R value of 21% with solvent (*17*). For the wild-type human SOD $P6_3$ crystal form, we collected 140,309 observations of 17,554 unique reflections representing 89.8% of the diffraction data to 2.4 Å resolution. The orientation was determined with Crowther's rotation function as implemented in MERLOT [P. M. D. Fitzgerald, *J. Appl. Crystallogr.* **21**, 273 (1988)] with the use of the "humanized" bovine enzyme as the search probe, and the position was found by means of an *R* factor search in XPLOR [A. T. Brünger, J. Kuriyan, M. Karplus, *Science* **235**, 458 (1987)]. The initial *R* factor after rigid group refinement was 43%, and the current *R* factor after conventional refinement with PROLSQ and one round of simulated annealing with XPLOR was 22.4% for the data between 5 and 2.4 Å resolution with overall deviations from ideal geometry of 0.04 Å for bond distances and 3.5° for bond angles. To evaluate the role of these residue interactions identified in the two human SOD structures in the stability of the SOD fold and its dimer assembly, the residue interactions at positions mutated in FALS patients were checked for conservation within the structures of bovine SOD refined to 1.8 Å resolution with an *R* value of 17% (S. Redford, D. McRee, J. Tainer, E. Getzoff, unpublished results) and yeast SOD refined to 1.7 Å resolution with an *R* value of 18% (H. Parge, J. Tsang, K. Slater, J. Valentine, J. Tainer, unpublished results). Buried surface areas, packing interactions, and residue pair contacts were quantitated as described (*26*).

19. E. D. Getzoff, J. A. Tainer, M. M. Stempien, G. I. Bell, R. A. Hallewell, *Proteins: Struct. Func. Genet.* **5**, 322 (1989).
20. E. D. Getzoff *et al.*, *Nature* **358**, 347 (1992).
21. A. E. Eriksson *et al.*, *Science* **255**, 178 (1992); N. S. Scrutton, M. P. Deonarain, A. Berry, R. N. Perham, *ibid.* **258**, 1140 (1992); D. Mendel *et al.*, *ibid.* **256**, 1798 (1992); W. S. Sandberg and T. C. Terwilliger, *ibid.* **245**, 54 (1989); D. E. McRee *et al.*, *J. Biol. Chem.* **265**, 14234 (1990).
22. The SOD activity was measured in partially purified enzyme protein preparation. The red cells, washed three times with phosphate-buffered saline in cold, were lysed with 50 mM potassium-phosphate buffer, pH 7.4, and the lysate was treated with 0.4 volume of an ethanol:chloroform (25:15, v/v) mixture to remove hemoglobin as described [J. M. McCord and I. Fridovich, *J. Biol. Chem.* **244**, 6049 (1969)]. The hemoglobin-free supernatant was mixed with potassium hydrogen phosphate (K_2HPO_4, final concentration of 300 mg/ml), and allowed to stand at room temperature. The clear supernatant obtained after centrifugation (5000*g* for 15 min) was cooled and treated with two volumes of acetone, and precipitated proteins were dissolved in 50 mM potassium-phosphate buffer, pH 7.8, reprecipitated with acetone, and dissolved in the buffer. Enzyme activity was assayed at 37°C as described [D. R. Spitz and L. W. Oberley, *Anal. Biochem.* **179**, 8 (1989)] by monitoring the reduction in blue formazan formation (L. W. Oberley and D. R. Spitz, in *CRC Handbook of Methods for Oxygen Radical Research*, R. Greenwald, Ed. (CRC Press, Boca Raton, FL, 1985), pp. 217–220). The assay mixture (1 ml) contained 50 mM potassium-phosphate buffer, pH 7.8, 56 μM nitroblue tetrazolium, 100 μM xanthine, 50 μM bathocuproine disulfonic acid (BCDA), 1 mM diethylene triamine pentaacetic acid, 130 μg of BSA, 1 unit of catalase, SOD-containing protein preparations at several concentrations, and an appropriate amount of xanthine oxidase to give ΔA/min of 0.025 to 0.035 at 560 nm. A unit of enzyme activity is defined as the amount of protein required for half-maximal inhibition of formazan formation. Assays were done in duplicate. Activity gels of red cell lysates were run as described [C. Beauchamp and I. Fridovich, *Anal. Biochem.* **44**, 276 (1971)]; Z. Iqbal *et al.*, unpublished data.
23. R. A. Hallewell *et al.*, *Nucleic Acids Res.* **13**, 2017 (1985); R. A. Hallewell, E. D. Getzoff, J. A. Tainer, unpublished data.
24. For SOD: R. A. Hallewell, D. Cabelli, E. D. Getzoff, unpublished data; for other Cu enzymes: M. Nishiyama *et al.*, *Protein Eng.* **5**, 177 (1992).
25. J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall, B. A. Freeman, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1620 (1990); C. F. Kuo, T. Mashino, I. Fridovich, *J. Biol. Chem.* **262**, 4724 (1987); W. H. Koppenol, J. J. Moreno, W. A. Pryor, H. Ischiropoulos, J. S. Beckman, *Chem. Res. Toxicol.* **5**, 834 (1992); C. C. Winterbourn, *Free Rad. Biol. Med.* **14**, 85 (1993); J. O. McNamara and I. Fridovich, *Nature* **362**, 20 (1993); S. Przedborski *et al.*, *J. Neurosci.* **12** (5), 1658 (1992).
26. Y. S. Ho, A. J. Howard, J. D. Crapo, *FEBS Lett.* **229**, 256 (1988); G. E. O. Borgstahl *et al.*, *Cell* **71**, 107 (1992).
27. K. Hjalmarsson, S. L. Marklund, Å. Engström, T. Edlund, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6340 (1987).
28. I. Ceballos *et al.*, *Free Rad. Res. Commun.* **12–13**, 571 (1991); T. Siddique *et al.*, unpublished data.
29. T. Yoneda, S. Inagaki, Y. Hayashi, T. Nomura, H. Takagi, *Brain Res.* **582**, 342 (1992).
30. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989), pp. 1.13–1.14.

