Requirements for LTP Induction by Pairing in Hippocampal CA1 Pyramidal Cells

HUAN-XIN CHEN, NIKOLAI OTMAKHOV, AND JOHN LISMAN
Volcan Center for Complex Systems, Biology Department, Brandeis University, Waltham, Massachusetts 02454

Chen, Huan-Xin, Nikolai Otmakhov, and John Lisman. Requirements for LTP induction by pairing in hippocampal CA1 pyramidal cells. J. Neurophysiol. 82: 526–532, 1999. The induction of long-term potentiation (LTP) in the hippocampal CA1 region requires both presynaptic activity and large postsynaptic depolarization. A standard protocol for inducing LTP using whole-cell recording is to pair low-frequency synaptic stimulation (100–200 pulses, 1–2 Hz) with a depolarizing voltage-clamp pulse (1–3 min duration). In this standard protocol, a Cs⁺-based internal solution is used to improve the fidelity of the depolarization produced by voltage-clamp. In an attempt to induce LTP more rapidly, we tried to induce LTP by pairing high-frequency stimulation (200 pulses, 20–100 Hz) with a short depolarization (~15 s). Surprisingly, we found that this protocol failed to induce LTP, even though large LTP (~300% of baseline) could be induced by a subsequent standard protocol in the same cell. Pairing brief high-frequency stimulation at the beginning of a long depolarization (3 min) also did not induce LTP. However, the same high-frequency stimulation at the end of the long depolarization did induce LTP. When similar experiments were done with a K⁺-based internal solution, pairing high-frequency stimulation with a short depolarization did induce LTP. This indicates that the requirement for long depolarization is related to the use of Cs⁺. We speculate that, when recording is made with Cs⁺, a tetanus given at the beginning of depolarization initiates a process that inhibits N-methyl-d-aspartate (NMDA)-dependent LTP. This inhibitory process itself decays away during prolonged depolarization.

INTRODUCTION

Long-term potentiation (LTP) of the Schaffer collateral synapses in the CA1 region of the hippocampus is the primary model system for the study of the associative synaptic modification thought to underlie learning and memory (Bliss and Collingridge 1993). The form of LTP at these synapses has a Hebbian property: synapses are strengthened if there is both presynaptic activity and substantial postsynaptic depolarization (Brown et al. 1990). The requirement for postsynaptic depolarization is due to the properties of the N-methyl-d-aspartate (NMDA) channel. To open, the Mg²⁺ block of these channels must be relieved by depolarization (Mayer et al. 1984; Nowak et al. 1984). Once NMDA channels open, they allow the influx of Ca²⁺ that triggers synaptic strengthening (Bliss and Collingridge 1993). A commonly used protocol for inducing LTP is to give “tetanic” stimulation in which a large number of axonal inputs is stimulated at high frequency (100 Hz) for 1 s. This synaptic input produces the postsynaptic depolarization required to open the NMDA conductance, but the quantitative properties and mechanism of this depolarization are not yet established. What is clear is that many factors are likely to be involved, including temporal and spatial summation of excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs, respectively), the triggering of back-propagating Na⁺ spikes (Magee and Johnston 1997), the triggering of bursts (Thomas et al. 1998), and the function of a variety of other voltage-dependent conductances (Magee 1998; Magee et al. 1998).

