

Long-Term Potentiation Induced by θ Frequency Stimulation Is Regulated by a Protein Phosphatase-1-Operated Gate

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Long-term potentiation (LTP) can be induced in the Schaffer collateral→CA1 synapse of hippocampus by stimulation in the θ frequency range (5–12 Hz), an effect that depends on activation of the cAMP pathway. We investigated the mechanisms of the cAMP contribution to this form of LTP in the rat hippocampal slice preparation. θ pulse stimulation (TPS; 150 stimuli at 10 Hz) by itself did not induce LTP, but the addition of either the β -adrenergic agonist isoproterenol or the cAMP analog 8-bromo-cAMP (8-Br-cAMP) enabled TPS-induced LTP. The isoproterenol effect was blocked by postsynaptic inhibition of cAMP-dependent protein kinase. Several lines of evidence indicated that cAMP enabled LTP by blocking postsynaptic protein phosphatase-1 (PP1). Activators of the cAMP pathway reduced PP1 activity in the CA1 region and increased the active form of inhibitor-1, an endogenous inhibitor of PP1. Postsynaptic injection of activated inhibitor-1 mimicked the LTP-enabling effect of

cAMP pathway stimulation. TPS evoked complex spiking when isoproterenol was present. However, complex spiking was not sufficient to enable TPS-induced LTP, which additionally required the inhibition of postsynaptic PP1. PP1 inhibition seems to promote the activation of Ca^{2+} /calmodulin-dependent protein kinase (CaMKII), because (1) a CaMKII inhibitor blocked the induction of LTP by TPS paired with either isoproterenol or activated inhibitor-1 and (2) CaMKII in area CA1 was activated by the combination of TPS and 8-Br-cAMP but not by either stimulus alone. These results indicate that the cAMP pathway enables TPS-induced LTP by inhibiting PP1, thereby enhancing Ca^{2+} -independent CaMKII activity.

Key words: LTP induction; cAMP-dependent protein kinase; inhibitor-1; protein phosphatase-1; Ca^{2+} /calmodulin-dependent protein kinase; gating; complex spikes

Many synapses in the CNS can be strengthened or weakened by repetitive stimulation. This phenomenon has been intensively studied in the rodent hippocampus, particularly at the synapse between the pyramidal cells of areas CA3 and CA1 (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999), and many of these studies have investigated how particular patterns of stimulation give rise to different forms of synaptic plasticity. Such factors as the frequency, intensity, and duration of the stimulation determine whether the synapse is strengthened or weakened, as well as the persistence of the synaptic change. Remarkably, even when two different patterns induce seemingly similar synaptic changes, they may do so via different molecular mechanisms. Efforts to understand the role of the pattern of stimulation in synaptic plasticity may prove particularly valuable, because pronounced spontaneous frequency variations characterize hippocampal neuronal activity in behaving animals (Fenton and Muller, 1998). Studies in hippocampal slices have established that the pattern of stimulation determines the mechanism of synaptic change, so it is reasonable to speculate that the natural patterns of the hippocampus *in vivo* may give rise to multiple forms of synaptic plasticity.

An important principle of pattern-dependent plasticity is the relationship between stimulus frequency and the direction of synaptic change (Dudek and Bear, 1992; Katsuki et al., 1997). In slices of rat hippocampus, low-frequency stimulation (1–3 Hz; LFS) produces a long-term depression of synaptic transmission (LTD).

Conversely, long-term potentiation (LTP) is induced when stimulation is delivered at higher frequencies (30–100 Hz; HFS). The crossover or neutral frequency, at which repetitive stimulation does not modify synaptic strength, is ~ 10 Hz at this synapse. However, the relationship between the stimulation frequency and the direction of the change in synaptic strength is sensitive to certain pharmacological manipulations, including the inhibition of protein kinases or phosphatases and the activation of β -adrenergic receptors (Coussens and Teyler, 1996; Thomas et al., 1996; Katsuki et al., 1997). In both mouse and rat hippocampal slices, LTP can be induced by stimulation at the neutral frequency in the presence of a β -adrenoceptor agonist, an effect that is mediated by the cAMP-signaling pathway (Thomas et al., 1996).

This pharmacological modification of the relationship between pattern and LTP touches on a relatively unexplored issue in LTP research. The intact hippocampus is richly innervated by other nuclei, such as the noradrenergic locus ceruleus. These extrinsic inputs may provide another level of control over hippocampal synaptic plasticity, in addition to any intrinsic variations in the frequency of hippocampal activity. Under certain conditions extrinsic input could be a major determinant of synaptic change, such as when a synapse is being activated at a rate near the neutral frequency. The slice data suggest that an increase in noradrenergic input could induce LTP at such synapses. In the behaving rat, a predominant component of hippocampal activity occurs near the neutral frequency, in the range of 5–12 Hz (θ frequency). Therefore, variations in noradrenergic tone may exert an important influence on synaptic plasticity *in vivo*.

Most investigations of LTP in hippocampal slices have used multiple trains of HFS for induction, usually delivered at 100 Hz. We and others have shown a requirement for the cAMP pathway in HFS-induced LTP and have demonstrated the existence of a postsynaptic gating mechanism that regulates the induction of LTP (Frey et al., 1993; Blitzer et al., 1995, 1998; Otmakhova et al., 2000).

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The gating mechanism comprises two antagonistic pathways. The cAMP arm favors LTP induction and includes adenylyl cyclase, cAMP, and cAMP-dependent kinase (PKA). Opposing the cAMP pathway is calcineurin, a Ca^{2+} /calmodulin-activated protein phosphatase. These pathways converge on the regulatory protein inhibitor-1, which in its phosphorylated form inhibits protein phosphatase-1 (PP1). The postsynaptic Ca^{2+} entry that accompanies HFS is thought to activate both arms of the gating mechanism, as well as the LTP-signaling pathway that begins with Ca^{2+} /calmodulin-dependent protein kinase (CaMKII) (Lisman, 1994). The same gating mechanism might explain how β -adrenergic stimulation enables neutral frequency stimulation to induce LTP. The source of postsynaptic cAMP in this case would be β -receptor-activated adenylyl cyclases, whereas the Ca^{2+} influx associated with θ frequency stimulation would activate both calcineurin and CaMKII.

The cAMP pathway influences the behavior of many membrane channels and signaling systems, so it is likely to contribute to synaptic plasticity in ways that are independent of the PP1 gate. Recent work in mouse hippocampus has shown a cAMP-mediated increase in postsynaptic excitability that is important in θ pulse stimulation (TPS)-induced LTP (Thomas et al., 1998; Winder et al., 1999). These studies establish that TPS must evoke postsynaptic burst spiking for LTP to occur and that β -adrenergic stimulation enhances this activity. The mechanism underlying this effect of the cAMP pathway is not well understood, but it may be mediated by an increase in the activity of MAP kinase (ERK). The present study was conducted to investigate the mechanisms underlying the contribution of the cAMP pathway to TPS-induced LTP.

