

Calcium Signaling at Single Mossy Fiber Presynaptic Terminals in the Rat Hippocampus

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Liang, Yong, Li-Lian Yuan, Daniel Johnston, and Richard Gray. Calcium signaling at single mossy fiber presynaptic terminals in the rat hippocampus. *J Neurophysiol* 87: 1132–1137, 2002; 10.1152/jn.00661.2001. We investigated internal Ca^{2+} release at mossy fiber synapses on CA3 pyramidal neurons (mossy fiber terminals, MFTs) in the hippocampus. Presynaptic Ca^{2+} influx was induced by giving a brief train of 20 stimuli at 100 Hz to the mossy fiber pathway. Using Ca^{2+} imaging techniques, we recorded the Ca^{2+} response as $\Delta F/F$, which increased rapidly with stimulation, but was often accompanied by a delayed peak that occurred after the train. The rise in presynaptic $[\text{Ca}^{2+}]_i$ could be completely blocked by application of 400 μM Cd^{2+} . Furthermore, the evoked Ca^{2+} signals were reduced by group II mGluR agonists. Under the same experimental conditions, we investigated the effects of several agents on MFTs that disrupt regulation of intracellular Ca^{2+} stores resulting in depletion of internal Ca^{2+} . We found that ryanodine, cyclopiazonic acid, thapsigargin, and ruthenium red all decreased both the early and the delayed increase in the Ca^{2+} signals. We applied D,L-2-amino-5-phosphonovaleric acid (D,L-APV; 50 μM) and 6,7-Dinitroquinoxaline-2,3-dione (DNQX; 20 μM) to exclude the action of *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors. Experiments with alternative lower affinity indicators for Ca^{2+} (fura-2FF and calcium green-2) and the transient K^+ channel blocker, 4-aminopyridine were performed to control for the possible saturation of fura-2. Taken together, these results strongly support the hypothesis that the recorded terminals were from the mossy fibers of the dentate gyrus and suggest that a portion of the presynaptic Ca^{2+} signal in response to brief trains of stimuli is due to release of Ca^{2+} from internal stores.

INTRODUCTION

The mossy fiber synapse has a number of unusual features, including large-sized terminals (3–8 μm), multiple release sites (up to 37), and a proximal termination zone along the apical dendrites of CA3 pyramidal neurons (Chicurel and Harris 1992; Claiborne et al. 1986). The large size and well-defined terminal zone for mossy fiber synapses make them ideal for studying presynaptic Ca^{2+} responses. The initial characterization of Ca^{2+} signals at single mossy fiber terminals was accomplished by Regehr and Tank through localized perfusion of the AM ester form of the fluorescent calcium indicator fura-2 to the mossy fiber tracts (Regehr and Tank 1991b). This method for selective labeling and measuring changes in $[\text{Ca}^{2+}]_i$ of presynaptic terminals has now been applied to many other synapses in hippocampus and cerebellum (Regehr and

Atluri 1995; Regehr and Tank 1991a,b; Sabatini and Regehr 1998; Wu and Saggau 1994). Recently, direct electrical recordings have also been made from single presynaptic mossy fiber boutons (Geiger and Jonas 2000).

