

Calcium Release from Presynaptic Ryanodine-Sensitive Stores Is Required for Long-Term Depression at Hippocampal CA3–CA3 Pyramidal Neuron Synapses

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Although Ca²⁺ release from internal stores has been proposed to be important for the induction of long-term synaptic plasticity, the importance of Ca²⁺ stores localized in presynaptic terminals remains unclear. Here, we have selectively applied pharmacological antagonists to either the presynaptic or postsynaptic cell in paired whole-cell recordings from hippocampal CA3 pyramidal neurons in slice culture. We demonstrate directly the necessary role of presynaptic, but not postsynaptic, ryanodine-sensitive Ca²⁺ stores in the induction of NMDA receptor (NMDAR)-dependent long-term depression (LTD). Using two-photon laser scanning microscopy, we further find that release from the ryanodine-sensitive stores during prolonged synaptic stimulation generates a slowly rising Ca²⁺ signal in the presynaptic terminal that is required for the induction of LTD. Moreover, this form of LTD has a significant presynaptic component of expression because it causes a marked decrease in the rate of release from CA3 neuron presynaptic terminals of FM 1-43, a fluorescent probe of synaptic vesicle cycling. Thus, Ca²⁺ release from presynaptic ryanodine-sensitive stores is critical in the induction of a presynaptic component of NMDAR-dependent LTD.

Key words: long-term depression; CA3; internal calcium stores; ryanodine-sensitive stores; hippocampus; synaptic plasticity

Introduction

Ca²⁺ release from internal stores, mediated by either the IP₃ receptor (IP₃R) or ryanodine receptor (RyR) Ca²⁺ release channel, amplifies and dynamically regulates cytosolic Ca²⁺ levels (Berridge et al., 2003). In neurons, Ca²⁺ release from internal stores has been implicated in postsynaptic signaling, presynaptic control of transmitter release, and long-term synaptic plasticity (Berridge, 1998; Fitzjohn and Collingridge, 2002), although its role in these events remains controversial. This is partly because of technical difficulties that limit direct measurement of store-dependent Ca²⁺ signals in small dendritic processes and presynaptic terminals in brain slices. Here, we take advantage of recent advances in imaging techniques to investigate the role of Ca²⁺ release in long-term depression (LTD) at the CA3–CA3 pyramidal neuron synapse in hippocampal slice culture (Montgomery et al., 2001).

A role for Ca²⁺ release from internal stores has been previously suggested for LTD at several hippocampal synapses (Reyes and Stanton, 1996; Wang et al., 1997; Caillard et al., 2000; Nishiyama et al., 2000). At least two forms of hippocampal LTD have

been identified: an NMDA receptor (NMDAR)-dependent form (Stanton and Sejnowski, 1989; Dudek and Bear, 1992; Mulkey and Malenka, 1992) and a metabotropic glutamate receptor (mGluR)-dependent form (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997). In both cases, a rise in postsynaptic Ca²⁺ is required for the induction of plasticity (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997). Moreover, at CA3–CA1 synapses in acute hippocampal slices, IP₃R-mediated Ca²⁺ release in the postsynaptic CA1 pyramidal neuron (Reyes and Stanton, 1996; Wang et al., 1997; Caillard et al., 2000; Nishiyama et al., 2000) and RyR-mediated Ca²⁺ release in either the postsynaptic CA1 neuron (Reyes and Stanton, 1996; Wang et al., 1997; Caillard et al., 2000; Nishiyama et al., 2000) or presynaptic CA3 neuron (Reyes and Stanton, 1996) have been implicated in the induction of NMDAR-dependent LTD. The importance of Ca²⁺ stores has also been shown genetically because deletion of the RyR3 ryanodine receptor subunit abolishes LTD at CA3–CA1 synapses (Futatsugi et al., 1999). However, a specific Ca²⁺ signal attributable to RyR- or IP₃R-mediated Ca²⁺ release during the induction of LTD has not yet been identified, nor has the presynaptic contribution of Ca²⁺ stores been directly investigated.

We have focused on the importance of Ca²⁺ stores in the induction of LTD at the excitatory collateral synapse formed between CA3 pyramidal neurons in organotypic slice cultures of the rat hippocampus (Stoppini et al., 1991; Debanne et al., 1998; Montgomery and Madison, 2002). Dual whole-cell patch-clamp recordings from pairs of synaptically connected CA3 neurons allowed us to deliver pharmacological agents selectively into ei-

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ther the presynaptic or postsynaptic cell. Two-photon laser scanning microscopy (TPLSM) permitted us to image Ca²⁺ in individual presynaptic terminals of CA3 neurons during the induction of LTD.

Here, we confirm that robust, NMDAR-dependent LTD exists at CA3–CA3 synapses (Montgomery and Madison, 2002) and show that the induction of this LTD requires Ca²⁺ release from presynaptic ryanodine-sensitive stores. Moreover, we provide direct evidence that NMDAR-dependent LTD produces a change in presynaptic function, based on two-photon imaging of the release of the fluorescent styryl dye FM 1-43 (Betz and Bewick, 1992) from presynaptic terminals. We suggest that the RyR-dependent Ca²⁺ signal may gate the response of the presynaptic terminal to a retrograde message, permitting only those terminals that have been activated to undergo LTD. Given the autoassociative nature of the CA3–CA3 connection, this form of LTD may be required to prevent the saturation of transmission at this synapse because of facilitatory processes such as LTP.

Materials and Methods

Slice culture preparation. Slice cultures were made using the general membrane insert technique (Stoppini et al., 1991). Postnatal day 8 Sprague Dawley rats were decapitated, and their brains were quickly removed and placed in chilled minimal essential medium (catalog #12370-037; Invitrogen, San Diego, CA) supplemented with 1% penicillin–streptomycin solution (catalog #15140-122; Invitrogen). The hippocampus was carefully dissected and cut into 400- μ m-thick slices using a tissue chopper. Three slices were placed on one Millicell CM membrane insert (catalog #PICM03050; Millipore, Bedford, MA), the underside of which was bathed in 1 ml of warmed 5% CO₂ equilibrated culture medium [50% minimal essential medium (catalog #12370-037; Invitrogen), 25% HBSS (catalog #24020-117; Invitrogen), 25% horse serum (catalog #A-3311-L; HyClone, Logan, UT), 0.5% L-glutamine solution (catalog #25030-081; Invitrogen), and 1% penicillin–streptomycin solution (catalog #15140-122; Invitrogen)]. The slice cultures were first placed in a 37°C incubator for 2 d and then moved to a 34°C incubator. Slice cultures were fed by exchanging the culture medium (1 ml; formulation described above) every 2–3 d. Experiments were performed on slice cultures after 1–6 weeks *in vitro*.

Electrophysiology. Paired whole-cell recordings were obtained with an Axoclamp-2A amplifier (presynaptic cell; Axon Instruments, Foster City, CA) and an EPC-9 amplifier (postsynaptic cell; HEKA Elektronik, Lambrecht/Pfalz, Germany). Patch pipettes were filled with the following internal solution (in mM): 115 KMeSO₄, 20 KCl, 10 HEPES, 4 MgCl₂, 4 Na₂ATP, 10 NaPhosphocreatine, 0.4 Na₂GTP, and 0.1 EGTA, titrated to pH 7.2–7.3 with KOH [all reagents were purchased from Sigma (St. Louis, MO), except KMeSO₄ from ICN Biomedicals (Cleveland, OH)]. In some experiments, the internal solution was supplemented with one of the following: 2–5 μ M thapsigargin (0.1–0.3% DMSO), 30 μ M cyclopiazonic acid (CPA; 0.3% DMSO), 300 μ M ryanodine (0.3% DMSO), 0.3% DMSO, or 400 μ M ruthenium red. Recordings were held \sim 1 hr before application of LTD induction protocols to allow sufficient time for drug diffusion throughout the cell. Based on imaging experiments, in which we tracked the time course of diffusion of the calcium dye Oregon Green BAPTA-1 to presynaptic terminals and distal postsynaptic dendrites, a 1 hr period results in a concentration of dye at distal dendrites and presynaptic terminals equal to \sim 50–60% of the concentration in the patch pipette. Because most of the drugs we used were similar in size or smaller than this calcium dye (molecular weight, 1114 Da), a 1 hr time period should be adequate for a sufficient concentration of drug to reach its target.

