L-Type Calcium Channels Are Required for One Form of Hippocampal Mossy Fiber LTP

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Kapur, Ajay, Mark F. Yeckel, Richard Gray, and Daniel Johnston. L-type calcium channels are required for one form of hippocampal mossy fiber LTP. *J. Neurophysiol.* 79: 2181–2190, 1998. The requirement of postsynaptic calcium influx via L-type channels for the induction of long-term potentiation (LTP) of mossy fiber input to CA3 pyramidal neurons was tested for two different patterns of stimulation. Two types of LTP-inducing stimuli were used based on the suggestion that one of them, brief high-frequency stimulation (B-HFS), induces LTP postsynaptically, whereas the other pattern, long high-frequency stimulation (L-HFS), induces mossy fiber LTP presynaptically. To test whether or not calcium influx into CA3 pyramidal neurons is necessary for LTP induced by either pattern of stimulation, nimodipine, a L-type calcium channel antagonist, was added during stimulation. In these experiments nimodipine blocked the induction of mossy fiber LTP when B-HFS was given [34 ± 5% (mean ± SE) increase in control versus 7 ± 4% in nimodipine, $P < 0.003$]; in contrast, nimodipine did not block the induction of LTP with L-HFS (107 ± 10% in control vs. 80 ± 9% in nimodipine, $P > 0.05$). Administration of nimodipine after the induction of LTP had no effect on the expression of LTP. In addition, B- and L-HFS delivered directly to commissural/associational fibers in stratum radiatum failed to induce a *N*-methyl-D-aspartate-independent form of LTP, obviating the possibility that the presumed mossy fiber LTP resulted from potentiation of other synapses. Nimodipine had no effect on calcium transients recorded from mossy fiber presynaptic terminals evoked with the B-HFS paradigm but reduced postsynaptic calcium transients. Our results support the hypothesis that induction of mossy fiber LTP by B-HFS is mediated postsynaptically and requires entry of calcium through L-type channels into CA3 neurons.

INTRODUCTION

Long-term potentiation (LTP) is regarded widely as a mechanism involved in memory formation in the central nervous system (Bliss and Collingridge 1993). LTP has been studied predominantly in the CA1 region of the hippocampus, and it has been shown that LTP of Schaffer collateral input to CA1 pyramidal neurons is induced after sufficient influx of Ca^{2+} through either postsynaptic *N*-methyl-D-aspartate (NMDA)-receptor–mediated channels (Collingridge 1985; Madison et al. 1991) or postsynaptic voltage-gated Ca^{2+} channels (Grover and Teyler 1990). In the CA3 region of the hippocampus, multiple forms of LTP can be induced depending on the subset of synapses being activated; LTP of commissural/associational synapses (C/A) is dependent on activation of NMDA receptors, whereas LTP of mossy fiber synapses does not depend on activation of NMDA receptors (Harris and Cotman 1986; Johnston et al. 1992b; Zalutsky and Nicoll 1990). The mechanism underlying induction of the NMDA-receptor–independent form of LTP at mossy fiber synapses is controversial—results conflict over whether the site of induction occurs at a presynaptic or postsynaptic locus. More specifically, it has been shown that both postsynaptic depolarization (Jaffe and Johnston 1990; Urban and Barrionuevo 1996) and postsynaptic Ca^{2+} influx (Williams and Johnston 1989; Yeckel et al. 1997) are necessary for the induction of mossy fiber LTP. Other results suggest, however, that postsynaptic changes are not required and that the induction of mossy fiber LTP is a presynaptic event (Katsuki et al. 1991; Langdon et al. 1995; Zalutsky and Nicoll 1990). More recently, another study sought to reconcile these differences (i.e., postsynaptic vs. presynaptic induction) by proposing that mossy fiber LTP can be induced either post- or presynaptically, depending on whether the stimulation protocol used many short bursts of pulses (brief high-frequency stimulation, B-HFS) or a few long bursts of pulses (long high-frequency stimulation, L-HFS), respectively (Urban and Barrionuevo 1996). In the experiments presented here, we examine this issue further by investigating the role of L-type voltage-gated Ca^{2+} channels in mossy fiber LTP induced by B- and L-HFS.

The highest density of L-type Ca^{2+} channels on CA3 pyramidal neurons, as measured by immunohistochemistry, occurs on somata and proximal dendrites (Hell et al. 1993; Westenbroek et al. 1990). Fluorescence imaging experiments have shown Ca^{2+} entry into the soma of CA3 neurons in culture to occur predominantly through L-type channels (Elliott et al. 1995). On the basis of the apparent overlap between the distribution of L-type channels and termination of mossy fiber input and previous findings showing a role for these channels in the induction of a NMDA-receptor–independent LTP at Schaffer collateral-CA1 pyramidal cell synapses (Grover and Teyler 1990), we tested the hypothesis that Ca^{2+} influx through L-type channels is necessary for the induction of the postsynaptic form of mossy fiber LTP (Johnston et al. 1992a). To test this, we used the L-type channel antagonist nimodipine to determine whether these channels contribute to mossy fiber LTP induced with either B- or L-HFS protocols.

Nimodipine had no effect on baseline synaptic responses (Castillo et al. 1994; Taube and Schwartzkroin 1986) or on presynaptic Ca^{2+} transients evoked with the B-HFS paradigm in mossy fiber terminals. Application of nimodipine during B-HFS, however, blocked the induction of mossy fiber LTP. Nimodipine did not block the induction of mossy fiber LTP induced with L-HFS. Given that B-HFS–induced mossy fiber LTP is blocked by postsynaptic hyperpolarization (Jaffe and Johnston 1990; Urban and Barrionuevo 1996), antagonists of ionotropic glutamate receptors (e.g., kynurenate) block postsynaptic depolarization and, corre-

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spondingly, the induction of mossy fiber LTP with B-HFS (Urban and Barrionuevo 1996), and chelation of postsynaptic Ca^{2+} during B-HFS blocks mossy fiber LTP (Yeckel et al. 1997), our results are consistent with the hypothesis that postsynaptic Ca^{2+} influx through L-type channels is required for the induction of one form of mossy fiber LTP. Induction of LTP with L-HFS was insensitive to nimodipine, so this LTP may be triggered presynaptically or by an increase in postsynaptic Ca^{2+} through some other mechanism.

METHODS

Preparation of slices

Hippocampal slices (400 μm) were prepared from male Sprague-Dawley rats weighing 50–80 g (21–28 day). An anesthetic consisting of a mixture of ketamine (10 mg), xylazine (2 mg), and acepromazine (1 mg) was injected intraperitoneally, and rats were perfused with a cold oxygenated solution (~2°C; see further text). Slices were obtained from approximately the middle third of hippocampi in both hemispheres, incubated in a holding chamber heated to 37°C for 40 min, and then stored at room temperature (~24°C) for the remainder of the experiment (≤6 h). The holding chamber was continuously bubbled with 95% O_2-5% CO_2 and contained the bathing solution described below minus the γ-aminobutyric acid-A ($GABA_A$)-receptor antagonists and the (+)-MK-801 hydrogen maleate (MK-801) but with 25 μM (±)-2-amino-5-phosphonovaleric acid (APV) added. Individual slices were transferred for recording into a submerged chamber held at 30 ± 1°C. A Zeiss Axioskop fitted with a ×40 water-immersion objective and differential interference contrast optics was used to view the slices.

