

Regulation of Synaptic Strength by Protein Phosphatase 1

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Summary

We investigated the role of postsynaptic protein phosphatase 1 (PP1) in regulating synaptic strength by loading CA1 pyramidal cells either with peptides that disrupt PP1 binding to synaptic targeting proteins or with active PP1. The peptides blocked synaptically evoked LTD but had no effect on basal synaptic currents mediated by either AMPA or NMDA receptors. They did, however, cause an increase in synaptic strength following the induction of LTD. Similarly, PP1 had no effect on basal synaptic strength but enhanced LTD. In cultured neurons, synaptic activation of NMDA receptors increased the proportion of PP1 localized to synapses. These results suggest that PP1 does not significantly regulate basal synaptic strength. Appropriate NMDA receptor activation, however, allows PP1 to gain access to synaptic substrates and be recruited to synapses where its activity is necessary for sustaining LTD.

Introduction

The mechanisms of intracellular postsynaptic signaling at excitatory synapses in the mammalian brain are of great interest because of their importance in influencing synaptic strength during various forms of synaptic plasticity. Over the last few years, evidence has accumulated from a variety of cell systems indicating that intracellular signaling molecules (in particular, protein kinases and protein phosphatases) are located at appropriate cellular microdomains through their association with specialized targeting subunits or anchoring proteins (Fraser and Scott, 1999; Hubbard and Cohen, 1993). Such targeting facilitates the formation of signaling complexes, which position the enzymes adjacent to the appropriate protein targets and provides substrate specificity to these broadly acting enzymes. The importance of such targeting mechanisms in the regulation of key physiolog-

ical events in mammalian tissue, however, remains largely unexplored.

How the targeting of signaling proteins to excitatory synapses is modified to influence synaptic strength has only recently begun to be analyzed, often in the context of elucidating the mechanisms of synaptic plasticity. Experiments using pharmacological inhibitors or genetic disruptions have implicated a myriad of signaling proteins. In particular, numerous protein kinases have been implicated in the triggering of long-term potentiation (LTP) (Malenka and Nicoll, 1999; Sanes and Lichtman, 1999). These include PKA, PKC, and CaMKII, all of which can modulate glutamate receptor function by phosphorylation of specific AMPA receptor (AMPA) subunits (Soderling and Derkach, 2000). PKA appears to be anchored adjacent to AMPARs via AKAP79 (A-kinase anchoring protein) (Colledge et al., 2000), while its ability to modulate NMDA receptor (NMDAR) function may be due to its binding to the scaffolding protein yotiao (Fraser and Scott, 1999). CaMKII can bind directly to the intracellular tail of NMDAR subunits (Bayer et al., 2001; Leonard et al., 1999; Strack and Colbran, 1998), positioning it in an ideal site to respond to the calcium entry that is the essential trigger for LTP. Furthermore, recent work indicates that the subcellular localization of CaMKII at synapses can be dramatically modified by activity, in particular, NMDAR stimulation (Shen and Meyer, 1999).

Compared to LTP, much less work has been performed on the signaling cascades involved in the triggering of long-term depression (LTD). For NMDAR-dependent LTD in the hippocampus, a predominant hypothesis is that the triggering of this form of LTD requires the activation of a protein phosphatase cascade involving calcineurin (PP2B) and protein phosphatase 1 (PP1) (Lisman, 1989; Mulkey et al., 1993, 1994). PP1 appears to be targeted to appropriate subcellular domains in neurons by a family of targeting/anchoring proteins which include spinophilin/neurabin II (Allen et al., 1997; Hsieh-Wilson et al., 1999), neurabin I (MacMillan et al., 1999; McAvoy et al., 1999), neurofilament-L (NF-L) (Terry-Lorenzo et al., 2000), and yotiao (Westphal et al., 1999).

Whether PP1 localization at excitatory synapses, due to its interactions with one or more of these proteins, is important for the triggering of LTD is unknown, as is whether synaptic activity dynamically modulates the actions and/or location of PP1 at synapses. It is also not known whether PP1 is constitutively active at synapses and thereby functions to limit synaptic strength, as has been suggested by work on isolated cells (Westphal et al., 1999; Yan et al., 1999). Furthermore, recent work has suggested that in neurons expressing NMDAR-dependent LTD, there are two additional forms of LTD that do not require PP1 activity: mGluR LTD, which is triggered by activation of postsynaptic group I mGluRs (Huber et al., 2000; Oliet et al., 1997; Palmer et al., 1997), and chemLTD, which is triggered by bath application of NMDA (Lee et al., 1998).

To further explore the role of PP1 in LTD and specifically evaluate the importance of its interactions with

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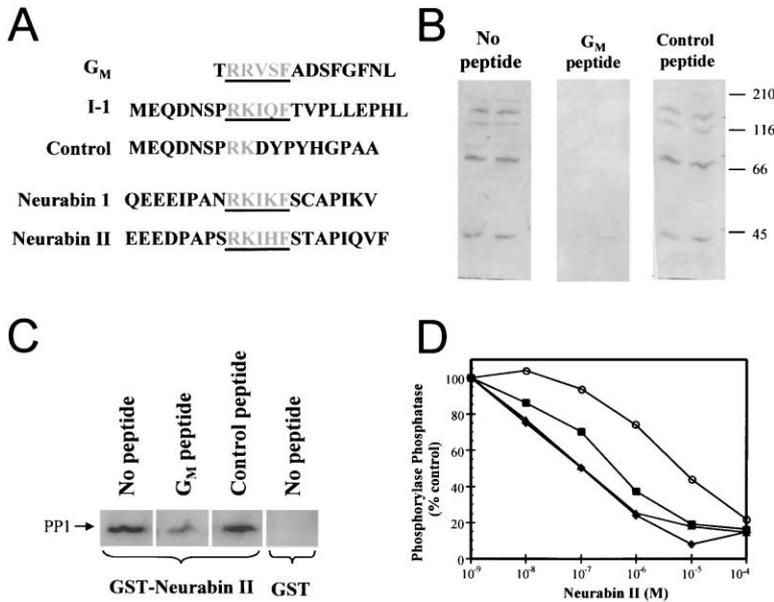


Figure 1. Effects of Gm and I-1 Peptides on the Formation of Neuronal PP1 Complexes

(A) The PP1 binding RKIXF sequences of the Gm peptide, I-1 peptide, control peptide, neurabin I, and neurabin II are illustrated with the RKIXF binding motif underlined.

(B) The gels illustrate the overlay of rat brain deoxycholate extracts with DIG-labeled PP1 in the presence or absence of 25 μ M Gm or control peptide as described in Experimental Procedures.

(C) The gels illustrate the cosedimentation of PP1 using GST-neurabin II (354-494) alone or with 25 μ M Gm or control peptide. The lack of cosedimentation of PP1 with GST alone is also shown.

(D) The graph shows the effects on PP1 activity of neurabin II alone (\blacklozenge), or with the control peptide (\blacktriangle), the I-1 peptide (\blacksquare), or the Gm peptide (\circ). This graph is a representative example of at least three separate experiments carried out in duplicate.

neuronal targeting proteins, as well as whether it constitutively regulates synaptic strength, we have taken three complementary approaches. First, we have studied the effects of peptides that disrupt the interactions of PP1 with its cognate targeting proteins on these three different forms of LTD, as well as their effects on basal AMPAR- and NMDAR-mediated synaptic currents. Second, we have examined the synaptic effects of directly loading CA1 pyramidal cells with active PP1. Third, we have examined activity-dependent changes in the distribution of endogenous PP1 in cultured hippocampal neurons.

Results

Biochemical Characterization of Two PP1 Binding Peptides

More than thirty PP1 binding or "targeting" proteins have been identified, including several that are found at excitatory synapses, where they are thought to play key roles in synaptic growth and function. These include: inhibitor-1 (I-1), neurabin I, spinophilin/neurabin II, yotiao, and NF-L (Allen et al., 1997; Endo et al., 1996; Hsieh-Wilson et al., 1999; Hubbard and Cohen, 1993; Oliver and Shenolikar, 1998; Price and Mumby, 1999; Terry-Lorenzo et al., 2000). Several of these reside within the PSD and share a 5 amino acid motif (R/K,K/R,I/V,X,F), which represents the core PP1 binding site (Egloff et al., 1997; Liu et al., 2000) (Figure 1A). To test the importance of PP1 targeting at the synapse, we used two different RKIXF motif-containing peptides derived from known PP1 binding proteins, Gm and I-1 (Hubbard and Cohen, 1993; Price and Mumby, 1999) (Figure 1A), in the intracellular loading experiments described below. We also used a control peptide based on a polymorphism in the human *I-1* gene (S. Shenolikar, unpublished results), a frameshift within the RKIXF sequence that disrupts PP1 binding. The Gm peptide, derived from the glycogen-targeting subunit found in skeletal muscle (Egloff et al.,

1997), has been shown to modify NMDAR function in HEK293 cells by displacing PP1 from yotiao (Westphal et al., 1999). The I-1 peptide contains an analogous binding motif critical for PP1 inhibition by I-1 (Endo et al., 1996). A similar peptide, based on spinophilin, modulated AMPAR function when introduced into dissociated striatal neurons (Yan et al., 1999). Importantly, none of these peptides had an effect on the enzymatic activity of PP1 in vitro (data not shown).

To test the peptides' ability to disrupt PP1 binding to endogenous targeting proteins, we performed several different biochemical assays. First, we used the peptides (25 μ M) to compete for binding of purified recombinant PP1 α to proteins present in the rat brain deoxycholate extract by a Far Western or overlay assay. The denaturation of PP1 binding proteins in SDS-containing buffer focuses attention on a subset of PP1 binding motifs, in particular, the RKIXF motif (Beullens et al., 2000), and the SDS-PAGE allows us to analyze PP1 binding to different PP1 binding proteins simultaneously. Without competing peptide, PP1 bound to at least four major bands (Figure 1B), all of which represented components of neuronal PP1 complexes (data not shown). Previous work had identified several of these proteins as NF-L (70 kDa), spinophilin/neurabin II (140 kDa), neurabin I (190 kDa), and yotiao (230 kDa) (Terry-Lorenzo et al., 2000). When Gm peptide was added to the overlay assay, binding of PP1 to these proteins was severely diminished, but the control peptide had no effect on PP1 binding (Figure 1B). (The I-1 peptide showed slightly weaker competition in this assay.)