MATERIALS AND METHODS

Electrophysiology. Dissection and recording methods were similar to those described previously (Blitzer et al., 1995). Intracellular recordings of area CA1 neurons from male Sprague Dawley rats (125–200 gm) were obtained in a submersion chamber at 31°C. Slices were superfused with a solution containing (in mM) NaCl (118), KCl (3.5), MgSO_4 (1.3), CaCl_2 (3.5), NaH_2PO_4 (1.25), NaHCO_3 (24), and glucose (15) and bubbled with 95% O_2 /5% CO_2 . Cells in the stratum pyramidale were impaled with sharp electrodes containing 3 M KCl ($R_c = 60$ – 90 M Ω), and field recordings were made with electrodes (2 M NaCl; $R_c = 2$ – 5 M Ω) placed in the stratum radiatum. Isoproterenol, 2-amino-5-phosphonovaleic acid (APV), and 8-bromo-cAMP (8-Br-cAMP) were applied in the superfusate. Thiophosphorylated inhibitor-1 (I-1), recombinant T35A nonphosphorylatable I-1, autocamtide-3, its control peptide (gifts of A. P. Braun and H. Schulman), and the Rp isomer of cyclic adenosine-3',5'-monophosphothioate (Rp-cAMPS) were included in the intracellular electrode and allowed to diffuse into the cell; in these experiments, TPS was delivered 40 min after impalement. For synaptic stimulation, monophasic, constant-current pulses (100 μsec in duration) were delivered to the Schaffer collaterals (stratum radiatum in area CA3). Test pulses were usually delivered in series of four pulses (5 sec apart), with the series separated by 5 min, and the EPSPs within each series were averaged. For some experiments (see Fig. 5), test stimuli were given every 30 sec, and the EPSPs were not averaged. EPSP amplitude and maximum initial slope (defined as the greatest slope within any 1 msec interval between the stimulus artifact and the EPSP peak) were measured either off-line for the averaged waveforms or on-line for the individual EPSPs in some experiments (see Fig. 5). TPS consisted of a single train of 150 stimuli delivered at 10 Hz. For most experiments that included intracellular recording, the stimulus intensity was adjusted to produce a 10–15 mV intracellular EPSP. However, in experiments designed to induce repetitive postsynaptic spiking (see Fig. 5), a stronger stimulus was used during TPS, sufficient to evoke single spikes reliably during pre-TPS test pulses. This stimulus was approximately twice as strong as that used during TPS in the other experiments. For those experiments using only field recording, the stimulus during TPS was adjusted to evoke a 1 mV field potential during test pulses. Data were analyzed by Student's *t* test or by ANOVAs followed by Newman–Keuls *post hoc* comparisons. Summary data are presented as group means with SE bars.

CaMKII activity. Hippocampal slices were incubated with 8-Br-cAMP for 2–5 hr before recording. Control slices were stimulated with TPS alone, 8-Br-cAMP alone, or neither. The slices were removed from the recording chamber and placed on a cold plate within 2–3 min after TPS, and the CA1 region was dissected out and frozen at -70°C . Extracts from individual CA1 regions were assayed for CaMKII activity essentially as described (Mayford et al., 1995). Tissue was homogenized in a buffer containing 50 mM HEPES, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 15 mM sodium pyrophosphate, 25 mM NaF, 75 $\mu\text{g/ml}$ leupeptin, 0.1 mg/ml aprotinin, 0.4 mM DTT, and 0.1 mM PMSF. Protein levels were assayed by Lowry assay using BSA as the standard. The enzyme reaction mix consisted of 50 mM

HEPES, pH 7.5, 10 mM MgCl_2 , 100 $\mu\text{g/ml}$ BSA, 200 $\mu\text{g/ml}$ leupeptin, 0.4 mM DTT, 0.6 mM EGTA, 0.2 mM EGTA, 2 μM Wipitide (PKA inhibitor; American Peptide Co.), 2 μM PKC (19–36) peptide (PKC inhibitor; American Peptide Co.), 200 μM ATP, 100 $\mu\text{Ci/ml}$ [γ - ^{32}P]ATP, and 20 μM autocamtide II (Calbiochem, La Jolla, CA), with either 1 mM CaCl_2 and 5 $\mu\text{g/ml}$ calmodulin (total activity) or 2 mM EGTA (Ca^{2+} -independent activity). Enzyme reactions were performed in quadruplicate at 30°C for 1 min in a final volume of 50 μl . The reaction was initiated by the addition of 2 μg of CA1 extract and terminated by the addition of an equal volume of 10% ice-cold TCA. Protein was pelleted, and the supernatant was spotted onto Whatman P81 filter paper and washed three times for 5 min with water. The amount of ^{32}P incorporated into substrate peptide was determined by liquid scintillation counting.

Inhibitor-1 immunoblotting. Hippocampal slices were placed in a submersion chamber bubbled with 95% O_2 /5% CO_2 at room temperature, and were exposed to either 8-Br-cAMP (1 mM for >2 hr) or isoproterenol (1 μM for 10 min). Immediately after treatment, the slices were frozen, and the CA1 region was dissected out and stored at -70°C . The tissue samples were lysed in 100 μl of lysis buffer (50 mM Tris, 4 mM EGTA, 10 mM EDTA, 15 mM Na phosphate, 100 mM β -glycerol phosphate, 10 mM NaF, 0.1 mM pepstatin, 1 mM PMSF, and 2 mM benzamide, pH 7.5) and ground three times for 30 sec each with a pellet pestle (Kontes glassware). The resulting lysate was spun down at $15,000 \times g$ at 4°C for 10 min, and the supernatant was then assayed for total protein concentration. Twenty micrograms of protein from each sample were run on a 15% SDS-PAGE gel. The samples were transferred onto a polyvinylidene difluoride membrane overnight (25 A constant current) and then blotted with an antibody to either phosphorylated DARPP-32/I-1 or recombinant human I-1. The monoclonal antibody to phosphorylated DARPP-32/I-1 was provided by G. L. Snyder and P. Greengard (Snyder et al., 1992). Both blots were developed using ECL and then digitized with a densitometer and analyzed using ImageQuant.

Protein phosphatase-1 activity. Hippocampal slices were treated and dissected as described for the I-1 immunoblot (above). Individual CA1 regions were homogenized in 50 mM Tris, pH 7.5, 0.2 mM EDTA, 2 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin, and 10 nM okadaic acid. Phosphatase activity of 20 ng of CA1 extract was measured with the Protein Phosphatase Assay System (Life Technologies, Gaithersburg, MD) in a final concentration of 3.3 nM okadaic acid. Each group was run in quadruplicate. By the use of this method, inclusion of 100 nM thiophosphorylated I-1 inhibited $>85\%$ of phosphatase activity, confirming the selectivity of the system for PP1.

