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Induction of Hippocampal Long-Term Depression Requires Release of Ca^{2+} from Separate Presynaptic and Postsynaptic Intracellular Stores

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Studies have suggested that an increase in intracellular [Ca^{2+}] is necessary for the induction of both long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission, and that release of Ca^{2+} from intracellular storage pools can be necessary to induce LTP. We investigated whether release of Ca^{2+} from intracellular stores also is required for the induction of LTD at Schaffer collateral–CA1 synapses in hippocampal slices. Both thapsigargin (1 μM) and cyclopiazonic acid (1 μM), compounds that deplete all intracellular Ca^{2+} pools by blocking ATP-dependent Ca^{2+} uptake into intracellular compartments, blocked the induction, but not maintenance, of LTD by low-frequency stimulation (LFS) (1 Hz/15 min) without affecting baseline synaptic transmission. Washout of the reversible inhibitor cyclopiazonic acid restored the ability to induce LTD. In contrast, thapsigargin did not block *depotentiation* of LTP by 1 Hz LFS, suggesting that LTP causes a reduction in the threshold [Ca^{2+}] necessary for LTD. Selective depletion of the ryanodine receptor-gated Ca^{2+} pool by bath application of ryanodine (10 μM) also blocked the induction of LTD, indicating a requirement for Ca^{2+}-induced Ca^{2+} release. Impalement of CA1 pyramidal neurons with microelectrodes containing thapsigargin (500 nM to 200 μM) prevented the induction of LTD at synapses on that neuron without blocking LTD in the rest of the slice. In contrast, similar filling of CA1 pyramidal neurons with ryanodine (2 μM to 5 mM) did not block the induction of LTD. From these data, we conclude that the induction of LTD requires release of Ca^{2+} both from a *presynaptic* ryanodine-sensitive pool and from *postsynaptic* (presumably IP_3-gated) stores.

Key words: calcium; CA1; cyclopiazonic acid; hippocampus; inositol triphosphate; learning and memory; long-term depression; ryanodine; synaptic plasticity; thapsigargin

Synapses in hippocampus and neocortex are capable of exhibiting both long-term potentiation (LTP) and long-term depression (LTD) of synaptic strength, depending on patterns of synaptic activation. Although LTP is the more studied phenomenon, there is growing interest in the roles LTD may play in bidirectional regulation of synaptic strength and in the cellular mechanisms of both the induction and expression of LTD (Stanton, 1996). In studies to date, relatively high levels of presynaptic activity that are coupled with postsynaptic *inactivity* were necessary to induce LTD (Stanton and Sejnowski, 1989; Artola et al., 1990; Stäubli and Lynch, 1990; Mulkey and Malenka, 1992), in contrast to the Hebbian pairing of strong presynaptic and postsynaptic activity needed for LTP (Gustaffson et al., 1987).

Two forms of long-lasting synaptic depression can be induced by single-pathway stimulation. One is a *de novo* LTD from baseline synaptic strength, whereas the second is a stimulus-induced reversal of LTP, which has been called depotentiation. Prolonged low-frequency stimulation (LFS) (1Hz/15 min) is one method that reliably elicits *de novo* LTD in area CA1 of hippocampal slices, especially in relatively young animals (<30 d old) (Dudek and Bear, 1993; Velíšek et al., 1993; Bolshakov and Siegelbaum, 1994). Depotentiation LTD has been elicited by the same LFS given 30–60 min after the induction of LTP (Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Wexler and Stanton, 1993) and also by shorter, theta frequency stimuli (5 Hz/1 min) given within 15 min of the induction of LTP (Stäubli and Lynch, 1990; Stäubli et al., 1995). It has been demonstrated that this latter stimulus paradigm is not capable of eliciting *de novo* LTD and is only effective in causing depotentiation within a rather brief 15 min time window after LTP. This raises the question of whether these two forms of LTD are mediated by distinct cellular mechanisms or whether they represent the same phenomenon induced to varying degrees by different stimulus paradigms.

Recent work has found that, similar to LTP, the induction of LTD is dependent on an increase in postsynaptic intracellular [Ca^{2+}] (Mulkey and Malenka, 1992) and on the activation of both NMDA (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Wexler and Stanton, 1993) and metabotropic subtypes of glutamate receptors (Stanton et al., 1991; Bashir et al., 1993; Kato, 1993; Wexler and Stanton, 1993; O'Mara et al., 1995). Because some metabotropic glutamate receptors are coupled via G-proteins to inositol triphosphate (IP_3)-triggered release of Ca^{2+} from intracellular stores in endoplasmic reticulum, it is a reasonable, but untested, hypothesis that Ca^{2+} release from these stores is necessary for the induction of LTD. However, there is a second, distinct pool of intracellularly stored Ca^{2+} that is released by caffeine and by the plant alkaloid ryanodine, and also mediates Ca^{2+}-triggered Ca^{2+} release (Fleischer and Inui, 1989; McPherson et al., 1991).

To examine the necessity of these intracellular Ca^{2+} stores for the induction of LTD, we used thapsigargin and cyclopiazonic acid, two inhibitors of endosomal Ca^{2+}-ATPase activity that deplete all intracellular Ca^{2+} stores (Seidler et al., 1989; Thastrup et al., 1990), as well as ryanodine to selectively deplete just the Ca^{2+}-activated pool, to determine their ability to impair the induction of both *de novo* LTD and depotentiation. In addition, we used intracellular infusion of thapsigargin or ryanodine into

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single CA1 pyramidal neurons to assess which *postsynaptic* Ca^{2+} pools are needed to induce LTD. We report here that release of Ca^{2+} from *presynaptic* ryanodine pools and from *postsynaptic* IP_3-gated stores appears to be conjointly necessary for the induction of LTD at Schaffer collateral synapses in hippocampal field CA1.

MATERIALS AND METHODS

Experiments were performed on 400-μm-thick transverse hippocampal slices obtained from male Sprague Dawley rats (21 ± 2 d old). Slices were cut simultaneously using a spring-loaded mechanism that rapidly forced a parallel grid of 20-μm-diameter wires through the tissue. They were placed in an interface recording chamber at 34°C and perfused at a rate of 3 ml/min with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126, $NaHCO_3$ 26, NaH_2PO_4 1.25, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, D-glucose 10 (bubbled with 95% O_2/5% CO_2), pH 7.4. Two separate inputs of Schaffer collateral–commissural axons were isolated by placing stimulated electrodes on opposite sides of the recording site, verified as separate inputs by a lack of paired-pulse interactions, and alternately stimulating each 30 sec throughout the experiment with a bipolar stainless steel electrode (25 μm tip diameter, Frederick Haer) delivering 150 μsec pulses. Evoked EPSPs (50% of maximum amplitude, 1–2 mV) were recorded in the apical dendritic field in stratum radiatum for a stable baseline period of at least 30 min, and then *de novo* LTD was induced by a 1 Hz/15 min train of LFS. In depotentiation experiments, LTP first was induced by two sets of high-frequency theta burst stimulus trains (HFS) (four trains of 100 Hz/5 pulse bursts spaced 200 msec apart, 10 bursts per train) applied at 5 min intervals. Thereafter, depotentiation LTD was induced in one of two ways: either the same 1 Hz/15 min LFS train was given 60 min after the second HFS or two 5 Hz/1 min theta frequency trains were applied 10 and 15 min after HFS, respectively. In some slices in each group, a second synaptic pathway that did not receive any LFS or HFS stimulation was monitored to verify that LTD was homosynaptic. In experiments in which thapsigargin was bath applied, control slices (Fig. 1*A*) came from the same rats and were recorded from in a paired manner to ensure that these slices were capable of exhibiting LTD.