Drugs and solutions

The bathing solution contained (in mM) 124 NaCl, 2.5 KCl, 25 $NaHCO_3$, 4 $MgCl_2$, 5 $CaCl_2$, and 10 dextrose, bubbled with 95% O_2-5% CO_2. NMDA receptor antagonists APV (50 μM) and MK-801 (20 μM), plus $GABA_A$ receptor antagonists (−)-bicuculline methiodide (10–20 μM) and picrotoxin (10 μM) were always present in the bathing solution during recording. The increased concentrations of Mg^{2+} and Ca^{2+} served to prevent epileptiform activity in the disinhibited slices. Where specified, nimodipine (10 μM) was added to the bathing solution. Nimodipine was prepared from 10 mM stock solution dissolved in 100% ethanol (0.1% final concentration). The perfusion solution contained (in mM) 125 choline Cl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 7 $MgCl_2$, 1 $CaCl_2$, 10 dextrose, 10 kynurenic acid, and 2.5 L-ascorbic acid and was bubbled with 95% O_2-5% CO_2. The solution used in the patch pipettes for perforated patch recordings contained (in mM) 120 K-gluconate, 20 KCl, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 2 $MgCl_2$, 4 ATP (disodium salt), 0.3 GTP [Tris (hydroxymethyl)aminomethane salt], and 7 phosphocreatine (pH 7.3 KOH). Electrode tips were filled with this solution by suction and then backfilled with the same solution containing 275 μg/ml nystatin in 0.55% dimethyl sulfoxide (DMSO). A fresh stock solution of nystatin (5 mg in 100 μl DMSO) was prepared for every experiment. APV, MK-801, and nimodipine were from Research Biochemicals International. Bicuculline, picrotoxin, and nystatin were from Sigma Chemicals.

Recording and stimulation

Perforated patch-clamp recordings were obtained from visually identified CA3 pyramidal neurons within the top ~70 μm of the slice (Fig. 1*A*). Recordings were made from cell bodies located in stratum pyramidale adjacent to s. lucidum or in the middle of s. pyramidale. Patch electrodes were pulled from borosilicate glass (Fisherbrand) and had a tip diameter of ~2.5 μm and resistance of 3–6 MΩ. In a subset of experiments, electrodes were coated with silicone elastomer (Sylgard) and fire-polished to reduce pipette

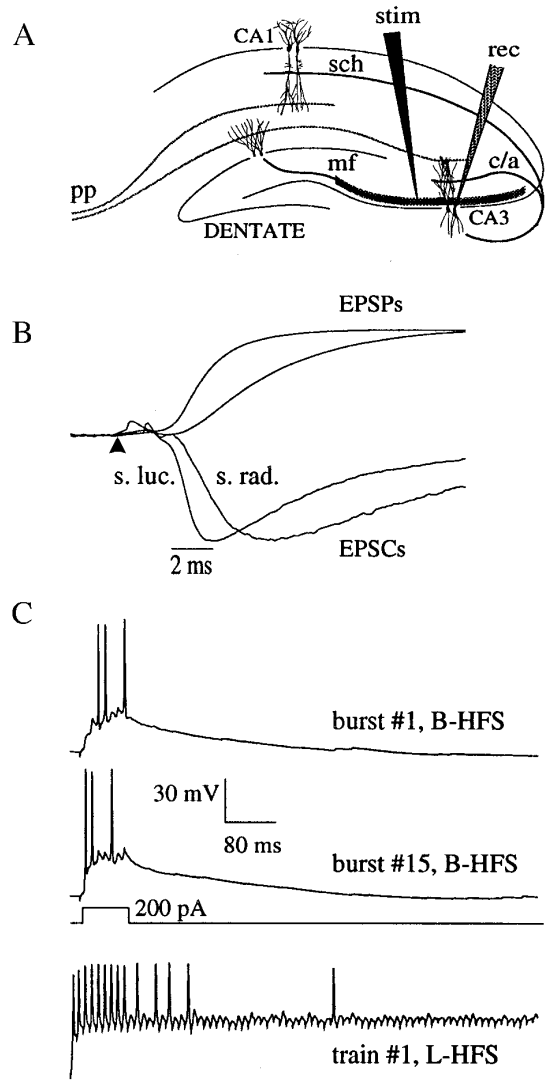

FIG. 1. Electrode locations and synaptic responses. *A*: schematic of hippocampal slice showing placement of electrodes. Perforated-patch recordings were obtained from CA3 pyramidal neurons in the region of the slice indicated by the shading. A saline-filled glass micropipette placed in stratum lucidum 50–200 μm from the recording site was used to stimulate mossy fibers. mf, mossy fibers; c/a, commissural/associational fibers; pp, perforant path; sch, Schaffer collaterals; stim, stimulating electrode; rec, recording electrode. *B*: kinetics of excitatory postsynaptic potentials (EPSPs) and excitatory postsynaptic currents (EPSCs) obtained by stimulation in s. lucidum and s. radiatum. Rise time of EPSPs and EPSCs was significantly faster when stimulation was in s. lucidum (see Table 1). Response amplitudes have been normalized for comparison purposes, and shock artifacts have been clipped. ▲, stimulus occurs. *C*: representative responses recorded during brief high-frequency stimulation (B-HFS; *top 2 traces*) and long high-frequency stimulation (L-HFS; *bottom trace*). *Top trace* is the 1st of 15 bursts (7 pulses at 100 Hz) and the 2nd trace is the 15th burst. Bursts were repeated at 5-s intervals. A 200-pA depolarizing pulse was given in conjunction with the synaptic stimulation to ensure generation of action potentials. *Bottom trace* is the 1st of 3, 1-s trains (100 Hz). Trains were repeated at 10-s intervals, and no depolarizing current was injected.

capacitance. Electrodes were advanced into the bathing solution with a slight positive pressure; pressure was increased before entering the slice, and when an electrode abutted a targeted cell, slight suction was applied to the pipette and a gigaohm seal was formed. Nystatin was then allowed to perforate the membrane, causing the series resistance to decrease to 10–30 MΩ within 1–5 min. In cases where the resistance did not decrease within 5 min, a slight positive pressure was applied to force more nystatin molecules toward the tip. Occasionally, positive pressure ruptured the membrane, as evidenced by membrane being ejected from the electrode or by a pressure wave travelling through the cell. The recording then was presumed to be whole cell and the experiment was terminated.

The majority of recordings (~90%) were made with a SEC 05L amplifier (Adams and List Associates) in bridge or discontinuous voltage-clamp modes; in the remaining experiments an Axoclamp 2A amplifier was used while in bridge mode. The capacity compensation circuitry of the SEC 05L amplifier allowed sampling rates of ≥20 kHz in discontinuous voltage-clamp mode with series resistances ≤25 MΩ. Bridge balance was adjusted before recording and not changed during the experiment. The initial resting membrane potential (V_m) of the cells was between −70 and −80 mV, and cells were discarded if V_m became more positive than −55 mV during the course of the experiment. In most cases, however, V_m stayed below −60 mV. The input resistance of the cells was 160 ± 6 MΩ (mean ± SE; $n = 35$).