Patch pipettes were pulled to resistances between 3 and 6 M Ω , and access resistances of recordings were between 8 and 30 M Ω . Experiments in which postsynaptic access resistance was not stable were not included in the analysis. Monosynaptic connections were identified by their constant short latency [$<$ 3 msec between action potential (AP) peak and EPSC rise]. Individual slice cultures were cut out of the membrane using

a scalpel and placed in a submerged perfusion chamber in artificial CSF (ACSF) solution (in mM: 119 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.25 Na₂HPO₄, 26.2 NaHCO₃, and 10 glucose) equilibrated with 95% O₂/5% CO₂. Slices were perfused at a rate of 2 ml/min. In some experiments, one of the following drugs was added to the ACSF: 50 μ M D-APV, 200 μ M 1-aminoinidan-1,5-dicarboxylic acid (AIDA), 5 μ M nitrendipine, 2–3 μ M CPA, 50 μ M nickel chloride, or 10 μ M ryanodine. In experiments using bath application of CPA and ryanodine, slice cultures were perfused with these agents for 20–30 min before the LTD induction protocol to ensure adequate delivery. All experiments using nitrendipine were conducted in a darkened room with the nitrendipine-containing ACSF shielded from light. Thapsigargin, ryanodine, and ruthenium red were purchased from Calbiochem (La Jolla, CA). CPA and nickel chloride were purchased from Sigma. D-APV, AIDA, and nitrendipine were purchased from Tocris (Bristol, UK).

Baseline recordings were made by firing a single AP in the presynaptic cell at 0.1 Hz by injecting 10–15 msec current steps (1 nA), while maintaining the postsynaptic cell in voltage-clamp mode at -70 mV. LTD was induced by switching the postsynaptic cell into current-clamp mode and firing the presynaptic cell at 5 Hz for 3 min (total, 900 APs). The recordings were then returned to the baseline configuration for the duration of the recording. Traces were acquired using Pulse (HEKA), digitized at 5 kHz and filtered at 2 kHz. Because of the variability in the size of the unitary EPSC in paired recording experiments, we used a sliding boxcar window to average 20 successive EPSCs (representing a 200 sec time period) and have displayed the mean EPSC amplitude versus time by shifting the boxcar over each successive EPSC. LTD was measured by averaging 20 data points centered on the time point 15 min after the end of the induction protocol. This analysis was done for each pair, and the average reported is the mean value from the different paired recording experiments. All mean values from paired recording experiments reported either in bar graphs or in the text are calculated from the 15 min time point, as described above. In some experiments, presynaptic neurons were stimulated using an ACSF-filled glass extracellular electrode placed in the CA3 cell body layer. In extracellular stimulation experiments, LTD was analyzed as the value of the data point 15 min after the end of the induction protocol. Errors are reported as SEM. Statistical significance was assessed using Student's *t* test, except as noted in the text and figure legends. All experiments were performed at room temperature.

Imaging. Calcium imaging was performed by patch clamping individual CA3 pyramidal neurons in whole-cell mode using the same internal solution described above, with the following change: EGTA was replaced with 500 μ M Oregon Green 488 BAPTA-1 (Molecular Probes, Eugene, OR). Based on fluorescence intensity, we estimate that the dye concentration at the presynaptic terminal was \sim 300 μ M. In some experiments, we used the lower-affinity calcium dye Fluo-5F at a concentration of 300 μ M in the patch pipette to explore the effects of buffer affinity and concentration on the measured calcium signals. In experiments in which we examined the importance of calcium stores, the internal solution was supplemented with one of the following: 30 μ M CPA (0.3% DMSO), 300 μ M ryanodine (0.3% DMSO), or 0.3% DMSO.

After \sim 1 hr loading, a visual search of the basal dendritic region of the cell revealed portions of the axonal arbor. Following the course of an axon branch would often lead to presynaptic varicosities along its length. TPLSM was performed using a BX50WI upright microscope with a 60 \times , 0.9 numerical aperture objective (Olympus, Melville, NY) and a 1024-MP system (Bio-Rad, Hercules, CA) with a Tsunami Ti:sapphire laser (Spectra-Physics, Mountain view, CA) tuned to 812 nm. Images were acquired using LaserSharp software (Bio-Rad).

Each frame encompassing a presynaptic terminal was 128 \times 128 pixels and was acquired once every 10 sec. These sequences of images of fluorescence intensity were exported and analyzed using custom-written software in Interactive Data Language (Research Systems, Boulder, CO) to measure baseline fluorescence and fluorescence changes. One region of interest (ROI) was centered on the terminal and a second ROI off the cell to record the background. This background was subtracted from the terminal signal to obtain the fluorescence value. Fast and slow phases of the calcium rise were measured as the difference between the fluorescence values at time points specified (see Fig. 4E). For each trace, fluo-

rescence values for three consecutive time points (10 sec intervals) were averaged during the baseline (indicated by “1”) (see Fig. 4E), at the peak of the fast phase (“2”), and at the end of the slow phase (“3”) during the 5 Hz train and after decay of the Ca^{2+} signal back to baseline after the train (“4”). The amplitude of the fast phase was measured as the difference between the resultant values at times 2 and 1 (fast phase, 2–1). The amplitude of the slow phase was measured as the difference between the resultant average values at times 3 and 2 (slow phase, 3–2). All averages reported are the mean value of the different experiments, and the error is SEM.

Preparation, acquisition, and analysis of FM 1-43 imaging data were performed using a protocol similar to one published previously (Zakharenko et al., 2001). Slice cultures were loaded by bath application of FM 1-43 ($25 \mu\text{M}$) for 2 min with no stimulation, followed by a 2 min loading stimulation (10 Hz). After a total of 4 min of exposure to FM 1-43, slices were washed with ACSF containing the sulfonated β -cyclodextrin ADVASEP-7 ($100 \mu\text{M}$; Cydex, Overland Park, KS) for 20 min to remove any extracellular dye (Kay et al., 1999). Dye release was then determined using 1.5 Hz stimulation in the continued presence of ADVASEP-7, following the same protocol published previously (Zakharenko et al., 2001).

Results

NMDAR-dependent LTD is present at CA3–CA3 synapses

Although LTD has been documented and studied at a variety of central synapses, relatively little is known about LTD at recurrent CA3–CA3 synapses in the hippocampus. We recorded from pairs of synaptically connected CA3–CA3 pyramidal neurons in organotypic slice cultures of the rat hippocampus using a protocol similar to that developed by Madison and colleagues (Pavlidis and Madison, 1999; Pavlidis et al., 2000; Montgomery et al., 2001). Stimulation of the presynaptic cell that evoked a single AP elicited an EPSC in the postsynaptic cell. The amplitude of this EPSC was quite variable (range, ~ 10 – 300 pA), even in a single synaptic pair, most likely because of the probabilistic nature of release from a relatively small number of presynaptic terminals (Fig. 1A, left).

When the presynaptic neuron was stimulated at a low frequency of 0.1 Hz, the mean amplitude of the postsynaptic response was stationary over time (Fig. 1A, left). However, when we stimulated the presynaptic neuron at a frequency of 5 Hz for a period of 3 min (900 APs), a protocol used to evoke LTD at CA3–CA1 synapses in acute hippocampal slices (Bolshakov and Siegelbaum, 1994), we observed a robust and long-lasting decrease in the size of the evoked EPSC that lasted at least 30 min (Fig. 1A). On average, 15 min after the induction of LTD, the magnitude of the evoked EPSC was significantly reduced to $32 \pm 7\%$ ($n = 11$ pairs; $p < 0.001$) of its baseline value (Fig. 1B, C; see Fig. 3C). Because of the variability in the size of the unitary EPSC, we used a boxcar average to display the time course of the EPSC amplitude during the experiments (Fig. 1C). LTD was only observed when the postsynaptic cell was allowed to depolarize under current-clamp conditions during the induction protocol; when the postsynaptic cell was voltage clamped to -70 mV during the induction protocol, no LTD was observed (data not shown). This result indi-