It is known that both endoplasmic reticulum (ER) and mitochondria are widely distributed within neurons, being present in dendrites and dendritic spines, axons and presynaptic nerve terminals, and in growth cones (Cheng and Reese 1985; Chicurel and Harris 1992; Dailey and Bridgman 1989; Deitch and Banker 1993; Kanaseki et al. 1998; Levesque et al. 1999), and Ca^{2+} pumps and internal ligand-gated Ca^{2+} channels, including ryanodine receptors (RyRs) and inositol (1,4,5)-trisphosphate receptors (IP_3Rs), participate in Ca^{2+} signaling under certain conditions (Berridge 1998). The spatiotemporal distribution of intracellular free Ca^{2+} levels is critically involved in higher brain activities including learning and memory (Alkon et al. 1998; Bliss and Collingridge 1993; Finkbeiner and Greenberg 1997; Teyler et al. 1994). During neuronal activation, elevation of cytoplasmic $[\text{Ca}^{2+}]_i$ is initially generated by Ca^{2+} influx through voltage- or ligand-gated Ca^{2+} channels. Subsequently, activation of IP_3Rs and RyRs on the endoplasmic reticulum membrane leads to Ca^{2+} release from intracellular stores (calcium-induced calcium release, CICR), which further amplifies and/or prolongs Ca^{2+} signals in specific subcellular compartments (Pozzan et al. 1994; Sorrentino and Volpe 1993; Sutko and Airey 1996). Electron microscope studies have provided suggestive but limited evidence showing the presence of mitochondria and ER in mossy fiber terminals (Chicurel and Harris 1992). Immunohistochemical staining experiments have shown clear differences in the localization of IP_3R and RyR. In the hippocampus, IP_3R is most concentrated in pyramidal cells of CA1. By contrast, RyR staining is much greater in area CA3 than CA1 and is particularly prominent in the granule cell layer of the dentate gyrus (Sharp et al. 1993). Further study suggested that of the three isoforms of RyR (RyR1, RyR2, and RyR3), RyR2 mRNA was shown to be widely expressed in the rat CNS at high concentrations, and high levels of RyR2 mRNA signal were detected in the dentate gyrus and area CA3 (Zhao et al. 2000).

Little is known about the possible contribution of internal calcium release to the rise in presynaptic calcium at the mossy fiber synapse. There have been, however, previous studies in area CA1 suggesting that intracellular calcium release may

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have an effect on synaptic transmission. Depleting the stores or blocking CICR reduces the transients and also reduces a form of short-term synaptic plasticity, paired-pulse facilitation of excitatory postsynaptic potentials (EPSPs) (Emptage et al. 2001). Thapsigargin, which depletes intracellular calcium stores, has been shown to block long-term potentiation elicited by weak stimuli, but not that induced with strong stimuli suggesting that calcium release may, under some conditions, play a role in induction of synaptic plasticity (Behnisch and Reymann 1995). Anoxia causes an increase in the frequency of spontaneous miniature excitatory postsynaptic currents measured in CA1 neurons; this increased frequency of release could be blocked by agents that decrease the release of intracellular calcium (Katchman and Hershkowitz 1993). Reyes and Stanton found, also in area CA1, that presynaptic blockade of ryanodine receptors, or postsynaptic blockade of inositol triphosphate, triggered release of internal calcium could block the induction of long-term depression (Reyes and Stanton 1996).

In the present study, we applied optical recording techniques to characterize the presynaptic Ca^{2+} signal at single mossy fiber terminals in rat hippocampal slices by loading synapses with fura-2AM or other permeant indicators. We found that in response to a brief train of 20 stimuli at 100 Hz, presynaptic $[Ca^{2+}]$ increased and decayed rapidly, and the decay phase of $[Ca^{2+}]$ was often accompanied by a delayed peak. The existence of the second peak was variable, often apparent

early in an individual recording, and then fading with time. In other experiments, the second peak was not apparent unless the number of stimuli in the train was increased (although we report here only results with a 20-stimuli train). This indicated to us that this component of the Ca^{2+} signal was under some level of physiological control and worthy of further study. Our data suggest that both the initial and the secondary peaks of Ca^{2+} reflect contributions of Ca^{2+} release from internal stores and that Ca^{2+} release from this ryanodine-sensitive store contributes significantly to the rise in preterminal $[Ca^{2+}]$.

METHODS

Preparation of hippocampal slices

Hippocampal slices were prepared from Sprague-Dawley rats (5–7 wk) using standard procedures (Magee and Johnston 1995). All experimental procedures were approved by the Animal Research Committee of Baylor College of Medicine. An anesthetic consisting of a mixture of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml), and acepromazine (1.4 mg/ml) was injected intraperitoneally, and rats were perfused with a cold ($\sim 2^{\circ}C$) oxygenated cutting solution described below. Slices were cut on a Vibratome at a thickness of 350 μm , incubated in a holding chamber heated to $35^{\circ}C$ for 20 min, and then stored at room temperature ($\sim 22^{\circ}C$). The holding chamber was continuously bubbled with 95% O_2 -5% CO_2 and contained the bathing solution described below.