Mossy fiber axons were stimulated with a glass microelectrode (~5-μm tip diam) filled with bathing solution and with a fine tungsten rod glued to its side for bipolar stimulation. The electrode was placed in s. lucidum (typically 30 μm from the edge of s. pyramidale), at a lateral distance of 50–200 μm from the recording electrode and within the top 50 μm of the surface of the slice (Fig. 1*A*). Test pulses were delivered at 0.1 Hz using stimulus intensities of 20–70 μA. A hyperpolarizing current pulse (300 ms, 20 pA) was injected into the cell between test pulses to monitor input and series (electrode) resistance. The mean peak amplitude of excitatory postsynaptic potentials (EPSPs) used in the LTP experiments was 11 ± 0.5 mV (range 5–17 mV, $n = 35$). At the start of the experiment, a conditioning paradigm was used to facilitate the blocking action of MK-801 on NMDA receptors: 10 single pulses were delivered to the mossy fibers paired with a 10-ms, 1.5-nA depolarizing current pulse injected into the postsynaptic neuron to evoke a single action potential (10-s interstimulus interval).

LTP-inducing stimuli consisted of one of the following two paradigms (Fig. 1*C*): *1*) B-HFS; 15 bursts of seven stimuli (100 Hz) repeated every 5 s. Concomitant with the stimulation pulses, depolarizing current injection (70 ms, 200 pA) was given to ensure that there was adequate postsynaptic depolarization during the presynaptic activity (i.e., Hebbian activity) (see Jaffe and Johnston 1990). *2*) L-HFS; three trains of 100 stimuli (100 Hz) given 10 s apart. No depolarizing current was injected into the cell during the trains. For both protocols, the membrane potential was −70 to −75 mV between the trains.

Analysis

The initial slope of the rising phase of the EPSP was used to quantify the magnitude of LTP. The slopes of mossy fiber-evoked EPSPs were computed for a 1-ms window starting approximately at the onset of the response. The slopes of EPSPs evoked by stimulation of C/A axons were similarly determined, but for a 2-ms window because they had a considerably slower rise time. The average value of the slope determined for a 10-min baseline recording period was normalized to one; normalization was performed before averaging across experiments. Peak amplitudes of EPSPs also were measured, and their values correlated positively with those of EPSP slope values (data not shown). LTP was defined as a >20% increase in the slope of the EPSP averaged during the interval of 21–24 min after B-HFS or L-HFS magnitude of potentiation is reported as means ± SE.

TABLE 1. *Characteristics of MF and C/A responses*

	Responses, ms[1]	*n*	Range[1]	Stimulation Site
EPSC 20–80% rise time	0.8 ± 0.1	18	0.4–1.4	s. lucidum[2]
	1.8 ± 0.1	11	1.3–3	s. radiatum[3]
EPSP 20–80% rise time	2.1 ± 0.1	35	1.4–3.6	s. lucidum[2]
	4.1 ± 0.2	6	3.5–4.8	s. radiatum[4]
	5.6 ± 0.2	7	4.5–6.7	s. radiatum[5]
EPSP expected peak latency	2.8 ± 0.1	35	2–3.9	s. lucidum[2]
	5.6 ± 0.3	6	4.6–6.9	s. radiatum[4]
	7.1 ± 0.4	7	5.2–8.9	s. radiatum[5]
EPSP peak amplitude	11 ± 1	35	5–17	s. lucidum[2]
	11 ± 1	13	6–17	s. radiatum[6]
Onset latency[7]	2 ± 0.0	33	1.5–2.5	s. lucidum[2]
	2.7 ± 0.1	13	2.1–3.7	s. radiatum[6]

Values are means ± SE. EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; MF, mossy fiber; C/A, commissural/associational. [1] Amplitude values are in millivolts. [2] Stimulation in stratum lucidum was 30–70 μm from the edge of s. pyramidale. [3] In three cells, stimulation in s. radiatum was 150–199 μm from s. pyramidale. In the rest, it was 200–300 μm. [4] Stimulation in s. radiatum 150–199 μm from s. pyramidale. [5] Stimulation in s. radiatum 200–300 μm from s. pyramidale. [6] Stimulation in s. radiatum 150–300 μm from s. pyramidale. There was no significant difference between the 150–199 and 200–300 μm groups. [7] Latency measured from the end of stimulus artifact to onset of response.

To determine series resistance, the change in voltage caused by the onset and offset of a hyperpolarizing current pulse (measured during a 2-ms interval immediately after the onset and offset of the pulse) was averaged and divided by the magnitude of the current pulse. Input resistance was determined from the steady-state voltage change produced by the same current pulse minus the series resistance. In early experiments, changes in series resistance of >10 MΩ were found to be correlated inversely with significant changes in the slope and peak of EPSPs. Coating electrodes with Sylgard and using the electrode capacitance compensation circuitry of the SEC amplifier significantly reduced the sensitivity of both the slope and peak amplitude measurements to changes in series resistance within the range of 15–40 MΩ.

Identification of mossy fiber-evoked EPSPs/excitatory postsynaptic currents (EPSCs) was based on the speed of the rising phase of the EPSPS/EPSCs (Jonas et al. 1993; Williams and Johnston 1991). This was determined by measuring the 20–80% rise time of the EPSPs/EPSCs (Table 1) and by calculating the ''expected peak latency'' of EPSPs. The expected peak latency was computed by dividing the EPSP peak amplitude by the initial EPSP slope. With a linear rising phase, this gives a 0–100% rise time for the EPSP. In some experiments, the rising phase of the EPSP could be differentiated into a fast component, presumably representing the mossy fiber input, and a slower rising component thought to represent C/A input due to stimulation of fibers of passage and/or pyramidal cells near the stimulating electrode. Such experiments were included only if the amplitude of the slower component was a small fraction of the total EPSP amplitude, and in these cases, the amplitude of the fast component was used to compute the expected peak latency. The expected peak latency allowed normalization across experiments, and because values differed significantly for responses evoked by stimulation of presumed mossy fiber axons versus responses evoked by stimulation of fibers in s. radiatum, it provided a reliable metric for determining which subset of synapses were being excited (see Table 1). Experiments of mossy fiber LTP were included if the following criteria were met: expected peak latency was ≤4 ms; series resistance changed ≤10

MΩ for non-Sylgarded and noncapacity-compensated electrodes and ≤15 MΩ for sylgarded and capacity-compensated electrodes; input resistance changed <50 MΩ during the course of an experiment; and $V_m \leq -55$ mV.