RESULTS

TPS-induced LTP requires the postsynaptic cAMP pathway

The initial experiments were designed to establish the conditions for inducing LTP in adult rat hippocampal slices using TPS and β -adrenergic stimulation (TPS-LTP). The combination of TPS (150 stimuli delivered to the Schaffer collaterals at 10 Hz) and isoproterenol (1 μM) reliably produced LTP (Fig. 1A,B; intracellular EPSP slope = $173 \pm 8\%$ of baseline at 30 min). During isoproterenol application, the EPSP was depressed in some slices; however, no persistent effect of isoproterenol alone was observed after 30 min of washout (Fig. 1B). When isoproterenol was omitted, there was a nonsignificant tendency for TPS alone to depress synaptic transmission (intracellular slope = $85 \pm 11\%$ of baseline at 30 min; $n = 6$).

β -Adrenergic stimulation increases neuronal cAMP production in the CA1 region, and postsynaptic effects of β -adrenergic agonists have been attributed to increased cAMP in CA1 pyramidal cells (Segal et al., 1981; Madison and Nicoll, 1986; Pedarzani and Storm, 1993). If the β -adrenergic contribution to TPS-induced LTP (TPS-LTP) is also mediated by the cAMP-dependent inhibition of PP1, then direct activation of the cAMP pathway should also enable TPS-LTP. This prediction was tested by delivering TPS in the presence of the membrane-permeable analog 8-Br-cAMP. By itself, 8-Br-cAMP had no effect on synaptic efficiency ($97 \pm 6\%$ at 30 min; $n = 3$). However, 8-Br-cAMP enabled TPS to induce LTP (Fig. 1C; $156 \pm 12\%$ at 30 min; $n = 7$).

The cAMP pathway exerts both presynaptic and postsynaptic effects that are likely to influence synaptic plasticity at the CA3→CA1 synapse (Madison and Nicoll, 1986; Ma et al., 1999). To determine whether the LTP-promoting effect of cAMP pathway activation was mediated by the postsynaptic cAMP pathway, we included the inhibitory cAMP analog Rp-cAMPS in the intracellular electrode and stimulated with TPS and isoproterenol. In these cells, the synaptic response was potentiated only transiently and returned to near-baseline levels within 30 min. Thus, activation of

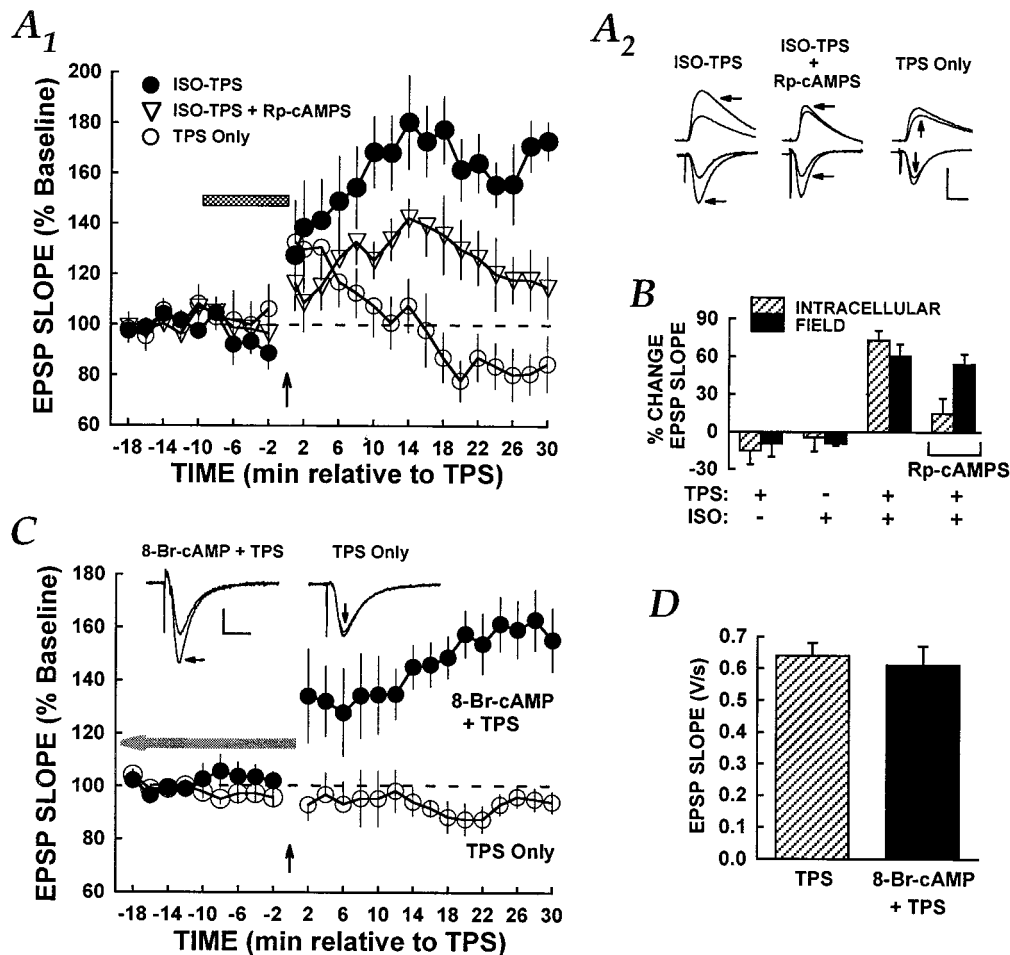


Figure 1. The induction of LTP by activation of the postsynaptic cAMP pathway paired with θ frequency synaptic stimulation. *A*, Postsynaptic blockade of the cAMP pathway prevents TPS-LTP. *A*₁, The graph shows the time course of the mean intracellular EPSP slope; the arrow denotes the time of TPS. In cells exposed to 1 μ M isoproterenol in the superfusate (horizontal bar), TPS induced a slowly developing LTP (filled circles; $n = 6$). When TPS was delivered in the absence of isoproterenol (open circles; $n = 6$), a nonsignificant trend toward synaptic depression was observed (ANOVA, $p > 0.10$). Intracellular injection of Rp-cAMPS (filled triangles; $n = 5$) blocked LTP after a transient potentiation. All groups differed from one another over the last 5 min of recording (final 3 time points; Newman–Keuls test, all p values < 0.05). *A*₂, Sample traces, with top and bottom panels showing intracellular and corresponding field potentials, respectively, are presented. Traces were obtained during the baseline period and at 30 min after TPS (arrow). Calibration: 20 mV intracellular, 500 μ V extracellular; 5 msec. Note that the inhibition of LTP by Rp-cAMPS was restricted to the recorded cell. *B*, Summary of intracellular and field results from all slices is shown. The data were obtained 30 min after TPS. *C*, The pairing of TPS with 8-Br-cAMP induces LTP. 8-Br-cAMP (500 μ M) was applied in the superfusate for 30 min (indicated by the gray horizontal arrow), ending with the delivery of TPS (black arrow). LTP was induced only when TPS was paired with 8-Br-cAMP. *Inset*, Representative traces are shown. Calibration: 200 μ V; 5 msec. *D*, 8-Br-cAMP does not regulate NMDA receptor-mediated EPSPs ($n = 5$). Field recordings were obtained in low Mg^{2+} (nominally 50 μ M) and in the presence of 10 μ M DNQX. EPSPs were recorded during a train of TPS before 8-Br-cAMP application and after 8-Br-cAMP washout (combined as control, filled bar) and in the presence of 500 μ M 8-Br-cAMP (hatched bar). The data indicate the mean slope of the final 10 stimuli in the train. There were no statistically significant group differences (paired t test, $p > 0.10$). ISO, Isoproterenol.