31. This paper is dedicated to the memory of Forbes H. Norris, Jr., and patients we lost to ALS. We thank our teachers, A. Ahmed, S. D. Cook, P. Tsairis, and W. K. Engel, who taught us the care of patients with ALS and who encouraged this research; N. Siddique, J. Braun, J. Sacks, P. Casey, J. Richman, H. Mitsumoto, and R. Tandan for help in obtaining blood samples and pedigree information; M. E. Pique for help with computer graphics; and J. Rimmler and P. Pate for help with linkage analysis. Supported by the Les Turner ALS Foundation (T.S.), the NINDS (T.S., M.A.P.-V., H.-X.D., R.P.R.), the NIGMS (J.A.T., R.A.H., E.D.G.) and CRC grant (A.D.R., T.S., M.A.P-V.), the ALS Association (T.S., G.D.), the Gisela Fund for ALS research (T.S.), the Muriel Heller Fellowship Fund for ALS Research (W.-Y.H.), the Muscular Dystrophy Association (T.S., M.A.P.-V., A.H.), H.C. and F. Wenske Foundation (T.S.), the Searle Family Fund for Neurological Disorders (T.S.), the Vena E. Schaaf ALS Research Fund (T.S.), and Atropos Genetic Engineering, Inc. (R.A.H.). Atomic coordinates have been deposited in the Brookhaven Protein Databank code 1SPD.

23 March 1993; accepted 19 July 1993

An Essential Role for Protein Phosphatases in Hippocampal Long-Term Depression

Rosel M. Mulkey, Caroline E. Herron, Robert C. Malenka*

The effectiveness of long-term potentiation (LTP) as a mechanism for information storage would be severely limited if processes that decrease synaptic strength did not also exist. In area CA1 of the rat hippocampus, prolonged periods of low-frequency afferent stimulation elicit a long-term depression (LTD) that is specific to the stimulated input. The induction of LTD was blocked by the extracellular application of okadaic acid or calyculin A, two inhibitors of protein phosphatases 1 and 2A. The loading of CA1 cells with microcystin LR, a membrane-impermeable protein phosphatase inhibitor, or calmodulin antagonists also blocked or attenuated LTD. The application of calyculin A after the induction of LTD reversed the synaptic depression, suggesting that phosphatase activity is required for the maintenance of LTD. These findings indicate that the synaptic activation of protein phosphatases plays an important role in the regulation of synaptic transmission.

Activity-dependent long-term changes in synaptic efficacy are of fundamental importance for the development of neural circuits and for information storage in the nervous system. Long-term potentiation in area CA1 of the hippocampus has been an intensively studied form of activity-dependent synaptic plasticity primarily because it can be elicited reliably in vitro in isolated slices of the hippocampus. Consequently, some of the biochemical steps responsible for its induction and maintenance are well characterized (*1*).

Several different forms of LTD in the hippocampus have been observed (*2*), although the underlying biochemical mechanisms are not known. Recently a form of LTD that, like LTP, is restricted to activated synapses has been described (*3*, *4*). This homosynaptic LTD requires activation of postsynaptic *N*-methyl-D-aspartate (NMDA) receptors (*3*, *4*) and a change in the postsynaptic Ca^{2+} concentration (*4*). We have examined biochemical processes underlying this form of LTD and find that LTD requires serine-threonine protein phosphatase activity.

Synaptic transmission between Schaffer collateral-commissural afferent fibers and CA1 pyramidal cells in rat hippocampal slices was studied with standard extracellular and whole-cell recording techniques (*5*). After LTD was saturated with repetitive periods (2 to 6 min) of 1-Hz stimulation (*4*), LTP-inducing high-frequency tetanuses (100 Hz, 1 s) increased synaptic strength beyond the original baseline value (Fig. 1). If the induction of LTP had not caused a reversal of the processes responsible for LTD, it would not have been possible to re-elicit LTD. Instead, additional episodes of 1-Hz stimulation reduced synaptic strength to its original minimal saturated level (10 of 12 experiments) (Fig. 1). Consistent with this finding, previous work has

Departments of Psychiatry and Physiology, University of California, San Francisco, CA 94143–0984.

*To whom correspondence should be addressed.

demonstrated that prolonged periods of 1-Hz stimulation may cause a "de-potentiation" of LTP (6).

