Ryanodine produces a low frequency stimulation-induced NMDA receptor-independent long-term potentiation in the rat dentate gyrus *in vitro*

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1. The induction of long-term potentiation (LTP) was investigated in the rat dentate gyrus in the presence of ryanodine, an agent which is known to selectively bind to the ryanodine receptor (RyR) Ca^{2+} channels which regulate Ca^{2+} release from intracellular Ca^{2+} stores.

2. In control media, high frequency stimulation (HFS) induced LTP, and prolonged low frequency stimulation (LFS) induced long-term depression (LTD), of field excitatory postsynaptic potentials (EPSPs) and patch clamped excitatory postsynaptic currents (EPSCs).

3. In the presence of ryanodine, at a threshold concentration of about 1 μM, HFS-induced LTP was inhibited, whereas LFS (5 Hz, 900 pulses) now induced LTP.

4. The *N*-methyl-D-aspartate receptor (NMDAR) antagonist D-2-amino-phosphonopentanoate (D-AP5), at both 50 and 200 μM, did not prevent the induction of LTP by 5 Hz LFS in the presence of ryanodine. This demonstrates the NMDAR independence of LTP induction in the presence of ryanodine. Furthermore, D-AP5 reversed the block of HFS-induced LTP by ryanodine.

5. The induction of LTP by 5 Hz LFS in the presence of ryanodine was blocked by lowering extracellular Ca^{2+}, or by rapidly buffering intracellular Ca^{2+} to very low levels with BAPTA.

6. The induction of LTP by 5 Hz LFS was inhibited by the L-type voltage-gated Ca^{2+} channel blocker nifedipine, and also by Ni^{2+}, a commonly used T-type voltage-gated Ca^{2+} channel blocker.

7. The 5 Hz LFS-induced LTP in the presence of ryanodine was inhibited by the metabotropic glutamate receptor (mGluR) antagonist (+)-α-methyl-4-carboxyphenylglycine (MCPG).

8. The 5 Hz LFS-induced LTP in the presence of ryanodine was blocked by Ruthenium Red, an agent known to block RyR channel opening, and also by thapsigargin, an agent known to block ATP-dependent Ca^{2+} uptake into endoplasmic reticulum.

9. The results of the present studies emphasize the importance of intracellular Ca^{2+} stores in the induction of LTP.

Synaptic transmission in the hippocampus mediated by α-amino-3-hydroxy-5-methylisoxazolepropionic acid receptors (AMPARs) can undergo considerable long-term plasticity, with high frequency stimulation (HFS) of 50–400 Hz inducing long-term potentiation (LTP) of the test excitatory postsynaptic potential/current (Bliss & Collingridge, 1993). The induction of LTP is known to depend on a rise in the concentration of intracellular Ca^{2+}. Thus buffering postsynaptic intracellular Ca^{2+} to very low levels with Ca^{2+} chelators prevented the induction of LTP (Lynch, Larson, Kelso, Barrionuevo & Schottler, 1983). Ca^{2+} influx from the extracellular media is known to be necessary for the induction of LTP, with the amplitude of potentiation being proportional to the concentration of extracellular Ca^{2+} (Mulkeen, Anwyl & Rowan, 1988). Although it has not been established with certainty, the Ca^{2+} influx responsible for LTP induction is thought to occur via the *N*-methyl-D-aspartate receptors (NMDARs), as antagonists of the highly Ca^{2+}-permeable NMDARs block the induction of LTP (Collingridge, Kehl & McLennan, 1983). Moreover, block of the NMDARs also partially prevented the HFS-induced rise in Ca^{2+} concentration observed in dendritic spines (Alford, Frenguelli, Schofield & Collingridge, 1993).

There is increasing evidence that intracellular Ca^{2+} stores may also play a major role in the induction of LTP. Thus thapsigargin, an agent known to deplete intracellular Ca^{2+} stores by inhibiting the endoplasmic reticulum (ER) Ca^{2+} pump, blocked the induction of LTP in CA1 hippocampus (Harvey & Collingridge, 1992). In addition, dantrolene, an agent known to block the release of Ca^{2+} from intracellular stores (Desmedt & Hainaut, 1977), was reported to inhibit the induction of LTP following a prolonged 3 h perfusion (Obenhaus, Mody & Baimbridge, 1989), although a shorter perfusion time of dantrolene was not observed to inhibit LTP induction in the dentate gyrus (O'Mara, Rowan & Anwyl, 1995). In addition, ryanodine and thapsigargin both partially inhibited the HFS-induced rise in the Ca^{2+} concentration in dendritic spines in CA1, experimental evidence leading to the theory that Ca^{2+} entry via the NMDAR results in Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores and subsequent induction of LTP (Alford *et al.* 1993).

The ryanodine receptor (RyR) is located on the intracellular ER membrane. It functions as a ligand-operated Ca^{2+} channel, with certain agonists, including Ca^{2+} itself, activating channel opening and causing Ca^{2+} release, a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (Miller, 1991). The plant alkaloid ryanodine has a high and a low affinity binding site on the RyR. Low concentrations of ryanodine (from nanomolar to ~10 μM) have been shown to bind to the high affinity site and lock the channel in a subconductance state (Meissner, 1986; McPherson *et al.* 1991), while higher concentrations of ryanodine (> 100 μM) bind to the low affinity site and result in channel block (McPherson *et al.* 1991). Ryanodine has been shown in several studies to prevent the caffeine-induced rise in intracellular Ca^{2+}, an action postulated to be the result of ryanodine greatly increasing the passive Ca^{2+} exchange between the Ca^{2+} store and the cytosol, thus preventing net Ca^{2+} accumulation by the store (Thayer, Hirning & Miller, 1988; Friel & Tsien, 1992).

In the present study, possible roles of intracellular Ca^{2+} stores in the induction of LTP were investigated by determining the action of ryanodine on the induction of LTP and long-term depression (LTD) in the dentate gyrus *in vitro*. The dentate gyrus has a particularly high-density expression of RyR, with a prominence in the dendritic spines (Sharp, McPherson, Dawson, Aoki, Campbell & Snyder, 1993).

METHODS

All experiments were carried out on transverse slices of the rat hippocampus (Wistar strain; body weight, 70–150 g). The brains were rapidly removed after decapitation and placed in cold oxygenated (95% O_2–5% CO_2) media. Slices were cut at a thickness of 350 μm using a Campden vibroslice, and placed in a holding chamber containing oxygenated media at room temperature (20–22 °C). The slices were then transferred as required to a recording chamber for submerged slices and continuously superfused at a rate of 5 ml min^{-1} at 30–32 °C.

The control media contained (mM): NaCl, 120; KCl, 2·5; NaH_2PO_4, 1·25; $NaHCO_3$, 26; $MgSO_4$, 2·0; $CaCl_2$, 2·0; and D-glucose, 10. All solutions contained 100 μM picrotoxin (Sigma) to block $GABA_A$-mediated activity. Additional drugs used were D-2-amino-phosphonopentanoate (D-AP5, Tocris Cookson, Bristol, UK), (+)-α-methyl-4-carboxyphenylglycine (MCPG, Tocris Cookson), ryanodine (Calbiochem), Ruthenium Red (Calbiochem) and nifedipine (Sigma). The latter was applied in 0·02 % DMSO. The patch clamp electrode, resistance 5–8 MΩ, contained (mM): potassium gluconate, 130; KCl, 10; EGTA, 10; $CaCl_2$, 1; $MgCl_2$, 3; Hepes, 20; Na_2ATP, 2; NaGTP, 0·5; and 2(triethylamino)-*N*-(2,6-dimethylphenyl) acetamide, 5; pH 7·2 (using KOH). Such an intracellular medium was calculated to give a free internal concentration of Ca^{2+} of 17·5 nM. In certain experiments, the rapid Ca^{2+} chelator BAPTA (20 mM) was added to the patch pipette in order to buffer the intracellular Ca^{2+} to below 10 nM.

