

The Maintenance of LTP at Hippocampal Mossy Fiber Synapses Is Independent of Sustained Presynaptic Calcium

Wade G. Regehr* and David W. Tank
Biophysics Research Department
AT & T Bell Laboratories
Murray Hill, New Jersey 07974

Summary

We have examined the role of presynaptic residual calcium in maintaining long-term changes in synaptic efficacy observed at mossy fiber synapses between hippocampal dentate granule cells and CA3 pyramidal cells. Calcium concentrations in individual mossy fiber terminals in hippocampal slice were optically measured with the calcium indicator fura-2 while stimulating the mossy fiber pathway and recording excitatory postsynaptic potentials extracellularly. Short-term synaptic enhancement was accompanied by increased presynaptic residual calcium concentration. A 2-fold enhancement of transmitter release was accompanied by a 10–30 nM increase in residual calcium. Following induction of mossy fiber LTP, transiently elevated presynaptic calcium decayed to prestimulus levels, whereas enhancement of synaptic transmission persisted. Our results demonstrate that, despite an apparent strong sensitivity of synaptic enhancement to presynaptic residual calcium levels, sustained increases in presynaptic residual calcium levels are not responsible for the maintained synaptic enhancement observed during mossy fiber LTP.

Introduction

There is now substantial evidence that the baseline or residual level of intracellular free calcium ($[Ca^{2+}]_{Res}$) present in a presynaptic terminal before the invasion of an action potential can strongly modulate the amount of transmitter released (Connor et al., 1986; Delaney et al., 1989). At the crayfish neuromuscular junction, trains of action potentials in the presynaptic terminal produce a buildup of $[Ca^{2+}]_{Res}$ that decays following termination of the train (Delaney et al., 1989). Recent experiments suggest that this calcium buildup is necessary and sufficient for the expression of two forms of activity-dependent, short-term synaptic enhancement: augmentation and posttetanic potentiation (Delaney and Tank, 1991). It is conceivable that this same basic mechanism of $[Ca^{2+}]_{Res}$ -induced changes of transmitter release underlies some forms of short-term and long-term synaptic enhancement observed in mammalian nervous systems.

Presynaptic changes in transmitter release have been implicated in the long-term potentiation (LTP) of synaptic transmission in the Schaffer collateral system in hippocampal region CA1. The induction of LTP

in region CA1 is dependent on the activation of N-methyl-D-aspartate (NMDA) receptors (Collingridge et al., 1983) on the postsynaptic CA1 pyramidal cell. However, quantal analysis suggests that the site of the expression of synaptic enhancement resides in the presynaptic terminal (Bekkers and Stevens, 1990; Malinow and Tsien, 1990), where a change in the quantal parameter p has been interpreted as a presynaptic change in the probability of release following the induction of LTP (Bekkers and Stevens, 1990). While the interpretations of these results are somewhat controversial (Kauer et al., 1988; Muller et al., 1988), they suggest that, even in forms of LTP in which there is clearly a postsynaptic site of induction (Lynch et al., 1983), the expression of LTP may be presynaptic.

Presynaptic changes in transmitter release have also been implicated in synaptic enhancement at the mossy fiber synapses in hippocampal region CA3. The mossy fibers are granule cell axons that make en passant synaptic connections with spines located on the proximal dendrites of CA3 pyramidal cells (Amaral, 1979; Claiborne et al., 1986). High frequency mossy fiber activation produces a long-term enhancement of synaptic strength in the granule cell to CA3 pyramidal cell connection, which we will refer to as mossy fiber LTP (Barrionuevo et al., 1986). Unlike the more widely studied form of LTP that involves the NMDA receptor (Bliss and Lomo, 1973; for reviews see Brown and Zador, 1990; Nicoll et al., 1988), mossy fiber LTP does not involve NMDA receptors and is not blocked by NMDA receptor antagonists (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990). There is controversy over the conditions necessary for induction of LTP (Jaffe and Johnston, 1990; Williams and Johnston, 1989; Zalutsky and Nicoll, 1990), but again there is evidence that the expression of mossy fiber LTP involves a presynaptic change. Paired-pulse facilitation is persistently modified following induction of LTP at these synapses, suggesting a change in the probability of release (Zalutsky and Nicoll, 1990).

By combining high resolution microfluorimetry with electrophysiological recordings, we have directly measured $[Ca^{2+}]_{Res}$ in individual mossy fiber presynaptic terminals during frequency-dependent, short-term synaptic enhancement and LTP induction. To label specifically the presynaptic terminals with a calcium indicator molecule, we have used our localized fura-2 perfusion method in mammalian brain slice (Regehr and Tank, 1991). Here we report results pertinent to understanding the involvement of $[Ca^{2+}]_{Res}$ in the maintenance of mossy fiber LTP. We observed that there is a buildup of $[Ca^{2+}]_{Res}$ produced by presynaptic action potentials which correlates with enhanced transmitter release produced during high frequency-induced, short-term synaptic enhancement. We characterized the relationship between $[Ca^{2+}]_{Res}$ and the amount of synaptic enhancement in order to establish

* Present address: Department of Physiology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104.