the postsynaptic cAMP pathway by β -adrenergic stimulation was necessary for TPS to generate LTP. The effect of Rp-cAMPS, together with the mimicry of the isoproterenol effect by 8-Br-cAMP, indicates that the only contribution of β -adrenergic stimulation to TPS-LTP is the activation of the postsynaptic cAMP pathway.

Ca^{2+} influx through NMDA channels is required for the induction of LTP by a variety of stimulus protocols (Larson and Lynch, 1988; Perkel et al., 1993; English and Sweatt, 1996; Thomas et al., 1996). The previously identified role of cAMP in the induction of LTP—as an inhibitor of postsynaptic PP1 activity—places it downstream of Ca^{2+} influx. However, there are reports of cAMP-dependent regulation of NMDA channels (Raman et al., 1996; Westphal et al., 1999) and increased Ca^{2+} entry through NMDA channels would be likely to promote LTP. To test whether cAMP-dependent NMDA receptor regulation contributes to TPS-LTP, the NMDA component of the EPSP was isolated by recording in a low- Mg^{2+} solution containing the AMPA receptor antagonist DNQX (10 μ M). TPS was delivered to the slices in the presence or

absence of 500 μ M 8-Br-cAMP. The mean amplitude of the final 10 field EPSPs was determined, because any effect of accelerated recovery of NMDA conductance should be most pronounced late in the TPS train. 8-Br-cAMP had no significant effect on the NMDA-mediated EPSP (Fig. 1*D*), suggesting that the enhancement of NMDA-mediated currents by cAMP is not likely to play a major role in TPS-induced LTP.

In the mouse hippocampus, TPS-induced LTP requires complex spiking during TPS, an effect that occurs in the absence of β -receptor activation but that is accentuated and prolonged by isoproterenol (Thomas et al., 1998; Winder et al., 1999). In field recordings obtained during TPS, we found that TPS alone did not evoke complex spikes, or even single spikes (Fig. 2*A*). However, complex potentials were apparent during TPS when isoproterenol was present. As noted previously (Winder et al., 1999), the negativity of these spikes suggests that they are dendritic potentials, presumably resulting from backpropagation from the soma. Complex waveforms were exhibited within 30 stimuli (the earliest sample obtained during TPS), and the number of discrete potentials

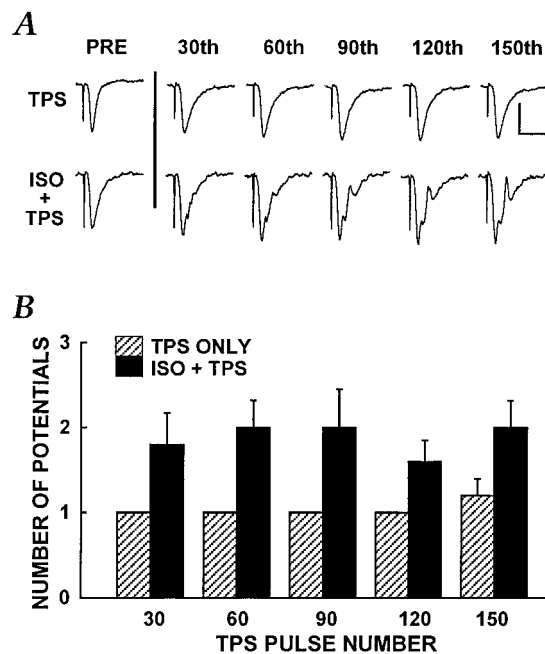


Figure 2. TPS evokes complex potentials when paired with isoproterenol. *A*, Representative field traces recorded immediately before the start of TPS (PRE) and at every 30th stimulus during TPS. In the absence of isoproterenol (top records), no spikes were seen during TPS. The increase in the duration of the EPSP relative to the baseline was typically observed in this experiment. When isoproterenol was present (bottom traces), the unitary EPSP was replaced by a complex waveform, which included spike potentials. Calibration: 500 μ V; 10 msec. *B*, Summary of the number of discrete components in sampled field traces during TPS in the absence (hatched bars; $n = 5$) and presence (filled bars; $n = 5$) of 1 μ M isoproterenol. The groups differed significantly ($p < 0.02$), and there was no patterning effect during TPS ($F < 1$).

per trace remained quite constant for the remainder of the train (Fig. 2*B*). These results indicate that, as with mouse hippocampus, the facilitation of repetitive spiking may be an important factor in the ability of β -adrenergic stimulation to enable LTP in the rat hippocampus.

Activation of the cAMP pathway suppresses PP1 activity and phosphorylates inhibitor-1

Previous work from this lab established that the cAMP pathway participates in HFS-induced LTP by inhibiting the activity of PP1 (Blitzer et al., 1998). A piece of evidence suggesting that the cAMP pathway may serve a similar role in TPS-LTP is the ability of phosphatase blockers to mimic the LTP-enabling effect of β -adrenergic stimulation in this induction protocol (Coussens and Teyler, 1996; Thomas et al., 1996).

To test whether cAMP-mediated phosphatase inhibition might underlie the role of the cAMP pathway in TPS-induced LTP, we first assayed PP1 activity in the CA1 region of slices exposed to 8-Br-cAMP (Fig. 3*A*). A reliable reduction in PP1 activity was produced by 8-Br-cAMP relative to unstimulated controls. This result is consistent with the hypothesis that activation of the postsynaptic cAMP pathway contributes to TPS-induced LTP by inhibiting PP1.