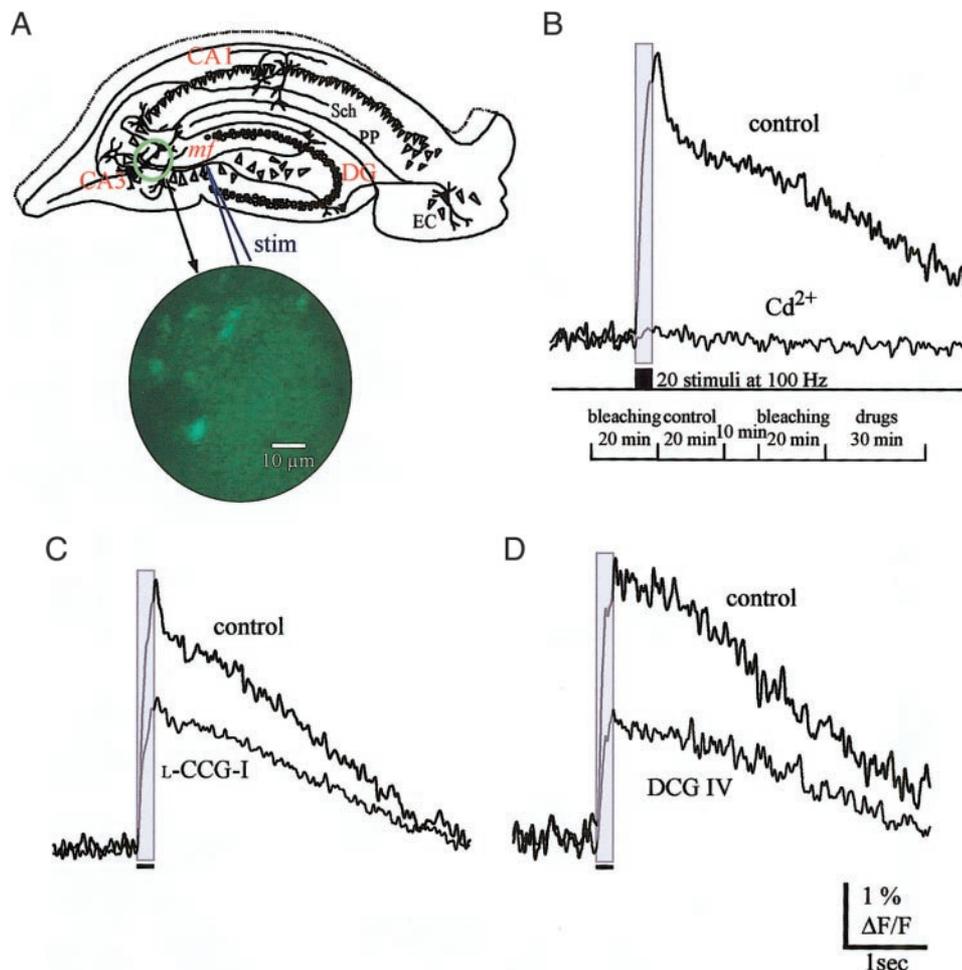


FIG. 1. Detection of Ca^{2+} signals in single mossy fiber terminals. *A*: schematic of hippocampal slice showing stimulating (stim) and recording site (green circle). *Bottom image* shows several mossy fiber terminals (MFTs) loaded with fura-2AM (bright green spots). *B*: presynaptic Ca^{2+} influx induced through voltage-dependent Ca^{2+} channels by stimulating the mossy fibers can be completely blocked by $400 \mu M Cd^{2+}$. The *bottom traces* show frequency of stimuli and time course of a typical recording procedure. First, bleaching traces (no stimulation and normal saline) were recorded for 20 min (10 traces, 5-s trace acquired every 2 min), then responses from 20 stimuli for 20 min (in normal saline) were recorded (10 traces), followed by bath application of the drug. After waiting 10 min, a new sequence of bleaching traces (10 traces) were collected, then the Ca^{2+} responses were measured in the presence of the drug for 30 min (15 traces). *C* and *D*: effects of bath-applied group II mGluR agonist L-CCG-I ($10 \mu M$) or DCG IV ($10 \mu M$) to MFTs after blocking presynaptic $GABA_B$ receptors with $1 \mu M CGP 55845$. Both L-CCG-I and DCG IV decreased presynaptic calcium responses significantly.

