Ryanodine Receptors Contribute to cGMP-Induced Late-Phase LTP and CREB Phosphorylation in the Hippocampus

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INTRODUCTION

Long-term potentiation (LTP) in the CA1 region of hippocampus has an early phase and a late phase that have been hypothesized to correspond to analogous phases of memory (Frey et al. 1988, 1993; Huang et al. 1996). The two phases overlap, but they can be distinguished by parametric, pharmacological, and genetic manipulations. Early-phase LTP (E-LTP) is typically induced by 1 or 2 trains of 100-Hz, 1-s stimulation separated by 20 s, lasts 1–2 h, and is not dependent on activation of cAMP-dependent protein kinase (PKA) or protein synthesis. By contrast, late-phase LTP (L-LTP) is typically induced by 3 or 4 trains of stimulation separated by 5–10 min, lasts more than 3 h, and requires PKA activation as well as protein and RNA synthesis (for review, see Huang et al. 1996). During the induction of L-LTP, PKA and (MAPK) are thought to activate the transcription factor cAMP response element-binding protein (CREB), leading to gene induction (Bourtchouladze et al. 1994; Impney et al. 1996, 1998; Matthes et al. 1997; but see Gass et al. 1998).

A number of studies have shown that nitric oxide (NO) is involved in E-LTP, perhaps as a retrograde messenger. NO is thought to contribute to E-LTP in part by activating guanylyl cyclase and cGMP-dependent protein kinase (PKG; for review, see Hawkins et al. 1998). Recently, we found that in addition to being involved in E-LTP, the NO-cGMP-PKG signaling pathway contributes to CREB phosphorylation and L-LTP in hippocampus, evidently acting in parallel with PKA and MAP kinase (Lu et al. 1999). NO, cGMP, and PKG have also been shown to trigger gene induction via CREB phosphorylation in other systems (Ding et al. 1997; Gudi et al. 1996, 1997, 1999; Haby et al. 1994; Ohki et al. 1995; Penina and Enkelopov 1993). PKG can phosphorylate CREB directly at the same site as PKA in vitro and in transfected kidney cells (Colbran et al. 1992; Gudi et al. 1996), but it is not known whether this occurs in neurons. Alternatively, PKG might act indirectly. In this paper, we have investigated the possibility that the NO-cGMP-PKG pathway contributes to CREB phosphorylation and L-LTP indirectly by causing or amplifying intracellular Ca2+ release.

Intracellular Ca2+ release is regulated by inositol-1,4,5-trisphosphate (IP3) receptors and ryanodine receptors for which the endogenous ligand is cyclic ADP-ribose (cADPR) (for reviews, see Berridge 1998 and Lee 1997). Ryanodine receptors are expressed in pyramidal cells in hippocampus (Furuichi et al. 1994; Giannini et al. 1995), and a variety of evidence suggests that they may contribute to hippocampal E-LTP (Balschun et al. 1999; Harvey and Collingridge 1992; Obenaus et al. 1989; Szinyei et al. 1999; Wang and Kelly 1997; Wang et al. 1996), L-LTP (Behnisch and Reymann 1995), and CREB-mediated gene expression (Hardingham et al. 2001). NO can stimulate ryanodine receptors through cGMP and PKG, which phosphorylates and activates the synthetic enzyme for cADPR, ADP-ribose cyclase (Galiome et al. 1993; Lee 1994; Willmott et al. 2000). We therefore investigated the possible role in NO- and cGMP-dependent L-LTP of Ca2+ release from ryanodine-sensitive intracellular stores.

METHODS

Male C57BL6 mice, aged 6–9 wk, were housed and killed in accordance with the guidelines of the Health Science Division of Columbia University. The brain was quickly removed and immersed...
in ice-cold artificial cerebrospinal fluid (ACSF) bubbled with a gas mixture of 95% O₂-5% CO₂. The hippocampus was dissected, and 400-μM transverse slices were prepared. The slices were incubated in an interface recording chamber maintained at 28.5 ± 0.5°C for ≥1.5 h before recording and were constantly superfused with gas-saturated ACSF at 1–1.5 ml/min. The composition of the ACSF was as follows (in mM): 124 NaCl, 4.4 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26 NaHCO₃, and 10 glucose.