While a number of studies have used a similar Far Western or overlay assay to analyze PP1 binding to cellular proteins, we previously showed that recombinant PP1 is modified in the RKIXF binding site (Endo et al., 1996). Thus, we used a second assay, which analyzed the ability of the neuronal PP1 binding protein spinophilin/neurabin II to recruit native PP1 from a rat brain extract. Using a recombinant polypeptide encompassing the PP1 binding domain, we showed that GST-

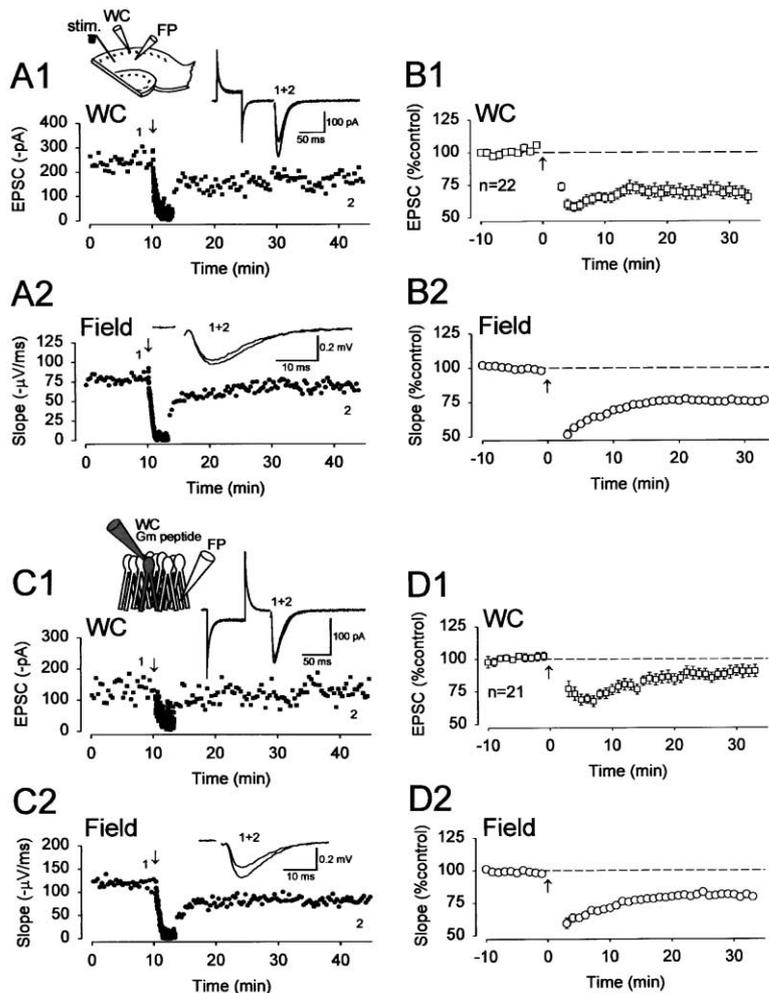


Figure 2. Loading CA1 Pyramidal Cells with Gm Peptide Inhibits LTD

(A) Panels 1 and 2 show an example of LTD during simultaneous whole-cell (A1) and extracellular field (A2) recordings. In this and all subsequent figures, sample traces were taken at the time indicated by the numbers on the graph.

(B) Panels 1 and 2 show the summary ($n = 22$) of control experiments in which whole-cell recordings were made with pipettes containing standard solution.

(C and D) Panels show an example (C1 and C2) and summary ($n = 21$) (D1 and D2) of experiments in which whole-cell pipette solution contained Gm peptide.

neurabin II (354–494), but not control GST, bound and sedimented rat brain PP1. The addition of Gm peptide (25 μM) resulted in significantly reduced PP1 binding (Figure 1C). The I-1 peptide yielded similar results but was slightly less effective than the Gm peptide.

Recent studies (Bollen, 2001) show that with the exception of yotiao (Westphal et al., 1999), all known PP1 binding proteins interact through multiple domains to inhibit the activity of the PP1 catalytic subunit against the *in vitro* substrate phosphorylase α . Thus, in a final assay, we analyzed the ability of the peptides to displace native PP1 from recombinant GST-neurabin II (354–494) and thereby attenuate the inhibition of phosphorylase phosphatase activity (Hsieh-Wilson et al., 1999; MacMillan et al., 1999). (It is important to note that this assay does not reflect the physiological action *in situ* of PP1 bound to neurabin II/spinophilin. Indeed, cellular studies indicate that targeted PP1 is constitutively active [Westphal et al., 1999; Yan et al., 1999], and we have found that immunoprecipitation of neurabin I or II yields protein complexes containing highly active PP1. This assay was used because it is quantitative and is a reliable readout of peptide efficacy). Both peptides diminished the effectiveness of GST-neurabin II (354–494) to inhibit PP1 activity. This assay also provides a quantitative compari-

son of the two peptides as disruptors of PP1 complexes containing neurabin II. The I-1 peptide decreased the IC_{50} for PP1 inhibition by GST-neurabin II (354–494) by 3-fold, while the Gm peptide shifted the dose-response curve for GST-neurabin II (354–494) by more than 10-fold (Figure 1D). Together, these data demonstrate that the Gm and I-1 peptides both compete for PP1 binding to targeting/anchoring proteins such as spinophilin/neurabin II and, therefore, should displace PP1 from appropriate synaptic sites, albeit with slightly differing efficacies.

Gm and I-1 Peptides Block LTD

To study the role of PP1 targeting proteins in synaptically evoked NMDAR-dependent LTD (termed simply LTD), we filled CA1 pyramidal cells with the Gm or I-1 peptides by adding them to the whole-cell pipette solution. To ensure that LTD was induced in the cells surrounding the one from which we recorded, we simultaneously recorded a field EPSP by placing a pipette in stratum radiatum adjacent to the whole-cell recording pipette. LTD was reliably induced in both the cells recorded with standard whole-cell pipette solution and in the adjacent population of cells (Figures 2A and 2B; whole-cell EPSC, $-30\% \pm 5\%$; field EPSP $-24\% \pm 2\%$,

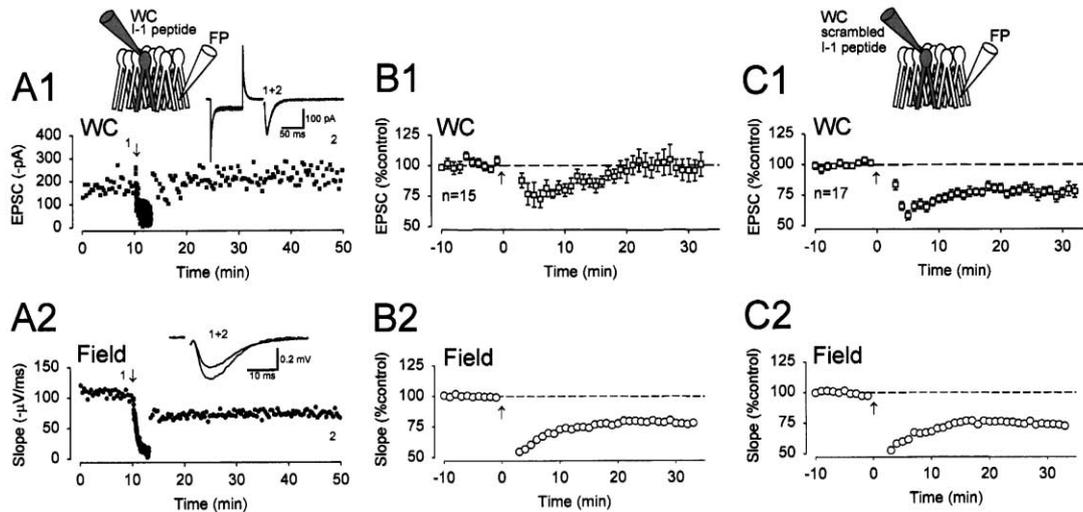


Figure 3. Loading CA1 Pyramidal Cells with I-1 Peptide Blocks LTD While Control I-1 Peptide Does Not

(A) Panels 1 and 2 show an example of an experiment in which LTD was blocked in a cell infused with the I-1 peptide (A1), but not in the simultaneously recorded field EPSP (A2).
 (B) Panels 1 and 2 show the summary ($n = 15$) of experiments in which the whole-cell pipette solution contained I-1 peptide.
 (C) Panels 1 and 2 show the summary of experiments ($n = 17$) in which the whole-cell pipette solution contained the control I-1 peptide.

$n = 22$). When cells were filled with the Gm peptide, LTD was significantly reduced or blocked ($-9\% \pm 4\%$, $n = 21$, $p < 0.01$ compared to control cells), while LTD monitored using the field EPSP was normal ($-19\% \pm 2\%$, $n = 21$) (Figures 2C and 2D). Similarly, LTD was blocked in cells filled with the I-1 peptide ($-2\% \pm 8\%$, $n = 15$), while the simultaneously recorded field EPSP showed normal LTD ($-23\% \pm 2\%$) (Figures 3A and 3B). In contrast, cells filled with the control mutant I-1 peptide exhibited clear LTD ($-23\% \pm 4\%$, $n = 17$, $p < 0.01$ compared to cells filled with I-1 peptide, field EPSP $-26\% \pm 2\%$; see Figure 3C). Thus, two different peptides that interfere with the binding of PP1 to targeting proteins blocked LTD while a control peptide did not.

Gm and I-1 Peptides Do Not Block mGluR LTD or chemLTD

In addition to expressing NMDAR-dependent LTD, CA1 pyramidal cells express a form of LTD that is dependent on metabotropic glutamate receptor (mGluR) activation (mGluR LTD) and does not appear to depend on the activation of protein phosphatases (Bolshakov et al., 2000; Oliet et al., 1997). Therefore, if the peptides specifically disrupted PP1 targeting, they should have no effect on mGluR LTD. To test this prediction, we elicited mGluR LTD by bath application of the group I mGluR agonist DHPG ($100 \mu\text{M}$) (Huber et al., 2000; Palmer et al., 1997). Figure 4A shows that this reliably elicited LTD in both the whole-cell (WC) and field recordings (WC, $-39\% \pm 3\%$; field, $-34\% \pm 4\%$, $n = 6$) and that the mGluR antagonist LY341495 blocked the actions of DHPG (WC, $4\% \pm 1\%$; field, $-8\% \pm 1\%$, $n = 3$). Loading cells with either Gm or I-1 peptide, however, had no effect on mGluR LTD (see Figure 4B; Gm peptide, $-39\% \pm 8\%$, $n = 6$; I-1 peptide, $-35\% \pm 7\%$, $n = 6$).