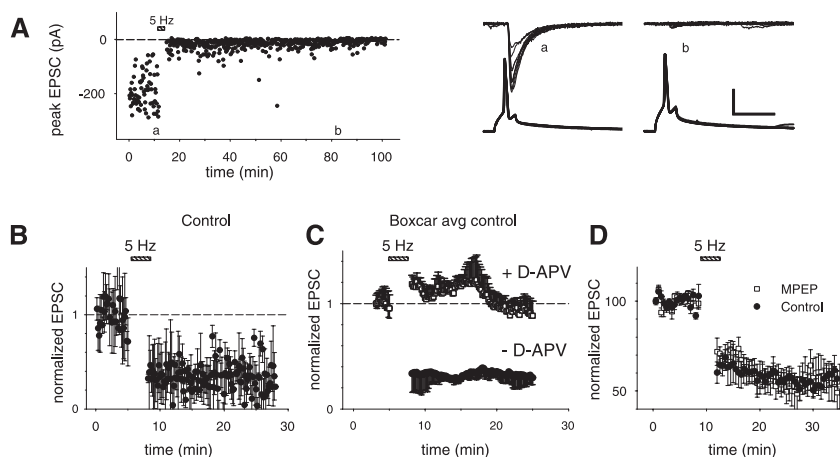


Figure 1. CA3–CA3 synapses exhibit NMDAR-dependent LTD. *A*, Left, Peak EPSCs recorded from a synaptically connected pair of CA3–CA3 pyramidal neurons before and after induction of LTD (induction protocol shown by striped bar). Letters a and b mark time periods during the recording from which sample traces are shown (on the right). Right, Presynaptic and postsynaptic responses before (a) and after (b) induction of LTD. The bottom traces are current-clamp records showing an overlay of 10 consecutive APs elicited in the presynaptic cell by current injection. The top traces are voltage-clamp records ($V_m = -70$ mV) from the postsynaptic cell showing an overlay of 10 consecutive EPSCs. Calibration: vertical, 40 mV, 100 pA; horizontal, 20 msec. *B*, Averaged (normalized) peak EPSC during experiment before and after LTD induction ($n = 11$ pairs). *C*, Sliding boxcar average (window size, 20 points) of data in the presence (top trace) and absence (bottom trace) of $50 \mu\text{M}$ D-APV. *D*, LTD at CA3–CA3 synapses does not require activation of mGluR5 receptors. Averaged (normalized) peak EPSCs before and after induction of LTD (induction stimulation shown by bar) in the presence (open squares; $n = 6$) and absence (closed circles; $n = 6$) of $10 \mu\text{M}$ MPEP. In this experiment, extracellular field stimulation was used to elicit EPSCs and induce LTD. No sliding boxcar was used.

cates that the induction of LTD at CA3–CA3 synapses requires postsynaptic activity.

To distinguish whether this form of LTD requires NMDAR or mGluR activation, we applied specific pharmacological antagonists. When the 5 Hz stimulation protocol was applied in the presence of the NMDAR antagonist D-APV ($50 \mu\text{M}$), the induction of LTD was blocked. The size of the evoked EPSC was not significantly different from its baseline value before the 5 Hz stimulation protocol (EPSC amplitude was $94 \pm 23\%$ of baseline; $n = 5$ pairs; $p > 0.33$) (Fig. 1C; see Fig. 3C). In contrast, inhibition of group I mGluRs using either the mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP; $10 \mu\text{M}$) (Fig. 1D) (Gasparini et al., 1999) or the mGluR1 antagonist AIDA ($200 \mu\text{M}$) (see Fig. 3C) (Moroni et al., 1997) had no effect on the magnitude of LTD measured 15 min after its induction. Thus, in the presence of either MPEP or AIDA, the 5 Hz stimulation reduced the EPSP to $54 \pm 9\%$ (Fig. 1D) ($p < 0.05$; $n = 6$) or $48 \pm 18\%$ of baseline (see Fig. 3C) ($p < 0.05$, $n = 3$), respectively. This is in agreement with the recent findings of Montgomery and Madison (2002) that CA3–CA3 LTD induced by a prolonged 15 min period of 1 Hz stimulation is NMDAR dependent but mGluR independent.

LTD depends on ryanodine-sensitive Ca^{2+} stores

Although the presence of Ca^{2+} stores and their role in synaptic plasticity is well documented at parallel fiber–Purkinje cell synapses (Finch and Augustine, 1998; Inoue et al., 1998; Takechi et al., 1998; Daniel et al., 1999) and in the generation of large-amplitude miniature IPSCs (Llano et al., 2000) in the cerebellum, their importance at pyramidal neuron synapses in the hippocampus is more controversial (Harvey and Collingridge, 1992; Emptage et al., 1999, 2001; Nakamura et al., 1999; Svoboda and Mainen, 1999; Rose and Konnerth, 2001). We have therefore tested for the presence and role of Ca^{2+} stores in the induction of LTD at the CA3–CA3 synapse. First, we used a pharmacological

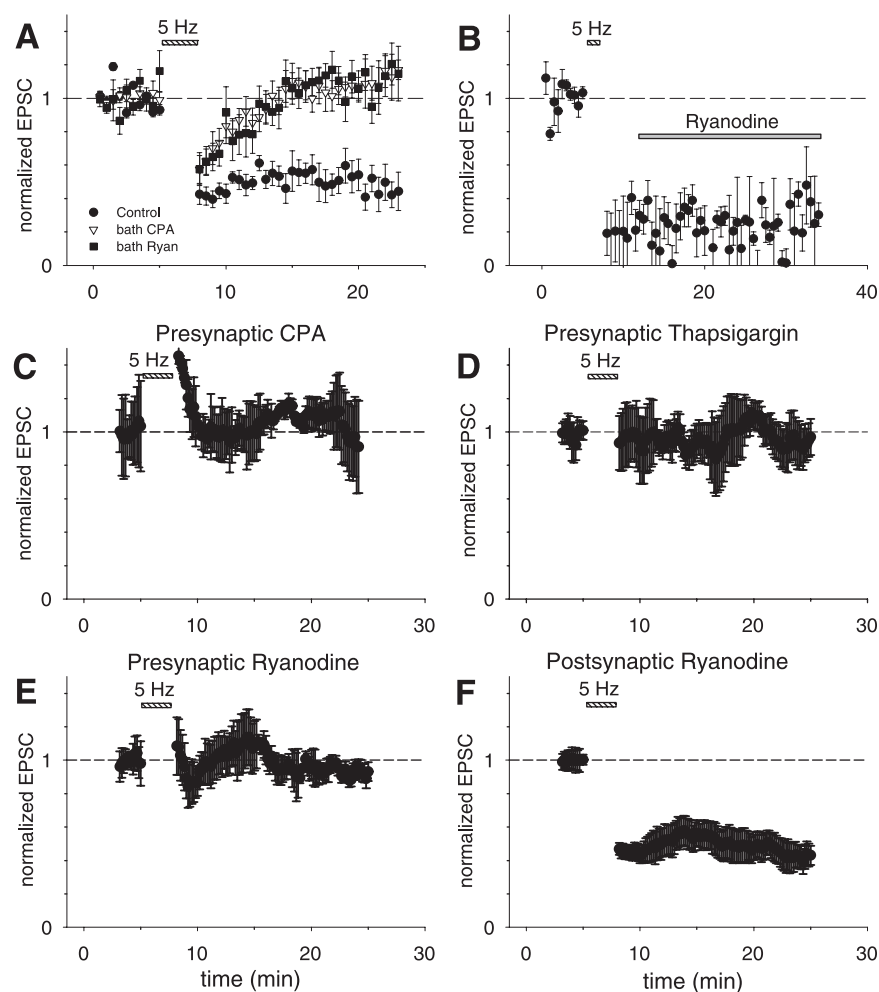


Figure 2. LTD requires presynaptic, but not postsynaptic, ryanodine-sensitive Ca²⁺ stores. *A*, Average (normalized) peak EPSC elicited by extracellular stimulation before and after LTD induction in normal external solution (filled circles; $n = 8$ cells), in presence of external CPA ($2\text{--}3\ \mu\text{M}$; open inverted triangles; $n = 10$ cells), or in presence of external ryanodine ($10\ \mu\text{M}$; filled squares; $n = 8$ cells). *B*, Bath application of ryanodine ($10\ \mu\text{M}$; $n = 4$ cells) 5 min after the end of the LTD induction protocol does not inhibit LTD. *C–F*, Results from boxcar averaged paired recordings showing effects of selective presynaptic or postsynaptic application of antagonists through recording electrodes. *C*, Presynaptic application of CPA ($30\ \mu\text{M}$; $n = 4$ pairs). *D*, Presynaptic application of thapsigargin ($2\text{--}5\ \mu\text{M}$; $n = 5$ pairs). *E*, Presynaptic application of ryanodine ($300\ \mu\text{M}$; $n = 4$ pairs). *F*, Postsynaptic application of ryanodine ($300\ \mu\text{M}$; $n = 4$ pairs).

approach, involving bath application of Ca²⁺ store antagonists and extracellular field stimulation, previously used to demonstrate a role for Ca²⁺ release in the induction of LTD at CA3–CA1 synapses (Reyes and Stanton, 1996).

In these pharmacological experiments, we used extracellular field stimulation of CA3 inputs at 5 Hz for 3 min, which produced a robust LTD of very similar magnitude to that obtained using paired recordings as described above. On average, the evoked EPSC was reduced to $44 \pm 6\%$ of its baseline value 15 min after induction of LTD ($n = 8$ cells; $p < 0.0001$) (Fig. 2*A*). To test for a role of Ca²⁺ stores in LTD, we applied the LTD induction protocol in the presence of CPA ($2\text{--}3\ \mu\text{M}$), an inhibitor of the smooth endoplasmic reticulum Ca²⁺-ATPase (SERCA) pump that depletes both ryanodine-sensitive and IP₃-sensitive stores (Kurebayashi and Ogawa, 1991). Under these conditions, the induction of LTD was blocked. The size of the evoked EPSC measured 15 min after the induction protocol was not significantly different from the baseline EPSC amplitude ($117 \pm 11\%$ of baseline; $n = 10$ cells; $p > 0.15$) (Fig. 2*A*).

