTD and GluR1 Dephosphorylation: Effects of Inhibitors of Protein Phosphatases 1, 2A, and 2B

In a final series of experiments, we examined the effects of protein phosphatases (PPs) on chemLTD and the dephosphorylation of GluR1. Okadaic acid is a potent, cell-permeable inhibitor of PP1 and PP2A (Ishihara et al., 1989; Cohen et al., 1990) that has been used in previous studies of LTD (Mulkey et al., 1993; Kirkwood and Bear, 1994; O'Dell and Kandel, 1994). Following a 2–4 hr preincubation in okadaic acid (1 μM in 0.1% dimethyl sulfoxide [DMSO]), chemLTD measured 86% ± 1% of baseline at 60 min post-NMDA (n = 8), which is not significantly different from same day controls treated only with DMSO (Figure $5B_1$; Table 1). Robust chemLTD was observed even when the okadaic acid concentration was increased to 10 μM (82% ± 3% of baseline at 60 min post-NMDA, n = 6; Table 1). The experiments were also repeated using calyculin A, a more potent inhibitor of PP1/2A (Ishihara et al., 1989). Preincubating slices in 1 μM calyculin A for ≥1 hr also did not affect chemLTD expression (Figure $5B_3$; Table 1).

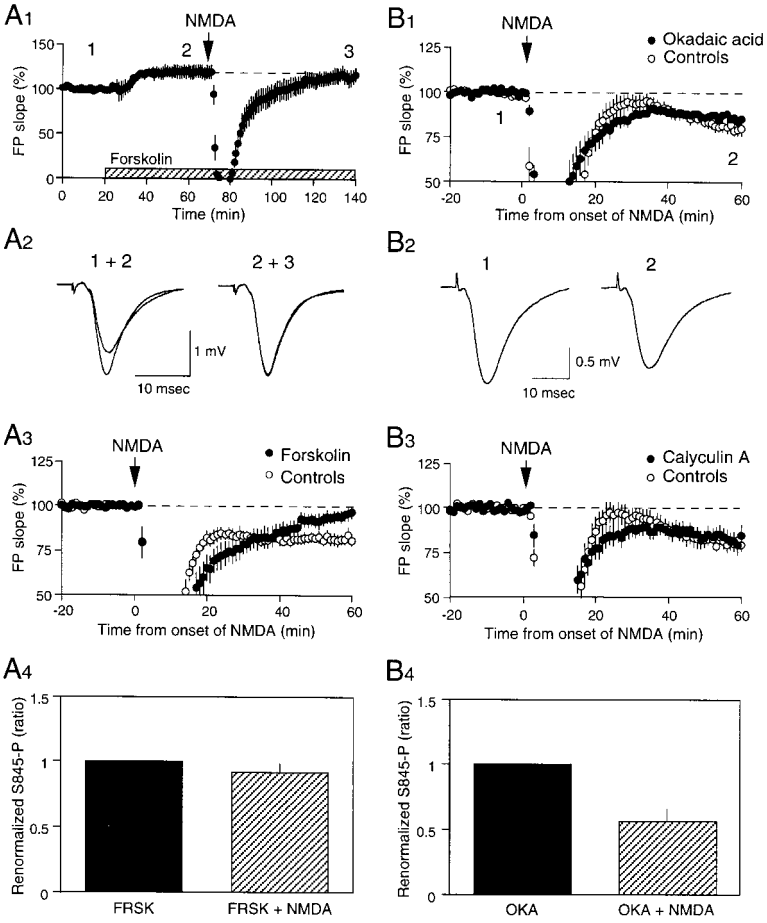

Figure 5. Chem-LTD and Dephosphorylation of GluR1 on Ser-845 Are Prevented by a PKA Activator but Are Not Affected by a PP1/2A Inhibitor

(A) Chem-LTD and NMDA-induced dephosphorylation of GluR1 are prevented by a PKA activator.

(A_1) Bath application of forskolin (50 μM) directly potentiates synaptic transmission, which stabilizes in 20–30 min. NMDA application (20 μM, 3 min) in the presence of forskolin failed to produce chemLTD.

(A_2) FPs (averages of four consecutive traces) from a representative case, taken at the time points indicated in (A_1).

(A_3) Comparison of chemLTD in forskolin with same day control chemLTD. Data are normalized to the 20 min baseline period immediately prior to NMDA application. Chem-LTD in forskolin (95% ± 3% of baseline at 60 min post-NMDA, n = 6) is significantly less than control (82% ± 3% of baseline, n = 7, $p < 0.01$).

