

Glutamate Iontophoresis Induces Long-Term Potentiation in the Absence of Evoked Presynaptic Activity

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Summary

Protocols that induce long-term potentiation (LTP) typically involve afferent stimulation. We tested the hypothesis that LTP induction does not require presynaptic activity. The significance of this hypothesis is underscored by results suggesting that LTP expression may involve activity-dependent presynaptic changes. An induction protocol using glutamate iontophoresis was developed that reliably induced LTP in hippocampal slices without afferent stimulation. Iontophoresis LTP was Ca^{2+} dependent, was blocked by MK-801, and occluded tetanus-induced LTP. Iontophoresis LTP was induced when excitatory postsynaptic potentials were completely blocked by adenosine plus tetrodotoxin. Our results suggest constraints on the involvement of presynaptic mechanisms and putative retrograde messengers in LTP induction and expression; namely, these processes must function without many forms of activity-dependent presynaptic processes.

Introduction

High frequency stimulation (HFS) of presynaptic fibers in several brain regions can induce a long-lasting enhancement of synaptic transmission known as long-term potentiation (LTP; Bliss and Lømo, 1973; Lee, 1982; Bindman et al., 1987, *J. Physiol.*, abstract). In the CA1 region of the hippocampus the induction of LTP by HFS involves Ca^{2+} entry into postsynaptic neurons through the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors. During HFS, the depolarization produced by summed excitatory postsynaptic potentials (EPSPs) relieves the voltage-dependent Mg^{2+} block of the NMDA receptor-ion channel (Coan et al., 1987). The resulting increase in intracellular free Ca^{2+} appears to be a critical trigger in the induction of LTP (Lynch et al., 1983). This "NMDA/ Ca^{2+} " hypothesis of LTP induction (Bliss and Lynch, 1988) is supported by experiments which demonstrated that LTP induction is blocked by competitive and noncompetitive NMDA receptor antagonists (Collingridge et al., 1983; Coan et al., 1987), by postsynaptic injections of Ca^{2+} chelators (Lynch et al., 1983), and by postsynaptic application of inhibitors of Ca^{2+} -dependent enzymes (Malenka et al., 1989; Malinow et al., 1989; del-Cerro et al., 1990).

Considerable evidence indicates that LTP induction requires postsynaptic processes (Lynch et al., 1983; Malinow and Miller, 1986; Wigström and Gustafsson, 1986; Bliss and Lynch, 1988; Malenka et al., 1988). Al-

though persistent changes associated with LTP expression may reside in either pre- or postsynaptic neurons (Foster and McNaughton, 1991; Kullmann and Nicoll, 1992; Larkman et al., 1992; Liao et al., 1992; Manabe et al., 1992), certain studies indicate that changes mediating LTP expression occur in presynaptic terminals (i.e., increased probability of neurotransmitter release; Bekkers et al., 1990; Malinow and Tsien, 1990; Malgaroli and Tsien, 1992; however, see Foster and McNaughton, 1991; Manabe et al., 1992). This suggests the intriguing possibility that retrograde messengers may be required for events underlying LTP induction and/or expression (Williams et al., 1989; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992). An important issue concerning the role of putative retrograde messengers is their mode of action on presynaptic terminals. With respect to the NMDA/ Ca^{2+} hypothesis, one view suggests that during induction, a retrograde messenger is released from postsynaptic neurons and initiates presynaptic processes that mediate LTP expression. In the context of the synapse-specific nature of LTP expression (Andersen et al., 1977; Lynch et al., 1977), the function of retrograde messengers may require that they interact with activity-dependent presynaptic processes. Thus both the NMDA/ Ca^{2+} hypothesis and the current speculation about the function of retrograde messengers highlight the importance of understanding the role of presynaptic activity in LTP induction. In support of these hypotheses, many protocols that induce NMDA receptor-dependent (Andersen et al., 1977; Lynch et al., 1977) or NMDA receptor-independent (Williams et al., 1989; Aniksztejn and Ben-Ari, 1990; Grover and Teyler, 1990b; Kullmann and Nicoll, 1992) LTP in CA1 hippocampal neurons appear to require evoked stimulation of presynaptic afferents.

One prediction of the NMDA/ Ca^{2+} hypothesis is that the induction of LTP should occur with exogenous application of NMDA in the absence of presynaptic activity and endogenous neurotransmitter release. Such an observation would illustrate that presynaptic activity is involved in LTP induction only to deliver transmitter to postsynaptic receptors and that other presynaptic processes that accompany release (e.g., the activation of Ca^{2+} -dependent enzymes or presynaptic autoreceptors) do not play an integral role in LTP expression. The studies herein test the hypothesis that evoked presynaptic activity and transmitter release are not required in the induction of some types of LTP and address the contribution of presynaptic events such as transmitter release and action potentials in the induction of LTP. In particular, we have sought to determine whether presynaptic activity is required only to provide glutamate for postsynaptic receptor activation, or are there other factors associated with presynaptic activity required for LTP induction. Our results indicate that mechanisms in-

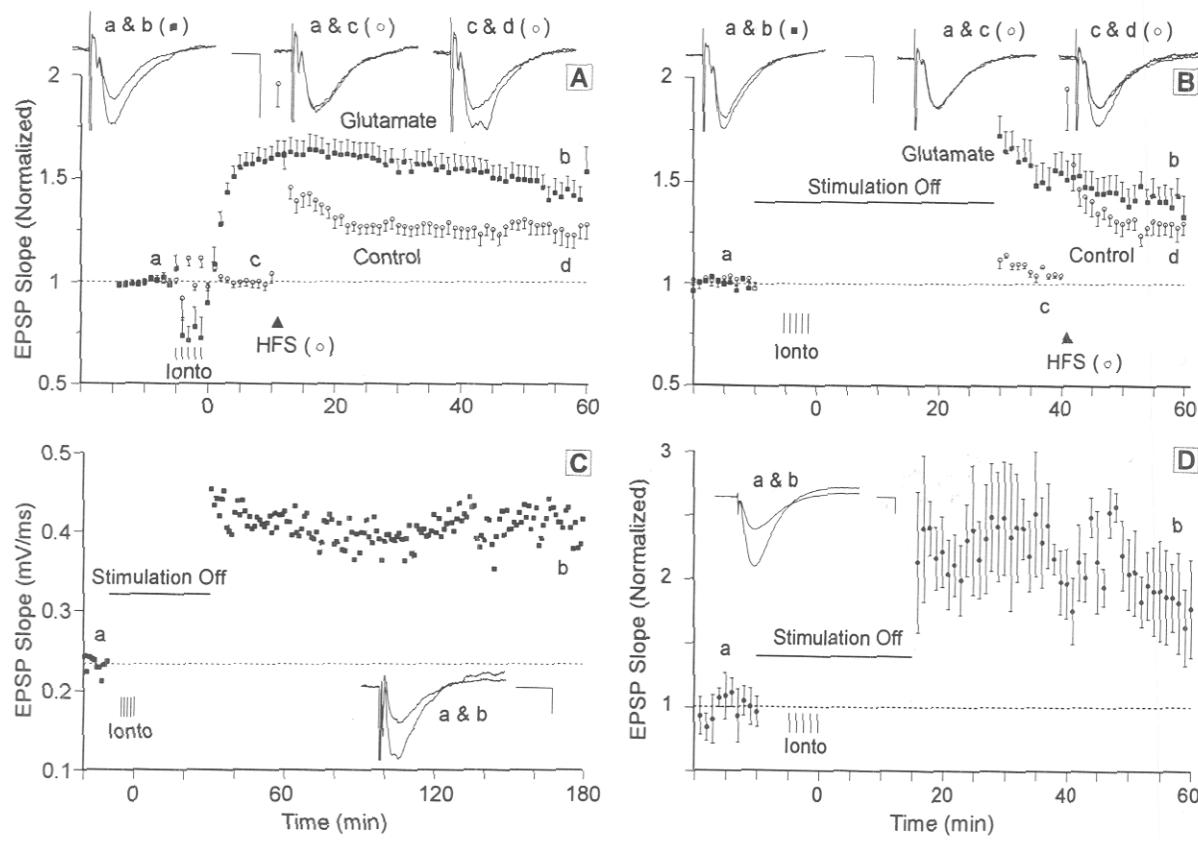


Figure 1. Repeated Glutamate Iontophoresis Potentiates Synaptic Transmission (Ionto-LTP)