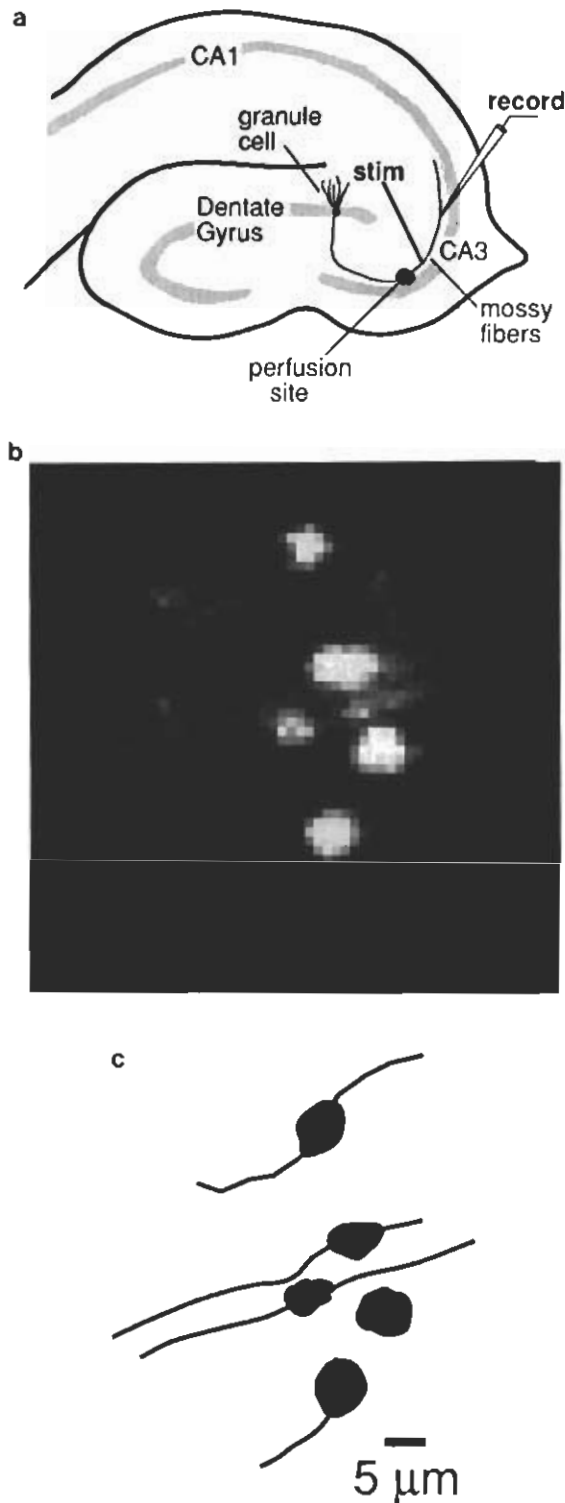


Figure 1. Localized Perfusion Labeling of Mossy Fiber Terminals (a) Schematic of hippocampal slice showing the local perfusion site, stimulus-electrode placement, and recording-electrode placement. (b) Fluorescence image of presynaptic terminals located in the stratum lucidum approximately 600 μm from the perfusion site ($40\times$ 1.3 NA Nikon objective, exposure 2 s, 380 nm illumination). (c) Camera lucida drawing of (b) showing the individual presynaptic terminals and associated axons that are resolved.

a calibration curve for the strength of the effect. We demonstrate that synaptic enhancement is very sensitive to $[\text{Ca}^{2+}]_{\text{res}}$ and that $[\text{Ca}^{2+}]_{\text{res}}$ levels are substantially increased by even brief stimulus trains (The detailed form of the relationship between $[\text{Ca}^{2+}]_{\text{res}}$ and synaptic release and its similarity to that observed in invertebrate systems will be described elsewhere). With the calibration curve as a reference, we induced LTP in mossy fiber synapses and determined whether changes in $[\text{Ca}^{2+}]_{\text{res}}$ were sufficient to account for LTP. Our results indicate that elevated $[\text{Ca}^{2+}]_{\text{res}}$ does not contribute to the prolonged phase of mossy fiber LTP.

Results

Simultaneous Fluorescence Imaging and Field Potential Measurement

To investigate the role of $[\text{Ca}^{2+}]_{\text{res}}$ in the maintenance of LTP, we developed a method that allowed accurate, reproducible measurements of presynaptic $[\text{Ca}^{2+}]_{\text{res}}$ while simultaneously measuring the strength of monosynaptic connections. We have combined two complementary methods to accomplish this goal: calcium indicator loading by localized perfusion of the acetoxymethyl (AM) ester derivative of fura-2, and local stimulation and extracellular recording of fluorescently visualized mossy fibers.

The localized perfusion method we developed (Regehr and Tank, 1991) is based upon providing a local continuous stream of extracellular labeling solution containing fura-2 AM by microperfusion methods. Nerve cell processes near the perfusion site fill with fura-2. Since intracellularly trapped fura-2 diffuses, filling the cytoplasm, cellular processes distant from the perfusion site are labeled by anatomical specificity usually obtained only by intracellular microinjections. When applied to the mossy fiber tract in hippocampal region CA3 of the guinea pig (Figure 1a), a fluorescent band extending parallel to the cell body layer is produced by labeled projections of mossy fibers (see Figure 2A in Regehr and Tank, 1991). When observed with high power, high numerical aperture objectives (Nikon UVF $40\times$ 1.3 NA), individual terminals can be easily imaged. A fluorescence image containing several mossy fiber terminals filled with fura-2 by this method is shown in Figure 1b. A corresponding camera lucida drawing created from the fluorescence image is shown in Figure 1c.