Whole-cell recordings from dentate granule cells were made using an Axopatch-1D amplifier (3 kHz low-pass Bessel filter), as described previously (O'Connor, Rowan & Anwyl, 1995). The capacitative current was always electronically cancelled and the series resistance (8–20 MΩ, as measured directly from the amplifier) compensated by 60–70%. The mean input resistance was 239 ± 24 MΩ, and the mean resting potential was −69 ± 4 mV. The input resistance was monitored continuously, and the recording terminated if it varied by more than 10%. Recordings of the amplitude of field excitatory postsynaptic potentials (EPSPs) were made using a low resistance (~1 MΩ) glass pipette placed in the medial perforant pathway. Excitatory postsynaptic currents (EPSCs) and field EPSPs were recorded in response to stimulation of the medial perforant pathway at a control frequency of 0·033 Hz, with the stimulation intensity adjusted to evoke an EPSC which was about 30% of the maximum amplitude, usually about 50–100 pA for AMPA EPSCs, equivalent to intracellular EPSPs of 5–10 mV and 1·5 mV for field EPSPs. LTP was induced by high frequency stimulation consisting of eight trains, each of eight pulses at 200 Hz with an intertrain interval of 2 s, under current clamp conditions for the duration of the tetanus in all experiments. The magnitude of LTP was measured at 20–35 min post-HFS. The amplitude of LTP had stabilized by 20 min following an initial larger short-term potentiation, and no difference was found between measurements of LTP at 20–35 min post-HFS. Control LTD was evoked by LFS, 900 stimuli at 5 Hz, and the magnitude measured 20–30 min post-LFS. Full experiments were carried out providing that certain criteria were met. These included a resting potential of at least −65 mV, a high input resistance (at least 200 MΩ) and a low threshold and steep input–output curve for the EPSCs.

Recordings were analysed using the Strathclyde electrophysiological software (Dr J. Dempster, University of Strathclyde, Glasgow, UK). Values are means ± S.E.M., and Student's *t* test was used for statistical comparisons.

RESULTS

Ryanodine blocks HFS-induced LTP

In control media, HFS resulted in the induction of LTP of AMPAR-mediated EPSCs, measuring 160 ± 6% ($n = 5$) at 25 min post-stimulation (Fig. 1), and of field EPSPs, measuring 152 ± 10% ($n = 5$) at 30 min post-stimulation. Ryanodine was found to block the HFS induction of LTP. This effect of ryanodine is shown in Fig. 1, in which HFS failed to induce LTP of the EPSC in the presence of

ryanodine (20 μM ryanodine in the patch pipette), the amplitude of the EPSC measuring 106 $\pm$ 1% of control ($n = 5$) at 30 min post-stimulation ($P < 0{\cdot}001$). Note also that short-term potentiation (STP) was blocked by this concentration of ryanodine, the EPSC measuring 99 $\pm$ 6% at 2 min post-stimulation compared with 179 $\pm$ 15% in control media ($P < 0{\cdot}001$).

The threshold extracellular concentration of ryanodine generating a block of induction of LTP of the field EPSP was about 100 nM. Thus HFS applied after 2 h extracellular perfusion of 100 nM ryanodine resulted in a complete inhibition of the induction of LTP of the field EPSP, which measured 97 $\pm$ 2% ($n = 5$) at 20 min post-stimulation ($P < 0{\cdot}001$). This low concentration of ryanodine did not completely inhibit the induction of STP, which had an amplitude of 113 $\pm$ 8% ($n = 5$) at 5 min post-stimulation and a duration of 5–10 min. Higher concentrations of ryanodine (1, 4 and 20 μM) blocked the induction of both STP and LTP of the field EPSP. For example, the amplitude of STP and LTP at 5 and 30 min post-HFS measured 103 $\pm$ 7% and 91 $\pm$ 9% ($n = 5$), respectively, in 1 μM ryanodine ($P < 0{\cdot}001$). Extracellular perfusion of ryanodine, at concentrations up to 100 μM, did not alter the amplitude of the test field EPSP.

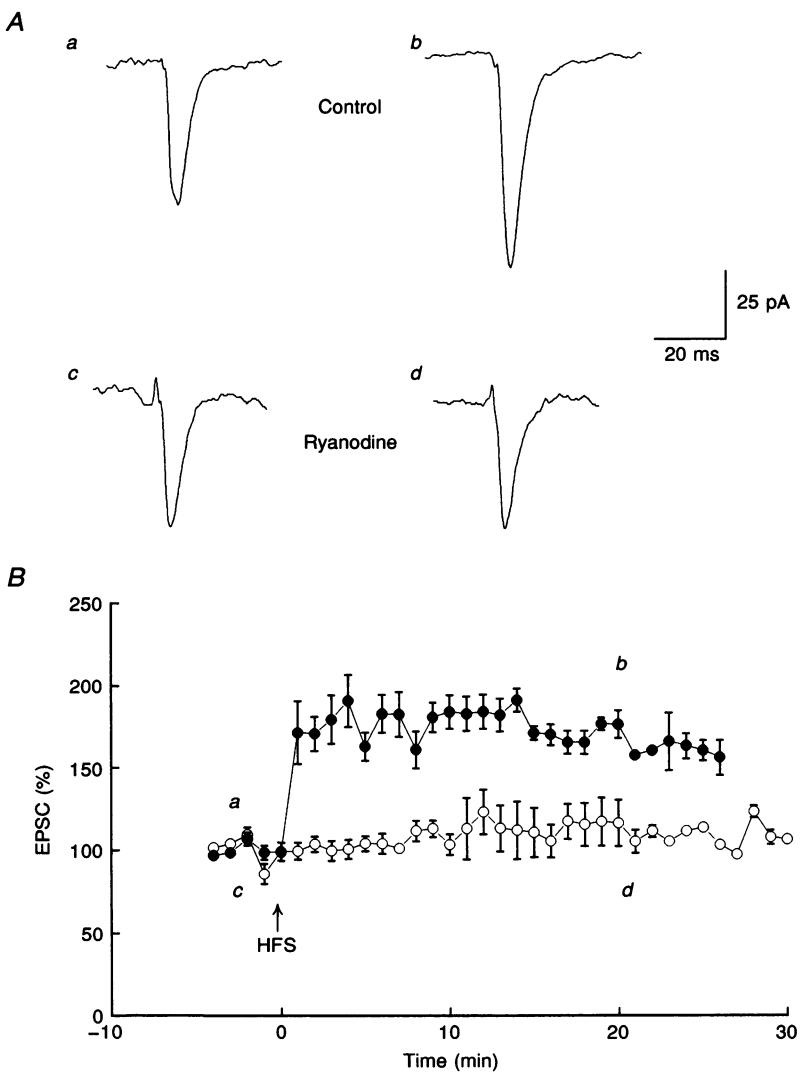

Figure 1. Inhibition of high frequency stimulation-induced LTP by ryanodine

A, original unaveraged records of excitatory postsynaptic currents (EPSCs) recorded under whole-cell patch clamp conditions from the cell bodies of dentate gyrus cells. EPSCs recorded 5 min prior to HFS in control media (*a*) and in the presence of ryanodine (*c*). EPSCs recorded 25 min post-HFS in control media (*b*) and ryanodine (*d*). Note the induction of LTP in control media, but not in ryanodine. Ryanodine (20 μM) was present in the patch pipette. *B*, the extent and time course of LTP induction following HFS (arrow) in control media (●), the EPSC measuring 160% ($n = 5$) of control at 25 min post-stimulation, and the inhibition of LTP induction following HFS in the presence of ryanodine (○), the EPSC measuring 106% ($n = 5$) of control at 25 min post-stimulation. The whole-cell recording mode was made 10 min prior to application of HFS. Each data point shown is the mean of 2 recordings of EPSCs.