(A_4) Changes in Ser-845 phosphorylation after chemLTD in slices pretreated with 50 μM forskolin for 50 min prior to chemLTD (20 μM NMDA, 3 min; FRSK + NMDA) induction compared to slices treated with forskolin alone (FRSK). Preincubation in forskolin prevented dephosphorylation of Ser-845 by NMDA.

(B) Chem-LTD and NMDA-induced dephosphorylation of GluR1 are not prevented by a PP1/2A inhibitor.

(B_1) Preincubation of slices for ≥3 hr in 1 μM okadaic acid (with 0.1% DMSO) did not affect chemLTD (86% ± 1% of baseline at 60 min post-NMDA, n = 8) compared with the same day controls (80% ± 3% of baseline, n = 7) incubated in the solvent (0.1% DMSO).

(B_2) FPs (averages of four consecutive traces) taken before and after chemLTD as indicated in (B_1).

(B_3) Preincubation of slices for ≥2 hr in 1 μM calyculin A also did not affect chemLTD (80% ± 6%, n = 6) compared with same day DMSO control (78% ± 5%, n = 4).

(B_4) Okadaic acid (1 μM, ≥3 hr) treatment prior to chemLTD induction does not prevent dephosphorylation of Ser-845. Phosphorylation level of GluR1 at Ser-845 in OKA + NMDA group is normalized to OKA-only controls.

These findings were surprising in light of previous works suggesting a role of PP1/2A in homosynaptic LTD (Mulkey et al., 1993; Kirkwood and Bear, 1994). However, they did suggest that if dephosphorylation of GluR1 is the expression mechanism of chemLTD, it should be similarly resistant to these PP inhibitors. Indeed, consistent with its lack of an effect on chemLTD, okadaic acid did not prevent the NMDA-induced dephosphorylation of Ser-845 (Figure 5B_4). Treatment of the slices with okadaic acid (1 μM) increased the phosphorylation of both serine 831 and 845, suggesting that PP1 or PP2A regulates GluR1 phosphorylation (Table 2). However, NMDA still produced a specific decrease in the phosphorylation of Ser-845, with little effect on the phosphorylation of Ser-831 (Table 2). These results indicate that PP1/2A do not play an essential role in the observed NMDA receptor–dependent decrease in Ser-845 phosphorylation.

Calcineurin (PP2B) is a calcium-activated protein phosphatase that has been proposed to be a part of the signaling pathway that triggers LTD (Lisman, 1989; Mulkey et al., 1994). PP2B is inhibited by the immunosuppressive drug FK506 (Kunz and Hall, 1993). To examine the possible contribution of PP2B to chemLTD and GluR1 dephosphorylation, slices were preincubated for 2–4 hr in 50–100 μM FK506. Although the magnitude of chemLTD was reduced compared with same day controls, significant chemLTD still occurred in the treated slices (Table 1). Biochemical experiments showed that the incubation of hippocampal slices with 100 μM FK506 had little effect on the basal phosphorylation of GluR1 on both Ser-845 and Ser-831 and that FK506 did not significantly block the NMDA receptor–dependent dephosphorylation of Ser-845 (Table 2). Treatment of slices with another inhibitor of PP2B, cyclosporin A (250 μM, n = 4), was similarly ineffective on the basal phosphorylation of GluR1 and on the NMDA-stimulated dephosphorylation of Ser-845 (data not shown). Thus, PP2B also does not appear to be required for chemLTD or GluR1 dephosphorylation.

The findings that neither okadaic acid nor calyculin inhibit chemLTD are in register with biochemical data

Table 1. Effects of Drug Treatments on the Magnitude of Chem-LTD and Homosynaptic LTD

Drug Treatment	Magnitude of Chem-LTD (% of baseline)		Magnitude of Homosynaptic LTD (% baseline)	
	Drug-Treated Slices (n)	Same Day Vehicle Controls (n)	Drug-Treated Slices (n)	Same Day Vehicle Controls (n)
Forskolin (50 μM, 50 min wash on)	95 ± 3[ab] (6)	82 ± 3 (7)	89 ± 3[a] (6)	73 ± 4 (6)
8-bromo-cAMP (100 μM, 30 min wash on)	96 ± 1[a] (6)	78 ± 3 (7)	84 ± 2[a] (4)	70 ± 1 (4)
Okadaic acid (1 μM, ≥3 hr preincubation)	86 ± 1 (8)	80 ± 3 (7)	122 ± 5 (4)	—
Okadaic acid (1 μM, ≥1 hr preincubation)	82 ± 3 (6)	85 ± 3 (4)	106 (1)	—
Calyculin A (1 μM, ≥1 hr preincubation)	80 ± 6 (6)	78 ± 5 (4)	—	—
FK506 (50–100 μM, ≥3 hr preincubation)	91 ± 3[a] (7)	74 ± 4 (7)	89 ± 1[a] (8)	77 ± 2 (8)