We next investigated whether ryanodine-sensitive Ca²⁺ stores are involved in the induction of LTD, as has been previously shown for CA3–CA1 synapses (Reyes and Stanton, 1996; Wang et al., 1997; Caillard et al., 2000; Nishiyama et al., 2000). When the 5 Hz stimulation protocol was applied in the presence of $10\ \mu\text{M}$ ryanodine, a concentration that depresses Ca²⁺ release selectively from ryanodine-sensitive stores (Zucchi and Ronca-Testoni, 1997), LTD was inhibited. The size of the evoked EPSC measured 15 min after the induction protocol was not significantly different from the baseline value ($115 \pm 16\%$ of baseline; $n = 8$ cells; $p > 0.25$) (Fig. 2*A*). To distinguish whether ryanodine-sensitive stores are necessary for the induction or expression of LTD, we applied ryanodine to the slice 5 min after the end of the induction protocol. This treatment did not reverse LTD. Thus, the magnitude of the EPSC remained depressed at $33 \pm 9\%$ of its baseline value 15 min after the induction protocol ($n = 4$; $p < 0.001$) (Fig. 2*B*). This finding is consistent with a previous study at CA3–CA1 synapses that demonstrated a requirement for ryanodine-sensitive stores during the induction, but not the expression, of LTD (Reyes and Stanton, 1996). Taken together, these experiments strongly suggest that ryanodine-sensitive Ca²⁺ stores are required for LTD induction at CA3–CA3 synapses.

Presynaptic, but not postsynaptic, ryanodine-sensitive stores are required for LTD

Studies using bath application of Ca²⁺ store antagonists cannot determine whether the stores important for LTD are located presynaptically, postsynaptically, or in glia, all of which contain internal Ca²⁺ stores that are thought to affect synaptic transmission (Reyes and Stanton, 1996; Wang et al., 1997; Emptage et al., 1999, 2001; Llano et al., 2000; Liang et al., 2002). Here, we have tested directly the potential presynaptic role of ryanodine-sensitive Ca²⁺ stores required for the induction of LTD by applying drugs to the presynaptic cell through the whole-cell recording electrode.

Our experiments provide several lines of evidence that indicate that presynaptic ryanodine-sensitive Ca²⁺ stores are necessary for the induction of LTD. First, when we applied to the presynaptic cell CPA (at a concentration of $30\ \mu\text{M}$ in the presynaptic electrode), the induction of LTD was blocked; the average evoked EPSC measured 15 min after the induction protocol was $101 \pm 24\%$ of baseline ($n = 4$ pairs; $p > 0.70$) (Figs. 2*C*, 3*C*). In contrast, inclusion of the DMSO vehicle (see Materials and Methods) by itself in the presynaptic electrode had no effect on LTD; the average EPSC measured 15 min after the induction protocol was reduced to $40 \pm 14\%$ of baseline ($n = 4$ pairs; $p < 0.001$). Second, presynaptic application of another SERCA pump inhib-

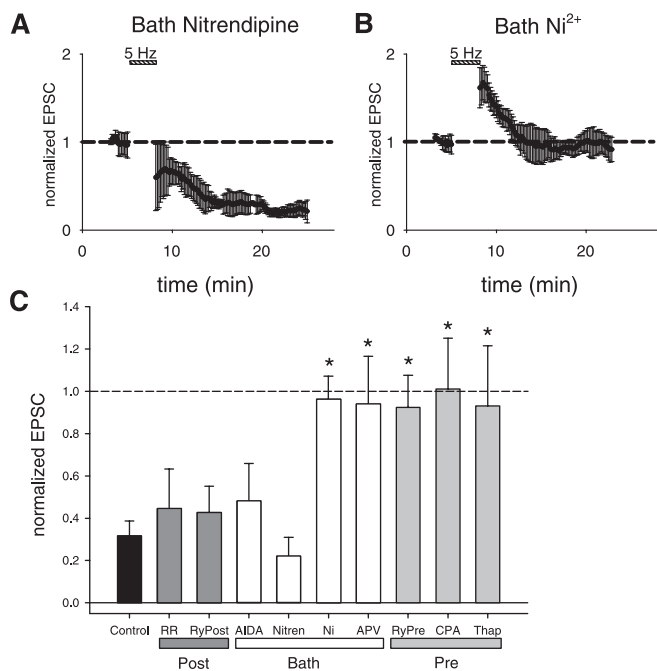


Figure 3. Pharmacological analysis of effects of calcium channel antagonists, glutamate receptor antagonists, and disruption of calcium stores on induction of LTD. *A, B*, Results from boxcar averaged paired recordings showing effects of selective voltage-gated calcium channel antagonists. *A*, Bath applied nitrendipine (5 μM) does not prevent the induction of LTD ($n = 3$ pairs). *B*, Bath applied Ni²⁺ (50 μM) prevents the induction of LTD ($n = 6$ pairs). *C*, Summary of pharmacology experiments. Each bar plots mean reduction in EPSC after induction of LTD. Error bars indicate SEM. Control, Normal extent of LTD in the absence of pharmacological agents; RR, LTD with postsynaptic ruthenium red (400 μM; $n = 3$); RyPost, LTD with postsynaptic ryanodine (300 μM; $n = 4$); AIDA, LTD with bath-applied AIDA (200 μM; $n = 3$); Nitren, LTD with bath-applied nitrendipine (5 μM; $n = 3$); Ni, LTD with bath-applied Ni²⁺ (50 μM; $n = 6$); APV, LTD with bath-applied D-APV (50 μM; $n = 5$); RyPre, LTD with presynaptic ryanodine (300 μM; $n = 4$); CPA, LTD with presynaptic CPA (30 μM; $n = 4$); Thap, LTD with presynaptic thapsigargin (2–5 μM; $n = 5$). The asterisk indicates that an agent caused a significant inhibition in the amount of LTD (ANOVA, $p < 0.002$; with *post hoc* Bonferroni test, $p < 0.05$).

itor, thapsigargin (2–5 μM), also prevented the induction of LTD, with an average EPSC response equal to $93 \pm 28\%$ of baseline ($n = 5$ pairs; $p > 0.53$) (Figs. 2*D*, 3*C*). Finally, application of ryanodine (300 μM in the pipette) to the presynaptic cell also blocked the induction of LTD, with an average response that was $92 \pm 15\%$ of baseline ($n = 5$ pairs; $p > 0.55$) (Figs. 2*E*, 3*C*). (We used 300 μM ryanodine in the pipette because 10 μM, the concentration used in bath applications, was ineffective, presumably because of loss of the membrane-permeable compound as it diffused from the whole-cell pipette in the soma down the axon to the presynaptic terminals).

In contrast to the inhibitory effect of presynaptic ryanodine, application of ryanodine (300 μM in the postsynaptic electrode) to the postsynaptic cell did not alter the induction of LTD. The average EPSC 15 min after the induction protocol was reduced to $43 \pm 12\%$ of baseline ($n = 4$ pairs; $p < 0.01$) (Figs. 2*F*, 3*C*). The lack of an effect of postsynaptic ryanodine is unlikely to reflect its failure to diffuse into the dendrites because we observed rapid diffusion of a variety of dyes (see below) into distal dendrites and presynaptic ryanodine was effective in blocking LTD. In addition, we found that postsynaptic application of ruthenium red (400 μM in the pipette), a hydrophilic membrane-impermeant inhibitor of Ca²⁺ release from ryanodine-sensitive stores that prevents LTD at other hippocampal synapses (Caillard et al., 2000), failed to block LTD (EPSC reduced to $45 \pm 19\%$ of baseline; $n = 3$, $p <$

0.01) (Fig. 3*C*). These experiments strongly suggest that the requirement for functional ryanodine-sensitive Ca²⁺ stores during the induction of CA3–CA3 LTD is limited to the presynaptic cell. A lack of involvement of postsynaptic ryanodine-sensitive Ca²⁺ stores in LTD was previously reported at CA3–CA1 synapses (Reyes and Stanton, 1996; cf. Nishiyama et al., 2000). However, our results do not exclude the possibility that ryanodine-insensitive Ca²⁺ stores in the postsynaptic cell are necessary for LTD.

