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A Role for Ca^{2+} Stores in Kainate Receptor-Dependent Synaptic Facilitation and LTP at Mossy Fiber Synapses in the Hippocampus

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Summary

Compared with NMDA receptor-dependent LTP, much less is known about the mechanism of induction of NMDA receptor-independent LTP; the most extensively studied form of which is mossy fiber LTP in the hippocampus. In the present study we show that Ca^{2+}-induced Ca^{2+} release from intracellular stores is involved in the induction of mossy fiber LTP. This release also contributes to the kainate receptor-dependent component of the pronounced synaptic facilitation that occurs during high-frequency stimulation. We also present evidence that the trigger for this Ca^{2+} release is Ca^{2+} permeation through kainate receptors. However, these novel synaptic mechanisms can be bypassed when the Ca^{2+} concentration is raised (from 2 to 4 mM), via a compensatory involvement of L-type Ca^{2+} channels. These findings suggest that presynaptic kainate receptors at mossy fiber synapses can initiate a cascade involving Ca^{2+} release from intracellular stores that is important in both short-term and long-term plasticity.

Introduction

There is a major effort directed at exploring the molecular mechanisms responsible for long-term potentiation (LTP) of glutamatergic synaptic transmission, since this provides the best experimental model for understanding the synaptic basis of learning and memory (Bliss and Collingridge, 1993). Two distinct forms of LTP have been described in the vertebrate CNS, which are distinguished on the basis of their requirement for the synaptic activation of N-methyl-D-aspartate (NMDA) receptors (Collingridge et al., 1983; Harris and Cotman, 1986). The best characterized form of NMDA receptor-independent LTP is at mossy fiber synapses in the hippocampus. Originally, it was believed that the induction of mossy fiber LTP was independent of the activation of glutamate receptors (Nicoll and Malenka, 1995).

More recent studies, however, have established a role for the kainate class of glutamate receptors in both the induction of mossy fiber LTP and in synaptic facilitation of mossy fiber synaptic transmission (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001a, 2001b; Schmitz et al., 2001). However, the subtype of kainate receptor involved has been the subject of considerable controversy. Pharmacological evidence has suggested a critical role of the GLU_{K5} (IUPHAR nomenclature; previously known as GluR5, iGlu5) (Lodge and Dingledine, 2000) subtype since the selective GLU_{K5} receptor antagonist LY382884 blocks both processes (Bortolotto et al., 1999; Lauri et al., 2001a, 2001b). However, the role of GLU_{K5} receptors has been disputed (Nicoll et al., 2000; cf. Bortolotto et al., 2000) since broad spectrum glutamate receptor antagonists, such as kynurenic acid and CNQX, have been reported not to block the induction of mossy fiber LTP (Ito and Sugiyama, 1991; Castillo et al., 1994; Weisskopf and Nicoll, 1995; Yeckel et al., 1999), despite being active at GLU_{K5} receptors (Bortolotto et al., 1999). Furthermore, the relatively low levels of GLU_{K5} mRNA expression (Bahn et al., 1994; Paternain et al., 2000) have been used to argue against a role for this subtype (Nicoll et al., 2000). Finally, gene knockouts support a role for GLU_{K6} (GluR6) but not GLU_{K5} receptors in mossy fiber LTP and synaptic facilitation (Contractor et al., 2001). Recently it has been reported that neither mossy fiber LTP nor homosynaptic facilitation is affected in the GLU_{K2} (KA2) knockout (Contractor et al., 2003).

There is evidence that Ca^{2+} stores are involved in synaptic plasticity at a variety of synapses in the CNS (see, Frenguelli et al., 1996; Berridge, 1998). For example, at Schaffer collateral-commissural inputs onto CA1 neurons, NMDA receptor-dependent LTP is inhibited by dantrolene (Obenaus et al., 1989), thapsigargin (Harvey and Collingridge, 1992; Bortolotto and Collingridge, 1993; Behnisch and Reymann, 1995), and ryanodine (Raymond and Redman, 2002). These effects are likely to be due to the magnification of the synaptic Ca^{2+} transient by release of Ca^{2+} from intracellular stores (Alford et al., 1993; Emptage et al., 1999). Ca^{2+} stores have also been shown to contribute to Ca^{2+} transients during brief high-frequency activation of mossy fiber terminals (Liang et al., 2002). However, depletion of Ca^{2+} stores has been reported to have no effect on low-frequency transmission or paired-pulse facilitation at

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this synapse (Carter et al., 2002). Thus, their role in short-term plasticity at mossy fiber synapses is unclear. Furthermore, their role in mossy fiber LTP has not been explored.

The present study was initiated to attempt to understand how the GLU_{K5} receptor antagonists LY382884, kynurenate, and CNQX were able to inhibit mossy fiber LTP in our previous study (Bortolotto et al., 1999), whereas kynurenate and CNQX have been reported to be ineffective against mossy fiber LTP in other studies (e.g., Ito and Sugiyama, 1991; Castillo et al., 1994; Weisskopf and Nicoll, 1995; Yeckel et al., 1999; but see Urban and Barrionuevo, 1996). One difference between our previous study and several others (e.g., Yeckel et al., 1999) is that we usually use a lower divalent cation concentration in our medium (2 mM Ca^{2+} plus 1 mM Mg^{2+} [Ca 2:Mg 1] compared with, for example, 4 mM Ca^{2+} plus 4 mM Mg^{2+} [Ca 4:Mg 4]). To our initial surprise, we found that the ability of LY382884 to block the induction of mossy fiber LTP was lost when the extracellular divalent cation concentration was changed from Ca 2:Mg 1 to Ca 4:Mg 4. Furthermore, the ability of LY382884 to inhibit the facilitation of AMPA receptor-mediated EPSCs during a high-frequency train (five stimuli at 50 Hz; hereafter referred to as synaptic facilitation) was strongly suppressed. In an attempt to understand the mechanism underlying this divalent cation dependence, we explored the roles of Ca^{2+}-induced Ca^{2+} release from intracellular stores in both synaptic facilitation and the induction of mossy fiber LTP. We also explored the possible involvement of Ca^{2+}-permeable kainate receptors using philanthotoxin (PhTx). We found that, in Ca 2:Mg 1, a component of synaptic facilitation is sensitive to ryanodine and PhTx and that the induction of mossy fiber LTP is completely blocked by these agents. In contrast, in Ca 4:Mg 4, these compounds have less effect on synaptic facilitation and no longer affect the induction of LTP. We find that this loss of sensitivity is due to an alternative pathway, involving L-type Ca^{2+} channels, that is able to compensate for the kainate receptor-mediated pathway, when the Ca^{2+} concentration is raised.

These results reconcile several of the emerging controversies concerning both the role of kainate receptors and Ca^{2+} stores in synaptic function at mossy fiber synapses. They indicate a novel synaptic mechanism whereby Ca^{2+} permeation through kainate receptors leads to Ca^{2+} release from intracellular stores, which is involved in both a component of synaptic facilitation and the induction of mossy fiber LTP.

Results

The Sensitivity of Mossy Fiber LTP and Synaptic Facilitation to LY382884 Depends on the Extracellular Divalent Cation Concentration

Elevated extracellular concentration of divalent cations is frequently used to reduce excitability when recording in the CA3 region of the hippocampus (e.g., Zalutsky and Nicoll, 1990). Surprisingly, we found that changing the extracellular divalent cation concentration in the perfusing medium from Ca 2:Mg 1 to Ca 4:Mg 4 completely eliminated the ability of LY382884 to inhibit mossy fiber LTP. Thus, a tetanus delivered in the presence of 10 μM LY382884 in Ca 2:Mg 1 induced no LTP, whereas when the tetanus was redelivered following a switch to elevated divalent cations, mossy fiber LTP was induced in the presence of LY382884 (Figure 1A). This alteration in divalent cations resulted in simultaneous changes in both Ca^{2+} concentration and the Ca:Mg ratio. To establish which was the more critical parameter, we investigated the effects of two additional divalent cation combinations (Ca 2:Mg 2 and Ca 4:Mg 2). LY382884 fully blocked the induction of LTP in Ca 2:Mg 2 but had no effect in Ca 4:Mg 2 (Figure 1B). Therefore, the critical parameter was Ca^{2+} concentration, not Ca:Mg ratio. Pooled data for all four conditions showing that LY382884 blocks LTP in Ca 2 but not Ca 4, irrespective of the Ca:Mg ratio is illustrated in Figure 1B.

Next we investigated the effects of altered divalents on the sensitivity of synaptic facilitation to LY382884, by making whole-cell recordings from CA3 pyramidal neurons in hippocampal slices (Figure 2). LY382884 inhibited synaptic facilitation (quantified throughout, unless otherwise stated, for the fifth EPSC in a 50 Hz train) by 48% ± 7% in Ca 2:Mg 1; $n = 10$, $p < 0.0001$), consistent with previous findings (Lauri et al., 2001a, 2001b). Increasing the concentrations of both Ca^{2+} and Mg^{2+} to 4 mM increased synaptic facilitation from 351% ± 39% to 502% ± 64% ($n = 7$; $p < 0.05$). However, in the presence of Ca 4:Mg 4, LY382884 inhibited synaptic facilitation by only 24% ± 6% ($n = 13$, $p < 0.05$); a difference in sensitivity that was highly significant ($p < 0.01$; Figures 2A and 2B).