[a] Significantly different from same day controls (t test, $p < 0.05$).
[b] Not significantly different from baseline.

showing that these PP1 inhibitors fail to prevent the dephosphorylation of the AMPA receptor following NMDA treatment. However, FK506 partially inhibits chemLTD, while it appears to have no effect on GluR1 phosphorylation. This difference could be explained if the dephosphorylation of AMPA receptors at the Schaffer collateral synapses was more sensitive to the drug than the dephosphorylation of the larger pool of receptors detected biochemically. Alternatively, it is possible that FK506 masks the full expression of chemLTD by enhancing synaptic transmission via a mechanism other than phosphorylation of GluR1 (e.g., Enan and Matsumura, 1991).

Discussion

In the previous paper, we showed that treating hippocampal slices with NMDA causes long-term synaptic depression that is associated with a persistent dephosphorylation of a PKA substrate: Ser-845 on the GluR1 subunit of the AMPA receptor (Lee et al., 1998). In the present study, we confirmed the involvement of PKA substrates in the expression of homosynaptic LTD. Of particular significance was the finding that the activation of postsynaptic PKA interferes with the expression of both chemLTD and homosynaptic LTD. In addition, PKA activators could prevent the dephosphorylation of GluR1 on Ser-845 associated with chemLTD. Taken together, our findings are consistent with the hypothesis that dephosphorylation of Ser-845 on GluR1 is one mechanism of LTD expression.

NMDA-Stimulated Dephosphorylation of the AMPA Receptor Correlates with NMDA-Stimulated Long-Term Synaptic Depression

We previously showed that brief application of NMDA to hippocampal slices causes rapid and persistent dephosphorylation of the AMPA receptor GluR1 subunit at Ser-845 (Lee et al., 1998). Because Ser-845 is a substrate of PKA, we expected that the PKA activators would prevent the dephosphorylation, and the data obtained in this study confirmed this prediction. Unexpected, however, were the effects of phosphatase inhibitors. There is evidence that NMDA receptor stimulation can activate PP1 and that PP1 dephosphorylation of postsynaptic substrates is crucial for induction of some forms of synaptic depression (Mulkey et al., 1993; Bear and Malenka, 1994; Kirkwood and Bear, 1994; O'Dell and Kandel, 1994). Okadaic acid is a potent inhibitor of PP1, so we expected this drug to block the NMDA-stimulated dephosphorylation of the AMPA receptor. However, although an increase in basal phosphorylation was observed in okadaic acid–treated slices, NMDA application still induced GluR1 dephosphorylation. Inhibitors of PP2B (calcineurin), a phosphatase involved in PP1 regulation, neither increased basal phosphorylation of the receptor nor affected the dephosphorylation stimulated by NMDA. These findings do not rule out a role for PP1 and PP2B in the regulation of AMPA receptor phosphorylation, but they do suggest that other phosphatases are also involved. An obvious candidate is PP2C, which is also highly enriched in the brain, especially in the hippocampus (Cohen, 1989; Abe et al., 1992).

Although the inability of okadaic acid to inhibit NMDA-induced dephosphorylation of GluR1 was unexpected, these data did lead to a strong prediction. If chemLTD is related to the dephosphorylation of GluR1, as we have hypothesized, then it too should be insensitive to PP1 inhibitors. The electrophysiological experiments confirmed this prediction. Neither the PP1 inhibitors okadaic acid (1 and 10 μM) nor calyculin A (1 μM) had any significant effect on NMDA-stimulated LTD. Chem-LTD also still occurred in the presence of a PP2B inhibitor, although the magnitude was reduced by this drug treatment.

The ineffectiveness of PP inhibitors contrasts sharply with the effects of PKA activators on NMDA-stimulated LTD. Bath application of PKA activators could completely inhibit the expression of chemLTD. Moreover, intracellular injection of a PKA activator confirmed that the critical PKA substrate is in the postsynaptic neuron. The fact that chemLTD could be blocked by activators

Table 2. Effects of Drug Treatments on Phosphorylation of the AMPA Receptor GluR1 Subunit at Ser-845 and Ser-831