**Electrophysiological experiments**

To record the field excitatory postsynaptic potential (EPSP), a glass micropipette filled with ACSF (1–5 MΩ resistance) was placed in the stratum radiatum of the CA1 region and a bipolar tungsten stimulating electrode was placed along the Schaffer collateral fibers. The stimulation intensity was adjusted to produce an EPSP with a slope that was about 35% of maximum at the beginning of each experiment. The test stimulation was delivered once per minute (0.017 Hz). For inducing LTP, either single or multiple trains of stimulation at 100 Hz for 1 s were delivered at the same intensity as the test stimulation. Multiple trains were delivered at 5-min intervals.

NO solution was prepared as previously described (Zhuo et al. 1993). Briefly, NO gas was bubbled to saturation (approximately 3 mM; MacIntyre et al. 1991) in helium-saturated distilled water and diluted to 0.1–1.0 μM in helium-saturated ACSF containing 30 unit/ml of superoxide dismutase to protect NO from inactivation by superoxide. The NO solution was prepared immediately before use and injected directly into the recording chamber for about 2 min before the resumption of perfusion with normal ACSF, in which NO has a half-life of approximately 30 s (Palmer et al. 1987). The following drugs were used: ryanodine and thapsigargin (RBI, Natick, MA), forskolin (Calbiochem, La Jolla, CA), and 8-Br-cGMP (Biolog). The drugs were prepared as stock solutions and diluted in ACSF immediately before application. Ryanodine, thapsigargin, and forskolin were prepared in DMSO. The final concentration of the DMSO was 0.01–0.1%.

Data are shown as mean ± SE of the percent of baseline EPSP slope. Data were analyzed using either t-tests to compare two conditions or analysis of variance (ANOVA) followed by planned comparisons of multiple conditions, and P < 0.05 was considered significant.

**Immunocytochemical experiments**

Hippocampal slices were prepared and treated with tetrac stimulation and/or drugs exactly as described for the electrophysiological experiments. Either 1 min or 60 min after the treatment, the slices were washed three times in PBS, permeabilized in 0.3% Triton X-100 in PBS for 60 min at room temperature, and washed three times in PBS again. The free aldehydes were quenched in 50 mM ammonium chloride in PBS for 20 min. Nonspecific antibody binding was blocked by incubation in 10% goat serum in PBS for 60 min at room temperature. The slices were then incubated with primary antibody, rabbit polyclonal anti-phospho-CREB (Upstate Biotechnology, Lake Placid, NY), diluted 1:100 in 10% goat serum in PBS at 4°C for 36 h. This antibody is thought to be relatively selective for phospho-CREB, although it may have some cross-reactivity with the related molecules cAMP response element modulator (CREM) and activating transcription factor 1 (ATF-1) (Ginty et al. 1993). The slices were washed six times in PBS, for 2 h each time. The slices were incubated in goat anti-rabbit antibody conjugated with Cy3 (Jackson ImmunoResearch, West Grove, PA), diluted 1:100 in 10% goat serum overnight at 4°C. They were then washed in PBS six times, for 2 h each time.

The slices were viewed on a Zeiss Axiovert 100 inverted microscope coupled to a Biorad MRC1000 laser confocal scanning system with which we were able to make optical sections of the less damaged interior of the slice. Images were taken using a 5×, 0.25 n.a. water-immersion objective, and Kalman averages of five scans were collected for each image. The mean pixel intensity in the entire CA1 cell body area and in an apical dendritic area of CA3 that was relatively free of cell bodies was measured using Biorad Comos software. The ratio of intensities in the two areas was determined in each slice to normalize for differences in background fluorescence. These values were in turn normalized to the values obtained from untreated control slices from the same animal. There was also a high level of immunofluorescence in the dentate gyrus cell body area, but this appeared to be quite variable and was not analyzed in this study. All data are presented as mean ± SE percent of control. The experimental data were analyzed by a two-way ANOVA (treatment and time) followed by planned comparisons of individual conditions. The specificity of the immunofluorescence was confirmed by omitting the primary antibody, which resulted in a significant reduction in fluorescence intensity.