Another form of LTD reported to be independent of PP1 activity is termed chemLTD, which is induced by

bath application of NMDA (Kameyama et al., 1998; Lee et al., 1998). The lack of effect of PP1 inhibitors on chemLTD is surprising since chemLTD and LTD are mutually occluding, which suggests a common expression mechanism (Lee et al., 1998). Loading cells with either the Gm or I-1 peptide had no effect on chemLTD (see Figure 4C; Gm peptide, $-46\% \pm 7\%$, $n = 6$; I-1 peptide, $-46\% \pm 6\%$, $n = 6$). Thus, the peptides specifically inhibited the form of LTD that previously was shown to be blocked by PP1 inhibitors (Mulkey et al., 1993, 1994).

Gm and I-1 Peptides Do Not Affect Basal AMPAR EPSCs or NMDAR EPSCs

A critical question for understanding the postsynaptic actions of PP1 on synaptic transmission and plasticity is whether basal synaptic strength is regulated by PP1 activity, as suggested by recent studies on isolated cells (Westphal et al., 1999; Yan et al., 1999). To address this, we recorded AMPAR-mediated EPSCs (AMPA EPSCs) while infusing cells with the Gm or I-1 peptides. Figure 5 shows that neither peptide had a significant effect on AMPAR EPSCs (Gm peptide, $11\% \pm 8\%$, $n = 11$; I-1 peptide, $12\% \pm 7\%$, $n = 9$, measured 15–20 min after breakin) when compared to recordings made with the pipette solution alone ($15\% \pm 7\%$, $n = 9$). Importantly, the effects of the peptides on AMPAR EPSCs were measured over a time course that was sufficient to block LTD (Figures 2 and 3).

We also examined the effects of the peptides on basal NMDAR-mediated EPSCs (NMDAR EPSCs), which were recorded in the presence of NBQX ($10 \mu\text{M}$) and low Mg^{2+} (0.1 mM). This is important since any effect on NMDAR function might impair the induction of LTD (Malenka and Nicoll, 1993). Similar to the lack of effect on AMPAR EPSCs, we found that the peptides had no significant effect on basal NMDAR EPSCs when compared to recordings made with the pipette solution alone (Figure

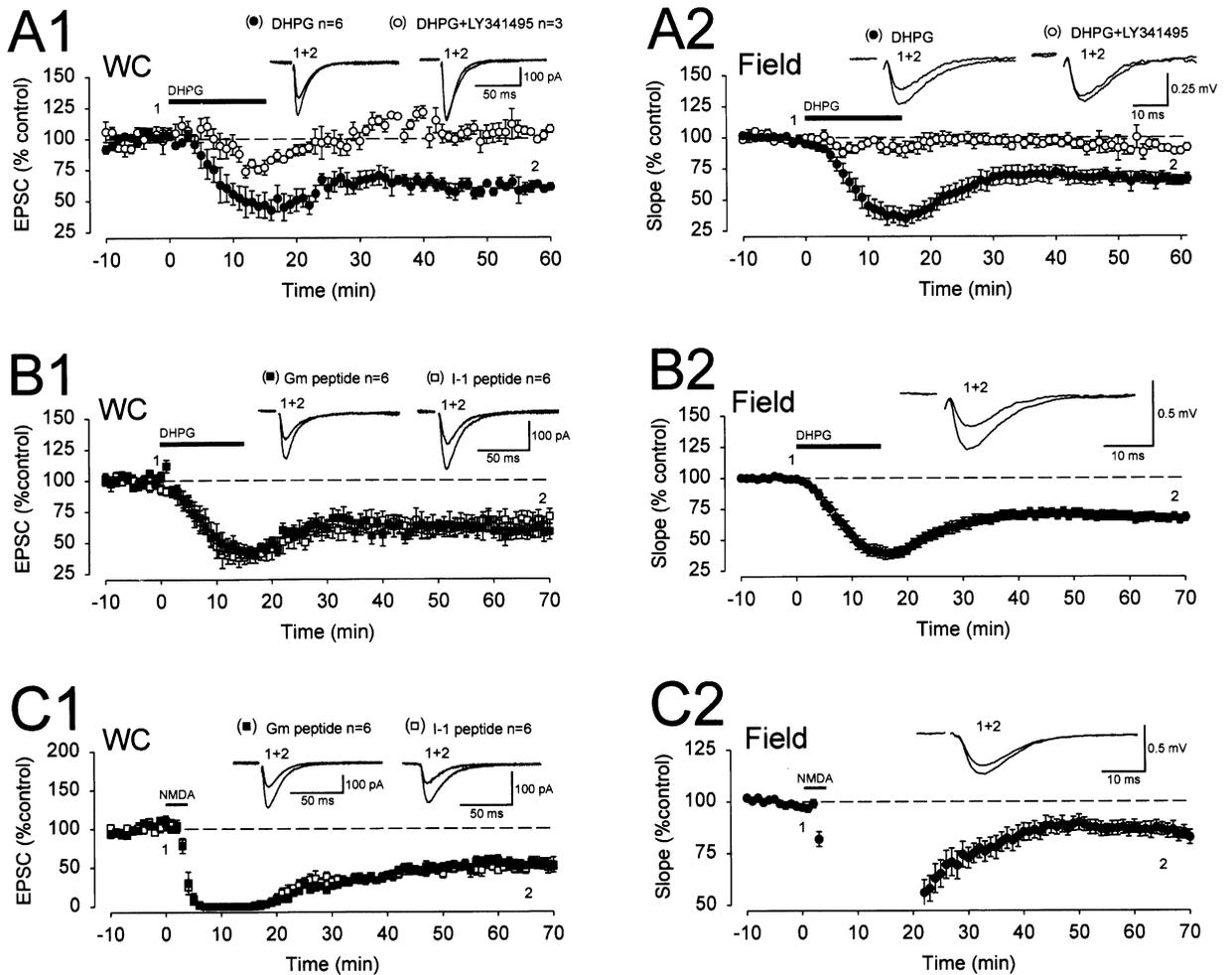


Figure 4. mGluR LTD and chemLTD Are Not Affected by Either Gm or I-1 Peptides

(A) Panels 1 and 2 show the summary of experiments demonstrating that bath application of DHPG (100 μ M) induces LTD ($n = 6$), which is blocked by the mGluR antagonist LY341495 (100 μ M) ($n = 3$).
 (B) Panel 1 shows the summary of experiments demonstrating that loading cells with Gm ($n = 6$) or I-1 ($n = 6$) peptides does not inhibit mGluR LTD. Field EPSP recordings from these experiments ($n = 12$) were combined to construct the graph in (B2).
 (C) Panel 1 shows the summary of experiments demonstrating that chemLTD elicited by bath application of NMDA is not blocked by Gm ($n = 6$) or I-1 ($n = 6$) peptides. Field EPSP recordings from these experiments ($n = 12$) were combined to construct the graph in (C2).

6) (Gm peptide, $32\% \pm 16\%$, $n = 5$; I-1 peptide, $19\% \pm 5\%$, $n = 4$; control, $33\% \pm 7\%$, $n = 9$). Thus, in contrast to previous suggestions (Westphal et al., 1999; Yan et al., 1999), interference with PP1 targeting does not influence basal synaptic transmission mediated by AMPARs or NMDARs at excitatory synapses on CA1 pyramidal cells.

Effects of PP1 on Basal Synaptic Strength and LTD

The lack of effect of the Gm and I-1 peptides on basal synaptic strength suggests either that PP1 is not constitutively active at synapses during low levels of synaptic activity or that it does not have access to the appropriate synaptic substrates. To help distinguish these possibilities, we examined the effect of loading cells with active PP1, a manipulation that we expected to decrease synaptic strength. Surprisingly, over the course of 50 min, PP1 had no effect on AMPAR EPSCs (Figure 7A) ($-8\% \pm 9\%$ change from baseline, $n = 7$). We next tested whether synaptic activity of the sort that elicits LTD

might be required for PP1 to affect synapses. Figure 7B shows that there was no significant effect of loading cells with PP1 following a strong LTD induction protocol (5 Hz, 3 min, while holding cells at -50 mV) (Inactive PP1, $-32\% \pm 4\%$, $n = 7$; PP1, $-38\% \pm 5\%$, $n = 7$). However, PP1 caused a large enhancement of the LTD elicited by a weaker induction protocol (5 Hz, 1.5 min; Figure 7C) (Inactive PP1, $-22\% \pm 5\%$, $n = 7$; PP1, $-48\% \pm 7\%$, $n = 7$). We also observed the same effect of PP1 when an even shorter induction protocol was used (5 Hz, 0.5 min) (Inactive PP1, $-14\% \pm 6\%$, $n = 6$; PP1, $-32\% \pm 7\%$, $n = 7$). This enhancing effect of PP1 on LTD required NMDAR activation since an LTD induction protocol in the presence of D-APV (100 μ M) had no lasting effect on synaptic strength in PP1 loaded cells (Figure 7D; $-7\% \pm 6\%$, $n = 4$). These results indicate that the lack of effect of PP1 on basal synaptic strength cannot be explained by the fact that PP1 did not reach the sampled synapses at a sufficiently high

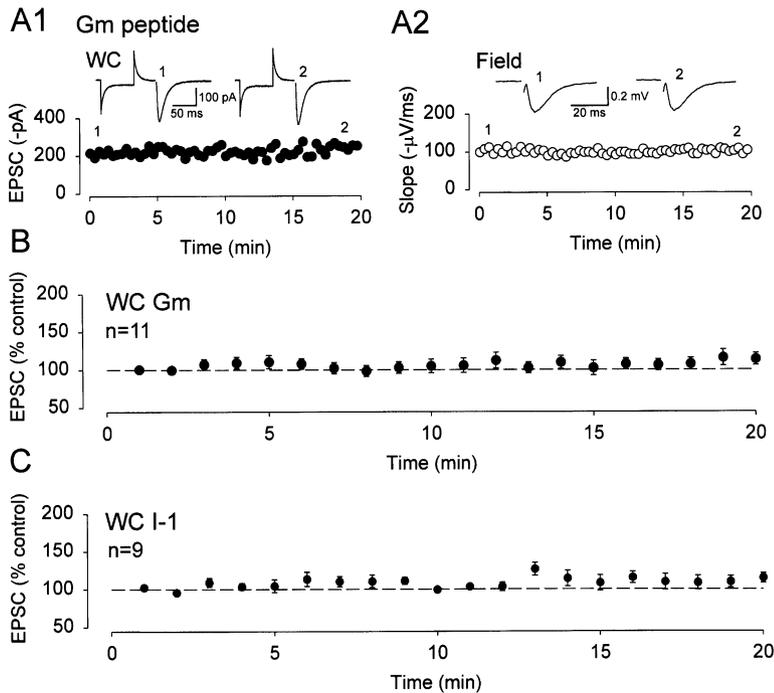


Figure 5. Gm and I-1 Peptides Do Not Affect Basal AMPAR EPSCs

(A) Panels 1 and 2 show an example in which AMPAR EPSCs (A1) and field EPSPs (A2) were monitored while infusing Gm peptide. (B and C) These graphs show the summary of experiments in which Gm (B) ($n = 11$) or I-1 (C) ($n = 9$) peptides were infused while monitoring AMPAR EPSCs.

concentration. Instead, they suggest that for PP1 to regulate synaptic strength, NMDARs must be activated.