Phosphorylation of Ser-845				
Drug Treatment	n	NMDA Alone (% of vehicle[a] control)	Drug Alone (% of vehicle[a] control)	NMDA + Drug (% of drug alone)
Forskolin (50 μM for 50 min)	6	62 ± 4[b]	911 ± 190[b]	92 ± 7
8-bromo-cAMP (100 μM for 30 min)	7	53 ± 7[b]	103 ± 13	73 ± 9[c]
Okadaic acid (1 μM for ≥3 hr)	4	38 ± 25[b]	2013 ± 834[b]	56 ± 10[c]
FK506 (50–100 μM for ≥2 hr)	5	78 ± 8[b]	67 ± 14[b]	50 ± 13[c]
Phosphorylation of Ser-831				
Drug Treatment	n	NMDA Alone (% of vehicle[a] control)	Drug Alone (% of vehicle[a] control)	NMDA + Drug (% of drug alone)
Forskolin (50 μM for 50 min)	6	85 ± 5	78 ± 6[b]	103 ± 8
8-bromo-cAMP (100 μM for 30 min)	7	101 ± 11	102 ± 10	95 ± 5
Okadaic acid (1 μM for ≥3 hr)	4	79 ± 24	587 ± 142[b]	94 ± 14
FK506 (50–100 μM for ≥2 hr)	5	138 ± 25	117 ± 26	89 ± 10

[a] Vehicle solution was ACSF alone for 8-bromo-cAMP, ACSF + 0.1% DMSO for forskolin and okadaic acid, and ACSF + 0.1%–0.2% EtOH for FK506 experiments.
[b] Significantly different from vehicle-treated controls (one way ANOVA, Student Newman-Keuls, $p < 0.05$).
[c] Significantly different from drug-along controls (one way ANOVA, Student Newman-Keuls, $p < 0.05$).

of PKA strengthens the conclusion that chemLTD is a form of synaptic plasticity rather than some deterioration of slices caused by NMDA treatment. Moreover, these data are in excellent agreement with the biochemical results, which showed that the dephosphorylation of GluR1 can be inhibited by PKA activators.

The effects of NMDA treatment on AMPA receptor phosphorylation correlate well with the effects of NMDA on synaptic transmission. The NMDA treatments that produce LTD (e.g., 20 μM for 3 min, with or without a chase with AP5) produce dephosphorylation of GluR1; a treatment that fails to produce LTD (e.g., 10 μM NMDA) fails to produce dephosphorylation. Pretreatments with AP5 or forskolin block both LTD and the dephosphorylation. Pretreatments with PP1 and PP2B inhibitors block neither LTD nor dephosphorylation. Taken together, the results provide strong support for the hypothesis that one mechanism of chemLTD expression is the dephosphorylation of the GluR1 subunit of the postsynaptic AMPA receptor. Such a postsynaptic change would be expected to cause a depression of synaptic transmission without a change in paired pulse facilitation, which is precisely what we observe with chemLTD (Lee et al., 1998).

Dephosphorylation of a Postsynaptic PKA Substrate Controls Expression of NMDA Receptor–Dependent Homosynaptic LTD

Homosynaptic LTD in CA1 is induced with LFS of the Schaffer collaterals. Although there are multiple routes of induction and expression of homosynaptic LTD in CA1, one well-characterized form depends on NMDA receptor activation for induction (Dudek and Bear, 1992) and may be expressed by a postsynaptic modification (Oliet et al., 1996; Kandler et al., 1998). Previously, evidence was presented suggesting that this form of homosynaptic LTD shares the same expression mechanism as chemLTD (Lee et al., 1998). Specifically, it was shown that (1) chemLTD does not occur at synapses already depressed by LFS; (2) little if any homosynaptic LTD occurs at synapses depressed previously by NMDA treatment; and (3) induction of chemLTD, like homosynaptic LTD, can unsaturate previously established LTP.

The current experiments provide further support for the hypothesis that homosynaptic LTD and chemLTD share common mechanisms. Most importantly, we confirmed involvement of a postsynaptic PKA substrate in the expression of homosynaptic LTD. Previously, it had been reported that bath application of a PKA activator (Mulkey et al., 1994) and a PKA inhibitor (Brandon et al., 1995), as well as genetic manipulations of PKA subunit expression (Brandon et al., 1995; Qi et al., 1996), interferes with the induction of homosynaptic LTD with LFS. However, our experiments are the first to demonstrate that the PKA substrate crucial for homosynaptic LTD is in the postsynaptic neuron and that it is involved specifically in LTD expression. Intracellular injection of a PKA activator (Sp-cAMPS) both inhibited LTD induction and completely reversed previously established LTD. Moreover, injection of a PKA inhibitor (PKI) produced synaptic depression that occluded LTD. Since dephosphorylation of a PKA site on the AMPA receptor is very likely to be a mechanism for chemLTD expression, we suggest that it also could be a mechanism for homosynaptic LTD expression.