The most likely mechanism for the cAMP-induced decrease in PP1 activity involves inhibitor-1. Biochemical experiments have established that PKA phosphorylates I-1, which is a direct and specific inhibitor of PP1 (Shenolikar, 1994), and this pathway was identified as the mechanism of cAMP-dependent HFS-induced LTP (Blitzer et al., 1998). If the role of the cAMP pathway in TPS-LTP is mediated by the I-1 mechanism, then β -adrenergic receptor agonists and cAMP pathway activators should increase I-1 phosphorylation in the CA1 region. To test this prediction, isoproterenol or 8-Br-cAMP was applied to hippocampal slices, followed by determination of total and phosphorylated I-1 in CA1. Both

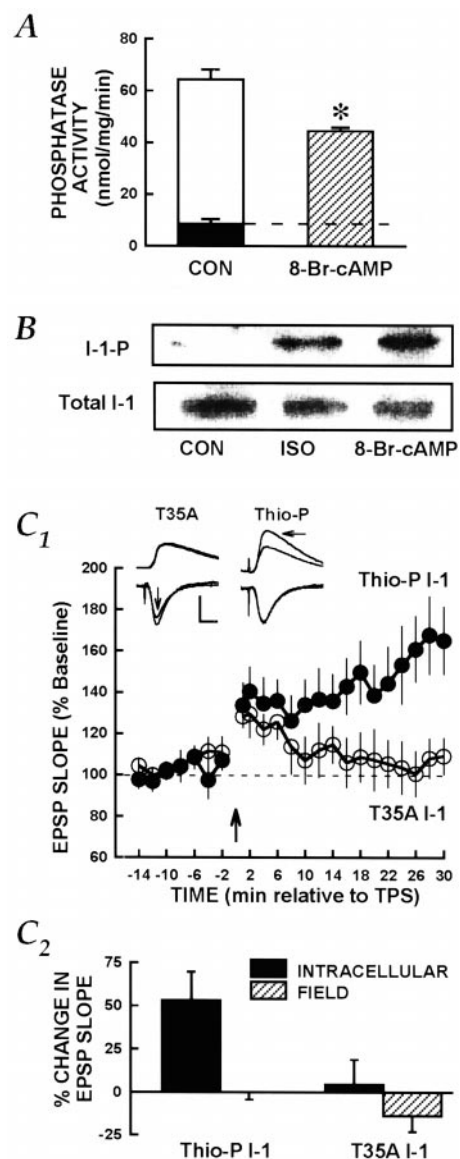


Figure 3. The role of protein phosphatase-1 in TPS-induced LTP. *A*, Protein phosphatase activity in CA1 is inhibited by activation of the cAMP pathway. In slices exposed to 1 mM 8-Br-cAMP for 30 min, total phosphatase activity was significantly lower than that in untreated controls ($p < 0.05$). Similar results were obtained in two independent experiments. The selectivity of the assay for PP1 is shown by the ability of 100 nM thiophosphorylated inhibitor-1 (I-1-P) to block >85% of total phosphatase activity, indicated by the black bar and dashed line. *B*, Endogenous protein phosphatase inhibitor-1 is phosphorylated by stimulation of the cAMP pathway. Tissue homogenates were probed with a monoclonal antibody recognizing either the phosphorylated form of inhibitor-1 selectively (top gel) or both the phosphorylated and nonphosphorylated inhibitor-1 (bottom gel). Isoproterenol (10 μ M) and 8-Br-cAMP (500 μ M) increased the levels of Thr³⁵-phosphorylated I-1 4.04 (\pm 2.28)-fold and 2.75 (\pm 0.62)-fold, respectively, relative to unstimulated tissue and normalized for total I-1 levels. Both isoproterenol and 8-Br-cAMP increased the level of thiophosphorylated inhibitor-1 (top gel) without significantly changing the total amount of inhibitor-1. Similar results were obtained in two other experiments. *C*, Postsynaptic inhibition of PP1 mimics cAMP pathway activation in TPS-LTP. *C*₁, The time course of LTP induced by TPS paired with thiophosphorylated I-1 is shown. The time of TPS is indicated by the arrow. Thiophosphorylated I-1 (Thio-P I-1; 10 μ M; $n = 9$) or inactive, nonphosphorylatable I-1 (T35A I-1; 10 μ M; $n = 8$) was applied in the intracellular electrode. A slowly developing LTP was induced by TPS only when thiophosphorylated I-1 was present, with the two groups differing significantly over the last three time points of the experiment (ANOVA, $p < 0.05$). Inset, Sample intracellular and field traces (top and bottom panels, respectively) from a T35A I-1 experiment (left) and a Thio-P I-1 experiment (right) are shown. Presentation details are as described in Figure 1. No field LTP was obtained in any of the slices in this experiment. Calibration: 10 mV intracellular, 250 μ V extracellular; 5 msec. *C*₂, Summary data of intracellular and field data at 30 min after TPS are shown. The intracellular data were derived from the experiment shown in *C*₁. CON, Control.