PP1 Modulates Extrasynaptic but Not Synaptic AMPARs

Previous studies have shown that peptide mediated disruption of PP1 targeting elicited an increase in the responses to exogenously applied NMDA (Westphal et al., 1999) or AMPA (Yan et al., 1999). This raises the possibility that PP1 may have different effects on extrasynaptic glutamate receptors versus synaptic glutamate receptors. To test this hypothesis, we loaded cells with PP1 while simultaneously monitoring AMPAR EPSCs and the inward current generated by puffing kainate on the soma to activate extrasynaptic AMPARs (Figure 8). Loading cells with inactive PP1 had no effect on either the kainate-induced currents ($107\% \pm 4\%$ of baseline) or EPSCs ($101\% \pm 9\%$ of baseline, $n = 8$) (Figure 8A). In contrast, when cells were loaded with active PP1 (Figure 8B), the kainate responses were significantly reduced ($60\% \pm 18\%$ of baseline, $n = 9$), while the EPSCs were unaffected ($98\% \pm 15\%$ of baseline). These results suggest that while PP1 has direct access to extrasynaptic AMPARs, synaptic AMPARs are protected from the actions of PP1 during basal levels of synaptic activity.

Targeted PP1 Is Required to Maintain LTD

The results thus far suggest that the regulation of synaptic strength by PP1 requires NMDAR activation and that disrupting PP1 targeting blocks LTD. How, then, do alterations in PP1 targeting result in the block of LTD yet have no effect on basal synaptic transmission? Analogous with work on the activity-dependent translocation of CaMKII (Shen and Meyer, 1999), one possibility is that the pattern of synaptic activation used to induce LTD results in PP1 recruitment to the appropriate synaptic sites. A mutually nonexclusive alternative is that LTD

induction may modify the functional architecture of synaptic PP1 complexes such that PP1 can dephosphorylate the protein substrates required for LTD. To test this hypothesis, we performed two pathway experiments in which we initially monitored field EPSPs and induced LTD in one pathway ($-25\% \pm 5\%$, $n = 5$), with the other pathway serving as a control ($-5\% \pm 2\%$) (Figure 9A). We then obtained a whole-cell recording in the same region of the slice, and EPSCs were evoked in the same two pathways using the same or lower stimulus intensity. This procedure allowed us to compare the effect of the Gm peptide at synapses in which LTD was induced and control synapses on the same cell. Infusing the Gm peptide caused a growth of the EPSC in the pathway in which LTD was induced ($38\% \pm 13\%$, $n = 5$), but not in the control pathway ($4\% \pm 8\%$) (Figures 9B and 9C). Importantly, LTD in the surrounding cells, as measured with the field potential recording, was maintained throughout the duration of the experiment (Figure 9A).

Similar results were obtained when the whole-cell recordings were made using pipettes filled with the I-1 peptide (Figure 10). Again, LTD was first induced in one pathway ($-23\% \pm 4\%$, $n = 6$), but not the control pathway ($-2\% \pm 3\%$) (Figure 10A), and then whole-cell recordings were made. Infusing the I-1 peptide caused a clear growth of the EPSC in the depressed pathway ($62\% \pm 24\%$, $n = 6$), but not in the control pathway ($5\% \pm 4\%$) (Figures 10B and 10C). These results suggest that the prior induction of LTD results in changes in synaptic PP1 function and/or recruitment that allow it to play a more effective role in the triggering, as well as maintenance, of subsequent LTD.

Activity-Dependent Recruitment of PP1 to Synapses

Our results thus far demonstrate that the NMDAR activation during LTD induction may either physically recruit

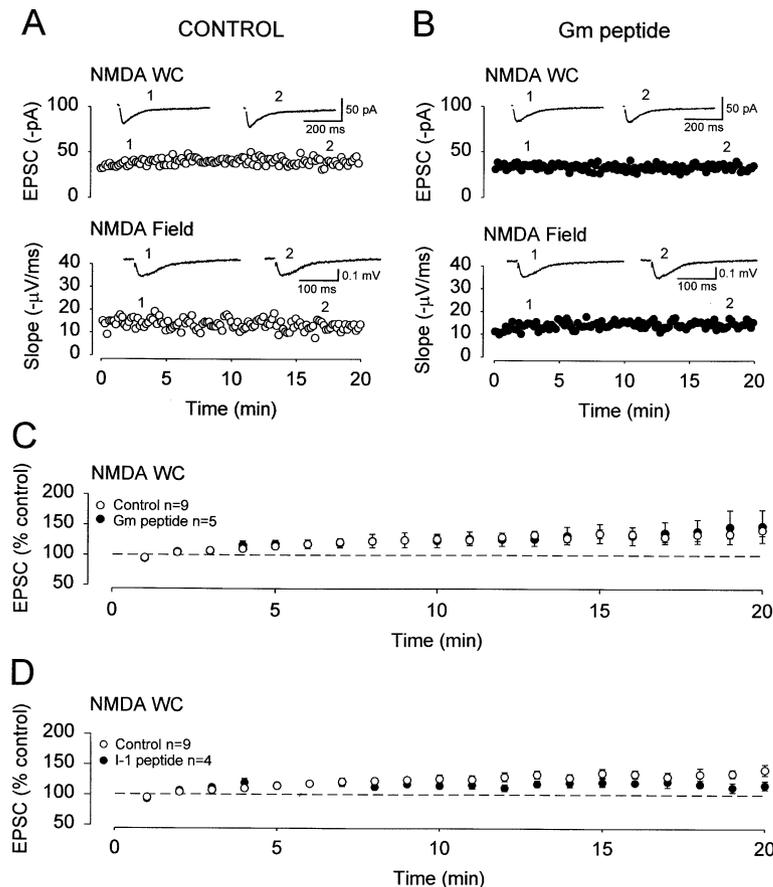


Figure 6. Gm and I-1 Peptides Do Not Affect Basal NMDAR EPSCs

(A) Panel shows an example of an experiment in which NMDAR EPSCs and NMDAR field EPSPs were simultaneously recorded using control recording solutions.

(B) Panels show an example of an experiment in which the cell was infused with Gm peptide while recording NMDAR EPSCs and NMDAR field EPSPs.

(C and D) These graphs show a summary of experiments in which cells were loaded with Gm (C) ($n = 5$) or I-1 (D) ($n = 4$) peptides. For comparison, a summary graph of experiments in which standard whole-cell pipette solution was used to monitor NMDAR EPSCs ($n = 9$) is also shown. The runup of the NMDAR EPSCs observed in both control and peptide-filled cells is likely due in large part to the fact that the whole-cell solution, which leaked out of the pipette tip, depressed synaptic transmission and that recovery from this depression was still occurring after break-in.

active PP1 to appropriate synaptic sites and/or modify the molecular architecture at the synapse such that appropriately targeted PP1 can act on relevant synaptic substrates. To more directly examine the first of these alternatives, we examined whether the location of endogenous PP1 in cultured hippocampal neurons was modified by synaptic activity. Immunocytochemical staining of nonactivated neurons with an antibody to PP1 α , a major neuronal isoform of PP1, revealed a modest, diffuse signal above background with a few isolated small puncta, presumably representing clustered PP1 (Figure 11A). Stimulating the cells at 5 Hz for 3 min, a protocol previously shown to elicit LTD in these cells (Carroll et al., 1999), caused a significant increase in the level of PP1 immunofluorescence (Figure 11A), presumably because of a redistribution and clustering of PP1. This stimulation-induced increase in PP1 immunoreactivity required NMDAR activation as it was blocked by APV (Figure 11A).

While these results indicate that synaptic activity can affect the subcellular distribution of PP1, the important question for LTD mechanisms is whether PP1 is recruited to synapses in an activity-dependent manner. To address this issue, we identified synapses using a synaptophysin antibody and calculated the percentage of synapses with detectable levels of PP1. Synaptic stimulation caused a significant increase in this measure (Figure 11B), likely because of a redistribution of PP1 to synaptic locations. These changes were blocked by D-APV (Figure 11B), indicating that they required NMDAR activation.

Discussion

We have shown that two different peptides that inhibit the binding of PP1 to proteins thought to be required for its synaptic targeting block synaptically evoked NMDAR-dependent LTD, but not mGluR LTD or chemLTD, two forms of synaptic plasticity that do not require PP1 activity. Surprisingly, these peptides had no effect on basal synaptic responses mediated by either AMPARs or NMDARs. However, at synapses at which LTD had been induced, the peptides caused an increase in synaptic strength. Similarly, loading cells with active PP1 had no effect on basal synaptic strength but greatly enhanced LTD. We also found that synaptic activation of NMDARs in cultured hippocampal neurons caused a recruitment/redistribution of PP1 to synapses. These results are consistent with the hypothesis that the pattern of synaptic activity used to induce LTD causes a modification of the synaptic molecular architecture such that appropriately targeted PP1 has access to the substrates relevant for LTD. They also are consistent with the proposal that LTD induction causes a recruitment of PP1 to the activated synapses. Finally, they suggest that preserving active PP1 at the appropriate site contributes to the maintenance of LTD, at least for the first 30 min or so after it is triggered.