Because of the complexity of the postsynaptic processes that affect depolarization during tetanus-induced LTP, many investigators have sought to evoke LTP in a simpler and more defined way by using what is termed a “pairing protocol.” This protocol circumvents the complexities of synaptically induced postsynaptic depolarization by simply imposing depolarization by current injection through the microelectrode (Gustafsson et al. 1987) or by voltage clamp, using the somatic patch electrode (Malinow 1991). Typically Cs⁺ is used as the major internal cation because it blocks K⁺ channels and makes it possible to achieve a larger and more uniform dendritic depolarization. Because postsynaptic depolarization is produced by the clamp rather than by synaptic stimulation, only a few input fibers need to be stimulated (Kullmann and Nicoll 1992; Malinow 1991). This improves accuracy with which synaptic currents can be quantified, because small currents reduce voltage-clamp errors. A further aspect of standard pairing protocols is that a much lower frequency of synaptic stimulation (0.1–2 Hz) is used (Colino et al. 1992; Malinow and Tsien 1990; Manabe et al. 1992; Perkel and Nicoll 1993) than during tetanic stimulation (100 Hz). This produces an additional simplification by avoiding the transient forms of presynaptic plasticity that occur during high-frequency stimulation. A consequence of using low-frequency stimulation is that the depolarization during pairing must be long. Typically, this depolarization is applied for more than one minute (Colino et al. 1992; Manabe et al. 1992; Otmakhov et al. 1997).

The experiments reported began with the attempt to devise a much briefer pairing protocol that more closely resembles the duration of depolarization during tetanically induced LTP. Based on what is known about LTP, we reasoned that it should be possible to induce LTP by tetanic stimulation during a brief depolarizing voltage-clamp pulse. We found, however, that it is not possible to do so. Our results further show that this surprising inability to induce LTP is related to the use of Cs⁺ as the major internal cation.

METHODS

Transverse hippocampal slices were prepared from male Long-Evans rats (14–19 days old) as described previously (Otmakhov et al. 1997). Pressurized solutions were delivered through a peristaltic pump, and the temperature was maintained at 35 ± 1°C. The slice was mounted in a recording chamber, which was continuously perfused with artificial cerebrospinal fluid (ACSF) equilibrated with 5% CO₂ and 95% O₂ at a flow rate of 1.5 ml/min. The ACSF contained (in mM) 124 NaCl, 3 KCl, 1.25KH₂PO₄, 26 NaHCO₃, 26 MgCl₂, 2 CaCl₂, and 5 glucose. The pH was 7.35. The ACSF was delivered to the slice by gravity, and the water bath temperature was maintained at 37°C. Slice recording was performed in a Michigan Scientific voltage-clamp amplifier (model 1800, Michigan Scientific Instruments, Inc.). Signals were filtered (3-kHz low-pass), digitized (10-kHz sampling rate), and stored for offline analysis.
paired with a short depolarization (high-frequency tetani (50 pulses at 50 or 20 Hz per each; 4 s intervals) stopped altogether. The specific LTP protocols were
stimuli in each pathway was swi t ha3s interval between pathways.
collaterals. Stimuli (100 m/s) were delivered alternatively to each input pathway through current output isolation units. The interval between stimuli in each pathway was 6 s with a 3 s interval between pathways. Stimulus intensity was adjusted to produce an excitatory post-

In all pairing protocols used for inducing LTP, a total of 200 stimuli were delivered to the test pathway during the depolarization. During all induction protocols, stimulation of the control pathway was stopped altogether. The specific LTP protocols were J) four brief high-frequency tetani (50 pulses at 50 or 20 Hz per each; 4 s intervals) paired with a short depolarization (~15 s to 0 mV); 2) four brief high-frequency tetani (50 pulse of 50 or 20 Hz per each; 4 s intervals) paired with a long depolarization (~3 min to 0 mV) given either at the beginning or at the end of the long depolarization; 3) the standard pairing protocol: low-frequency stimulation (200 pulses, 1.4 Hz) paired with a long depolarization (~3 min to 0 mV).

Data were acquired using a 486 PC computer, Labmaster DMA ADC and program written in Axobasic. The amplitude of a synaptic response was calculated as the difference between the average of data points in a window before the stimulus and in a window around the peak of the synaptic response. The average of responses during a 5 min period before LTP induction was taken as the baseline, and all values were normalized to this baseline. The level of LTP was calculated from this normalized data as the average (over a 3 min period) at the times after LTP induction indicated in each case. Values were expressed as means ± SE. Two-tail paired and unpaired t-test were used for calculation of the statistical significance of differences. Drugs used included d-2-amino-5-phosphonovaleric acid (d-AP5, Research Biochemicals International) and picrotoxin (Sigma).

