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NMDA Induces Long-Term Synaptic Depression and Dephosphorylation of the GluR1 Subunit of AMPA Receptors in Hippocampus

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Summary

Brief bath application of N-methyl-D-aspartate (NMDA) to hippocampal slices produces long-term synaptic depression (LTD) in CA1 that is (1) sensitive to postnatal age, (2) saturable, (3) induced postsynaptically, (4) reversible, and (5) not associated with a change in paired pulse facilitation. Chemically induced LTD (Chem-LTD) and homosynaptic LTD are mutually occluding, suggesting a common expression mechanism. Using phosphorylation site–specific antibodies, we found that induction of chem-LTD produces a persistent dephosphorylation of the GluR1 subunit of AMPA receptors at serine 845, a cAMP-dependent protein kinase (PKA) substrate, but not at serine 831, a substrate of protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII). These results suggest that dephosphorylation of AMPA receptors is an expression mechanism for LTD and indicate an unexpected role of PKA in the postsynaptic modulation of excitatory synaptic transmission.

Introduction

It is now well established that the effectiveness of synapses can be altered by activity. At many glutamatergic synapses in the CNS, long lasting (>1 hr) modifications can be produced reliably by the appropriate experimental manipulations of presynaptic and postsynaptic activity. The modifications thus produced are termed long-term depression, or LTD, in cases of synaptic decrement and long-term potentiation, or LTP, in cases of synaptic enhancement. In the well-studied CA1 region of the hippocampus, there are distinct types of LTP and LTD, and these have different induction mechanisms (e.g., Grover and Teyler, 1990; Bolshakov and Siegelbaum, 1994; Huber et al., 1995; Oliet et al., 1997). However, the most prominent—or at least the most commonly studied—forms of LTP and LTD require the activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors for induction (Collingridge et al., 1983; Dudek and Bear, 1992; reviewed by Bear and Abraham, 1996). These forms of LTP and LTD occur at the same Schaffer collateral synapses and may result from the bidirectional regulation of common molecular targets (Mulkey and Malenka, 1992; Dudek and Bear, 1993; Mulkey et al., 1993, 1994; Stevens and Wang, 1994; Heynen et al., 1996; Oliet et al., 1996). A key unanswered question is the identity of these targets.

Although both LTP and LTD depend upon (Lynch et al., 1983; Mulkey and Malenka, 1992) and are triggered by (Malenka et al., 1988; Neveu and Zucker, 1996) a rise in postsynaptic [Ca^{2+}], they are induced with different types of NMDA receptor activation. Strong, brief (≤2 sec), high-frequency (≥50 Hz) synaptic stimulation is optimal for LTP induction, whereas weak, prolonged (≥30 sec), low-frequency (<10 Hz) stimulation is optimal for LTD induction. Available data suggest that the different Ca^{2+} signals produced by the different types of stimulation activate distinct biochemical cascades. For example, activation of postsynaptic calcium/calmodulin-dependent protein kinase II (CaMKII) is necessary (Malenka et al., 1989; Malinow et al., 1989) and sufficient (Petit et al., 1994; Lledo et al., 1995) to induce LTP. In contrast, there is evidence that LTD induction requires activation of a protein phosphatase cascade (Mulkey et al., 1993, 1994). A current working hypothesis is that LTP and LTD result from the phosphorylation and dephosphorylation, respectively, of a common set of synaptic phosphoproteins (Bear and Malenka, 1994).

A synaptic phosphoprotein of particular interest in this regard is the AMPA receptor, which mediates rapid excitatory transmission in the CNS. AMPA receptors are tetrameric or pentameric complexes of four homologous subunits (GluR1–GluR4) that combine to form different AMPA receptor subtypes (Seeburg, 1993; Hollmann and Heinemann, 1994). The AMPA receptor GluR1 subunit is one of the most abundantly expressed subunits in the hippocampus and neocortex and, in combination with GluR2, is thought to constitute the majority of AMPA receptor complexes in these regions (Wenthold et al., 1996). The GluR1 subunit has been demonstrated to be directly phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and CaMKII in vivo and in vitro (McGlade-McCulloh et al., 1993; Blackstone et al., 1994; Tan et al., 1994; Roche et al., 1996; Barria et al., 1997a, 1997b; Mammen et al., 1997). PKA specifically phosphorylates serine 845, while PKC and CaMKII phosphorylate serine 831, both contained in the C-terminal tail of the GluR1 subunit (Roche et al., 1996; Barria et al., 1997a; Mammen et al., 1997). Phosphorylation of both of these serine residues potentiates AMPA receptor function (Roche et al., 1996; Barria et al., 1997a). These results suggest that AMPA receptor phosphorylation and dephosphorylation could contribute to the expression mechanisms of LTP and LTD, respectively (Raymond et al., 1993; Roche et al., 1994). Unfortunately, monitoring changes in AMPA receptor phosphorylation during LTP and LTD has been technically difficult, since only a small fraction of synapses are affected by the electrical stimulation typically used to elicit synaptic plasticity.

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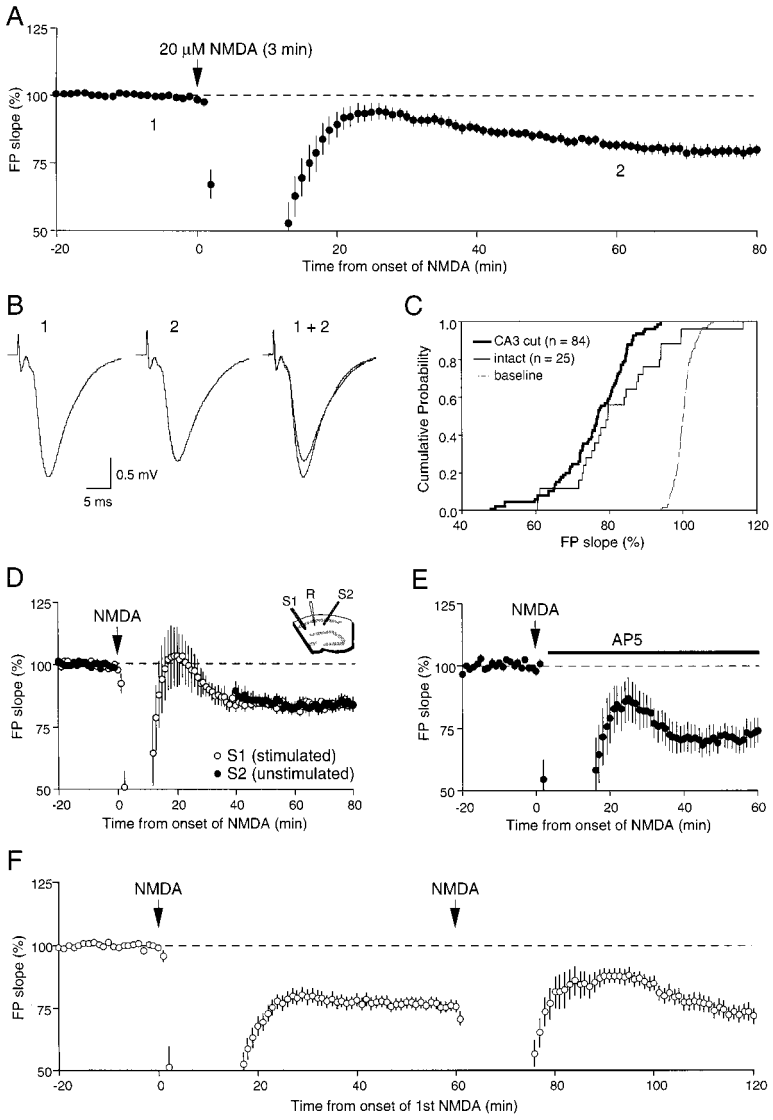

Figure 1. NMDA Produces LTD

(A) Bath application of NMDA can produce LTD. 20 μM NMDA for 3 min produced a stable LTD of evoked responses in CA1 hippocampal slices in which CA3 was surgically removed (n = 20).