Specificity of Peptide Actions

Using three different biochemical assays, we showed that the two RKIXF-containing peptides, Gm and I-1,

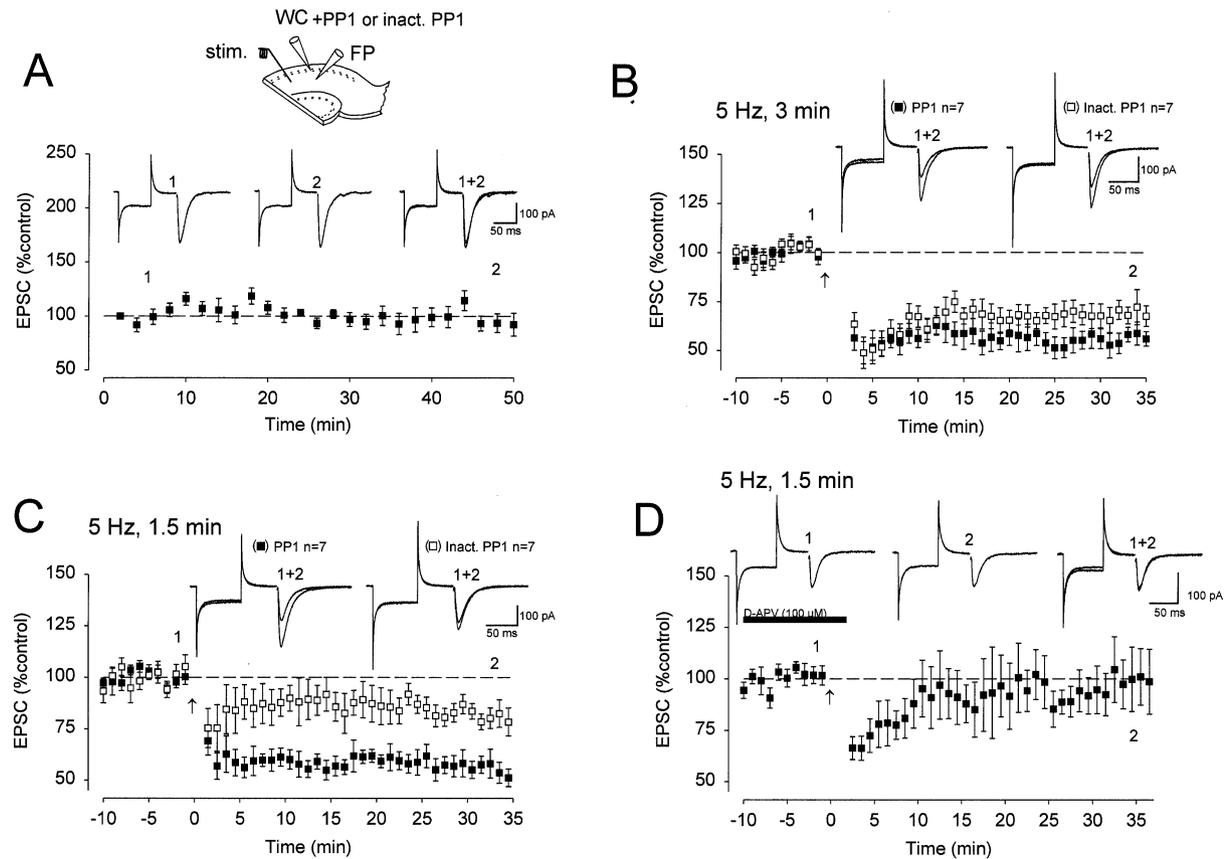


Figure 7. Loading Cells with PP1 Does Not Affect Basal Synaptic Strength But Enhances LTD

(A) The graph shows a summary of experiments ($n = 7$) in which AMPAR EPSCs were monitored while infusing active PP1 ($n = 7$).

(B and C) These graphs show a summary of experiments in which LTD was elicited by a strong (B) or weak (C) induction protocol in cells loaded with active ($n = 7$) or inactive ($n = 7$) PP1.

(D) Graph shows a summary ($n = 4$) of experiments demonstrating that application of D-APV blocks the enhancement of LTD elicited by loading cells with PP1.

disrupted PP1 binding to a number of targeting proteins previously identified as the major PP1 binding proteins in deoxycholate extracts of rat brain (Terry-Lorenzo et al., 2000). Focusing on the interactions of PP1 with spinophilin/neurabin II, which is concentrated at excitatory synapses within the PSD (Allen et al., 1997; Feng et al., 2000; Hsieh-Wilson et al., 1999), both peptides prevented PP1 binding to a recombinant neurabin II peptide. Interestingly, preformed PP1 complexes from rat brain isolated on microcystin-LR-Sepharose were more resistant to disruption by the Gm and I-1 peptides (data not shown). This is consistent with previous observations that PP1 regulators demonstrate multiple interactions with the PP1 catalytic subunit (Connor et al., 2000; Endo et al., 1996). Thus, while the RKIXF sequence is pivotal and sufficient for PP1 binding, the RKIXF-containing peptides may be more effective in preventing the formation of new PP1 complexes than disrupting existing PP1 holoenzymes.

NF-L and yotiao are different from the two neurabins in that these PP1 binding proteins lack the RKIXF motif. Earlier studies (Westphal et al., 1999) showed that the Gm peptide could inhibit PP1 association with yotiao, and our data suggest that PP1 association with the

RKIXF peptides prevents its recruitment by a variety of regulators: those containing this motif, as well as those, like NF-L, that do not. Emerging evidence suggests that sequences flanking the RKIXF-motif contribute to the affinity or specificity of PP1 binding peptides; thus, longer peptides, while more effective in disrupting specific neuronal PP1 complexes, may have differing effects in modulating PP1-mediated events in cells. In this regard, the Gm peptide, though a more effective reagent for disrupting PP1 binding in vitro, was somewhat less effective than the I-1 peptide when introduced into CA1 neurons. The reason for this difference is unknown but may include turnover, affinity, accessibility, and specificity. Finally, numerous studies have shown that targeting subunits inhibit PP1 activity against the commonly used in vitro substrate, phosphorylase a. In contrast, where the physiological substrate and its relevant phosphorylation sites are known, the targeting subunits enhanced the activity of PP1 (Liu and Brautigan, 2000; Moorhead et al., 1998). The fact that peptides that disrupt PP1 binding to spinophilin/neurabin II or yotiao increase AMPAR and NMDAR responses (Westphal et al., 1999; Yan et al., 1999) provides further evidence that PP1 is active when bound to its targeting partners.

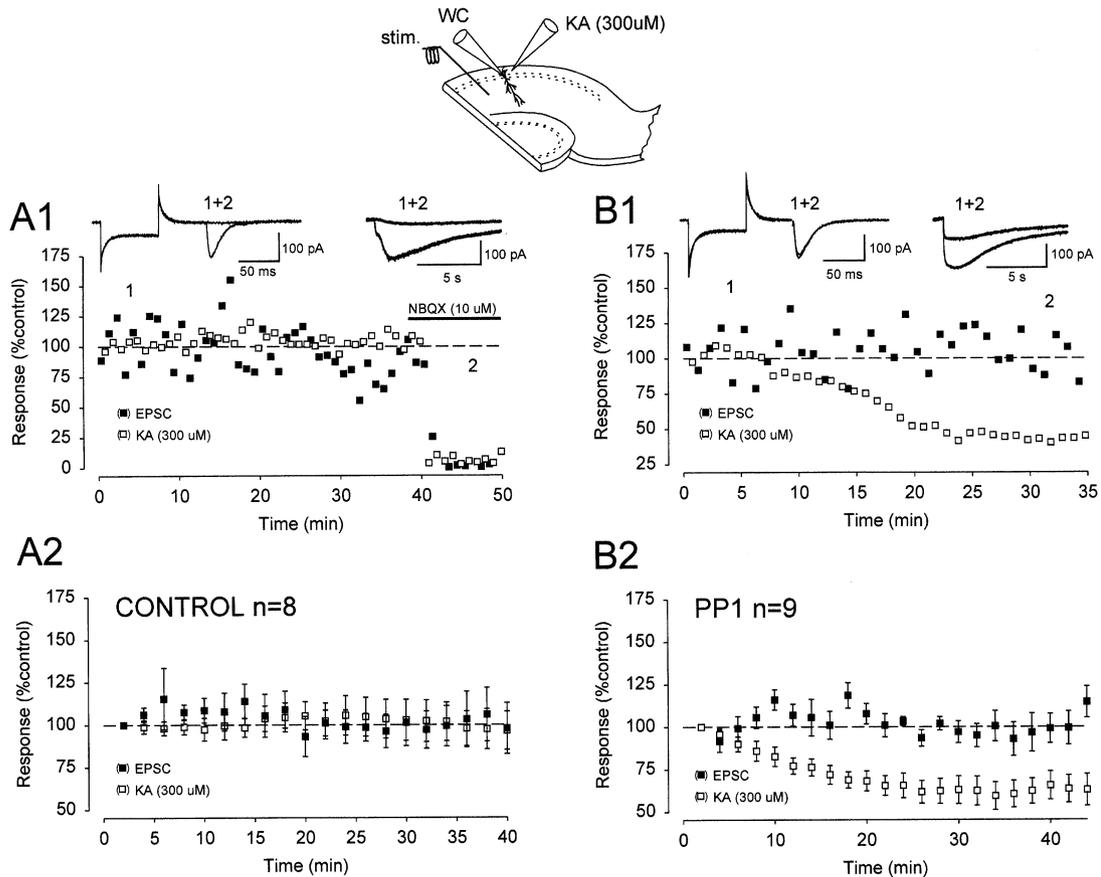


Figure 8. PP1 Depresses Responses of Extrasynaptic AMPARs But Not EPSCs

(A) Panels 1 and 2 show an example (A1) and summary ($n = 8$) (A2) of effects of loading cells with inactive PP1 on responses to kainate (applied to the soma) and AMPAR EPSCs.

(B) Panels 1 and 2 show an example (B1) and summary ($n = 9$) (B2) of effects of loading cells with active PP1 on responses to kainate and AMPAR EPSCs.