LFS (5 Hz) induces LTP in the presence of ryanodine

In control media, LFS, consisting of 900 pulses at 5 Hz, induced LTD of both the EPSC and the field EPSP. Thus the amplitude of the EPSC was depressed to 54 ± 3% of control ($n = 5$) at 20 min post-stimulation (Fig. 2). In contrast, in the presence of ryanodine, 5 Hz LFS resulted in the induction of LTP. The effect of 20 μM ryanodine applied intracellularly from the patch pipette is shown in Fig. 2, in which 5 Hz stimulation induced a rapid onset LTP of the EPSC that measured 160 ± 13% and 148 ± 28% ($n = 5$) at 5 and 20 min post-stimulation, respectively, which was a significant enhancement from control levels ($P < 0{\cdot}001$).

The threshold extracellular concentration of ryanodine for such LFS-induced LTP of the field EPSP was about 1 μM. A concentration of 100 nM ryanodine had no effect on 5 Hz-induced LTD, the field EPSP measuring 68 ± 3% ($n = 5$) of control levels at 30 min post-stimulation. In the presence of 1 μM ryanodine, a 5 Hz, 900 pulse stimulation resulted in the EPSP having an mean amplitude of 102 ± 2% at

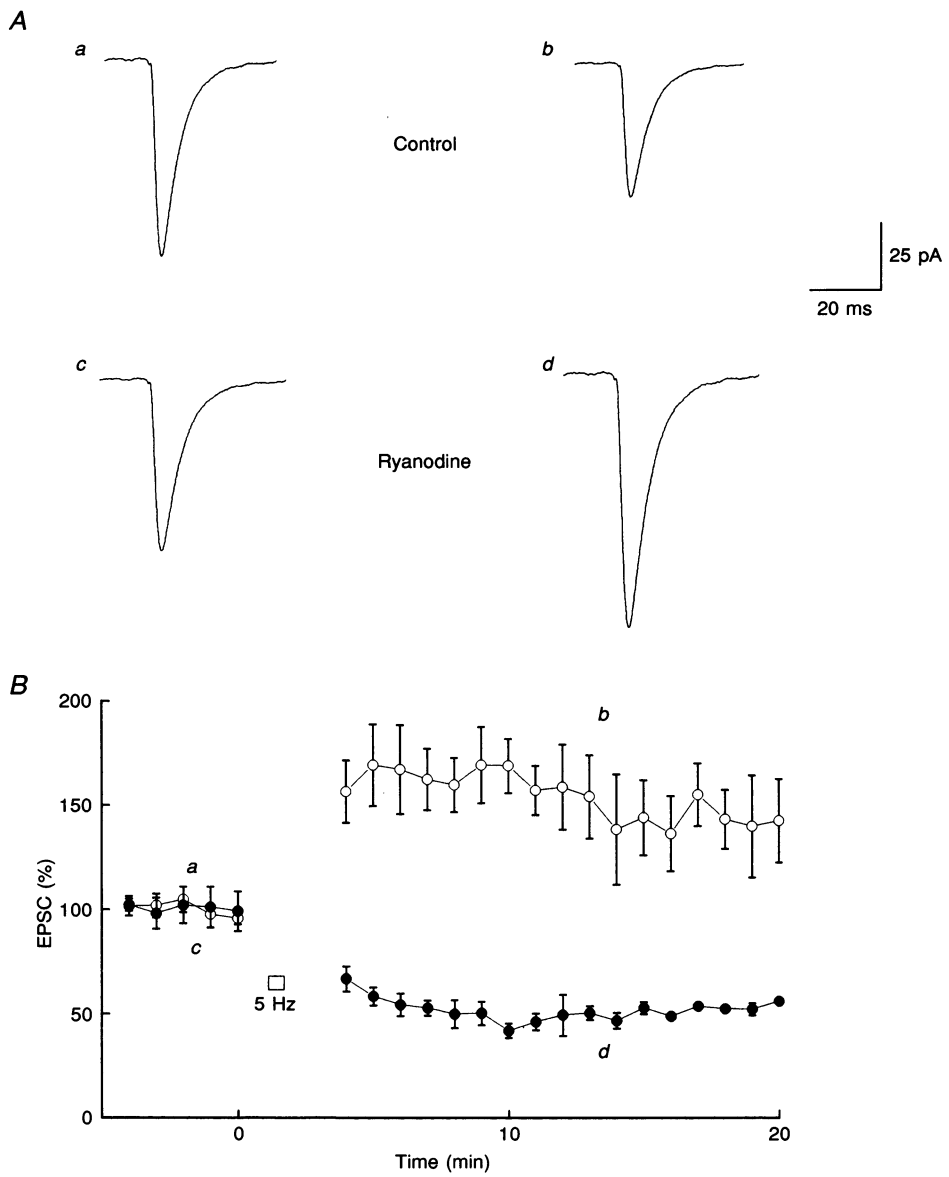

Figure 2. Induction of LTP by low frequency (5 Hz) stimulation

A, original unaveraged records of EPSCs. EPSCs recorded 5 min prior to 5 Hz, 900 pulse stimulation in control media (*a*) and in the presence of ryanodine (*c*; 20 μM in pipette). EPSCs recorded following 5 Hz, 900 pulse stimulation in control media (*b*) and in ryanodine (*d*). Note the induction of LTD in control media and LTP in ryanodine. *B*, the extent and time course of the changes in the EPSC following 5 Hz stimulation (open bar). In control media (●), 5 Hz, 900 pulse stimulation induced LTD averaging 54% ($n = 5$) at 20 min post-stimulation. In the presence of ryanodine (○), the stimulation induced LTP measuring 148% ($n = 5$) at 20 min post-stimulation. Each data point shown is the mean of 3 recordings of EPSCs.

30 min post-stimulation, a significant enhancement from the control LTD ($P < 0{\cdot}001$). In the presence of 4 μM ryanodine, 5 Hz stimulation induced LTP, the EPSP measuring 148 ± 5% ($n = 5$) at 30 min post-stimulation, which was a significant enhancement from control levels ($P < 0{\cdot}001$).

The generation of the 5 Hz-induced LTP and the block of HFS-induced LTP by ryanodine are shown in Fig. 3*A*. This figure shows an experiment in which extracellular perfusion of 4 μM ryanodine had no effect on the amplitude of the test EPSP, and HFS resulted in a complete absence of STP or LTP. In five similar experiments the EPSP measured 102 ± 3% and 98 ± 2% at 2 and 20 min post-stimulation ($n = 6$), which was a significant inhibition ($P < 0{\cdot}001$). However, subsequent application of 5 Hz stimulation resulted in LTP measuring 148 ± 5% ($n = 6$).