Pre- and postsynaptic calcium measurements

Slices were prepared for optical recordings as for other experiments, but, with the following additional steps. A stock solution of fura-2 AM (Molecular Probes) was made by dissolving 50 μg fura-2 AM in 40 μl DMSO with 20% (w/v) Pluronic F-127 (Molecular Probes). This stock solution was added to 3 ml of oxygenated artificial cerebrospinal fluid (ACSF), which then was sonicated for 10 s and spun for 15 s in a low-speed benchtop centrifuge. Individual slices were removed from the holding chamber and incubated in a 35-mm Petri dish containing ACSF and 8.3 μM fura-2 AM. Incubation times of 10–15 min at 30°C were used to preferentially load mossy fiber terminals for the presynaptic calcium measurements; much longer incubation times (2–3 h) were necessary to load CA3 pyramidal neurons for the postsynaptic calcium measurements. The ACSF contained (in mM) 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, and 10 dextrose and was superfused with 95% O_2-5% CO_2. The slice was rinsed gently ≥10 times in ACSF and then transferred to a chamber on the stage of the microscope where it was perfused with the bathing solution used in the other experiments. The following drugs were added to the bathing solution to block synaptic transmission during these experiments: 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 50 μM (±)-APV, 20 μM MK-801, 10 μM bicuculline methiodide, and 10 μM picrotoxin. Nimodipine (10 μM) was washed into the bath after obtaining control measurements.

Mossy fiber terminals were located under fluorescent illumination appropriate for fura-2 in the narrow band along the proximal apical dendrites of the CA3 neurons and optical signals in response to synaptic stimulation were collected from them with a photodiode as described elsewhere (Gray et al. 1996). A diaphragm in the light path was used to illuminate a single or at most a few mossy fiber terminals. A tungsten bipolar stimulating electrode was placed in the granule cell layer, and a B-HFS pattern of stimulation was used to activate mossy fibers terminals. For measurement of postsynaptic Ca^{2+} signals, the photodiode was placed over a CA3 neuron, which then was antidromically activated with the tungsten stimulating electrode placed in the fimbria. All optical traces collected with the photodiode were corrected for autofluorescence and bleaching.

RESULTS

Identification of mossy fiber-evoked EPSPs

Perforated-patch recordings of mossy fiber-evoked EPSPs/EPSCs were obtained from CA3 pyramidal neurons in hippocampal slices. Mossy fibers were stimulated with a glass micropipette placed ~50–200 μm from the recording site in s. lucidum. To isolate the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated component of the EPSP, the bathing solution contained the NMDA-receptor antagonists APV and MK-801, and the $GABA_A$-receptor antagonists bicuculline and picrotoxin (see METHODS). Mossy fiber-evoked EPSPs/EPSCs have been shown to have faster rise and decay times than C/A fiber-evoked EPSPs/EPSCs (Williams and Johnston 1991). Because the rise time is a more sensitive indicator of electrotonic distance from soma than the decay time (Johnston and Brown 1983; Williams and Johnston 1991), we used it as the defining characteristic of a mossy fiber-evoked response. EPSPs/EPSCs with the fastest rise times were evoked by stimulating with low stimulus intensities in s. lucidum (Fig. 1*B*, Table 1). EPSPs/EPSCs with slower rise times also could be evoked from sites in s. lucidum itself, as well as from sites in s. radiatum (Fig. 1*B*, Table 1). Slow EPSPs/EPSCs evoked from s. lucidum could reflect stimulation of association fibers traversing s. lucidum and/or direct activation of CA3 pyramidal cell dendrites and, consequently, excitation of recurrent axons projecting to the cell being recorded. Appropriate positioning of the stimulating electrode in s. lucidum and adjustment of the stimulus intensity, however, resulted in responses with fast kinetics in ~75% of the slices. All LTP experiments were done while in current clamp because the slope/amplitude of EPSPs were found to be less sensitive to changes in series resistance than EPSC slope/amplitude. In a number of experiments, responses were recorded in voltage clamp to evaluate the kinetics of EPSCs and whether they were consistent with mossy fiber activation.

Two measures were used to quantify the rate of rise of EPSPs: the 20–80% rise time and the expected peak latency (0–100% rise time, see METHODS). As shown in Table 1, the 20–80% rise time of presumed mossy fiber-evoked EPSPs was 2.1 ± 0.1 ms (range 1.4–3.6 ms, $n = 35$), and their expected peak latency was 2.8 ± 0.1 ms (range 2.0–3.9 ms, $n = 35$). For s. radiatum evoked EPSPs, the 20–80% rise time was 5.6 ± 0.2 ms (range 4.5–6.7, $n = 7$), and the expected peak latency was 7.1 ± 0.4 ms (range 5.2–8.9, $n = 7$), when the distance of stimulation from the pyramidal cell layer was >200 μm. Stimulation in s. radiatum closer to the pyramidal cell layer (150–200 μm), evoked slightly faster responses; the 20–80% rise time was 4.1 ± 0.2 ms (range 3.5–4.8, $n = 6$) while the expected peak latency was 5.6 ± 0.3 ms (range 4.6–6.9, $n = 6$). In summary, the expected peak latency of presumed mossy fiber responses is <4 ms, whereas that of s. radiatum-evoked responses is ≥4.6 ms. Experiments in which EPSPs had expected peak latencies >4 ms were not included in the analysis even when the site of stimulation was in s. lucidum.

Effect of nimodipine on B-HFS–induced LTP

In control experiments B-HFS induced mossy fiber LTP in 6 of 7 slices. Mean potentiation 21–24 min after B-HFS was 34 ± 5% ($n = 7$; Figs. 2 and 5). As reported previously (Jaffe and Johnston 1990; Urban and Barrionuevo 1996), B-HFS–induced LTP did not exhibit a pronounced posttetanic potentiation (PTP). To test whether L-type Ca^{2+} channels are required for the induction of mossy fiber LTP with B-HFS, the L-type channel antagonist nimodipine (10 μM) was added to the bathing solution for the duration of the experiment. In the presence of nimodipine, LTP was never observed (0/6 slices). Mean potentiation 21–24 min after B-HFS was 7 ± 4% ($n = 6$) (Figs. 2 and 5), which was significantly smaller in magnitude than control ($P < 0.003$, two-tailed unpaired *t*-test).

Although LTP was not induced with B-HFS in the presence of nimodipine, a short-term potentiation (STP), which decayed to baseline in ~20 min, was observed. Because nimodipine was present throughout the experiment, it could have blocked the expression of LTP if the contribution of