To determine whether changing the divalents from Ca 2:Mg 1 to Ca 4:Mg 4 inhibited kainate receptor activation directly, we applied a low concentration of kainate (50 nM), which facilitates mossy fiber transmission evoked by low-frequency stimulation (Kehl et al., 1984; Lauri et al., 2001a, 2001b; Schmitz et al., 2001). Kainate receptor-mediated synaptic facilitation was enhanced in high divalents (Ca 4:Mg 4 versus Ca 2:Mg 1), consistent with the change in probability of release (Pr), but the sensitivity of kainate facilitation to LY382884 was unaltered (Figures 2C and 2D). Therefore, the reduced sensitivity to LY382884 is not due to block of kainate receptors by high divalents.

We also extended the range of divalent cation concentrations used to study synaptic facilitation. We found that in the lower divalent ratio (Ca 2:Mg 2 or Ca 4:Mg 4) there is considerably more kainate receptor-independent synaptic facilitation, consistent with a lower initial Pr. However, the sensitivity to LY382884 again correlated with the Ca^{2+} concentration per se rather than the Ca:Mg ratio (Figure 2E).

Ca^{2+} Release from Intracellular Stores Contributes to Synaptic Facilitation

Given the complex factors that contribute to synaptic facilitation, and in particular the dependence on Ca^{2+} concentration, we wondered to what extent release of Ca^{2+} from intracellular stores contributed to the kainate receptor-dependent component of synaptic facilitation (Figure 3). We therefore examined the effects of thapsigargin, which depletes intracellular stores in neurons by preventing their refilling (Irving et al., 1992). We found that bath application of 4 μM thapsigargin had no con-

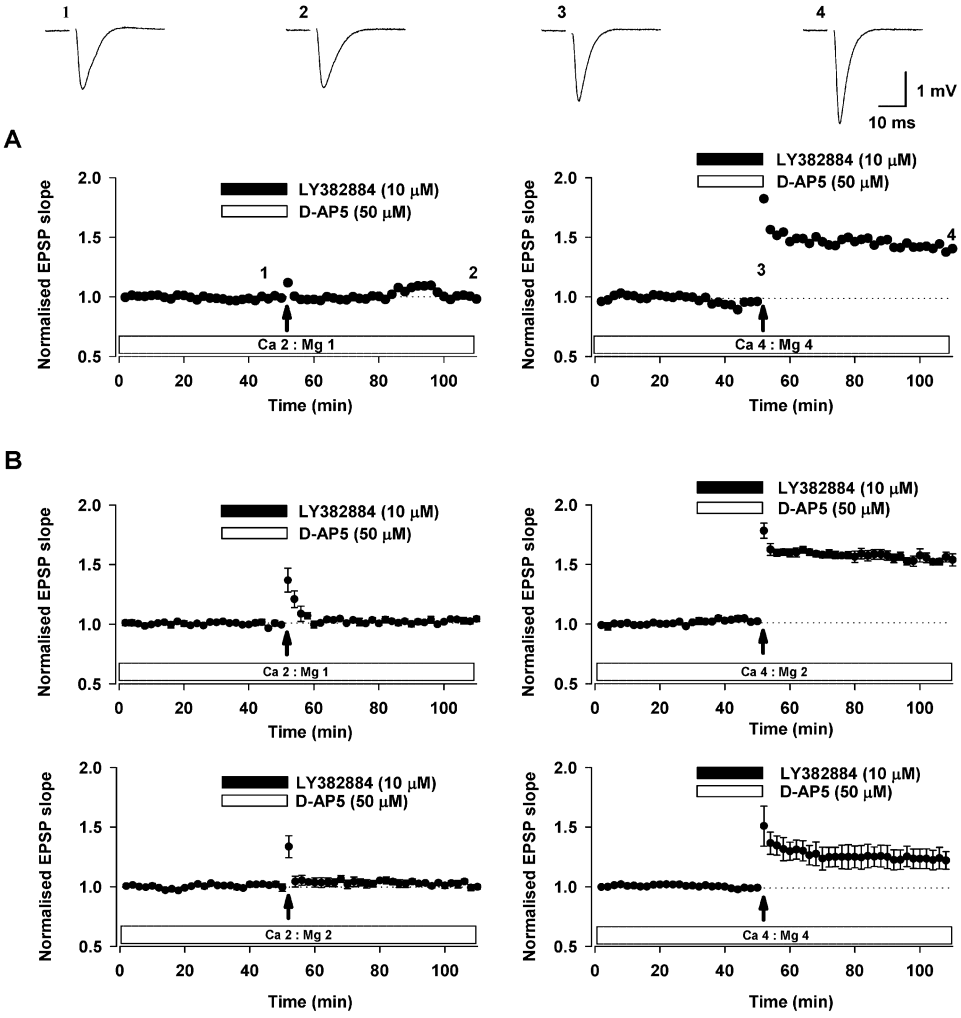


Figure 1. The Ability of LY382884 to Inhibit the Induction of Mossy Fiber LTP Is Dependent on the Ca^{2+} Concentration

(A) A single experiment that shows the effects of delivering a tetanus (100 Hz, 1 s, test intensity; arrow) in the presence of LY382884 (10 μM), first in the presence of Ca 2:Mg 1 and then in the presence of Ca 4:Mg 4. The traces are averages of four successive responses obtained at the times indicated (1–4).

(B) Pooled data (n = 4 for each) showing the effects of LY382884 under the four different divalent cation concentrations. In this and all subsequent LTP experiments, D-AP5 (50 μM) was present during the tetani to ensure that only NMDA receptor-independent LTP was studied.

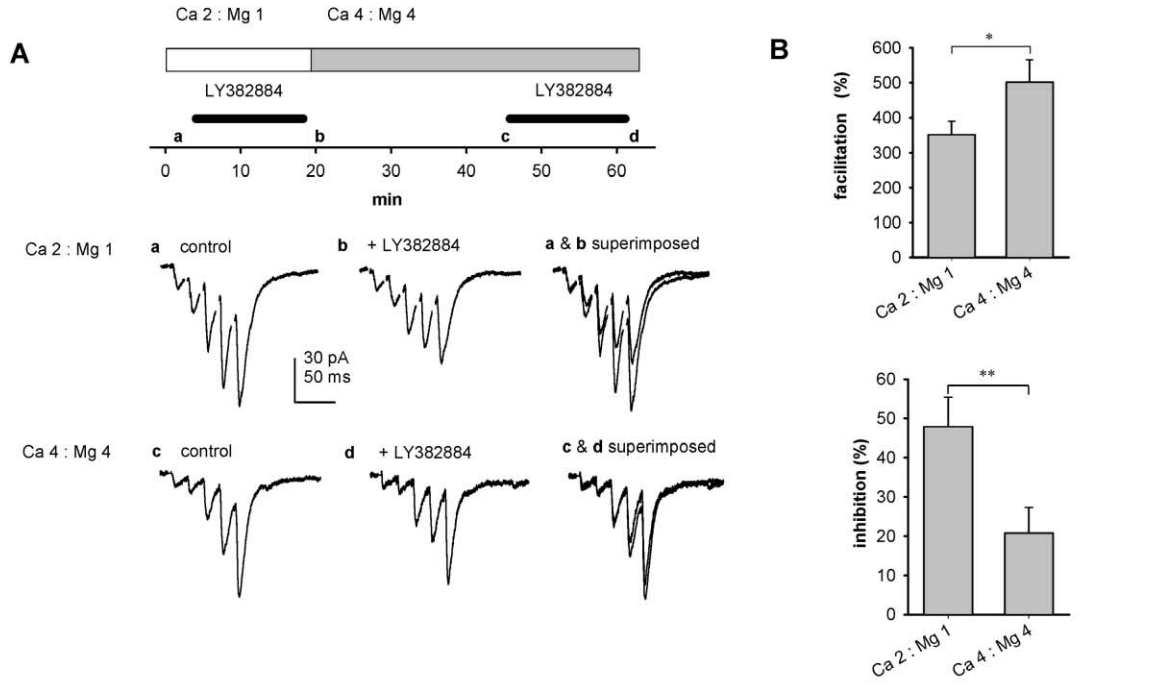
sistent effect on basal synaptic transmission but inhibited synaptic facilitation by 34% ± 6% (n = 9; $p < 0.05$; Figures 3A and 3E).