Intracellular evoked EPSPs in CA1 pyramidal neurons (RMP = −64 ± 1.2 mV; R_N = 45.8 ± 1.7 MΩ) impaled with sharp microelectrodes (80–130 MΩ, 2 M K-acetate) were recorded using an amplifier with an active bridge balance circuit (AxoClamp 2A). Initial slope of the EPSP (V/sec) was measured to assess changes in excitatory synaptic strength and each experiment normalized to its pre-LFS baseline amplitude for comparison across experiments. To determine whether LTD had been induced, the five EPSPs immediately preceding LFS were averaged and compared with the mean of five EPSPs starting 60 min after LFS, using a paired *t* test. In all intracellular experiments, a second synaptic input that did not receive LFS was monitored to ensure that intracellular LTD was homosynaptic. Stock solutions of thapsigargin, cyclopiazonic acid, and ryanodine (RBI) were dissolved in dimethyl sulfoxide (DMSO) and diluted either in ACSF for bath application (1–10 μM in 0.05% DMSO) or in 2 M K-acetate for intracellular infusion (500 nM to 2 μM in 0.025% DMSO; 200 μM to 5 mM in 5% DMSO). Intracellular and extracellular control experiments used equal concentrations of DMSO vehicle alone, and expression of LTD was unaffected by this vehicle at any concentration used.

For intracellular dye filling experiments, electrodes were back filled with a solution containing 5 mM ryanodine in 5% DMSO, 5 mM Lucifer yellow (Sigma; Li^+ salt), and 250 mM LiCl as the primary electrolyte (100–150 MΩ). The normal experimental protocol for testing blockade of LTD by ryanodine was used, consisting of a 30 min pre-experiment infusion period, followed by 90 min total experimental time, during which dye and drug continued to leak into the cell. Slices then were transferred to a second perfusion chamber mounted on an upright fluorescence microscope (Zeiss Axioskop) and imaged using an excitation wavelength of 470 ± 35 nm, a long-pass emission filter wavelength of 515 nm, and a dichroic wavelength of 505 nm. Images were viewed with a 40× water-immersion Achroplan objective (Zeiss) and digitized with a Photometrics charged-coupled device camera, and background fluorescence was subtracted off-line using IPLab image analysis software.

RESULTS

Figure 1 illustrates our initial experiments to determine whether depletion of intracellular Ca^{2+} stores impairs the ability to induce LTD at Schaffer collateral–commissural synapses in field CA1 of hippocampal slices. In control slices (Fig. 1*A, n* = 5), LFS (1 Hz/15 min, *solid bar*) elicited marked LTD of synaptic transmission (−39.5 ± 9%, each point is the mean ± SEM, $p < 0.05$, paired *t* test, compared with pre-LFS baselines), which was confined to the stimulated input (control input change = −3.7 ± 1.9%). The insets in Figure 1, *A* and *B*, illustrate representative Schaffer collateral-evoked extracellular EPSPs recorded in one slice 15 min before and 30 min after the application of LFS and, in the control slice, show a marked LTD of both initial negative slope and EPSP amplitude.

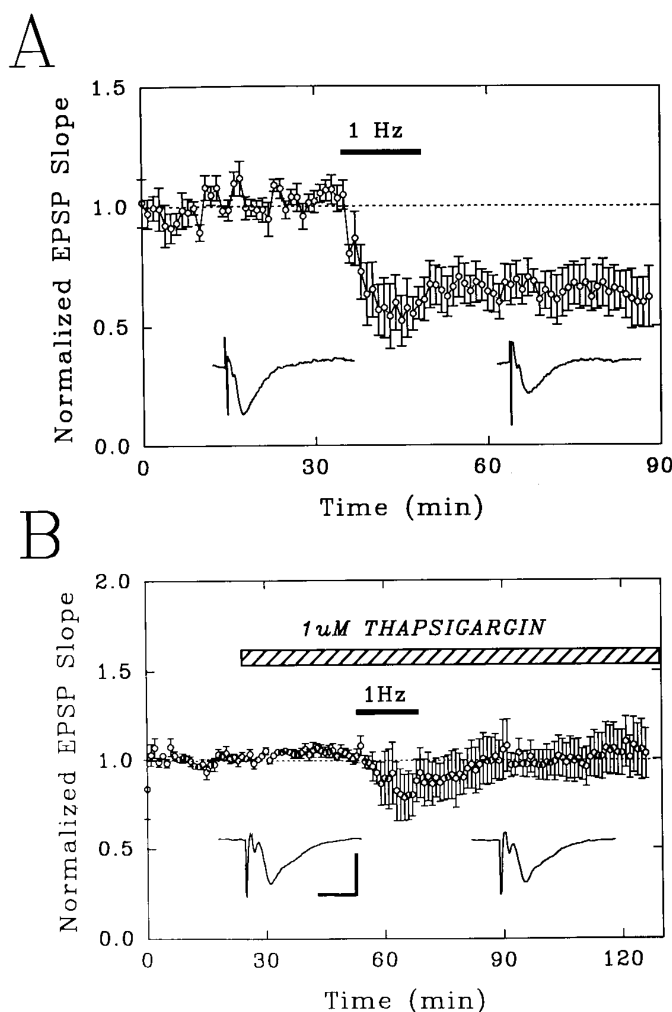


Figure 1. Depletion of intracellular Ca^{2+} stores with thapsigargin blocks induction of LTD. *A*, Time course of LTD of Schaffer collateral–CA1 synaptic transmission in untreated, control hippocampal slices induced by LFS (1 Hz/15 min, *solid bar*). *Inset traces* are averages of four evoked EPSPs recorded in stratum radiatum immediately before (*left trace*) and 30 min after (*right trace*) LFS showing marked LTD of synaptic transmission ($p < 0.05$ for all slices, compared with pre-LFS baseline slopes). A second, independent control input that did not receive LFS did not exhibit any persistent change in synaptic strength (data not shown). Each point is the mean ± SEM of five slices. *B*, Bath application of thapsigargin (1 μM, *hatched bar*), which depletes intracellular Ca^{2+} stores, 30 min before LFS (1 Hz/15 min, *solid bar*) completely blocked the induction of LTD at Schaffer collateral–CA1 synapses ($p > 0.20$, compared with pre-LFS baseline slopes). *Inset traces* are averages of four evoked EPSPs before (*left trace*) and 30 min after (*right trace*) LFS. Each point is the mean ± SEM of six slices. (Calibration: 2 mV/5 msec, same for all traces.)

In separate slices from the same rats recorded from in parallel,

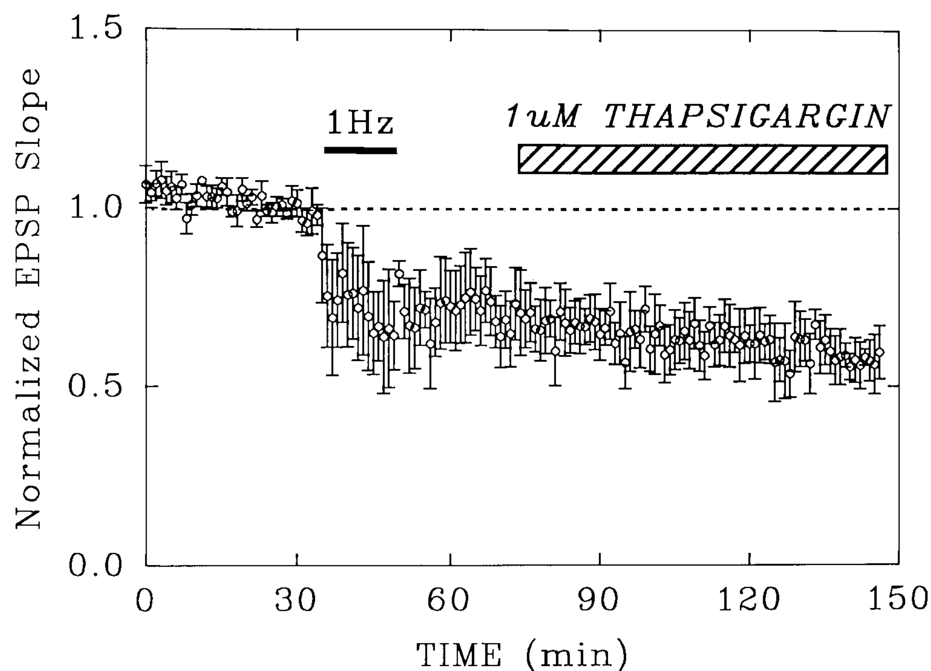


Figure 2. Thapsigargin does not affect the maintenance of LTD. Time course of experiments (n = 5) in which thapsigargin (1 μM, *hatched bar*) was bath-applied 30 min *after* the induction of LTD. Schaffer collateral–commissural LFS was given first (1 Hz/15 min, *solid bar*) and induced significant LTD of synaptic transmission. Thirty minutes after the end of LFS, thapsigargin was bath-applied and remained throughout the experiment. In contrast to induction, maintenance of LTD was unaffected by depletion of intracellular Ca^{2+} stores with thapsigargin. (Each point is the mean ± SEM.)