LTD depends on T- or R-type, but not L-type, voltage-gated Ca²⁺ channels

RyRs can participate in a form of Ca²⁺ store mobilization known as Ca²⁺-induced Ca²⁺ release (CICR). In this process, Ca²⁺ influx through a plasma membrane channel triggers the subsequent release of Ca²⁺ from internal stores through RyRs. In cardiac muscle, Ca²⁺ influx through the L-type voltage-gated Ca²⁺ channel (VGCC) has been shown to be the dominant mechanism for providing RyRs with this trigger source of Ca²⁺ (Ogawa et al., 1999). However, additional sources, including influx through low voltage-activated T-type Ca²⁺ channels, have been implicated under certain situations (Ogawa et al., 1999). To test what mechanisms may be at work in CA3–CA3 synapses during the induction of LTD, we repeated our paired whole-cell recording induction protocol with the L-type VGCC antagonist nitrendipine present in the bath solution. Application of nitrendipine (5 μM) did not prevent the induction of LTD, because the average EPSC was reduced to $22 \pm 9\%$ of baseline ($n = 3$ pairs; $p < 0.01$) (Fig. 3*A*, *C*). In contrast, bath application of 50 μM Ni²⁺, a concentration that is thought to be a relatively selective T- or R-type VGCC antagonist (Fox et al., 1987; Turner et al., 1995; Huguenard, 1996), prevented the induction of LTD. Fifteen minutes after the LTD induction protocol was delivered in the presence of Ni²⁺, the EPSC remained at $96 \pm 11\%$ of its initial value ($n = 6$ pairs; $p > 0.57$) (Fig. 3*B*, *C*). This is in accordance with evidence that T-type VGCCs are required for the induction of LTD at several other synapses in the hippocampus (Christie et al., 1997; Oliet et al., 1997; Wang et al., 1997).

Although N- and P/Q-type VGCCs are believed to mediate fast synaptic transmission between most central neurons, less is known about the role of T- and R-type VGCCs (Turner et al., 1995; Meir et al., 1999). In light of our data demonstrating that Ni²⁺ can prevent LTD induction, we examined its effect on basal synaptic transmission at CA3–CA3 synapses. We discovered that 50 μM Ni²⁺ inhibited the EPSC to $68 \pm 7\%$ ($n = 4$ cells; $p < 0.01$) of its initial level (data not shown), suggesting that T- or R-type VGCCs might contribute to transmitter release at this synapse. This result complicates the interpretation of our finding that Ni²⁺ blocked the induction of LTD. Thus, blockade of the T- or R-type channels could act to block induction of LTD either by inhibiting Ca²⁺ release from internal presynaptic stores or by reducing the extent of postsynaptic activity required to trigger postsynaptic signal transduction cascades (because of the reduction in the EPSC). Results presented below using Ca²⁺ imaging, however, do suggest a contribution of T- or R-type channels to Ca²⁺ release from presynaptic stores.

Presynaptic terminal Ca²⁺ rises in two phases during the LTD protocol

To test directly whether or not the LTD induction protocol mobilizes Ca²⁺ from internal presynaptic stores, we used TPLSM to image Ca²⁺ transients in individual presynaptic terminals from CA3 pyramidal neurons. We loaded presynaptic CA3 neurons with the Ca²⁺ indicator Oregon Green 488 BAPTA-1 (500 μM) in

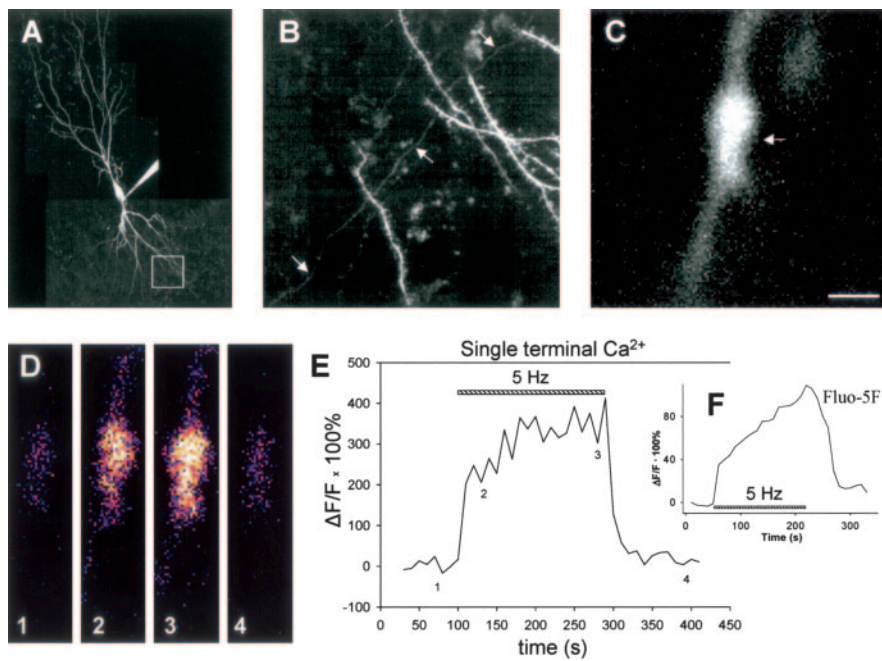


Figure 4. Presynaptic terminal Ca^{2+} rises in two distinct phases during the LTD induction protocol. *A*, Image of a CA3 pyramidal neuron filled with Oregon Green 488 BAPTA-1. The white box delineates the approximate region shown at higher magnification in *B*. Scale bar (in *C*), 50 μm . *B*, Basal dendritic region showing thin, smooth axons and thicker, spiny dendrites. The arrows point to an example of one axon crossing from top right to bottom left of field. Scale bar (in *C*), 10 μm . *C*, High-magnification view of presynaptic terminal (arrow). Scale bar, 1 μm . *D*, Single images taken from the terminal shown in *C*, before (1), during the beginning (2), during the end (3), and after (4) the LTD induction protocol. Fluorescence changes coded with a thermal look-up table, small changes coded by “cooler” colors, and larger changes coded by “warmer” colors are shown. Scale bar (in *C*), 1 μm . *E*, Ca^{2+} rise measured as $\Delta F/F$ (change in fluorescence intensity divided by initial baseline fluorescence intensity) in the terminal during LTD induction protocol. The striped bar marks delivery of LTD induction protocol (5 Hz stimulation for 3 min). The numbers along the trace show times at which images in *D* were obtained. *F*, Inset, Presynaptic Ca^{2+} signal during induction of LTD detected in a cell loaded with 300 μM Fluo-5F, a relatively low-affinity Ca^{2+} dye. A similar fast and slow component to the Ca^{2+} signal is detected. Data were averaged from seven cells.

the pipette solution under whole-cell recording conditions. After ~ 1 hr of whole-cell recording, portions of the axonal arbor could be observed interspersed among spiny basal dendrites (Fig. 4*A*). Based on their small diameter, smooth appearance, and presence of varicosities, axons were clearly distinguishable from dendrites (Fig. 4*B, C*). A previous study on the same preparation has shown that the vast majority of these varicosities are presynaptic terminals of *en passant* synapses (Emptage et al., 2001).

Imaging of presynaptic terminals of a CA3 neuron during the LTD induction protocol (APs at 5 Hz for 3 min) revealed a large increase in terminal Ca^{2+} with two distinct phases (Figs. 4*D, E*, 5*A*). A fast rising phase is complete within 10 sec of the start of the 3 min period of 5 Hz stimulation (the time resolution of these experiments). During this fast phase, the fluorescence signal significantly increased by $248 \pm 25\%$ ($n = 11$; $p < 0.001$) over its initial resting level. This fast phase is then followed by a slow continuous rise in Ca^{2+} levels over the remaining 3 min period of 5 Hz stimulation. During the slow phase, the fluorescence signal significantly increased by an additional $121 \pm 48\%$ ($n = 11$; $p < 0.05$) over its resting level. To examine whether the slow phase was an artifact of altered Ca^{2+} buffering attributable to the high concentration in the patch pipette of Oregon Green BAPTA-1, which has a high Ca^{2+} affinity of 170 nM, we repeated the measurements with a lower concentration of the low-affinity Ca^{2+} dye Fluo-5F ($K_d = 2.3 \mu\text{M}$). We observed a rapid and slow phase of the Ca^{2+} transient in the presynaptic terminals during the 3 min period of 5 Hz stimulation using 300 μM Fluo-5F in the

pipette solution (the lowest concentration that gave a reasonable signal in the presynaptic terminal), essentially identical to the results with Oregon Green BAPTA-1 (Fig. 4*F*). Thus, we conclude that the slow rise in Ca^{2+} represents a genuine signal that is not an artifact of our measuring conditions.