(B) Representative FPs (average of four consecutive traces) before and after induction of chem-LTD, taken at time points indicated in (A).

(C) Cumulative probability of the magnitude of chem-LTD measured 1 hr post-NMDA application in CA3 cut (n = 84) and intact (n = 25) hippocampal slices compared with baseline (n = 109).

(D) Chem-LTD is stimulation-independent. The inset shows the experimental configuration, in which two independent inputs (S1 and S2) were stimulated in alternation (n = 5). Stimulation to S2 was turned off immediately prior to NMDA application and was resumed 30–40 min later, while S1 was stimulated at baseline frequency (0.033 Hz) throughout the duration of the experiment. Both S1 and S2 depressed to the same level.

(E) Chem-LTD is not due to tonic activation of NMDA receptors. 100 μM AP5 was applied immediately after NMDA, as indicated by the dark bar, to block any possible tonic activation of NMDA receptors (n = 6). Chem-LTD was not affected by this manipulation.

(F) Chem-LTD is saturable. A second application of NMDA given 1 hr after the first did not produce any further depression (n = 22).

The electrical stimulation requirements for LTD induction in vitro appear to be less stringent than those for LTP induction. Although 900 pulses at 1–3 Hz remains the standard protocol for inducing LTD (Dudek and Bear, 1992), several other types of stimulation can be equally effective (e.g., Debanne et al., 1994; Cummings et al., 1996). Presumably, any stimulation protocol that selectively activates the appropriate biochemical cascade in the postsynaptic neuron should be sufficient to induce LTD. We were interested in the possibility that LTD could be induced in slices of the hippocampus simply by pharmacologically activating NMDA receptors. Our motivation for attempting this approach was to develop a paradigm in which LTD is induced at synapses throughout the slice instead of being confined to only those synapses that are within reach of a stimulating electrode. This paradigm could then be used for biochemical detection of changes in AMPA receptor phosphorylation.

Here, we present evidence (1) that brief bath application of NMDA produces a form of LTD in CA1 (chem-LTD) that is similar to homosynaptic LTD produced by 1 Hz stimulation and (2) that using this technique, we can detect a persistent dephosphorylation of the GluR1 subunit of the AMPA receptor. Our results suggest that dephosphorylation of the GluR1 subunit of the AMPA receptors may, at least in part, mediate NMDA receptor–dependent LTD.

Results

Brief Application of NMDA Causes Synaptic Depression

Hippocampal slices were prepared from young (postnatal day [P] 21–35) male Long-Evans rats, and synaptic field potentials (FPs) in stratum radiatum were recorded in response to baseline stimulation of the Schaffer collaterals every 30 sec. After collecting stable baseline responses, we attempted to induce LTD by bath applying low concentrations of NMDA. We failed to observe any lasting effects of brief applications of 5 or 10 μM NMDA; however, a 3 min application of 20 μM NMDA consistently produced a long lasting depression of the evoked

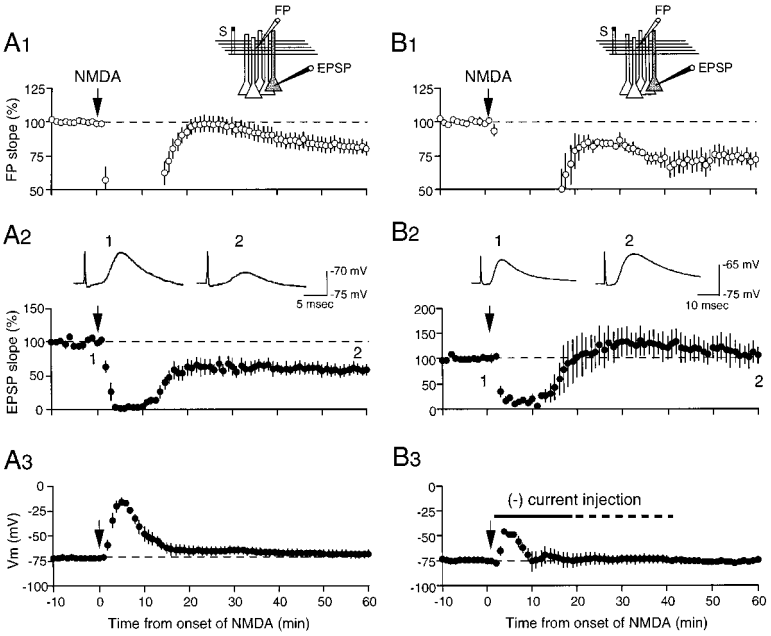

Figure 2. Chem-LTD Is Observed Intracellularly as a Decrease in EPSP Slope that Can Be Blocked by (−) Current Injection

(A) Chem-LTD of the FP (A_1) correlates with chem-LTD of the EPSP slope (A_2). The inset shows the preparation in which simultaneous intracellular and extracellular recordings were made in CA1. (A_3) shows average changes in the membrane potential during and after NMDA application (n = 11).

(B) When NMDA application was quickly followed by a (−) current injection to partially block the large depolarization (B_3), the EPSP did not depress (B_2), while depression was still observed in the FPs (B_1; n = 4 cells). Traces in (A_2) and (B_2) are intracellularly recorded postsynaptic potentials taken at the times indicated.

synaptic response (Figure 1). This effect of NMDA was observed reliably with an extracellular [Ca^{2+}] of 4 and 2.5 mM but not with 1 mM Ca^{2+} ([Mg^{2+}] = 1.5 mM in all cases; data not shown). Therefore, we used 20 μM NMDA for 3 min in the presence of 2.5 mM Ca^{2+} for all subsequent studies of chemically induced LTD (chem-LTD).

Figure 1 illustrates the basic effect of a 3 min bath application of 20 μM NMDA on excitatory synaptic transmission in CA1. NMDA transiently abolishes synaptic responses, owing to depolarization of the neurons in the slice, and this is followed by a recovery and relaxation to a stable response that is depressed, relative to the initial baseline value. Chem-LTD could be elicited in intact slices (82% ± 3% of baseline at 60 min post-NMDA, n = 25), although it was slightly more reliable in slices in which CA3 was surgically removed (76% ± 1%, n = 84; Figure 1C). Chem-LTD was not associated with a change in the fiber volley (e.g., Figure 1B). Chem-LTD typically was stable 60 min post-NMDA application (e.g., Figures 1A and 1D–1F); therefore, we used the magnitude of chem-LTD at this time point for statistical comparisons. At 60 min post-NMDA, there was no change in paired pulse facilitation (data not shown), suggesting that the effect may not be accounted for by a change in the presynaptic glutamate release probability. Although the effect of age on chem-LTD was not studied systematically, posthoc analysis of our experiments revealed that chem-LTD magnitude in slices prepared from animals during the fourth postnatal week (P21–P28, 75% ± 1% of baseline, 1 hr post-NMDA, n = 79) was significantly greater than in slices prepared from animals during the fifth postnatal week (P29–P35, 82% ± 2% of baseline, 1 hr post-NMDA, n = 30, $p < 0.01$).