(A) Glutamate iontophoresis by the iontop-protocol (closed squares; $n = 16$) paired with 1 Hz stimulation caused a long-term enhancement of synaptic transmission. Control NaCl iontophoresis (open circles; $n = 10$) did not potentiate synaptic transmission; however, subsequent HFS (closed triangle) to control slices produced LTP. In this and following figures, EPSP slopes are normalized to mean baseline values. Each data point represents the mean \pm SEM of all experiments for a given condition; within each experiment, six consecutive EPSPs were averaged to give a single slope value. The rate of afferent stimulation was 0.1 Hz. Repeated glutamate iontophoresis (five repeated 10 s pulses at 60 s intervals) is indicated by vertical bars. Time = 0 denotes the end of iontophoresis in all experiments. The EPSPs shown above each panel are averages of six responses at the designated time from a representative experiment. Scale bars are 10 ms (horizontal) and 0.5 mV (vertical) in all figures, except where noted.

(B) Glutamate iontophoresis (closed squares; $n = 4$) in the absence of afferent stimulation enhanced synaptic transmission for at least 1 hr. Afferent stimulation was stopped 5 min prior to and not resumed until 30 min following iontophoresis. Control iontophoresis of NaCl (open circles; $n = 4$) in the absence of afferent stimulation did not potentiate EPSPs; subsequent HFS (closed triangle) to control slices produced LTP.

(C) Ionto-LTP can be observed for at least 3 hr. The iontop-protocol in the absence of afferent stimulation was used (see [B] above). (D) Whole-cell recordings of ionto-LTP. CA1 neurons ($n = 3$) were voltage-clamped at -80 mV. Stimulation was turned off 5 min before and not resumed until 15 min after iontophoresis (vertical bars). During the iontop-protocol, recordings were switched to current-clamp mode, initially at -80 mV. During iontophoretic pulses, pyramidal neurons were depolarized to -20 to 0 mV. Scale bar: 50 nA, 10 ms.

volved in the induction of LTP occur in the absence of certain types of presynaptic activity and endogenous glutamate release and suggest certain constraints about the underlying mechanisms associated with LTP induction in the hippocampus.

Results

Glutamate Iontophoresis Enhances

Synaptic Transmission

A first step in examining the requirement of presynaptic activity in LTP was to establish conditions for activating postsynaptic neurons in the absence of afferent stimulation. We chose to iontophorese excitatory

amino acids rather than to bath apply them because the former provides better spatial and temporal control of receptor stimulation. We initially chose to iontophorese L-glutamate, with the expectation that its rapid reuptake (Stallcup et al., 1979) should not produce substantial synaptic depression, and we reasoned that short (10 s) pulses of this mixed agonist should reduce NMDA and/or non-NMDA receptor desensitization (Trussell and Fischbach, 1989; Clark et al., 1990). Anticipating that a critical threshold for postsynaptic Ca^{2+} may be more efficiently reached with repeated short pulses of glutamate iontophoresis, we tested the ability of five consecutive glutamate pulses (10 s each) to produce synaptic potentiation. Extracellular Ca^{2+} was maintained at 2 mM to avoid

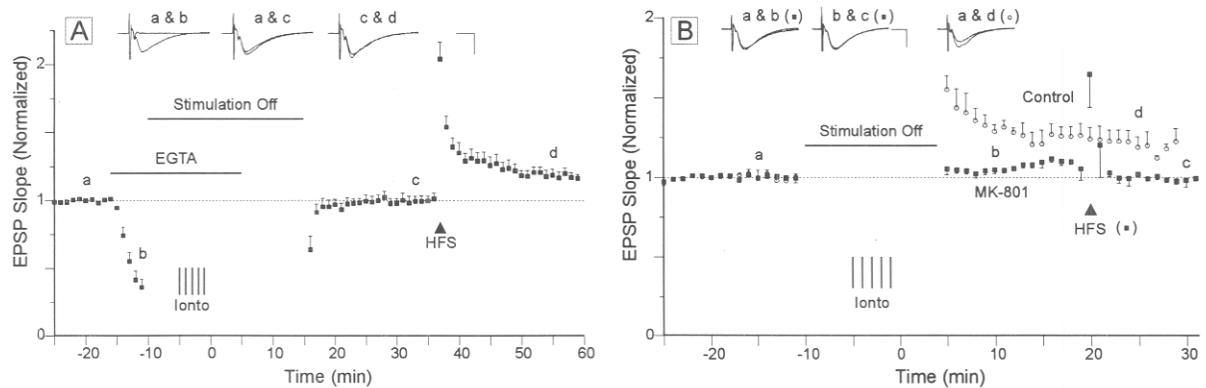


Figure 2. Extracellular Ca^{2+} and NMDA Receptor Activity Are Required for Ionto-LTP

(A) Ionto-LTP was blocked when extracellular Ca^{2+} was reduced. Slice medium containing $100 \mu\text{M}$ EGTA plus 3 mM extracellular Mg^{2+} (no added extracellular Ca^{2+}) was applied 14 min prior to glutamate iontophoresis (closed squares; $n = 6$) and washed off 5 min following iontophoresis. Afferent stimulation was stopped 5 min prior to and not resumed until 15 min following iontophoresis; subsequent HFS (closed triangle) produced LTP, demonstrating that EGTA treatments were reversible.

(B) Ionto-LTP is blocked by MK-801. Afferent stimulation was stopped 5 min prior to and not resumed until 5 min following iontophoresis. Slices preincubated in MK-801 ($10 \mu\text{M}$; closed squares; $n = 5$) for at least 30 min prior to and throughout the recording session failed to produce ionto-LTP. Following iontophoresis, HFS (closed triangle) failed to produce LTP in the presence of MK-801. Control slices (no MK-801) from the same hippocampi and analyzed in the same session displayed ionto-LTP (Control; open circles; $n = 4$).

the reported effects of nonphysiologically high extracellular Ca^{2+} on increasing NMDA receptor desensitization (Clark et al., 1990), or presynaptic processes such as increased spontaneous release (Hubbard et al., 1968). Figure 1A shows that repeated glutamate iontophoresis (ionto-protocol; see Experimental Procedures), paired with afferent stimulation, enhanced synaptic transmission by $54\% \pm 6.8\%$ ($n = 16$) when measured 45 min after iontophoresis. D-Glutamate was used in three experiments, and the remaining experiments used L-glutamate. Experiments using L- or D-glutamate produced similar levels of potentiation and were therefore combined (Figure 1A). Afferent stimulation (1 Hz) was given during the glutamate pulses of the ionto-protocol, and EPSPs were recorded at 0.1 Hz before and after this protocol. We observed that synaptic transmission was attenuated during and for approximately 2 min after iontophoresis. The EPSP slope recovered after iontophoresis, reached baseline in 1–2 min, continued to increase for 5–10 min, and remained enhanced for at least 60 min following iontophoresis. This long-term enhancement of synaptic transmission produced by glutamate iontophoresis will be referred to as ionto-LTP.