The fast Ca^{2+} signal depends partially on T- or R-type VGCCs, and the slow phase depends on ryanodine-sensitive stores

To explore a role for release from internal stores in the terminal Ca^{2+} signal, we imaged the rise in presynaptic Ca^{2+} during the LTD induction protocol with CPA added to the pipette solution at the same concentration (30 μM) that blocked LTD in our paired recording experiments (Fig. 5*B, F*). Application of CPA did not affect the initial fast phase of the Ca^{2+} signal. In the presence of CPA, the Ca^{2+} signal rose initially by $252 \pm 35\%$ ($n = 15$; $p < 0.001$) above its resting level, similar to the value of 248% measured without CPA (see above) or to the value of $279 \pm 39\%$ ($n = 5$; $p < 0.001$) obtained with a pipette solution containing DMSO, the solvent used to dissolve CPA. However, CPA completely blocked the late slow increase in Ca^{2+} , converting it to a slow decline. In the presence of CPA, the fluorescence decreased by $46 \pm 20\%$ from its initial peak value during this slow decline ($n = 15$). This is in contrast to, and significantly different from, the slow increase of 121% deter-

mined with our normal pipette solution in the absence of CPA (see above; $p < 0.01$) or to the slow increase of $178 \pm 99\%$ ($n = 5$; $p < 0.01$) observed using the DMSO solution. Presynaptic application of ryanodine (300 μM in the pipette), at a concentration that blocked LTD, also significantly blocked the slow phase of the Ca^{2+} increase, converting it to a slowly declining phase (decreasing by $88 \pm 24\%$; $n = 5$; $p < 0.05$) while leaving the fast phase unaffected compared with control ($247 \pm 39\%$ increase; $n = 5$, $p > 0.98$) (Fig. 5*C, F*). In parallel experiments using the low-affinity calcium indicator Fluo-5F described above, bath application of ryanodine (10 μM) also blocked the slow phase but not the fast phase of the Ca^{2+} signal (data not shown).

What might provide the Ca^{2+} trigger for the RyR-mediated slow rise in terminal Ca^{2+} ? In light of our pharmacological experiments on LTD, we tested the involvement of NMDARs or T-type/R-type VGCCs using bath application of APV or Ni^{2+} , respectively. Because LTD is blocked by D-APV, a finding normally attributable to postsynaptic NMDAR activation, we wondered whether presynaptic NMDARs might also be involved, as has been recently suggested for cerebellar LTD (Casado et al., 2002). However, we found that D-APV (50 μM), applied in the bath at the same concentration that blocked LTD, had no effect on either the fast phase ($286 \pm 35\%$ increase; $n = 3$; $p > 0.47$) or slow phase ($72 \pm 48\%$ increase; $n = 3$; $p > 0.62$) of the Ca^{2+} signal compared with control (Fig. 5*D, F*). In contrast, bath application of the T- and R-type VGCC antagonist Ni^{2+} at the same concentration that blocked LTD (50 μM) significantly

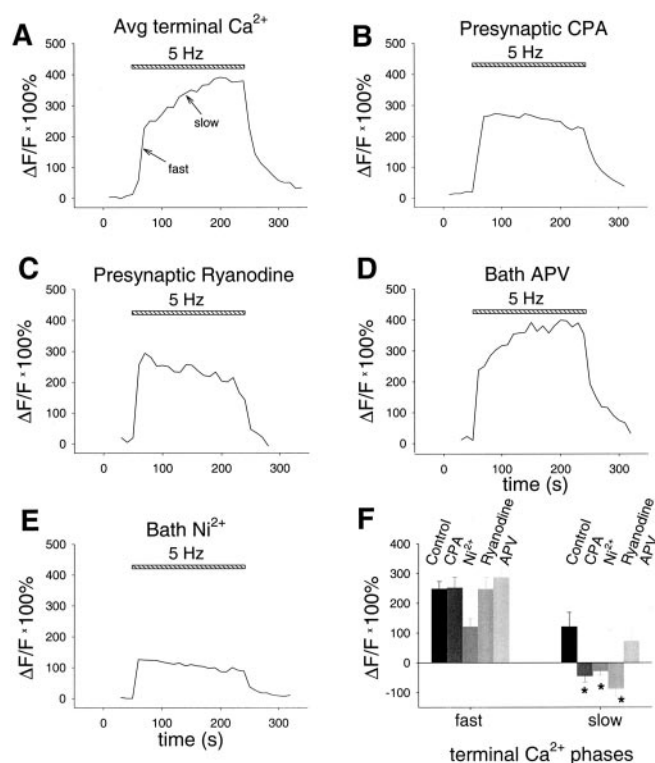


Figure 5. Slow phase of Ca^{2+} signal depends on release from presynaptic ryanodine-sensitive stores, and the fast phase depends partially on influx via T-type or R-type VGCCs. *A*, Average presynaptic terminal Ca^{2+} rise during LTD induction protocol ($n = 11$). Arrows point to fast and slow phases. Selective presynaptic application of CPA ($30 \mu\text{M}$; $n = 15$; *B*) or ryanodine ($300 \mu\text{M}$; $n = 5$; *C*) selectively inhibits the slow phase. *D*, Bath application of *D*-APV ($50 \mu\text{M}$) does not affect either the fast or slow phase ($n = 3$). *E*, Bath application of Ni^{2+} ($50 \mu\text{M}$) prevents the slow phase and partially inhibits the fast phase ($n = 4$). *F*, Summary data from all terminal imaging experiments. The asterisk indicates condition that is significantly different from control (*post hoc* Fisher LSD test; $p < 0.05$).

reduced the initial fast phase of the Ca^{2+} signal by $\sim 50\%$ ($121 \pm 27\%$ increase; $n = 4$; $p < 0.05$) and completely blocked the slow rise in Ca^{2+} , once again converting it to a slow decline ($28 \pm 14\%$ decline; $n = 4$; $p < 0.05$) (Fig. 5*E,F*).

Presynaptic Ca^{2+} stores do not contribute to maintained transmitter release during induction of LTD

What role might Ca^{2+} release from presynaptic stores play in the induction of LTD? It is generally agreed that release from Ca^{2+} stores does not contribute to transmitter release evoked by single APs with low-frequency (~ 0.1 Hz) stimulation (Reyes and Stanton, 1996; Emptage et al., 2001; Carter et al., 2002). However, it has been suggested that Ca^{2+} release from internal stores may be involved in short-term synaptic plasticity observed at higher stimulus frequencies (Emptage et al., 2001) (but see Carter et al., 2002). Therefore, Ca^{2+} release from presynaptic stores may be necessary for maintaining appropriate levels of transmitter release required to produce sufficient postsynaptic activation to induce LTD during the 5 Hz stimulation protocol.

To test this idea, we measured EPSP size during the 5 Hz protocol, either in the presence or absence of antagonists of Ca^{2+} release (Fig. 6*A₁–A₃*). Even in the absence of antagonists, there is a progressive decline in the EPSP amplitude to a steady-state level during the 5 Hz stimulation, presumably resulting from short-term depression because of synaptic vesicle depletion. We characterized several properties of the postsynaptic response during 5

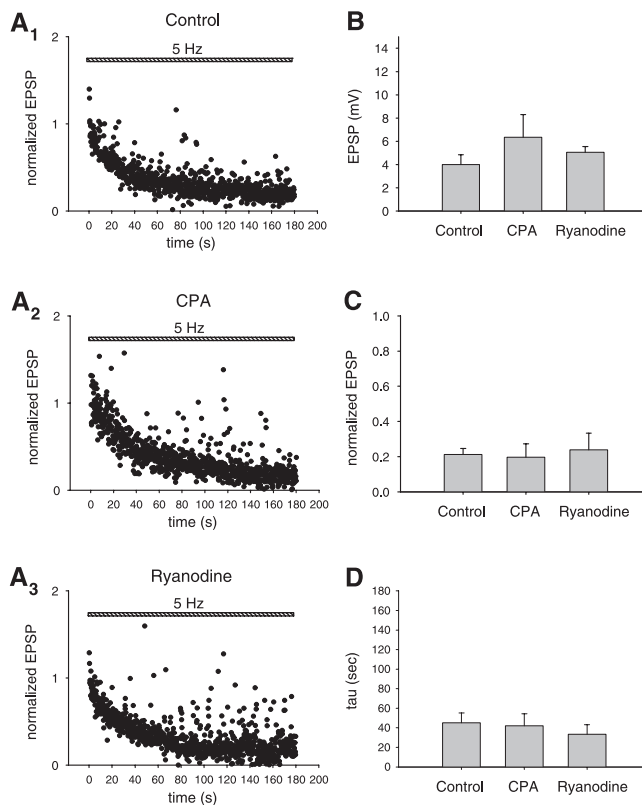


Figure 6. Inhibition of ryanodine-sensitive Ca^{2+} stores does not reduce postsynaptic depolarization during LTD induction. *A₁–A₃*, Average (normalized) peak EPSP during LTD induction protocol (striped bar): *A₁*, control conditions; *A₂*, in the presence of external CPA ($2\text{--}3 \mu\text{M}$); *A₃*, in the presence of external ryanodine ($10 \mu\text{M}$). *B*, Size of initial EPSP (average of first 4 responses) during LTD protocol under the three conditions shown in *A* (ANOVA; $p > 0.40$). *C*, Normalized size of steady-state EPSP size at the end of the train (average of last 50 responses) during LTD protocol (ANOVA; $p > 0.90$). *D*, Time constant of decay (τ) of EPSP size during LTD induction protocol (ANOVA; $p > 0.83$).