The fact that chem-LTD was reliably observed in slices with CA3 removed suggests that NMDA is exerting its effect by a direct action on the CA1 neuropil. However, this effect of NMDA could be to promote depression of only those synapses that have received baseline electrical stimulation. Therefore, we tested whether Chem-LTD occurs in the absence of baseline stimulation. In one series of experiments (n = 5), two stimulating electrodes were used to activate independent inputs to the same population of CA1 neurons (Figure 1D). Stimulation of one input (S2) was turned off immediately prior to NMDA application and resumed 30–40 min post-NMDA. We found that the magnitude of chem-LTD measured 80 min post-NMDA in the unstimulated input (S2: 84 ± 3% of baseline slope) was not different from the control input (S1), which received baseline stimulation during the NMDA application (84 ± 1% of baseline slope). In a second series of experiments, stimulation was turned off in both inputs during NMDA application (data not shown). The resulting chem-LTD (81% ± 7% of baseline, n = 4) was not different from the same day control slices that received stimulation (83% ± 1% of baseline, n = 4). Therefore, we conclude that induction of chem-LTD does not require electrical activation of presynaptic inputs to CA1 neurons.

Preincubation with 100 μM 2-amino-5-phosphonovaleric acid (AP5) completely prevented establishment of chem-LTD (n = 2; data not shown), confirming that the NMDA is acting at NMDA receptors to produce the synaptic depression. The simplest interpretation of the data is that transient NMDA receptor activation initiates a process that produces stable depression of synaptic transmission in CA1. However, an alternative explanation could be that NMDA does not wash out of the slice, and it is the tonic activation of NMDA receptors that is responsible for the apparent reduction in synaptic effectiveness. To rule out this possibility, we performed a series of experiments (n = 6) in which the NMDA was immediately chased with 100 μM AP5 (Figure 1E). We found that chasing with AP5 had no significant effect (FP slope at 60 min post-NMDA = 74% ± 5% of baseline), confirming that expression or maintenance of chem-LTD does not require NMDA receptor activation.

One obvious worry is that chem-LTD is simply a reflection of the excitotoxic actions of NMDA on neurons in CA1. As a first test of this possibility, we examined the effects of repeated applications of NMDA. We reasoned that if chem-LTD was caused by damage to CA1 neurons, then repeated exposure to NMDA would produce cumulative decreases in the size of the evoked responses until they disappeared altogether. In this series of experiments (n = 22), the FP slope was depressed to 76% ± 2% of the baseline value 60 min after the first application of NMDA (Figure 1F). A second application of NMDA, however, failed to produce any significant additional depression (72% ± 3% of the initial baseline at 60 min after the second exposure to NMDA; 94% ± 4% of the renormalized baseline). These data suggest that chem-LTD, like homosynaptic LTD, is saturable.

Induction of Chem-LTD Is Postsynaptic and Voltage-Dependent

Another way to rule out excitotoxic cell death as the basis for chem-LTD is to record from individual neurons during and after NMDA application. Therefore, we combined extracellular recordings from stratum radiatum with intracellular recordings from CA1 pyramidal cells using sharp electrodes (Figure 2A). NMDA application resulted in a transient depolarization of the cell, which often resulted in spontaneous spiking or, in some cases, bursting, followed by a depolarization block that lasted for ~10 min. Both FPs and excitatory postsynaptic potentials (EPSPs) recovered as membrane potential repolarized, and both showed long lasting depression (FP: 80% ± 4% of baseline, measured 1 hr post-NMDA; EPSP: 59% ± 8% of baseline, n = 11 cells). On average, there was a trend toward an increase in input resistance (134% ± 17% of baseline) and a slight depolarization of the membrane potential (ΔV_m = 4.5 ± 2.5 mV) 60 min after NMDA application. However, these changes were not statistically significant (paired t test, $p > 0.05$) and did not correlate with the change in the evoked synaptic response. In cases where the cell remained slightly depolarized 1 hr after NMDA application, the EPSP slope was still significantly depressed when current was injected through the microelectrode to bring the membrane potential back to the baseline level. Therefore, EPSP depression seems not to be a mere reflection of the slight depolarization after NMDA treatment. The fact that input resistance of the cells was not significantly changed after NMDA application argues against a possible toxic effect of NMDA at the concentration used for producing chem-LTD. Other electrophysiological measures of neuronal health, like action potential morphology, were also not changed by induction of chem-LTD (data not shown).

To address the question of whether NMDA triggers LTD by a direct action on postsynaptic NMDA receptors, we took advantage of the fact that the conductance of open NMDA receptor channels is voltage dependent in normal extracellular [Mg^{2+}] (Mayer et al., 1984; Nowak et al., 1984). Thus, we reasoned that if NMDA triggers chem-LTD directly, then it should be possible to block the effect intracellularly with a hyperpolarizing current injection. The results of this experiment are illustrated in Figure 2B. Up to −1.5 nA of current was injected manually to dampen the depolarization caused by the NMDA. No depression of the EPSP slope was observed in these cells (106% ± 15% of baseline, measured 1 hr post-NMDA, n = 4 cells), despite clear evidence for chem-LTD in the simultaneously recorded FPs (72% ± 5%). These data do not rule out possible contributions of unknown factors released from the neuropil in response to the massive depolarization of the slice by NMDA. However, they do suggest that postsynaptic, voltage-dependent processes are necessary for chem-LTD induction. The voltage dependence of chem-LTD may explain why 10 μM NMDA failed to produce depression consistently in our pilot studies. The maximal depolarization during 10 min of 10 μM NMDA averaged 23 ± 11 mV (from a resting V_m of −70 ± 4 mV, n = 4) compared with 61 ± 2 mV (from a resting V_m of −72 ± 2 mV, n = 11) during 20 μM NMDA.

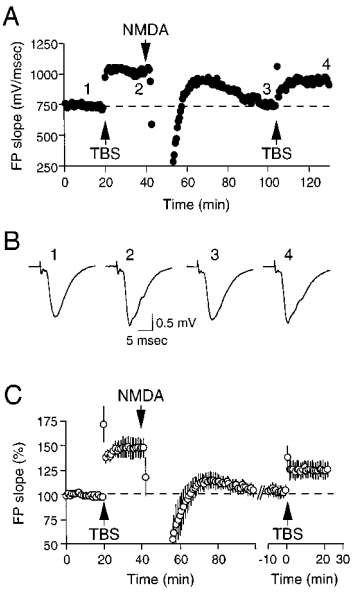

Figure 3. Chemically Depressed Synapses Can Be Potentiated

(A) An example experiment in which chem-LTD is reversed by TBS-induced LTP. After a stable baseline, four episodes of TBS were delivered to saturate LTP. Bath application of NMDA 20 min after LTP induction produced depotentiation that could be reversed by a subsequent TBS.

(B) Field potential traces (averages of four consecutive traces) taken at times indicated in (A).

(C) Averages of all of the experiments (n = 6).