Given that a network of protein kinases appears to play an important role in LTP (1), an attractive hypothesis is that LTD results from a relative increase in protein phosphatase activity compared with protein kinase activity (7). Therefore we examined the effects of specific serine-threonine protein phosphatase inhibitors on LTD (8) (Fig. 2). Okadaic acid (1 μM), a specific inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A) (9), completely blocked LTD [4 ± 5% (mean percent change from baseline ± SEM); n = 10]. In contrast, the application of 1-nor-okadaone (1 μM), a compound with physical and chemical properties similar to those of okadaic acid but lacking any phosphatase inhibitory activity (9), had no effect on the ability to generate LTD (−27 ± 3%; n = 3) (Fig. 2B). Calyculin A (1 μM), which is structurally distinct from okadaic acid and a more potent inhibitor of PP1 and PP2A (10), also blocked the generation of LTD completely (12 ± 6%; n = 12) (Fig. 2C) (11, 12). As a final control we tested the effects of the impermeant protein phosphatase inhibitor, microcystin LR (13). Consistent with its inability to cross cell membranes (13), LTD was elicited in all slices treated with microcystin LR (n = 6) (14).

Long-term potentiation may be maintained, at least in part, by a persistent increase in kinase activity (1, 15). To investigate whether, similarly, LTD is expressed when critical phosphoproteins are maintained in the dephosphorylated state, we monitored simultaneously synaptic transmission in response to stimulation of two independent afferent fiber bundles and compared the effects of calyculin A (1 μM) application on a control path and a path in which LTD had been elicited previously.

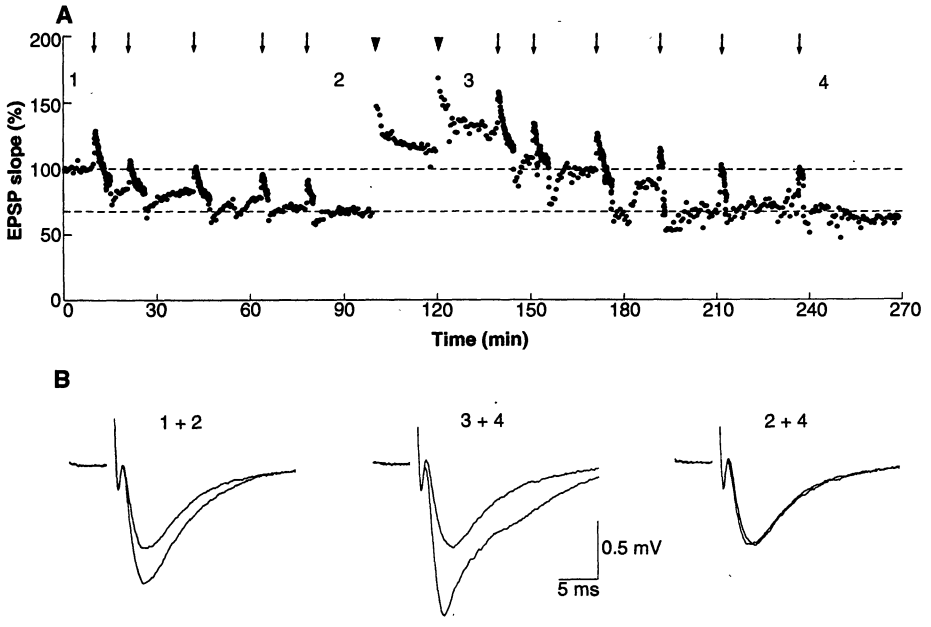

Fig. 1. Long-term depression and LTP are reversible modifications of synaptic mechanisms. (**A**) Plot of a typical experiment in which a 1-Hz stimulation (2 to 5 min) was applied repetitively (arrows) to saturate LTD. At 100 and 120 min LTP was induced by tetanic stimulation (100 Hz for 1 s, arrowheads), increasing synaptic strength above the baseline value. At 140 min, episodes of 1-Hz stimulation (2 to 5 min) depressed synaptic strength to the value preceding the induction of LTP. (**B**) Field EPSPs (average of three successive sweeps) were taken at the indicated times.