The localized perfusion method for filling mossy fiber terminals with fura-2 not only loaded the structures of interest with a fluorescent calcium indicator molecule, it also enabled us to use a new method for local stimulation and recording that provided good measures of monosynaptic connection strength. In general, the measurement of monosynaptic mossy fiber postsynaptic potentials (PSPs) is complicated by the recurrent excitatory connections within hippocampal region CA3 (Miles and Wong, 1987). As shown in Figure 1a, we were able to use a somewhat different stimulus electrode placement than has been generally

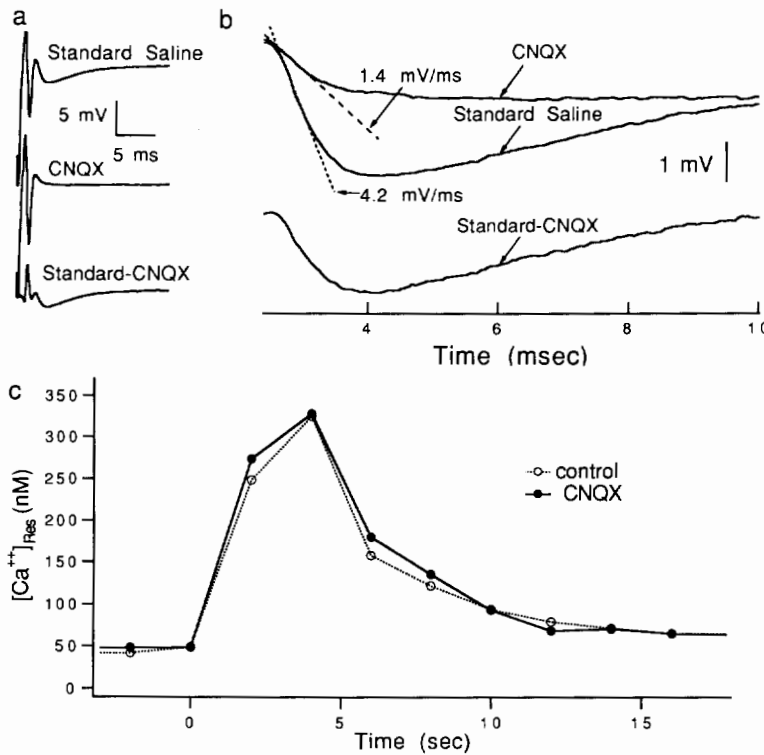


Figure 2. Effects of CNQX on Evoked Synaptic Currents and Presynaptic Calcium Accumulations at Mossy Fiber Terminals (a) Effect of CNQX on field PSPs measured in response to 0.2-Hz mossy fiber stimulation. (b) Expanded view of the field potential recordings shown in (a), with the lines used to determine slope shown. (c) Calcium accumulations in an individual presynaptic terminal in response to 10-Hz, 4-s stimulation in standard ACSF and in the presence of ACSF containing CNQX.

used in previous studies of mossy fiber terminals. Rather than placing a large stimulus electrode in the cell body layer of the dentate gyrus, we placed a small stimulus electrode in the fluorescent fura-2-labeled mossy fiber tract 500–1000 μm from the terminals to be imaged. The fluorescence of mossy fibers was also used to position an extracellular field potential recording electrode near the stimulated terminals. Under these conditions of electrode placement, field potential recordings consisted of large presynaptic volleys that peaked 1.0–1.5 ms after stimulation, followed by the field PSP (Figure 2a). The PSP slope was measured approximately 2.5–3.0 ms after the stimulus pulse, as shown in Figure 2b. We sought to minimize the contribution of disynaptic connections by placing the recording electrode very near the stimulus electrode and measuring the initial PSP slope. If the recording electrode was moved from the mossy fiber tract, the presynaptic volley amplitude dropped off rapidly with distance, as did the field PSP (data not shown). To minimize disynaptic and non-mossy fiber synaptic currents further, the saline contained high divalent ion concentration along with 50–100 μM DL-AP5 (DL-2-amino-5-phospho-pentanoic acid) and 50 μM picrotoxin. Elevated divalents increased the postsynaptic cell threshold. The presence of the γ -aminobutyric acid type A (GABA_A) antagonist picrotoxin eliminated the possibility that PSP changes were due to a decrease in GABA_A inhibition. The presence of DL-AP5 eliminated the possibility of potentiation of associational responses contributing to potentiation.

In the presence of the AMPA antagonist CNQX

(6-cyano-7-nitroquinoxaline-2,3-dione; RBI 20 μM), the presynaptic volley remained, but the PSP was blocked, as shown in Figures 2a and 2b. Our slope measurement was made up of a component produced by the synaptic current field PSP and a CNQX-insensitive component produced by the afferent volley current that contributed a small constant to all measurements.

Calcium concentration measurements were made on individual mossy fiber presynaptic terminals using the ratio of fura-2 fluorescence produced by 340 nm and 380 nm excitation (Grynkiewicz et al., 1985). As shown in Figure 2c, the calcium concentration computed from intensity values located within an individual terminal showed a transient 300 nM increase in response to 10-Hz, 4-s stimulation of the mossy fiber pathway. The increase subsequently decayed to the prestimulus level. The presence of 10 μM CNQX did not alter this calcium accumulation (Figure 2c) while blocking most of the postsynaptic current (Figure 2b), directly confirming that the fura-2 label is located in the presynaptic terminal.

Images such as that shown in Figure 1b illustrate that the quantity of light at an image location corresponding to a presynaptic terminal contains a component of out of focus fluorescence produced by terminal and non-terminal mossy fiber regions. If our measurements predominantly represent the change in [Ca²⁺]_{Res} in individual terminals, then as the stimulus current intensity is gradually increased, individual terminals should abruptly show a calcium response when the threshold for the individual fiber is reached.