**RESULTS**

**Tetanic L-LTP involves intracellular Ca²⁺ release**

We first examined the effect on tetanic L-LTP of thapsigargin, an inhibitor of Ca²⁺-ATPase that depletes intracellular Ca²⁺ stores (Alford et al. 1993; Thastrup et al. 1990). Four trains of 100-Hz, 1-s tetanization at 5-min intervals induced robust L-LTP (191 ± 17% of baseline 180–185 min after the last tetanus, n = 5; Fig. 1A). This potentiation was significantly reduced by perfusion with thapsigargin (10 μM) for 40 min before the tetanic stimulation in interleaved experiments [120 ± 6%, n = 4; t(7) = 3.61, P < 0.01 compared with no drug], suggesting that the L-LTP involves Ca²⁺ release from intracellular stores.

Thapsigargin prevents Ca²⁺ uptake into both IP3- and ryano-dine-sensitive intracellular stores. The ryanodine receptor channels can be blocked more selectively by prolonged application (>30 min) of relatively high concentrations (4–20 μM) of ryanodine, which allows binding of the agonist to a low affinity site (McPherson et al. 1991; Meissner 1986; Rousseau et al. 1987). We found that, like thapsigargin, perfusion with ryanodine (10 μM) for 40 min before the tetanization significantly reduced L-LTP induced by four trains [133 ± 3%, n = 4; t(7) = 3.02, P < 0.05 compared with no drug]. The effects of ryanodine and thapsigargin were not significantly different. Ryanodine also reduced L-LTP induced by three-train tetanization [ryanodine: 150 ± 12%, n = 6; no drug: 230 ± 28%, n = 6; t(10) = 2.65, P < 0.05; Fig. 1B]. These results support the idea that L-LTP involves Ca²⁺ release from intracellular stores and suggest that most of that release may be through ryanodine receptor channels.

In addition, thapsigargin and ryanodine reduced an early phase of the potentiation produced by three- or four-train tetanization (Fig. 1, A and B), which consists of a combination of E-LTP and an intermediate phase of potentiation (I-LTP) that is induced by multiple train tetanization and is PKA-dependent but protein synthesis–independent (Winder et al. 1998). We therefore examined whether these drugs also reduce E-LTP induced by one-train tetanization. One-train tetanization induced significant potentiation 1 h after the tetanus (150 ± 11%, n = 5; Fig. 1C). However, unlike potentiation induced by multiple tetani, the E-LTP induced by one-train tetanus was not affected by perfusion with either thapsigargin (10 μM) or ryanodine (10 μM) for 40 min before the tetanus.
These results suggest that E-LTP does not require Ca\(^{2+}\) release from intracellular stores under the conditions of these experiments, and therefore imply that the reduction in the early phase of potentiation seen in Fig. 1, A and B, is an effect on I-LTP. In addition, these results indicate that thapsigargin and ryanodine do not have nonspecific effects that interfere with potentiation in general.

One-train tetanization induced E-LTP 1 h after the tetanus but almost no L-LTP 3 h after the tetanus (106 ± 5%, n = 5; Fig. 1D). As another way to test the possible involvement of ryanodine receptors in L-LTP, we examined whether activation of those receptors could convert the E-LTP to L-LTP. Brief application of ryanodine at relatively low concentrations (from submicromolar to 1 \(\mu M\)) has been shown to activate ryanodine receptor channels by binding to a high affinity site (Fleischer et al. 1985; McPherson et al. 1991; Meissner 1986). Perfusion with ryanodine (1 \(\mu M\)) for 10 min before a single tetanus produced stable L-LTP that was significantly greater than that produced by one-train tetanus alone [161 ± 19%, n = 6; \(t(9) = 2.53, P < 0.05\) compared with no drug]. These results support the involvement of ryanodine receptors in the induction of L-LTP.