Importantly, none of the peptides had any effect on PP1 catalytic activity (analyzed *in vitro*) and thus, must have exerted their effects via a mechanism distinct from the standard PP1 inhibitors previously found to block LTD (Mulkey et al., 1993, 1994).

The inability of the RKIXF-containing peptides to disrupt preformed PP1 complexes *in vitro*, in contrast to their ability to compete for PP1 binding to newly presented targeting proteins, suggests a dynamic association of PP1 with targeting proteins *in vivo* such that the peptides disrupt or interfere with the binding of relevant PP1 targeting proteins. Thus, the simplest explanation for the actions of the peptides is that they interfered with the activity-dependent recruitment or modulation of PP1 complexes at critical synaptic sites during the LTD induction protocol and that the initial maintenance of LTD requires the sustained PP1 binding to its targeting protein (as well as its catalytic activity). Such a hypothesis also explains why the peptides blocked LTD and enhanced synaptic strength at previously depressed synapses but had no effect on basal synaptic responses. Another possible explanation for our results is that targeted PP1 activity was inhibited by high concentrations of phosphorylated I-1. It seems unlikely, however, that there could be sufficient phosphorylated

I-1 to block the actions of the exogenous PP1 that was present in the pipette solution at high concentrations. Furthermore, this hypothesis does not explain the activity-dependent redistribution of PP1 to synapses observed in cultured neurons

A limitation of our study is that the Gm and I-1 peptides do not distinguish the binding of PP1 to specific targeting proteins and thus, our results do not allow us to determine which PP1 targeting protein(s) is particularly important for LTD. One prominent candidate is spinophilin/neurabin II, the genetic deletion of which results in the almost complete inhibition of LTD (Feng et al., 2000).

Lack of Effect of Gm and I-1 Peptides or PP1 on Basal Synaptic Transmission

Previous work has used similar or identical peptides to examine the role of PP1 in controlling the function of AMPARs (Yan et al., 1999) and NMDARs (Westphal et al., 1999). Specifically, in acutely dissociated neostriatal neurons, the rundown of inward currents generated by activation of AMPARs with kainate was blocked by a peptide that interferes with the binding of PP1 to spinophilin (Yan et al., 1999). This suggested that active PP1 bound to spinophilin negatively regulates AMPAR function in these cells. Similarly, in HEK293 cells ex-

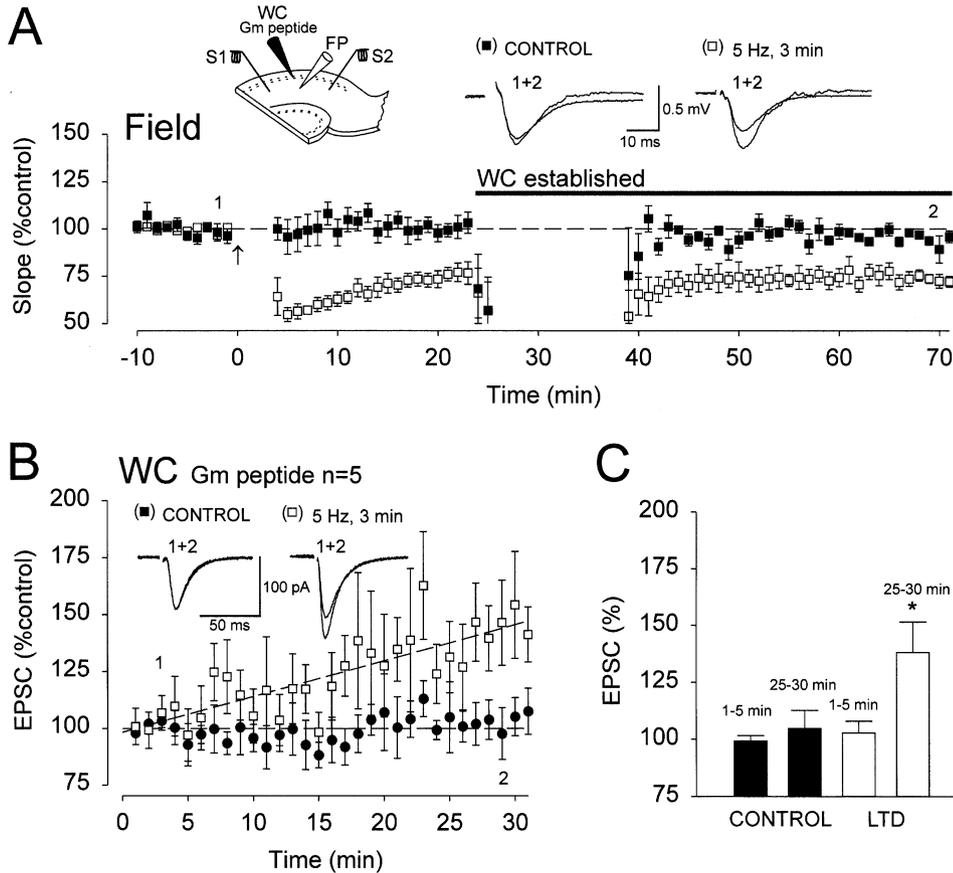


Figure 9. Infusing Cells with Gm Peptide Increases AMPAR EPSCs at Synapses Expressing LTD

(A) Graphs show a summary of experiments ($n = 5$) from field EPSP recordings. LTD was induced in one pathway (\square) while the other pathway (\blacksquare) served as a control. Whole-cell recordings were then established using pipettes filled with solutions containing the Gm peptide. The absence of points during WC establishment is the period in which the field was depressed when the whole-cell pipette was lowered into the slice. Once the field recovered from the depression, WC configuration was attained. Note that after establishing the whole-cell recordings, LTD was maintained in the test pathway.

(B) Graphs show summary of changes in the AMPAR EPSCs recorded from the two pathways.

(C) Bars show summary of changes in the amplitude of AMPAR EPSCs in the two pathways during the first 1–5 min of recording and 25–30 min after breakin ($*p < 0.05$).

pressing NMDARs and yotiao, infusion of the Gm peptide was found to cause a runup of the inward currents generated by application of NMDA (Westphal et al., 1999). These results suggested that PP1 activity, in this case associated with yotiao, also negatively regulates NMDARs. We were therefore surprised to find that the Gm and I-1 peptides had minimal effect on basal synaptic currents mediated by either AMPARs or NMDARs. One important difference between these previous studies and our experiments is that we examined the synaptic currents generated by AMPARs and NMDARs, not the inward currents generated by application of exogenous ligands to isolated cells. The regulation of PP1 localization and activity may be significantly different at intact synapses, compared to its properties in heterologous cells or at extrasynaptic receptors.

The lack of effect of PP1 itself on basal synaptic strength lends further support to the idea that synaptic substrates such as AMPARs are relatively inaccessible to PP1 actions during basal levels of activity. PP1 was able, however, to strongly depress the responsiveness

of extrasynaptic AMPARs, indicating that the molecular architecture at excitatory synapses plays a critical role in controlling the modulation of AMPARs and perhaps other important synaptic proteins. Together, these results suggest that constitutive PP1 activity does not play a significant role in the regulation of AMPAR or NMDAR function at excitatory synapses on CA1 pyramidal cells. Consistent with this conclusion, we previously found that infusing cells with the PP1 inhibitor microcystin LR had no detectable effect on AMPAR EPSCs (Issac and Malenka, unpublished observations) or on NMDAR EPSCs (R. Mulkey and R.C.M., unpublished observations).

Activity Affects the Synaptic Actions and Location of PP1

Although loading cells with PP1 had no effect on basal synaptic strength, it did significantly enhance the magnitude of LTD in an NMDAR-dependent manner. This suggests that NMDAR activation during the LTD induction protocol modified the molecular architecture of the syn-

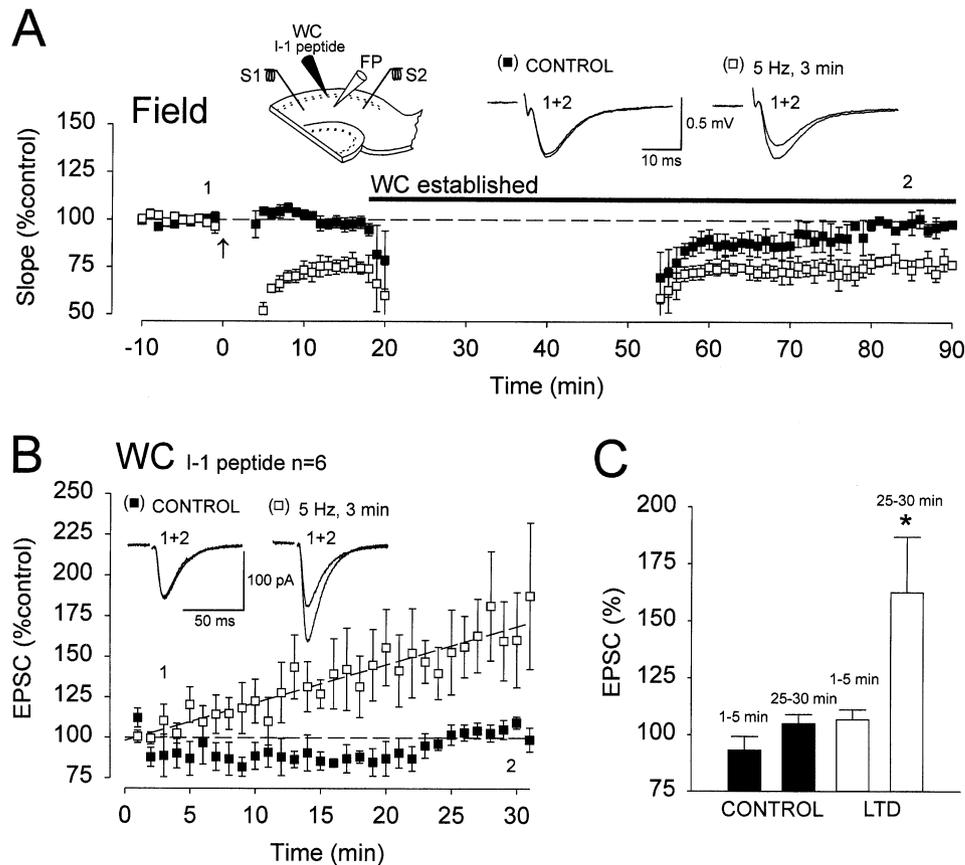


Figure 10. Infusing Cells with I-1 Peptide Increases AMPAR EPSCs at Synapses Expressing LTD

(A) Graphs show summary of experiments ($n = 6$) from field EPSP recordings. LTD was induced in one pathway (\square) while the other pathway (\blacksquare) served as a control. Whole-cell recordings were then established using pipettes filled with solutions containing the I-1 peptide. The absence of points during WC establishment is the period in which the field was depressed when the whole-cell pipette was lowered into the slice. Once the field recovered from the depression, WC configuration was attained. Note that after establishing the whole-cell recordings, LTD was maintained in the test pathway.