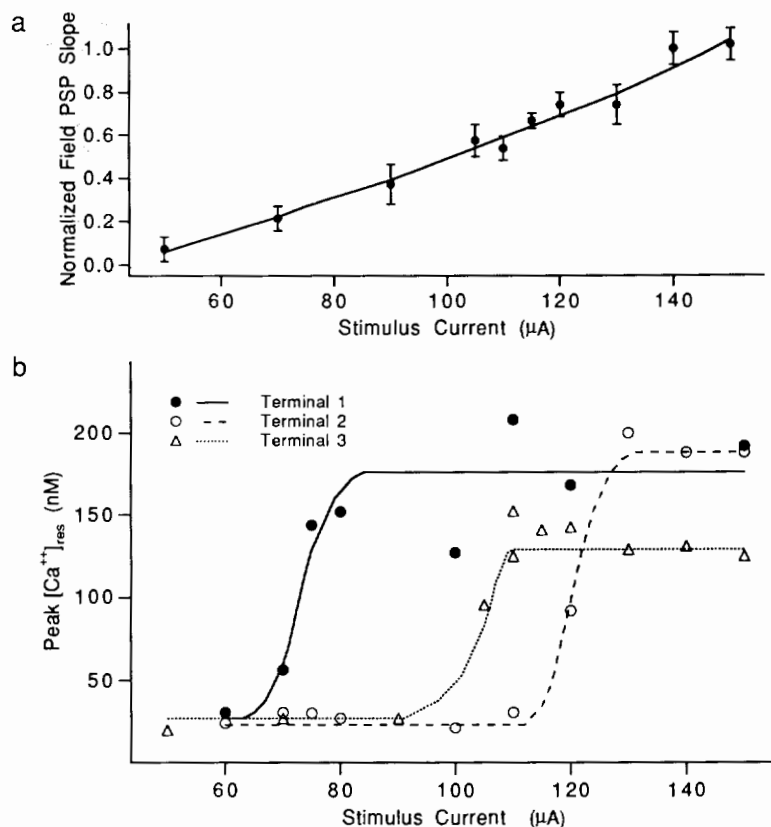


Figure 3. Individual Mossy Fiber Terminals Show Calcium Accumulations at Different Levels of Stimulus Current

(a) Field PSP slope versus stimulus intensity measured in response to a series of 2-Hz, 20-s stimulus trains. (b) Calcium accumulations in three individual mossy fiber terminals versus stimulus intensity for the same trains as in (a).

In contrast, the field potential, an average over many synapses, will change amplitude gradually. To test this and establish that we were recording from individual terminals, we stimulated with trains of increasing stimulus current and recorded the field potential while simultaneously measuring the $[Ca^{2+}]_{res}$ in several individual terminals located in the same field of view, but located along different mossy fibers. A representative experiment is shown in Figure 3. The field potential response increased in a graded manner (Figure 3a), whereas the peak $[Ca^{2+}]_{res}$ measured in three terminals increased abruptly (Figure 3b). Such abrupt increases were observed in 10 of 10 terminals in 6 different hippocampal brain slices. Occasionally, large variations in the amplitude of accumulations were observed around threshold, similar to that shown for terminal 1 in Figure 3a in response to stimuli of 100 µA, 110 µA, and 120 µA.

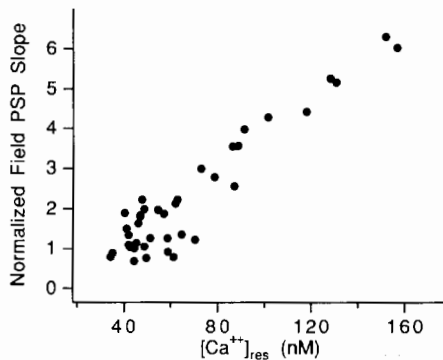
The Sensitivity of Synaptic Enhancement to Intraterminal Calcium Concentration

As previously described at invertebrate synapses (Delaney and Tank, 1991; Delaney et al., 1989), the increases in $[Ca^{2+}]_{res}$ produced by mossy fiber stimulation that did not induce LTP, such as that shown in Figure 2c, were accompanied by increases in the strength of synaptic connections, as measured by the slope of the field PSP. There is strong experimental evidence in the invertebrate system that the calcium

changes are a causal link in the expression of the synaptic enhancement. We therefore interpreted the calcium changes we observed in the mossy fiber terminals as being responsible for the observed synaptic enhancement, and we experimentally determined the sensitivity of the relationship.

A brief, high frequency stimulation of mossy fibers in low calcium saline does not produce LTP, but does produce a transient enhancement of transmission and a concomitant transient rise in $[Ca^{2+}]_{res}$. The transient synaptic enhancement has been previously studied for mossy fiber terminals (Griffith, 1990) and is characterized by decay components that may be equivalent to augmentation and posttetanic potentiation, two forms of enhancement that have been well documented at the frog neuromuscular junction (Magelby, 1987). By simultaneously measuring both $[Ca^{2+}]_{res}$ and the normalized field PSP slope at various times during the decay, a graph of synaptic enhancement versus $[Ca^{2+}]_{res}$ was generated (Figure 4). The relationship between the enhancement of field PSP slope and $[Ca^{2+}]_{res}$ was well fitted by a linear function, qualitatively similar to what is observed at the crayfish neuromuscular junction (Delaney and Tank, 1991). Figure 4 demonstrates a tremendous sensitivity of release to $[Ca^{2+}]_{res}$, with an increase of 17 ± 4 nM ($n = 7$ slices; range 8 to 35 nM), corresponding to a 2-fold enhancement of release.