RESULTS

Whole-cell recordings were made under visual control from CA1 pyramidal cells located 30–90 μm beneath the slice surface under visual control using infrared dark-field illumination and a charge-coupled device (CCD) TV camera. The patch electrodes were made from hard borosilicate glass and filled with (in mM) 120 Cs-gluconate, 10 CsCl, 10 HEPES, 8 NaCl, 0.2 EGTA, 2 MgATP, 0.3 Na3GTP, and 10 phosphocreatine (pH 7.3 with CsOH, osmolarity 290–300 mosM), or 125 K-glucolate, 10 HEPES, 8 NaCl, 0.2 EGTA, 2 MgATP, 0.3 Na3GTP, and 10 phosphocreatine (pH 7.3 with KOH, osmolarity 290–296 mosM). The electrodes had resistance 3–5 MΩ when filled with internal solution. Whole-cell recording was made in voltage-clamp mode using an Axopatch-1D (Axon Instruments, Foster City, CA) and cells were voltage-clamped at −68 mV. To evoke synaptic current, two glass electrodes filled with ACSF (300 KΩ) were placed in the dentrite region 70 and 150 μm away from the cell body layer to stimulate two separate groups of Schaffer collaterals. Stimuli (100 μs) were delivered alternatively to each input pathway through current output isolation units. The interval between stimuli in each pathway was 6 s with a 3 s interval between pathways. The input resistance ranged from 6 to 12 MΩ. Ten minutes after 20 Hz tetani given during short depolarization, the level of potentiation was 116 ± 11% in the test and 122 ± 5% in control input. Ten minutes after 20 Hz tetani given during short depolarization, the potentiation was 138 ± 10% in the test and 119 ± 8% in the control input. The standard pairing protocol given later in the same experiments produced robust synapse-specific LTP (300 ± 7% in the test and 116 ± 8% in the control input, n = 8, 30 min after induction).

There are two major differences between the brief protocol and the standard protocol: the length of the depolarization and the frequency of synaptic stimulation. We next sought to determine which of these differences is important. In the experiments illustrated in Fig. 2, pairing was done with the long depolarization (~3 min) used in the standard pairing protocol, but synaptic stimulation was done using brief periods of high-frequency (50 Hz) stimulation. Two variants of this experiment were done, one in which the high-frequency stimulation was given at the beginning of the long depolarization, the other in which the same high-frequency stimulation was given at the end of the long depolarization. When stimulation was given at the beginning of the depolarization (Fig. 2A), there was virtually no potentiation (101.5 ± 15.7%, at 20 min after pairing, n = 5). However, when stimulation was given at the end of the depolarization (Fig. 2B), large input-specific LTP was induced (243 ± 22% in the test and 101 ± 11% in the control at 20 min after induction of LTP, n = 7). In a separate set of experiments when 20 Hz tetani were given at the end of the long depolarization, strong and synapse-specific LTP was also induced (197 ± 6% in the test and 102 ± 5% in the control at 20 min
after induction of LTP, \( n = 7 \). These results indicate that LTP can be induced by a high-frequency stimulation during pairing, but that stimulation must be preceded by a long depolarization.

The LTP that can be induced by the standard low-frequency pairing protocol can be blocked by 2-amino-5-phosphonovaleric acid (APV), a blocker of NMDA channels (\( n = 4 \), data not shown). To check whether the LTP produced by high frequency given at the end of a long depolarization was also dependent on the NMDA channels, we repeated these experiments in the presence of APV (50 \( \mu \)M). Figure 3A shows that this type of LTP was dependent on NMDA channels. We also tested whether the small, transient potentiation produced by pairing high-frequency stimulation with a brief depolarization was NMDA dependent. Figure 3B shows that it was not sensitive to APV (50 \( \mu \)M; 126 \( \pm \) 6\%, 10 min after tetanus). This level of potentiation was not significantly different from the level of potentiation produced in control ACSF (\( P > 0.05 \)). It should also be noted that the NMDA-independent potentiation was not input specific (123 \( \pm \) 9\% potentiation on the control input 10 min after tetanus).