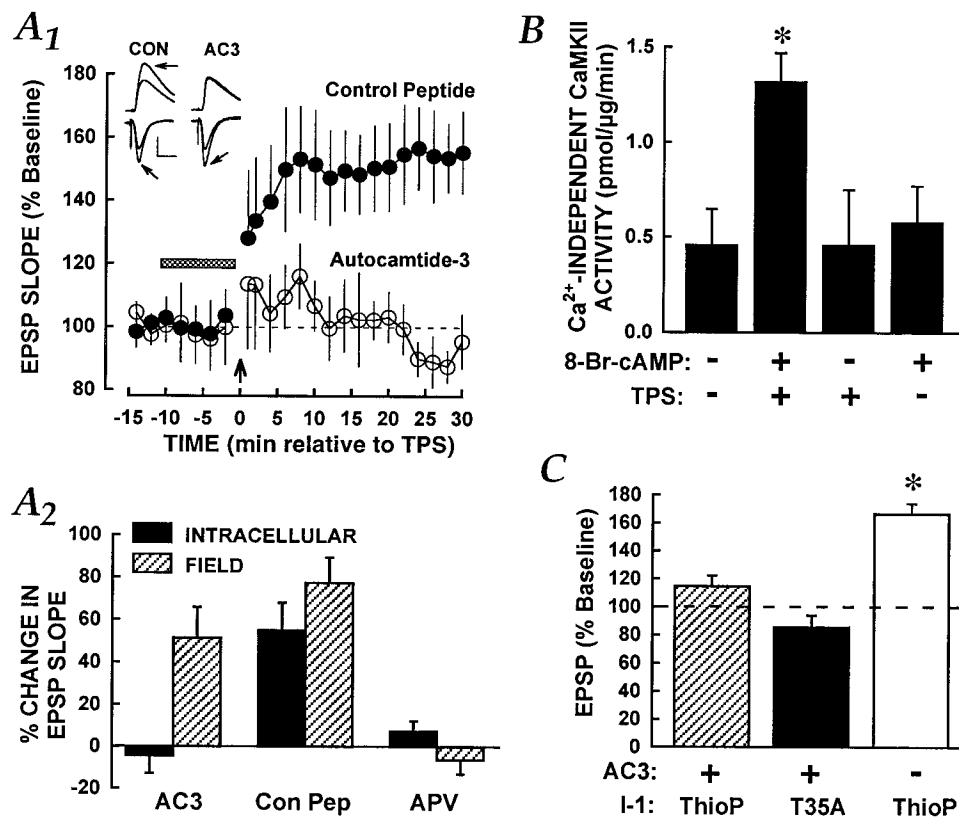


Figure 4. CaMKII integrates TPS and cAMP pathway stimulation. *A*, Postsynaptic CaMKII activity is required for TPS-LTP. *A*₁, Time course graph of intracellular EPSPs is shown. The horizontal bar indicates isoproterenol application, and the arrow near the x-axis shows the time of TPS. The intracellular electrode contained either inactive control peptide (5 mM; filled symbols; $n = 5$) or the CaMKII inhibitor autocamtide-3 (AC3; 5 mM; open symbols; $n = 5$). Normal LTP was obtained in the control cells, but LTP was absent in cells injected with AC3. The groups differed significantly over the last three time points (ANOVA, $p < 0.01$). Inset, Representative intracellular and field EPSPs from a control peptide experiment (left) and from an AC3 experiment (right) are shown. Presentation details are as described in Figure 1. Calibration: 10 mV intracellular, 250 μ V extracellular; 10 msec. *A*₂, Summary graph of data recorded at 30 min after TPS is shown. The filled bars represent changes in intracellular synaptic strength, and the hatched bars represent field data. Con Pep, Peptide control for AC3. The only group to exhibit intracellular LTP was the combined TPS plus Con Pep group, which was significantly different from both other groups (Newman-Keuls test, p values < 0.05). *B*, TPS paired with 8-Br-cAMP (500 μ M) increases Ca^{2+} -independent CaMKII activity. Similar results were obtained in three independent experiments, one of which is shown here. The slices that were exposed to the combination treatment showed significantly greater CaMKII activity in the absence of Ca^{2+} than did all other groups (Newman-Keuls test, $*p < 0.05$), which did not differ among themselves. No group differences in total CaMKII activity were observed (ANOVA, $p > 0.20$; mean activity = 3.29 ± 0.39 pmol μ g⁻¹ min⁻¹ pooled across groups). *C*, The blockade of LTP by a CaMKII inhibitor is resistant to PP1 suppression. Summary of results from experiments in which LTP was induced using TPS, with data taken from the final three time points (26–30 min after TPS), is shown. Substances were applied in the intracellular recording electrode. Autocamtide-3 (2.5 mM) was combined either with nonphosphorylatable T35A I-1 (T35A; filled bar; $n = 6$) or with thiophosphorylated I-1 (ThioP; hatched bar; $n = 6$). In other cells, thiophosphorylated I-1 was presented with the control peptide for autocamtide-3 (2.5 mM; open bar; $n = 6$). The asterisk indicates a significant increase in EPSP slope above baseline (ANOVA, $p < 0.05$).

isoproterenol and 8-Br-cAMP increased Thr³⁵-phosphorylated I-1 levels, by fourfold and threefold, respectively (Fig. 3B).

Inhibitor-1 substitutes for the cAMP pathway in TPS-LTP

If phosphorylation of I-1 mediates the isoproterenol effect in TPS-LTP, then postsynaptic application of phosphorylated I-1 should mimic the effect of isoproterenol by enabling TPS to induce LTP. When cells were recorded with electrodes containing recombinant, constitutively active thiophosphorylated I-1, TPS alone was sufficient to induce LTP (Fig. 3C; $165 \pm 16\%$ at 30 min). In control cells injected with the nonphosphorylatable T35A mutant of I-1, TPS induced only a transient potentiation, which returned to baseline within 10 min ($109 \pm 9\%$ at 30 min). Thiophosphorylated I-1 had no effect on the EPSP when TPS was omitted ($103 \pm 11\%$ at the corresponding time point; $n = 3$). The ability of activated I-1 to substitute for β -adrenergic stimulation provides strong support for I-1 as the mediator of the cAMP pathway contribution to TPS-LTP.

CaMKII integrates TPS and the cAMP pathway in TPS-induced LTP

Many studies have identified CaMKII as an essential component of the LTP-signaling pathway, at least during the induction phase (Lledo et al., 1995; Otmakhov et al., 1997). The induction of LTP

by trains of HFS is accompanied by increased Thr²⁸⁶ phosphorylation of CaMKII and the generation of Ca^{2+} -independent CaMKII activity (Malinow et al., 1989; Fukunaga et al., 1995; Blitzer et al., 1998). CaMKII is a substrate for PP1 (Strack et al., 1997), and the cAMP-regulated phosphatase gate described for HFS-induced LTP acts directly on CaMKII (Blitzer et al., 1998).

The role of CaMKII in TPS-LTP and its possible interaction with cAMP-sensitive phosphatase activity were explored in a series of experiments. First, we determined whether postsynaptic CaMKII activity is required for TPS-LTP by recording from cells with electrodes filled with either the selective CaMKII inhibitor autocamtide-3 or an inactive control peptide (Braun and Schulman, 1995). Autocamtide-3 blocks CaMKII and protein kinase C with IC₅₀ values of ~ 3 and ~ 500 μ M, respectively. On the basis of previous work with inhibitors introduced through intracellular electrodes (Hvalby et al., 1994; Blitzer et al., 1995), we estimate that our electrode concentration of 5 mM yielded an intracellular concentration of ~ 50 μ M, which is expected to effectively block CaMKII with little effect on protein kinase C. TPS paired with isoproterenol induced LTP in the cells injected with the control peptide, but no LTP was seen in cells recorded with autocamtide-3 (Fig. 4A). In addition, LTP was blocked by the bath-applied NMDA antagonist APV (Fig. 4A2). These results identify

CaMKII as a necessary signaling component in TPS-LTP and suggest that, in common with several other forms of LTP, CaMKII is activated by Ca^{2+} influx through NMDA channels.

We next asked whether the stimulation of the cAMP pathway could enable TPS-LTP by activating CaMKII. Total and Ca^{2+} -independent CaMKII activities were measured in homogenates from area CA1 of slices exposed to either TPS, 8-Br-cAMP, or both. Only the combination treatment increased Ca^{2+} -independent CaMKII activity in the CA1 region above the level observed in unstimulated control slices (Fig. 4B). Ca^{2+} -independent CaMKII activity was not increased above basal by treatment of the slices with 8-Br-cAMP or TPS alone. None of the groups differed with respect to total CaMKII activity. Thus, the detection of coincident TPS and β -adrenergic activity, which induces LTP, is complete at the level of CaMKII.