The observed relationship between PSP enhance-



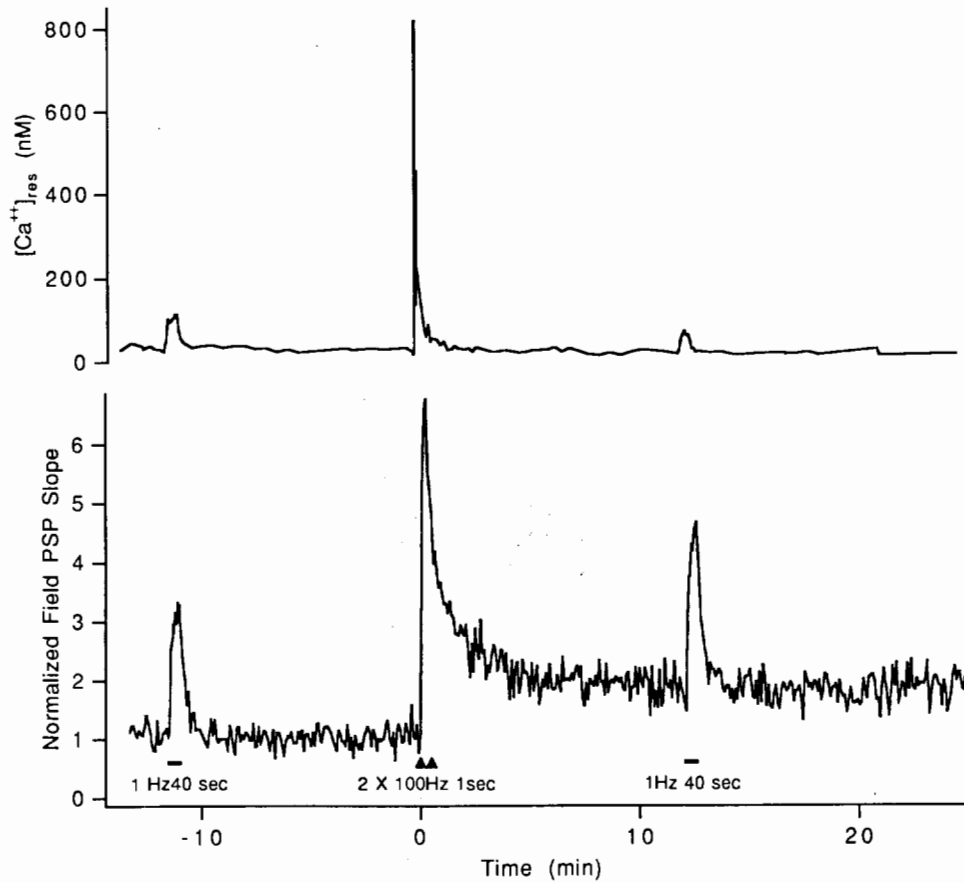
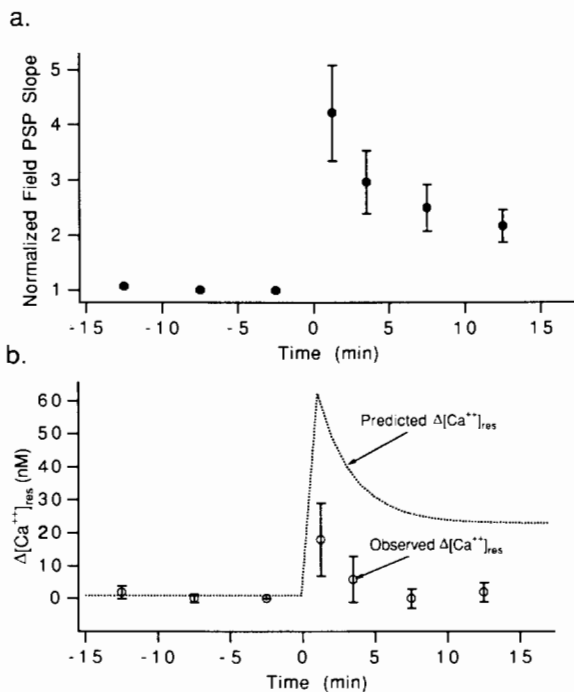


Figure 5. Residual Calcium Levels and Field PSP Amplitude versus Time for a Representative Mossy Fiber LTP Experiment



here. First, there is no washout or alteration of calcium currents, a phenomenon commonly observed with patch-electrode internal dialysis. Second, internal dialysis may also alter the intrinsic calcium buffer composition, since freely mobile calcium-binding proteins can also be washed out. Finally, loading of the terminal by applying the membrane-permeant derivative fura-2 AM at a site distant from the synapse eliminates the washout of important subcellular biochemistry and endogenous buffers while still providing an optimized fluorescence signal-to-noise ratio.

Figure 6. Summary of Observed Field Potentials and Observed and Expected Presynaptic Calcium Levels for 7 Experiments Similar to That Shown in Figure 5

For each experiment field PSPs (a) and $\Delta[Ca^{2+}]_{res}$ (b) (measured relative to the -5 to 0 min time interval) were averaged over the following time intervals (in min): -15 to -10, -10 to -5, -5 to 0, 0.5 to 2, 2 to 5, 5 to 10, and 10-15, with the LTP-inducing stimulus train occurring at $t = 0$. For each time interval, the average of 7 experiments and the SEM are plotted against the median time of the interval. The calcium that would have been necessary to produce the observed enhancement if $[Ca^{2+}]_{res}$ -induced synaptic enhancement were responsible is also plotted (dotted line) in (b).

One of the principle results of this work is to show the extreme sensitivity of short-term synaptic enhancement to the level of $[\text{Ca}^{2+}]_{\text{Res}}$. The data in Figure 4 demonstrate that even a change in $[\text{Ca}^{2+}]_{\text{Res}}$ of 50 nM correlates with a severalfold enhancement of release. This is particularly important when one considers the dependence of calcium accumulation on frequency that is observed at this synapse. When the background firing rate of a granule cell is only 0.33 Hz, presynaptic calcium levels are increased approximately 20 nM (data not shown) and synaptic efficacy can be increased 2-fold. For short, high frequency bursts, there can be large calcium increases and concomitant large synaptic enhancements.