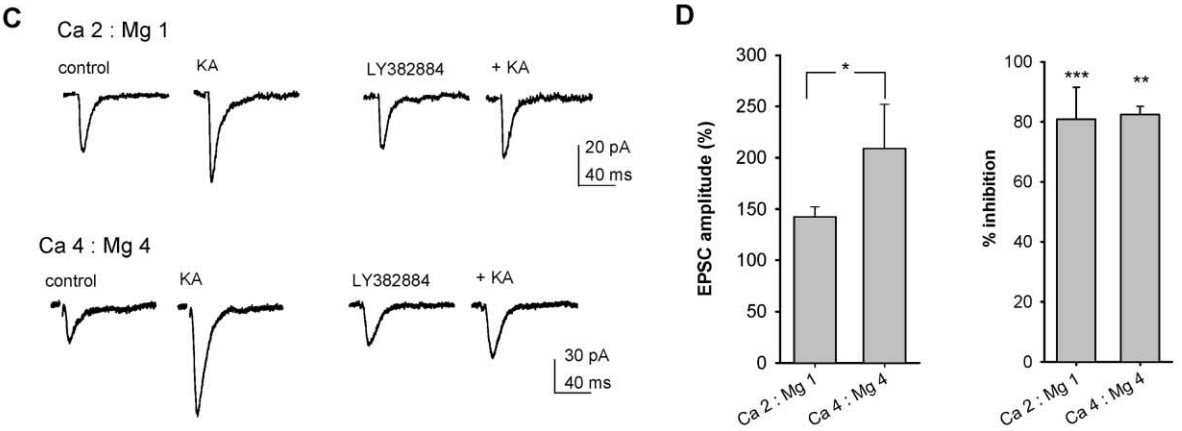
This intracellular release of Ca^{2+} could be triggered either by IP_3, perhaps via a metabotropic kainate receptor (Rodríguez-Moreno and Lerma, 1998), or via Ca^{2+}-induced Ca^{2+} release. Previously we found that calphostin C, which inhibits the function of the metabotropic kainate receptor (Rodríguez-Moreno and Lerma, 1998), did not influence synaptic facilitation (Lauri et al., 2001a). We therefore investigated the effects of ryanodine, which selectively inhibits Ca^{2+}-induced Ca^{2+} release (Berridge, 1998). Consistent with the action of thapsigargin, 10 μM ryanodine had no effect on basal synaptic transmission but inhibited synaptic facilitation by 38% ± 6% (n = 22; $p < 0.005$; Figures 3B and 3E). In the presence of ryanodine, LY382884 had little or no additional effect (Figures 3C and 3E). Conversely, in the presence of Ca 4:Mg 4, which greatly reduces the LY382884-sensitive component of synaptic transmission, ryanodine did not significantly antagonise synaptic facilitation (2% ± 10% inhibition; n = 9; $p > 0.05$; Figures 3D and 3E). These data suggest that ryanodine is acting selectively on the kainate receptor-mediated component of synaptic facilitation.

The effect of ryanodine, in Ca 2:Mg 1, was apparent by the second stimulus (17% ± 10% inhibition of facilitation; $p < 0.05$; Figure 3F), which suggests that Ca^{2+} release from intracellular stores occurs rapidly and therefore can contribute to paired-pulse facilitation at mossy fibers, even at short intervals.

Philanthotoxin Antagonizes Synaptic Facilitation

The finding that both the actions of LY382884 and ryanodine show a similar sensitivity to the divalent cation concentration, and that their effects occlude, led us to wonder how these two components are linked. One possible mechanism is that Ca^{2+} enters via Ca^{2+}-permeable


A
Ca 2 : Mg 1
Ca 4 : Mg 4
LY382884
LY382884
a
b
c
d
0
10
20
30
40
50
60
min
Ca 2 : Mg 1
a control
b + LY382884
a & b superimposed
30 pA
50 ms
Ca 4 : Mg 4
c control
d + LY382884
c & d superimposed
B
facilitation (%)
600
500
400
300
200
100
0
*
Ca 2 : Mg 1
Ca 4 : Mg 4
inhibition (%)
60
50
40
30
20
10
0
**
Ca 2 : Mg 1
Ca 4 : Mg 4


C
Ca 2 : Mg 1
control
KA
LY382884
+ KA
20 pA
40 ms
Ca 4 : Mg 4
control
KA
LY382884
+ KA
30 pA
40 ms
D
EPSC amplitude (%)
300
250
200
150
100
50
0
*
Ca 2 : Mg 1
Ca 4 : Mg 4
% inhibition
100
80
60
40
20
0
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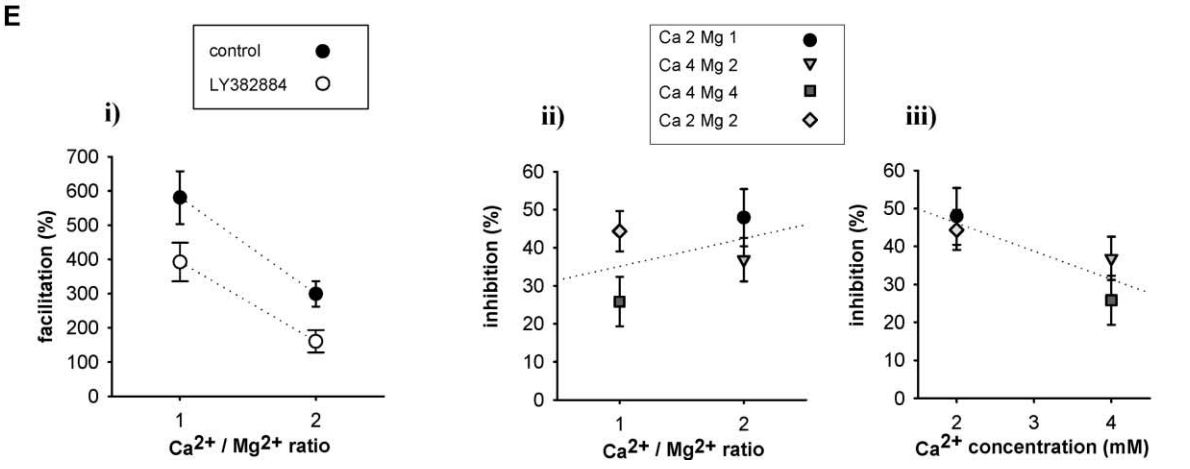
Ca 2 : Mg 1
Ca 4 : Mg 4


E
control
LY382884
i)
facilitation (%)
700
600
500
400
300
200
100
0
1
2
Ca2+ / Mg2+ ratio
Ca 2 Mg 1
Ca 4 Mg 2
Ca 4 Mg 4
Ca 2 Mg 2
ii)
inhibition (%)
60
50
40
30
20
10
0
1
2
Ca2+ / Mg2+ ratio
iii)
inhibition (%)
60
50
40
30
20
10
0
2
3
4
Ca2+ concentration (mM)

kainate receptors, and that this Ca^{2+} is the trigger for Ca^{2+}-induced Ca^{2+} release. To test this possibility, we used philanthotoxin-433 (PhTx), which selectively blocks unedited, Ca^{2+} permeable glutamate receptors (see Fletcher and Lodge, 1996). Using our standard recording medium (Ca 2:Mg 1), we found that 3 μM PhTx had no effect on the first AMPA receptor-mediated EPSC but inhibited facilitation of all subsequent EPSCs during the 50 Hz train (Figures 4A and 4B; fifth EPSC in the train inhibited by 42% ± 6%, $n = 6$, $p < 0.001$). These results are most simply interpreted by the existence of Ca^{2+}-permeable GLU_{K5}-containing kainate receptors that selectively cause the release of Ca^{2+} from intracellular stores, and which, in turn, contributes to the kainate receptor-dependent component of synaptic facilitation. Consistent with this hypothesis, PhTx inhibited kainate-induced facilitation to similar extent as did LY382884 (Figure 4C). Ryanodine also inhibited kainate-induced facilitation, but to a slightly smaller extent (Figure 4D). In both cases, the level of inhibition was similar in Ca 2:Mg 1 and Ca 4:Mg 4 (Figures 4C and 4D).

Ca^{2+} Release from Intracellular Stores Contributes to Mossy Fiber LTP

Given the association between synaptic facilitation and mossy fiber LTP (Lauri et al., 2001a), it follows from these findings that Ca^{2+} release from intracellular stores may also be involved in the induction of mossy fiber LTP, depending on the divalent cation concentration used. We therefore tested the ability of ryanodine to block the induction of mossy fiber LTP under the two sets of conditions. In the presence of Ca 2:Mg 1, ryanodine completely prevented the induction of mossy fiber LTP in a reversible manner. Basal synaptic transmission and preestablished LTP were unaffected (Figures 5A and 5B). In contrast, in the presence of Ca 4:Mg 4, mossy fiber LTP was reliably induced in the presence of ryanodine (Figures 5C and 5D).

Philanthotoxin Blocks the Induction of Mossy Fiber LTP

The prediction from these results is that PhTx will block the induction of mossy fiber LTP in Ca 2:Mg 1 but not in Ca 4:Mg 4. Because the ability of PhTx to block Ca^{2+}-permeable receptors is strongly use dependent (Toth et al., 2000), we delivered a series of pulses (30 shocks at 50 Hz, delivered five times at 2 min intervals) prior to the tetanus, to preblock PhTx-sensitive channels. Under these conditions in Ca 2:Mg 1, 3 μM PhTx fully inhibited the induction of mossy fiber LTP in a reversible manner (Figures 6A and 6B). However, as predicted, PhTx was ineffective in Ca 4:Mg 4 (Figures 6C and 6D). These results suggest a model for the induction of mossy fiber LTP whereby the synaptic activation of presynaptic GLU_{K5}-containing kainate receptors leads to Ca^{2+} permeation through these kainate receptors, which then triggers Ca^{2+} release from intracellular stores. This then triggers the induction of mossy fiber LTP. However, in Ca 4:Mg 4, this pathway is bypassed and the necessary Ca^{2+} signal can instead be provided by an alternative pathway.

L-Type Ca^{2+} Channels Can Compensate for Kainate Receptors

This left the question as to the identity of the alternative pathway. It seemed feasible that a voltage-gated Ca^{2+} channel might provide this Ca^{2+} source. We thought it unlikely to involve N- or P-type calcium channels, since blocking these did not interfere with LTP, even though neurotransmitter release was greatly depressed (Castillo et al., 1994). However, L-type Ca^{2+} channels have been implicated in one form of mossy fiber LTP (Kapur et al., 1998). We therefore determined the sensitivity of mossy fiber LTP to the L-type Ca^{2+} channel blocker nifedipine (10 μM). We found that nifedipine alone had no effect on either basal synaptic transmission or on mossy fiber LTP, studied either in Ca 2:Mg 1 or Ca 4:Mg 4 (Figure 7A). However, in the presence of nifedipine, LY382884 was able to fully block the induction of mossy fiber LTP in Ca 4:Mg 4 (Figure 7B). This shows that in elevated divalents, Ca^{2+} entry via L-type Ca^{2+} channels can compensate for Ca^{2+} entry associated with kainate receptor activation. Therefore, under these conditions, LTP is only blocked when both L-type Ca^{2+} channels and kainate receptors are inhibited.