The LTP induced by 5 Hz LFS in ryanodine was occluded by prior LTP induced in control media by HFS (Fig. 3*B*). In these experiments, LTP of the field EPSP was initially induced by HFS in control media (mean LTP at 25 min post-stimulation was 152 ± 5%, $n = 5$). Following perfusion of 4 μM ryanodine, 5 Hz LFS failed to induce LTP, a small depotentiation was induced instead. Such experiments demonstrate that the maintenance mechanisms of the

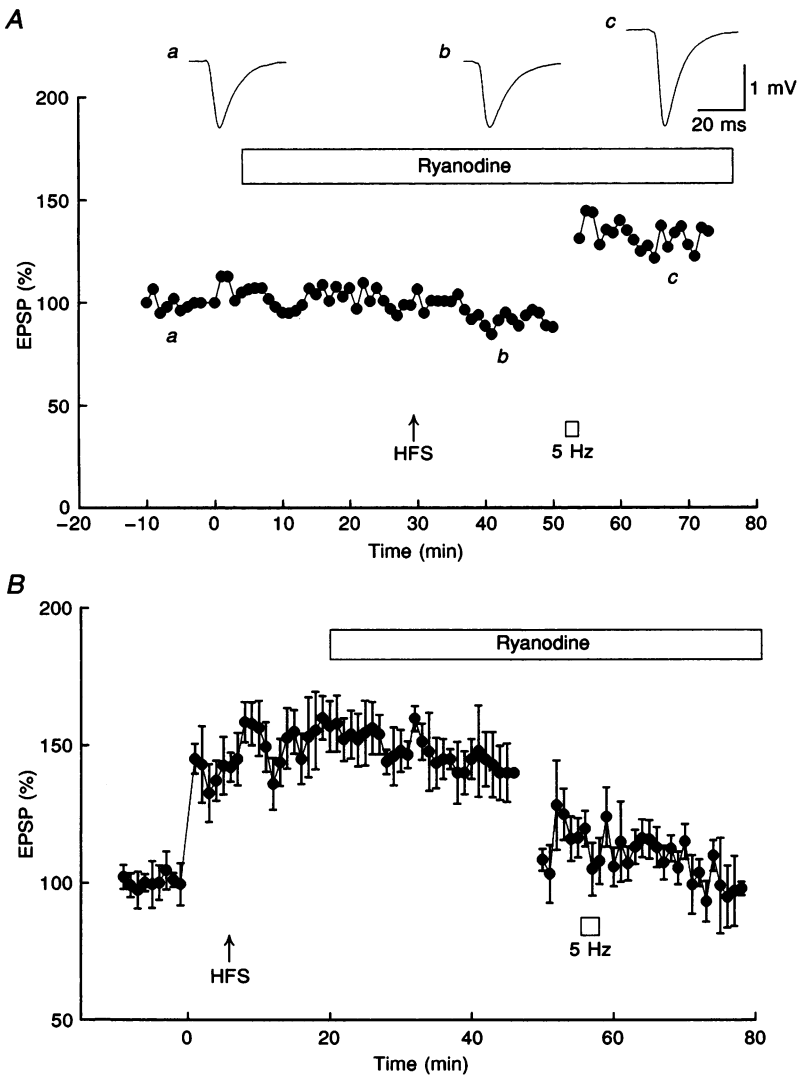

Figure 3. LFS-induced LTP can be evoked despite block of HFS-induced LTP in the presence of ryanodine, but HFS-induced LTP in control media occludes LFS-induced LTP in the presence of ryanodine

A, the graph shows an example of an experiment in which perfusion of ryanodine (4 μM) did not alter the amplitude of the field EPSP, and application of HFS (arrow) failed to induce LTP. However, stimulation of 900 pulses at 5 Hz (open bar), did induce LTP measuring 142%, at 20 min post-stimulation. *B*, an occlusion experiment in which application of HFS induced LTP of 152 ± 5% ($n = 5$) in control media. Subsequent application of 5 Hz LFS in the presence of ryanodine (4 μM) failed to induce LTP, instead it induced a small depotentiation.

control HFS-induced LTP and the LFS-induced LTP in ryanodine are identical.

LFS-induced LTP in the presence of ryanodine is not blocked by D-AP5

The dependency of the LFS-induced LTP in the presence of ryanodine was investigated by applying 5 Hz stimulation in the presence of the NMDAR antagonist D-AP5. The NMDAR independence of the 5 Hz LTP induction in the presence of ryanodine was demonstrated by the experiments shown in Fig. 4*A*. Application of D-AP5 (50 μM) was initially shown to inhibit the induction of LTP by HFS in control media, the EPSP measuring 105 ± 6% ($n = 6$) at 20 min post-stimulation, which was a significant block ($P < 0{\cdot}001$). LFS (5 Hz) was then applied in the presence of 4 μM ryanodine, resulting in the induction of LTP of 144 ± 5% ($n = 5$) at 35 min post-stimulation, a value not significantly different from that in the absence of D-AP5 (148 ± 5%).

It is possible that the rise in the concentration of intracellular Ca^{2+} generated by tetanic stimulation is greater with ryanodine present due to non-functioning intracellular stores. This may result in LTP, in the presence of ryanodine, being induced by a much smaller influx of Ca^{2+} through the NMDAR than in control media. To eliminate this possibility, complete block of NMDAR was ensured by raising the concentration of D-AP5 to 200 μM. Figure 4*B* shows that even this very high concentration of D-AP5 did not block the induction of LTP in the presence of ryanodine, with 5 Hz LFS inducing LTP measuring 142 ± 3% ($n = 5$).