It should be noted that it has been shown previously

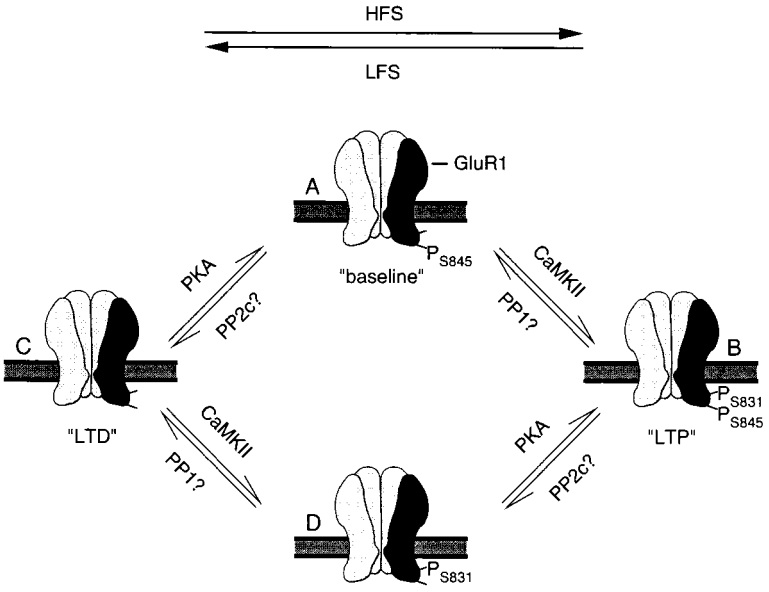

Figure 6. A Possible Model for Bidirectional Synaptic Plasticity in CA1

The model is designed to take into account the following four observations regarding NMDA receptor–dependent synaptic plasticity in CA1. First, from a baseline state, synapses can be potentiated in response to HFS and depressed in response to LFS. Second, LTP and LTD can be reversed by LFS and HFS, respectively. Third, expression of LTP from baseline depends on phosphorylation of a postsynaptic CaMKII (but not PKA) substrate. Fourth, expression of LTD from baseline depends on dephosphorylation of a PKA substrate. According to the model, HFS can cause synapses to grow more effective in two distinct ways, depending on the initial state of the synapses. For example, from a depressed state, HFS can cause "dedepression" via phosphorylation of a postsynaptic PKA substrate, while from a baseline state, HFS can cause "potentiation" via phosphorylation of a postsynaptic CaMKII substrate. Similarly, LFS can cause synapses to become weaker in two different ways. For example, from a potentiated state, LFS can cause "depotentiation" via dephosphorylation of a postsynaptic CaMKII substrate, and from a baseline state, LFS can cause "depression" via dephosphorylation of a postsynaptic PKA substrate. The mechanism proposed to implement the model is based on observed changes in phosphorylation of the GluR1 subunit of postsynaptic AMPA receptors following induction of LTD and LTP. The phosphatases involved in depression and depotentiation are not known with certainty.

that postsynaptic inhibition of PKA, as well as displacement of the enzyme from anchoring sites in the postsynaptic density, lead to a gradual decrease in the amplitude of spontaneous excitatory postsynaptic currents mediated by native AMPA receptors (Rosenmund et al., 1994). Our data strongly suggest that this rundown of postsynaptic AMPA receptor–mediated currents is a direct result of dephosphorylation of the AMPA receptor and, furthermore, that this may be a mechanism for homosynaptic LTD.

Chem-LTD and Homosynaptic LTD: Common Expression Mechanisms with Distinct Induction Mechanisms?

Despite their similarities, chemLTD and homosynaptic LTD differ substantially with respect to the effects of PP1 inhibitors. A number of laboratories have shown that LFS does not produce normal LTD in slices treated with PP1 inhibitors (Mulkey et al., 1993, 1994; Kirkwood and Bear, 1994; O'Dell and Kandel, 1994), and we confirmed this (Table 1). This finding contrasts with the absence of an effect of either okadaic acid or calyculin A on chemLTD. In addition, the partial blockade of homosynaptic LTD by bath-applied PKA activators contrasts with the complete block of chemLTD by these drugs (Table 1).

Although it is possible that chemLTD and homosynaptic LTD are nonoverlapping phenomena, most evidence suggests otherwise. In particular, the fact that they are mutually occluding suggests that they utilize a common saturable mechanism (Lee et al., 1998). Moreover, expression of both forms apparently depends on persistent dephosphorylation of a postsynaptic PKA substrate. How, then, are we to account for the differential sensitivity to PP1 inhibitors and bath-applied PKA activators?