NO- and cGMP- but not cAMP-induced L-LTP requires intracellular Ca\(^{2+}\) release

We have previously found that, like ryanodine, brief perfusion with NO before a single tetanus could also produce stable L-LTP similar to that produced by three-train tetanization (Lu et al. 1999). We therefore examined whether ryanodine receptors are involved in that NO-induced L-LTP. Perfusion with ACSF containing dissolved NO gas (0.1–1 \(\mu M\)) for 3 min before a single tetanus produced robust L-LTP (194 ± 16%, n = 5; Fig. 2A). Like tetanic L-LTP, the NO-induced L-LTP was significantly reduced by perfusion with ryanodine (10 \(\mu M\)) for 40 min before the tetanus [119 ± 12%, n = 4; \(t(7) = 3.52, P < 0.01\) compared with no drug]. These results suggest that

![Diagram](https://example.com/diagram.png)
NO activates ryanodine receptors to produce L-LTP and are consistent with the idea that this pathway also plays a role in tetanic L-LTP.

NO contributes to L-LTP in part by activating soluble guanylyl cyclase and producing cGMP (Lu et al. 1999). We therefore examined whether ryanodine receptors act downstream of cGMP as well as NO during the induction of L-LTP. Perfusion with the membrane permeable analog 8-Br-cGMP (1 μM) for 10 min before and after a single tetanus produced robust L-LTP (169 ± 8%, n = 7; Fig. 2B). This cGMP-induced L-LTP was significantly reduced by perfusion with ryanodine (10 μM) for 40 min before the tetanus [114 ± 6%, n = 4; t(9) = 4.37, P < 0.01 compared with no drug], whereas ryanodine perfusion alone had no effect on the baseline EPSP. These results suggest that ryanodine receptors play a role in cGMP-induced L-LTP as well as NO-induced L-LTP.

The NO-cGMP pathway is also thought to contribute to E-LTP (Hawkins et al. 1998). To examine whether ryanodine receptors are required for that effect as well, we modified the previous protocol for cGMP-induced E-LTP (Zhuo et al. 1994a) for use in mice. A weak tetanus (50 Hz, 0.2 s) produced short-term potentiation (STP) but almost no E-LTP 1 h after the tetanus (113 ± 4%, n = 6; Fig. 2C). Perfusion with 8-Br-cGMP (1 μM) for 10 min before the weak tetanus produced reliable E-LTP (145 ± 8%, n = 7) that was not significantly reduced by prolonged perfusion with ryanodine (10 μM). E-LTP does not require ryanodine receptors under the conditions of these experiments.

The NO-cGMP-PKG pathway is thought to act in parallel with the cAMP-PKA pathway during the induction of L-LTP (Lu et al. 1999). We therefore examined whether cAMP-induced L-LTP also involves intracellular Ca²⁺ release. Perfusion with the adenylyl cyclase activator forskolin (50 μM) for 15 min induced slow onset L-LTP that was not significantly reduced by prolonged perfusion with thapsigargin (10 μM).

FIG. 2. Nitric oxide (NO)- and cGMP-, but not cAMP-induced L-LTP require intracellular Ca²⁺ release. A: perfusion with NO-containing solution (0.1–1.0 μM, open bar) for 3 min before 1-train tetanization induced L-LTP that was blocked by prolonged perfusion with ryanodine (10 μM). B: perfusion with 8-Br-cGMP (1 μM, open bar) for 10 min before and after 1-train tetanization induced L-LTP that was blocked by prolonged perfusion with ryanodine (10 μM). Perfusion with ryanodine alone had no effect on the baseline EPSP. C: weak tetanic stimulation (50 Hz, 0.2 s) induced rapidly decaying short-term potentiation, but little E-LTP. Perfusion with 8-Br-cGMP (1 μM) for 10 min before and after the weak tetanus induced reliable E-LTP. However, unlike cGMP-induced L-LTP, this cGMP-induced E-LTP was not significantly reduced by prolonged perfusion with ryanodine (10 μM). D: perfusion with the adenylyl cyclase activator forskolin (50 μM, black bar) for 15 min induced slow onset L-LTP that was not significantly reduced by prolonged perfusion with thapsigargin (10 μM).
Perfusion with thapsigargin (10 μM) for 50 min before the forskolin application did not significantly reduce this potentiation (165 ± 13%, n = 5). These results suggest that unlike cGMP-induced L-LTP, cAMP-induced L-LTP does not require intracellular Ca\(^{2+}\) release.

cGMP- but not cAMP-induced CREB phosphorylation requires intracellular Ca\(^{2+}\) release