Iontophoresis of NaCl (1 M) using the same ionto-protocol produced no detectable persistent change in EPSPs ($n = 10$; Figure 1A, Control). This control excludes factors such as direct depolarization by iontophoresis current or increased neurotransmitter release under hyperosmotic conditions as potential artifacts in ionto-LTP. In these controls, each slice subjected to NaCl iontophoresis displayed HFS-induced LTP (HFS-LTP) ($28\% \pm 4\%$ potentiation) following tetanic stimulation (100 Hz for 1 s, given twice with a 20 s interval).

These results show that glutamate iontophoresis

can enhance synaptic transmission; however, this potentiation may have resulted from glutamate-induced depolarization of postsynaptic neurons paired with stimulation of presynaptic fibers. Figure 1B shows that ionto-LTP can be induced when afferent stimulation is stopped 5 min prior to and not resumed until 30 min following iontophoresis (stimulation also remained off during the ionto-protocol); levels of potentiation were $41\% \pm 11\%$ ($n = 5$) at 60 min post-iontophoresis. This result shows that afferent stimulation is not required for the induction of ionto-LTP. Moreover, the data indicate that the induction/maintenance of ionto-LTP does not require afferent stimulation in the first 30 min following induction. When glutamate was replaced with NaCl, the ionto-protocol occasionally produced slight potentiation ($6\% \pm 1\%$ potentiation, $n = 4$); however, this potentiation returned to baseline within 10 min (see Figure 1B, Control). This small but transient potentiation appeared to result from interrupting afferent stimulation for 30–40 min and was not observed when stimulation was interrupted for less than 15 min (see Figure 3). Each slice in these control experiments displayed HFS-LTP ($27\% \pm 6\%$ potentiation, $n = 4$). Although we generally conducted the experiments for 1 hr following iontophoresis, ionto-LTP can be stable for at least 3 hr (Figure 1C).

We considered the possibility that the induction of ionto-LTP could in part result from changes in the input resistance or resting potential of postsynaptic neurons. These possibilities were explored with whole-cell recordings in voltage-clamp mode (Figure 1D). Ionto-LTP induced with the stimulation-off protocol (cells were switched to current-clamp mode during the ionto-protocol) was observed to be robust ($77\% \pm 4\%$, $n = 3$) at 60 min following iontophoresis,

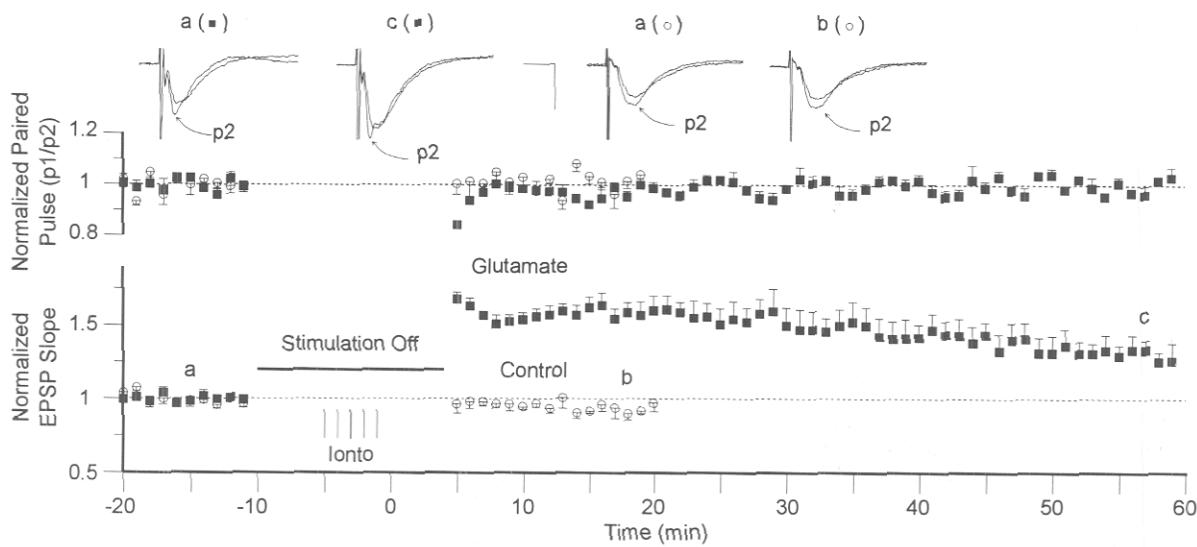


Figure 3. Ionto-LTP Does Not Affect Paired-Pulse Facilitation

In these experiments test stimuli (0.1 Hz) were interspersed with a pulse (p2) that was preceded (40 ms) by a conditioning pulse (p1). The bottom panel shows the normalized EPSP slopes of test stimulus responses, and the top panel shows the ratio of p2/p1 normalized to baseline values. This p2/p1 ratio reveals the degree of PPF. Following glutamate iontophoresis (closed squares; $n = 4$), EPSP slopes were enhanced, indicating ionto-LTP induction (bottom panel), whereas the p2/p1 ratio did not change significantly, indicating no effect on PPF. Control NaCl iontophoresis (open circles; $n = 4$) did not produce ionto-LTP and did not affect PPF ratios. Representative waveforms show p1 and p2 superimposed.

whereas the input resistance changed very little ($1\% \pm 12\%$). Although the series resistance increased slightly ($15\% \pm 14\%$), this result would lead to an underestimation of ionto-LTP. Ionto-LTP was also observed in field EPSPs monitored with extracellular recording electrodes in these same experiments (results not shown). These data indicate that ionto-LTP does not result simply from changes in these passive biophysical properties of postsynaptic neurons.

Ionto-LTP and HFS-LTP Share Certain Properties

Is extracellular Ca^{2+} important for the induction of ionto-LTP? In this context, HFS-LTP requires that Ca^{2+} enter postsynaptic cells, since LTP induction can be blocked by postsynaptic injection of Ca^{2+} chelators (e.g., EGTA; Lynch et al., 1983), or by reducing extracellular Ca^{2+} (Dunwiddie and Lynch, 1978). The interpretation of LTP induction experiments performed in the absence of extracellular Ca^{2+} is complicated by the fact that these conditions also block evoked neurotransmitter release, which is the induction stimulus. We have taken advantage of applying neurotransmitters directly to postsynaptic cells via iontophoresis to test the necessity of extracellular Ca^{2+} in the induction of LTP. When we reduced extracellular Ca^{2+} with a perfusion medium containing no added Ca^{2+} plus 3 mM Mg^{2+} and 0.1 mM EGTA (Figure 2A), glutamate iontophoresis did not enhance synaptic transmission ($0 \pm 4\%$ potentiation, $n = 6$). At the time indicated by (b) in Figure 2A (4 min after EGTA addition and 1 min before stimulation was interrupted), EPSPs were undetectable. Although slices did not produce ionto-LTP by glutamate iontophoresis in EGTA, following

EGTA washout they did display HFS-LTP in the presence of 2 mM extracellular Ca^{2+} ($20\% \pm 2\%$ potentiation, $n = 6$; Figure 2A). We observed that when the ionto-protocol was conducted in EGTA, that subsequent ionto-LTP or HFS-LTP was more difficult to obtain compared with controls (see Figure 2A). This may be due to an occlusion-like effect, as had been observed for the occlusion of HFS-LTP by NMDA iontophoresis (Huang et al., 1992) or inhibitors of nitric oxide synthase activity (Izumi et al., 1992), or deleterious effects of nominally Ca^{2+} -free solutions on neuronal viability (Malgaroli and Tsien, 1992). These results indicate that extracellular Ca^{2+} is necessary for the induction of ionto-LTP.