Chemically Depressed Synapses Can Be Potentiated

We next sought to determine if chemically depressed synapses could be potentiated. We adopted the approach used to address this same question following induction of homosynaptic LTD (Dudek and Bear, 1993; Mulkey et al., 1993; Heynen et al., 1996). The idea was first to saturate LTP with repeated tetani and then to induce LTD. If a subsequent high-frequency tetanus caused LTP from the depressed baseline, it would suggest that (1) induction of LTD had "unsaturated" LTP,

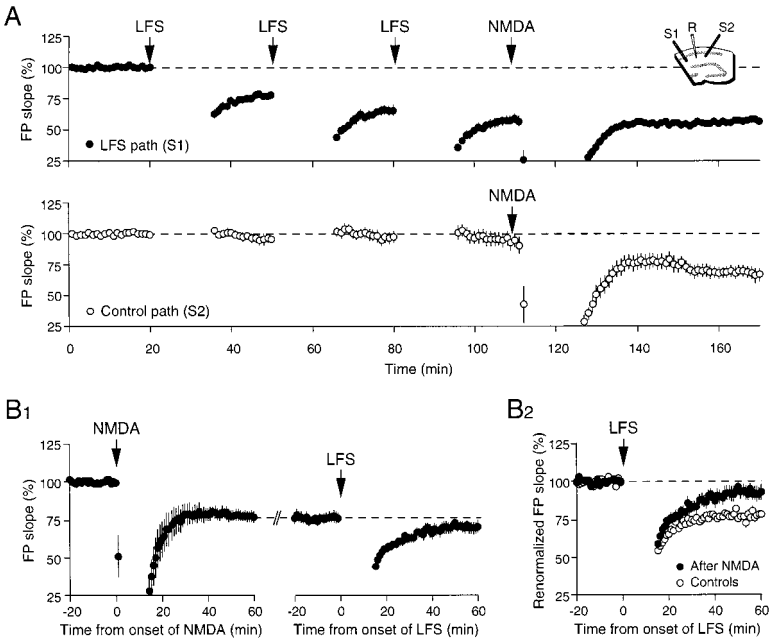

Figure 4. Homosynaptic LTD and Chem-LTD Can Mutually Occlude Each Other

(A) Saturation of homosynaptic LTD can occlude chem-LTD. Responses to two independent pathways converging on a common population of CA1 neurons were monitored (inset). Three episodes of 1 Hz stimulation (3 × 900 pulses) were given to one pathway (S1; closed symbols) to saturate homosynaptic LTD, and NMDA was applied. NMDA did not produce any significant chem-LTD in the pathway expressing homosynaptic LTD (S1), while chem-LTD was expressed in the naive path (S2) (n = 8).

(B_1) One application of 20 μM NMDA for 3 min was given to saturate chem-LTD. After the chem-LTD stabilized, 1 Hz stimulation (900 pulses, 15 min) was delivered. LFS produced no significant further depression (n = 8).

(B_2) Homosynaptic LTD induced after chem-LTD, renormalized to 20 min pre-NMDA baseline (n = 8; closed symbols), is compared with homosynaptic LTD from same day control slices (n = 5; open symbols).

and (2) the same synapses that had undergone LTD could subsequently exhibit LTP.

To saturate LTP, four 2 sec trains of theta burst stimulation (TBS) were delivered to the Schaffer collaterals. Previous work had shown that the LTP produced by this stimulation protocol is nearly maximal under the conditions of our experiments (Dudek and Bear, 1993), and we confirmed this in our study. TBS led to LTP that stabilized at a value of 142% ± 6% (20 min post-TBS, n = 13) of the preconditioning baseline response. In control experiments (n = 7), a second episode of TBS delivered 1 hr later increased the response by only 8% ± 4% (relative to the baseline period immediately prior to the second TBS). A very different result was obtained if chem-LTD was induced between the first and second episodes of TBS. As shown in Figure 3, NMDA application 20 min after the first TBS caused chem-LTD of the response from the potentiated baseline; and now the second TBS 1 hr later caused the response to increase by 28% ± 6% (n = 6). Although the second TBS after induction of chem-LTD was unable to restore fully the response to the LTP saturation level, it did cause significantly more LTP than in controls (t test, $p < 0.03$). The inability to fully repotentiate the synapses in these experiments is not surprising, given the well-documented "metaplastic" effects of prior, strong NMDA receptor activation on LTP (Bear and Abraham, 1996). This fact does not diminish the significance of the observation that potentiation consistently did occur in response to TBS after chem-LTD induction. The data show that synapses that have undergone chem-LTD can be potentiated by TBS.

Chem-LTD and NMDA Receptor–Dependent Homosynaptic LTD Are Mutually Occluding

Like homosynaptic LTD, chem-LTD is saturable, reversible, and dependent upon postsynaptic NMDA receptor activation. We next wished to determine if chem-LTD and homosynaptic LTD share a common mechanism. Without knowing a priori the mechanism for either form of LTD, the best approach to address this question is to see if saturation of one form of LTD occludes induction of the other form of LTD at the same synapses.

The first series of experiments was designed to test if saturation of homosynaptic LTD occludes chem-LTD (Figure 4A). The baseline responses to stimulation of two independent inputs to a common population of CA1 neurons were monitored in alternation. One input (S1) received three 15 min trains of 1 Hz stimulation (3 × 900 pulses) to induce homosynaptic LTD (Dudek and Bear, 1993), and then NMDA was bath applied to the slice. No chem-LTD was induced in the previously depressed path (S1) (105% ± 7% of renormalized baseline, measured 1 hr post-NMDA, n = 8), contrasting with the usual chem-LTD in the control path (S2) (69% ± 3% of renormalized baseline, 1 hr post-NMDA, n = 8). Thus, saturation of homosynaptic LTD in a population of synapses prevents the induction of chem-LTD at this population of synapses but not at other "naive" synapses converging on the same group of postsynaptic neurons. The occlusion of chem-LTD by prior saturation of homosynaptic LTD argues strongly against excitotoxicity as a mechanism for the observed synaptic depression. Instead, these data suggest that 1 Hz stimulation of the Schaffer collaterals and NMDA produce synaptic depression via a common mechanism.

In a complementary set of experiments, we tested if prior induction of chem-LTD interferes with induction of homosynaptic LTD. As shown in Figure 4B, LFS delivered ≥60 min after induction of chem-LTD had little if any effect on synaptic transmission (93% ± 5% of renormalized baseline, 1 hr post-LFS, n = 8; paired t test, $p > 0.1$). This result contrasts with the usual homosynaptic LTD observed in interleaved control slices (78% ± 2% of baseline, 1 hr post-LFS, n = 5; paired t test, $p < 0.01$). The small residual homosynaptic LTD