The late, protein synthesis–dependent phase of LTP is thought to involve induction of immediate early genes via phosphorylation of the transcription factor CREB, mediated in part via PKA (Bourtchouladze et al. 1994; Impey et al. 1996, 1998; Matthies et al. 1997; but see Gass et al. 1998). We have previously found that the NO-cGMP-PKG signaling pathway contributes to CREB phosphorylation during the induction of L-LTP, evidently acting in parallel with the cAMP-PKA pathway (Lu et al. 1999). Because cGMP- but not cAMP-induced L-LTP involves intracellular Ca\(^{2+}\) release, we investigated the possible role of Ca\(^{2+}\) release in CREB phosphorylation by each of these pathways.

We examined CREB phosphorylation by measuring phospho-CREB immunofluorescence in hippocampal slices that had received the same treatments described for the electrophysiological experiments. The slices were fixed either 1 or 60 min after the treatments, stained with an antibody for CREB phosphorylated at Ser-133, and viewed on a confocal microscope. One minute after the end of three-train tetanization, the intensity of phospho-CREB immunofluorescence in the CA1 cell body area was significantly increased compared with that in untreated control slices from the same animals (148 ± 8% of control, n = 8, F(1.72) = 22.60, P < 0.01; Fig. 3). This increase in immunofluorescence was significantly reduced by perfusing the slices with ryanodine (10 μM) for 40 min before the tetanization (112 ± 5%, n = 6, F = 5.47, P < 0.05 compared with no drug). Similarly, there was an increase in phospho-CREB immunofluorescence 1 min after 8-Br-cGMP paired with one-train tetanization (169 ± 14%, n = 7, F = 41.00, P < 0.01), and this increase was also blocked by perfusion with 10 μM ryanodine (97 ± 11%, n = 7, F = 22.20, P < 0.01 compared with no drug). Sixty minutes after
three trains of tetanization, phospho-CREB immunofluorescence was maintained at nearly the same level as at 1 min (154 ± 11%, n = 9, F = 32.01, P < 0.01), consistent with similar studies on hippocampal slices (Lu et al. 1999) and cultured hippocampal neurons (Bito et al. 1996). Results with the various drug treatments were also similar to results 1 min after the treatments (3 train + ryanodine: 105 ± 9%, n = 6, F = 10.56, P < 0.01 compared with no drug; 8-Br-cGMP + 1 train: 154 ± 6%, n = 12, F = 42.83, P < 0.01; 8-Br-cGMP + 1 train + ryanodine: 104 ± 7%, n = 5, F = 10.96, P < 0.01 compared with no drug). These results suggest that CREB phosphorylation by either three-train tetanization or 8-Br-cGMP paired with one-train tetanization involves Ca2+ release from ryanodine-sensitive intracellular stores.

We also examined cAMP-induced CREB phosphorylation, and found that there was a significant increase in phospho-CREB immunofluorescence 1 min after perfusion with forskolin (50 μM) for 15 min (175 ± 15%, n = 6, F = 41.07, P < 0.01). This increase was not significantly reduced by perfusion with thapsigargin (10 μM) for 50 min before the forskolin application (147 ± 14%, n = 6). Again, results 60 min after these treatments were similar to results 1 min after the treatments (forskolin: 147 ± 16%, n = 6, F = 16.15, P < 0.01; forskolin + thapsigargin: 143 ± 13%, n = 6). These immunocytochemical results are very similar to the electrophysiological results on L-LTP, and support the idea that cGMP and cAMP act through different pathways to cause CREB phosphorylation during the induction of L-LTP. More specifically, they suggest that cGMP and PKG, but not cAMP and PKA act indirectly through intracellular Ca2+ release.

The increase in phospho-CREB immunofluorescence shown in Fig. 3 occurs in the postsynaptic (CA1) cell bodies, suggesting that the gene induction critical for L-LTP may occur postsynaptically. To test that idea in another way, we investigated whether the presynaptic cell bodies are necessary for cGMP-induced L-LTP. In slices from which the CA3 region had been surgically removed, 8-Br-cGMP paired with one-train tetanus still produced robust L-LTP (197 ± 19%, n = 6). These results suggest that gene induction in the postsynaptic but not the presynaptic neurons is critical for the induction of L-LTP by cGMP.