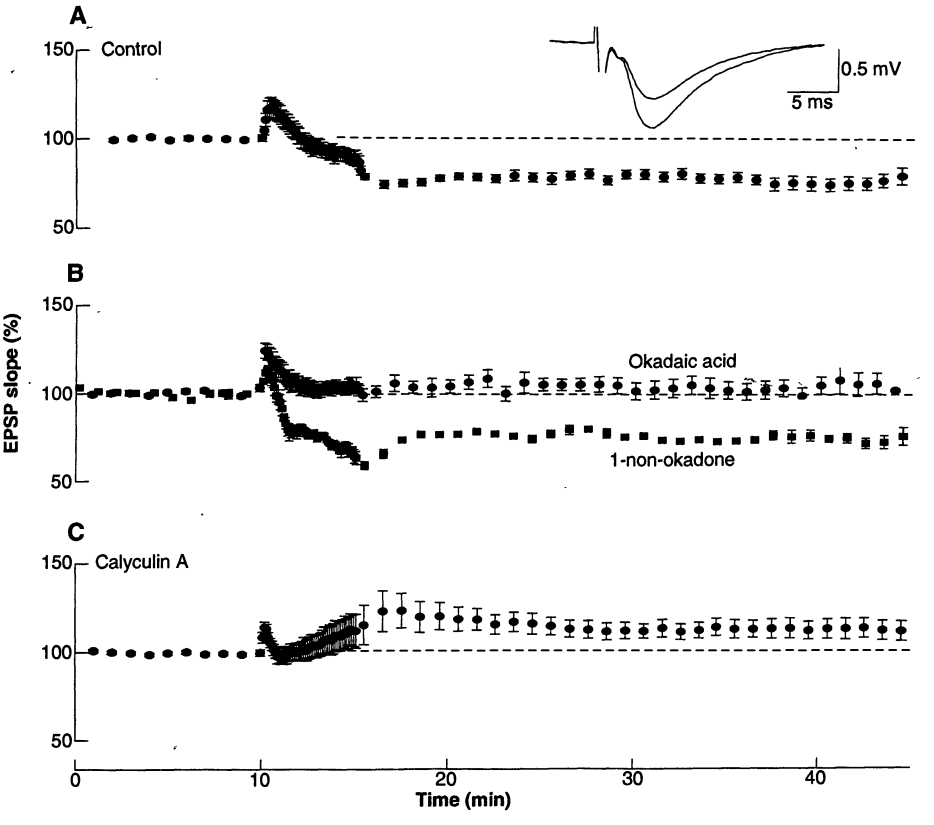

Fig. 2. Effects of the protein phosphatase inhibitors okadaic acid and calyculin A on the induction of LTD. Low-frequency stimulation (1 Hz for 5 min) was applied to hippocampal slices. (**A**) Control (−27 ± 4%; n = 16). The insets (average of three successive sweeps) were taken from a control experiment before and 30 min after LTD induction. (**B**) Slices incubated in (circles) okadaic acid (1 μM; n = 10) or (squares) 1-nor-okadaone (1 μM; n = 3). (**C**) Slices incubated in calyculin A (1 μM; n = 12).

The application of calyculin A had no effect on synaptic transmission monitored in the control path (−1 ± 4%) but caused an increase in synaptic strength in the path in which LTD had been induced (24 ± 5%) (Fig. 3, A and C). A shift to the left in the input-output curve after calyculin A application in the depressed but not the control path (Fig. 3B) demonstrated that the observed increase in synaptic strength in the depressed path did not result from an effect on fiber excitability (*11*). Consistent with previous experiments (Fig. 2C), in all slices tested (n = 6) bath application of calyculin A for 40 min was effective in preventing the subsequent induction of LTD by 1-Hz stimulation in either the control (Fig. 3A) or test path (*16*).

Both LTP (*1*) and homosynaptic LTD (*4*) are dependent on a change in the postsynaptic Ca^{2+} concentration elicited by the activation of NMDA receptors. Therefore, a change in the magnitude of the synaptic current mediated by NMDA receptors might significantly affect the direction of change in synaptic efficacy caused by a fixed pattern of afferent activity. Bath application of calyculin A (1 μM; n = 4) had no discernible effect on the magnitude of the excitatory postsynaptic potential (EPSP) mediated by NMDA receptors that was recorded in the presence of the non–NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and low extracellular magnesium (0.1 mM). Moreover, whole cell voltage-clamp recordings revealed that the voltage depen-

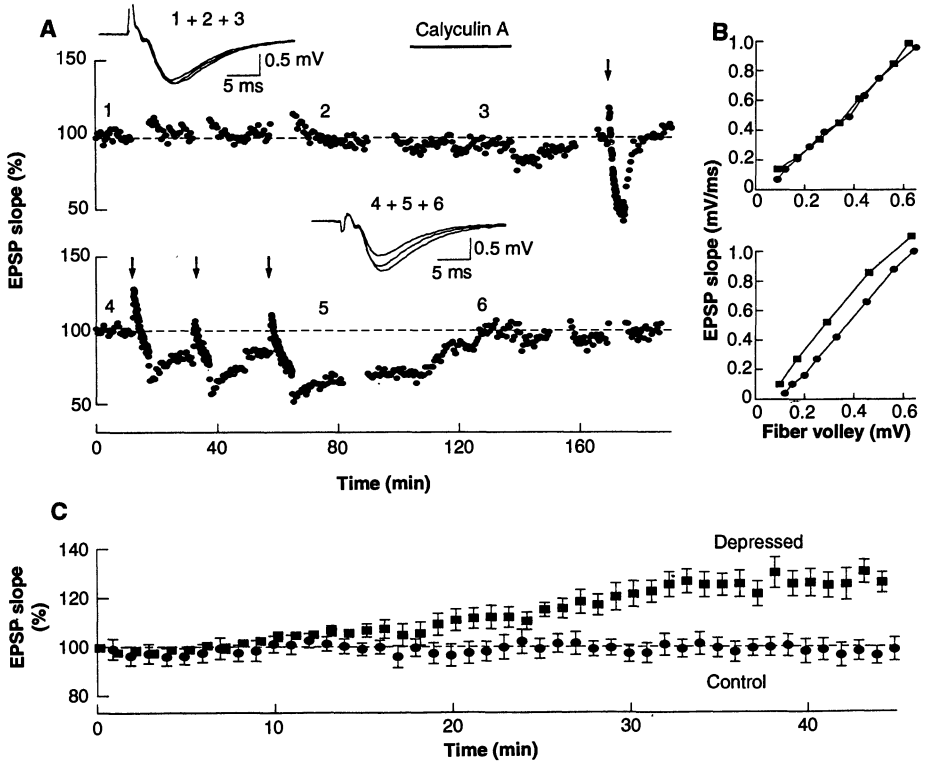

Fig. 3. Effects of calyculin A on existing LTD. (**A**) Field EPSPs were recorded from a single site in response to the alternating stimulation of two independent afferent-fiber bundles. Long-term depression was induced in one pathway by stimulation at 1 Hz for 5 min (arrows). Calyculin A (1 μM) was then applied to the slice (solid bar). Sample traces (average of three successive sweeps) were taken at the indicated times. (**B**) Input-output curves generated immediately before (circles) and after (squares) calyculin application. The curves were generated during the gaps in the plots of the complete experiment (A). (**C**) Summary graph (n = 6) of the depressed (squares) and control (circles) paths. For each experiment, the control path was normalized to the original baseline and the depressed path was normalized to the baseline obtained 10 min before calyculin A application.