The model assumes a role of L-type Ca^{2+} channels presynaptically, as opposed to a postsynaptic involvement that has been observed under different experimental conditions (Kapur et al., 1998). We therefore examined whether synaptic facilitation was affected by nifedipine and whether nifedipine affected the sensitivity of synaptic facilitation to LY382884 (Figure 8). Nifedipine had little effect on synaptic facilitation per se in either Ca 2:Mg 1 or Ca 4:Mg 4. However, it greatly enhanced sensitivity to LY382884 in Ca 4:Mg 4, such that LY382884

Figure 2. The Ability of LY382884 to Inhibit Mossy Fiber Synaptic Facilitation Is Dependent on the Ca^{2+} Concentration

(A) A single example showing the effect of 10 μM LY382884 on the facilitation of mossy fiber EPSCs in the presence of Ca 2:Mg 1 and in the presence of Ca 4:Mg 4. In this and subsequent examples, the traces are averages of five to seven sweeps showing the response to five shocks delivered at 50 Hz. Note that LY382884 is far more effective in the lower divalent cation concentration.

(B) Pooled data showing that synaptic facilitation ($n = 7$) is significantly ($p < 0.05$) enhanced in the higher divalent cation concentration, whereas the sensitivity of synaptic facilitation to antagonism by LY382884 (Ca 2:Mg 1, $n = 10$; Ca 4:Mg 4, $n = 13$) is significantly ($p < 0.01$) reduced.

(C) Single examples showing that LY382884 inhibits facilitation of mossy fiber synaptic transmission induced by 50 nM kainate in the presence of Ca 2:Mg 1 and in the presence of Ca 4:Mg 4. In this and subsequent examples, the traces are averages of five to seven sweeps showing the response to single shock stimulation before and in the presence of kainate.

(D) Pooled data showing that kainate facilitation ($n = 8$) is significantly ($p < 0.05$) enhanced in the higher divalent cation concentration, whereas the sensitivity of synaptic facilitation to antagonism by LY382884 is unaltered.

(E) Graphs to show synaptic facilitation (i) and its inhibition by LY382884 as a function of divalent ratio (ii) or Ca^{2+} concentration (iii). Note that percent inhibition by LY382884 correlated strongly with Ca^{2+} concentration ($r^2 = 0.99$; $p < 0.05$), but not with divalent ratio ($r^2 = 0.63$). The lines (in ii and iii) are best-fit linear regressions between the four divalent cation concentrations.

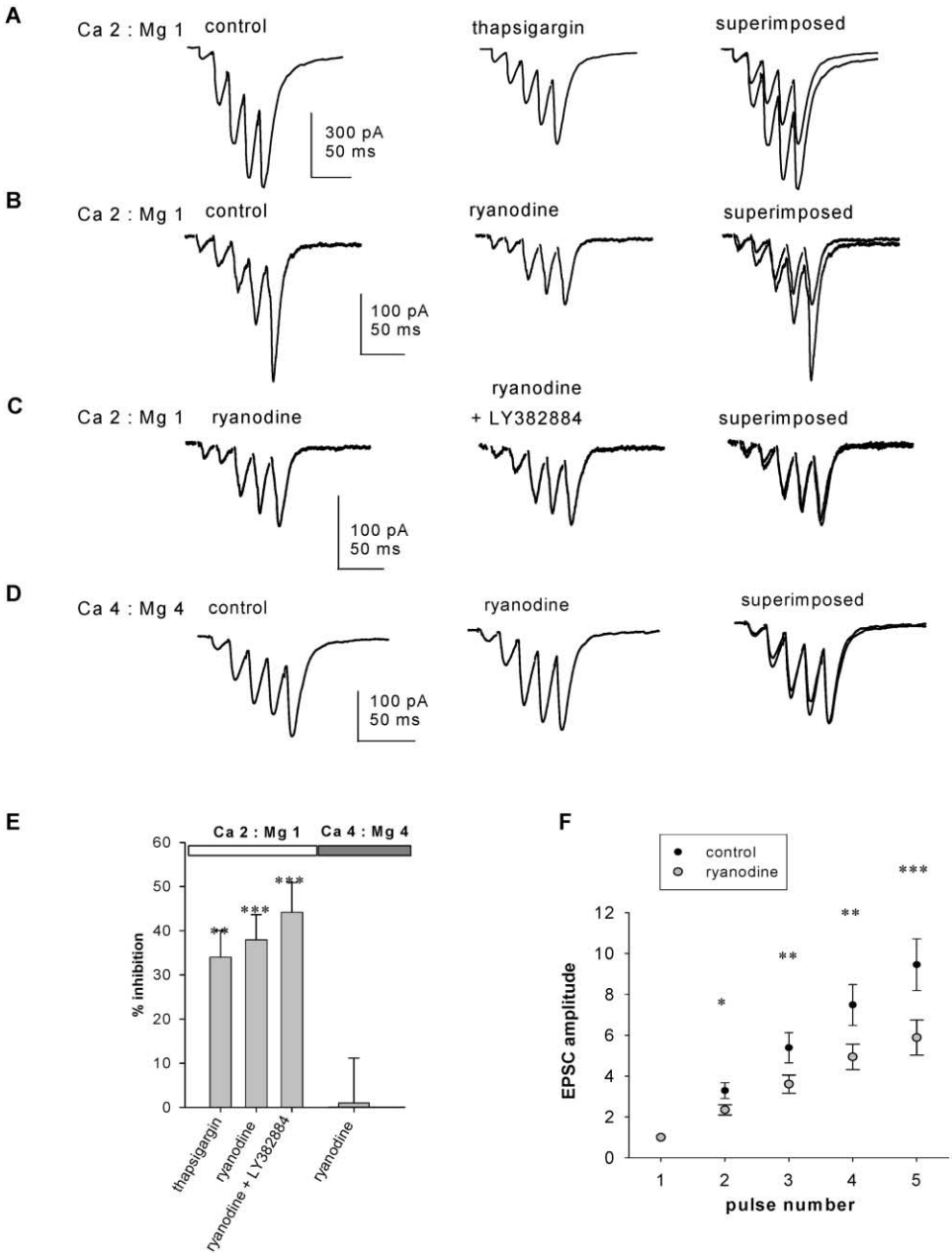


Figure 3. A Role for Ca^{2+}-Induced Ca^{2+} Release in Synaptic Facilitation

(A) Example traces from a single experiment showing that thapsigargin (4 μM) inhibits synaptic facilitation.

(B) Example traces from a single experiment showing that ryanodine (10 μM) inhibits synaptic facilitation.

(C) Example traces from a single experiment showing that LY382884 has no further affect when applied in the presence of ryanodine (same neuron as illustrated in [B]).

(D) Example traces from a single experiment showing that ryanodine does not inhibit synaptic facilitation in elevated divalents.

(E) Quantification of the effects of thapsigargin and ryanodine. Each histogram plots the percent inhibition of synaptic facilitation of the fifth EPSC in the train. Note that LY382884 (10 μM), applied in the presence of ryanodine, had no additional effect.

(F) Quantification of the effects of ryanodine on each EPSC during the 50 Hz train. The data are normalized to the first EPSC in each train.

became as effective as it is in Ca 2:Mg 1. This suggests that Ca^{2+} entry via presynaptic L-type Ca^{2+} channels can partially compensate for the requirement for kainate receptor activation in elevated divalents and fully explains the differential sensitivity of synaptic facilitation to LY382884 under these different divalent cation concentrations.

Discussion

In the present study we have identified several new properties of synaptic transmission at the mossy fiber-CA3 pathway in the hippocampus. We have (i) found that Ca^{2+}-induced Ca^{2+} release contributes to synaptic responses induced by repetitive stimulation, (ii) found