It is of interest to know whether the properties of the synaptically evoked current during pairing give any hint about why LTP occurs under some conditions and not others. Figure 4A shows the currents during each of four tetani that were given early (left column) or late (right column) during a long depolarization. The records were from the same cell and the same input. Figure 4B shows an example from another cell, but where different inputs were used for early and late responses. These currents have the slow kinetics characteristic of the NMDA current and are almost completely blocked by APV (Fig. 4C). It can be seen that the current is larger when the tetani are given early than when they are given late during the depolarization. Similar results were obtained in all cells examined. One curious feature of these records that might be sig-

---

FIG. 1. High-frequency synaptic stimulation paired with a brief depolarization did not induce long-term potentiation (LTP), whereas a subsequent standard protocol induced robust LTP. A: individual experiment in which high-frequency stimulation (4 trains with 4 s interval, each contains 50 pulse, 50 Hz) was given to the test pathway (●) paired with 15 s depolarization to 0 mV (at arrow). ○, control pathway not stimulated during the depolarization. Subsequently (at bar) a standard pairing protocol (200 pulses, 1.4 Hz, holding potential, 0 mV) was applied to the test pathway. Inset: diagram representing the 4 short high-frequency trains. B: averages of excitatory postsynaptic currents (EPSCs; 20 successive responses) in the test (●) and control (○) pathway taken at the times marked by letters in A. C: summary data (\( n = 6 \)).

FIG. 2. High-frequency stimulation given at the end, but not the beginning of a long depolarization, could induce LTP. A: summary data (\( n = 7 \)) when high-frequency stimulation was given at the beginning of a long depolarization (3 min). ● and ○, test pathway and control pathway, respectively. B: summary data (\( n = 5 \)) when high-frequency stimulation given at the end of the long depolarization. Insets in A and B are the diagrams of the protocols.
significant is that the tetanus given early sometimes evoked currents with seemingly supralinear summation on the rising edge, followed by a rapid collapse of the current. These features were not observed when the tetanus was given at the end of the depolarization. Possible interpretations of these complex kinetics are given in the DISCUSSION. An additional feature of these experiments is that the total outward membrane current falls gradually during a long depolarization (Fig. 4).

In a final series of experiments, we sought to determine whether the failure of the short depolarization protocol might be related to the use of Cs\(^+\) as the major internal cation. To examine this issue, we changed the internal solution to one containing K\(^+\) instead of Cs\(^+\). Figure 5 shows that, with the use of this solution, it became possible to induce LTP by pairing high-frequency stimulation (50 Hz) with a brief (15 s) depolarization to 0 mV. Although the amount of LTP is large (217 ± 21.6%, \(n = 6\)), it is significantly smaller than that induced by the standard pairing protocol with Cs\(^+\) as the internal cation (307 ± 36%, \(P < 0.05\)).

**FIG. 4.** Synaptic currents induced by the trains of high-frequency stimulation given during depolarization, and the holding current during depolarization. A: comparison of synaptic currents induced by 2 protocols given to same inputs in the same neuron at different times during long depolarization. The diagram at the top indicates the protocols. Left: responses evoked by 4 trains of high-frequency stimulation (50 pulse, 50 Hz) given at the beginning of depolarization, which did not induce LTP. Right: responses induced by 4 trains of high-frequency stimulation given at the end of depolarization, which did induce LTP. B: another comparison of synaptic currents induced by 2 protocols given in same cell but different inputs at different times. Left: the synaptic currents evoked by the trains of high-frequency stimulation paired with short depolarization, which did not induce LTP. Right: the synaptic current induced by trains of high-frequency stimulation given at the end of depolarization, which did induce LTP (note that stimulus artifacts were not picked up by this electrode). C: synaptic current induced by 1 train of high-frequency stimulation during the short high-frequency pairing protocol was blocked by APV (50 \(\mu M\)). D: holding current during long depolarization (from -68 to 0 mV). Bars over holding current trace indicate where high-frequency stimulations were given.