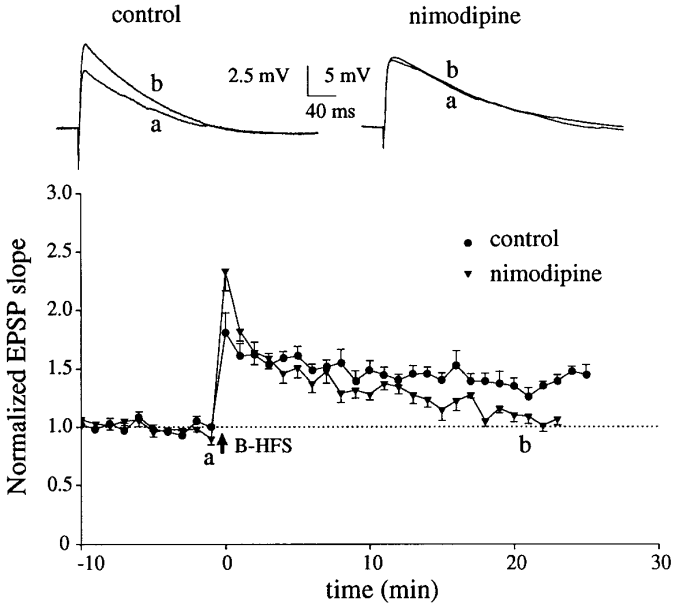

FIG. 2. Nimodipine blocks the induction of B-HFS–induced long-term potentiation (LTP). In control slices ($n = 7$), B-HFS produced stable potentiation of the EPSP slope and peak (not shown), that persisted for >20 min. In the presence of nimodipine ($n = 6$), there was a transient increase after B-HFS that returned to baseline after ~20 min (B-HFS was given at the arrow). Responses from 1 control experiment and 1 with nimodipine present recorded at the time points indicated by the letters are shown above the graph. Each trace is the average of 6 consecutive trials recorded at 0.1 Hz. Scale bars: horizontal axis: 40 ms; vertical axis: 5 mV for nimodipine and 2.5 mV for control.

L-type channels to the EPSP amplitude increased after HFS. To distinguish between the effect of nimodipine on the induction versus the expression of mossy fiber LTP, nimodipine was applied starting 10 min after B-HFS. Results indicate that nimodipine did not affect the expression of LTP (Fig. 3). Mean potentiation 21–24 min after B-HFS was 73 ± 15% ($n = 4$). In these experiments, the concentration of APV was increased to 100 μM and 0.1% ethanol also was present in the bath during B-HFS to control for its

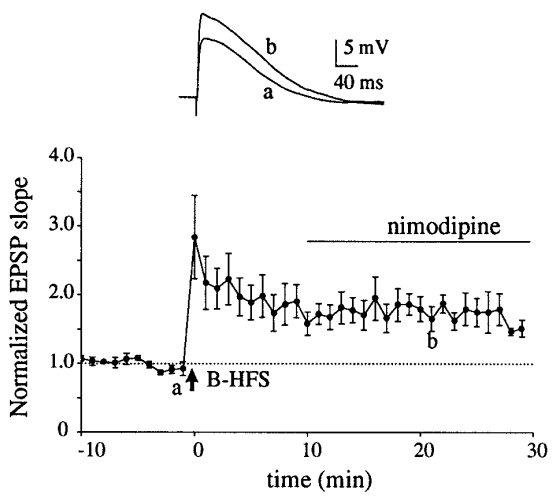

FIG. 3. Nimodipine does not block the expression of B-HFS–induced LTP. Nimodipine added to the bathing solution 10 min after delivery of B-HFS (↑) failed to block LTP ($n = 4$). Representative traces from an individual experiment at the time points indicated by the letters.

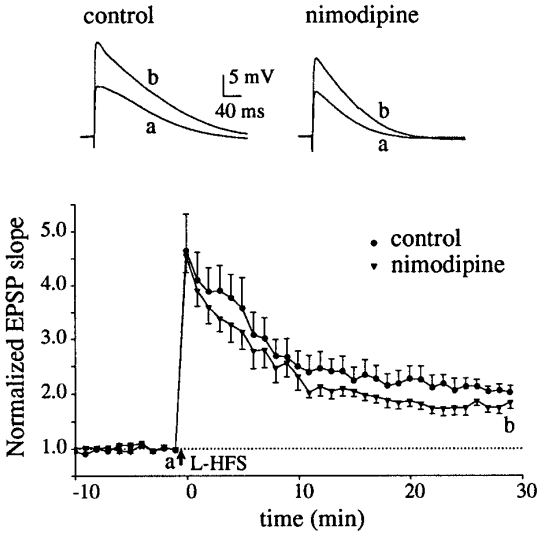

FIG. 4. Nimodipine does not block the induction of L-HFS–induced LTP. In control experiments ($n = 7$) as well as experiments done in the presence of nimodipine ($n = 7$), LTP persisted for ≥30 min. L-HFS was delivered at the arrow. Responses from one control experiment and one with nimodipine present recorded at the time points indicated by the letters. Each trace is the average of 6 consecutive trials recorded at 0.1 Hz.

presence in the previous nimodipine experiments. This suggests that Ca^{2+} influx through L-type channels triggers the induction of a slowly developing form of LTP, as has been observed at the Schaffer collateral synapses in the CA1 region (Grover and Teyler 1990).

Effect of nimodipine on L-HFS–induced LTP

In control experiments, L-HFS induced LTP in 7/7 slices. Mean potentiation 27–30 min after L-HFS was 107 ± 10% ($n = 7$; Figs. 4 and 5). In the presence of nimodipine, mean potentiation was 80 ± 9% ($n = 7$; Figs. 4 and 5), and LTP

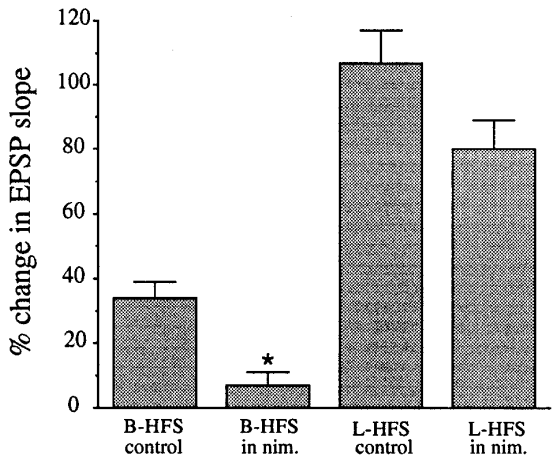

FIG. 5. Effect of nimodipine on the magnitude of potentiation produced by B- and L-HFS. Average increase in EPSP slope 21–24 min after B-HFS and 27–30 min after L-HFS was calculated for control and nimodipine experiments. For B-HFS, the slope was potentiated 34 ± 5% ($n = 7$) under control conditions and by only 7 ± 4% ($n = 6$) in nimodipine. *, significant difference from control ($P < 0.003$, 2-tailed unpaired t-test). For L-HFS, the slope was potentiated by 107 ± 10% ($n = 7$) under control conditions and by 80 ± 9% (n = 7) in nimodipine. This difference was not significant ($P > 0.05$, 2-tailed unpaired t-test).