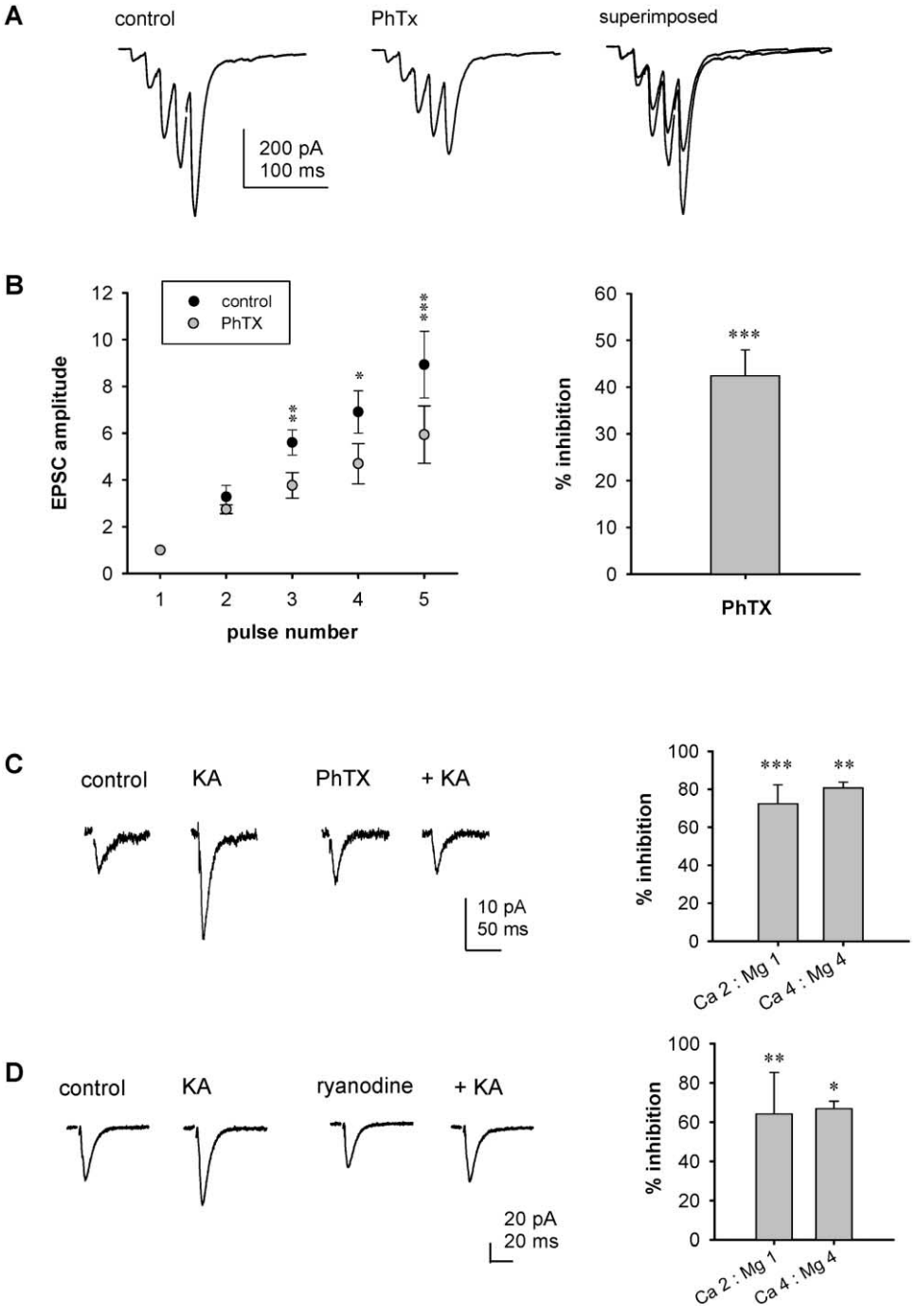


Figure 4. Evidence that Ca^{2+}-Permeable Receptors Contribute to Synaptic Facilitation

(A) Example traces from a single experiment showing that PhTx (3 μM) inhibits synaptic facilitation.

(B) Quantification of the effects of PhTx on each EPSC during the 50 Hz train. The data are normalized to the first EPSC in each train. The histogram quantifies the percent inhibition, measured using the fifth EPSC in the train (n = 6).

(C) PhTx inhibits 50 nM kainate-induced facilitation in Ca 2:Mg 1. The graph plots the percent inhibition in Ca 2:Mg 1 (n = 8) and Ca 4:Mg 4 (n = 3).

(D) Ryanodine inhibits 50 nM kainate-induced facilitation in Ca 2:Mg 1. The graph plots the percent inhibition in Ca 2:Mg 1 (n = 9) and Ca 4:Mg 4 (n = 5).

that release from Ca^{2+} stores is involved in the induction of mossy fiber LTP, and (iii) presented evidence that Ca^{2+} permeation through kainate receptors is a trigger for Ca^{2+} release from intracellular stores. We have also shown that the involvement of GLU_{K5}-containing kainate receptors, in both synaptic facilitation and mossy fiber

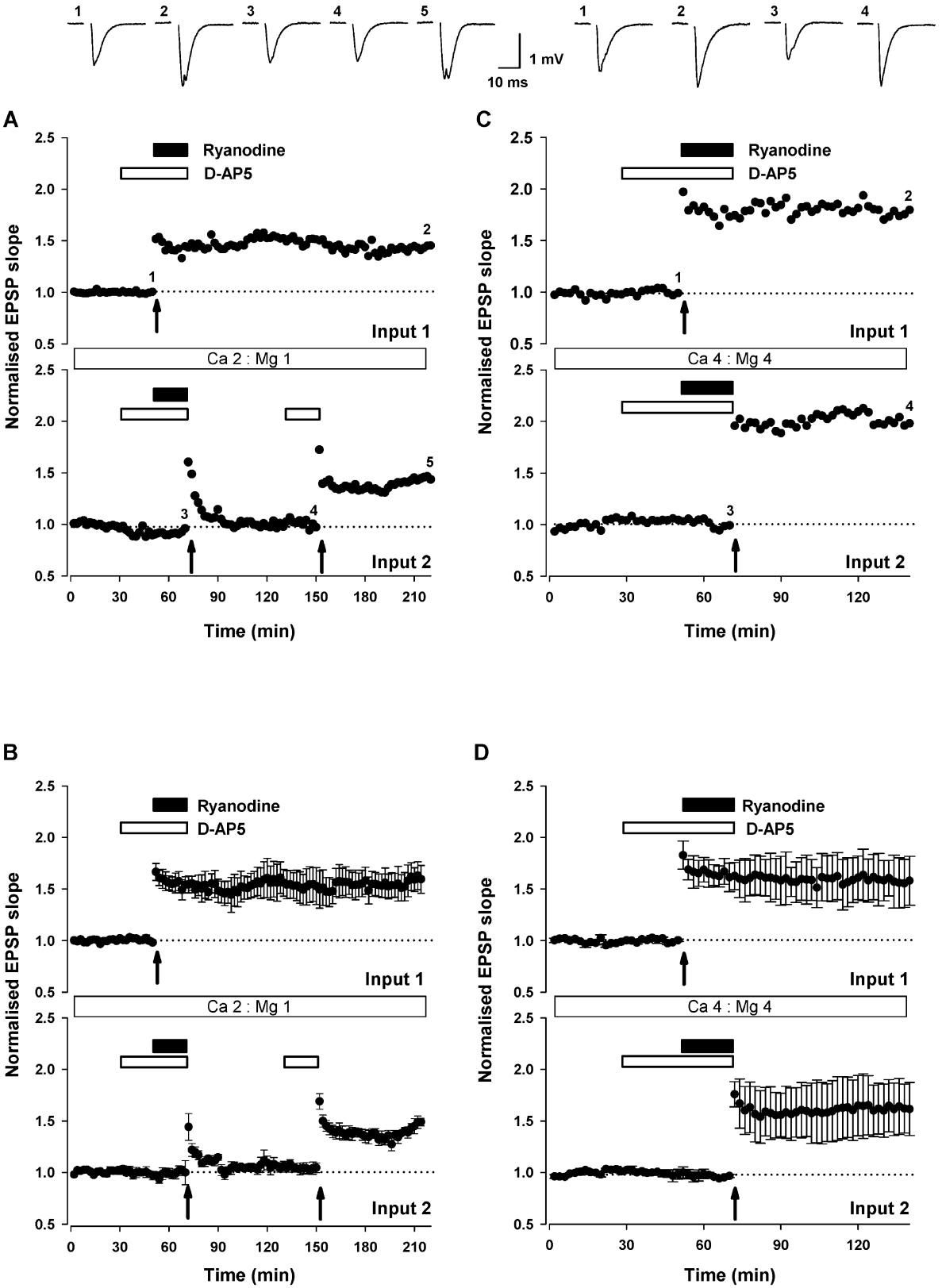


Figure 5. A Role for Ca^{2+}-Induced Ca^{2+} Release in Mossy Fiber LTP

(A) A single example to show that ryanodine (10 μM) blocks the induction of mossy fiber LTP, in Ca 2:Mg 1. In input 1, the tetanus was delivered immediately before application of ryanodine and in input 2 tetani were delivered immediately before and 90 min following washout of ryanodine. Note that the effects of ryanodine were reversible and that ryanodine did not affect preestablished LTP.

(B) Pooled data of four experiments performed as in (A).

(C) A single example to show that ryanodine (10 μM) does not block the induction of mossy fiber LTP in elevated divalents (Ca 4:Mg 4).

(D) Pooled data of four experiments performed as in (C).