**DISCUSSION**

We have investigated the requirements for inducing LTP using variants of the standard pairing protocol. In the standard protocol, depolarization is imposed on the postsynaptic neuron by voltage clamping the membrane to 0 mV while stimulating the input axons at fairly low frequency (0.1–2 Hz) for several minutes (Colino et al. 1992; Manabe et al. 1992; Otmaukhov et al. 1997). Generally, Cs\(^+\) has been used as the major internal cation to block several K\(^+\) channels and thereby make it easier to impose depolarization. We have found that if we modified the standard protocol and used high-frequency (20–100 Hz) stimulation given during a brief (15 s) depolarization to 0 mV, LTP cannot be induced. This failure is not simply due to the use of high-frequency stimulation because LTP can be induced by high-frequency stimulation given at the end of a long depolarization. Furthermore, we have found that the failure to induce LTP during brief depolarization is related to the use of Cs\(^+\)-based internal solution. When K\(^+\) is the major cation, high-frequency stimulation given during a brief depolarization does induce LTP. Thus it would appear that Cs\(^+\) somehow prevents LTP induction when...
short high-frequency pairing protocol was given to the test pathway (●). ○, control pathway. Inset: diagram of protocol. B: averages of EPSCs (20 successive responses) in test (●) and control pathway (○) taken at the time marked by letters in A. C: summary data of experiments as shown in A (n = 6). ● and ○, test and control pathways, respectively.

These findings are quite surprising and cannot be simply explained in terms of what is known about LTP. Cs+ is used to block K+ channels and should therefore enhance depolarization. It has been shown that depolarization up to the reversal potential for EPSC should enhance LTP induction, not inhibit it (Perkel et al. 1993). Because we do pairing at a potential somewhat negative of reversal potential, any dendritic depolarization may occur during synaptic stimulation (due to clamp failure) should cause the voltage to become closer to the reversal potential and therefore lead to larger potentiation rather than the very small potentiation we observed. The use of brief high-frequency stimulation should bring about facilitation of transmitter release and enhance LTP induction, not inhibit it.

Unfortunately, we are unable to provide any clear explanation of our findings and can only point out several possibilities. A key unresolved question is how the NMDA current itself is affected by various induction conditions. The total synaptically evoked current during pairing can be measured and gives hints about what might be going on. This current is inward and appears larger when high-frequency stimulation is given during a brief depolarization (or early during a long depolarization) than when the same stimulation is given toward the end of a long depolarization (Fig. 4). A curious and unexplained feature of this current is that it often has an accelerating rising edge and a transient character. The inward current is strongly reduced by APV, suggesting that it is either largely NMDA current or consists of both NMDA current and components triggered as a consequence of current through the NMDA channel. On the assumption that the current is primarily NMDA current, one might ask why the current has such complex kinetics (Fig. 4). Recent work showed that the NMDA conductance is enhanced by intracellular Na+ (Yu and Salter 1998). So it is possible that the odd kinetics of the current during the tetanus are the consequence of Na+-dependent regulation. If the total current is indeed largely NMDA current, then the failure to induce LTP during brief depolarization can certainly not be attributed to a reduced NMDA conductance, because the total synaptic current is larger at early times (Fig. 4). If anything, the results raise the possibility that there could be an optimal NMDA-mediated Ca2+ entry and that if this optimum is exceeded, smaller LTP will be induced (Lisman 1985).