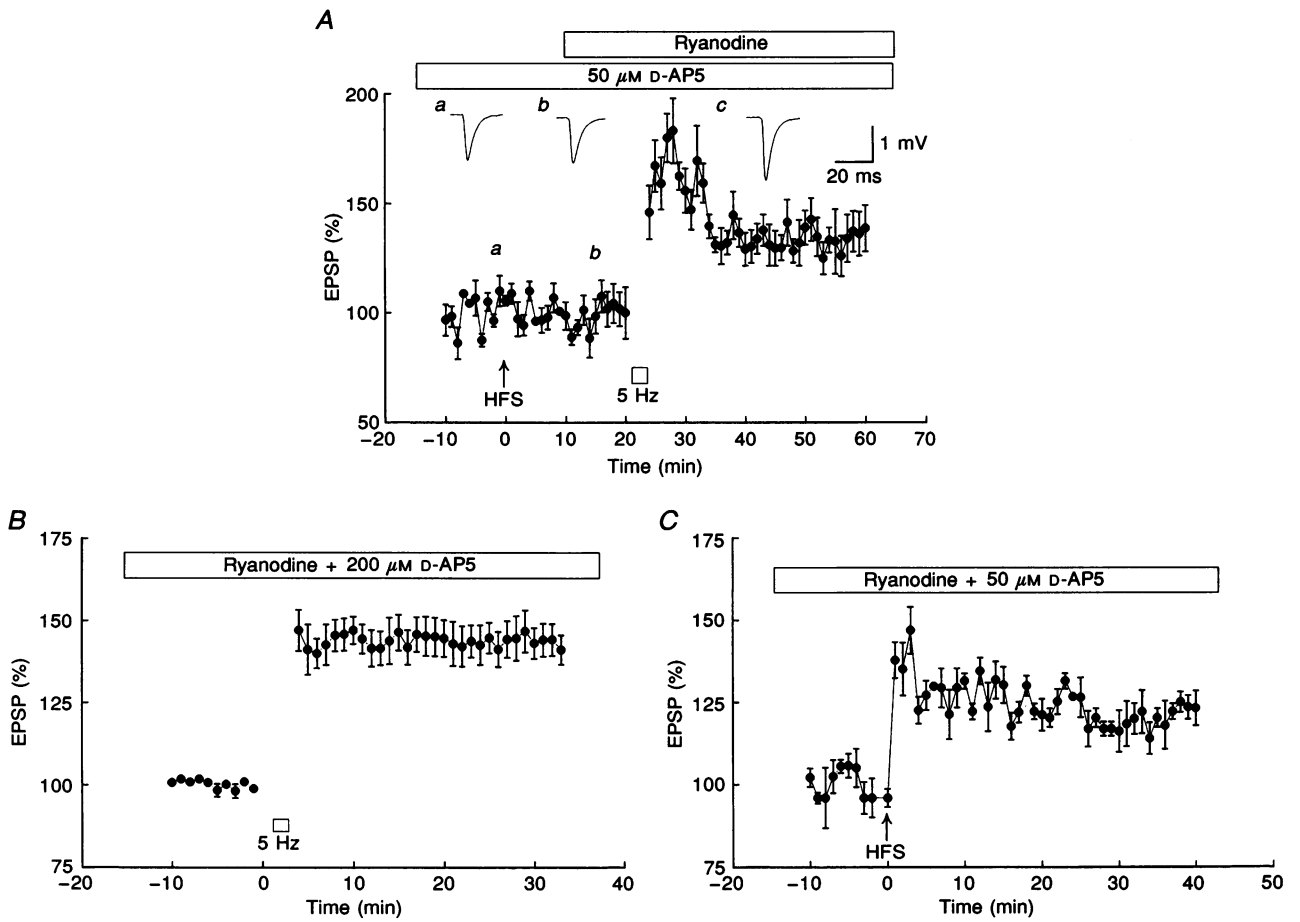

Figure 4. In the presence of ryanodine, LFS-induced LTP of the field EPSP is independent of NMDAR activation, but HFS does induce LTP if NMDAR activation is completely blocked with D-AP5

A, D-AP5 (50 μM) blocked the induction of LTP by HFS, the EPSP measuring 105% at 20 min post-stimulation, but did not occlude the 5 Hz, 900 pulse, LFS-induced LTP in the presence of ryanodine (4 μM), with the LTP averaging 140% ($n = 6$) at 35 min post-stimulation. *B*, D-AP5 (200 μM) did not block the induction of the 5 Hz, 900 pulse, LFS-induced LTP in the presence of ryanodine, which measured 142% ($n = 5$). *C*, HFS does induce LTP in ryanodine in the presence of the NMDAR antagonist D-AP5. The graph shows that HFS in ryanodine (4 μM) plus D-AP5 (50 μM) induced LTP of the field EPSP of 135% ($n = 6$) at 35 min post-stimulation.

D-AP5 reverses the ryanodine block of HFS-induced LTP

In the presence of both ryanodine (4 μM) and the NMDAR antagonist D-AP5 (50 μM), LTP measuring 135 ± 14% ($n = 6$) at 35 min post-stimulation was induced by HFS (Fig. 4*C*), a value significantly higher than that in the presence of ryanodine alone in which HFS-induced LTP was completely blocked (101 ± 2%; $P < 0{\cdot}05$). These experiments demonstrate that with ryanodine present, an HFS-induced LTP was inhibited by the activation of NMDARs.

LFS-induced LTP in the presence of ryanodine is dependent on an influx of Ca^{2+}

The role of Ca^{2+} in the 5 Hz-induced LTP in the presence of ryanodine was investigated in two ways: (1) intracellular application of the rapid Ca^{2+} chelator BAPTA; and (2) reducing extracellular Ca^{2+}.

Using patch clamp recordings of the EPSC, LFS was applied 10 min after the application of 20 mM BAPTA from the patch pipette in the presence of ryanodine (20 μM in the patch pipette). LFS (5 Hz) failed to induce LTP under such conditions, the EPSC measuring 97 ± 4% ($n = 5$) of control levels at 30 min post-stimulation, which was a significant inhibition of the 5 Hz-induced LTP ($P < 0{\cdot}001$; Fig. 5*A*).

In extracellular recordings, reducing extracellular Ca^{2+} from the control concentration of 2·0 mM to 0·8 mM also resulted in an inhibition of the 5 Hz-induced LTP in the presence of ryanodine. Thus the field EPSP measured 86 ± 5% ($n = 5$) of control levels at 30 min post-stimulation, a significant inhibition of the 5 Hz-induced LTP ($P < 0{\cdot}001$; Fig. 5*B*).

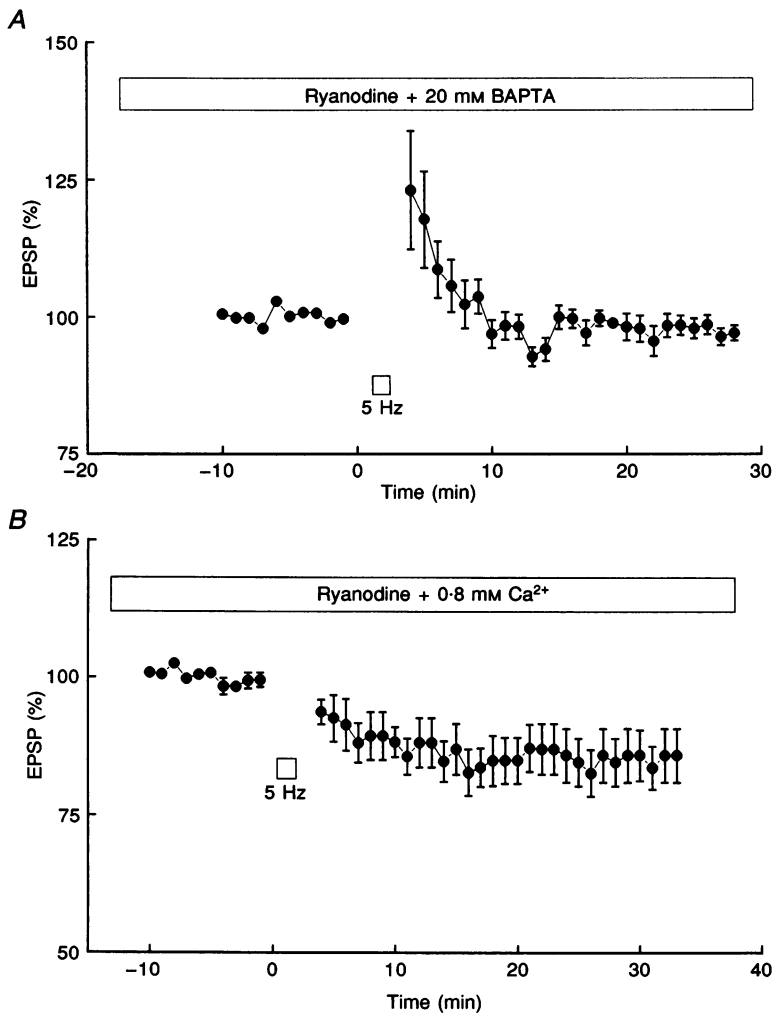

Figure 5. LFS (5 Hz)-induced LTP of the patch clamped EPSC in the presence of ryanodine is inhibited by buffering intracellular Ca^{2+} to a low level and also in low extracellular Ca^{2+}

A, in the presence of 20 mM BAPTA (included in the patch pipette), 5 Hz LFS with ryanodine present (20 μM in the pipette) did not induce LTP of the EPSC, which measured 97% ($n = 5$) at 25 min post-stimulation. *B*, 5 Hz LFS applied in the presence of ryanodine (4 μM) in 0·8 mM extracellular Ca^{2+} did not induce LTP of the field EPSP, but rather an LTD, the EPSP measuring 86 ± 5% ($n = 5$) at 30 min post-stimulation.