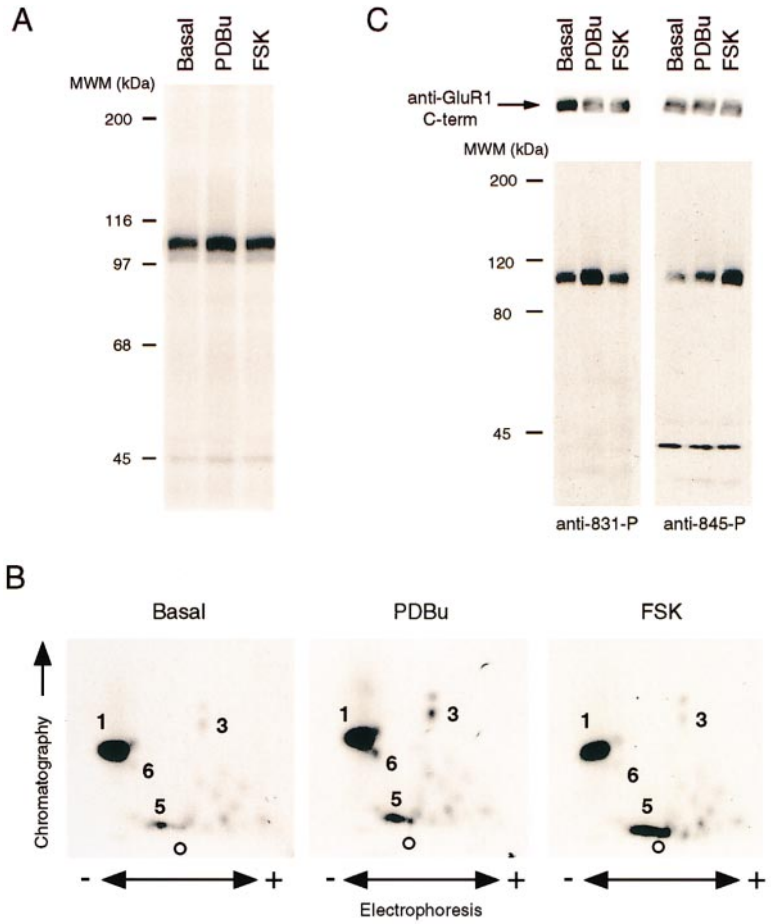

Figure 5. Phosphorylation of GluR1 in Hippocampal Slices

(A) To determine the basal phosphorylation of GluR1, hippocampal slices were preincubated with [^{32}P]-orthophosphate. The GluR1 subunit was then immunoprecipitated and analyzed for phosphorylation by SDS-PAGE and autoradiography. Protein kinase–induced phosphorylation of GluR1 was measured by treating [^{32}P]-orthophosphate-labeled slices with phorbol esters (PDBu) or forskolin (FSK) prior to immunoprecipitation.

(B) GluR1 was excised from the gel in (A) and digested with trypsin, and the resulting phosphopeptides were analyzed by two-dimensional TLC. Characterized phosphopeptides are numbered as previously described (Blackstone et al., 1994).

(C) Membrane preparations of unlabeled hippocampal slices were isolated and run on SDS-PAGE gels, transferred to PVDF, and then analyzed for GluR1 phosphorylation by immunoblot techniques using the phosphorylation site–specific antibodies (anti-831-P and anti-845-P) and phosphorylation-independent antibodies (anti-GluR1 C-term).

that remains after chem-LTD is similar to the residual chem-LTD that can be induced with a second application of NMDA (94% ± 4%; see Figure 1F). Taken together, the data suggest that chem-LTD and homosynaptic LTD utilize a common, saturable expression mechanism.

Characterization of the Phosphorylation of the GluR1 Subunit in Hippocampal Slices

We next sought to determine if chem-LTD is associated with a change in phosphorylation of the AMPA receptor. To examine the phosphorylation of the GluR1 subunit in hippocampal slices, we prelabeled slices with [^{32}P]-orthophosphate and immunoprecipitated the GluR1 subunit using anti-GluR1 antibodies. Similar to what has been observed in transfected cells (Roche et al., 1996) and neurons in culture (Blackstone et al., 1994), GluR1 is basally phosphorylated in hippocampal slices (Figure 5A). Treatment of the hippocampal slices with forskolin to activate PKA or with phorbol esters to activate PKC qualitatively increased the phosphorylation of GluR1 (Figure 5A). To compare the phosphorylation of GluR1 isolated from hippocampal slices with previous results on the phosphorylation of GluR1 in culture (Blackstone et al., 1994; Roche et al., 1996), we performed phosphopeptide map analysis of the phosphorylation sites. The phosphorylated GluR1 was excised from the gel and digested with trypsin, and the resulting phosphopeptides were subjected to two-dimensional thin layer chromatography (TLC; Figure 5B). The phosphopeptide maps generated were essentially identical to maps previously obtained from cells in culture (Blackstone et al., 1994; Roche et al., 1996; Mammen et al., 1997). GluR1 was basally phosphorylated on one major phosphorylation site (phosphopeptide 1). Phorbol ester treatment increased the phosphorylation of two phosphopeptides (phosphopeptides 3 and 6) that have been shown previously to contain serine 831 and to be major PKC and CaMKII sites on GluR1 (Roche et al., 1996; Mammen et al., 1997). Treatment of the slices with forskolin increased the phosphorylation of GluR1 on a phosphopeptide (phosphopeptide 5) that has been shown previously to contain serine 845 and to be a major PKA site in GluR1 (Roche et al., 1996). These results demonstrate that these sites are phosphorylated in hippocampal slice preparations.

To further investigate the phosphorylation of GluR1 in the hippocampal slices, we also used phosphorylation site–specific antibodies generated against the major PKA site (serine 845) and the major PKC and CaMKII site (serine 831) of GluR1 (Mammen et al., 1997). These antibodies recognize GluR1 only when these sites are specifically phosphorylated. Immunoblots of membrane proteins from hippocampal slices with the phosphorylation site–specific antibodies demonstrated that serine

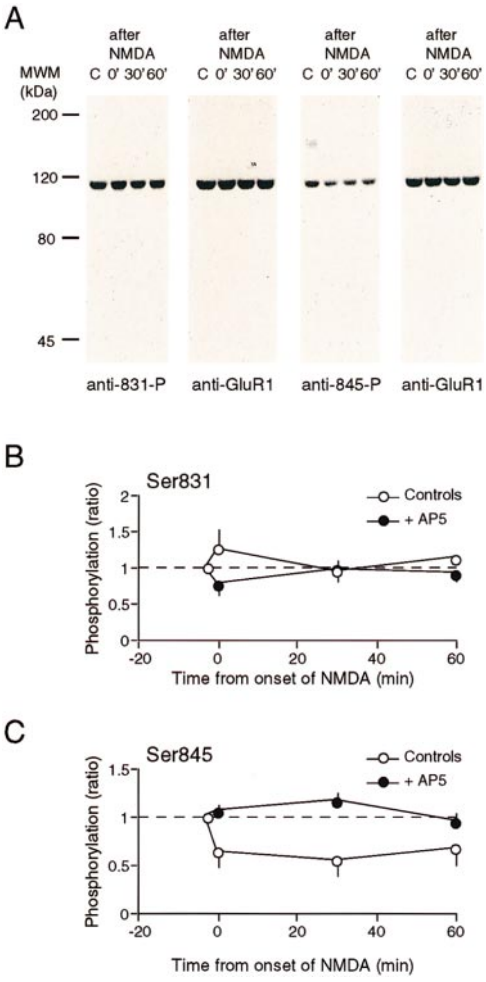

Figure 6. Persistent Dephosphorylation of the AMPA Receptor GluR1 Subunit during Chemically Induced LTD in Hippocampal Slices

(A) Membrane fractions (10 μg) were made from control hippocampal slices (lane labeled C) and chem-LTD-induced slices taken at indicated times (lanes labeled 0', 30', and 60') after the application of 20 μM NMDA for 3 min. The membrane fractions were run on SDS-PAGE gels and transferred to PVDF membranes that were first analyzed with the phosphorylation site–specific antibodies (anti-831-P and anti-845-P) and then stripped and reprobed with a GluR1 C-terminal antibody (anti-GluR1).