To test the importance of postsynaptic Ca^{2+} influx mediated by NMDA receptor activity in the induction of ionto-LTP, slices were incubated in the noncompetitive antagonist MK-801 (10 μM ; Coan et al., 1987) for 60 min prior to iontophoresis. Slices incubated in MK-801 failed to produce ionto-LTP (Figure 2B); levels of potentiation ($0 \pm 4\%$, $n = 5$) were not significantly different from baseline. MK-801 also effectively blocked the induction of conventional LTP induced by tetanic stimulation (Figure 2B). Slices from the same hippocampi but incubated in normal medium displayed ionto-LTP ($26\% \pm 7\%$ potentiation, $n = 4$; Figure 2B, Control). These results demonstrated that NMDA receptor activation is required for the induction of ionto-LTP. Taken together, our data with EGTA and MK-801 suggest that the induction of ionto-LTP, like HFS-LTP, involves postsynaptic Ca^{2+} -activated mechanisms.

Prior studies have used the analysis of paired-pulse

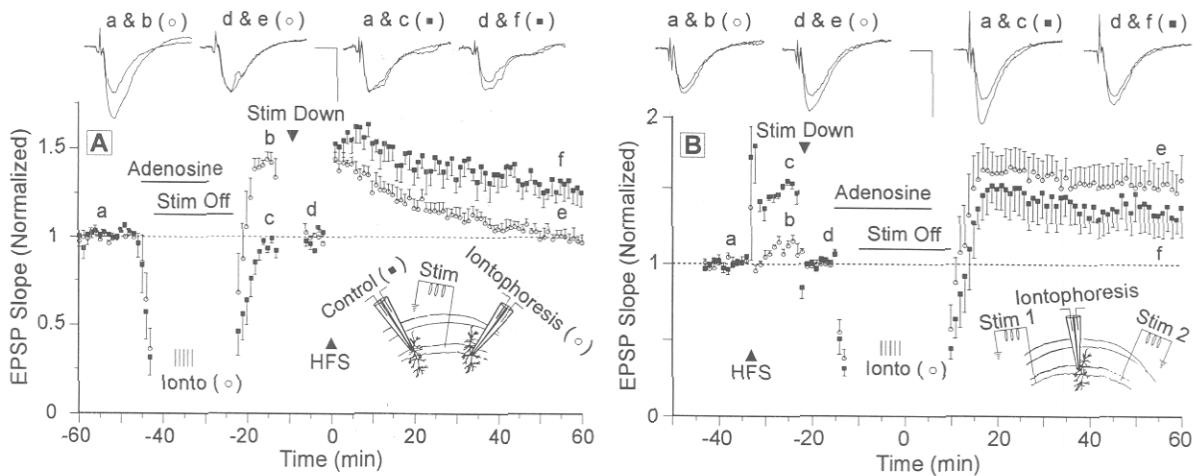


Figure 4. Interaction between Ionto- and HFS-LTP

(A) Ionto-LTP occludes HFS-LTP. Two recording electrodes were used (see cartoon inset): one was for recording plus iontophoresis (open circles), and a second for recording only (Control; closed squares) was positioned 2–3 mm from the iontophoresis electrode. Adenosine (200 μ M) was applied from 8 min before to 5 min after iontophoresis. Following iontophoresis, stimulation intensity was decreased (closed inverted triangle) to produce EPSPs with slopes equivalent to baseline levels at $t = -50$ min, and a new baseline was established. Five minutes later, HFS (closed triangle) was delivered to afferents projecting to both recording electrodes. HFS induced LTP at the control electrode (closed squares; $n = 5$), but only decremental potentiation at the iontophoresis electrode (open circles; $n = 5$).

(B) HFS-LTP partially occludes ionto-LTP. In these experiments one iontophoresis and two stimulating electrodes were used (see cartoon inset). Following baseline, HFS was delivered to one pathway (closed squares) to generate HFS-LTP. The second pathway (open circles) was not tetanized. Fifteen minutes later, the stimulus intensity was decreased in the potentiated pathway (closed squares) so that EPSP slopes were equivalent to baseline values. A new baseline was established (5 min) to which subsequent EPSPs were normalized. Adenosine was applied, stimulation was turned off, and iontophoresis was delivered as in (A). Ionto-LTP was produced in both pathways but was significantly reduced ($p < 0.05$; $n = 4$) in the tetanized pathway.

facilitation (PPF) as a measure of possible presynaptic changes that could mediate the expression of HFS-LTP (Muller and Lynch, 1989; Zalutsky and Nicoll, 1990). For example, PPF is unaffected by HFS-LTP in area CA1, and this result has been interpreted as supporting evidence that certain presynaptic changes (e.g., residual Ca^{2+} ; see Zucker, 1987) are not responsible for LTP expression. Likewise, we tested the possibility that the expression of ionto-LTP may be accompanied by changes in PPF. Values of PPF, with an interstimulus interval of 40 ms, remained unchanged following the induction of ionto-LTP (Figure 3). Although PPF was slightly attenuated for the first minute after afferent stimulation was resumed, it returned to baseline values even though ionto-LTP lasted for 60 min. Control slices subjected to NaCl iontophoresis displayed no change in PPF, even during the first minute of resumed stimulation. PPF measured with an interstimulus interval of 100 ms was also not affected by ionto-LTP (data not shown). The absence of changes in PPF during ionto-LTP expression indicates one more similarity between it and HFS-LTP.

Like HFS-LTP, ionto-LTP requires extracellular Ca^{2+} and NMDA receptor activation and does not affect PPF. But do HFS- and ionto-LTP operate through similar biochemical processes? An often used strategy to test whether different induction protocols share common mechanisms is to determine whether the extent of potentiation is additive when two induction protocols are given in series. If two protocols share com-

mon mechanisms, then the first protocol will “occlude” subsequent potentiation by a second protocol (Gustafsson et al., 1987; Kauer et al., 1988; Huang et al., 1992). Occlusion experiments were conducted to demonstrate that ionto-LTP and HFS-LTP involve similar underlying cellular and molecular mechanisms. When ionto-LTP was induced first (in the presence of adenosine; see below), subsequent attempts to induce HFS-LTP produced only short-term potentiation, which decayed to baseline within 40 min (Figure 4A). On the other hand, when HFS-LTP was induced first, ionto-LTP was only partially occluded ($17\% \pm 1\%$; Figure 4B); however, this effect was statistically significant ($p < 0.05$). These two-way occlusion experiments indicate that ionto- and HFS-LTP share common mechanisms, but that ionto-LTP may activate molecular pathways that are not totally engaged by HFS-LTP.

Ionto-LTP Can Be Induced When Action Potentials and Evoked Transmitter Release Are Inhibited

Building upon the similarities between ionto- and HFS-LTP, we further tested the necessity of presynaptic activity for the induction of ionto-LTP. Although ionto-LTP is induced in the absence of afferent stimulation, there may be endogenous neurotransmitter release produced by spontaneous quantal release and/or spontaneous action potentials that occur during the ionto protocol. Endogenous glutamate release by such processes, which might be augmented by glutamate iontophoresis (Malgaroli and Tsien, 1992),

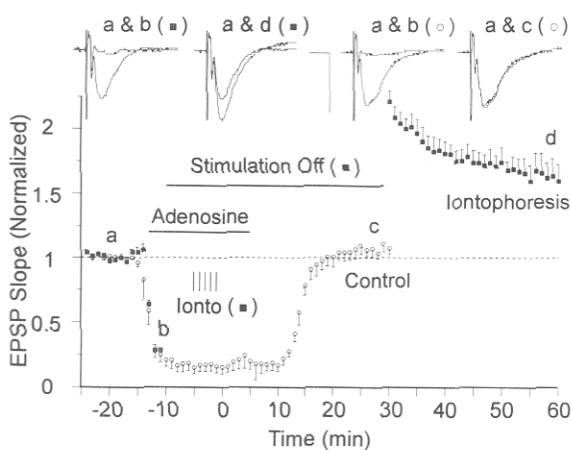


Figure 5. Adenosine Does Not Block the Induction of Ionto-LTP
Ionto-LTP was not prevented when presynaptic neurotransmitter release was inhibited by adenosine ($200 \mu\text{M}$). Adenosine (horizontal bar) was applied 8 min prior to and was washed off 5 min after iontophoresis. Afferent stimulation was stopped 5 min prior to and not resumed until 30 min following glutamate iontophoresis (closed squares; $n = 5$). Adenosine's inhibition of basal synaptic transmission in control slices not receiving glutamate iontophoresis (open circles; $n = 5$) reversed within 15 min.