Drugs and solution

The cutting solution contained (in mM) 110 choline chloride, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 1.3 ascorbate, 3 pyruvate, and 7 dextrose, bubbled with 95% O₂-5% CO₂ during whole cutting process. The bathing solution contained (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 2 MgCl₂, and 10 dextrose, bubbled with 95% O₂-5% CO₂ continuously. Where specified, one of the following drugs was included in the media: L-CCG-I (Tocris) or D,L-2-amino-5-phosphonovaleric acid (D,L-APV; Tocris) were prepared from a stock solution dissolved in 1 molar equivalent (1 eq.) NaOH; ryanodine (Sigma), cyclopiazonic acid (Sigma), thapsigargin (Alomone labs), 6,7-Dinitroquinoxaline-2,3-dione (DNQX; Sigma), or CGP 55845 (Tocris), prepared from a stock solution dissolved in 20, 30, 10, 20, or 10 mM DMSO, respectively; DCG IV (Tocris) or ruthenium red (Sigma), prepared from a concentrated stock solution in water. The glass stimulating electrode solution contained (in mM) 149 NaCl, 5 KCl, 10 HEPES, 2 CaCl₂, 1 MgCl₂, and 10 dextrose (pH 7.3).

Fluorescence imaging

In our experiments, the loading of mossy fiber terminals in hippocampal slices with cell-permeant fura-2AM took advantage of two previous findings (Gray et al. 1996; Regehr and Tank 1991b). First, CA3 neurons in the hippocampal slice do not load well with fura-2AM; second, presynaptic terminals tend to load well with fluorophores. We were therefore able to identify and locate mossy fiber terminals (MFTs) using fluorescence illumination.

For loading of slices with dye, 50 μg fura-2 acetoxyethyl ester (fura-2AM) (Molecular Probes) was added to 40 μl of 20% Pluronic F-127 (Molecular Probes) in DMSO (Sigma) to make 1.25 mM stock solution as described (Gray et al. 1996; Saggau et al. 1999). Individual slices were removed from the holding chamber and placed in a 35-mm Petri dish containing artificial cerebrospinal fluid (ACSF) and 8.3 μM cell-permeant fura-2AM for 10–15 min at 30°C. After loading, individual slices were then rinsed gently at least 10 times in ACSF and transferred to a chamber on the stage of the microscope. The recording chamber was continuously perfused with bathing solution at 30–32°C. A Zeiss Axioskop, fitted with a ×40 Zeiss water-immersion objective (N.A. 0.75) and differential interference contrast (DIC) optics, was used to view slices. MFTs were located using fluorescence illumination (380 nm), and single terminals were isolated by closing a diaphragm in the light path to a spot diameter of 10–15 μm (Fig. 1A). Light emission of 510 nm was measured with a photodiode (Hamamatsu S1336-18BK) over the terminal through a 1-μm pinhole to reduce collection of scattered light. Relative changes in [Ca²⁺]_i were quantified as changes in ΔF/F, where F is fluorescence intensity before stimulation (after subtracting autofluorescence), and ΔF is the change from this value during neuronal activity (corrected for bleaching during optical recording). The bleaching correction was determined by measuring fluorescence in the MFT without stimulation. Tissue autofluorescence and background fluorescence were subtracted by measuring fluorescence at a parallel location in the slice that was away from the loaded MFT. The terminal was stimulated with a glass microelectrode (2–4 μm diam) filled with HEPES-buffered internal solution and placed in stratum lucidum approximately 50 μm from the selected terminal (Fig. 1A) by giving a brief train of 20 stimuli at 100 Hz, which was the minimum stimulus to reliably produce a delayed peak in the Ca²⁺ signal.

Statistics

All numerical values are represented as means ± SE, and the number of experiments (n) refers to the number of slices investigated. The differences between the experimental groups were evaluated

using Student's paired *t*-test. In all cases a probability value of <0.05 was considered statistically significant.