(B) Dephosphorylation of GluR1 on Ser-831 during chem-LTD. Phosphorylation of GluR1 at Ser-831 was analyzed using phosphorylation site–specific antibody from slices taken at various time points before or after 3 min application of 20 μM NMDA in the absence (control, n = 4; open symbols) or presence (+AP5, n = 4; closed symbols) of 100 μM AP5. The relative amount of GluR1 phosphorylation during NMDA treatment was analyzed by determining the ratio of the signals for the phosphorylation site–specific antibody and the C-terminal antibody at each time point.

(C) Dephosphorylation of GluR1 on Ser-845 during chem-LTD. There was a persistent decrease in phosphorylation on Ser-845 after chem-LTD (control, n = 4; open symbols) that was completely blocked by incubating slices in 100 μM AP5 prior to and during NMDA treatment (+AP5, n = 4; closed symbols).

845 and serine 831 have a significant level of basal phosphorylation in hippocampal slices (Figure 5C). Treatment of the slices with forskolin specifically increased phosphorylation of serine 845, while phorbol ester treatment increased the phosphorylation of serine 831 (Figure 5C). Phosphorylation of serine 831 has previously been shown to be decreased by the treatment of slices with the CaMKII inhibitor KN62, suggesting that CaMKII also phosphorylates this site in hippocampal slices (Mammen et al., 1997).

Chemically Induced LTD Promotes Dephosphorylation of GluR1

We next examined the phosphorylation of the AMPA receptor GluR1 subunit using the phosphorylation site–specific antibodies before and after chem-LTD induction (Figure 6A). Immunoblots of membrane proteins isolated from hippocampal slices before and after NMDA treatment with the anti-serine 831 antibody demonstrated that the phosphorylation of serine 831 does not change during chem-LTD (Figure 6A). In contrast, phosphorylation of GluR1 on the major PKA site, detected using the anti-phosphoserine 845 antibody, was dramatically and reproducibly reduced after NMDA treatment (Figure 6A). Immunoblot analysis with phosphorylation-independent antibodies to GluR1 demonstrated that the overall level of GluR1 does not change during chem-LTD.

The averages of the time course of NMDA-induced GluR1 dephosphorylation is shown in Figures 6B and 6C. NMDA-induced dephosphorylation of Ser 845 was rapid and, similar to the synaptic depression, was still observed 60 min after LTD induction (Figure 6C). In contrast, no significant changes in serine 831 phosphorylation were observed (Figure 6B). As seen with the NMDA receptor–dependent chem-LTD, this decrease in GluR1 phosphorylation was blocked by pretreatment of the slices with AP5 (100 μM). AP5 by itself had little effect on the basal level of serine 845 phosphorylation (Figure 6C).

Electrophysiological analysis showed that synaptic depression was observed following treatment with 20 μM NMDA, but not with 10 μM NMDA, and that the LTD was not affected by chasing the 20 μM NMDA with AP5 (Figure 7A). If dephosphorylation of GluR1 is an expression mechanism for the observed synaptic depression, it should be affected similarly by these different treatments. As shown in Figure 7B, treatment of hippocampal slices with 10 μM NMDA failed to produce significant dephosphorylation of Ser 845 (85% ± 8% of control at 60 min post-NMDA, $n = 7$; paired t test, $p > 0.1$), contrasting with the large dephosphorylation caused by 20 μM (59% ± 7% of control, $n = 11$; $p < 0.01$). Furthermore, chasing 20 μM NMDA with AP5 (100 μM) had no effect on the dephosphorylation of GluR1 (61% ± 5% of control, $n = 7$; $p < 0.01$). Thus, the dephosphorylation of GluR1 after NMDA treatment is strongly correlated with the LTD of synaptic responses in terms of time course, sensitivity to NMDA concentration, and the effects of AP5 treatments.

Discussion

We have shown that a brief bath application of NMDA can induce a long lasting depression of excitatory synaptic transmission in the CA1 region of the hippocampus. NMDA acts directly on the CA1 neuropil to produce chem-LTD, as it occurs in the absence of CA3 and does

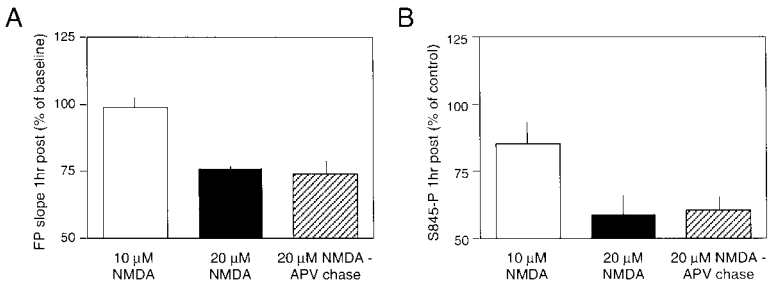

Figure 7. Comparison of Synaptic Depression Induced by NMDA and Dephosphorylation of GluR1 at Ser-845

(A) Magnitude of synaptic depression expressed as FP slope (percentage of baseline) measured 1 hr after NMDA application under different conditions. Bath application of 10 μM NMDA for 10 min did not produce significant synaptic depression (n = 9), while 20 μM NMDA for 3 min did produce significant synaptic depression (n = 84). The synaptic depression persisted even when NMDA application was chased by 100 μM AP5 (n = 6).

(B) Phosphorylated Ser-845 on the GluR1 subunit measured 1 hr after NMDA application in various conditions. Consistent with the effect on synaptic transmission, 10 μM NMDA for 10 min did not produce a significant decrease in Ser-845 phosphorylation (n = 7), while 20 μM NMDA for 3 min did produce a significant dephosphorylation (n = 11). The dephosphorylation of Ser-845 persisted even when 20 μM NMDA (3 min) was chased by 100 μM AP5 (n = 7).

not require evoked synaptic transmission. Like homosynaptic LTD, chem-LTD is (1) saturable, (2) induced postsynaptically, (3) reversible, and (4) sensitive to postnatal age. These similarities, coupled with the fact that NMDA receptor–dependent homosynaptic LTD and chem-LTD mutually occlude one another, strongly suggest that both forms of LTD use common expression mechanisms. This new paradigm offers an alternative to tetanic stimulation as a means to induce NMDA receptor–dependent LTD. Thus, chem-LTD may be useful for distinguishing pharmacological or genetic effects on the mechanisms of LTD from effects on the responses to electrical stimulation used to induce LTD. In addition, since chem-LTD occurs at synapses throughout the hippocampal slice, it should be useful for the study of biochemical and biophysical correlates of synaptic plasticity.

Using the chem-LTD procedure, we examined biochemically one candidate mechanism for synaptic depression, dephosphorylation of AMPA receptors. We find that dephosphorylation of Ser 845, but not Ser 831, of the GluR1 subunit correlates with synaptic LTD under a number of different conditions. Phosphorylation of Ser 845 has been shown previously to potentiate glutamate-evoked currents at AMPA receptors (Roche et al., 1996), and dephosphorylation of this site should have the opposite effect. Thus, the dephosphorylation of Ser 845 of the GluR1 subunit of the AMPA receptor may significantly contribute to the NMDA receptor–dependent LTD.