could engage activity-dependent presynaptic processes in a manner analogous to evoked release. Such inadvertent pairing may be sufficient to induce iono-LTP. To address the possible contribution of presynaptic neurotransmitter release and glutamate receptor activation in iono-LTP, the strategy illustrated in Figure 5 was employed. We used the transient but reversible application of adenosine ($200 \mu\text{M}$ for 20 min; see Dunwiddie, 1990; Mitchell et al., 1993), which severely attenuated or eliminated EPSPs during glutamate iontophoresis but does not block postsynaptic receptors (Proctor and Dunwiddie, 1987). In these experiments glutamate iontophoresis was also applied in the absence of afferent stimulation. Under these conditions the iono-protocol enhanced synaptic transmission for at least 60 min; levels of potentiation were $56\% \pm 12\%$ ($n = 5$). Control experiments in which adenosine was applied without iontophoresis produced only reversible attenuation of EPSPs lasting approximately 15 min ($n = 5$; Figure 5, Control) with no detectable long-term change in basal synaptic transmission.

Although the above data demonstrate that iono-LTP can be induced when synaptic transmission is blocked, factors involved in induction, such as putative retrograde messengers, could still require, or interact with, action potential-mediated presynaptic processes. We used tetrodotoxin (TTX) to block evoked and spontaneous action potentials by inhibiting voltage-dependent Na^+ channels. Since the effects of TTX were often not totally reversible under our conditions, a recording pipette was positioned at a site distant from the iontophoresis pipette to provide a second, unpotentiated response to measure the extent of TTX

reversibility in each slice. When TTX ($0.25 \mu\text{M}$) was applied to slices, presynaptic fiber volleys and EPSPs were undetectable within 10 min, even when the intensity of afferent stimulation was increased 2-fold (Figure 6A, inset waveforms). At this time, afferent stimulation was turned off for 5 min and then the iono-protocol was applied. The return to normal, TTX-free medium was timed so that it reached the recording chamber shortly after the last iontophoresis pulse. When stimulation was resumed 30 min after iontophoresis, the EPSPs at either recording electrode had not completely recovered from TTX applications; in the absence of glutamate iontophoresis (Figure 6B), the extent of recovery between the two recording electrodes was statistically indistinguishable ($p > 0.5$;

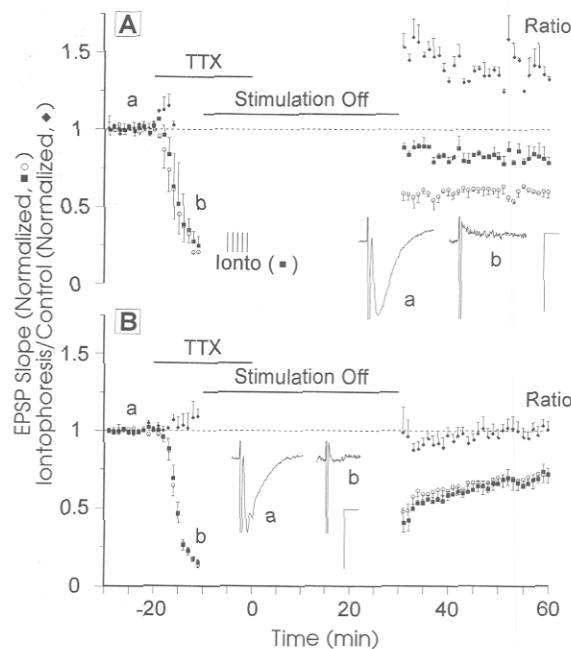


Figure 6. Ionto-LTP Is Induced in the Presence of TTX
Two recording electrodes were used in these experiments (see Figure 4A). The iontophoresis electrode (Ionto; closed squares) was the standard double-barreled pipette used for iontophoresis and recording, and a second electrode (control; open circles) for recording only was used to measure TTX reversibility (see cartoon inset in Figure 4A). Ratios of normalized EPSP slopes recorded by the iontophoresis electrode relative to the control electrode were calculated for each time point (ratio = iontophoresis + control; closed diamonds); representative EPSPs recorded at the iontophoresis electrode before (a) and during (b) TTX applications (waveforms are the average of 6 traces for [a] and a single trace for [b]) are shown.

(A) TTX ($250 \mu\text{M}$) was applied for 20 min as indicated by the horizontal bar. Synaptic responses to afferent stimulation were not detectable following 10 min of TTX application, and afferent stimulation was turned off. The iono-protocol (vertical bars) was begun 5 min after afferent stimulation was turned off; TTX-free medium was returned to the slice chamber immediately after glutamate iontophoresis, and afferent stimulation was resumed 30 min later ($n = 4$).

(B) The same procedure was used as outline in (A), except that glutamate iontophoresis was omitted ($n = 4$).

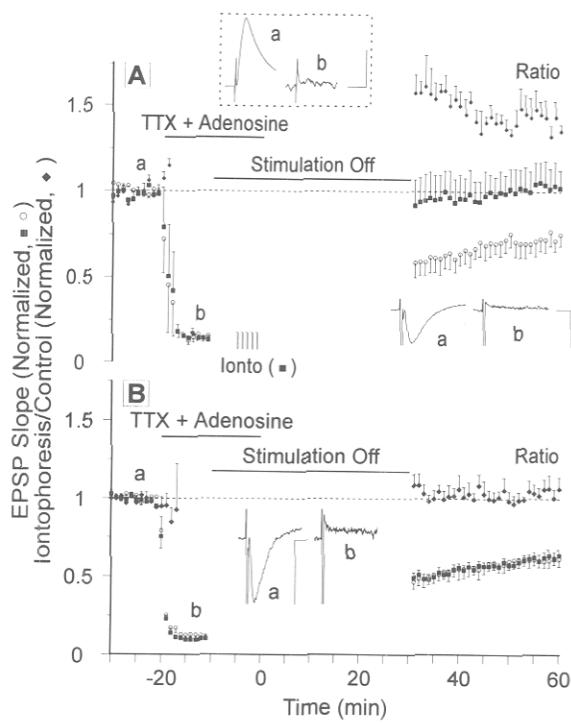


Figure 7. Ionto-LTP Is Induced in the Presence of TTX plus Adenosine

(A) The same procedures were used as in Figure 6, except that adenosine ($200 \mu\text{M}$) was included with TTX (250nM) application (horizontal bar). Under these conditions, the iontoprotocol (vertical bars) produced ionto-LTP, as indicated by the normalized iontophoresis/control ratios ($n = 5$; closed diamonds). EPSPs did not completely recover from TTX plus adenosine treatments for either the iontophoresis (closed squares) or control pipette (open circles). The boxed inset displays waveforms of whole-cell recordings (current-clamp) showing that EPSPs were undetectable in TTX plus adenosine (scale bar, 20 ms, 5 mV [a]; 20 ms, 0.1 mV [b]).

(B) The same procedure was used as outlined in (A), except glutamate iontophoresis was omitted ($n = 5$); EPSP slope values remained equally attenuated at the iontophoresis (closed squares) and control pipettes (open circles) following TTX plus adenosine washout.

$n = 4$). The range of EPSP slope recoveries was 58%–87% in these experiments. We used the ratio of normalized EPSP slopes measured by the iontophoresis electrode relative to control electrode as a measure of potentiation induced by the ionto-protocol. Examination of these ratios revealed that glutamate iontophoresis potentiated EPSP slopes 34% ($n = 2$) in the presence of TTX.