RESULTS

The rapid rise in presynaptic [Ca²⁺]_i in response to MF stimulation appeared to result from activation of voltage-dependent Ca²⁺ channels because it was completely blocked by 400 μM Cd²⁺ (Fig. 1B). To test whether the recorded terminals were from the mossy fibers of the dentate gyrus and the evoked calcium signals were from mossy fiber presynaptic terminals, we sequentially co-stained slices with calcium green and the Zn²⁺ fluorescent dye TFLZn. While there was a high background fluorescence with TFLZn, significant co-staining was evident (data not shown). In addition, we applied the group II mGluR agonist L-CCG-I (10 μM) or DCG IV (10 μM) to MFTs after blocking presynaptic GABA_B receptors with 1 μM CGP 55845. Both L-CCG-I and DCG IV decreased presynap-

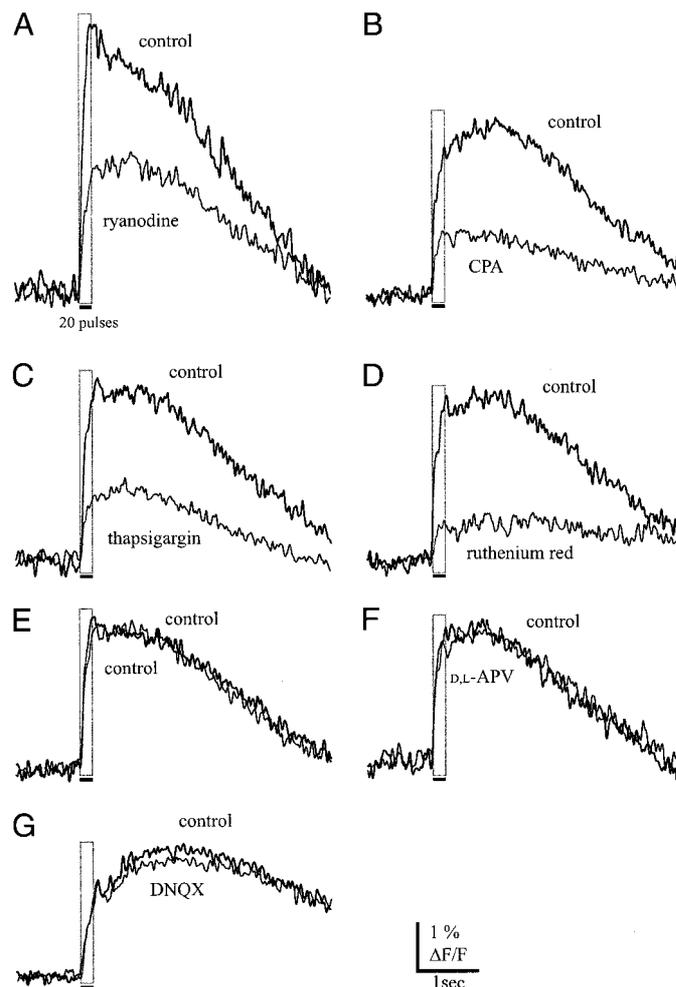


FIG. 2. Effects of bath-applied ryanodine (20 μM; A), CPA (30 μM; B), thapsigargin (10 μM; C), and ruthenium red (100 μM; D) on Ca²⁺ responses after giving a brief train of 20 stimuli. The Ca²⁺ signals from mossy fiber terminals were decreased significantly after wash-in one of the above agents for 30 min (60 min for thapsigargin). But under control condition (E) and in the presence of D,L-APV (50 μM; F) and DNQX (20 μM; G), the Ca²⁺ signals did not change. These results exclude the action of *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors on the evoked Ca²⁺ signals and indicate the stability of the recordings.