bath application 30 min before LFS of 1 μM thapsigargin (Fig. 1*B*, *hatched bar*), a concentration that has been reported previously to block the induction of LTP in CA1 (Harvey and Collingridge, 1992), had no effect on baseline Schaffer collateral synaptic transmission. It did, however, block the induction of LTD by LFS (1 Hz/15 min, *solid bar*) in all six slices tested (1.5 ± 11%, $p > 0.20$, paired t test, compared with pre-LFS baseline). These results are consistent with a requirement for the release of Ca^{2+} from intracellular stores as one component in the induction of LTD by LFS. In contrast to induction, in slices in which thapsigargin was bath applied 30 min after establishment of LTD (Fig. 2, *hatched bar*, n = 5), it did not affect the maintenance phase of LTD. Thus, induction, but not maintenance, of LTD by LFS was blocked by depleting intracellular Ca^{2+} stores with thapsigargin.

We also tested cyclopiazonic acid, a second compound that depletes intracellular Ca^{2+} by *reversibly* inhibiting the Ca^{2+}-ATPase in the endoplasmic reticulum (Seidler et al., 1989) for its ability to prevent the induction of LTD. Figure 3 illustrates these experiments (n = 6) in which 1 μM cyclopiazonic acid (*hatched bar*) was bath applied 30 min before LFS. In the presence of cyclopiazonic acid, the first LFS (1 Hz/15 min, *first solid bar*) of Schaffer collateral–commissural axons failed to induce significant LTD of synaptic transmission (−7 ± 6% change from pre-LFS baseline). Fifteen minutes after the end of LFS, cyclopiazonic acid was washed out for 30 min, a time period twice as long as that shown previously to be sufficient for >80% refilling of calcium stores (Janssen and Sims, 1993; Maggi et al., 1995), and a second, identical LFS (1 Hz/15 min, *second solid bar*) was applied. After drug washout, the second LFS now evoked marked LTD of synaptic transmission (−48.1 ± 7.5%; $p < 0.05$, paired t test, compared with pre-LFS baseline EPSP slopes), which was confined to the stimulated input.

There is another form of LTD, known as depotentiation, which is the reversal of LTP by LFS (Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Wexler and Stanton, 1993). Although depotentiation has similarities to *de novo* LTD (Wexler and Stanton, 1993; Stanton, 1995), it still is unclear whether the two represent the same set of cellular phenomena (Stäubli et al., 1995; Wagner and Alger, 1995). Therefore, we also tested the requirement for intact intracellular Ca^{2+} stores in depotentiation LTD. Figure 4 summarizes experiments in which LTP was induced by two sets of HFS applied 15 min apart (*TET*, 100 Hz/5 pulse theta bursts, interburst interval 200 msec, 10 bursts/train × 2 trains). After short-term potentiation had decayed to a marked, stable LTP lasting 30 min, 1 μM thapsigargin was bath applied for an additional 30 min (*hatched bar*), and then LFS (1 Hz/15 min, *solid bar*) was given to the same synaptic input. In contrast to our findings with *de novo* LTD, marked depotentiation still could be induced in the presence of thapsigargin (−45 ± 12%, $p < 0.05$, paired t test, compared with pre-LFS baseline amplitudes, n = 5) and did not differ in amplitude from control depotentiation LTD (Fig. 4, *inset bars*, $p > 0.20$, Student's t test). These data suggest that after recent LTP, LTD can be induced without requiring the release of Ca^{2+} from intracellular stores.

Although the previous results suggest that depotentiation LTD does not require intact intracellular Ca^{2+} stores, studies by Stäubli and Lynch (1990) and Stäubli and colleagues (1995) found that stimuli at theta rhythm frequency (5 Hz) also can induce depotentiation of LTP, with some interestingly different properties. Unlike LFS-induced (1 Hz) depotentiation, theta depotentiation can be induced only within a brief 15 min time window after the induction of LTP. Therefore, we also tested the necessity for intracellular Ca^{2+} stores to the induction of depotentiation by theta frequency stimulation. Figure 5*A* summarizes data from control, untreated hippocampal slices in which LTP was induced in one Schaffer collateral–CA1 synaptic pathway (*TET*, *open triangle*) and followed by application of theta frequency stimulation (5 Hz/1 min, *solid triangles*). Under these conditions, 5 Hz theta stimulation produced marked depotentiation LTD (−38 ± 12%, $p < 0.05$, paired t test, compared with pre-theta, post-LTP baselines, n = 5). In contrast, Figure 5*B* illustrates five experiments in which 1 μM thapsigargin (*hatched bar*) was bath applied starting 1 min after the induction of LTP (*TET*) to avoid blocking LTP but to allow 10 min for thapsigargin to deplete intracellular Ca^{2+}

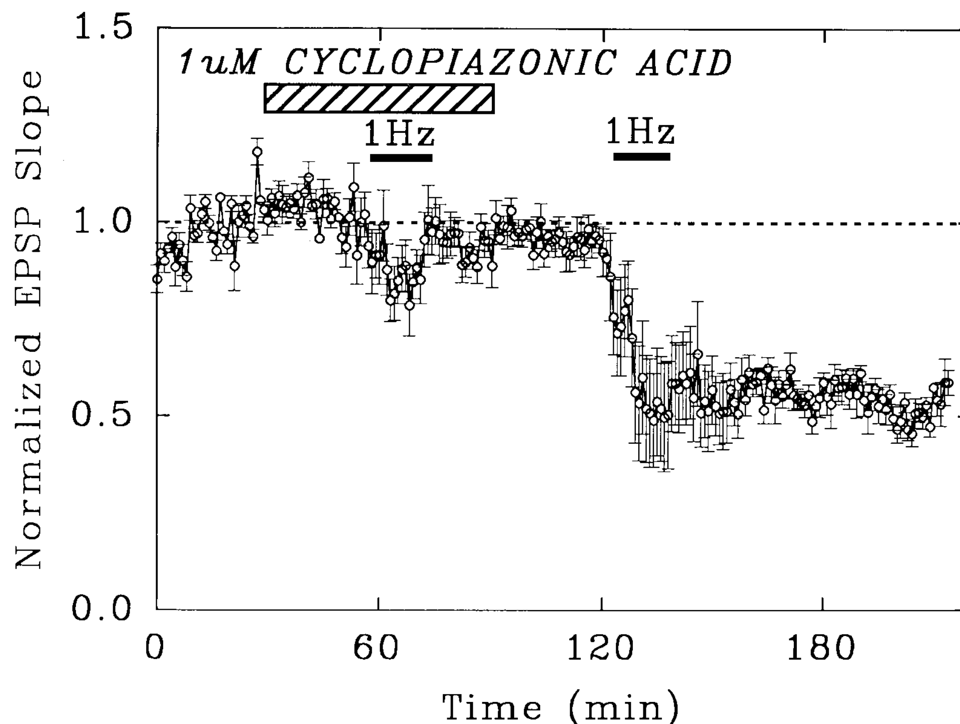


Figure 3. Cyclopiazonic acid, a reversible depleter of intracellular Ca^{2+} stores, reversibly blocks the induction of LTD. Time course of experiments (n = 6) in which cyclopiazonic acid (1 μM, *hatched bar*), a reversible endosomal Ca^{2+}-ATPase inhibitor, was bath-applied 30 min before application of LFS. In the presence of cyclopiazonic acid, the first LFS (1 Hz/15 min, *solid bar*) of Schaffer collateral–commissural axons failed to induce significant LTD of synaptic transmission. Fifteen minutes after the end of LFS, the inhibitor was washed out for 30 min, and a second, identical LFS (1 Hz/15 min, *second bar*) was applied. After drug washout, the second LFS now evoked marked LTD of synaptic transmission ($p < 0.05$, paired t test, compared with pre-LFS baseline EPSP slopes), which was confined to the stimulated input (control input data not shown). (Each point is the mean ± SEM.)

stores before theta stimulation. Theta depotentiation was blocked by thapsigargin (−5 ± 8%, n = 5), suggesting that, under some conditions, depotentiation can still require release of stored Ca^{2+}.