The combined application of TTX plus adenosine will inhibit many forms of presynaptic activity, including action potentials, neurotransmitter release, voltage-sensitive Ca^{2+} channels (N-type), and activation of adenylate cyclase. Indeed, the application of a TTX plus adenosine cocktail caused the rapid inhibition of EPSPs (Figure 7), whereas presynaptic volleys were inhibited at a rate similar to TTX alone. Increasing afferent stimulation 2-fold in the presence of TTX plus adenosine demonstrated that presynaptic volleys and

EPSPs were undetectable under these conditions. Subsequent whole-cell recordings in current-clamp mode revealed that evoked synaptic transmission was undetectable even when the stimulus intensity was increased 50% and recording amplification was increased 50-fold (Figure 7A, boxed inset). After TTX plus adenosine applications had been in progress for 10 min, afferent stimulation was stopped for 5 min prior to, and not resumed until 30 min following, glutamate iontophoresis. The return to TTX- and adenosine-free medium was timed so that it reached the chamber shortly (<1 min) after the last iontophoresis pulse. Upon resuming afferent stimulation, comparisons of normalized EPSP slope ratios between iontophoresis and control recording electrodes demonstrated that glutamate iontophoresis resulted in $35\% \pm 9\%$ ($n = 3$) potentiation. Control experiments in which iontophoresis had been omitted (Figure 7B) showed that TTX plus adenosine treatments produced the same levels of EPSP attenuation at control and iontophoresis electrodes ($p > 0.5$; $n = 5$). One of the experiments represented in Figure 7 was conducted with D -glutamate; the results with D -glutamate were within the range of values obtained with L -glutamate, and the data for both glutamate isomers were combined.

In summary, our experiments show that glutamate iontophoresis reliably produced LTP in the CA1 region of hippocampal slices. Excluding the experiments done in EGTA or MK-801, glutamate iontophoresis produced LTP in 36 out of 46 slices (78%). Glutamate iontophoresis produced ionto-LTP under conditions of afferent stimulation in 16 out of 20 slices (80%), in the absence of afferent stimulation in 5 out of 6 slices (83%), in the presence of adenosine without

Table 1. Summary of Group Data

Experimental Group	% Potentiation		
	Mean	SEM	n
Stimulation-on	43	6	20
Stimulation-off	41	11	6
Adenosine ^a	65	9	6
EGTA ^a	0	4	6
MK-801 ^a	0	2	5
TTX ^a	24	7	4
TTX + adenosine ^a	26	8	5
Occlusion: ionto-LTP (after HFS-LTP) ^a	34	14	5
Occlusion: HFS-LTP (after ionto-LTP) ^a	2	4	4
Stimulation-off (whole-cell recording)	77	4	3

^a These groups used the stimulation-off ionto-protocol.

afferent stimulation in 6 out of 6 slices (100%), in the presence of TTX in 2 out of 4 slices (50%), and in the combined presence of TTX plus adenosine in 3 out of 5 slices (60%). Slices treated with EGTA or MK-801 did not display ionto-LTP. A summary of group data including all experiments, some of which displayed potentiation not meeting the criteria for ionto-LTP (i.e., $\geq 20\%$ potentiation for 60 min), is shown in Table 1. Potentiation in each experimental group was significantly different from its appropriate control ($p < 0.05$; Student's *t* test, or paired-sample *t* test for occlusion experiments). Analysis of variance indicated a significant difference among the experimental groups (whole-cell recording group excluded). Post hoc comparisons using the Newman-Keuls test indicated significant differences ($p < 0.05$) between the following groups: adenosine (stimulation-off) versus stimulation-on, EGTA, and MK-801; stimulation-on versus EGTA and MK-801. The stimulation-on group was not significantly different from the stimulation-off group.

Discussion

These studies demonstrate that LTP is produced by repeated iontophoresis of glutamate onto CA1 pyramidal neurons. The ionto-protocol has allowed us to test the requirement of certain forms of presynaptic activity in the induction of LTP. Ionto-LTP does not require afferent stimulation or spontaneous action potentials and appears not to require presynaptic neurotransmitter release. Ionto-LTP requires extracellular Ca^{2+} and NMDA receptor activation, since it is blocked by extracellular EGTA or MK-801 applications, respectively. Although ionto-LTP induction does not require evoked presynaptic activity, it shares many properties with HFS-LTP. We have attempted to exclude potential artifacts that could contribute to the induction of ionto-LTP. Whole-cell recordings demonstrated that the induction of ionto-LTP occurs without significant changes in series or input resistance of postsynaptic neurons. Control experiments with NaCl (1 M) iontophoresis did not produce short-term potentiation or even posttetanic potentiation, indicating that the passage of iontophoretic current (Dudar, 1974) or nonphysiological hypertonic effects (Malgarnoli and Tsien, 1992) are unlikely to contribute to the induction of ionto-LTP.

Prior studies have shown that transient applications of NMDA can produce LTP (Malenka, 1991); this type of LTP does not require afferent stimulation during NMDA iontophoresis, but does require elevated extracellular Ca^{2+} (6 mM; however, see McGuinness et al., 1991). At lower extracellular Ca^{2+} (2–4 mM), NMDA iontophoresis produced only short-term potentiation lasting 20–40 min. Thibault et al. (1989) induced LTP under conditions of high extracellular Ca^{2+} (6.3 mM) and low Mg^{2+} (20 μM) by applying a cocktail containing NMDA, glycine, and spermidine. The use of high extracellular Ca^{2+} and/or low extracellular Mg^{2+} in hippocampal slice experiments is thought to allow more Ca^{2+} entry through NMDA receptors so that an LTP threshold can be reached (Malenka, 1991). The interpretation of results from experiments on these types of LTP is complicated, since manipulations that increase the effective concentration of extracellular Ca^{2+} have been shown to alter several properties of synaptic transmission. For example, increasing Ca^{2+} from 2 to 6.6 mM increases spontaneous miniature EPSP (mEPSP) frequency from 2.3 to 4.3 Hz (Hubbard et al., 1968), and increasing Ca^{2+} from 1 to 10 mM decreases the $t_{1/2}$ of NMDA receptor desensitization from 2.8 to 1.1 s (Clark et al., 1990). Moreover, elevated extracellular Ca^{2+} alone (>4 mM) has been shown to produce long-lasting synaptic potentiation (Williams and Bliss, 1988; Grover and Teyler, 1990a). To avoid the possible complexities in interpreting results from experiments using elevated Ca^{2+} , we maintained an extracellular Ca^{2+} concentration (2 mM) commonly found in the rat hippocampus (Alger et al., 1984).

An important issue to address before ionto-LTP can be used to explore mechanistic properties of HFS-LTP is whether or not these two forms of LTP operate by common mechanisms. Our results from occlusion experiments indicate that the induction of ionto-LTP completely occludes subsequent attempts to induce HFS-LTP. On the other hand, HFS-LTP only partially occluded ionto-LTP. These results indicate that ionto- and HFS-LTP share common mechanisms, but that ionto-LTP may activate molecular pathways that are not totally engaged by HFS-LTP. One possible interpretation of these findings is that glutamate iontophoresis produces greater intracellular Ca^{2+} concentration increases compared with HFS-LTP by activating greater numbers of receptors for a longer period of time, which could result in levels of postsynaptic depolarization that would increase Ca^{2+} influx via voltage-dependent Ca^{2+} channels. This could lead to greater intracellular Ca^{2+} concentration increases in synaptic spines compared with HFS-LTP and produce greater synaptic strengthening. Glutamate iontophoresis may also potentiate weaker or different populations of synapses that are not brought to the LTP threshold by HFS. Despite this difference, occlusion experiments indicate that ionto- and HFS-LTP appear to operate through shared biochemical pathways, and thus we believe that ionto-LTP can be used to explore the role of pre- and postsynaptic mechanisms responsible for the induction and expression of LTP.