(B) Graphs show summary of changes in the AMPAR EPSCs recorded from the two pathways.

(C) Bars show a summary of changes in the amplitude of AMPAR EPSCs in the two pathways during the first 1–5 min of recording and 25–30 min after breakin ($*p < 0.05$).

apses such that PP1 could now access the critical synaptic substrates. A useful analogy can be made to the voltage-dependent potentiation of L-type Ca^{2+} channels in skeletal muscle which is due to PKA (Johnson et al., 1994; Sculptoreanu et al., 1993). A peptide that blocks the binding of PKA to an AKAP substantially reduced the potentiation but had no effect on the basal properties of the Ca^{2+} channels (Johnson et al., 1994). Similarly, loading cells with the catalytic subunit of PKA had no effect on the level or voltage dependence of basal Ca^{2+} channel activity but did rescue the depolarization-induced potentiation in the presence of the peptide inhibitor. Thus, PKA anchoring is not required to maintain the basal level of activity of the Ca^{2+} channels, and PKA requires depolarization of the membrane to exert its effects, perhaps because of a voltage-dependent conformational change in the Ca^{2+} channel itself (Johnson et al., 1994).

Our findings in cultured hippocampal neurons suggest that NMDAR activation also results in the redistribution or recruitment of PP1 to synapses, presumably because

PP1 can now bind to a synaptic targeting protein. A similar activity-dependent recruitment to synapses occurs to CaMKII (Shen and Meyer, 1999), likely as a consequence of its binding to the intracellular tails of NMDAR subunits (Bayer et al., 2001; Leonard et al., 1999; Strack and Colbran, 1998). These findings raise the intriguing possibility that the pattern of synaptic activity controls synaptic strength by strongly influencing the composition of the intracellular signaling cascades found at individual excitatory synapses.

Protein Phosphatase Activity and LTD

In contrast to LTP, the triggering of which has been suggested to involve a number of intracellular signaling cascades (Sanes and Lichtman, 1999), a single hypothesis involving a protein phosphatase cascade has dominated the thinking about the induction of LTD (Lisman, 1989). Specifically, it was proposed that a modest rise in calcium preferentially activates calcineurin, which dephosphorylates I-1 and results in an increase in the activity of PP1 via a mechanism of disinhibition. Consistent

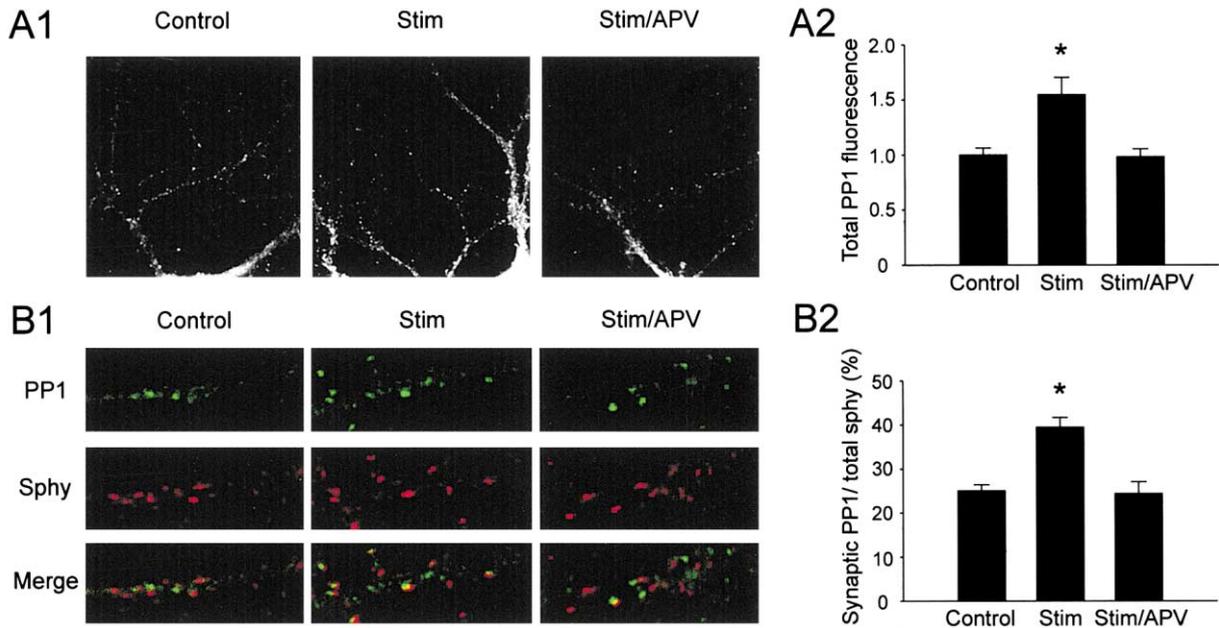


Figure 11. Synaptic Activation of NMDARs Recruits PP1 to Synapses

(A) Panels 1 and 2 show examples (A1) and quantitation (A2) of PP1 immunoreactivity in control, stimulated, and stimulated in the presence of D-APV (100 μ M) cultures. (* $p < 0.01$.)

(B) Panels 1 and 2 show examples (B1) and quantitation (B2) of percentage of synapses (defined by synatophysin puncta) that contain PP1 in control, stimulated, and stimulated in the presence of D-APV cultures. (* $p < 0.01$.)

with this hypothesis, a number of different calcineurin and PP1 inhibitors were found to block or inhibit LTD when loaded into CA1 pyramidal cells (Mulkey et al., 1993, 1994). Evidence was also presented that the maintenance of LTD, at least over the course of 20–40 min, required persistent phosphatase activity since application of calyculin A caused an increase in synaptic strength in a previously depressed pathway, but not in the simultaneously recorded control pathway (Mulkey et al., 1993). Our results using the Gm and I-1 peptides confirm this result and extend it by demonstrating that the binding of PP1 to a cognate targeting protein is required for the maintenance of PP1 activity during LTD. Biochemical measurements have also shown persistent protein phosphatase activity following the generation of LTD in the hippocampus *in vivo*, although the increase in PP1 activity lasted somewhere between 5–35 min following the induction of LTD, while PP2A activity remained elevated for over 1 hr (Thiels et al., 1998).

Two important questions remain about the role of PP1 in LTD. First, is PP1 activity absolutely required for LTD? It is now clear that there are multiple forms of LTD, some of which do not require PP1 activity (Bear and Linden, 2001; Bolshakov et al., 2000), and that these may even coexist at the same set of excitatory synapses (Oliet et al., 1997). A particularly surprising result is that chemLTD in the hippocampus, which has been reported to mutually occlude with synaptically evoked LTD (Lee et al., 1998), is not blocked by PP1 inhibitors (Kameyama et al., 1998) or by the Gm or I-1 peptides. This suggests that the repetitive synaptic activation of NMDARs used to induce LTD may modify intracellular signaling cascades in a manner distinct from that which occurs fol-

lowing continual activation of NMDARs by an exogenous ligand. Indeed, the endocytosis of AMPARs caused by bath application of NMDA to cultured neurons (and which is thought to contribute to the expression of LTD, see below) was not blocked by PP1 inhibitors (Beattie et al., 2000) (but see Ehlers, 2000). Nevertheless, both present and previous results (Mulkey et al., 1993, 1994) indicate that the appropriate targeting and activity of PP1 appears to be essential for synaptically induced NMDAR-dependent LTD. A useful analogy can be made to the properties of CaMKII, the activation of which is strongly influenced by temporal properties of the calcium transients that normally activate it (De Koninck and Schulman, 1998), and the translocation of which to synaptic sites is influenced by the time course of its prior activation (Shen et al., 2000). This activity-dependent modulation of CaMKII and PP1 localization may provide additional flexibility to the intracellular signaling cascades activated during LTP and LTD, and may help in preserving the malleability of synapses which have previously been strengthened or depressed. It could also clearly play a significant role in mediating metaplasticity (Abraham and Bear, 1996).

A second important question is what are the critical substrates of PP1 that contribute to the expression of LTD? One prime candidate is the AMPAR itself, in particular, the GluR1 subunit. Both chemLTD and synaptically evoked LTD are accompanied by dephosphorylation of serine 845 on GluR1, a dephosphorylation that is maintained for at least 1 hr after LTD induction (Lee et al., 1998, 2000). Importantly, both synaptically evoked LTD and the dephosphorylation of serine 845 were blocked by the PP1/2A inhibitor okadaic acid (Lee et al., 2000).

Depotential is also blocked by PP1 inhibitors (O'Dell and Kandel, 1994), although this results in the dephosphorylation of a different site, serine 831, on GluR1 (Lee et al., 2000).

The phosphorylation state of GluR1 influences the single channel conductance of AMPARs (Derkach et al., 1999; Soderling and Derkach, 2000), a mechanism that likely contributes to LTP (Benke et al., 1998), as well as their peak open channel probability (Banke et al., 2000). LTD, however, does not appear to involve a change in single channel conductance (Lüthi et al., 1999), and changes in AMPAR open channel probability during LTD have not been examined. Strong evidence has been presented indicating that LTD involves the endocytosis of synaptic AMPARs (Carroll et al., 2001, 1999; Lüscher et al., 1999; Man et al., 2000). The intracellular signaling cascades involved in the enhancement of AMPAR endocytosis following NMDAR activation have recently been studied in cultured neurons, and the actions of pharmacological inhibitors support a critical role for both calcineurin (Beattie et al., 2000; Ehlers, 2000) and perhaps PP1 (Ehlers, 2000) (but see Beattie et al., 2000) in this process.