There are at least two separate intracellular Ca^{2+} pools, and release from these pools is gated by different second messengers. Release from one store is triggered by IP_3, whereas a second is activated by Ca^{2+} influx and selectively released by ryanodine and caffeine. We bath applied ryanodine to hippocampal slices to selectively deplete this second pool as a test of its necessity for the induction of LTD. Figure 6 summarizes the time course of these experiments (n = 5) in which ryanodine (10 μM, *hatched bar*) was bath applied to slices 30 min before the application of LFS. After depletion of ryanodine receptor-gated Ca^{2+} stores, LFS did not elicit any significant LTD of synaptic transmission measured 30 min after the end of LFS (−8 ± 8.6%, $p > 0.20$, paired t test, compared with pre-LFS baseline). Control LTD in paired recordings from slices from the same rats was −36.5 ± 7% ($p < 0.05$, paired t test). These data suggest that Ca^{2+}-activated Ca^{2+} release from ryanodine receptor-gated pools is necessary for the induction of LTD. However, they do not specify whether it is presynaptic and/or postsynaptic ryanodine stores that are involved.

Although the previous results suggest that release of Ca^{2+} from intracellular stores is necessary for the induction of LTD, they do not address the location of these stores. To determine whether *postsynaptic* Ca^{2+} stores are necessary to induce LTD, we included thapsigargin in intracellular microelectrodes (resistance 80–130 MΩ) and impaled pyramidal neurons in field CA1. Figure 7 illustrates the results of these experiments in which intracellular and extracellular Schaffer collateral-evoked EPSPs were recorded simultaneously. After impaling a CA1 pyramidal neuron, at least 30 min was allowed to permit diffusion of thapsigargin into the cell and to acquire baseline Schaffer collateral-evoked EPSP recordings. Soon after impalement, large evoked (and sometimes spontaneous) depolarizations and multiple action potentials typically were observed, which disappeared 5–10 min later. This is consistent with previous reports that these depleters cause a slow leakage into the cytoplasm of Ca^{2+} from intracellular stores, which transiently activates voltage-dependent calcium currents (Hoth and Penner, 1992; Luckhoff and Clapham, 1994) and is one independent indication that thapsigargin was entering the cell. Thirty minutes after stabilization of the impalement and baseline recording of Schaffer collateral-evoked EPSPs, LFS (1 Hz/15 min, *solid bar*) was applied to one synaptic input, while a second input served as control.

Figure 7*A* plots intracellular EPSP slopes over time in CA1 pyramidal neurons impaled with electrodes containing either 500 nM or 2 μM thapsigargin (n = 5). Infusion of thapsigargin into the postsynaptic neuron converted normal LTD into a slowly decaying depression that completely reversed by 1 hr after LFS. Because these concentrations of thapsigargin caused a much slower decay in LTD than observed in previous extracellular experiments, we also tested the effects of a 100-fold higher concentration of thapsigargin (200 μM) in the intracellular recording electrode (Fig. 7*B*, n = 6). Impalement and intracellular infusion of thapsigargin with the higher electrode concentration produced complete blockade of LTD and a much more rapid return to baseline EPSP amplitudes. In contrast, extracellular field EPSPs recorded simultaneously and pooled over all experiments (Fig. 7*C*, n = 11) exhibited robust, stable LTD that persisted throughout the recording period (−29.4 ± 5.5%, $p < 0.05$, paired t test, compared with pre-LFS baselines). The insets in each figure illustrate either intracellular or extracellular EPSP immediately before (*Pre-LFS*) and 60 min after (*Post-LFS*) the application of LFS. Taken together, our experiments support the conclusion that release of Ca^{2+} from *postsynaptic* stores is a necessary requirement for the induction of LTD by LFS.

Although the above experiments indicate a requirement for intact postsynaptic Ca^{2+} stores in the induction of LTD, they do not preclude an additional role for presynaptic stores, nor do they help to determine selective involvement of either ryanodine or

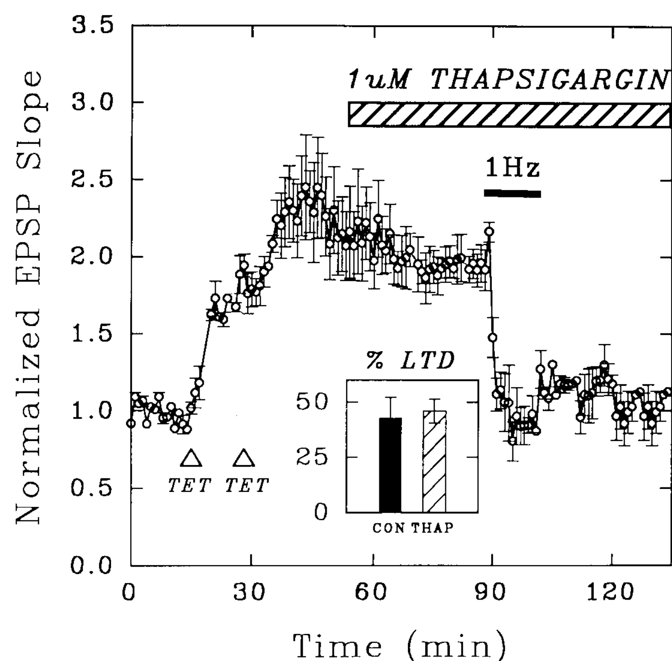


Figure 4. Thapsigargin does not block 1 Hz-induced depotentiation of established LTP. Time course of experiments (n = 5) in which saturating LTP was induced before depleting intracellular Ca^{2+} stores with bath-applied thapsigargin (1 μM, *hatched bar*). High-frequency theta burst stimulations (*TET*, trains of 100 Hz/5 pulse bursts at a 200 msec interburst interval) (see Materials and Methods) were applied twice 15 min apart, which induced marked LTP of Schaffer collateral-evoked EPSPs recorded in stratum radiatum. Thirty minutes after the second tetanus, 1 μM thapsigargin was bath-applied (*hatched bar*). After allowing an additional 30 min for thapsigargin to deplete intracellular Ca^{2+} stores, LFS (1 Hz/15 min, *solid bar*) was given to the same potentiated pathway and induced significant depotentiation LTD ($p < 0.05$ compared with pre-LFS baseline slopes). (Each point is the mean ± SEM.)

IP_3-gated calcium pools. To address these issues, we filled a separate group of CA1 pyramidal neurons with ryanodine, using the same experimental protocol as in the thapsigargin intracellular experiments. Even more so than with thapsigargin, inclusion of ryanodine in the intracellular recording electrode (concentration range 5 μM to 5 mM) transiently evoked strong multiple bursts of action potentials riding on large depolarizations, probably indicative of more rapid release of Ca^{2+} from the ryanodine-sensitive stores and activation of voltage-dependent calcium conductances. These potentials confirmed that ryanodine entered the neuron and disappeared in 5–15 min, after which the recording of baseline EPSPs commenced.