The transient nature of the synaptically evoked current during pairing suggests a second interpretation in which the initial NMDA-mediated synaptic current triggers a dendritic Ca2+ spike (assuming that clamping is not adequate in the dendrites). There are indications that Ca2+ elevations can inhibit the NMDA conductance itself (Legendre et al. 1993), and this might explain why LTP induction was blocked. Ca2+ spikes might be much more difficult to induce when K+ channels are functional, and this might explain why LTP can be induced when K+ is the major internal cation. In a related way, it might be supposed that inactivation of the Ca2+ conductance by a long depolarization prevents initiation of a Ca2+ spike late during the depolarization, and this could explain why stimulation given late during a long depolarization can induce LTP.

It is possible that the failure to induce LTP by high-frequency stimulation given at the beginning of long depolarization (or during short depolarization) is related to both the high frequency and the timing when the tetanus is given. In this case, short low-frequency stimulation would induce LTP even if it is given at the beginning of long depolarization (or during short depolarization). Indeed the work has indicated that weak NMDA-dependent potentiation can be produced when a small number of stimuli are given at low frequency during brief depolarizations (Petersen et al. 1998). One possible explanation in terms of the Ca2+ spike model given above would be that only high-frequency stimulation can produce sufficient depolarization to trigger a Ca2+ spike.
Our work is not the first attempt to induce LTP by combining high-frequency stimulation with imposed depolarization. A number of works reported that LTP could be induced by pairing procedures of this kind (Hjelmstad et al. 1997; Kato et al. 1993; Manabe et al. 1993; Perkel and Nicoll 1993). These experiments were done using Cs⁺. However, the detailed timing of synaptic stimulation during the depolarization is not clear. These previous results may therefore not be incompatible with our findings. In a study done under experimental conditions (5-wk-old guinea pigs, at 32°C) (Chen et al. 1998) somewhat different from those used here (<3-wk-old rats, room temperature, Cs⁺ as the major intracellular cation was used in both cases), it was possible to induce LTP by delivering two 20 Hz trains given 10 s apart during continuous depolarization lasting overall ~15 s. It is unclear whether these technical differences are important. One previous paper reported that intracellular Cs⁺ can block tetanus-induced LTP (Haas and Rose 1984). Their results are consistent with ours, although they used sharp microelectrode recording, and depolarizing current was not injected during the tetanus.

Our results indicate that there are aspects of the standard pairing procedure that are not understood, specifically, the requirement for long depolarization. This raises the question of whether pairing protocols are a good model for the LTP induced by tetanic stimulation or whether there are fundamental differences. One difference is the size of the LTP produced by pairing and tetanus protocols. Both forms of LTP produced by pairing and tetanus protocols. Both forms of LTP differ in magnitude, there are no other reasons to think that fundamentally different mechanisms are involved in the LTP produced by pairing and tetanus protocols. Both forms of LTP are dependent on NMDA channels and can be blocked by kinase inhibitors (Otmakhov et al. 1997).

In summary, we have studied the requirements for inducing LTP by pairing protocols. We unexpectedly found that when a tetanus is given at the beginning, but not at the end of depolarization, LTP induction is inhibited. The generation of this transient inhibition is related to the use of Cs⁺ as a major intracellular cation. These results are not expected from the known properties of LTP and suggest that there is a process that can powerfully regulate LTP induction that has not yet been identified.

The authors thank Dr. Nonna Otmakhova for useful comments.

This work was supported by National Institute of Neurological Disorders and Stroke Grants 5 R01 NS-35083 and 5 R01 NS-27337. The authors gratefully acknowledge the support of the W. M. Keck Foundation. Address reprint requests to J. Lisman.

Received 21 December 1998; accepted in final form 21 April 1999.

REFERENCES


Otmakhov, N., Griffith, L. C., AND Lisman, J. E. Postsynaptic inhibitors of calcium/calmodulin-dependent protein kinase type II block induction but not