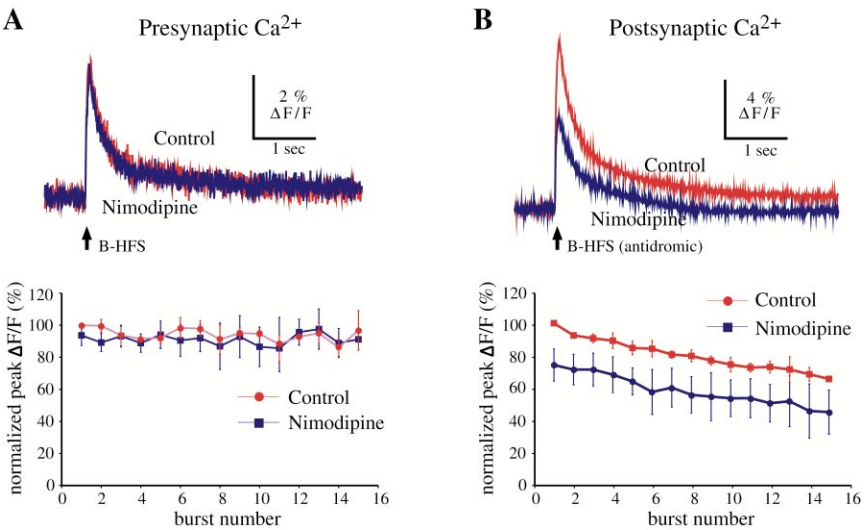

FIG. 6. Effect of nimodipine on Ca^{2+} entry into mossy fiber terminals (MFTs) and CA3 somata. *A*: fura-2 signals from a single MFT in response to a burst of 7 stimuli at 100 Hz applied to the granule cell body layer before (red trace) and 20 min after perfusion of nimodipine (10 μM; blue trace). Summary (mean ± SE) of peak fura-2 signals from 4 MFTs during each of 15 bursts of stimuli applied before (red line) and in the presence of nimodipine (blue line) is shown below. *B*: fura-2 signals from the CA3 cell body layer before (red trace) and after (blue trace) perfusion of nimodipine. CA3 neurons were antidromically activated by burst stimulation in the fimbria. Summary of peak fura-2 signals from 3 recordings from the CA3 cell body layer before (red line) and after (blue line) perfusion of nimodipine is below. Traces are averages of all 15 stimulus bursts comprising the B-HFS paradigm.

again was induced in every case (7/7). Although mean potentiation was smaller in the presence of nimodipine, this difference was not significant ($P > 0.05$, 2-tailed unpaired *t*-test). This is consistent with previous results showing that nimodipine does not prevent L-HFS–induced mossy fiber LTP in the guinea pig (Castillo et al. 1994).

The time course and magnitude of L-HFS–induced LTP appeared to be quite different from that of B-HFS–induced LTP, as noted previously (Urban and Barrionuevo 1996). Specifically, there was a large PTP/STP after L-HFS that declined to a stable value in ~10–20 min (Fig. 4). Such a pronounced PTP/STP was not seen with B-HFS (Figs. 2 and 3). The magnitude of potentiation 21–24 min after HFS was also significantly greater with L-HFS (120 ± 20%, $n = 7$ vs. 34 ± 5%, $n = 7$; $P < 0.007$, two-tailed unpaired *t*-test). Response amplitude increased by >200% immediately after L-HFS, resulting in suprathreshold EPSPs for a period of 2–10 min after HFS. Because it is likely that voltage-dependent Na^+ currents contributed to the rising phase of these EPSPs, the change in EPSP slope is probably overestimated. After ~10 min, responses decreased to levels subthreshold for spiking but still remained relatively large (typically 20 mV).

Effect of nimodipine on presynaptic calcium transients in mossy fiber terminals

To test whether nimodipine could have blocked B-HFS–induced LTP by reducing Ca^{2+} influx into presynaptic mossy fiber terminals (MFTs), we looked at the effects of nimodipine on Ca^{2+} transients in MFTs. Slices were loaded with the Ca^{2+} indicator dye fura-2 AM, and optical signals were recorded from MFTs in response to stimulation of mossy fibers. The B-HFS paradigm was used to activate MFTs (in 1 cell, 5 bursts were given instead of 15). In 4 of 4 cells the Ca^{2+} transients elicited in MFTs were not affected by nimodipine (10 μM; Fig. 6, *A* and *B*). As a control, we looked at the effects of nimodipine on Ca^{2+} transients in CA3 pyramidal neurons, where L-type channels are known to be activated by Na^+ action potentials. Nimodipine reduced antidromically activated Ca^{2+} transients in CA3 pyramidal neurons by ~20% (Fig. 6, *C* and *D; n* = 3).

Stimulation in s. radiatum

To determine whether NMDA-receptor independent forms of LTP at C/A synapses contributed to our measurements, we tried to induce LTP at these synapses by stimulating in s. radiatum (typically at a distance of 200 μm from the suprapyramidal edge of s. pyramidale). B- and L-HFS protocols were used but with modifications designed to enhance the probability of inducing LTP. In the B-HFS protocol, the magnitude of the depolarizing current injected into the cell during the stimulus bursts was increased from 200 pA to 1.5 nA; using the L-HFS protocol, in 3 of 6 cells, a 2-nA depolarizing current pulse was injected during the 1-s stimulus train (Fig. 7*A*). This was done to evoke a comparable amount of depolarization and action potential generation with C/A stimulation as was observed with mossy fiber stimulation. Presumably, the larger $GABA_B$-receptor–mediated inhibitory postsynaptic potential evoked from s. radiatum stimulation sites made it harder for the cell to generate action potentials. In the presence of NMDA and $GABA_A$ receptor antagonists as before, LTP could not be induced with the modified versions of either B-HFS (mean potentiation 20 min after HFS was 1 ± 6%, $n = 6$) or L-HFS (0 ± 7%, $n = 6$) when the C/A fibers-were stimulated directly (Fig. 7*B*).

DISCUSSION

Role of L-type Ca^{2+} channels in B-HFS–induced mossy fiber LTP

The results of these experiments provide the first direct evidence for a role of L-type Ca^{2+} channels in hippocampal mossy fiber LTP. Previous studies have shown that induction of mossy fiber LTP using brief trains of high-frequency stimulation is enhanced significantly with postsynaptic depolarization and blocked altogether with postsynaptic hyperpolarization (Jaffe and Johnston 1990; Urban and Barrionuevo 1996). On the basis of these findings, it was hypothesized that mossy fiber LTP induced with B-HFS requires postsynaptic Ca^{2+} influx via voltage-gated Ca^{2+} channels (Johnston et al. 1992b). Our results support this hypothesis and suggest