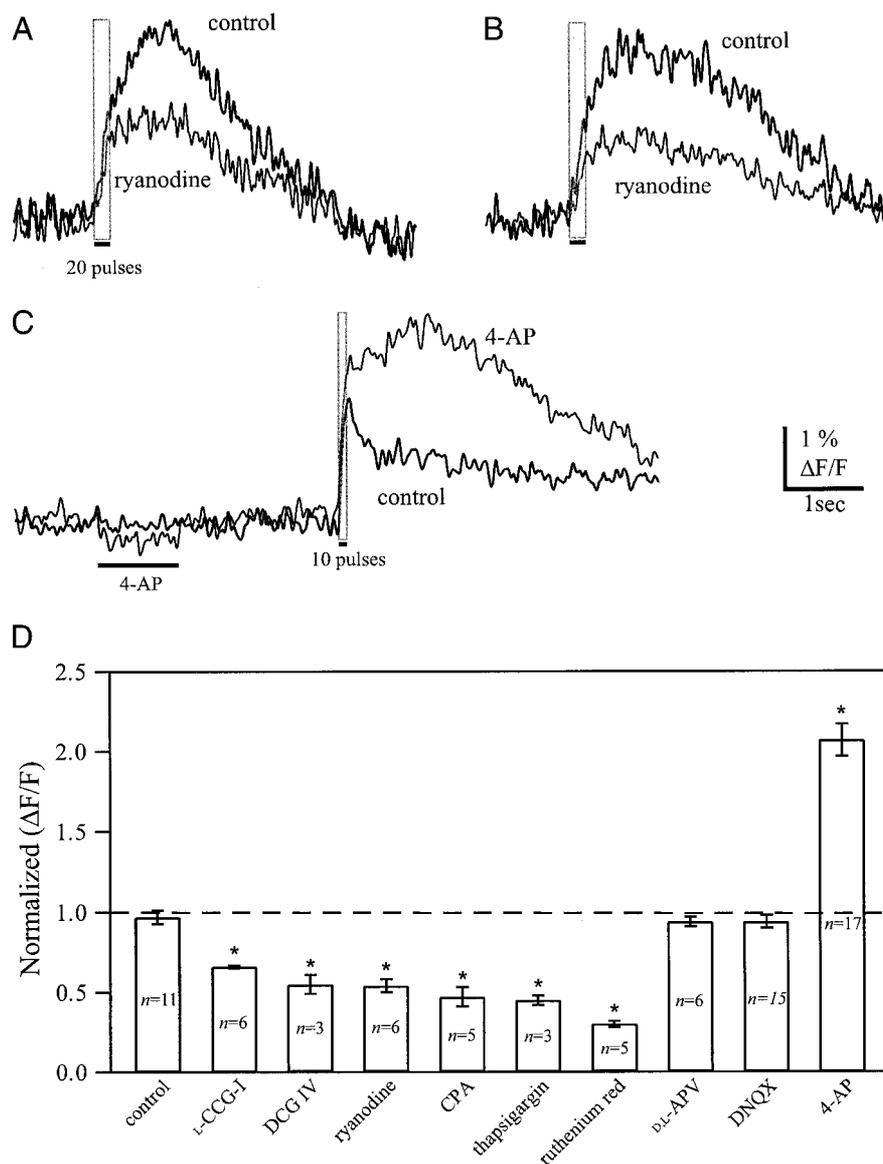


FIG. 3. To test for saturation of fura-2, we compared the effects of ryanodine ($20 \mu\text{M}$) on Ca^{2+} release at mossy fiber terminals loaded with indicators having different affinities for Ca^{2+} . *A*: calcium green-2AM ($K_d = 0.55 \mu\text{M}$). *B*: fura-2FF-AM ($K_d = 6 \mu\text{M}$). *C*: after focal application of 4-aminopyridine (4-AP; 1 mM), the transient K^+ channel blocker, Ca^{2+} signals increased. *D*: summary of the effects of all tested agents on calcium responses at single MFTs.

tic calcium influx to 66% ($n = 6$) or 55% ($n = 3$) of control, respectively (Fig. 1, *C* and *D*). The group II mGluR agonists have become useful tools in distinguishing MF synapses because they selectively inhibit MF synapses and do not affect C/A synapses (Kamiya et al. 1996; Yeckel et al. 1999). Under the same experimental conditions, we investigated the contribution of intracellular Ca^{2+} release to the total Ca^{2+} signal at mossy fiber presynaptic terminals by stimulating mossy fibers and bath applying agents known to interfere with intracellular Ca^{2+} signaling.