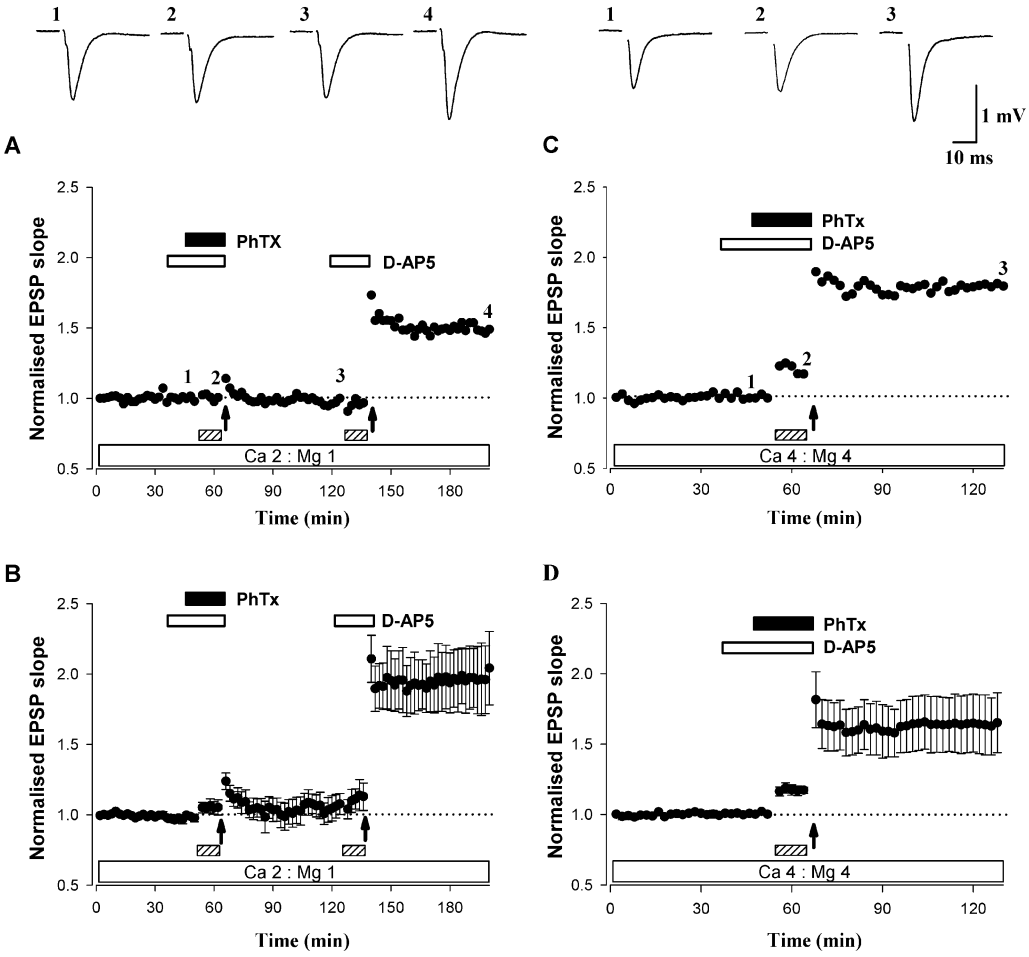


Figure 6. Evidence that Ca^{2+}-Permeable Receptors Are Involved in the Induction of Mossy Fiber LTP

(A) A single example to show reversible block of the induction of LTP by PhTx (3 μM), in Ca 2:Mg 1. In this, and all other LTP experiments using PhTx, additional stimuli (30 shocks at 50 Hz, delivered five times at 2 min intervals) were delivered prior to the tetanus (at the times indicated by hatched bars) because of the use-dependent nature of the block. This resulted in a small, transient enhancement of the synaptic response.

(B) Pooled data of four experiments performed as in (A).

(C) A single example to show that PhTx (3 μM) fails to inhibit the induction of LTP in elevated divalents (Ca 4:Mg 4).

(D) Pooled data of four experiments performed as in (C).

LTP, is affected by the extracellular Ca^{2+} concentration via a mechanism that involves L-type Ca^{2+} channels. This Ca^{2+} sensitivity may explain recent controversies concerning the role of kainate receptors in mossy fiber LTP.

Ca^{2+} Stores and Synaptic Facilitation at Mossy Fiber Synapses

Despite the high density of ryanodine receptors in the CA3 region of the hippocampus (Padua et al., 1992; Sharp et al., 1993), the role of Ca^{2+} stores in synaptic transmission and plasticity at mossy fiber synapses has only recently become the subject of investigation. In agreement with previous reports, we find that neither thapsigargin nor ryanodine significantly affected low-frequency synaptic transmission (Carter et al., 2002). Compounds that interfere with Ca^{2+} stores do, however, greatly affect the size of Ca^{2+} transients in mossy fiber terminals induced by high-frequency trains (Liang et al., 2002). These authors proposed that this source of Ca^{2+} might contribute to the pronounced facilitation of synaptic transmission that mossy fibers exhibit (e.g., Salin et al., 1996; Lauri et al., 2001a, 2001b). Our results provide direct support of this hypothesis.

The effect of ryanodine on synaptic facilitation was evident by the time of the second stimulus during a 50 Hz train. This suggests that Ca^{2+} stores can contribute to paired-pulse facilitation at mossy fiber synapses. A similar observation has been reported for associational synapses onto CA3 neurons (Emptage et al., 2001). In a previous study, ryanodine and thapsigargin did not affect paired-pulse facilitation of mossy fiber transmission (Carter et al., 2002). However, in that study, an elevated divalent cation concentration was used (Ca 3:Mg 2), which would have tended to suppress the relative contribution of the ryanodine-sensitive component. In the present study, the effect of ryanodine on the second response in the train shows that Ca^{2+}-induced

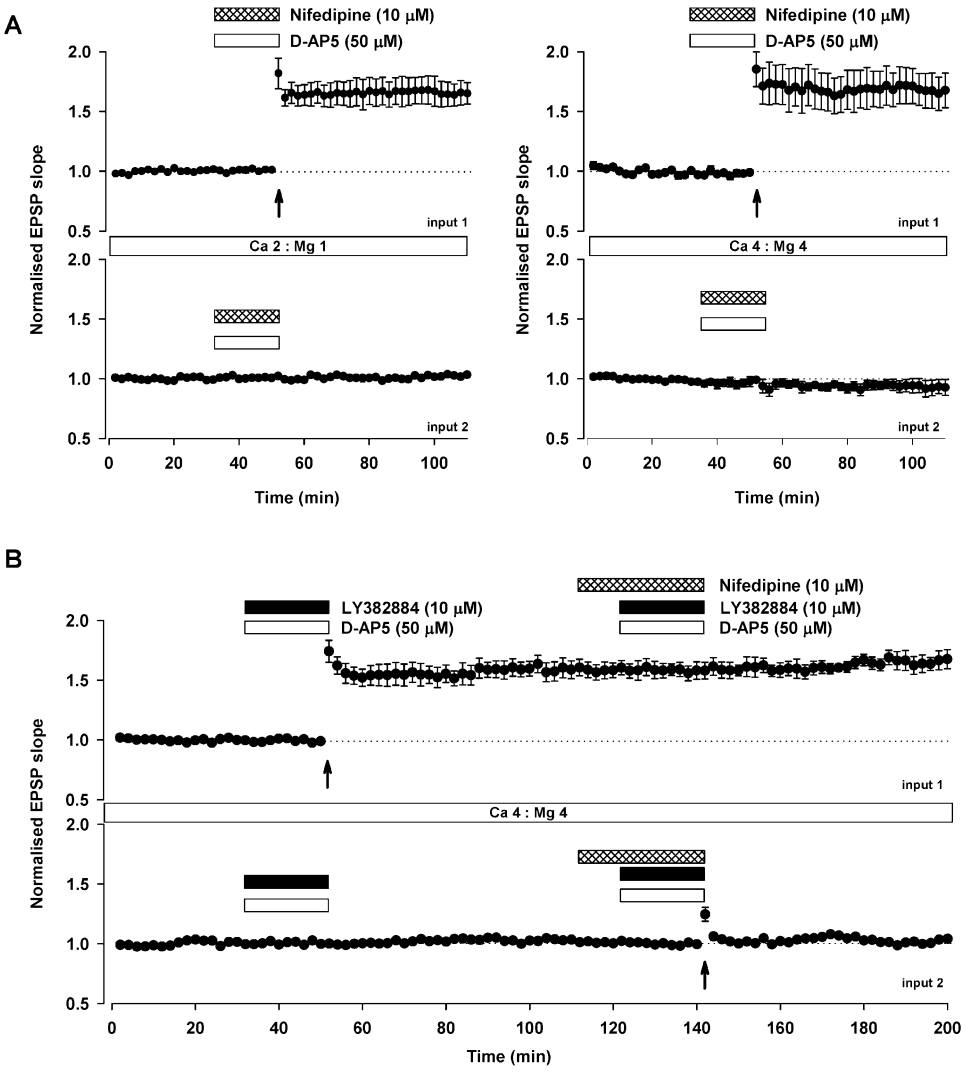


Figure 7. L-Type Ca^{2+} Channels Can Compensate for the Role of Kainate Receptors in Mossy Fiber LTP in Elevated Divalents

(A) Pooled data to show that nifedipine (10 μM) has no effect on mossy fiber LTP in Ca 2:Mg 1 (n = 5; left panel) or on mossy fiber LTP in Ca 4:Mg 4 (n = 5; right panel).

(B) Pooled data to show that in the presence of nifedipine LY382884 blocks mossy fiber LTP in Ca 4:Mg 4 (n = 5).

Ca^{2+} release can rapidly affect synaptic release (i.e., within 20 ms).

As discussed earlier, the relative size of the component of synaptic facilitation that involves release of Ca^{2+} from intracellular stores is determined by the divalent cation concentrations used. However, under the most physiological conditions that we used (Ca 2:Mg 1), the effect of ryanodine is fairly substantial. This suggests that Ca^{2+}-induced Ca^{2+} release is involved in the transfer of high-frequency information in the mossy fiber-CA3 pathway under normal conditions.