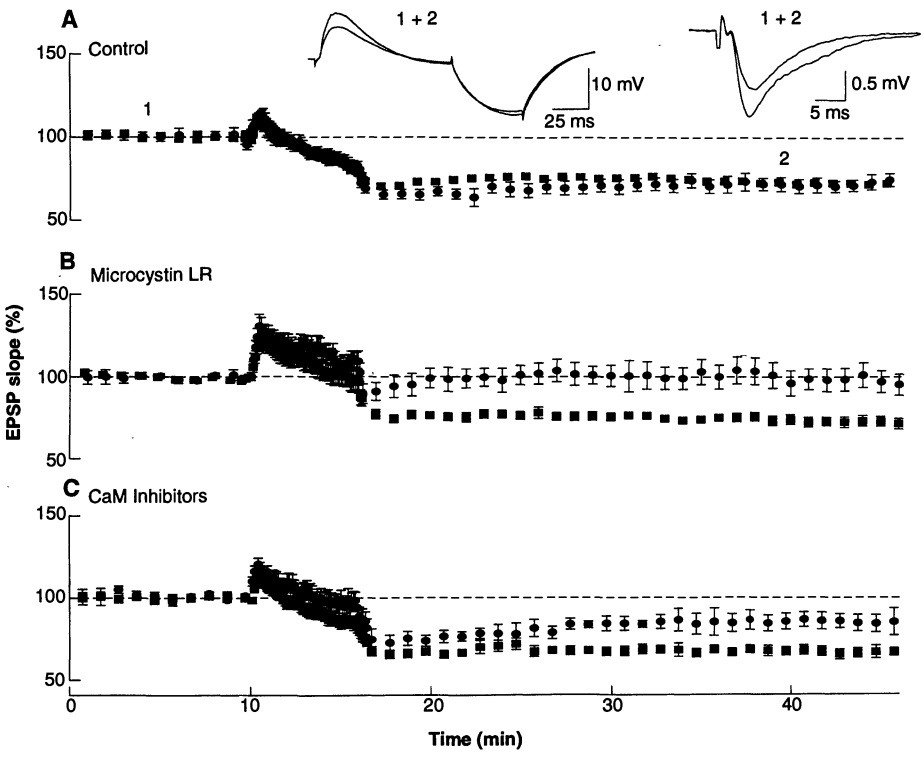

Fig. 4. Long-term depression is dependent on postsynaptic protein phosphatase activity and postsynaptic Ca^{2+}-CaM. (**A**) Control experiments (n = 24) in which EPSPs were monitored in (circles) a single cell and (squares) a population of cells in the same slice, with simultaneous whole cell and extracellular field recording. Long-term depression was induced by stimulation (1 Hz for 6 min). Sample traces (averages of three successive sweeps) were taken at the indicated times. (**B**) Experiments (n = 12) in which CA1 cells were loaded with microcystin LR (10 μM in patch pipette). (**C**) Experiments (n = 12) in which CA1 cells were loaded with CBP_{-3} (20 μM) or calmidazolium (2 μM).

dence of the excitatory postsynaptic current (EPSC) mediated by NMDA receptors also appeared normal in cells treated with calyculin A (*17*).

A critical question is whether the phosphatase inhibitors act pre- or postsynaptically to block LTD. We examined whether loading CA1 pyramidal cells with microcystin LR could block LTD (*18*). There was a strong correspondence between the magnitude of LTD elicited in the control cells (n = 24) and that measured in the simultaneously recorded field EPSP (cells: $-29 \pm 4\%$; fields: $-28 \pm 3\%$) (Fig. 4A). Long-term depression was blocked in cells that were loaded with microcystin LR ($-2 \pm 7\%$; n = 12) (Fig. 4B), although the 1-Hz stimulation induced LTD in surrounding cells, as evidenced by the decrease in the field EPSP ($-28 \pm 3\%$). Thus, the activity of a postsynaptic protein phosphatase appears to be critical for the induction of LTD.

How might protein phosphatase activity in the postsynaptic cell be regulated by synaptic activity? Because homosynaptic LTD depends on a change in the postsynaptic Ca^{2+} concentration (*4*), one possibility is that the Ca^{2+} complexes with calmodulin (CaM), leading to a change in protein phosphatase activity (*7, 19*). To test whether Ca^{2+}-CaM in the postsynaptic cell is required to generate LTD, we loaded CA1 cells with either a CaM-binding peptide (CBP_{-3}; 20 μM) (*20*) or calmidazolium (2 μM), both of which have been shown to block LTP (*21*). These agents significantly reduced the magnitude of LTD ($-14 \pm 5\%$; n = 12) compared with that observed in the field EPSP ($-33 \pm 3\%$; $P < 0.005$) (Fig. 4C) or in the control cells ($-29 \pm 4\%$; $P < 0.05$) (*22*).