One hypothesis that may reconcile many of the observations concerning the events contributing to the triggering and maintenance of LTD is that NMDAR-dependent activation of calcineurin initially triggers AMPAR endocytosis via dephosphorylation of endocytic proteins (Beattie et al., 2000), as has been proposed for endocytosis of presynaptic vesicles (Lai et al., 1999). Internalized AMPARs may then be stabilized intracellularly through the PP1-dependent dephosphorylation of specific residues on AMPAR subunits such as serine 845 on GluR1 (Ehlers, 2000) or serine 880 on GluR2, a residue which, when dephosphorylated, greatly increases the affinity of GluR2 for GRIP (Chung et al., 2000; Matsuda et al., 1999). Disruption of GRIP binding to AMPARs due to PKC-dependent phosphorylation of serine 880 has been suggested to be particularly important for the reinsertion of AMPARs into the postsynaptic membrane following their internalization (Daw et al., 2000). According to this hypothesis, the disruption of PP1 function/targeting blocks LTD expression by preventing the internal retention of endocytosed AMPARs, which consequently recycle back to the membrane surface. ChemLTD may not require PP1 activity because the prolonged activation of NMDARs forces the internalized AMPARs into a degradative pathway. However, LTD recently has been reported to be accompanied by increased phosphorylation of serine 880 on GluR2, an effect that was blocked by both NMDAR antagonists and the PP1 inhibitor okadaic acid (Kim et al., 2001). Furthermore, based on the effects of peptide inhibitors, these authors suggested that the interaction of GluR2 with PICK1, not GRIP, was particularly important for LTD. Clearly, further work needs to be done to clarify the exact role of the phosphorylation state of AMPAR subunits in mediating LTD.

Conclusion

By using peptides that disrupt the binding of PP1 to its cognate targeting proteins, but do not inhibit PP1 catalytic activity, we have provided further evidence for

a critical role for PP1 in the triggering and initial maintenance of NMDAR-dependent LTD. Our results suggest that like the extensively studied protein kinase CaMKII, synaptic activation of NMDARs may not only influence the catalytic activity of PP1, but also its subcellular and perhaps subsynaptic localization, which is likely critical for positioning it next to the appropriate substrates. This additional level of complexity in the control of signal transduction at synapses, while making the study of synaptic function more difficult, likely provides an important substrate for the extensive repertoire of plasticity mechanisms that appear to exist at individual excitatory synapses.

Experimental Procedures

Biochemistry

Peptides were synthesized by the BioPolymer Analysis Laboratory (University of Pennsylvania). Purity and concentration of the peptides were assessed by HPLC. Peptides were dissolved into 10 mM Tris-HCl (pH 7.5). PP1 overlay assays were done using standard techniques (Connor et al., 2000). Briefly, 40 μ g of a 1% deoxycholate extract from rat brain was separated on 1%–10% SDS-PAGE gel and electrophoretically transferred to a PVDF membrane. Following transfer, gels were blocked with dry milk and incubated with digoxigenin-conjugated PP1 (DIG-PP1), with or without 25 μ M of the competing peptide. PP1 binding was detected by Western blotting with an anti-digoxigenin antibody (Roche Biochemicals).

For cosedimentation or pull-downs of PP1 using recombinant GST-neurabin II (354–494), 2 μ g of recombinant GST-neurabin II was incubated with 10 μ l of glutathione-Sepharose beads (Pharmacia) for 30 min at 4°C. GST beads were washed four times with TBS (Tris-HCl [pH 7.5], 150 mM NaCl). Two hundred microliters of a 20 U/ml solution of PP1 (approximately 100 ng PP1) with or without 25 μ M peptide was added and the mixture was incubated at 4°C for 30 min. Beads were washed four times with 1 ml TBS. The PP1, which remained bound to the beads, was eluted with 25 μ l 2 \times SDS sample buffer. The eluted proteins were separated on a 10% SDS-PAGE gel and electrophoretically transferred to a PVDF membrane. PP1 was detected by Western blotting using an anti-PP1 antibody (Transduction Laboratories).

Protein phosphatase assays were carried out as described previously (Connor et al., 2000). Assays were run in a 60 μ g total volume containing 20 μ M 32 P-phosphorylase a as a substrate and 0.2 units of PP1 in a 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% 2-mercaptoethanol reaction buffer. Peptides were added to the PP1 dilution 15–60 min prior to use. GST-neurabin II (354–494) was preincubated with PP1 for 5 min prior to the addition of substrate. Following 10 min at 37°C, reactions were terminated using 200 μ l 20% TCA and 50 μ l 10 mg/ml BSA. The precipitated protein was sedimented by centrifugation and 32 P release was determined by scintillation counting of 200 μ l of the supernatant.

Electrophysiology

Hippocampal slices (400 μ m) were prepared from 2- to 4-week-old Sprague-Dawley rats using standard procedures, allowed to recover for a minimum of 1 hr, and then transferred to a submersion-type recording chamber mounted on an Olympus BX50WI microscope equipped with IR DIC optics, which allowed visualization of individual CA1 pyramidal cells with a 40 \times objective. The slices were perfused at room temperature (23°C) with a standard external solution that was bubbled continuously with 95% O₂ and 5% CO₂ and containing: 119 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, 11 mM glucose, and 0.1 mM picrotoxin. Field potential and whole-cell recording techniques were as previously described (Isaac et al., 1995; Selig et al., 1995). Whole-cell recording pipettes (2–4 M Ω) were filled with a solution containing: 117.5 mM CsMeSO₄, 10 mM HEPES, 0.5 mM EGTA, 8 mM NaCl, 10 mM glucose, and 2 mM Mg-ATP (pH 7.2 with CsOH, osmolality adjusted to 280–290 mOsm). When experiments were performed in the presence of DHPG, 0.3 mM GTP was added to the

pipette solution. Peptides from a stock solution were added to the pipette solution immediately before recording so that their final concentration was 100–200 $\mu\text{g/ml}$ (50–120 μM). PP1 was prepared from rabbit skeletal muscle (DeGuzman and Lee, 1988) and added to the pipette solution from a stock solution at a final concentration of 400 U/ml. To inactivate PP1, it was heated to 90°C for 60 min before adding it to the pipette solution. Cells were held at -65 to -75 mV during the recordings except where noted. Series and input resistances were monitored online throughout each experiment. Field potential recordings were made using whole-cell pipettes filled with 1 M NaCl. Stimulation of Schaffer collateral/commissural afferents was performed using stainless steel bipolar electrodes and was maintained at 0.05 Hz except during the LTD induction protocol.

To ensure stability of the recordings when monitoring basal AMPAR and NMDAR EPSCs, electrical stimulation was initiated before the whole-cell recording was established. After breakin, recordings were started within 1–3 min. During this time period, stimulation strength was adjusted, after which the first 6–9 responses were averaged and normalized to 100% for comparison to all subsequent responses. The variable runup of the AMPAR and NMDAR EPSCs observed in both control and peptide filled cells is likely due in large part to the fact that the whole-cell solution, which leaks out of the pipette tip, depresses synaptic transmission and that recovery from this depression was still occurring during the first minutes after breakin. For two pathway experiments (Figures 9 and 10), LTD was generated in one pathway using 5 Hz stimulation for 3 min. After LTD had stabilized (20–35 min after LTD induction), a whole-cell recording was established while maintaining electrical stimulation at the same stimulus strength. Field EPSPs were monitored throughout each experiment. Data are expressed as means \pm SEM. Significance was determined using Student's paired and unpaired *t* tests.

Cell Culture and Immunocytochemistry

Hippocampal cell cultures were prepared as described previously (Lüscher et al., 1999). Briefly, hippocampi were taken from P0 rat pups, and the dentate gyri were removed. Tissue dissociation was facilitated by papain treatment and followed by trituration with glass pipettes. Cells were plated on poly D-lysine coated cover slips and grown in neurobasal medium supplemented with B27. Media was changed on the day after plating and in part each week thereafter. Glial growth was inhibited by FUDR after one week in culture.

PP1 monoclonal antibody (BD Transduction Laboratory) was used at 1 $\mu\text{g/ml}$ for labeling endogenous PP1. Rabbit polyclonal anti-synaptophysin antibody (Zymed Laboratory) was used at 1 $\mu\text{g/ml}$. Two-week-old primary hippocampal neurons were field stimulated using an LTD protocol (Carroll et al., 1999) in HEPES buffered Ringer solution followed by fixation using 4% paraformaldehyde (in PBS) for 15 min. The cells were then incubated with permeabilizing blocking buffer (P buffer: 0.1% Triton X 100, 2% BSA in PBS) for at least 40 min. The primary antibodies (anti-PP1 and anti-synaptophysin) were then diluted in the P buffer and incubated with the cells for 1 hr before they were stained with secondary antibodies (Alexa 488 conjugated anti-mouse secondary antibody [Molecular Probes] for anti-PP1 antibody, and Alexa 568 conjugated anti-rabbit secondary antibody [Molecular Probes] for synaptophysin).

Labeled cells were imaged using a 63 \times oil immersion objective on a Zeiss Axioskop2. Images were obtained using a cooled CCD camera (Hamamatsu) and were analyzed using Metamorph Software (Universal Imaging). For individual experiments, cover slips from the same culture preparation were compared, and images for all conditions were analyzed using identical acquisition parameters. All data acquisition and analysis was performed blindly, without knowledge of the treatment history of the cover slip being examined. Images from each experiment were thresholded to subtract the average background fluorescence, and cell bodies were excluded from analysis. The total thresholded, fluorescently-labeled PP1 signal was measured automatically by the Metamorph software and divided by the total cell area, which was determined by setting a lower threshold level to measure background fluorescence. For each experiment, PP1 fluorescence in all cells was normalized by dividing by the average fluorescence of nonstimulated control cells.

For the experiments comparing colocalization of PP1 and synaptophysin, corresponding PP1 and synaptophysin images were

thresholded, and Metamorph software gave two sets of puncta with central x-y coordinates and equivalent radii. These two sets of data were fed into a custom written algorithm (Dr. Peng Liu, Intel Inc.), which calculated all the distances between any points from the PP1 image and any points from the synaptophysin image. If the distance between coordinates was smaller than or equal to the sum of the two radii of the two puncta being compared, those two puncta were considered colocalized. The "n" value given for each experiment refers to the number of cells analyzed.

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