The increase in constitutive CaMKII activity, restricted to slices receiving both TPS and 8-Br-cAMP, is consistent with the expected behavior of the phosphatase gate acting on CaMKII. TPS alone should activate the CaMKII pathway [probably to a submaximal level at 10 Hz; see Coomber (1998)] and produce a calcineurin-dependent increase in the activity of PP1, which would be free to dephosphorylate CaMKII. The application of 8-Br-cAMP alone should inhibit PP1, but in the absence of Ca^{2+} influx provided by TPS, no CaMKII activation will occur. Thus, the gating model predicts an increase in Ca^{2+} -independent CaMKII activity only after combined TPS and cAMP pathway stimulation, the former contributing Ca^{2+} for CaMKII autophosphorylation and the latter protecting the nascent autonomous CaMKII activity by phosphorylating I-1 and suppressing PP1. This model places PP1 upstream of CaMKII and predicts that phosphatase inhibition should not overcome the blockade of TPS-LTP by a CaMKII inhibitor. We tested this prediction by blocking postsynaptic CaMKII with autocamtide-3 and stimulating the cell with combined TPS and isoproterenol. This treatment blocked LTP even when PP1 was directly inhibited by the postsynaptic injection of thiophosphorylated I-1 (Fig. 4C). The requirement for CaMKII activity despite such aggressive inhibition of PP1 clearly indicates that CaMKII is downstream of PP1.

TPS-induced LTP does not require increased postsynaptic excitability

The induction of LTP by synaptic stimulation requires the pairing of presynaptic activity with postsynaptic depolarization (Bliss and Collingridge, 1993). The source of the depolarization is usually considered to be the summation of EPSPs, particularly those mediated by NMDA channels. A potential additional source of dendritic depolarization is the backpropagation of action potentials from the soma, which could contribute to LTP [Spruston et al. (1995); Magee and Johnston (1997); but see Gustafsson et al. (1987) for evidence that postsynaptic spiking does not contribute to LTP]. As seen in Fig. 2 and as observed in experiments on mouse hippocampus (Thomas et al., 1998; Winder et al., 1999), spiking activity can occur during a train of TPS.

Is an increase in postsynaptic excitability sufficient to induce LTP in rat hippocampus? We examined this issue by comparing spiking during TPS in cells injected with either thiophosphorylated inhibitor-1 or inactive T35A inhibitor-1. In preliminary experiments, a stimulus intensity adequate to induce LTP, which produced the complex field potentials shown in Figure 2, failed to reliably induce multiple action potentials in the recorded postsynaptic neuron during TPS. To obtain repetitive postsynaptic firing during TPS, the stimulus intensity had to be increased to a level sufficient for test pulses to elicit single action potentials (Fig. 5A). However, a comparison of the cells injected with Thio-P I-1 and T35A I-1 showed no differences in the number of action potentials evoked by stimuli during TPS, despite a clear group difference in the induction of LTP (Fig. 5A,B). Thus, repetitive postsynaptic firing was not sufficient to enable TPS-induced LTP, but the additional blockade of postsynaptic PP1 produced a lasting potentiation. In addition, because thiophosphorylated I-1 did not increase

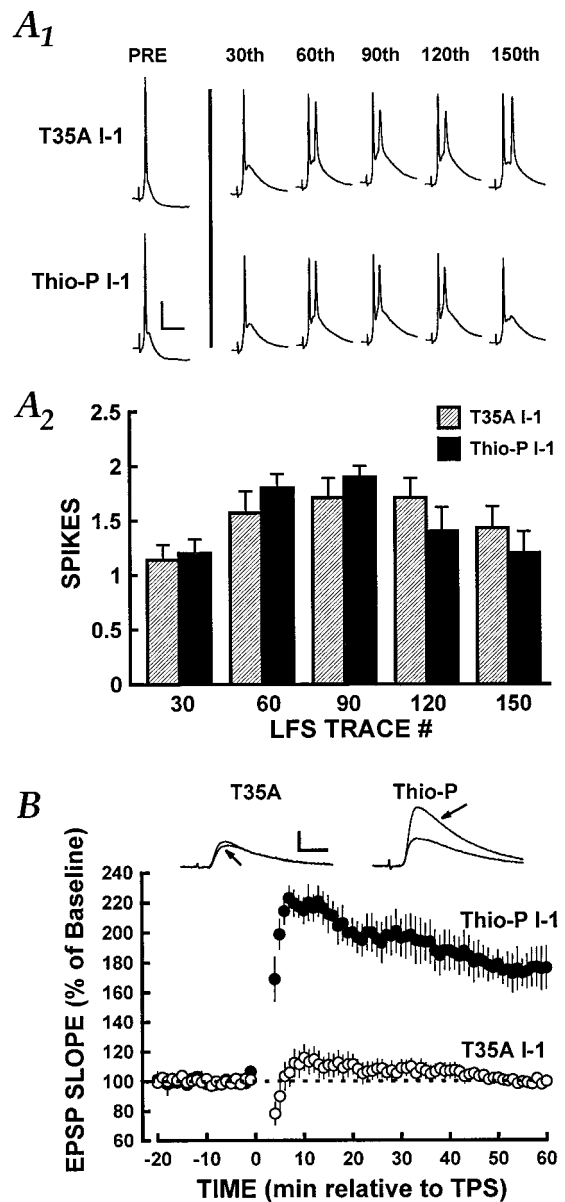


Figure 5. The direct inhibition of postsynaptic PP1 enables TPS-induced LTP without an increase in postsynaptic spiking. *A*, Postsynaptic activity during TPS was not increased by thiophosphorylated I-1. *A₁*, Samples of postsynaptic potentials recorded just before TPS (PRE) and at every 30th stimulus during TPS are shown. Double spikes were evoked during TPS in both groups. Of all TPS samples recorded ($n = 85$), only one stimulus produced three spikes, with all of the others yielding one or two. Calibration: 10 mV; 10 msec. *A₂*, Summary of the number of spikes produced by stimuli during TPS is shown. There were no statistically significant differences between cells recorded with thiophosphorylated I-1 and those recorded with T35A I-1. A patterning effect was obtained, with the number of spikes at trace 90 significantly higher than those at traces 30 and 150 (Newman-Keuls, $p < 0.01$ and 0.05 , respectively). *B*, TPS induces LTP when paired with intracellular application of thiophosphorylated I-1. Only cells impaled with Thio-P I-1 ($n = 10$) showed LTP after TPS. The rapid induction of LTP compared with that shown in Figure 3 is probably caused by the stronger stimulation used during TPS (see Materials and Methods). No lasting effect on synaptic efficiency was seen when TPS was delivered to cells impaled with electrodes containing inactive T35A I-1 ($n = 7$). The inset shows sample EPSPs obtained during the baseline period and at 60 min after TPS.

membrane excitability, the induction of burst firing by isoproterenol must be independent of the inhibition of postsynaptic PP1 by the cAMP pathway.