Our results demonstrate that serine-threonine protein phosphatase activity in the postsynaptic cell is required for the generation of homosynaptic LTD in CA1 pyramidal cells. Several lines of evidence suggest that PP1 is more likely than PP2A to affect LTD. Protein phosphatase 1 is found in high concentrations in isolated synaptic junctions (*19, 23*) and can dephosphorylate several phosphoproteins found at the synapse, including CaMKII (*19, 23*), a kinase thought to be important for LTP (*1, 24*). The activity of PP1 also can be indirectly regulated by the postsynaptic Ca^{2+} concentration, whereas there are no known mechanisms to regulate the activity of PP2A (*19*).

A model that is consistent with several of our results and with the known regulation of PP1 has been proposed by Lisman (*7*). Protein phosphatase 1 is inhibited by the phosphorylated form of inhibitor I (I-1), which is dephosphorylated by the Ca^{2+}-CaM–dependent protein phosphatase calcineurin (PP2B) (*19*). Therefore, the activation of PP2B by Ca^{2+}-CaM would dephosphorylate I-1, resulting in the activation (by way of disinhibition) of PP1. The Ca^{2+}-CaM complex is also required for LTP (*1*) but perhaps at a higher threshold concentration than is required for LTD (*7*). This difference might explain why loading cells with CaM inhibitors significantly reduced but did not completely block LTD, whereas these same inhibitors blocked LTP in a previous study (*21*).

Our data, combined with previous work on LTP (*1*), provide evidence that activity-dependent modulation of synaptic strength in CA1 pyramidal cells is at least partially controlled by the balance between the activities of critical protein kinases and serine-threonine protein phosphatases in the postsynaptic cell. A potential substrate for this biochemical competition is the synaptic glutamate receptor. The adenosine 3′,5′-monophosphate–dependent protein kinase (PKA) phosphorylates the glutamate receptor subunit GluR6, resulting in an enhancement of agonist-induced responses (*25*). However, it seems unlikely that this mechanism is important in mediating LTD or LTP in CA1 cells in situ because GluR6 is preferentially expressed in CA3 cells in the hippocampus (*26*) and strong activation of PKA did not block LTD (*27*). In isolated postsynaptic densities, endogenous CaMKII phosphorylates a GluR1 subunit that is part of the native synaptic glutamate receptor (*28*). This protein kinase also enhances responses to kainate in hippocampal neurons (*28*), as do phosphatase inhibitors (*28, 29*). Thus, a change in the phosphorylation state of GluR1 or related subunits could provide an efficient mechanism to control synaptic strength.

Our results by no means rule out an important role for presynaptic phosphatase activity in LTD. However, like LTP, homosynaptic LTD is induced postsynaptically (*4*), indicating that if presynaptic modifications occur during LTD, a "retrograde" messenger (*1*) must exist. The examination of LTP and LTD in parallel should facilitate the identification of phosphoproteins that are critical for the control of synaptic efficacy and whose phosphorylation states are regulated by synaptic activity.