A Possible Role for Ca^{2+}-Permeable Kainate Receptors in Ca^{2+}-Induced Ca^{2+} Release

Release of Ca^{2+} from intracellular stores in neurons can be triggered by several different mechanisms, including stimulation of PLC-coupled receptors leading to formation of IP_3 (Murphy and Miller, 1989; Irving et al., 1992) and Ca^{2+} entry through NMDA receptors acting on ryanodine receptors (Alford et al., 1993; Emptage et al., 1999; Rae et al., 2000). We wondered if there was a direct link between the activation of kainate receptors and the release of Ca^{2+} from stores, since this could explain why ryanodine and LY382884 both block the induction of mossy fiber LTP and the same component of synaptic facilitation. The most direct mechanism would be if the presynaptic GLU_{K5}-containing kainate receptors on mossy fiber terminals are directly permeable to Ca^{2+}. Thus, their activation could provide a localized Ca^{2+} flux specifically related to Ca^{2+} release from stores. In this context, a high proportion of the GLU_{K5} message that is expressed in dentate gyrus of adult rat hippocampus is unedited (Sommer et al., 1991; Bernard and Khrestchatisky, 1994). The high sensitivity of kainate-induced facilitation to PhTx is consistent with this hypothesis. However, we cannot fully discount other

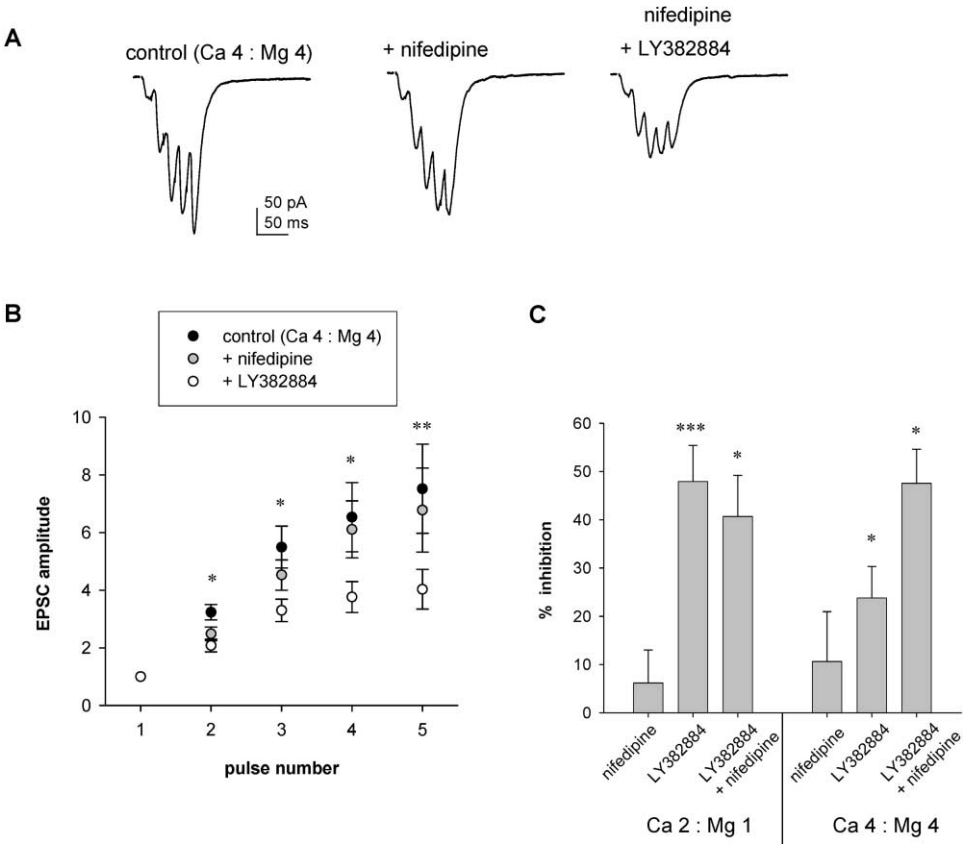


Figure 8. L-Type Ca^{2+} Channels Can Compensate for the Role of Kainate Receptors in Synaptic Facilitation in Elevated Divalents

(A) Example traces from a single experiment showing that nifedipine has little direct effect on, but enables LY382884 to substantially inhibit, synaptic facilitation in Ca 4:Mg 4.

(B) Quantification of the effects of nifedipine and nifedipine plus LY382884 on each EPSC during the 50 Hz train, in Ca 4:Mg 4. The data are normalized to the first EPSC in each train (n = 12).

(C) A comparison of the effects of nifedipine and LY382884 applied alone or in combination on synaptic facilitation in Ca 2:Mg 1 (n = 8) and Ca 4:Mg 4 (n = 12).

scenarios; for example, PhTx, by inhibiting GLU_{K5}-mediated depolarization of mossy fibers, reduces Ca^{2+} entry via voltage-gated Ca^{2+} channels, and that this Ca^{2+} entry is the trigger for Ca^{2+} release from stores rather than the Ca^{2+} permeating the kainate receptors per se. Another possibility is that PhTx is acting on another, as yet unidentified, channel at mossy fiber synapses.

The sensitivity to LY382884 parallels the sensitivity to PhTx, suggesting that the main function of GLU_{K5}-containing kainate receptors is to provide a Ca^{2+} trigger to release Ca^{2+} from intracellular stores. However, under all conditions examined, ryanodine was less effective than LY382884 or PhTx in inhibiting synaptic facilitation or kainate-induced facilitation. This suggests that GLU_{K5} receptors also contribute to a component of synaptic facilitation that does not involve Ca^{2+}-induced Ca^{2+} release. One possibility is these receptors contribute to depolarization of mossy fibers and thereby augment the action potential-dependent activation of voltage-gated Ca^{2+} channels. Recent evidence is consistent with this mechanism (Kamiya et al., 2002).

Kainate Receptors and the Induction of Mossy Fiber LTP

This study was initiated to explain why we found that the selective GLU_{K5} kainate receptor antagonist LY382884 blocked the induction of mossy fiber LTP, whereas other groups argued either that kainate receptors were not involved (Nicoll et al., 2000) or, if they were, that it was the GLU_{K6} subtype (Contractor et al., 2001). LY382884, when used at a concentration of 10 μM, antagonizes kainate responses on dentate granule cells but not CA3 neurons (Lauri et al., 2001a) and does not affect responses mediated by AMPA, NMDA, $GABA_A$, $GABA_B$, mGlu, muscarinic, adenosine, or adrenergic receptors (Bortolotto et al., 1999; Lauri et al., 2001b). Within the kainate receptor family, it is highly selective for both homomeric GLU_{K5} and heteromers containing GLU_{K5} subunits (Bortolotto et al., 1999). Recently, studies with knockout mice have further confirmed the selectivity of LY382884. This antagonist inhibits kainate currents in dorsal horn neurons in wild-type and $GLU_{K6}^{-/-}$ mice, but not in $GLU_{K5}^{-/-}$ mice (Kerchner et al., 2002). Interestingly, in that study the kainate receptor-mediated current density in dorsal horn neurons of $GLU_{K5}^{-/-}$ and wild-type mice was similar. This shows, therefore, that GLU_{K5}-dependent functions in wild-type mice can be compensated for in $GLU_{K5}^{-/-}$ mice. Similarly, we have observed that synaptic facilitation is similar in wild-type and $GLU_{K5}^{-/-}$ mice, in agreement with Contractor et al. (2001). However, we find that LY382884 inhibits synaptic facilitation at mossy fibers in wild-type but not in

$GLU_{K5}^{-/-}$ mice (S.E.L., J.T.R.I, and G.L.C., unpublished data). We can therefore reconcile the apparent controversy with the report of Contractor et al. (2001) in the following way: in wild-type animals GLU_{K5} receptors mediate a component of synaptic facilitation, but in $GLU_{K5}^{-/-}$ mice, this function is compensated for. This could occur if, for example, kainate receptors on mossy fibers are heteromeric assemblies containing GLU_{K5} subunits but that in GLU_{K5} knockouts, the remaining kainate receptor subunits still assemble into functional receptors (Huettner, 2001).

One piece of evidence that was cited against a role of GLU_{K5} containing kainate receptors in the induction of LTP is the low levels of expression of GLU_{K5} message in the hippocampus (Nicoll et al., 2000). However, dentate granule cells, like CA3 pyramidal neurons, express detectable levels of GLU_{K5} message (Bahn et al., 1994), and presynaptic kainate receptors would, most likely, need only one GLU_{K5} subunit to confer sensitivity to GLU_{K5} antagonists. Evidence for a role of GLU_{K5} receptors in mossy fiber LTP is further supported by the finding that both CNQX and kynurenic acid block its induction when applied at concentrations that also antagonise GLU_{K5} receptors (Bortolotto et al., 1999).

Extracellular [Ca^{2+}] Regulates the Sensitivity of LTP to LY382884, PhTx, and Ryanodine

One difference between our experiments and those of some groups with contrary findings was the divalent cation concentration in the bathing medium. We routinely use 2 mM Ca^{2+} (plus 1 mM Mg^{2+}) which, after allowing for buffering by other constituents in the aCSF, provides a Ca^{2+} activity similar to that measured in the extracellular space using ion-sensitive electrodes ($\sim$1.2 mM) (e.g., Heinemann et al., 1977; Nicholson et al., 1978). In contrast, other groups often use higher concentrations of Ca^{2+} and Mg^{2+} to suppress excitability (e.g., Zalutsky and Nicoll, 1990). Previously we found that elevated Ca^{2+} inhibited the GLU_{K5} receptor-mediated regulation of synaptic transmission at CA1 synapses (Clarke and Collingridge, 2002). Here we have found that the Ca^{2+} concentration dramatically affected whether LY382884 blocked the induction of mossy fiber LTP.