Hz stimulation, including the initial EPSP size, final EPSP size, and time constant of decay of the EPSP. Neither ryanodine nor CPA had any effect on synaptic transmission as measured by these three criteria (Fig. 6*B–D*). These data suggest that ryanodine-sensitive Ca^{2+} stores are not required for maintenance of transmitter release during the induction of LTD; rather, these stores must act at a step downstream from the depolarization and activation of the postsynaptic cell.

LTD expression involves a decrease in presynaptic release that is dependent on ryanodine-sensitive stores

The results from our paired recording and imaging experiments argue that presynaptic ryanodine-sensitive stores are required for a slowly rising phase of Ca^{2+} in the presynaptic terminal that is necessary for the induction of LTD. Given the localization of this slow Ca^{2+} signal, we next asked whether the expression of CA3–CA3 LTD might have a presynaptic component that requires ryanodine-sensitive stores for its induction. Here, we used the styryl dye FM 1-43, a marker of synaptic vesicle cycling dynamics (Betz and Bewick, 1992; Ryan et al., 1993), to track changes in presynaptic function that may be associated with NMDAR-dependent LTD.

We followed a loading protocol similar to one used previously to investigate presynaptic changes at CA3–CA1 synapses in acute hippocampal slices during LTP (Zakharenko et al., 2001) and during a form of LTD dependent on mGluRs (Zakharenko et al.,

FM 1-43 destaining from presynaptic terminals

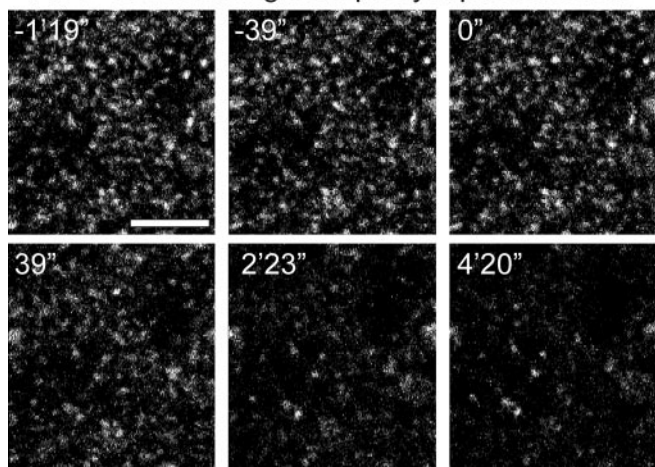


Figure 7. Activity-dependent destaining of FM 1-43 from presynaptic CA3 terminals. Images show FM 1-43 staining for one field of fluorescent puncta that had been previously loaded with dye. Time stamps are marked with reference to the beginning of the 1.5 Hz destaining stimulation, beginning at time 0" and continuing for 4'20". Scale bar, 10 μ m.

2002). FM 1-43 was first loaded into presynaptic terminals by applying the dye (10 μ M) in the bath solution during synaptic stimulation (10 Hz for 2 min), which triggers cycles of exocytosis and endocytosis that results in dye uptake into synaptic vesicles. The dye-loading stimulation was performed in the presence of D-APV (50 μ M) to block induction of synaptic plasticity at this time. After bath application of the cyclodextrin ADVASEP-7 to remove external dye that is nonspecifically bound to tissue (Kay et al., 1999), brightly fluorescent puncta were visible that corresponded to presynaptic terminals loaded with FM 1-43 (Fig. 7). Presynaptic terminals were then stimulated at 1.5 Hz in the absence of FM 1-43 (but in the presence of D-APV and ADVASEP-7) to unload dye through triggered exocytosis (Fig. 7). Previous studies showed that the rate of dye unloading provides a measure of the efficacy of transmitter release under these conditions (Zakharenko et al., 2001, 2002).

After induction of LTD with the 5 Hz stimulation protocol (no APV present), the rate of puncta destaining in the CA3 region of slice cultures was significantly decreased (Fig. 8A), consistent with a reduction in the rate of transmitter release. Bath application of D-APV (50 μ M) during the LTD stimulation protocol, which blocks the induction of LTD (Fig. 1D), also blocked the decrease in FM 1-43 destaining rate (Fig. 8B). To determine whether the induction of this presynaptic change in FM 1-43 release kinetics required Ca²⁺ release from presynaptic RyR-dependent stores, we delivered the LTD induction protocol in the presence of ryanodine (10 μ M in the bath solution). Under these conditions, both the induction of LTD and the depression of FM 1-43 release were blocked (Fig. 8C). These results demonstrate that NMDAR-dependent LTD at CA3–CA3 synapses involves altered presynaptic function, which requires Ca²⁺ release from ryanodine-sensitive stores.

Discussion

Our study reveals that the induction of LTD at CA3–CA3 synapses in the hippocampus requires presynaptic ryanodine-sensitive Ca²⁺ stores. Given the role for such stores during the induction of LTD at other hippocampal synapses (Reyes and Stanton, 1996; Wang et al., 1997; Caillard et al., 2000; Nishiyama et al., 2000), our results indicate the conservation of mechanisms

of LTD induction at diverse synapses. Dual whole-cell recordings provided direct evidence that the ryanodine-sensitive stores are required only in the presynaptic neuron and not in the postsynaptic neuron during LTD induction. Calcium imaging experiments demonstrated that the LTD induction protocol recruits a slowly rising component of the presynaptic Ca²⁺ signal that is attributable to calcium release from ryanodine-sensitive stores. Moreover, this signal is necessary for the induction of LTD and the expression of presynaptic changes in vesicle cycling associated with LTD. It is important to point out, however, that our results do not preclude a role for Ca²⁺ release from ryanodine-insensitive stores in the postsynaptic cell, for example because of IP₃R_s in the induction of LTD at these synapses.

Perhaps the simplest model that is consistent with our data are that LTD requires CICR from presynaptic Ca²⁺ stores through activation of RyRs themselves. Alternatively, the slow phase of the presynaptic Ca²⁺ signal may actually result from presynaptic IP₃R-dependent Ca²⁺ release. In this case, the inhibitory effect of ryanodine would be explained if there were a transient opening of the RyRs during the onset of ryanodine application that depleted the IP₃R-dependent Ca²⁺ stores because of the fact that RyRs and IP₃R_s can coexist in the same stores (Zacchetti et al., 1991; Wang et al., 1995). Finally, release from internal stores may require a cooperative interaction between RyRs and IP₃R_s (Ashby et al., 2003). According to this model, blockade of either type of release channel would abolish the slow phase.

Our attempts to directly assess the role of IP₃-sensitive stores in the induction of LTD were inconclusive. The IP₃R [and store-operated channel (SOC)] antagonist 2-aminoethoxydiphenyl borate (2-APB) (Maruyama et al., 1997; Ascher-Landsberg et al., 1999) was toxic to neurons in our slice cultures (concentrations from 5 to 250 μ M). Although we found that the IP₃R antagonist xestospongine C (10 μ M) did not block LTD at this synapse ($n = 2$; data not shown), the lack of adequate positive controls make this result difficult to interpret. However, our finding that group I mGluRs, the type most commonly linked to IP₃ production, do not participate in this form of LTD suggests that IP₃R-dependent Ca²⁺ release may not be involved here. This is further consistent with an electron-microscopic study showing a much higher level of expression of RyRs compared with IP₃R_s in axons of hippocampal neurons (Sharp et al., 1993). Thus, we believe that the simplest interpretation of our experiments is that induction of LTD requires CICR from presynaptic stores mediated by the RyRs themselves.

The slow time course of the Ca²⁺ signal associated with release from ryanodine-sensitive stores during the induction of LTD is puzzling because the rise in Ca²⁺ in response to CICR through RyRs is usually quite rapid (Bers, 2002). The RyRs or ryanodine-sensitive stores in CA3 terminals may exist initially in a nonfunctional state and may slowly activate during the 3 min period of synaptic stimulation during LTD induction. Kuba and colleagues (Narita et al., 1998, 2000) have reported a similar slow ryanodine-sensitive Ca²⁺ rise in presynaptic terminals of frog motoneurons during a 20–50 Hz train of stimulation, which they attribute to a "priming" effect of the tetanic stimulation. Liang et al. (2002) have imaged Ca²⁺ in hippocampal mossy fiber presynaptic terminals during stimulation with short trains of APs. They also suggest that regulation of presynaptic ryanodine-sensitive stores might explain the variable presence of a RyR-dependent delayed component to the Ca²⁺ signal measured in these terminals.