The approximately linear relationship between presynaptic calcium levels and synaptic enhancement shown in Figure 4 is also observed at the crayfish neuromuscular junction (Delaney and Tank, 1991; Delaney et al., 1989). At the crayfish neuromuscular junction, recent experiments strongly suggest that this observed relationship is not just a correlation, but rather that the calcium accumulation is a causal link in the expression of enhancement. In that system, linear dependence is observed up to large levels of enhancement. Compared with the $n = 3-4$ power law relationship suggested for the dependence of transmitter release on transient calcium current (for a review see Augustine et al., 1987), the linear dependence of synaptic enhancement on residual calcium argues that the calcium does not simply add to the transient influx and act at the same low affinity calcium-binding sites associated with the release site. Rather, it argues that the residual calcium acts at a higher affinity site, either associated with the release site or at an independent facilitatory site, for example, a system of calcium-dependent phosphorylation reactions that change the availability of synaptic vesicles for release. In the mossy fiber experiments we report here, a linear relationship is observed for enhancement that decays after a short, high frequency tetanus (Figure 4) and also during the very different conditions of low frequency trains (data not shown; Regehr et al., unpublished data). This suggests that, as at the crayfish neuromuscular junction, calcium accumulation in mossy fiber terminals is a causal link in the expression of some forms of short-term enhancement.

The high calcium sensitivity observed at mossy fiber terminals suggests that increases in $[\text{Ca}^{2+}]_{\text{Res}}$ could have been, in principle, a reasonable way for the maintenance of the 2-fold change in synaptic strength that is typical of the LTP observed in this system. The modest increase of 10–30 nM (Figure 6b) that would be necessary could be produced by any of a large number of biochemical alterations, including a change in calcium buffers or calcium pumps. Our basic result, however, is that no such sustained increase in residual calcium was observed within our experimental error. Increases in $[\text{Ca}^{2+}]_{\text{Res}}$ do not account for mossy fiber LTP. Since our experimental error is approximately 5 nM in the experiments summarized in Figure 6, it is

still possible that a sustained increase in presynaptic residual calcium could account for a small percentage of the synaptic enhancement. We estimate that for the 3–5 min period following tetanus, increases in $[\text{Ca}^{2+}]_{\text{Res}}$ could account for less than 30%, whereas for the 10–15 min period increases in $[\text{Ca}^{2+}]_{\text{Res}}$ could account for less than 25% of the observed synaptic enhancement.

The finding that mossy fiber LTP is independent of presynaptic calcium is not compromised by stimulus artifacts contributing to the PSP slope, nor is it dependent upon precise fura-2 calibrations.

Although field PSP slopes were measured in the presence of a constant stimulus artifact (Figure 2b) that was typically 20%–35% of the nonfacilitated PSP slope, this offset was present in both calibration curves and LTP measurements and thus does not alter our conclusions. Similarly, while we have converted all of our fura-2 ratio measurements to calcium concentrations, our basic conclusions are not altered by any potential inaccuracies in absolute calcium concentration values produced by using *in vitro* calibration procedures. Our quantitative estimates of the sensitivity of enhancement to presynaptic calcium levels (Figure 4) are, however, affected by the contributions of stimulus artifact to field potential measurements and uncertainties in fura-2 calibrations. Based on experiments in which CNQX was used to determine the nonsynaptic contribution to the PSP slope, we estimate that errors produced by the stimulus artifact in field PSP slope enhancement contribute less than 30% to the sensitivity measurements.

While our experiments demonstrate that sustained presynaptic residual calcium is not the mechanism for the maintenance of mossy fiber LTP, these data do not eliminate the possibility of other forms of calcium-dependent presynaptic enhancement at this synapse. Of particular interest is the possibility that an increase in presynaptic calcium current might account for the maintenance of mossy fiber LTP. The data presented here do not eliminate this possibility. Experiments addressing this question are made difficult by the highly nonlinear relationship expected between calcium current and transmitter release. For example, if transmitter release is dependent on the 4th power of the calcium current, then a calcium current increase of only 20% would produce a 2-fold enhancement of release. While, conceptually, changes in residual calcium accumulation that would be produced by such increased calcium currents could be used to determine the calcium influx indirectly (see, for example, Delaney et al., 1991), the experimental methods used in the present study did not provide sufficient reproducibility of calcium transients to resolve a 20% difference in calcium currents produced by a 1-Hz test train. However, we anticipate that further technical refinements and averaging will provide the stability necessary to use calcium accumulation as an indirect measure of changes in calcium influx.

Although calcium influx and $[\text{Ca}^{2+}]_{\text{Res}}$ control the neurosecretory process, there is increasing experi-

mental evidence that some forms of synaptic plasticity involve direct modifications of the biochemical apparatus which releases synaptic vesicles (Man-Son-Hing et al., 1989). Such Ca^{2+} -independent mechanisms may be responsible for the synaptic enhancement observed during mossy fiber LTP. For example, at both crayfish neuromuscular junctions (Delaney et al., 1991) and Aplysia sensory neuron terminals (Dale and Kandel, 1990), serotonin enhances the probability of release without changing $[\text{Ca}^{2+}]_{\text{Res}}$. For the crayfish, using calcium accumulation produced by short trains as an indirect measure of calcium influx suggested that the synaptic enhancement produced by serotonin also is not associated with an increase in presynaptic calcium current. These results directly demonstrate that the neurosecretory process can undergo multiple, independent forms of calcium-dependent and calcium-independent enhancement.