DISCUSSION

The present paper addresses some of the biochemical mechanisms that underlie LTP induced by θ frequency stimulation in combina-

tion with activation of the postsynaptic cAMP pathway. From the standpoint of the widely proposed relationship between synaptic plasticity and behavioral formation, the study of LTP induced by this pattern of stimulation should prove valuable. Hippocampus-based memory is likely to be better modeled by TPS-induced LTP than by the HFS protocols more typically used, because prolonged 100 Hz trains do not occur within natural *in vivo* firing patterns (Fenton and Muller, 1998; Dobrunz and Stevens, 1999). Rather, hippocampal activity recorded in behaving animals consists of short bursts of spikes interspersed with relatively long, quiescent intervals. The median frequency of the spikes within a burst, adjusted to 31°C, is \sim 11 Hz. The bursts are short ($<$ 15 pulses), and the interburst intervals are in the order of 0.7 sec. Dobrunz and Stevens (1999) have shown that such stimulation patterns, applied for 256 pulses, can induce LTP. However, our data and those of others (Thomas et al., 1996; Katsuki et al., 1997; Winder et al., 1999) suggest that the probability of LTP induction will be enhanced by coactivation of β -adrenergic receptors. Hippocampus activity in the θ range, coupled with locus ceruleus stimulation (Foote et al., 1983; Aston-Jones et al., 1994), should provide an effective stimulus for modifying synaptic efficiency.

TPS-induced LTP has been studied in detail in mouse hippocampal slices. However, the induction conditions and underlying mechanisms of TPS-induced LTP appear to be quite different from those observed in rat hippocampus. In mouse slices, a train of 150 stimuli delivered at 5 Hz induces LTP, whereas longer trains cause depotentiation (Thomas et al., 1996). These phenomena are linked to complex spike bursting that occurs during the first 150 pulses, followed by predominantly single spikes in response to subsequent stimuli. When isoproterenol is applied to the mouse hippocampus, the longer trains persist in eliciting complex spikes, and depotentiation is inhibited (Thomas et al., 1998). In contrast, in rat hippocampus, 150 pulses delivered at 10 Hz do not cause LTP on their own, nor do they evoke complex spike bursting. Furthermore, the inhibition of postsynaptic PP1 enables TPS to induce LTP without any increase in postsynaptic excitability. This finding does not exclude a role for postsynaptic burst firing in the induction of TPS-LTP; however, it indicates that some other process, which is inhibited by PP1 activity, contributes to TPS-LTP in the rat hippocampus. Our data suggest that this process is the regulation of postsynaptic CaMKII activity.

Studies that used widely spaced trains of HFS in the rat CA3 \rightarrow CA1 synapse have shown the importance of the indirect regulation of CaMKII by the cAMP pathway in LTP at the rat CA3 \rightarrow CA1 synapse (Blitzer et al., 1995, 1998). The LTP-induction model based on those results included a signaling pathway and a PP1-regulated gate, each incorporating Ca²⁺/calmodulin-dependent enzymes. The signaling pathway is activated by CaMKII, and the position of the gate is determined by the balance of adenylyl cyclase activity and calcineurin activity. When synaptic stimulation is relatively strong, such as that used in the spaced HFS experiments, high levels of Ca²⁺/calmodulin are attained that are sufficient to activate the signaling pathway (because of CaMKII activity) and keep the gate open (because of adenylyl cyclase activity and consequent PP1 inhibition).

How can this model handle LTP induction by weak synaptic stimulation paired with β -adrenergic stimulation? The abilities of different patterns of stimulation to activate calcineurin and adenylyl cyclase may offer a solution. Weak stimulation, exemplified by low-frequency stimulation of 1 Hz, can induce LTD in area CA1 via a calcineurin-dependent process (Mulkey et al., 1994). This effect is blocked by 8-Br-cAMP, confirming an antagonistic relationship between calcineurin and the cAMP pathway and indicating that such weak stimulation preferentially activates calcineurin. Much stronger synaptic stimulation is needed to activate the cAMP pathway fully; e.g., widely spaced triple trains of HFS are sufficient, but a single HFS train is not (Frey et al., 1993; Blitzer et al., 1995). With weak stimulation, the closed position of the gate would be favored, because calcineurin is not opposed by the cAMP pathway and PP1 is active. The concurrent activation of the cAMP pathway by some other

method, such as β -adrenergic stimulation, would open the PP1 gate and permit CaMKII-dependent signaling.

The application of the gating model to TPS-induced LTP is supported by the results presented in this paper. These include the blockade of TPS-LTP by inhibitors of the NMDA receptor, of CaMKII, and of PKA, the phosphorylation of endogenous I-1 by TPS paired with either isoproterenol or 8-Br-cAMP, the ability of postsynaptic thiophosphorylated I-1 to substitute for β -adrenergic stimulation, and the stimulation of Ca²⁺-independent CaMKII activity by TPS and 8-Br-cAMP applied together but not separately. It thus appears that two forms of cAMP-dependent LTP, one induced by strong synaptic stimulation and the other by more physiologically relevant stimulation coupled with an active postsynaptic cAMP pathway, use similar mechanisms.

The gate-related effect of the cAMP pathway on CaMKII is independent of the ability of isoproterenol to enhance burst firing during TPS (Fig. 1) (Winder et al., 1999), because direct postsynaptic inhibition of PP1 did not increase excitability during TPS (Fig. 5). However, these two cAMP-dependent processes could act in concert to strengthen postsynaptic CaMKII signaling. The backpropagation of even individual action potentials produces dendritic Ca²⁺ transients in CA1 neurons (Callaway and Ross, 1995; Spruston et al., 1995), and burst firing during a train of TPS might be expected to substantially increase the Ca²⁺ signal. An additional contribution of repetitive firing to Ca²⁺ influx is suggested by the recent demonstration of a postsynaptic Na⁺-dependent increase in NMDA receptor-mediated currents (Yu and Salter, 1998). Under some conditions, such as in the mouse hippocampus, the rise in dendritic Ca²⁺ concentrations produced by bursting may be sufficient to enable TPS-induced LTP, perhaps by activating Ca²⁺-dependent adenylyl cyclases. The Ca²⁺ entry that accompanies widely spaced trains of HFS increases postsynaptic cAMP in this manner (Blitzer et al., 1995), and the backpropagation of bursts into the dendrites during TPS may similarly activate the cAMP pathway. However, our data in the rat hippocampus show that the Ca²⁺ entry associated with TPS-induced bursting is not adequate to enable LTP, and additional inhibition of postsynaptic PP1 activity is necessary. It seems reasonable that, under physiological conditions, cAMP-dependent processes are activated to a lesser degree than that achieved in slice experiments, so that both burst firing and phosphatase inhibition may contribute to the role of the cAMP pathway in LTP and memory.

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