Ryanodine ($20 \mu\text{M}$), which blocks ryanodine receptors in the endoplasmic reticulum and prevents Ca^{2+} release in high concentration (Mattson et al. 2000; Sutko et al. 1997), decreased the amplitude of the Ca^{2+} signals. After bath application for 30 min, the fluorescent signal ($\Delta F/F$) induced by stimulating mossy fiber was decreased from 3.22 ± 0.64 to 1.73 ± 0.34 (mean \pm SE, $n = 6$; $P < 0.01$; Fig. 2*A*). Cyclopiazonic acid (CPA $30 \mu\text{M}$), a specific inhibitor of Ca^{2+} -ATPase in the ER that blocks Ca^{2+} uptake and depletes Ca^{2+} stores without increasing intracellular IP_3 levels (Markram et

al. 1995; Plenge-Tellechea et al. 1997; Seidler et al. 1989), decreased the amplitude of the Ca^{2+} signals from terminals. After washing in CPA for 30 min, the fluorescent signal ($\Delta F/F$) induced by stimulating mossy fiber was decreased from 2.93 ± 0.31 to 1.45 ± 0.31 ($n = 5$; $P < 0.01$; Fig. 2*B*). Thapsigargin ($10 \mu\text{M}$) can deplete ER Ca^{2+} by specific inhibition of ER Ca^{2+} -ATPase (Markram et al. 1995; Mattson et al. 2000; Thastrup et al. 1990; Treiman et al. 1998). After bath application for 60 min, the Ca^{2+} signal amplitude decreased from 4.56 ± 0.2 to 2.06 ± 0.19 ($n = 3$; $P < 0.01$; Fig. 2*C*). Ruthenium red ($100 \mu\text{M}$), a Ca^{2+} antagonist that inhibits the ryanodine receptor and the mitochondrial Ca^{2+} uniporter (Chen and MacLennan 1994; Ma 1993; Moore 1971), decreased the amplitude of the Ca^{2+} signals significantly. After perfusing ruthenium red for 30 min, the fluorescent signal ($\Delta F/F$) was changed from 2.58 ± 0.18 to 0.76 ± 0.04 ($n = 5$; $P < 0.01$; Fig. 2*D*). We also measured the half decay time for both control and with blockers. We found that there were no statistical differences before and after blockers. Surprisingly, drugs that affect Ca^{2+} release also decreased the initial peak of the

terminal Ca^{2+} signal, suggesting that release from intracellular stores is rapid and participates in the formation of the initial peak.

Continuously perfusing normal external solution or applying DMSO (0.1%) after normal external solution in the same time period as drug application, the $\Delta F/F$ signals were stable during recording (Fig. 2E). In addition, we applied D,L-APV (50 μM) and DNQX (20 μM), the selective N-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonist, to exclude the action of NMDA and non-NMDA receptors on the evoked presynaptic Ca^{2+} signals (Fig. 2, F and G).

Alternative indicators with lower affinities for Ca^{2+} were used to address the concern that saturation of fura-2 might confound these results. Both calcium green-2AM ($K_d = 0.55 \mu\text{M}$; Fig. 3A) and fura-2FF-AM ($K_d = 6 \mu\text{M}$; Fig. 3B) showed changes similar to those measured with fura-2AM ($K_d = \text{approximately } 0.22 \mu\text{M}$; Fig. 2A) in response to application of ryanodine. 4-Aminopyridine (4-AP, 1 mM), which blocks transient K^+ channels, increased the presynaptic signal by $107 \pm 0.1\%$ ($n = 17$, $P < 0.01$; Fig. 3C), further suggesting that saturation of indicator was not occurring in these experiments. Figure 3D summarized the effects of all tested agents on calcium responses at single MFTs.