We have used the method of localized indicator loading to examine directly the role of $[\text{Ca}^{2+}]_{\text{Res}}$ in mossy fiber LTP. We have found that increases in $[\text{Ca}^{2+}]_{\text{Res}}$ are not responsible for the maintenance of the sustained synaptic enhancement. In the future, the ability to measure optically both calcium transients and chemical concentrations of other ions and biomolecules in individual mossy fiber presynaptic terminals contained intact within brain slice should contribute to a greater understanding of the processes responsible for the synaptic enhancement. We have already demonstrated that the methods we have developed and used here can be applied to the parallel fiber presynaptic terminals in the cerebellum (Regehr and Tank, 1991). It is likely that they are applicable to many other synaptic systems in brain slice or intact nervous systems.

Experimental Procedures

Brain Slice Preparation and Electrophysiology

Slice preparation and maintenance followed standard procedures (Regehr et al., 1989; Schwartzkroin, 1975). The artificial cerebral spinal fluid (ACSF) contained 124 mM NaCl, 3 mM KCl, 4 mM CaCl_2 , 4 mM MgCl_2 , 26 mM NaHCO_3 , 10 mM D-glucose, 50–100 μM DL-AP5 (Sigma), and 50 μM picrotoxin (Sigma). This ionic composition, with elevated divalents, increased action potential threshold so that field PSPs could be measured without contamination from a population spike. The presence of the GABA_A antagonist picrotoxin eliminated the possibility that PSP changes were due to a decrease in GABA_A inhibition, and the presence of DL-AP5 eliminated the possibility of potentiation of associational responses contributing to potentiation. In some experiments ACSF containing lower calcium concentration (2.5 mM CaCl_2) was used.

Stimulus electrodes were placed on the mossy fiber tract, either directly at the site of localized perfusion or displaced slightly toward the terminals to be imaged. They consisted of two parallel, laquer-coated tungsten electrodes (FHC #25-05-3), each cut across its axis to expose an approximately 40- μm diameter of metal. The pair was positioned with one wire on the mossy fiber tract and one wire situated just above the slice. Extracellular field potentials were recorded with glass microelectrodes filled with 2 M NaCl. Electrode impedance was 3–6 M Ω . Guided by the mossy fiber fluorescence, a recording electrode was positioned near the imaged terminals, 500–1000 μm from the stimulus electrodes. Field potential recordings were amplified (Neuro Data,

model IR-283 intracellular recording amplifier), filtered (Princeton Applied Research, model 113, preamp 0.03–3000 Hz), viewed on an oscilloscope (Tektronix 5223), and stored on tape (Neuro Data, Neurocorder Model DR-484). Recordings were subsequently digitized, stored, and analyzed on a microcomputer. The field PSP slope (see text) and presynaptic volley amplitude were determined in software for each trace. No detectable change in the presynaptic volley amplitude occurred during the course of an experiment. Test pulses were 0.2 Hz (A.M.P.I. Master 8 stimulator) except as noted in the text, and mossy fiber LTP was induced with either 1 or 2 100-Hz trains containing 100 pulses.

Microfluorometry of Calcium Ion Concentration

Mossy fiber terminals were labeled using a localized perfusion method described in detail elsewhere (Regehr and Tank, 1991). In brief, slices were bathed in a submersion chamber, maintained at 30°C–32°C, and perfused (1 ml/min) with ACSF equilibrated with 95% O_2 and 5% CO_2 . Small areas of the slice were locally perfused by a delivery pipette containing labeling solution and a nearby suction pipette. The labeling solution was prepared as follows: 50 μg of fura-2 AM (Molecular Probes, Eugene OR) was dissolved in a solution of 20 μl 75% DMSO, 25% pluronic acid. The mixture was added to 500 μl of ACSF, vortexed for 1 min, and filtered (0.2 μm Rainin #38–159 mm). The final solution contained 4% DMSO, pluronic acid, and 20 μM fura-2 AM. Labeling solution was prepared each day and used within 4 hr.

Perfusion was performed on the top surface of the slice. For imaging experiments the slices were then turned over and viewed through an inverted epifluorescence microscope (Zeiss IM35). Fura-2 measurements of intracellular calcium concentration were performed using the ratio method (Grynkiewicz et al., 1985) on images acquired with a computer-controlled (MacII fx), cooled CCD camera (CH220, Photometrics, Tucson, AZ) as described previously (Regehr et al., 1989). Image pairs were formed from the fluorescence ratio at 340 and 380 nm, with an exposure time of 360 ms for each wavelength and 140 ms between exposures. In some cases fiber tracts ran obliquely through the slice and labeled presynaptic terminals following perfusion were best observed on the slice surface opposite to that used for local perfusion.

Computer simulation of calcium dynamics (Sala and Hernández-Cruz, 1990) suggests that calcium gradients produced by transient membrane calcium currents will dissipate within 1 s for a 5- μm diameter spherical structure similar in size to a mossy fiber terminal. Thus, except during periods of tetanic stimulation and shortly thereafter, it is unlikely that our measurements of intracellular calcium concentration are corrupted by spatial gradients and accurately reflect the level of residual intracellular free calcium that influences cytosolic biochemistry underlying the enhancement of transmitter release. During the low frequency stimulus test trains (0.2 Hz) used during LTP experiments, fura-2 fluorescence measurements were made in the 1-s time period immediately before a test pulse, 4–5 s after the preceding test pulse. Spatial gradients are thus also not expected to be important in these measurements.

Acknowledgments

We thank Kerry Delaney for many helpful discussions and Alan Gelperin and Alan Kay for careful review of the manuscript.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received April 29, 1991.