Additional similarities between HFS- and ionto-LTP were revealed by an analysis of PPF, which in CA1 neurons is believed to rely on presynaptic mechanisms (Harris and Cotman, 1985) that involve residual Ca^{2+} in presynaptic terminals (Zucker, 1987). Manipulations that increase EPSPs by facilitating neurotransmitter release decrease PPF (Muller and Lynch, 1989; Muller et al., 1990), whereas manipulations that inhibit neurotransmitter release increase PPF (Dunwiddie and Haas, 1985). On the other hand, the attenuation

of evoked EPSPs by the action of glutamate antagonists at postsynaptic receptors does not affect PPF (Harris and Cotman, 1985). PPF is not changed following the induction of HFS-LTP in area CA1 (Muller and Lynch, 1989; Zalutsky and Nicoll, 1990; but also see Schultz and Johnston, 1992, Soc. Neurosci., abstract), and we find that ionto-LTP does not affect PPF. The observation that ionto-LTP does not affect PPF also suggests that changes in presynaptic processes may not play a major role in the expression of ionto-LTP.

In further testing the involvement of presynaptic activity in ionto-LTP, we attempted to diminish severely presynaptic neurotransmitter release by the extracellular application of adenosine. Adenosine activates A₂ receptors and stimulates adenylyl cyclase (van Calker et al., 1979). High affinity A_{2a} receptors appear to be absent or undetectable in the hippocampus (Jarvis et al., 1989); however, there is evidence suggesting that low affinity A_{2b} receptors are present in the hippocampus (Winder and Conn, 1993). The major site of adenosine action in the hippocampus is at A₁ receptors, where it inhibits several processes, including the evoked release of many transmitters (Proctor and Dunwiddie, 1987; Dunwiddie, 1990) as well as spontaneous mEPSPs (Branisteanu et al., 1989; Lambert and Teyler, 1991); voltage-dependent Ca²⁺ channel conductance, primarily N-type (Madison et al., 1987, Biophys. J., abstract; Gross et al., 1989); and adenylyl cyclase activity (van Calker et al., 1979). Adenosine also causes pyramidal neurons to hyperpolarize by activating K⁺ channels (Trussell and Jackson, 1985). The net result of adenosine acting at A₁ receptors is to reduce neuronal excitability and synaptic transmission without affecting the sensitivity of postsynaptic receptors to neurotransmitters (Proctor and Dunwiddie, 1987; Malenka and Kocsis, 1988). Our ability to produce reliable ionto-LTP during the reversible application of adenosine, when evoked EPSPs are completely blocked, indicates that the underlying induction mechanism(s) is unlikely to rely on N-type Ca²⁺ channels (Madison et al., 1987), adenylyl cyclase activities (van Calker et al., 1979), or presynaptic neurotransmitter release (Proctor and Dunwiddie, 1987).

It is interesting to note that adenosine has been used to block the induction of HFS-LTP at perforant path dentate gyrus synapses *in vivo* (Dolphin, 1983) and in CA1 *in vitro* (Arai et al., 1990; Mitchell et al., 1993). Although these studies differ in design, they suggest the involvement of an adenosine-sensitive mechanism that is necessary for the induction of HFS-LTP. In contrast, the failure of adenosine to block ionto-LTP indicates that the ionto-protocol may bypass adenosine-sensitive processes associated with HFS-LTP; namely adenosine's blockade of presynaptic neurotransmitter release.

Is afferent stimulation immediately before NMDA or glutamate iontophoresis required for LTP induction? Prior studies have shown that the enhanced synaptic transmission produced by glutamate applica-

tions does not require afferent stimulation during and 5 min after agonist applications (Kauer et al., 1988; Malenka, 1991). However, presynaptic stimulation may produce effects that last after the cessation of evoked transmitter release, since afferent stimulation has been shown to increase the frequency of miniature excitatory postsynaptic currents (mEPSCs) (Manabe et al., 1992), although it is unclear how long these increases persist. In an attempt to minimize such effects during the induction of ionto-LTP, we have extended the period in which evoked stimulation is turned off from 5 min prior to until 30 min after glutamate iontophoresis.

The induction of ionto-LTP in the presence of TTX, which inhibits voltage-dependent Na⁺ channels (Narahashi et al., 1964), indicates that presynaptic processes dependent on action potentials are not required for the mechanisms underlying the induction of ionto-LTP. TTX has been used to block action potentials in studies demonstrating increases in both the amplitude (Manabe et al., 1992) and the frequency (Malgarelli and Tsien, 1992) of spontaneous mEPSCs induced by the application of either NMDA or glutamate onto hippocampal neurons. We have not determined whether increased mEPSC frequency and/or amplitude occur following the induction of ionto-LTP. Using whole-cell recordings, we determined that TTX plus adenosine completely blocked evoked action potentials (as revealed by the absence of presynaptic fiber volleys) and evoked EPSPs, even when test stimulus intensity was increased 1.5-fold and recording amplification was increased 50-fold.

Is presynaptic activity involved in the induction of ionto-LTP? The results herein indicate that ionto-LTP does not require evoked presynaptic stimulation and is not blocked by the application of adenosine plus TTX, conditions that completely block evoked EPSPs. As discussed above, adenosine greatly attenuates, but may not completely block, spontaneous transmitter release or presynaptic Ca²⁺ currents (N-type). In addition to these inhibitory effects, glutamate applied during the ionto-protocol will have further inhibitory actions via presynaptic autoreceptors. For example, activation of presynaptic glutamate receptors (metabotropic and/or L-2-amino-4-phosphonobutyrate types) has been shown to inhibit transmitter release (Baskys and Malenka, 1991) and reduce both N- and L-type Ca²⁺ currents (Lester and Jahr, 1990; Trombley and Westbrook, 1992). Thus, under our conditions of inducing ionto-LTP, the known actions of adenosine plus TTX plus glutamate on presynaptic processes are all inhibitory. One interpretation of these findings is that many forms of presynaptic activity are not required for the induction of ionto-LTP. However, we cannot rule out the involvement of all forms of presynaptic activity in the induction of ionto-LTP, such as increases in spontaneous release and/or stimulation of G protein-dependent processes via activation of metabotropic glutamate receptors (Sladeczek et al.,

1985). Recent results by Kullman et al. (1992) showed that LTP induction by repeated postsynaptic depolarization (which is not blocked by aminophosphonovalerate) required simultaneous presynaptic stimulation (2 Hz). These studies showed that the same postsynaptic depolarization protocol produced only transient potentiation in the absence of presynaptic stimulation, even when extracellular Ca^{2+} was increased to 5 mM. Kullman et al. (1992) did nothing to reduce spontaneous release in the latter experiments, thus suggesting that spontaneous transmitter release in the presence of elevated Ca^{2+} was insufficient to induce LTP. Although we cannot rule out an involvement of spontaneous release in the induction of iono-LTP, by analogy with the findings of Kullman et al. (1992), we believe that increases in spontaneous release do not play an important role in the induction of iono-LTP. To our knowledge, experiments demonstrating a causal relationship between increases in spontaneous release and the induction of LTP have yet to be demonstrated. Previous experiments using hypertonic solutions or applications of putative retrograde messengers to increase spontaneous release have not reported long-term increases in the potentiation of evoked synaptic transmission (Fatt and Katz, 1952; Bekkers and Stevens, 1989; O'Dell et al., 1991; Malgaroli and Tsien, 1992). We are currently examining changes in spontaneous release during the induction and expression of iono-LTP.