Chem-LTD: Excitotoxicity or Synaptic Plasticity?

A major concern with bath application of NMDA is that it may produce irreversible neuronal damage (reviewed by Choi, 1992, 1994). It is therefore conceivable that chem-LTD could be a result of pre- or postsynaptic cell death or irreversible damage to dendritic spines bearing NMDA receptors. However, several lines of evidence argue against excitotoxicity as the explanation for chem-LTD. First, the NMDA concentration and duration we used (20 μM for 3 min) is well below that required to elicit excitotoxicity under normoxic conditions (Hartley and Choi, 1989; Schurr et al., 1995; Lu et al., 1996; Pizzi et al., 1996). Second, it has been shown that a large percentage of neurons are spared from death if exposure to an excitotoxin is followed by incubation in an NMDA receptor antagonist (e.g., Hartley and Choi, 1989). However, we found that chasing NMDA with AP5 caused no reduction in the magnitude of chem-LTD (Figure 1E). Third, chem-LTD occurred reliably in slices with CA3 removed (Figure 1C). This finding rules out excitotoxic death of presynaptic CA3 neurons as a cause for chem-LTD in CA1. In addition, no change in the Schaffer collateral fiber volley was observed after chem-LTD, indicating that the same number of presynaptic axons was recruited by baseline stimulation before and after NMDA treatment. Fourth, chem-LTD saturated upon repeated exposures to NMDA (Figure 1F). This finding makes excitotoxicity unlikely as an explanation for chem-LTD, unless only a subset of synapses or neurons are susceptible to excitotoxic damage. Fifth, chem-LTD is expressed in the synaptic responses of individual cells recorded intracellularly (Figure 2), showing that the reduction in the FP was not accounted for solely by a loss of responsive postsynaptic neurons. In addition, the cells expressing chem-LTD did not show a decrease in input resistance or changes in action potential morphology, which normally occur during necrosis (Choi, 1992). Sixth, chem-LTD can be reversed (Figure 3), showing that at least some synapses undergoing chem-LTD are still viable and able to support LTP. Seventh, and finally, the saturation of homosynaptic LTD by the delivery of LFS to one set of synapses occludes chem-LTD at that set of synapses but not at others on the same postsynaptic neurons (Figure 4A). The saturation of homosynaptic LTD would not be expected to confer selective protection of a synapse from excitotoxic damage caused by NMDA.

Taken together, these considerations lead us to conclude that chem-LTD is not a manifestation of excitotoxicity. Rather, the results suggest that chem-LTD is a form of synaptic plasticity that utilizes the same saturable expression mechanism(s) as homosynaptic LTD.

AMPA Receptor Dephosphorylation and LTD

Protein kinases and protein phosphatases have been implicated in many forms of synaptic plasticity, including LTP and LTD (Bear and Malenka, 1994; Roche et al., 1994; Nicoll and Malenka, 1995). However, the protein kinase and phosphatase substrates that are relevant to the regulation of synaptic efficacy have not been

identified. Biochemical analysis of these phosphoproteins has been difficult, owing to the fact that only a small fraction of the synapses are modulated by electrically induced LTP and LTD. Chemical induction of LTD circumvents this limitation, however. Using this procedure, we have shown that synaptic depression is associated with a rapid and persistent dephosphorylation of the GluR1 subunit of AMPA receptors.

Previous studies have demonstrated that GluR1 is phosphorylated by PKA, PKC, and CaMKII in vitro and in vivo (McGlade-McCulloh et al., 1993; Blackstone et al., 1994; Tan et al., 1994; Roche et al., 1996; Barria et al., 1997a, 1997b; Mammen et al., 1997). PKA phosphorylates serine 845, while PKC and CaMKII phosphorylate serine 831, both of which are found in the intracellular C-terminal tail of GluR1 (Roche et al., 1996; Barria et al., 1997b; Mammen et al., 1997). CaMKII has been shown to play a central role in LTP (Malenka et al., 1989; Malinow et al., 1989; Ito et al., 1991; Silva et al., 1992), and recent results have reported that GluR1 is phosphorylated by CaMKII during LTP (Barria et al., 1997a). A variety of studies have suggested that LTD is a reversal of LTP, owing to the bidirectional control of the phosphorylation of substrate proteins such as the AMPA receptor (Dudek and Bear, 1993; Mulkey et al., 1993; Roche et al., 1994). We therefore initially expected that chem-LTD might induce the dephosphorylation of serine 831, the major CaMKII site on GluR1. We were surprised to find that chem-LTD had no effect on the phosphorylation of serine 831, but in contrast, serine 845, the major PKA site, was specifically dephosphorylated.

Previous work has shown that the phosphorylation of serine 845 by PKA potentiates recombinant AMPA receptors (Roche et al., 1996). PKA has also been demonstrated to potentiate native AMPA receptors in cultured hippocampal neurons (Greengard et al., 1991; Wang et al., 1991; Rosenmund et al., 1994), indicating a role for PKA in the modulation of excitatory synaptic transmission. Although potentiation of AMPA receptor function in hippocampal slices has not been observed using activators of PKA (Chavez-Noriega and Stevens, 1992, 1994), this discrepancy may be due to the complex regulation of the phosphorylation of AMPA receptors by PKA in situ. Physiological studies in hippocampal neurons have suggested that synaptic AMPA receptors are constitutively phosphorylated (and therefore potentiated) by PKA, owing to the specific targeting of PKA to the synapse by the A kinase anchoring proteins (AKAPs; Rosenmund et al., 1994). Our finding that GluR1 is significantly phosphorylated on serine 845 under basal conditions is consistent with the idea that synaptic AMPA receptors in hippocampal slices are constitutively phosphorylated by PKA at a high level. Taken together, the data suggest that dephosphorylation of the AMPA receptors at the PKA site should lead to a depression of baseline synaptic transmission in CA1. We therefore propose that dephosphorylation of serine 845 on the GluR1 subunit of postsynaptic AMPA receptors is a mechanism for NMDA receptor–dependent LTD. Consistent with this hypothesis, it has been shown very recently that NMDA receptor–dependent LTD is associated with a decrease in postsynaptic responses to glutamate (Kandler et al., 1998).

Conclusions

We conclude that synaptic depression produced in the hippocampus by brief bath application of NMDA is likely to be accounted for by the same expression mechanism as homosynaptic LTD. Chem-LTD correlates with a specific dephosphorylation of the major PKA site of the AMPA receptor GluR1 subunit. Previous work has shown that a consequence of dephosphorylation of this site on the AMPA receptor is a depression of synaptic transmission. Thus, our results suggest that dephosphorylation of the AMPA receptor is a mechanism for NMDA receptor–dependent LTD. In the following paper, we demonstrate that activators of PKA can prevent the NMDA-stimulated dephosphorylation of GluR1 as well as expression of both chem-LTD and homosynaptic LTD (Kameyama et al., 1998 [this issue of Neuron]). These results reveal an unexpected role of PKA in hippocampal LTD and indicate that protein phosphorylation of AMPA receptors may be an important mechanism underlying synaptic plasticity in the brain.