One possible explanation is that PP1 inhibitors do not affect the mechanism of homosynaptic LTD per se but alter the cellular responses to LFS (e.g., Herron and Malenka, 1994; Blitzer et al., 1995; Thomas et al., 1996). An alternative is that whereas chemLTD and homosynaptic LTD share a common expression mechanism (e.g., dephosphorylation of GluR1), they have distinct induction mechanisms. For example, LFS might trigger dephosphorylation via the selective activation of PP2B and PP1, as has been suggested (Lisman, 1989; Bear and Malenka, 1994; Mulkey et al., 1994). In contrast, NMDA treatment might inhibit or displace postsynaptic PKA, allowing constituitively active protein phosphatases to produce a net dephosphorylation. In this context, it is worth pointing out that PP1 and the constituitively active protein phosphatase PP2C have broad and overlapping substrate specificity (Cohen, 1989). According to this scheme, PP1/2A or PP2B inhibitors may be more efficient in blocking homosynaptic LTD, while PKA activators may inhibit chemLTD more effectively, which is what we observed.

Dissecting the Relationship of LTD and LTP

NMDA receptor activation is necessary for the induction of several types of long-term synaptic change, including specific forms of LTP (Collingridge et al., 1983), LTD (Dudek and Bear, 1992), and depotentiation (the reversal of LTP) (Fujii et al., 1991). The simplest model relating these types of plasticity is the bidirectional regulation of a single phosphoprotein, such as the AMPA receptor. Our results suggest that dephosphorylation of the PKA

site on GluR1 may be one mechanism for LTD. Does PKA phosphorylation of this same site account for LTP?

High-frequency stimulation (HFS), which induces LTP, causes an elevation of cAMP in CA1 (Stanton and Sarvey, 1985) that is NMDA receptor–dependent (Chetkovich and Sweatt, 1993). There is considerable evidence that this biochemical response contributes to the establishment of protein synthesis–dependent, late phase LTP via PKA regulation of gene expression (Frey et al., 1993; Matthies and Reymann, 1993; Bourtchuladze et al., 1994; Impey et al., 1996). However, a role for postsynaptic PKA involvement in the induction and initial expression of LTP is still unclear. PKA inhibitors can block LTP induction by certain types of tetanic stimulation, but this effect may be indirect, accounted for by the release from the inhibition of PP1 (Blitzer et al., 1995, 1998; Thomas et al., 1996). And, although activation of PKA with forskolin produces a rapid enhancement of synaptic transmission in CA1, there are conflicting data regarding the extent to which this effect reflects a postsynaptic modification. In cultured hippocampal neurons, forskolin has been shown to enhance the amplitude of miniature postsynaptic currents by increasing the mean open probability of glutamate-gated, non-NMDA channels (Greengard et al., 1991; Wang et al., 1991), but in hippocampal slices, forskolin was found to have a negligible postsynaptic effect (Chavez-Noriega and Stevens, 1994; Rosenmund et al., 1994). Consistent with the latter observations, in our experiments, we did not typically observe a potentiation of basal synaptic transmission when postsynaptic cells were injected with a cAMP analog.

These data suggest that in hippocampal slices, the effect on synaptic transmission of phosphorylating the PKA sites on postsynaptic AMPA receptors may be saturated under basal conditions. Therefore, LTP of synaptic transmission from the "naive" or "baseline" state is unlikely to be accounted for by the PKA phosphorylation of postsynaptic AMPA receptors. This mechanism for synaptic potentiation is only available after the PKA sites have been dephosphorylated, which occurs following the induction of LTD. Indeed, we find that postsynaptic injection of cAMP does produce potentiation selectively at synapses that had previously undergone LTD.

In contrast to the situation with PKA, there is very strong evidence that postsynaptic CaMKII is essential for the induction of LTP from the baseline state (reviewed by Lisman, 1994). The AMPA receptor is also a substrate of CaMKII, and phosphorylation of GluR1 by CaMKII can also enhance glutamate-evoked currents through AMPA receptors (Tan et al., 1994; Yakel et al., 1995). Recent studies have reported that LTP-inducing stimulation in CA1 leads to CaMKII-dependent phosphorylation of GluR1 (Barria et al., 1997), suggesting that CaMKII phosphorylation of GluR1 is a mechanism for at least the initial expression of LTP.

Taken together, the data support the idea that NMDA receptor–dependent LTD and LTP can result from the bidirectional modification of AMPA receptor phosphorylation. However, since different phosphorylation sites are implicated in LTD and LTP, a new model is suggested based on the four possible phosphorylation states of GluR1 (Figure 6). If it is assumed that the effects of phosphorylating the PKA site (Ser-845) are usually saturated under baseline conditions (Figure 6A), then synaptic transmission may be modified either by the phosphorylation of the CaMKII site, causing potentiation (Figure 6B), or by dephosphorylation of the PKA site, causing depression (Figure 6C). It is interesting to note that the model suggests that the reversal of LTP with LFS (depotentiation) and the reversal of LTD with HFS (dedepression) may or may not "reset" the synapse to its original state (Figure 6D).