Implicit in recent suggestions that retrograde messengers are involved in the induction of HFS-LTP (Williams et al., 1989; O'Dell et al., 1991; Schuman and Madison, 1991) is the notion that the action of retrograde messengers on presynaptic terminals is dependent on evoked presynaptic activity. The finding that afferent stimulation 5 min before and 30 min after glutamate iontophoresis is not required for the induction of iono-LTP suggests that one criterion retrograde messengers should fulfill is to engage presynaptic processes that are independent of afferent stimulation. Moreover, if retrograde messengers are involved in the induction of iono-LTP, their action is not blocked by TTX and adenosine. Studies implicating arachidonic acid as a retrograde messenger have shown that afferent stimulation is required for the induction of arachidonic acid-mediated long-term synaptic enhancement (Williams et al., 1989). If arachidonic acid is also involved in iono-LTP, then its action may be working by a different mechanism that is not dependent on afferent stimulation. Recent experiments on the potential retrograde messenger nitric oxide (Bohme et al., 1991; Schuman and Madison, 1991; Haley et al., 1992) suggest that nitric oxide acts only as an initial messenger in LTP induction, since inhibitors of nitric oxide synthase administered after LTP induction do not affect LTP expression. Our data suggest that nitric oxide, or any other putative retrograde messenger, should be able to potentiate synaptic transmission when applied in the absence of affer-

ent stimulation and/or when transmitter release and presynaptic action potentials are blocked.

One possible interpretation of recent LTP experiments (Bonhoeffer et al., 1989; Schuman and Madison, 1991, Soc. Neurosci., abstract, and Cold Spring Harbor, 1992, abstract) is that retrograde messengers can diffuse from potentiated synapses to nearby synapses to induce LTP. On the other hand, it is generally accepted that LTP can be specifically induced in one of two independent but convergent pathways to the same postsynaptic neurons (Dunwiddie and Lynch, 1978). One explanation consistent with these two observations is that retrograde messengers must interact with presynaptic processes that are activity dependent to produce the synapse specificity associated with LTP (Schuman and Madison, 1991). Our data suggest that evoked presynaptic activity may not be the single factor determining synapse specificity and that other mechanisms which are independent of presynaptic activity should be explored.

Glutamate iontophoresis using the iono-protocol is an efficient means of inducing LTP. Our results oppose the hypothesis that presynaptic transmitter release and certain types of presynaptic activity are required for the induction of this type of LTP. Evoked stimulation is not required for its induction, and induction is refractory to adenosine's inhibition of presynaptic neurotransmitter release and TTX's blockage of voltage-dependent Na^+ channels. The precise actions of glutamate iontophoresis on pre- and/or postsynaptic mechanisms that contribute to the induction of iono-LTP are not completely understood. Since glutamate is a mixed agonist, postsynaptic depolarization produced by its activation of AMPA/kainate receptors should facilitate Ca^{2+} influx through NMDA receptors and voltage-dependent Ca^{2+} channels, as well as the activation of metabotropic glutamate receptors. In this context, the activation of metabotropic glutamate receptors is required for the induction of LTP in the dorsal septal nucleus (Zheng and Gallagher, 1992) and increases the magnitude of LTP induced in the CA1 region of the hippocampus (McGuinness et al., 1991b; Aniksztajn et al., 1992). In addition, application (20 min) of trans-ACPD to hippocampal slices produces a slowly developing long-term enhancement in synaptic transmission in area CA1 that is insensitive to 2-amino-5-phosphonovalerate and requires intact afferent fibers from area CA3 (Bortolotto and Collingridge, 1993). Glutamate's activation of metabotropic receptors may play a role in the induction of iono-LTP; however, we have induced iono-LTP using D-glutamate (even in the presence of TTX plus adenosine), and D-glutamate is ineffective in activating metabotropic receptors expressed in *Xenopus* oocytes (Sugiyama et al., 1989). Clearly, additional experiments are required to determine which subtypes of glutamate receptors participate in the induction of iono-LTP. Our findings that glutamate iontophoresis can apparently replace the role of presynaptic mechanisms involved

in the induction and expression of LTP make iono-LTP an attractive model for future studies on the function of retrograde messengers and the role of postsynaptic mechanisms in LTP induction and expression.

Experimental Procedures

Brains were rapidly removed following decapitation of 3- to 12-week-old male Harlan Sprague-Dawley rats that had been deeply anesthetized with sodium pentobarbital. Hippocampi were dissected from the brains in ice-cold medium containing 2 mM Ca²⁺ and 2 mM Mg²⁺ (see below), sliced with a McIlwain tissue chopper (400 µm thick), and returned to the same ice-cold medium. The temperature of the medium was gradually brought to 25°C–30°C during the course of 1 hr. Individual slices were transferred to the recording chamber and allowed to equilibrate for 5 min in standard medium prior to electrode positioning; the temperature of the recording chamber was maintained at 31°C with a flow rate of 3 ml/min. The standard medium for electrophysiological recordings consisted of 124 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 2 mM NaH₂PO₄, 13–26 mM NaHCO₃, 10 mM dextrose, and 10 mM HEPES; it was continuously gassed with 5% O₂, 95% CO₂. Changes in the standard medium are noted in the text and figure legends. All chemicals were purchased from Sigma, except MK-801, which was a generous gift from Jarek Aronowski (University of Texas Medical School, Houston, TX).

Field potentials were recorded by standard techniques. Data were digitized (Nicolet 4094C digital oscilloscope) and transferred to a computer hard disk, and the EPSP slopes were computed on-line with custom software. Double-barreled, glass theta pipettes (Sutter, BT150-10) were used for simultaneous recording and iontophoresis in the same synaptic field. Recording barrels were filled with 3 M NaCl (1–6 MΩ), and the iontophoresis barrel was filled with 1 M glutamate (pH 7.4, 6–15 MΩ), unless otherwise noted. Schaffer collaterals in the stratum radiatum were stimulated at 0.05–0.1 Hz via a monopolar tungsten electrode; field EPSPs were 0.2–0.8 mV in amplitude. A 30 nA retaining current was applied to the iontophoresis barrel. Repeated iontophoresis of glutamate (iono-protocol) involved application of 1–2 µA for 10 s for a total of five pulses; each iontophoresis was separated by 60 s. The retaining current was reestablished during the interval between each iontophoresis and at the end of iono-protocol. In experiments examining the induction of LTP by electrical stimulation, high frequency stimulation (100 Hz for 1 s; HFS) was applied twice, separated by 20 s.

Whole-cell recordings were conducted according to published procedures (Sakmann and Neher, 1983; Blanton et al., 1989). Briefly, recordings are more successfully achieved with hippocampal slices prepared from 3.5- to 5-week-old rats. Patch pipettes were made from borosilicate glass containing a filling fiber (Garner Glass Co.; glass type, KG-33). The internal solution contained 122.5 mM cesium gluconate, 10 mM HEPES, 0.2 mM EGTA, 8 mM NaCl, 2 mM Mg-ATP, and 0.3 mM GTP. Care was taken to maintain the osmolarity (290–295 mosmol) of patch pipette solutions immediately before use; small aliquots of Mg-ATP and GTP stocks were stored at –80°C and thawed only once prior to use. Pipette resistances were 3–7 MΩ. The series and input resistance were monitored throughout the experiment by delivering small hyperpolarizing voltage steps (5–10 mV) before each test stimulus. Fast and slow capacitance transients were compensated, and the series resistance was compensated at 100%.

Experiments were excluded from analysis when HFS-LTP could not be induced in a control pathway, except for MK-801 experiments. This was to ensure that only healthy slices capable of supporting LTP were studied. The criteria for iono-LTP was an enhancement of synaptic transmission (initial EPSP slope) that was ≥20% of baseline values for ≥60 min, or ≥20% for ≥30 min for HFS-LTP. Mean values of potentiation ± SEM reported in the text were computed from the average values of potentiation

between 55 and 60 min following iontophoresis in slices that displayed iono-LTP, except for experiments using EGTA or MK-801, in which average values were obtained between 30–35 and 10–15 min post-iontophoresis, respectively.

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