Experimental Procedures

Slice Preparation for Electrophysiology

Hippocampal slices were prepared from P21–P35 male Long-Evans rats (Charles River). Each animal was anesthetized with methoxyflurane vapor and decapitated soon after the disappearance of any corneal reflexes. The brain was rapidly removed and immersed in ice-cold dissection buffer (composition in mM: sucrose, 212.7; KCl, 2.6; NaH_2PO_4, 1.23; $NaHCO_3$, 26; dextrose, 10; $MgCl_2$, 3; and $CaCl_2$, 1) bubbled with 95% O_2 and 5% CO_2. A block of hippocampus was removed and sectioned in the transverse plane into 400 μm thick slices with a microslicer (DTK 1000; Ted Pella, Redding, CA). In most cases, CA3 was surgically removed immediately after sectioning. The slices were gently transferred to an interface- or submersion-type holding chamber containing artificial cerebrospinal fluid (ACSF; composition 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) bubbled with 95% O_2 and 5% CO_2. The slices were left in the holding chamber at room temperature for ⩾1 hr to equilibrate. Slices were then gently transferred to a submersion-type recording chamber (Medical Systems, Greenvale, NY) continually perfused with 30°C oxygenated ACSF at a rate of 2 ml/min. Slices were left undisturbed in the recording chamber for ≥20 min prior to recording.

Electrophysiological Recordings

Synaptic responses were measured extracellularly in the stratum radiatum of CA1 using glass microelectrodes (borosilicate: outer diameter, 1.0 mm; inner diameter, 0.78 mm; Sutter Instrument, Novato, CA) filled with ACSF (1–2 MΩ). Synaptic responses were evoked by stimulating Schaffer collaterals with 0.2 msec pulses delivered using bipolar stimulating electrodes (outer diameter, 200 μm; Frederic Haer). Baseline responses were obtained by stimulating once every 30 sec using 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 sec.

Intracellular EPSPs were recorded using sharp glass electrodes (borosilicate, outer diameter: 1 mm; inner diameter: 0.58 mm; BF100–58–10, Sutter Instrument, Novato, CA), with tip resistance 70–150 MΩ, and filled with a 3 M KAc and 10 mM KCl solution. Resting membrane potential of the cells was ≤−65 mV (average -73 ± 1 mV, $n = 15$ cells). Input resistance of the cells was measured by passing −0.2 nA current through the electrode and was on average 60 ± 5 MΩ ($n = 15$ cells, range 30–90 MΩ).

To induce LTP, four episodes of TBS were delivered at 0.1 Hz using the same stimulation intensity as for baseline. TBS consists of ten stimulus trains delivered at 5 Hz, with each train consisting of four pulses at 100 Hz (Larson et al., 1986). Homosynaptic LTD was induced by delivering low-frequency stimulation (LFS; 900 pulses at 1 Hz) at the same stimulation intensity as baseline (Dudek and Bear, 1992). To induce chem-LTD, NMDA (Sigma; 200 μM in ACSF) was infused into the slice chamber perfusion line at one-tenth the ACSF flow rate (0.2 ml/min) to yield a final concentration of 20 μM in the recording chamber. The NMDA infusion lasted 3 min.

Electrophysiological Data Analysis

Evoked extracellular field potentials and intracellular EPSPs were digitized at 20 kHz and stored on an IBM-compatible 486 computer using Experimenter's Workbench (DataWave 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 the measurements 60 min after treatment (NMDA or LFS) using a t test.

Slice Preparation for Biochemical Experiments

Hippocampal slices were prepared as described above. The slices were collected in ACSF (composition in mM: NaCl, 124; KCl, 5; NaH_2PO_4, 125; $NaHCO_3$, 26; dextrose, 10; $MgCl_2$, 1.5; and $CaCl_2$, 2.5), 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 (well volume, 4 ml). After 3 min, the slices were gently rinsed with ACSF and transferred to another well containing standard ACSF solution. To determine the changes in GluR1 phosphorylation over time, slices were divided into four different groups (two to four hippocampal slices in each group): (1) controls, which did not get NMDA treatment, (2) a chem-LTD + 0 min group, which was removed quickly after the 3 min NMDA treatment, (3) a chem-LTD + 30 min group, which was left in normal ACSF solution for an additional 30 min after the 3 min NMDA treatment, and (4) a chem-LTD + 60 min group, which was left in ACSF for 60 min after the NMDA treatment. Slices in all groups were placed in microcentrifuge tubes and quickly frozen on dry ice after the removal of excess ACSF solution and stored at −80°C.

Homogenates of hippocampal slices were prepared by sonicating the one to four 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 amido-black method and loaded onto SDS-PAGE gels (7.5%) by using 10–20 μg of protein per lane, and the resulting gels were transferred to polyvinylidene difluoride (PVDF) membranes.

For metabolic labeling studies, hippocampal slices were prelabeled for 2 hr with 4 mCi/ml [^{32}P]-orthophosphate and treated for 15 min with or without 100 nM PDBu, 20 mM forskolin, and 50 mM isobutylmethylxanthine or vehicle solution as indicated. The membrane proteins were then harvested, and the GluR1 protein was isolated by immunoprecipitation using anti-GluR1 antibodies and the immunoprecipitate resolved by SDS-PAGE on 7.5% acrylamide gels as described previously (Roche et al. 1996; Mammen et al. 1997). Slices containing [^{32}P]-labeled GluR1 were excised from gels for phosphopeptide mapping.

Phosphopeptide Mapping

Gel slices containing immunoprecipitated GluR1 were digested with trypsin (0.3 mg/ml) and spotted onto a TLC plate (Roche et al., 1996; Mammen et al., 1997). The peptides were then resolved by electrophoresis in the first dimension in pyridine/acetic acid/H_2O, 1:19:89 (volume/volume) and ascending chromatography in the second dimension in pyridine/butanol/acetic acid/H_2O, 15:10:3:12 (volume/volume). The TLC plates were placed in phosphoimager cassettes and visualized on a Molecular Dynamics Phosphoimager using ImageQuant software.

Generation of Phosphorylation Site–Specific Antibodies

The phosphorylation site–specific antibodies against GluR1 were generated as previously described (Mammen et al., 1997). Briefly, the peptides LIPQQSINEAIK and KTLPRNSGAGAS, corresponding to amino acids 826–836 and 840–850 of GluR1, respectively, were synthesized with phosphoserines included at the S831 and S845 positions. Lysine residues were included to facilitate glutaraldehyde coupling to the carrier protein, thyroglobulin, and the resulting phosphopeptide–thyroglobulin mixtures were used for immunization of rabbits. Polyclonal anti-phosphopeptide antibodies were purified from sera by sequential chromatography on Affi-Gel 15 (Bio-Rad) columns covalently linked to phosphorylated and unphosphorylated peptides. Antibodies were first eluted from the phosphorylated peptide affinity columns with 100 mM glycine buffer (pH 2.7). The affinity-purified antibodies were dialyzed against PBS and then loaded onto unphosphorylated peptide affinity columns. The flow through fractions was collected and used for immunoblot analysis.

Immunoblot Analysis

PVDF membranes were incubated for 15 min with 25% methanol and 10% acetic acid. The membranes were then blocked with 1% 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 Tris-buffered saline 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-mercaptoethanol 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 by using the phosphorylation site–specific antibodies and the phosphorylation-independent C-terminal antibody.

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