REFERENCES AND NOTES

1. T. V. P. Bliss and G. L. Collingridge, *Nature* **361**, 31 (1993); D. V. Madison, R. C. Malenka, R. A. Nicoll, *Annu. Rev. Neurosci.* **14**, 379 (1991).
2. R. C. Malenka, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3121 (1993); A. Artola and W. Singer, *Trends Neurosci.*, in press.
3. S. M. Dudek and M. F. Bear, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4363 (1992).
4. R. M. Mulkey and R. C. Malenka, *Neuron* **9**, 967 (1992).
5. Standard procedures were used to record from CA1 pyramidal cells in hippocampal slices prepared from Sprague-Dawley rats (12 to 20 days) [R. C. Malenka, *ibid.* **6**, 53 (1991); A. Colino, Y.-Y. Huang, R. C. Malenka, *J. Neurosci.* **12**, 180 (1992)]. The composition of the perfusing solution (saturated with 95% O_2 and 5% CO_2) was 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose. For whole cell recordings, the patch-pipette solution contained 117.5 mM K gluconate, 17.5 mM potassium methylsulfate, 8 mM NaCl, 10 mM Hepes, 0.2 mM EGTA, 3 mM magnesium–adenosine triphosphate, and 0.2 mM guanosine triphosphate (pH = 7.2). Schaffer collateral-commissural fibers were stimulated at a base-line frequency of 0.1 Hz. Homosynaptic LTD was elicited by stimulation at 1 Hz for 5 to 7 min (*3, 4*). Experiments that required the stimulation of two independent populations of afferents were conducted and data were collected and analyzed as described [Y.-Y. Huang, A. Colino, D. K. Selig, R. C. Malenka, *Science* **255**, 730 (1992)]. The mean change in excitatory postsynaptic potential (EPSP) slope for each experiment was calculated by comparison of the average of all data points collected 25 to 30 min after LTD induction with the average collected during the initial 10-min base-line. A two-tailed Student's t test was used to test for significant differences between groups.
6. G. Barrionuevo, F. Schottler, G. Lynch, *Life Sci.* **27**, 2385 (1980); U. Staubli and G. Lynch, *Brain Res.* **513**, 113 (1990); S. Fujii, K. Saitoh, H. Miyakawa, K. Ito, H. Kato, *Brain Res.* **555**, 112 (1991).
7. J. Lisman, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9574 (1989).
8. Slices were incubated in phosphatase inhibitors (LC Laboratories, Woburn, MA) for 1 to 3 hours before transfer to the experimental chamber. Control slices were incubated for 1 to 3 hours in the solvent (0.1% dimethyl sulfoxide) used to dissolve the drugs. Experiments on control slices were interleaved with those on treated slices so that control slices were always tested on the same day as slices treated with phosphatase inhibitors.
9. P. Cohen, C. F. B. Holmes, Y. Tsukitani, *Trends Biochem. Sci.* **15**, 98 (1990); S. Nishiwaki *et al.*, *Carcinogenesis* **11**, 1837 (1990).
10. H. Ishihara *et al.*, *Biochem. Biophys. Res. Commun.* **159**, 871 (1989); M. Suganuma *et al.*, *Cancer Res.* **50**, 3521 (1990).
11. In initial experiments we observed that calyculin A could increase the fiber volley amplitude, indicating some direct effect on fiber excitability. This effect was often accentuated by the 1-Hz stimulation used to induce LTD. Therefore, the fiber volley amplitude was continuously measured and monitored on-line during the course of the experiment and kept constant by small adjustments in the stimulation voltage.
12. Because 1-Hz stimulation in the presence of calyculin A could enhance synaptic transmission, it is possible that the observed "block" of LTD by calyculin A was attributable to the generation of a superimposed potentiation. To test this possibility, in the presence of calyculin A we applied the NMDA-receptor antagonist D-APV (50 μM) to block activation and saturation of LTD mechanisms (*3, 4*) and then applied 1-Hz stimulation for several 5-min periods until the observed potentiation was saturated (n = 3). The D-APV was then washed out for a minimum of 20 min, and another 1-Hz 5-min stimulation was applied. [The results of additional experiments (n = 3) indicated that a 20-min wash was sufficient to eliminate D-APV and generate LTD in control slices]. If the block of LTD by calyculin A was attributable to the generation of an independent potentiation, LTD should have been elicited at this point in the experiment. In all experiments, neither LTD nor further potentiation was elicited by 1-Hz stimulation after D-APV washout. These results indicate that the effects of calyculin A on LTD were due to interference with an essential mechanism required to generate LTD.
13. R. E. Honkanen *et al.*, *J. Biol. Chem.* **265**, 19401 (1990); R. Matsushima *et al.*, *Biochem. Biophys. Res. Comm.* **171**, 867 (1990); C. MacKintosh, K.

A. Beattie, S. Klumpp, P. Cohen, G. A. Codd, *FEBS Lett.* **264**, 187 (1990).
14. The effects of phosphatase inhibitors on LTP have also been examined. In slices in which LTD was blocked by okadaic acid, high-frequency tetanuses (100 Hz, 1 s) elicited LTP (seven of nine experiments). Similarly, calyculin A has been reported not to block LTP [K. Fukunaga, L. Stoppini, E. Miyamoto, D. Muller, *J. Biol. Chem.* **268**, 7863 (1993)].
15. R. Malinow, D. V. Madison, R. W. Tsien, *Nature* **335**, 820 (1988); E. Klann, S.-J. Chen, J. D. Sweatt, *J. Biol. Chem.* **266**, 24253 (1991); J.-H. Wang and D.-P. Feng, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2576 (1992).
16. Because okadaic acid required long periods of bath application (>60 min) to block LTD, we did not repeat this two-pathway experiment using okadaic acid because any differential effect on synaptic transmission in the two paths would be difficult to distinguish from "drift" in the preparation.
17. We made whole cell voltage-clamp recordings with an Axoclamp 2A (Axon Instruments) using patch pipettes containing a cesium-based solution (*4*). Synaptic responses in the presence of CNQX (10 μM) and picrotoxin (25 μM) were recorded at holding potentials ranging from −80 to +40 mV. There were no significant differences in the current/voltage relation observed when recordings from control slices (n = 4) and calyculin A–treated slices (n = 4) were compared. The EPSC at −80 mV was 8 ± 4% (mean ± SD) and 11 ± 7% of the peak current in control cells and calyculin A–treated cells, respectively. At −30 mV the EPSC was 48 ± 9% and 47 ± 13% of the peak current in the two respective populations of cells.
18. In all experiments in which compounds were loaded directly into CA1 cells, an extracellular field EPSP was monitored simultaneously to ensure that the 1-Hz stimulation had elicited LTD in surrounding cells. Microcystin LR (10 μM in 0.5% ethanol) was added directly to the patch-pipette recording solution each day immediately before the beginning of experiments. Control cells were always examined on the same day with the same recording solution. In addition, the 1-Hz 6-min stimulation was always applied within 20 min of break-in, a period over which we have never observed washout of the ability to induce LTD. Only cells in which LTD was elicited in the simultaneously recorded field EPSP were included in the summary graphs.
19. S. Shenolikar and A. C. Nairn, *Adv. Second Mess. Phosphoprotein Res.* **23**, 1 (1991); P. Stemmer and C. B. Klee, *Curr. Opin. Neurobiol.* **1**, 53 (1991); S. I. Walaas and P. Greengard, *Pharmacol. Rev.* **43**, 299 (1991).
20. P. T. Kelly, R. P. Weinberger, M. N. Waxham, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4991 (1988).
21. R. C. Malenka *et al.*, *Nature* **340**, 554 (1989).
22. We also examined the effects of loading cells with calyculin A by adding it to our recording solution. This manipulation had significant effects on the magnitude of LTD [−18 ± 4% in the cells (n = 14) versus −31 ± 4% in the field EPSPs ($P < 0.05$) or −29 ± 4% in control cells (n = 24; $P < 0.05$)]. Two important differences in the properties of calyculin A and microcystin LR could explain the lower efficacy of calyculin A in blocking LTD when applied with a patch pipette. First, calyculin A is quite lipophilic and can partition into and cross cell membranes, whereas microcystin LR is hydrophilic and cell-impermeable. Thus, much higher concentrations of microcystin LR should reach critical synaptic sites. Second, microcystin LR, but not calyculin A, may inhibit the Ca^{2+}-CaM–dependent protein phosphatase PP2B in addition to PP1 and PP2A (*13*).
23. S. M. Shields, T. S. Ingebritsen, P. T. Kelly, *J. Neurosci.* **5**, 3414 (1985).
24. J. E. Lisman and M. A. Goldring, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5320 (1988).
25. L. A. Raymond, C. D. Blackstone, R. L. Huganir, *Nature* **361**, 637 (1993); L.-Y. Wang, F. A. Taverna, X.-P. Huang, J. F. MacDonald, D. R. Hampson, *Science* **259**, 1173 (1993).
26. J. Egebjerg, B. Bettler, I. Hermans-Borgmeyer, S. Heinemann, *Nature* **351**, 745 (1991).
27. C. E. Herron, R. M. Mulkey, R. C. Malenka, unpublished observations.
28. E. McGlade-McCulloh, H. Yamamoto, S.-E. Tan, D. A. Brickey, T. R. Soderling, *Nature* **362**, 640 (1993).
29. L.-Y. Wang, M. W. Salter, J. F. MacDonald, *Science* **253**, 1132 (1991).