LFS-induced LTP in the presence of ryanodine is induced by Ca^{2+} entry via voltage-gated Ca^{2+} channels

The role of voltage-gated Ca^{2+} channels in the induction of the LFS-induced LTP was examined by investigating the action of two known blockers of voltage-gated Ca^{2+} channels: Ni^{2+}, which is known to inhibit T-type channels, and nifedipine, which inhibits L-type channels. At a concentration of 50 μM, Ni^{2+} did not alter the amplitude of the test EPSP, which measured 98 $\pm$ 2% ($n = 5$) at 30 min following the perfusion of Ni^{2+}. However, Ni^{2+} was found to significantly inhibit the induction of LTP induced by LFS in the presence of ryanodine (4 μM), the EPSP measuring 99 $\pm$ 3% ($n = 7$) at 30 min following 5 Hz stimulation ($P < 0.001$; Fig. 6*A*). This inhibition of LTP by Ni^{2+} was not caused by a Ni^{2+}-induced frequency-dependent block of the EPSP during the 5 Hz stimulation. Thus the mean of ten EPSPs numbering 290–300, 590–600 and 890–900 during 5 Hz stimulation, measured 0·86, 0·73 and 0·66 mV, respectively, in control media, and 0·79, 0·69 and 0·65 mV, respectively, in Ni^{2+}. Similar results were obtained in a further two experiments.

Nifedipine did not alter the amplitude of the test EPSP, but did significantly inhibit the induction of LFS-induced LTP in the presence of ryanodine (4 μM). Thus the induction of LTP by 5 Hz LFS was almost completely abolished in the presence of both 10 μM nifedipine and ryanodine, the EPSP measuring 107 $\pm$ 3% at 30 min post-stimulation ($P < 0.001$; Fig. 6*B*). LTP (5 Hz) induction in the presence of ryanodine was also significantly reduced by 2 μM nifedipine, the EPSP measuring 132 $\pm$ 4% ($n = 6$) at 30 min post-stimulation ($P < 0.05$).

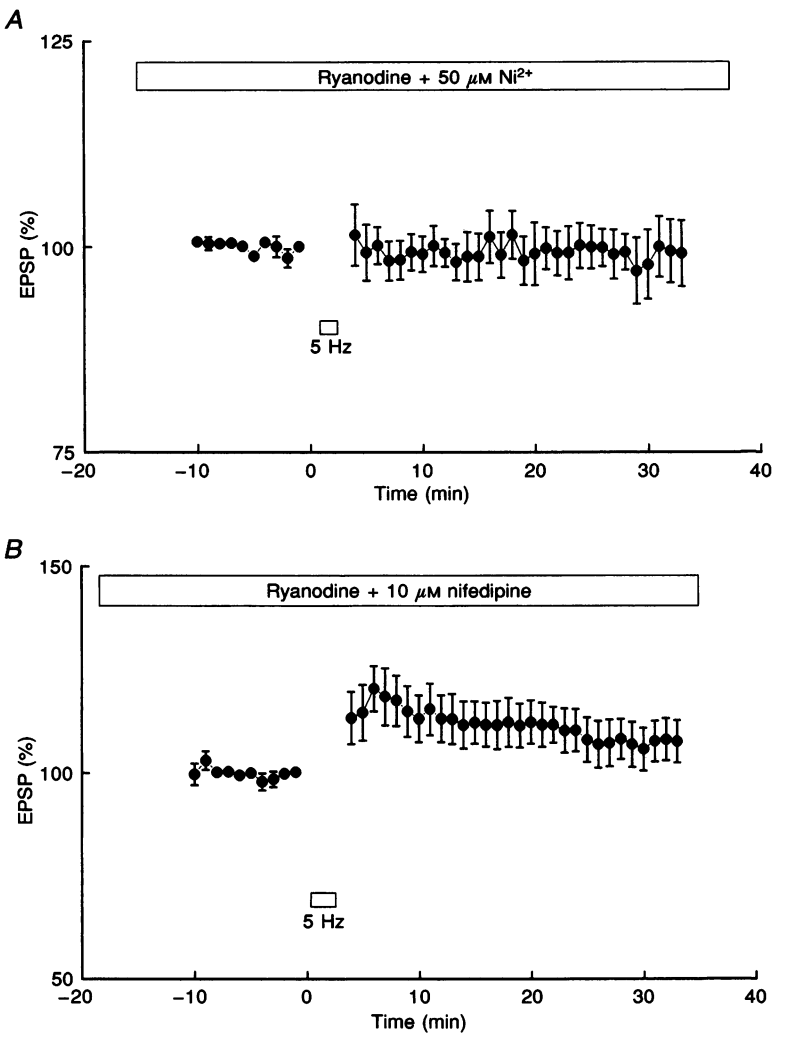

Figure 6. Both Ni^{2+} and nifedipine inhibit induction of LFS-induced LTP of the field EPSP in the presence of ryanodine

A, perfusion of Ni^{2+} (50 μM) did not alter the amplitude of the test EPSP in the presence of ryanodine, but did block the induction of LTP induced by LFS, the EPSP measuring 99% ($n = 5$) at 30 min post-LFS. *B*, perfusion of nifedipine (10 μM) reduced the amplitude of LTP induced by LFS to 107% ($n = 5$) at 30 min post-LFS.

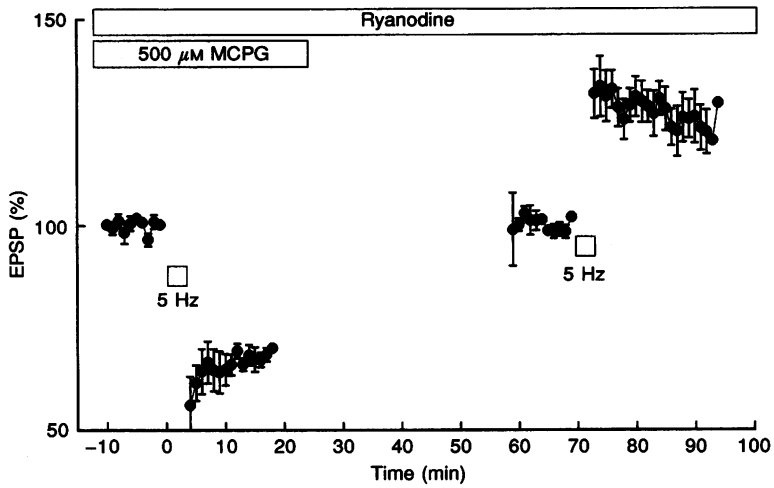

Figure 7. The 5 Hz LFS-induced LTP in the presence of ryanodine is mediated by activation of mGluRs

The graph shows that MCPG (500 μM) blocked the induction of LTP of the field EPSP by pulse stimulation at 5 Hz, in the presence of ryanodine (4 μM), with an LTD measuring 66% ($n = 5$) being induced at 20 min post-stimulation. Following washout of MCPG for 40 min, and adjustment of the afferent stimulation voltage in order to return the EPSP amplitude to its original level, the 5 Hz stimulation induced LTP measuring 131% ($n = 5$).