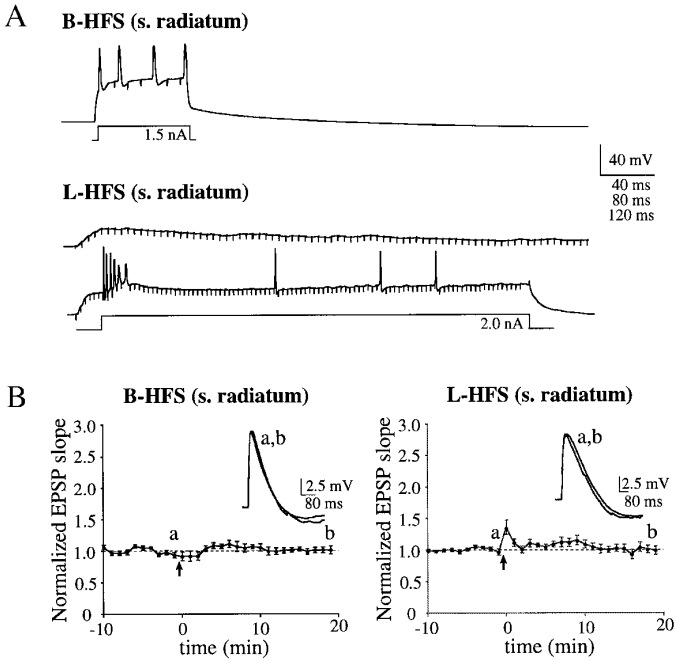

FIG. 7. *N*-methyl-D-aspartate (NMDA)-receptor–independent LTP could not be induced at commissural/associational synapses with either B- or L-HFS. *A*: responses recorded during B-HFS (*top*) or L-HFS (*bottom 2 traces*) evoked by stimulation of C/A fibers in s. radiatum. *Top*: single burst (of 15) with 1.5-nA current injected during the burst (instead of 200 pA as with mossy fiber stimulation) to evoke a comparable number of action potentials. *Middle*: 1 (of 3) 100-Hz, 1-s trains delivered to C/A fibers. This was done in 3 of 6 L-HFS experiments. In the remaining 3 experiments, a 2-nA current pulse was injected during the tetanus (*bottom*) to increase depolarization and action potential firing. *B*: summary of experiments indicating that neither B-HFS (*left, n* = 6) nor L-HFS (*right, n* = 6) could induce NMDA-receptor–independent LTP at the C/A synapses. ↑, HFS delivery. *Insets*: representative traces at the indicated time points from one experiment in each case. Each trace is the average of 6 consecutive trials recorded at 0.1 Hz.

that postsynaptic Ca^{2+} entry through L-type Ca^{2+} channels provides an essential route for this to occur. Further support for this finding comes from recent data from this laboratory showing that the Ca^{2+} chelator bis-(*o*-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid (BAPTA; 1–5 mM) also blocks B-HFS–induced LTP (Yeckel et al. 1997).

Immunohistochemical studies have shown that the greatest density of L-type Ca^{2+} channels occurs on the soma and proximal dendrites ($\leq$50 μm from soma) of CA3 neurons (Hell et al. 1993; Westenbroek et al. 1990). Consistent with the spatial distribution of L-type channels, fluorescence imaging of Ca^{2+} in CA3 pyramidal neurons in organotypic slice cultures has shown that L-type Ca^{2+} channels contribute significantly to somatic Ca^{2+} influx during postsynaptic depolarization (Elliott et al. 1995). In addition, it has been shown that L-type channels also are present in the postsynaptic densities of asymmetric synapses on CA3 pyramidal neurons (Hell et al. 1996). The proximity of L-type channels to mossy fiber synapses on the proximal dendrites enables the interaction between rises in postsynaptic Ca^{2+} during B-HFS and second-messenger cascades known to be triggered by Ca^{2+} and involved in synaptic plasticity (Roberson et al. 1996).

In addition to L-type channels, other Ca^{2+} channel subtypes have been observed near the base of CA3 pyramidal cell dendrites. For example, it has been shown that blockade of L-type channels reduces depolarization-induced Ca^{2+} influx into the soma of CA3 neurons by only ~50% and that somatic Ca^{2+} influx also can occur through P/Q type Ca^{2+} channels (Elliott et al. 1995). Furthermore, dense immunoreactivity for the neuronal class E Ca^{2+} channel α_1 subunit, thought to represent the R-type channel (Randall and Tsien 1995), also has been shown to be located primarily on the soma of CA3 pyramidal neurons (Yokoyama et al. 1995). Therefore it still must be determined whether these Ca^{2+} channel subtypes also are involved in the induction of mossy fiber LTP or whether L-type channels have a unique role in NMDA-receptor–independent synaptic plasticity in the CA3, as well as CA1 regions.

L-HFS–induced mossy fiber LTP

Although nimodipine reduced the magnitude of LTP induced with L-HFS, it was not significantly different from control LTP (see Fig. 4). Suppression of L-HFS–induced LTP with nimodipine, however, is consistent with the possibility that LTP induced with this protocol represents a composite of potentiation induced by both L- and B-HFS, with nimodipine blocking the B-HFS component of this potentiation. For example, if two forms of mossy fiber LTP exist (e.g., pre- and postsynaptic) and LTP induced with L-HFS exhibits both forms because the L-HFS pattern contains characteristics of the B-HFS pattern embedded in its longer bursts, it might be predicted that nimodipine would decrease the total magnitude of L-HFS–induced LTP by blocking the induction of the B-HFS form of LTP. The relative independence of L-HFS–induced LTP on Ca^{2+} influx through L-type channels does not preclude a requirement for postsynaptic Ca^{2+}, however, because previously it has been shown that postsynaptic injection of the Ca^{2+}-chelator BAPTA also blocks LTP induced with L-HFS, suggesting that other Ca^{2+} channel subtypes and/or Ca^{2+} release from internal stores also are involved in potentiation (Williams and Johnston 1989; Yeckel et al. 1997; but see Katsuki et al. 1991; Zalutsky and Nicoll 1990).

Differences in the sensitivity of LTP induction for B- and L-HFS to nimodipine also may be related to differential release of opioid neuropeptides. The greater number of consecutive stimuli used during L-HFS (100 vs. 7 for B-HFS) may elicit a greater contribution from the opioid receptor-coupled signaling pathway (Derrick and Martinez 1994), such that there is an increased contribution from opioid-triggered events that offset a need for Ca^{2+} influx through L-type channels. For example, it has been shown in a number of cell lines (Connor et al. 1994; Fields et al. 1994; Tang et al. 1994) that activation of opioid receptors can cause an elevation of cytosolic Ca^{2+} by an influx of Ca^{2+} from the extracellular space as well as release of Ca^{2+} from intracellular stores. Alternatively, opioids have been shown to enhance the accumulation of some second messengers, such as adenosine 3′,5′-cyclic monophosphate (cAMP) (Cruciani et al. 1993; Mehta and Strada 1994; Wang and Gintzler 1994, 1995), that are thought to be involved in mossy fiber LTP. More specifically, it has been suggested that cAMP is important for mossy fiber LTP (Huang et al. 1994; Weisskopf

et al. 1994), and, therefore, potentiation of adenylyl cyclase activity by opioids might bypass the need for large increases in postsynaptic Ca^{2+} during the induction of LTP. Blockade of mossy fiber LTP by opioid receptor antagonists supports the idea that opioids contribute to the induction of LTP at these synapses (Derrick et al. 1991; Ishihara et al. 1990; Martin 1983; Williams and Johnston 1996; but see Salin et al. 1995; Weisskopf et al. 1993).

Site of action of nimodipine

The presence of nimodipine during baseline recordings or after the induction of LTP had no effect on EPSP amplitude (Fig. 3), indicating that there is no effect on mossy fiber synaptic transmission at low frequencies of stimulation. This is consistent with previous results showing that another L-type antagonist, nifedipine ($\leq$30 μM), reduced mossy fiber responses by only 13 $\pm$ 3%, whereas the responses were almost completely blocked ($\sim$95%) by the P/Q-type antagonist ω-agatoxin-IVA and partially blocked ($\sim$75%) by the N-type antagonist ω-conotoxin-GVIA (Castillo et al. 1994). L-type antagonists also have been shown to have little or no effect on synaptic transmission between hippocampal CA3 and CA1 neurons (Horne and Kemp 1991; Kamiya et al. 1988; Wheeler et al. 1994). Therefore it appears unlikely that nimodipine could have blocked B-HFS–induced LTP by reducing glutamatergic synaptic transmission.