DISCUSSION

There are two principal results of this study. 1) Using optical methods, we recorded a Ca^{2+} response ($\Delta F/F$) at single synaptic boutons in stratum lucidum of the CA3 region in the hippocampus and stimulated the mossy fiber pathway. Because the evoked Ca^{2+} signals were reduced by group II mGluR agonists and the terminals co-stained for Zn^{2+} , the results strongly support the hypothesis that the recorded terminals were from the mossy fibers of the dentate gyrus. This entire Ca^{2+} response could be completely blocked by the Ca^{2+} channel blocker Cd^{2+} . 2) Several pharmacological agents thought to disrupt regulation of intracellular Ca^{2+} stores (resulting in depletion of internal Ca^{2+}) decreased the early and the delayed increase in the Ca^{2+} signals. Local application of 4-AP and indicators with lower affinities for Ca^{2+} were used to control for possible saturation of fura-2.

Our experiments suggest the following sequence of events. Stimulation of the mossy fiber pathway generated action potentials that propagated down the axon to the terminals. Action potentials directly caused $[\text{Ca}^{2+}]_i$ increases by opening voltage-gated Ca^{2+} channels located on mossy fiber terminals. This transiently increased Ca^{2+} concentration rapidly triggered more Ca^{2+} release from ER through ryanodine receptors. The triggered release could be inhibited by either ryanodine receptors blockers (ryanodine and ruthenium red) or specific inhibition of ER Ca^{2+} -ATPase (CPA and thapsigargin), which deplete the ER Ca^{2+} store. These results were in agreement with observations on Schaffer collateral boutons in cultured hippocampal slices (Emptage et al. 2001), in which they reported that action potentials reliably triggered large Ca^{2+} transients in boutons, due both to Ca^{2+} influx and to CICR from internal stores, depleting the stores (by CPA or thapsigargin) or blocking CICR (by ryanodine) reduced the evoked Ca^{2+} transients. Our proposed presynaptic mechanism is different from other reports on postsynaptic $[\text{Ca}^{2+}]_i$ changes (Finch and Augustine 1998; Kapur et al. 2001; Nakamura et al. 1999; Takechi et al. 1998). First, these reports all studied postsynaptic $[\text{Ca}^{2+}]_i$

changes, either in hippocampal pyramidal neurons or Purkinje cells in the cerebellum, all of which prominently express metabotropic glutamate receptor I (mGluRI) and inositol-1,4,5-trisphosphate (IP_3) receptors (Conn and Pin 1997; Walton et al. 1991); second, the results showed that the synaptically activated Ca^{2+} transients in these cells were mediated by activation of postsynaptic mGluRI and required IP_3 -mediated Ca^{2+} release from internal stores. We investigated Ca^{2+} release at presynaptic mossy fiber synapses on CA3 pyramidal neurons in the hippocampus, where the levels of RyRs are particularly high (Padua et al. 1992; Sharp et al. 1993). The Ca^{2+} channel blocker, Cd^{2+} (400 μM) could completely inhibit the rise of presynaptic $[\text{Ca}^{2+}]_i$. This is evidence that extracellular Ca^{2+} through voltage-gated Ca^{2+} channels on MFTs participated in the initial $[\text{Ca}^{2+}]_i$ increase. In addition, we applied D,L-APV and DNQX, the selective NMDA and non-NMDA receptor antagonists, to exclude the effects of NMDA and non-NMDA receptors on the evoked presynaptic Ca^{2+} signals.

Mossy fiber boutons terminate on the proximal portion of the apical dendrites of CA3 pyramidal neurons. There are multiple active zones at each bouton resulting in multiple release sites for neurotransmitter (Chicurel and Harris 1992; Claiborne et al. 1986). Two particularly noteworthy features of mossy fiber synaptic transmission are large facilitation and minimal depression during long-duration, high-frequency stimulation (Yeckel et al. 1999). One possibility is that the Ca^{2+} release described here contributes to the high capacity for neurotransmitter release at this synapse.

Although different mechanisms mediated the presynaptic and postsynaptic Ca^{2+} release from intracellular stores, our studies suggest that at mossy fiber presynaptic terminals, Ca^{2+} release from internal stores is an important component of the Ca^{2+} signal and contributes to the rise of presynaptic $[\text{Ca}^{2+}]_i$ during evoked activity. A recent report has also characterized mossy fiber-evoked Ca^{2+} release in postsynaptic CA3 neurons (Kapur et al. 2001).

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