30. We thank D. Brautigan, S. Kombian, S. Nicola, D. Spillane, and C. Stevens for helpful discussions. Supported by grants from the National Institute of Mental Health (MH45334), the McKnight Foundation, the National Alliance for Research on Schizophrenia and Depression (R.C.M.) and by a Research Scientist Award (MH00942) (R.C.M.) and an individual National Research Service Award (MH10306) (R.M.M.).

13 April 1993; accepted 21 June 1993

Dynamics of the Hippocampal Ensemble Code for Space

Matthew A. Wilson and Bruce L. McNaughton

Ensemble recordings of 73 to 148 rat hippocampal neurons were used to predict accurately the animals' movement through their environment, which confirms that the hippocampus transmits an ensemble code for location. In a novel space, the ensemble code was initially less robust but improved rapidly with exploration. During this period, the activity of many inhibitory cells was suppressed, which suggests that new spatial information creates conditions in the hippocampal circuitry that are conducive to the synaptic modification presumed to be involved in learning. Development of a new population code for a novel environment did not substantially alter the code for a familiar one, which suggests that the interference between the two spatial representations was very small. The parallel recording methods outlined here make possible the study of the dynamics of neuronal interactions during unique behavioral events.

The hippocampal formation is the highest level of association cortex (*1*) and is an essential component in memory encoding. Its role in the internal representation of space (*2*) is indicated by both neuropsychological studies (*3*) and the observation that its pyramidal cells ("place" cells) are selectively active in particular locations (*4*). At the computational level, the matrix-like organization of hippocampal circuitry has inspired the theory that it serves as a rapid autoassociative memory (*5, 6*). Finally, its neurons exhibit potential substrates of learning in the form of cooperative, long-term synaptic enhancement (*7*). Thus, the hippocampus represents a key to understanding biological and computational mechanisms of associative memory and the structure of cognitive representations.

The spatial and mnemonic dependence of hippocampal neuronal activity has been well characterized at the level of single cells (*8*); however, complete understanding of hippocampal function will require knowledge of the dynamics of ensemble encoding (*9*) of information across many neurons during relevant behavioral tasks, particularly in view of both the involvement of the hippocampus in memories that are unique to a trial and the low firing rates and intrinsic variability of single hippocampal cells. In the only published study to address whether experience is required for the initial establishment of hippocampal spatial representations, single-unit recordings failed to detect differences in the firing of most place cells between the first and subsequent entries of a rat into a specific location (*10*). Here, we provide evidence to the contrary and describe the dynamics of ensemble encoding of space in the hippocampus during a single episode of exploration in a novel environment.

Three rats were implanted with microdrive arrays (*11*) containing 12 four-channel recording electrodes (tetrodes), each capable of resolving activity from 5 to 20 single hippocampal neurons (*12*). The rats were trained over 10 days to forage for small, chocolate pellets in half of a rectangular apparatus (box A) (*13*). The other half of the apparatus (box B) was obscured from view by a partition.

While the rat sat quietly or slept outside the apparatus, the electrodes were lowered until stable recordings of well-isolated cells in the pyramidal layer of CA1 were obtained on all tetrodes. Most hippocampal cells become active during these conditions, even if they exhibit little or no activity during the particular behavioral task under study (*14, 15*). Eighty-two single units were identified in rat 1, 73 in rat 2, and 148 in rat 3. Of these, the numbers of pyramidal [complex spike (CS)] cells were 76, 71, and 141 for rats 1, 2, and 3, respectively. The remain-

Department of Psychology and Division of Neural Systems, Memory, and Aging, University of Arizona, Tucson, AZ 85724.