In Figure 8*A*, intracellular EPSPs are plotted from six pyramidal neurons impaled with a microelectrode containing 5 mM ryanodine, a 500-fold higher concentration than that which we bath applied to block LTD. In contrast to the effects of thapsigargin, infusion of the postsynaptic neuron with ryanodine did not prevent the induction of stable LTD by LFS (*solid bar*), which lasted over 1 hr after LFS (−45 ± 11%, $p < 0.05$, paired t test, compared with pre-LFS baselines). Corresponding extracellular EPSPs in the same slices (data not shown) exhibited LTD of population responses of a similar amplitude to intracellular LTD (−47 ± 12%, $p < 0.05$, paired t test, compared with pre-LFS baselines), and LTD amplitude in ryanodine-filled cells was not different from control intracellular LTD ($p > 0.20$, Student's t test). The lack of effect of postsynaptic ryanodine infusion leads us

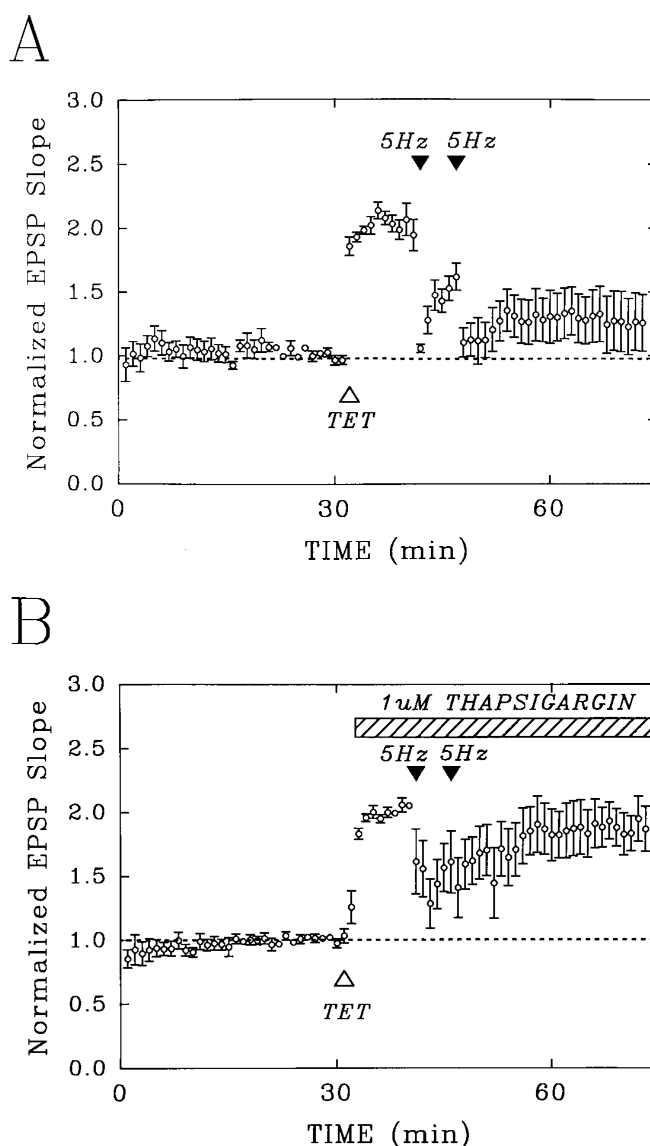


Figure 5. Thapsigargin does prevent 5 Hz theta frequency stimulation from depotentiating recently induced LTP. *A*, Time course of experiments demonstrating 5 Hz theta frequency depotentiation in control slices (n = 5). LTP was induced with high-frequency stimulation (*TET*), followed by theta frequency stimulation (5 Hz/1 min) 10 and 15 min after induction of LTP. Theta stimuli induced significant depotentiation LTD of this Schaffer collateral pathway ($p < 0.05$, paired t test comparing pre-LFS baseline EPSP slopes with 30 min post-LFS). (Each point is the mean ± SEM.) *B*, Identical experiments (n = 5) in which saturating LTP was induced at Schaffer collateral–CA1 synapses with high-frequency theta bursts as in Figure 5 (*TET*, 100 Hz/5 pulse bursts, 200 msec interburst interval), and thapsigargin (1 μM, *hatched bar*) was bath-applied starting 1 min after the second TET. Theta frequency stimulus trains (5 Hz/1 min) were given at 10 and 15 min after TET, and these stimuli were unable to induce depotentiation LTD of synaptic transmission.

to conclude that release of Ca^{2+} from a postsynaptic ryanodine-sensitive pool is *not* required to induce LTD.

However, although the transient appearance of presumed Ca^{2+}-activated depolarizations suggests effective drug infusion, our negative conclusion depends on assurance that ryanodine entered the postsynaptic neuron in sufficient concentration to deplete these stores. To ensure that this was the case, in some of the above experiments, the intracellular recording electrodes also

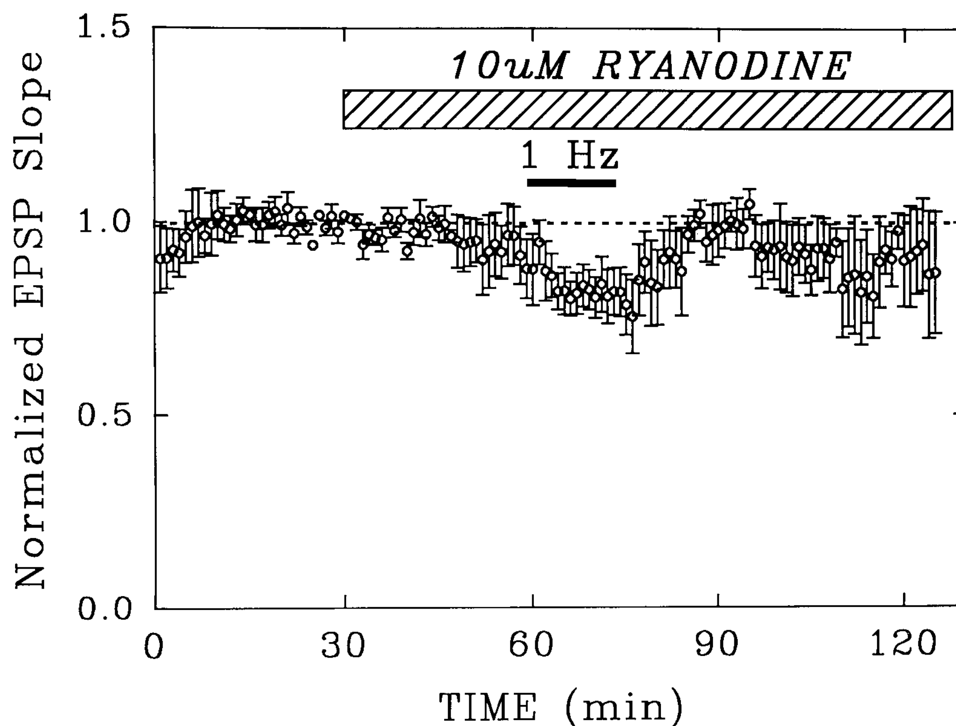


Figure 6. Bath application of ryanodine, a selective depleter of Ca^{2+}-dependent intracellular Ca^{2+} stores, also blocks the induction of LTD. Time course of experiments ($n = 5$) in which ryanodine (10 μM, *hatched bar*), a selective depleter of an intracellular Ca^{2+} pool that mediates Ca^{2+}-triggered release, was bath-applied 30 min before application of LFS. In the presence of ryanodine, LFS (1 Hz/15 min, *solid bar*) of Schaffer collateral–commissural axons did not elicit significant LTD of synaptic transmission ($p > 0.20$, paired t test comparing pre-LFS baseline EPSP slopes with 30 min post-LFS). (Each point is the mean ± SEM.)

contained the same concentration of the fluorescent dye Lucifer yellow (5 mM Li^{+} salt in 250 mM LiCl) to verify intracellular infusion from the pipette. Figure 8*B* shows a CA1 pyramidal neuron from one of these experiments, in which the intracellular recording electrode was removed at the end of the experiment and the slice transferred to another perfusion chamber for microscopic fluorescence imaging. Although dimmer than typical neurons filled with higher concentrations of Lucifer yellow by current pulse injection, both apical and basal dendrites of this neuron were clearly filled, the apical dendrites out to at least 400 μm from the cell soma. All of the neurons imaged in this manner, as well as other impalements using this concentration of dye, showed similar dendritic filling, indicating the likelihood of substantial infusion of ryanodine into the postsynaptic neuron. LTD with only LiCl in electrodes was no different in amplitude from control cells filled with 2 M K-acetate ($-39 \pm 14\%$, $n = 3$). Furthermore, two additional cells impaled with electrodes containing 2 M K-acetate, 5 mM ryanodine, and 5% DMSO exhibited similarly unimpaired LTD (-40 and -22% EPSP reduction 60 min after LFS).