Although nimodipine does not seem to affect synaptic transmission at low frequencies of stimulation, its effect on presynaptic Ca^{2+} dynamics in mossy fiber terminals had not been determined previously for higher frequencies of stimulation such as those used to induce LTP. Our results with fura-2 imaging of presynaptic terminals demonstrate that nimodipine does not affect Ca^{2+} influx into mossy fiber terminals even when the B-HFS pattern of stimulation is used. Consistent with previous results (Avery and Johnston 1996; Elliott et al. 1995), nimodipine does reduce postsynaptic Ca^{2+} influx into CA3 pyramidal neurons.

Additionally, it is not likely that nimodipine blocked the release of opioids from the presynaptic terminal. Although L-type channels have been shown to be required for peptide release stimulated with high K^+ solutions (Cazalis et al. 1987; Perney et al. 1986; Rane et al. 1987), L-type antagonists do not block the release of dynorphin from guinea pig hippocampal mossy fiber terminals elicited with electrical stimulation (Castillo et al. 1996; Simmons et al. 1995). In addition, the opioid antagonist naloxone has been shown to block mossy fiber LTP induced with L-HFS in rat hippocampal slices (Williams and Johnston 1996), and, by extension, if nimodipine was blocking the release of opioid peptides in our study, it would also have blocked L-HFS–induced LTP. Therefore it is unlikely that nimodipine was acting presynaptically to block the release of either glutamate or opioids. Taken together, the evidence supports the hypothesis that the site of action of nimodipine in these experiments is the postsynaptic CA3 neuron where L-type antagonists have been shown to reduce somatic Ca^{2+} influx (Fig. 6, *C* and *D*) (Elliott et al. 1995) and where L-type channels are present at a high density (Avery and Johnston 1996; Fisher et al. 1990; Mogul and Fox 1991).

Isolation of mossy fiber ltp

Selective activation of mossy fiber synapses is complicated by the number of convergent fiber systems in the hippocampus that can be activated incidentally with electrical stimulation as well as the powerful recurrent excitatory pathway activated when CA3 pyramidal neurons fire action potentials (see Claiborne et al. 1993; Henze et al. 1997). In most previous studies examining mossy fiber-evoked responses, stimulating electrodes were placed either in the inner molecular layer of the dentate gyrus to activate granule cells directly or in the hilus of the dentate gyrus to stimulate axons of the granule cells. In preliminary experiments, we attempted to excite mossy fiber synapses in the CA3 subregion by stimulating in the hilus of the dentate gyrus. This resulted in evoked EPSPs with several components, suggestive of excitation of multiple subsets of synapses either monosynaptically or polysynaptically. To circumvent this problem, we found that placing our stimulating pipette in s. lucidum, $\sim$50–200 μm lateral to the recording pipette, resulted in synaptic responses with fast rise times and few secondary components.

Despite this strategy, it is still possible that stimulation of mossy fibers in s. lucidum also resulted in activation of other fiber systems traversing this layer. For example, it is possible that C/A fibers also were activated, including during the LTP-inducing stimulation protocols. Therefore it is possible that B-HFS induced a NMDA-receptor–independent LTP of C/A synapses either exclusively or in addition to mossy fiber LTP. This form of LTP has been observed previously for Schaffer collateral input to CA1 pyramidal neurons and also has been shown to be sensitive to nifedipine, an L-type channel blocker (Grover and Teyler 1990; Teyler et al. 1994). Several observations, however, argue strongly against LTP of C/A synapses occurring under our experimental conditions. First, there was a graded fall-off in rise times of EPSPs/EPSCs with distance of stimulation from s. pyramidale. By choosing responses with only the fastest rise times, we tried to limit our study to mossy fiber responses, which are known to terminate proximally and to have fast rise times (Brown and Johnston 1983; Johnston and Brown 1983; Williams and Johnston 1991). It is possible that our stimulation protocol resulted in the activation of CA3 pyramidal neurons and their inputs onto basal dendrites of the cell we were recording from. However, when we stimulated in s. oriens to activate the axons of CA3 pyramidal cells, we evoked slow responses similar to those seen with s. radiatum stimulation (data not shown) and not the fast responses indicative of mossy fiber activation. Second, B- and L-HFS delivered directly to C/A fibers in s. radiatum did not induce a NMDA-independent form of LTP. Third, the time course of LTP differs when mossy fiber synapses are potentiated versus LTP induced at distal synapses by tetanization in s. radiatum. In particular, L-HFS–induced mossy fiber LTP produces a characteristically large increase ($\sim$200%) in the size of the response immediately after L-HFS, which decays to a plateau level of 50–100% in $\sim$10 min (Katsuki et al. 1991; Urban and Barrionuevo 1996; Zalutsky and Nicoll 1990) (Fig. 4). Such a large increase is not observed when L-HFS is delivered in s. radiatum (Zalutsky and Nicoll 1990) in the absence of NMDA-receptor antagonists. Fourth,

the NMDA-receptor-independent form of LTP observed in the CA1 region has a slow onset, reaching its maximum potentiation in ~10–20 min (Cavus and Teyler 1996). If we were blocking a similar form of LTP using NMDA-receptor antagonists and nimodipine, we would not have expected to see the residual STP present in our experiments.

Functional implications

Single-unit recordings in freely moving rats have shown that under some conditions granule cells can fire in brief bursts at an average rate of 0.15 ± 0.13 Hz (Jung and McNaughton 1993). In other laboratories under different conditions, granule cells can fire at high rates (≤80 Hz) for sustained periods of time (Buzsaki et al. 1983; Rose et al. 1983). These firing patterns are similar to B- and L-HFS, respectively, and suggest the possibility that patterns of granule cell firing associated with different behaviors can produce mossy fiber LTP by different mechanisms: one mechanism that requires activation of L-type Ca^{2+} channels and one that does not. Whatever the mechanism, LTP at mossy fiber synapses will result in a higher probability of firing action potentials in CA3 neurons from activity of granule cells. Such postsynaptic cell firing may provide an associative signal for inducing LTP at C/A or perforant path synapses as was recently demonstrated for Schaffer collateral synapses in CA1 (Magee and Johnston 1997; Markram et al. 1997). Mossy fiber synapses and the recurrent collateral circuitry in CA3 have been suggested to provide the substrates for autoassociative memory functions in the hippocampus (McNaughton and Morris 1987; Rolls 1996).

We thank Dr. Costa Colbert for computer assistance during the course of these experiments.

This work was supported by National Institutes of Health Grants MH-44754, MH-48432, and NS-11535 to D. Johnston and MH-11390 to M. Yeckel.

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Received 9 September 1997; accepted in final form 15 December 1997.

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