**Discussion**

Our results indicate that cGMP acts through intracellular Ca2+ release to contribute to CREB phosphorylation in the postsynaptic neurons during the induction of L-LTP. By contrast, previous experiments on hippocampal neurons in dissociated cell culture have indicated that NO, cGMP, and PKG act directly in the presynaptic neurons during the induction of E-LTP (Arancio et al. 1995, 1996, 2001). These results would seem to imply that the NO-cGMP-PKG pathway may act at two different sites (pre- and postsynaptic) to contribute to E-LTP and L-LTP. However, a few studies have suggested that NO, cGMP, and PKG may have postsynaptic as well as presynaptic actions during the induction of E-LTP (Arancio et al. 2001; Ko and Kelly 1999; Son et al. 1998). Furthermore, although we did not obtain evidence for a role of intracellular Ca2+ release in E-LTP (Figs. 1C and 2C), a number of other studies have (Balschun et al. 1999; Harvey and Collingridge 1992; Obenau et al. 1989; Szinyei et al. 1999; Wang and Kelly 1997; Wang et al. 1996), suggesting that the involvement of Ca2+ release in E-LTP may depend on the experimental conditions. In addition, our results (Fig. 1, A and B) suggest that Ca2+ release is involved in an intermediate phase of potentiation (I-LTP), which is thought to involve a balance between Ca2+-dependent kinases and phosphatases (Winder et al. 1998). Because the kinases have a lower Ca2+ sensitivity, Ca2+ release from intracellular stores may be necessary to activate them sufficiently for L-LTP.

Collectively, these results suggest the following more general hypothesis concerning the possible roles of NO, cGMP, and intracellular Ca2+ release in the induction of LTP. Tetanic stimulation causes an increase in postsynaptic Ca2+ from a variety of sources, including N-methyl-D-aspartate (NMDA) receptor-channels, voltage-dependent Ca2+ channels, and metabotropic glutamate receptors linked to the production of IP3 and release of Ca2+ from IP3-sensitive intracellular stores (Bortolotto and Collingridge 1993; Wilsch et al. 1998). The Ca2+ activates NO synthase, stimulating the production of NO which diffuses to presynaptic terminals and activates guanylyl cyclase and cGMP-dependent protein kinase, leading to a presynaptic component of E-LTP (Hawkins et al. 1998). In addition, NO can also activate postsynaptic guanylyl cyclase, PKG, and ADP-ribosycyclase, stimulating production of cADPR, which acts synergistically with cytoplasmic Ca2+ to cause release of Ca2+ from ryanodine-sensitive intracellular stores (Lee 1993; Lee et al. 1995). This synergism can create a positive feedback situation, thus amplifying Ca2+ signals from other sources (Alford et al. 1993; Bliss and Collingridge 1993; Empgate et al. 1999). When the Ca2+ signal is sufficiently large, it can trigger CREB phosphorylation and the induction of L-LTP in parallel with PKA.

The idea that NO, cGMP, and PKG act in part to regulate postsynaptic Ca2+ levels might help to explain a number of other seemingly conflicting findings on the roles of these molecules and intracellular Ca2+ release in several forms of synaptic plasticity in the hippocampus. These explanations are based on the concept that different levels and durations of postsynaptic Ca2+ elevation can produce different forms of plasticity, with a long, low Ca2+ elevation producing long-term depression (LTD), a brief, higher elevation producing either STP or E-LTP, and a longer or higher elevation producing I-LTP or L-LTP (Artola and Singer 1993; Bito et al. 1996; Yang et al. 1999). Activation of the NO-cGMP-PKG-RyR pathway would amplify the Ca2+ signal and thus lower the stimulation threshold for each of these forms of plasticity, and inhibition of the NO-cGMP-PKG-RyR pathway would have the opposite effect. According to this hypothesis, the postsynaptic NO-cGMP-PKG-RyR pathway can be thought of as an intrinsic modulatory system that may be necessary to achieve the proper Ca2+ level under many circumstances, but is not required under all circumstances.