Because ryanodine failed to block LTD when infused postsynaptically but was effective when bath applied, we conclude that Ca^{2+}-activated stores necessary to induce LTD are *presynaptic*. Furthermore, the combination of the extracellular ryanodine and intracellular thapsigargin data leads to the conclusion that an IP_3 receptor-gated pool is the *postsynaptic* calcium store contributor to the induction of LTD.

DISCUSSION

The results presented here show that the Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid, at concentrations that have been shown to deplete both IP_3 and ryanodine receptor-gated intracellular Ca^{2+} pools (Seidler et al., 1989; Thastrup et al., 1990) as well as block the induction of LTP (Harvey and Collingridge, 1992), also can prevent the *de novo* induction of LTD at Schaffer collateral–CA1 synapses in hippocampus. This blockade probably is not attributable to direct block of NMDA receptors, because 10-fold higher concentrations of thapsigargin have been shown previously not to directly interfere with NMDA-mediated responses at these same synapses (Harvey and Collingridge, 1992). Furthermore, because thapsigargin did not affect previously established LTD, we conclude that continued release of Ca^{2+} from intracellular stores is not needed for the maintenance of LTD (at least 60 min after induction).

Although thapsigargin causes a slow release of Ca^{2+} within neurons (Thastrup et al., 1990), we did not observe either persistent LTP or LTD of synaptic transmission with its application, probably because the kinetics of thapsigargin-induced Ca^{2+} release was too slow. However, we did observe a transient stimulus-evoked burst firing, consistent with previous reports of Ca^{2+}-mediated enhancement of voltage-dependent calcium conductances (Hoth and Penner, 1992; Luckhoff and Clapham, 1994). These probable calcium potentials lasted for 5–15 min after impalement and were larger and more rapid in onset in neurons filled with ryanodine instead of thapsigargin. As suggested for tetanus-induced LTP (Harvey and Collingridge, 1992), thapsigargin might be inhibiting the induction of LTD by preventing an LFS-induced Ca^{2+} signal through NMDA channels from being magnified by release of stored Ca^{2+}. Indeed, Alford et al. (1993) have shown that >50% of a tetanus-induced increase in postsynaptic $[Ca^{2+}]$ in CA1 pyramidal neurons is blocked by either thapsigargin or ryanodine. However, our experiments in which postsynaptic infusion of ryanodine did not block LTD are evidence that postsynaptic Ca^{2+}-triggered stores are not necessary for induction of LTD.

Evidence indicates that the activation of metabotropic subtypes of glutamate receptors, some of which are coupled to phosphoinositide-induced release of Ca^{2+}, is also necessary for the induction of LTD in hippocampus (Stanton et al., 1991; Bashir et al., 1993; Wexler and Stanton, 1993), dentate gyrus (O'Mara et al., 1995), and neocortex (Kato, 1993). Because postsynaptic infusion of thapsigargin did block