The finding that ryanodine and PhTx also block the induction of mossy fiber LTP, but only in Ca 2:Mg 1, provides an explanation for how extracellular Ca^{2+} regulates sensitivity of LTP to LY382884. Thus, in Ca 2:Mg 1, Ca^{2+} release from intracellular stores, triggered by Ca^{2+} permeation through kainate receptors, is a necessary trigger for the induction of mossy fiber LTP. However, in elevated Ca^{2+}, Ca^{2+}-induced Ca^{2+} release is no longer required for mossy fiber LTP. The finding that PhTx and ryanodine were able to still inhibit kainate facilitation in Ca 4:Mg 4 shows that kainate receptor-mediated Ca^{2+}-induced Ca^{2+} release can still operate under these conditions. We assume, therefore, that release occurs but is no longer necessary, since Ca^{2+} provided by other routes is now sufficient for the induction of mossy fiber LTP.

We have also investigated the mechanism responsible for LTP in the elevated divalent cation concentration of Ca 4:Mg 4. We found that L-type Ca^{2+} channels provided an alternative pathway, such that blockade of both kainate receptors and L-type Ca^{2+} channels is required to block the induction of mossy fiber LTP. Our data are consistent with L-type Ca^{2+} channels providing an alternate Ca^{2+} source in the presynaptic terminal. Previous work has shown that L-type channels have little involvement in mediating basal synaptic transmission at mossy fiber synapses, which is primarily mediated via P- and N-type channels (Castillo et al., 1994). Our data suggest that L-type Ca^{2+} channels can provide a Ca^{2+} influx during high-frequency stimulation. In contrast, these channels do not provide significant Ca^{2+} entry during low-frequency stimulation, presumably because of their slower activation kinetics, and therefore do not compensate for kainate-induced facilitation of mossy fiber transmission in elevated divalents. This explains why synaptic facilitation, but not kainate facilitation, is differentially affected by both LY382884 and ryanodine in Ca 2:Mg 1 versus Ca 4:Mg 4. We assume that in elevated Ca^{2+} there is a greater Ca^{2+} influx via these channels and that this occurs even if Mg^{2+} is also elevated due to the low sensitivity of L-type Ca^{2+} channels to extracellular Mg^{2+} (see McDonald et al., 1994). These data provide an interesting analogy to NMDA receptor-dependent LTP where the role of NMDA receptors can be compensated for, under certain circumstances, by activation of L-type Ca^{2+} channels (Grover and Teyler, 1990), although this occurs in the postsynaptic neuron.

On the Mechanism of Induction of Mossy Fiber LTP

There is a controversy as to whether the locus of induction of mossy fiber LTP is presynaptic (Zalutsky and Nicoll, 1990; Katsuki et al., 1991), postsynaptic (Yeckel et al., 1999), or both (Urban and Barrionuevo, 1996). Our experiments strongly support a presynaptic locus since LY382884 is selective for presynaptic kainate receptors at mossy fiber synapses at the concentration of 10 μM that we used (Lauri et al., 2001a, 2001b). This, of course, does not exclude the existence of a different form of mossy fiber LTP with a postsynaptic locus of induction, which might involve ryanodine-sensitive stores (Kapur et al., 2001) and L-type Ca^{2+} channels (Kapur et al., 1998), and which can be recorded under different experimental conditions. However, we shall limit our discussion of the mechanism of induction of mossy fiber LTP to the presynaptic terminal.

A simple scheme has been presented whereby mossy fiber LTP is induced by Ca^{2+} entry via voltage-gated Ca^{2+} channels activated during the high-frequency afferent volley that stimulates Ca^{2+}-sensitive adenylyl cyclase I to generate cAMP and activate PKA. PKA is then believed to modulate the release machinery, such that subsequently there is a greater probability of release per impulse (Nicoll and Malenka, 1995). We have previously added refinements to this scheme by demonstrating a role for kainate receptors in mossy fiber LTP (Bortolotto et al., 1999) and by demonstrating their presynaptic location (Lauri et al., 2001a). We and others have found evidence that an ion channel regulating mossy fiber resting membrane potential might be a target for PKA for the expression of mossy fiber LTP (Lauri et al., 2001b; Mellor et al., 2002). In this context, it has recently been suggested that I_h might be the site of expression of

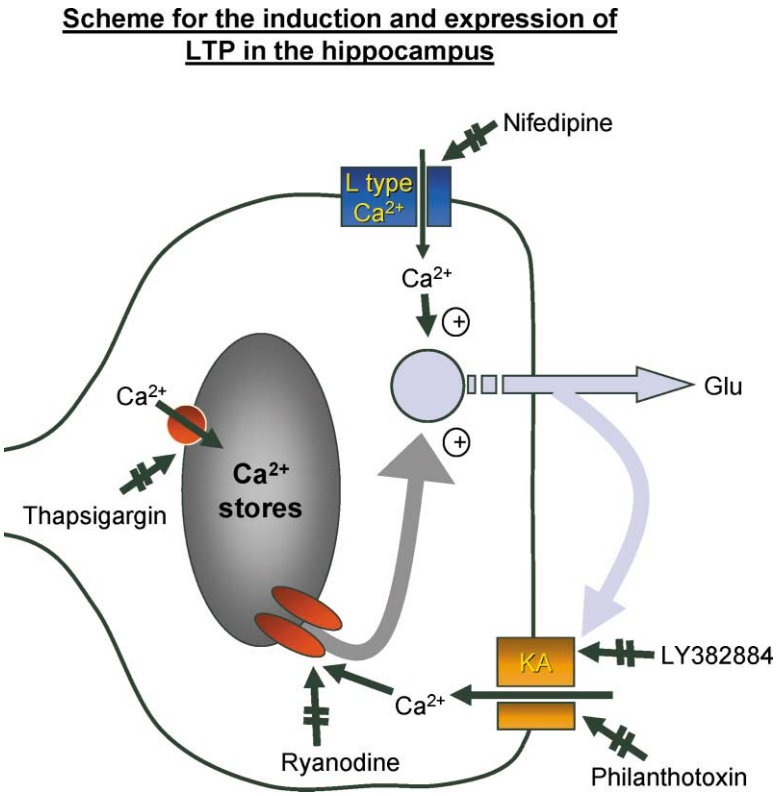


Figure 9. A Scheme for the Role of Kainate Receptors in Synaptic Facilitation and the Induction of Mossy Fiber LTP

Synaptically released L-glutamate feeds back onto GLU_{K5}-containing, Ca^{2+}-permeable presynaptic kainate receptors. Ca^{2+} permeating these receptors triggers release from internal stores that, in turn, results in a rapid facilitation of neurotransmitter release and initiates processes that result in LTP. In the presence of elevated Ca^{2+} in the bating medium, sufficient Ca^{2+} can enter via L-type Ca^{2+} channels during repetitive stimulation to provide an alternative Ca^{2+} source that contributes to synaptic facilitation and triggers the induction of LTP. This parallel pathway means that both L-type Ca^{2+} channels and kainate receptors need to be blocked to prevent the induction of LTP under these conditions. The site(s) of action of Ca^{2+} provided by these two different routes is not known, but one possibility is Ca^{2+}-sensitive adenylyl cyclase.

mossy fiber LTP (Mellor et al., 2002; but see Chevaleyre and Castillo, 2002). Alternatively, proteins directly involved in the release machinery, such as the PKA substrate RIMIα and the associated synaptic vesicle protein Rab3A, may be the effector targets (Castillo et al., 2002). We have now extended our knowledge of the induction process by demonstrating a role for Ca^{2+} stores in mossy fiber LTP and suggesting a role for Ca^{2+}-permeable kainate receptors in the triggering of Ca^{2+} release from these stores (Figure 9). It is tempting to speculate that the high levels of ryanodine receptors associated with mossy fibers (Padua et al., 1992; Sharp et al., 1993) relates to this role in synaptic plasticity.

Experimental Procedures

Experiments were performed on transverse rat hippocampal slices (400 μm) using standard techniques (Lauri et al., 2001a). Slices were perfused with extracellular solution containing 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$, 26 mM $NaHCO_3$, 10–15 mM D-glucose, and 2 mM $CaCl_2$ at room temperature. In some experiments, where indicated, a modified divalent cation solution was used (e.g., 4 mM $CaCl_2$, 4 mM $MgS0_4$). Field potential recordings were made using microelectrodes containing 4 M NaCl, and whole-cell recordings were made using patch electrodes (3–5 MΩ) containing 130 mM $CsMeSO_4$, 10 mM HEPES, 0.5 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, 5 mM QX-314, and 8 mM NaCl, 285 mOsm, pH 7.2. BAPTA (10 mM) was included in the patch solution for most experiments. Synaptic responses were evoked by stimulation of the dentate granule cell layer (mossy fiber pathway) at a baseline interval of 15–60 s. For whole-cell recordings, slices were obtained from 14- to 18-day-old animals and neurons were visualized using infrared microscopy, and ascorbate (1 mM), picrotoxin (PTX, 100 μM), and D-AP5 (50 μM) were included in the perfusate throughout recordings. In some experiments we added CGP 55845A at a concentration (1 μM) that completely blocks the synaptic activation of $GABA_B$ receptors (Davies et al., 1993). This had no effect on any parameter measured and so data obtained in the presence and absence of this antagonist were pooled. Data were collected and analyzed on-line using the LTP program (Anderson and Collingridge, 2001) (www.ltp-program.com). Data are expressed as mean $\pm$ SEM. Statistical significance was assessed using the Student's t test. (In the figures, statistical significance is denoted as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$). LY382884 can be synthesized according to Bleisch et al. (1997) and was used at a concentration of 10 μM throughout.

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