LFS-induced LTP in the presence of ryanodine is metabotropic glutamate receptor (mGluR) dependent

The dependency of the LFS-induced LTP in the presence of ryanodine was determined by investigating the action of the mGluR antagonist MCPG (500 μM). The 5 Hz LFS-induced LTP in the presence of ryanodine was dependent upon activation of mGluR, the LTP being inhibited by MCPG. Figure 7 shows that MCPG (500 μM) blocked the LTP of the EPSP induced by 5 Hz LFS, applied in the presence of ryanodine (4 μM), the stimulation inducing LTD as in control media (the EPSP measured 66 ± 2% of pre-stimulation value at 10 min post-stimulation), which was a significant reduction of the LTP ($P < 0{\cdot}001$). Following 40 min washout of the MCPG, repeating the 5 Hz stimulation with the ryanodine present resulted in the induction of LTP measuring 131 ± 4% ($n = 5$).

LFS-induced LTP in the presence of ryanodine is blocked by Ruthenium Red

Ruthenium Red is known to act as an antagonist at the RyR (Ma, Fill, Knudson, Campbell & Coronado, 1988; Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988). Ruthenium Red (20 μM) was applied intracellularly from the patch pipette. After establishing the whole-cell mode,

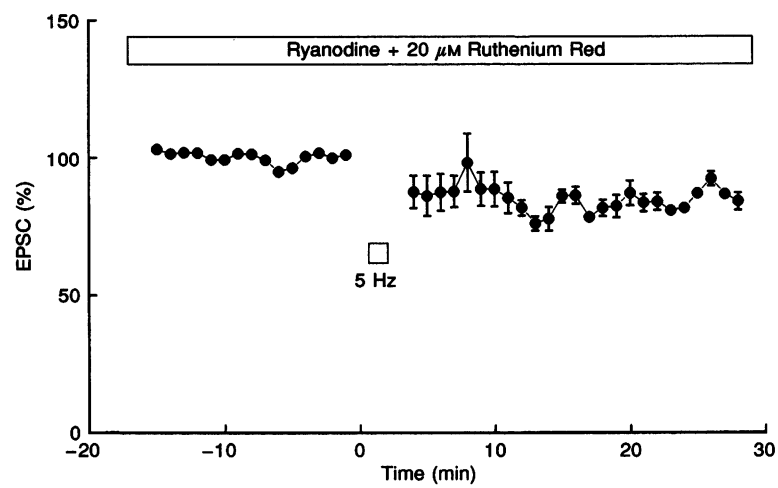

Figure 8. Ruthenium Red inhibits 5 Hz-induced LTP in the presence of ryanodine

The graph shows that 5 Hz, 900 pulses given in the presence of ryanodine (20 μM in the patch pipette) plus Ruthenium Red (20 μM) did not induce LTP of the patch clamped EPSC.

the EPSC was monitored for 15 min to allow diffusion of the dye into the cell. Ruthenium Red blocked the 5 Hz LFS-induced LTP in the presence of ryanodine (20 μM in the patch pipette) with the EPSC measuring 78 $\pm$ 4% ($n = 5$) 30 min following LFS, a value significantly reduced from that in the presence of ryanodine alone ($P < 0{\cdot}001$; Fig. 8).

LFS-induced LTP in the presence of ryanodine is blocked by thapsigargin

Thapsigargin is known to deplete intracellular Ca^{2+} stores by acting as an antagonist at the ATP-dependent Ca^{2+} uptake site on intracellular Ca^{2+} stores (Thastrup, Cullen, Droback, Hanley & Dawson, 1990). At a concentration of 2 μM it was found to prevent the induction of the LFS-induced LTP in ryanodine. Thus 5 Hz LFS applied in the presence of thapsigargin and ryanodine did not induce LTP, the EPSP measuring 84 $\pm$ 5% at 30 min post-stimulation, which was significantly reduced from the value in the presence of ryanodine alone ($P < 0{\cdot}005$; Fig. 9).

DISCUSSION

The present studies have shown that ryanodine greatly alters the induction requirements of LTP. Thus LTP in the presence of ryanodine was induced by 5 Hz LFS, but not by HFS. Moreover, LTP induction in the presence of ryanodine was independent of the activation of NMDAR, being unaffected by the NMDAR antagonist D-AP5, even at very high concentrations. NMDAR-dependent LTP induced by weak to moderate tetani has been shown previously to be fully abolished by 25–50 μM D-AP5 (Huang & Malenka, 1993; Hanse & Gustafson, 1995), although LTP induced by a very strong repeated tetanus was only completely blocked by higher concentrations of D-AP5 (100 μM) (Hanse & Gustaffson, 1995). In the present experiments, it is extremely unlikely that there is incomplete block of activation of NMDAR by the very high concentrations of D-AP5 (200 μM) during the relatively mild tetanus (5 Hz) applied in the presence of ryanodine. The restoration of HFS-induced LTP by D-AP5 with ryanodine present indicates that activation of NMDAR actually inhibits induction of LTP in the presence of ryanodine. Inhibition of LTP by excessive or inappropriate stimulation of NMDAR has been observed previously. For example, activation of the NMDAR prior to HFS, either with low Mg^{2+} (Coan, Irving & Collingridge, 1989) or with NMDA (Izumi, Clifford & Zorumski, 1992), was shown to inhibit the induction of LTP, and the induction of LTP could be restored by application of a low concentration of D-AP5 (Coan *et al.* 1989).

The inhibition of the 5 Hz-induced LTP in the presence of ryanodine by Ni^{2+} and nifedipine suggests that this form of LTP is induced by Ca^{2+} influx via voltage-dependent Ca^{2+} channels. The block of the 5 Hz-induced LTP, in the presence of ryanodine, by nifedipine strongly suggests that Ca^{2+} entry via L-type Ca^{2+} channels mediates, at least in part, this form of LTP induction, as nifedipine is a well-known HVA L-type Ca^{2+} channel blocker (Carbone & Swandulla, 1989), and L-type Ca^{2+} channels have been found on dendrites of hippocampal neurons using dendritic patch clamp recordings (Magee & Johnston, 1995). The dependency of LTP induction by LFS in the presence of ryanodine on Ca^{2+} influx via L-type Ca^{2+} channels is very different from the standard NMDAR-dependent HFS-induced LTP, which is not blocked by L-type Ca^{2+} channel blockers (Taube & Schwartzkroin, 1986; Grover & Teyler, 1990; Huang & Malenka, 1993). However, it has been shown previously that Ca^{2+} influx via L-type Ca^{2+} channels can induce LTP. Thus a relatively small NMDAR-insensitive component of HFS-induced LTP has been found to be blocked by L-type Ca^{2+} channel blockers (Grover & Teyler, 1990). Moreover, repeated depolarizing pulses paired with