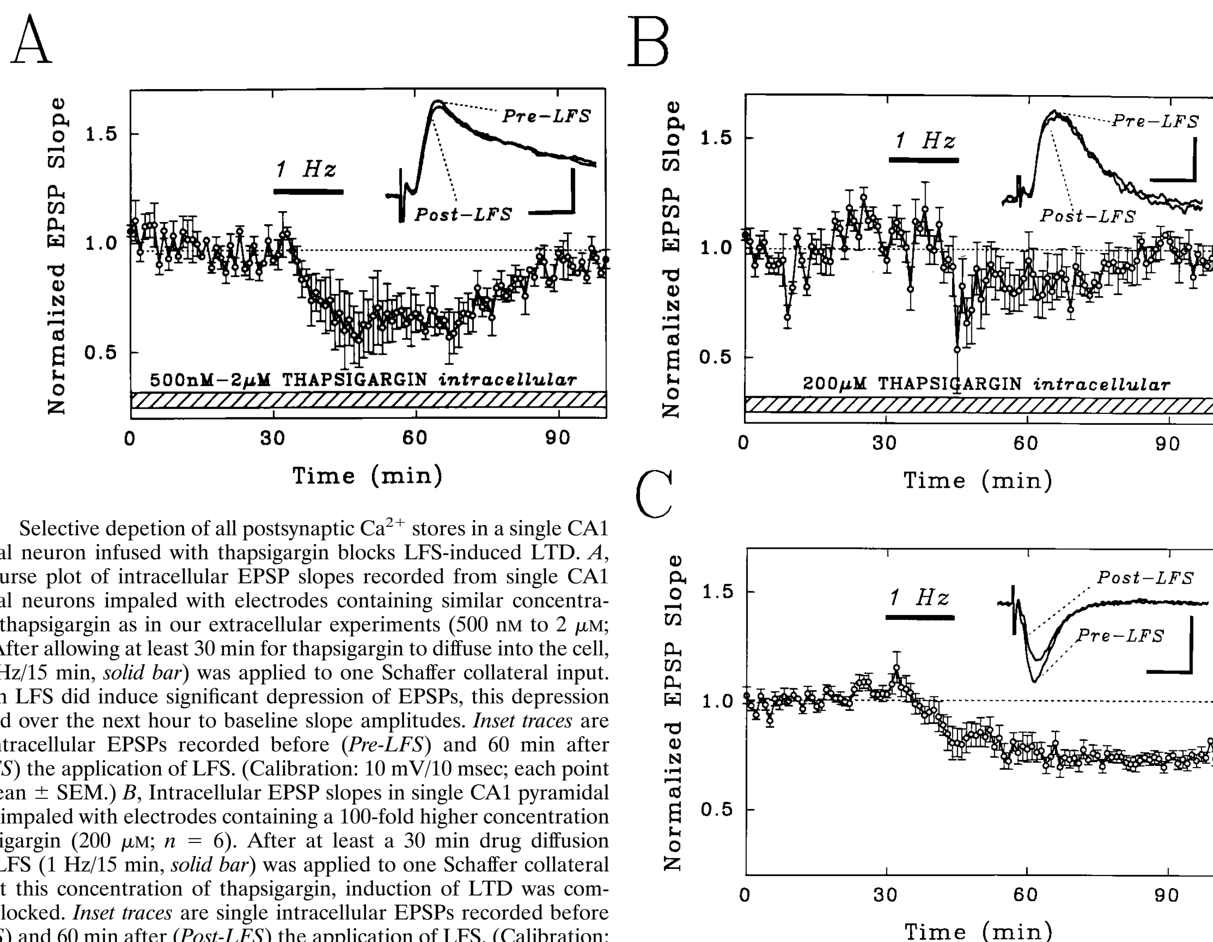


Figure 7. Selective depetion of all postsynaptic Ca^{2+} stores in a single CA1 pyramidal neuron infused with thapsigargin blocks LFS-induced LTD. *A*, Time course plot of intracellular EPSP slopes recorded from single CA1 pyramidal neurons impaled with electrodes containing similar concentrations of thapsigargin as in our extracellular experiments (500 nM to 2 μM; $n = 5$). After allowing at least 30 min for thapsigargin to diffuse into the cell, LFS (1 Hz/15 min, *solid bar*) was applied to one Schaffer collateral input. Although LFS did induce significant depression of EPSPs, this depression recovered over the next hour to baseline slope amplitudes. *Inset traces* are single intracellular EPSPs recorded before (*Pre-LFS*) and 60 min after (*Post-LFS*) the application of LFS. (Calibration: 10 mV/10 msec; each point is the mean ± SEM.) *B*, Intracellular EPSP slopes in single CA1 pyramidal neurons impaled with electrodes containing a 100-fold higher concentration of thapsigargin (200 μM; $n = 6$). After at least a 30 min drug diffusion period, LFS (1 Hz/15 min, *solid bar*) was applied to one Schaffer collateral input. At this concentration of thapsigargin, induction of LTD was completely blocked. *Inset traces* are single intracellular EPSPs recorded before (*Pre-LFS*) and 60 min after (*Post-LFS*) the application of LFS. (Calibration: 10 mV/10 msec; each point is the mean ± SEM.) *C*, In contrast to the intracellular blockade of LTD, simultaneous extracellular recordings of the Schaffer collateral-evoked population EPSPs from the experiments in *A* and *B* combined ($n = 11$) exhibited normal LFS-induced (1 Hz/15 min, *solid bar*) LTD that did not decay ($p < 0.05$, paired t test comparing pre-LFS baseline EPSP slopes with 30 min after LFS). *Inset traces* are single extracellular EPSPs recorded before (*Pre-LFS*) and 60 min after (*Post-LFS*) LFS. (Calibration: 2 mV/10 msec.)

the induction of LTD and ryanodine did not, we conclude that *postsynaptic* release of Ca^{2+} from *IP_3 receptor-gated pools*, perhaps in conjunction with metabotropic receptor-release of diacylglycerol, is needed to induce LTD.

Postsynaptic mechanisms in LTD

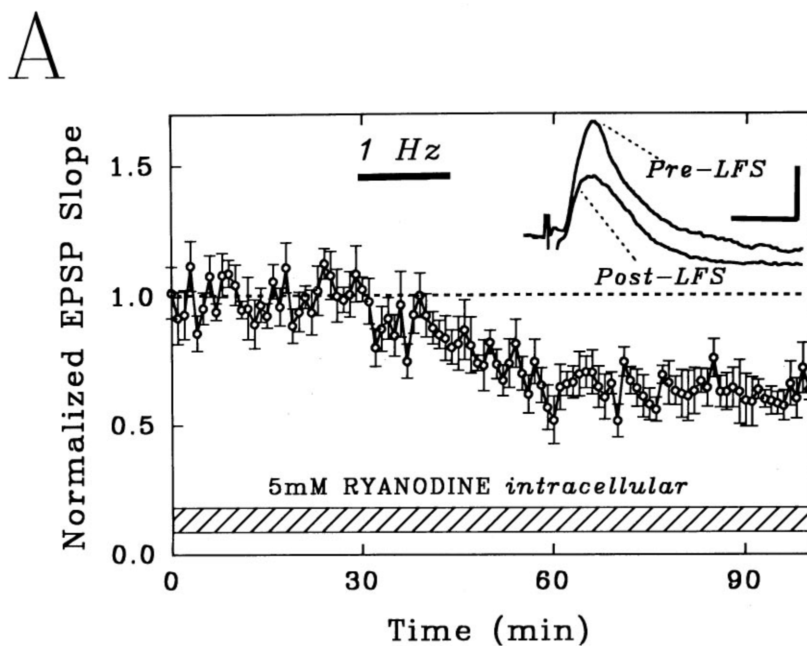
Several mechanisms have been suggested by which postsynaptic Ca^{2+} might trigger changes leading to LTD (see Fig. 9, *Postsynaptic*). One possibility is Ca^{2+}-activated serine/threonine protein phosphatases, because phosphatase inhibitors have been shown to block induction of LTD by LFS (Mulkey et al., 1993). Phosphatases could be responsible for dephosphorylating AMPA and/or NMDA receptors, reducing EPSPs at those synapses (Greengard et al., 1991; Wang et al., 1991). In addition, Mayford et al. (1995) found that overexpression in transgenic mice of a Ca^{2+}-independent, constitutively active form of CAM kinase II produced a threshold shift favoring induction of LTD by higher stimulus frequencies. However, we have shown recently that inhibition of *presynaptic*, but not postsynaptic, CAM kinase II blocks the induction of LTD (Stanton and Gage, 1996).

Nitric oxide (NO) is a recently proposed intercellular messenger, the synthesis of which also is stimulated by increases in postsynaptic Ca^{2+}. Izumi and Zorumski (1993) reported that inhibitors of nitric oxide synthase (NOS) block induction of hippocampal LTD. However, Cummings et al. (1994) failed to replicate this finding, leaving the role of Ca^{2+}-activated NOS uncertain. Lev-Ram et al. (1995) further muddied the NO waters by using caged, membrane-impermeant NO to demonstrate that postsynaptic, not presynaptic, NO may mediate induction of LTD in the cerebellum. Finally, Bolshakov and Siegelbaum (1994), using quantal analysis of Schaffer collateral–CA1 synapses in slices from young rats, concluded that LTD is induced postsynaptically, but expressed presynaptically, suggesting that a diffusable retrograde messenger may be involved in triggering LTD.

Our data from bath application of ryanodine also are consistent with a presynaptic site for at least some of the modification(s) underlying LTD. Although postsynaptic injection of ryanodine into single neurons did not block LTD, bath application of ryanodine, which permeates both presynaptic and postsynaptic membranes, did block the induction of LTD. Furthermore, the concentration of ryanodine used did not alter normal low-frequency baseline synaptic transmission, indicating that ryanodine was not simply blocking transmitter release. Thus, we concluded that *presynaptic ryanodine receptor-gated Ca^{2+} stores* also are necessary for the induction of LTD.

Presynaptic mechanisms in LTD

There are some suggestive data regarding presynaptic messengers and LTD (see Fig. 9, *Presynaptic*). An immunohistochemical study (Sharp et al., 1993) found that ryanodine receptor labeling is



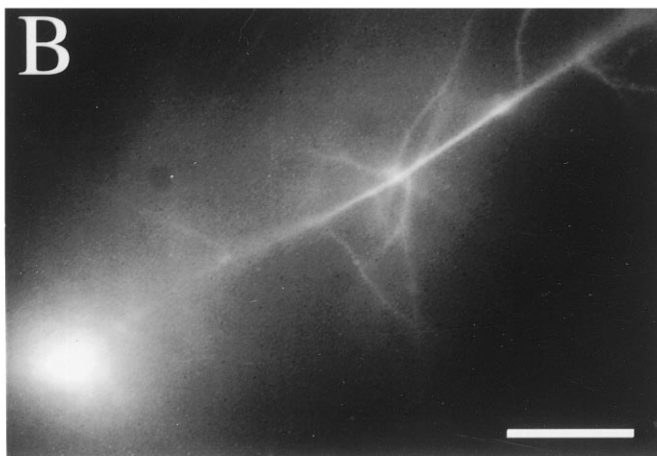


Figure 8. Selective intracellular depletion of postsynaptic ryanodine receptor-gated Ca^{2+} stores in a single CA1 pyramidal neuron does not block the induction of LTD. *A*, Time course of five experiments plotting the intracellular EPSP slope recorded from single CA1 pyramidal neurons impaled with electrodes containing 5 mM ryanodine plus 5 mM Lucifer yellow. After allowing 30 min for ryanodine diffusion, LFS (1 Hz/15 min, *solid bar*) was applied to one Schaffer collateral input. LFS induced significant LTD of intracellular EPSPs, which lasted at least 60 min after LFS. *Inset traces* are single intracellular EPSPs recorded before (*Pre-LFS*) and 60 min after (*Post-LFS*) the application of LFS. (Calibration: 10 mV/10 msec; each point is the mean ± SEM.) *B*, Fluorescent image of one of the CA1 pyramidal neurons from *A* after removal of the intracellular recording electrode, verifying diffusion of material well out into apical dendritic branches. (Calibration: 40 μm.)