While the data clearly support the hypothesis that LTP and LTD can result from changes in the phosphorylation of the GluR1 subunit of the AMPA receptor, it is equally clear that the model in Figure 6 is not a complete description of long-term plasticity in CA1. For example, the model does not account for the distinct mechanisms of NMDA receptor–independent LTD (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997), LTP (Grover and Teyler, 1990), and depotentiation (Staubli and Chun, 1996). Nonetheless, the model accounts well for the observed biochemical changes in AMPA receptor phosphorylation, as well as the effects of experimentally manipulating postsynaptic PKA and CaMKII. The model predicts that HFS and LFS can engage different mechanisms, depending on the initial state of the stimulated synapses. These different mechanisms may be differentially regulated, for example, by age or the history of synaptic activation. Variations in the relative expression of these mechanisms for plasticity could be an important source of variability between laboratories studying LTP and LTD. Perhaps more significantly, such differential regulation would provide a means for gain control of synaptic plasticity during development or different behavioral states.

Experimental Procedures

Slice Preparation for Electrophysiology

Hippocampal slices were prepared from 21- to 30-day-old male Long-Evans rats (Charles River) as described previously (Lee et al., 1998). CA_3 was surgically removed immediately after sectioning. The composition of artificial cerebrospinal fluid (ACSF) was as follows (in mM): NaCl, 124; KCl, 5; NaH_2PO_4, 1.25; $NaHCO_3$, 26; dextrose, 10; $MgCl_2$, 1.5; and $CaCl_2$, 2.5 and was bubbled with 95% O_2 and 5% CO_2. The slices were left in the holding chamber at room temperature for $\geq$1 hr to equilibrate. Slices were then gently transferred to a submersion-type recording chamber (Medical Systems, Greenvale, NY) and continually perfused with 30°C oxygenated ACSF at a rate of 2 ml/min. Slices were left undisturbed in the recording chamber for $\geq$20 min prior to recording.

Electrophysiological Recordings

Synaptic responses were measured extracellularly in the stratum radiatum of CA1 as described previously (Lee et al., 1998). Baseline synaptic responses were obtained by stimulating once every 30 s with a stimulation intensity (10–30 μA) that yielded a half maximal population EPSP slope. For two pathway experiments, two stimulating electrodes were placed on either side of the recording electrode to stimulate two independent pathways. The absence of cross-pathway paired pulse facilitation was the criterion used to determine that the pathways were independent from each other. The two inputs were stimulated in alternation every 15 or 20 s.

Intracellular EPSPs were recorded by using sharp glass as described previously (Lee et al., 1998). Resting membrane potential of the cells was ≤ -60 mV (average -69 ± 1 mV, $n = 37$ cells). Input resistance of the cells was measured by passing -0.1 nA current (100 msec) through the electrode and was on average 100 ± 6 MΩ

(range 47–190 MΩ). Spike accomodation was monitored by injecting +0.1 or +0.2 nA (1 s) current pulses.

Homosynaptic LTD was induced by delivering low-frequency stimulation (LFS, 900 pulses at 1 Hz) at the same stimulation intensity as baseline. Depending on the experimental conditions, LFS can induce two different types of homosynaptic LTD, one that depends upon NMDA receptor activation (Dudek and Bear, 1992) and another that is induced independent of NMDA receptor activation (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997). Under the conditions employed in this study, the induction of homosynaptic LTD is completely blocked by an NMDA receptor antagonist, provided that the LFS is applied within 6 hr of preparing the slices. In the experiments reported here, care was taken to study LTD in slices maintained ≥6 hr in vitro.

Data Analysis

Evoked extracellular field potentials and intracellular EPSPs were digitized at 20 kHz and stored on a 486 IBM-compatible computer with Experimenter's Workbench (BrainWave Systems, Boulder, CO) until further analysis. Initial slope of the recorded field potentials and intracellular EPSP were measured as an indicator of synaptic strength. Summary graphs were generated as follows: (1) the EPSP slope data for each experiment were expressed as percentages of the preconditioning baseline average, (2) the time scale in each experiment was converted to time from the onset of conditioning, and (3) the time-matched, normalized data were averaged across experiments and expressed as the means (±SEM). Statistical comparisons were made of 5 min averages of the measurements immediately before and 55–60 min after treatment (NMDA or LFS) using a t test.