This hypothesis is able to account for all of our results and most of the published results on the roles of NO, cGMP, and intracellular Ca2+ release in synaptic plasticity in hippocampus, some of which appear to be contradictory. For example, NO, cGMP, and intracellular Ca2+ are thought to be involved in LTD (Gage et al. 1997; Reyes and Stanton 1996; Reyes-Harde et al. 1999a,b; Santschi et al. 1999; Wu et al. 1997, 1998; Zhuo et al. 1994b) as well as E-LTP and L-LTP. Furthermore, in each case inhibitors or knock-outs block the...
plasticity under some experimental circumstances but not others (Balschun et al. 1999; Futatsugi et al. 1999; Hawkins et al. 1998; Kleppisch et al. 1999). The most frequent (although not universal) result is that the inhibitors are more effective with weaker induction protocols (Behnisch and Reymann 1995; Chetkovich et al. 1993; Haley et al. 1993; Lu et al. 1999; Malen and Chapman 1997; O’Dell et al. 1994; Zhuo et al. 1998). This pattern might be explained if the inhibitors are able to lower the intracellular Ca$^{2+}$ signal from above to below threshold for a particular type of plasticity with the weaker protocols but not with the stronger protocols. Another common pattern is that exogenous NO, cGMP analogs, or low levels of ryanodine can change a given type of stimulation from below threshold to above threshold for producing a particular type of plasticity (Gage et al. 1997; Lu et al. 1999; Malen and Chapman 1997; Reyes-Harde et al. 1999b; Son et al. 1998; Wang et al. 1996; Wu et al. 1998; Zhuo et al. 1993, 1994a,b). However, as with the antagonists, results with these exogenous agents have been variable, and in some studies they have not altered plasticity (Murphy et al. 1994; Schuman et al. 1994; Selig et al. 1996). This pattern might be explained if NO, cGMP, and ryanodine receptors act to boost intracellular Ca$^{2+}$ levels, in some cases from below to above threshold for a particular type of plasticity.

The idea that intracellular Ca$^{2+}$ release contributes to CREB phosphorylation during L-LTP might also help to explain why the induction of L-LTP typically requires three to four tetani separated by minutes, whereas the induction of E-LTP typically requires only one or two tetani separated by seconds. One possibility is that a brief, high rise in Ca$^{2+}$ is sufficient for E-LTP, but a more prolonged elevation in Ca$^{2+}$ is necessary for CREB phosphorylation and L-LTP. Consistent with this idea, Bito et al. (1996) found that electrical stimulation that produced a prolonged Ca$^{2+}$ elevation also produced prolonged CREB phosphorylation and gene activation in cultured hippocampal neurons, whereas stimulation that produced brief Ca$^{2+}$ elevation did not. In hippocampal slices, a single tetanus triggers Ca$^{2+}$ influx through NMDA receptor channels, producing a Ca$^{2+}$ elevation that lasts only 1–2 s (Regehr and Tank 1990). That Ca$^{2+}$ elevation can be prolonged by Ca$^{2+}$ release from ryanodine-sensitive intracellular stores and prolonged even further by multiple, spaced tetani, each of which triggers intracellular Ca$^{2+}$ release (Alford et al. 1993; Schiegg et al. 1995). This process could be more than additive if activation of the NO-cGMP-PKG-RyR pathway by the first tetanus enhanced the Ca$^{2+}$ signal produced by the second tetanus, and so forth (Yermolaieva et al. 2000).

Although this hypothesis could thus account for a wide range of results, it is highly simplified and does not include many other molecules and pathways that are also thought to play a role. For example, presynaptic ryanodine receptors are thought to contribute to several types of plasticity including facilitation, potentiation, and LTD (Emptage et al. 2001; Narita et al. 2000; Reyes and Stanton 1996; Reyes-Harde et al. 1999a), and might contribute to other forms of plasticity as well. Furthermore, L-LTP may also have a presynaptic component of expression (Bozagi et al. 2000; Ma et al. 1999; Sokolov et al. 1998). In addition, several important steps are still unknown. For example, we do not yet have direct evidence on how cGMP activates ryanodine receptors, or how intracellular Ca$^{2+}$ release leads to CREB phosphorylation. Additional experiments will be needed to address these issues.

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