extremely high in Schaffer collateral axons, whereas IP_3 receptors are much more prevalent in dendritic spines and cell somata of CA1 pyramidal neurons, consistent with our conclusions about the separate sites of actions for these two pools. As mentioned above, one putative retrograde intercellular messenger suggested to play a role in both LTP and LTD is NO. Pharmacological data has suggested that NO activation of an ADP-ribosyltransferase is necessary for induction of LTP (Schuman et al., 1994). However, NO also is known to cause the production of cGMP by a specific NO-sensitive guanylyl cyclase (Southam and Garthwaite, 1993). In turn, cGMP triggers production of another messenger, cADP-ribose, which is known to activate ryanodine receptors, and releases Ca^{2+} from this pool (Galione et al., 1993; Mészáros et al., 1993). Intriguingly, both the NO-sensitive guanylyl cyclase (Gukovskaya and Pandol, 1995) and cADP-ribose itself (through calmodulin) (Lee et al., 1994) are strongly inhibited by elevations in $[Ca^{2+}]$. Putting this all together, we suggest that NO might function selectively in presynaptic terminals that have been relatively inactive and, hence, have low $[Ca^{2+}]$, to cause the production of cGMP and cADP-ribose and release from ryanodine-sensitive Ca^{2+} stores. In fact, we have found recently that a selective inhibitor of NO-sensitive guanylyl cyclase also blocks the induction of hippocampal LTD but not LTP (A. Gage and P. Stanton, unpublished observations). Such a mechanism would allow the same intercellular messenger (NO) to play opposite roles in the presynaptic components of LTP and LTD by activating different second messenger systems as a function of activity-dependent presynaptic $[Ca^{2+}]$. Thus, a Hebbian pairing of high levels of presynaptic and postsynaptic activity triggers LTP, whereas lower presynaptic $[Ca^{2+}]$ and postsynaptic activation would lead to LTD. As one potential enzymatic target of the Ca^{2+} released from ryanodine stores, we have shown recently that selective inhibition of *presynaptic* CAM kinase II blocks the induction of hippocampal LTD (Stanton and Gage, 1996), consistent with the transgenic overexpression data of Mayford et al. (1995).

Depotentiation and the threshold for LTD

Previously, it has been shown that hyperpolarizing CA1 pyramidal neurons (Stanton and Sejnowski, 1989) or limiting the depolarization of more hyperpolarized visual cortical neurons (Artola et al., 1990) during low-frequency presynaptic activation can satisfy the conditions needed to induce LTD. This has led to suggestions

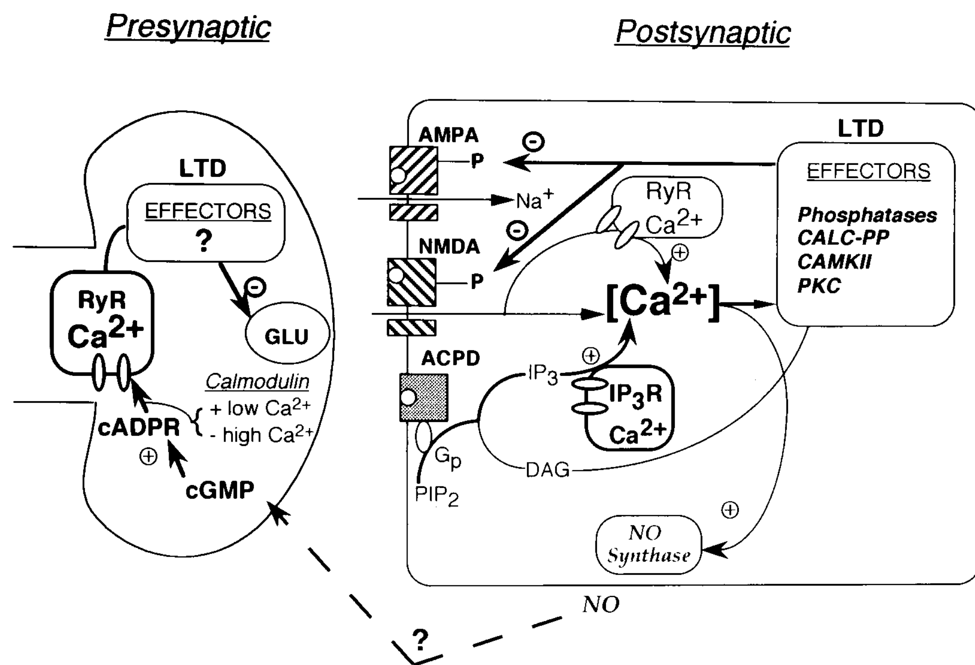


Figure 9. Presynaptic and postsynaptic Ca^{2+} pools, Ca^{2+}-activated messenger systems, and targets of modifications suggested to play a role in the induction and expression of LTD of synaptic transmission at Schaffer collateral–CA1 synapses. Abbreviations: ACPD, metabotropic glutamate receptors; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptors; CALC-PP, Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cADPR, cyclic adenosine diphosphate ribose; cGMP, cyclic guanine 3′,5′-monophosphate; DAG, diacylglycerol; GLU, glutamate; G_p, stimulatory G-protein; IP_3R, inositol triphosphate receptors; LTD, long-term depression; NMDA, *N*-methyl-D-aspartate receptors; NO, nitric oxide; P, phosphorylation site; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, Ca^{2+}/phospholipid-dependent protein kinase C; RyR, ryanodine receptors.

that a certain level of postsynaptic $[Ca^{2+}]$ increase, subthreshold to that needed for LTP, is required to induce LTD. Interestingly, we found that thapsigargin was less effective in impairing the induction of depotentiation of recently induced LTP, compared with *de novo* LTD. That is, thapsigargin could not prevent depotentiation induced by prolonged 1 Hz stimulation (900 stimuli), but still blocked depotentiation when shorter, 5 Hz theta trains were used (300 stimuli). Other reports indicate that the induction of LTP can lower the 1 Hz depotentiation threshold for hours (Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Wexler and Stanton, 1993; Bashir and Collingridge, 1994) but that shorter stimuli such as our 5 Hz theta trains only cause depotentiation for a brief time window (15 min) after LTP induction (Stäubli et al., 1995), suggesting that this stimulus may be less effective at raising intracellular $[Ca^{2+}]$. Behnisch and Reymann (1995) also found a similar threshold phenomenon for thapsigargin block of LTP in CA1 by weak, but not strong, tetanization. Therefore, we propose that the intracellular (probably postsynaptic) $[Ca^{2+}]$ threshold needed to induce LTD is reduced by the induction of LTP and then slowly increases in the first hours after LTP induction.

The LTP-induced lowering of LTD threshold could have a number of different explanations including (1) increased sensitivity of intracellular Ca^{2+} stores to Ca^{2+} and/or IP_3-stimulated release; (2) persistently increased basal intracellular $[Ca^{2+}]$, perhaps attributable to increased plasma membrane Ca^{2+} conductance (Hoth and Penner, 1992); (3) enhanced Ca^{2+} sensitivity of kinases (Mayford et al., 1995; Stanton, 1995) and/or phosphatases (Mulkey et al., 1993) mediating postsynaptic modifications leading to LTD; (4) enhanced sensitivity to dephosphorylation of recently LTP-phosphorylated sites on AMPA glutamate receptors (Greengard et al., 1991; Wang et al., 1991); and (5) enhanced sensitivity to Ca^{2+} of a retrograde messenger enzyme such as nitric oxide synthase (Izumi and Zorumski, 1993; Cummings et al., 1994; Schuman et al., 1994). In light of our demonstration that PKC activation is sufficient to lower LTD threshold (Stanton, 1995), the transgenic studies by Mayford et al. (1995) showing that constitutive CAMKII activation favors LTD and studies by Waxham and Aronowski (1993) demonstrating that CAMKII can be a substrate for PKC, we propose PKC phosphorylation of CAMKII, leading to an increase in its sensitivity to Ca^{2+}, as a possible mechanism underlying priming of LTD.

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