References

- Amaral, D. G. (1979). Synaptic extensions from mossy fibers of the fascia dentata. *Anat. Embryol.* 155, 241–251.
- Augustine, G. J., Charlton, M. P., and Smith, S. J. (1987). Calcium

- action in synaptic transmitter release. *Annu. Rev. Neurosci.* 10, 633-693.
- Barrionuevo, G., Kelso, S. R., Johnston, D., and Brown, T. H. (1986). Conductance mechanism responsible for long-term potentiation in monosynaptic and isolated excitatory synaptic inputs to hippocampus. *J. Neurophysiol.* 55, 540-550.
- Bekkers, J. M., and Stevens, C. F. (1990). Presynaptic mechanism for long-term potentiation in the hippocampus. *Nature* 346, 724-729.
- Bliss, T. V. P., and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* 232, 331-356.
- Brown, T. H., and Zador, A. M. (1990). *Hippocampus. The Synaptic Organization of the Brain* (New York: Oxford University Press).
- Claiborne, B. J., Amaral, D. G., and Cowan, W. M. (1986). A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus. *J. Comp. Neurol.* 246, 435-458.
- Collingridge, G. L., Kehl, S. J., and McLennan, H. (1983). The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurons in vitro. *J. Physiol.* 334, 19-31.
- Connor, J. A., Kretz, R., and Shapiro, E. (1986). Calcium levels measured in presynaptic neurone of *Aplysia* under conditions that modulate transmitter release. *J. Physiol.* 375, 625-642.
- Dale, N., and Kandel, E. R. (1990). Facilitatory and inhibitory transmitters modulate spontaneous transmitter release at cultured *Aplysia* sensorimotor synapses. *J. Physiol.* 421, 203-222.
- Delaney, K. R., and Tank, D. W. (1991). Calcium-dependent and calcium-independent enhancement of transmitter release at the crayfish neuromuscular junction studied with fura-2 imaging. *Ann. NY Acad. Sci.*, in press.
- Delaney, K. R., Zucker, R. S., and Tank, D. W. (1989). Calcium in motor nerve terminals associated with posttetanic potentiation. *J. Neurosci.* 9, 3558-3567.
- Delaney, K. R., Tank, D. W., and Zucker, R. S. (1991). Presynaptic calcium and serotonin-mediated enhancement of transmitter at crayfish neuromuscular junction. *J. Neurosci.*, in press.
- Griffith, W. H. (1990). Voltage-clamp analysis of posttetanic potentiation of the mossy fiber to CA3 synapse in hippocampus. *J. Neurophysiol.* 63, 491-501.
- Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985). A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440-3450.
- Harris, E. W., and Cotman, C. W. (1986). Long-term potentiation of guinea pig mossy fiber responses is not blocked by N-methyl-D-aspartate antagonists. *Neurosci. Lett.* 70, 132-137.
- Jackson, M. B., Konnerth, A., and Augustine, G. J. (1991). Action potential broadening and frequency-dependent facilitation of calcium signals in pituitary nerve terminals. *Proc. Natl. Acad. Sci. USA* 88, 380-384.
- Jaffe, D., and Johnston, D. (1990). Induction of long-term potentiation at hippocampal mossy fiber synapses follows a hebbian rule. *J. Neurophysiol.* 64, 948-960.
- Kauer, J. A., Malenka, R. C., and Nicoll, R. A. (1988). A persistent postsynaptic modification mediates long-term potentiation in the hippocampus. *Neuron* 1, 911-917.
- Lynch, G., Larson, J., Kelso, S., Barrionuevo, G., and Schottler, F. (1983). Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature* 305, 719-721.
- Magelby, K. L. (1987). *Short-Term Changes in Synaptic Efficacy. Synaptic Function* (New York: John Wiley and Sons).
- Malinow, R., and Tsien, R. W. (1990). Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* 346, 177-180.
- Man-Son-Hing, H., Zoran, M. J., Lukowiak, K., and Haydon, P. G. (1989). A neuromodulator of synaptic transmission acts on the secretory apparatus as well as on ion channels. *Nature* 341, 237-239.
- Miles, R., and Wong, R. K. S. (1987). Latent synaptic pathways revealed after tetanic stimulation in the hippocampus. *Nature* 329, 724-726.
- Muller, D., Joly, M., and Lynch, G. (1988). Contributions of quisqualate and NMDA receptors to the induction and expression of LTP. *Science* 242, 1694-1697.
- Nicoll, R. A., Kauer, J. A., and Malenka, R. C. (1988). The current excitement in long-term potentiation. *Neuron* 1, 97-103.
- Regehr, W. G., and Tank, D. W. (1991). Selective fura-2 loading of presynaptic terminals and nerve cell processes by local perfusion in mammalian brain slice. *J. Neurosci. Meth.* 37, 111-119.
- Regehr, W. G., Connor, J. A., and Tank, D. W. (1989). Optical imaging of calcium accumulation in hippocampal pyramidal cells during synaptic activation. *Nature* 341, 533-536.
- Sala, F., and Hernández-Cruz, A. (1990). Calcium diffusion modeling in a spherical neuron. *Biophys. J.* 57, 313-324.
- Schwartzkroin, P. A. (1975). Characteristics of CA1 neuron recorded intracellularly in the hippocampal in vitro slice preparation. *Brain Res.* 85, 423-436.
- Williams, S., and Johnston, D. (1989). Long-term potentiation of hippocampal mossy fiber synapses is blocked by postsynaptic injection of calcium chelators. *Neuron* 3, 583-588.
- Zalutsky, R. A., and Nicoll, R. A. (1990). Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science* 248, 1619-1624.