One possibility is that the stores are initially empty and then slowly fill as Ca²⁺ enters the terminal through VGCCs during the

5 Hz stimulation. Such an effect would be similar to the slow filling of cardiac Ca^{2+} stores that underlies a slow increase in cardiac contractility (staircase phenomenon) during a tetanus (Brotto and Creazzo, 1996; Huser et al., 1996). Alternatively, there may be a slow modulation of the RyRs themselves. Two particularly intriguing candidate modulators are nitric oxide and cyclic ADP-ribose, both of which are known to alter RyR function (Zucchi and Ronca-Testoni, 1997). Moreover, pharmacological blockade of either pathway inhibits the induction of LTD at CA3–CA1 synapses (Reyes-Harde et al., 1999). Differences in the basal state of RyR function could explain the conflicting results from different studies arguing either for (Emptage et al., 2001) or against (Carter et al., 2002) a role for store-mediated pre-synaptic Ca^{2+} release at pyramidal neuron synapses.

It is also possible that the slow Ca^{2+} signal is not directly attributable to release from RyR-sensitive stores. Rather, pharmacological interference with RyR-dependent store function might lead to secondary changes in Ca^{2+} signaling. For example, depletion of Ca^{2+} from RyR-sensitive internal stores during the 5 Hz stimulation may trigger the opening of plasma membrane SOCs, which could give rise to a slow Ca^{2+} signal (Bouron, 2000; Putney et al., 2001). We have tried to test this hypothesis with the SOC inhibitor 2-APB (Maruyama et al., 1997; Ascher-Landsberg et al., 1999), but, as reported above, this compound was toxic to our slice cultures at relevant concentrations. A second possibility is that the slow Ca^{2+} signal is attributable to release from mitochondrial stores (Babcock and Hille, 1998; Kaczmarek, 2000), the filling of which is indirectly affected by inhibition of RyR-sensitive stores (perhaps because of changes in resting Ca^{2+} levels).

What is the function of the slow ryanodine-sensitive rise in Ca^{2+} ? One potential clue is that the time course of this slow signal parallels the time course of LTD induction during the 3 min period of 5 Hz stimulation. Shorter periods of stimulation (e.g., 30 sec), which recruit only a small RyR-dependent Ca^{2+} signal, induce LTP (Winder et al., 1999), not LTD. Thus, the slow Ca^{2+} rise could serve as an integrator of synaptic activity, informing the presynaptic terminal as to the history of its activity over a time scale of tens of seconds to minutes. The importance of presynaptic signaling is underscored by our finding that LTD is associated with a diminished rate of FM 1-43 release from presynaptic terminals. Given the requirement of postsynaptic NMDAR activation for LTD induction, these results indicate the need for a retrograde messenger that conveys a postsynaptic signal to the presynaptic terminal. In this context, the RyR-dependent slow phase of $[\text{Ca}^{2+}]_i$ could act as a gate that restricts the action of the retrograde message to those terminals that are coactivated for prolonged periods with postsynaptic NMDAR stimulation. This may explain how a single retrograde messenger, such as nitric

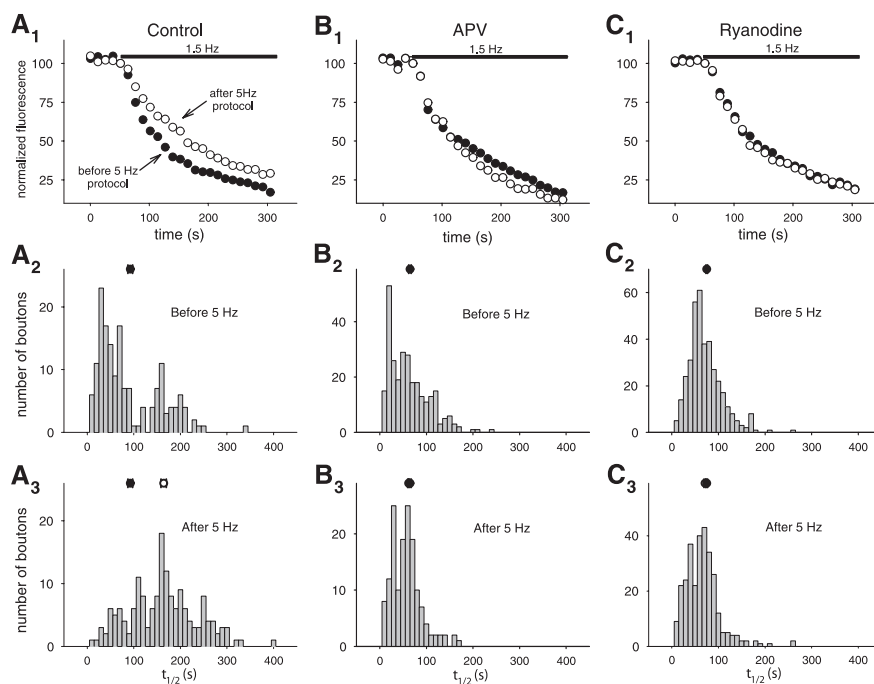


Figure 8. LTD expression involves a decrease in presynaptic FM 1-43 release that depends on ryanodine-sensitive stores. *A*₁, Decay of average fluorescence intensity of puncta during unloading of FM 1-43 with 1.5 Hz stimulation before (filled circles) and after (open circles) the 5 Hz LTD induction protocol ($n = 7$ slices). The black bar represents duration of 1.5 Hz unloading stimulation. *A*₂, Frequency histogram of unloading halftimes ($t_{1/2}$) for individual puncta before the LTD protocol. The filled circle on top shows the mean (and SEM) of the distribution ($n = 165$ puncta). *A*₃, Frequency histogram of halftimes after the LTD induction protocol (significantly different from distribution before 5 Hz stimulation; Kolmogorov–Smirnov 2-sample test; $p < 0.001$). The open circle shows the mean of the distribution ($n = 168$ puncta). The filled circle shows the mean of the distribution from *A*₂. *B*₁, Unloading of FM 1-43 when the LTD protocol was given in the presence of D-APV (50 μM ; $n = 4$ slices). *B*₂, Unloading half-time and the mean of the distribution (filled circle) before the LTD induction protocol ($n = 280$ puncta). *B*₃, Unloading time and the mean of the distribution (open circle) after the LTD induction protocol (Kolmogorov–Smirnov test; $p > 0.05$; $n = 150$ puncta). The filled circle shows the mean of the distribution from *B*₂. Note that the open and filled circles overlap. *C*₁, Unloading of FM 1-43 when the LTD protocol was given in the presence of ryanodine (10 μM ; $n = 4$ slices). *C*₂, Half-time ($t_{1/2}$) of unloading and the mean of the distribution (filled circle) before the LTD protocol ($n = 374$ puncta). *C*₃, Half-time and the mean of the distribution (open circle) after the LTD protocol (Kolmogorov–Smirnov test; $p > 0.05$; $n = 297$ puncta). The filled circle shows the mean of the distribution from *C*₂.

oxide, could mediate both LTP (Arancio et al., 1996) and LTD (Reyes-Harde et al., 1999): only LTD-inducing patterns of stimulation would produce a sufficiently prolonged period of presynaptic activity to elicit a sufficiently large ryanodine-sensitive Ca^{2+} signal to induce LTD, perhaps by gating the response of the presynaptic terminal to nitric oxide, producing depression rather than potentiation. [This explanation may not apply to forms of spike-timing-dependent LTD induced by shorter periods of stimulation (Nishiyama et al., 2000)]. It is unlikely that the slow presynaptic Ca^{2+} signal is itself generated by the retrograde messenger because the slow Ca^{2+} rise was not blocked by inhibition of NMDARs with D-APV (Fig. 5D).

The RyR-dependent presynaptic Ca^{2+} signal may also act to increase the synapse specificity of homosynaptic LTD. A freely diffusible retrograde messenger could impinge on both presynaptic terminals that are activated during the induction of plasticity and presynaptic terminals that are not active during the induction stimulation. If induction of presynaptic LTD required the conjoint action of the retrograde message with the slow Ca^{2+} signal, LTD would be restricted to only those synapses that were active during its induction. Recently, Nishiyama et al. (2000) proposed a similar role for postsynaptic RyR-sensitive stores because inclusion of ryanodine in the postsynaptic electrode blocked homosynaptic LTD in CA1 neurons induced by spike

timing and resulted in the appearance of heterosynaptic LTD. Our results and those of Stanton et al. (2001, 2003), demonstrating a presynaptic component to NMDAR-dependent LTD, raise the interesting question as to its behavioral relevance. Selective deletion of RyRs from presynaptic neurons using region-specific knock-outs would provide a potentially powerful system for exploring the cellular and behavioral consequences of this regulatory pathway.

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