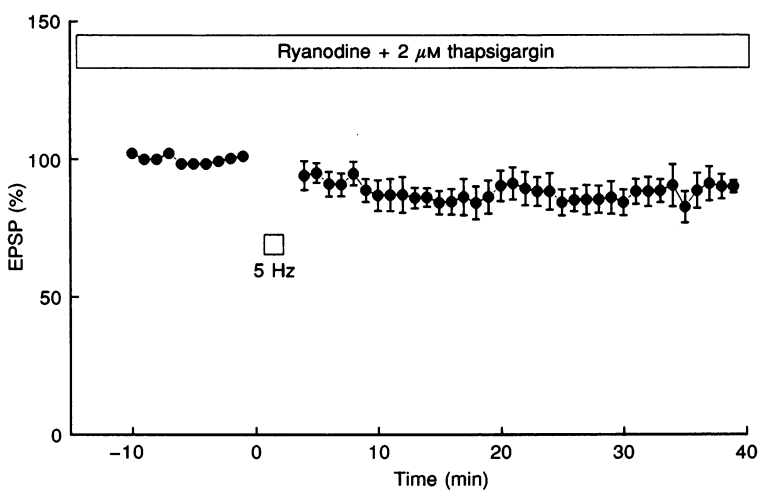

Figure 9. Thapsigargin blocks the induction of 5 Hz-induced LTP in ryanodine

The graph shows that 5 Hz, 900 pulses given in the presence of ryanodine (4 μM) plus thapsigargin (2 μM) did not induce LTP of the field EPSP.

low frequency synaptic stimulation induced a long-lasting NMDAR-independent potentiation which was dependent on Ca^{2+} influx via nifedipine-sensitive Ca^{2+} channels (Kullman, Perkel, Manabe & Nicoll, 1992). Recent evidence has demonstrated cross-signalling between the RyR and the L-type Ca^{2+} channel in both skeletal muscle (Nakal, Dirkson, Nguyen, Pessah, Beam & Allen, 1996) and cardiac muscle (Sham, Cleeman & Morad, 1995), with functional RyRs necessary for the initiation of L-type Ca^{2+} currents (Nakal *et al.* 1996). It is therefore possible that the activation of the L-type Ca^{2+} channels during LTP induction in the presence of ryanodine in the present studies results from such cross-signalling following ryanodine activation of the RyR. The abolition of the 5 Hz-induced LTP, with ryanodine present, following depletion of intracellular Ca^{2+} stores by thapsigargin provides further evidence that normally functioning intracellular Ca^{2+} stores are required for such LTP.

LFS-induced LTP in the presence of ryanodine was found to be inhibited by Ni^{2+}. This provides some evidence that Ca^{2+} entry via T-type Ca^{2+} channels is essential for the induction of such LTP, as T-type Ca^{2+} channels exhibit a greater sensitivity to Ni^{2+} (IC_{50} of ~50 μM) than most other Ca^{2+} channel subtypes (Fox, Nowycky & Tsien, 1987; Magee & Johnston, 1995). Ni^{2+}-sensitive low voltage threshold Ca^{2+} channels have been identified in hippocampal cells, including hippocampal dendrites (Mogul & Fox, 1991; Eliot & Johnston, 1994; Magee & Johnston, 1995). Moreover, subthreshold trains of synaptic potentials have also been shown to produce a Ni^{2+}-sensitive local increase in intradendritic Ca^{2+} concentration (Magee, Christofi, Miyakawa, Christie, Lasser-Ross & Johnston, 1995). It should be stressed that other Ca^{2+} channels, such as R-type channels in the cerebellum, are also fairly sensitive to a block by Ni^{2+} (Regan, 1991; Zhang *et al.* 1993). HFS-induced NMDAR-dependent LTP has also been shown to be blocked by Ni^{2+} (Ito *et al.* 1995), although control experiments for a frequency-dependent block of presynaptic Ca^{2+} channels by Ni^{2+} during HFS were not performed in these experiments. Thus it is possible that Ca^{2+} entry via Ni^{2+}-sensitive Ca^{2+} channels is required for both NMDAR and L-type Ca^{2+} channel-sensitive LTP induction.

The 5 Hz LFS-induced LTP in ryanodine was shown to be blocked by MCPG, demonstrating that such LTP induction is dependent on activation of mGluRs. There is now convincing evidence that standard NMDAR-dependent LTP induction requires activation of mGluRs. Thus the selective mGluR antagonist MCPG blocked the induction of LTP (Bashir *et al.* 1993), while the selective mGluR agonist 1-aminocyclopentane-(1*S*,3*R*)-dicarboxylate (ACPD) was found to both strongly potentiate HFS-induced LTP (McGuinness, Anwyl & Rowan, 1991) and to directly evoke an NMDAR-dependent (O'Connor *et al.* 1995) or NMDAR-independent (Bortolotto & Collingridge, 1993) long-lasting potentiation. One difference between the action of MCPG in the dentate gyrus in control media and that in ryanodine is that in the former, a blocking action on LTP was only observed following prior application of LFS (Wang, Rowan & Anwyl, 1995), whereas in the presence of ryanodine, such prior LFS was not necessary to obtain a block of LTP induction.

The results of the present studies suggest that ryanodine is altering the induction properties of LTP by binding to the high affinity site on the intracellular RyR on the RyR-sensitive Ca^{2+} store, and locking the RyR channel in an open state. Thus the concentrations of ryanodine effective in the present study (threshold of 100 nM to 1 μM) are similar to those shown previously to lock the RyR channel in a low conductance open-configuration state (Meissner, 1986; McPherson *et al.* 1991). The concentrations of ryanodine effective in the present studies are also much lower than those found previously to inhibit opening of the HVA dihydropyridine-sensitive Ca^{2+} channels (IC_{50} of 45 μM, with little effect under 10 μM; Valdivia & Coronado, 1989).

Intracellular Ca^{2+} stores could have a role in the induction of standard NMDAR-dependent LTP by acting as a store of Ca^{2+}, and releasing Ca^{2+} during HFS by calcium-induced calcium release (CICR). Evidence for this hypothesis has been presented in previous studies, which have shown that CICR contributes a substantial part of the intracellular rise in calcium following either HFS (Alford *et al.* 1993) or application of NMDA (Simpson, Challiss & Nahorski, 1993), with ryanodine or thapsigargin partially blocking such a rise in intracellular Ca^{2+}. The inhibition of HFS-induced LTP by ryanodine in the present studies may be generated by inhibition of CICR, if CICR is required for induction of HFS-induced LTP. Alternatively, intracellular Ca^{2+} stores could act as a sink for Ca^{2+} entering the cell following HFS. The block of HFS-induced LTP in the presence of ryanodine would then be caused by ryanodine preventing the stores acting as such a sink. Consequently, the Ca^{2+} influx via the NMDARs would result in the intracellular cytosol concentration of Ca^{2+} rising to a level which is excessively high for the induction of LTP. Experimental evidence for ryanodine preventing Ca^{2+} stores acting as a sink has been presented in previous studies (Thayer *et al.* 1988; Friel & Tsien, 1992; Kano, Garaschuk, Verkhratsky & Konnerth, 1995).

If the levels of Ca^{2+} entering the cell in the present experiments are reduced in the presence of ryanodine, either by blocking the NMDARs during HFS, or alternatively, by applying LFS, then the intracellular Ca^{2+} stores will not be required as a sink for Ca^{2+}, and thus LTP will be induced. This theory for the site of action of ryanodine is supported by the ability of Ruthenium Red to block the action of ryanodine in the present studies. Ruthenium Red has previously been shown to bind to the RyR and prevent channel opening, including that by ryanodine (Ma *et al.* 1988; Smith *et al.* 1988).

In conclusion, the results of the present experiments demonstrate the importance of intracellular Ca^{2+} stores, and in particular, the RyR located on such stores, in controlling the induction requirements of LTP.

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