Drug Preparation and Application

NMDA (Sigma) was dissolved in ACSF at a concentration of 200 μM and infused at one-tenth the ACSF flow rate (0.2 ml/min) to yield a final concentration of 20 μM in the recording chamber. Forskolin (BioMol) was dissolved in DMSO and diluted to the final concentration of 50 μM in ACSF (with 0.1% DMSO) and was bath applied for 50 min before experimental manipulations. 8-Bromo-cAMP (BioMol) was dissolved directly in ACSF at 100 μM and was applied for ≥35 min before the experimental manipulations. D,L-amino-5-phosphonovaleric acid (D,L-AP5; Sigma) was dissolved in ACSF at final concentrations and was applied for ≥30 min prior to experimental manipulations. Sp-cAMPS (BioMol) was dissolved in dH_2O, diluted to final concentration in the intracellular recording solution (3 M KAc and 10 mM KCl), and applied via the intracellular recording pipette. Okadaic acid and calyculin A (LC Laboratories) were dissolved in DMSO and further diluted with ACSF to a final concentration of 1 μM (with 0.1% DMSO). Slices were preincubated in okadaic acid and calyculin A for ≥3 hr and ≥1 hr, respectively, prior to being transferred into the recording chamber. FK506 was a gift from Fugisawa Pharmaceuticals. It was dissolved in ethanol at 50 mM, which was diluted to 50–100 μM (with 0.1%–0.2% ethanol) in ACSF. Slices were preincubated in FK506 for ≥4 hr. For all experiments in which inhibitors were bath applied or used for preincubation, statistical comparisons were made with same day controls treated only with vehicle solution. On any given day, vehicle- and drug-treated slices were studied in alternation so that there was not a systematic difference in the time spent in vitro.

Slice Preparation for Biochemical Experiments

Hippocampal slices were prepared from 21–30-day-old male Long-Evans rats (Charles River), and the CA_3 region was surgically removed as previously described (Lee et al., 1998). The slices were collected in ACSF (in mM: NaCl, 124; KCl, 5 NaH_2PO_4, 1.25; $NaHCO_3$, 26; dextrose, 10; $MgCl_2$, 1.5; and $CaCl_2$, 2.5) and transferred to a submersion-type holding chamber and incubated at room temperature in ACSF bubbled with 95% O_2 and 5% CO_2 for at least 30 min. The slices were then transferred to a 12-well multiwell plate with mesh inserts (Fisher Scientific, Pittsburgh, PA); each well was filled with ACSF. The multiwell plate was placed in a closed chamber supplied with humidified oxygenated atmosphere (95% O_2, 5% CO_2) and placed in a 30°C water bath. The slices were left to equilibrate for an additional 1 hr prior to the experiments.

Chem-LTD was induced by submerging the slices in 20 μM NMDA for 3 min. The slices were gently transferred by using the mesh inserts to a well containing 20 μM NMDA solution. After 3 min, the slices were transferred to another well containing standard ACSF solution. For various drug treatments, the slices were preincubated in the drugs prior to NMDA treatment.

Homogenates of hippocampal slices were prepared by sonicating the 1–4 slices on ice in 1 ml of resuspension (RS) buffer consisting of 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, 5 mM EGTA, 1 μM okadaic acid, and 10 U/ml aprotinin for 30 s. The homogenates were centrifuged at 12,000 × g for 5 min and the crude membrane pellets were resuspended in RS buffer and centrifuged again at the same speed. The pellets were then suspended in SDS sample buffer. The protein concentration was determined by amino black method and loaded onto SDS-PAGE gels (7.5%) with 10–20 μg of protein per lane, and the resulting gels were transferred to polyvinylidene difluoride (PVDF) membranes.

Immunoblot Analysis

PVDF membranes were incubated for 15 min with 25% methanol and 10% acetic acid (weight/volume). The membranes were then blocked with 1% bovine serum albumin (BSA) and 0.1% Tween-20 in PBS for 1 hr, incubated 90 min with the phosphorylation site–specific antibodies (150–500 mg/ml in blocking buffer), washed 3 × 5 min with blocking buffer, and incubated 90 min with horseradish peroxidase–conjugated anti-rabbit Ig (1:5000 in blocking buffer). After final washes in blocking buffer (3 × 10 min), the membranes were rinsed in TBS and immersed in chemiluminescence (ECL) detection reagent (DuPont NEN) for 15 s and exposed to XAR film. Exposure times ranged from 30 s to 5 min and were in the linear range for quantitative analysis. The phosphorylation site–specific antibodies were stripped from the membranes by incubating them in 62.5 mM Tris (pH 6.8), 2% SDS, and 0.7% 2 mercapto-ethanol and then reprobed with anti GluR1 C-terminal antibodies to estimate the total amount of GluR1. Immunoblots were analyzed on a Molecular Dynamics Personal Densitometer. The relative amount of GluR1 phosphorylation was analyzed by determining the ratio of the signals detected with the phosphorylation site–specific antibodies and the phosphorylation independent C-terminal antibody.

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