Protein Phosphatases Mediate Depotentiation Induced by High-Intensity Theta-Burst Stimulation

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INTRODUCTION

In hippocampal area CA1, synaptic strength was shown to be up- or down-regulated by previous synaptic activities. Brief high-frequency stimulation (HFS) produces a long-lasting increase in synaptic strength, i.e., long-term potentiation (LTP), while prolonged low-frequency stimulation (LFS) leads to a long-lasting decrease in synaptic strength, i.e., long-term depression (LTD) (for review see Bear and Malenka 1994; Bliss and Collingridge 1993; Linden 1994). We previously observed that the same theta-burst (TB) stimulation produced different synaptic plasticity depending on the stimulus intensity: low-intensity theta-burst (LBT) stimulation produced potentiation similar to other forms of synaptic plasticity in area CA1 (Barr et al. 1995). Here, we found that high-intensity theta-burst (HBT) stimulation produced LTD (Barria et al. 1997). In addition, moderate elevations of [Ca$^{2+}$]i, necessary for LTD induction (Perkel et al. 1993). Therefore, we investigated the mechanisms underlying high-intensity theta-burst (HBT) stimulation and found that HBT stimulation produced a long-lasting decrease in synaptic strength, i.e., LTD (for review see Bear and Malenka 1994; Bliss and Collingridge 1993; Linden 1994). We previously observed that the same theta-burst (TB) stimulation produced different synaptic plasticity depending on the stimulus intensity: low-intensity theta-burst (LBT) stimulation produced LTD (Mulkey et al. 1994). However, TB depotentiation and LTD stimulation could have different functional significance.

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excitatory postsynaptic potentials (fEPSPs). Synaptic responses were evoked using monopolar tungsten stimulating electrodes (A-M systems, Carlsborg, WA). Stimuli were square wave current pulses (0.1–0.2 ms duration) delivered at 1–2 stimuli/min. The stimulating electrodes were placed in the s. radiatum to activate the Schaffer Collateral pathway projecting to CA1. The basal synaptic response was chosen to be at 20–30% of the maximum rising slope of fEPSP from each slice, which fell within stimulus intensities of 40–80 μA. Tetanic stimulation was delivered by a TB stimulation pattern composed of 10 minitrains (TB-10) given at 200-ms intervals; each minitrain consisted of 4 pulses given at 100 Hz. LTP was elicited by TB stimuli at the stimulus intensity that gave the basal synaptic response (LI TB), while depotentiation was induced by TB stimuli at 11 TB stimuli at the stimulus intensity that gave the basal synaptic response (HI TB). In some cases, depotentiation was induced by five minitrains of TB stimulation (TB-5). This shorter (TB-5) stimulus was used to produce a lower magnitude of depotentiation that might be more sensitive to modulation by drugs. In some experiments, the HI TB stimuli were applied twice at 20-min intervals to monitor accumulative depotentiation.

**Drug application**

Stock (1–10 mM) solutions of okadaic acid, calyculin A (LC Laboratories, Woburn, MA), and rapamycin (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO; final concentration of DMSO between 0.075% and 0.1%). FK506 (Fujisawa Pharmaceuticals, Deerfield, IL) in an intravenous injection solution form (6 mM in 80% vol/vol ethanol) was used (final concentration of ethanol 0.66%). Stock solutions were kept as frozen aliquots, and each aliquot was thawed immediately before use. Slices were preincubated for 90–180 min in okadaic acid (1 μM), calyculin A (0.75 μM), FK506 (50 μM), or rapamycin (1 μM) before transferring to the recording chamber, after which slices were perfused with ACSF. 8-Br-cAMP (BioMol Research Laboratories, Plymouth Meeting, PA) was directly dissolved in ACSF at 300 μM and applied by bath-perfusion 35 min before HI-TB stimulation and throughout the experiments. 8-Br-cAMP was also applied together with 3-isobutyl-1-methylxanthine (IBMX, Sigma), a phosphodiesterase inhibitor, based on a previous report that 8-Br-cAMP blocked LTD when applied with IBMX (Mulkey et al. 1994). 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Sigma), an A₁ adenosine receptor antagonist, was included in control and test solution to avoid any confounding effect of IBMX on adenosine receptors. The stock solutions of IBMX (20 mM) and DPCPX (1 mM) were prepared by dissolving them in either DMSO (final concentration of DMSO 0.25%) or 0.1% NaOH, respectively. In control experiments, which were conducted in an interleaved manner, slices were exposed to the vehicle solvents (e.g., DMSO or ethanol) at the same concentration used during drug application.

**Data acquisition and analysis**

Field potentials were sequentially amplified by an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) and a DC amplifier (Warner Instrument, Hamden, CT) and digitized at 10 kHz by Lab PC+ (National Instruments, Austin, TX). Data were acquired and analyzed by programs designed by Dr. Jeffery L. Calton (Dartmouth College) using Labview software package (National Instruments). fEPSPs were quantified by measuring the slope at ±300 μs from the half-peak time of the rising response. The slope was determined by dividing the voltage difference between these two time points by 600 μs (Fig. 2A). Since the response at this time window gives near-linear rising phase, this measure is close to the true value of the rising slope of fEPSP. To make sure that any change in response was attributed to synaptic plasticity, we measured the fiber volley. The fiber volleys were quantified by measuring their amplitude relative to the baseline before the stimulus. We observed some changes in the magnitude of

![FIG. 1. A schematic diagram illustrating proposed cellular mechanisms for long-term potentiation (LTP) and long-term depression (LTD). Proposed pathways for LTD or LTD induction are shown with straight or dashed line arrows, respectively. Low-frequency stimulation (LFS) is thought to lead to LTD as follows. LFS acts through N-methyl-D-aspartate receptors (NMDA-R) to raise intracellular calcium concentrations slightly (low [Ca²⁺]), activates Ca²⁺/CaM activity. This, in turn, leads to preferential activation of calcineurin, because only moderate levels of Ca²⁺/CaM activity are required for calcineurin activation. Calcineurin then dephosphorylates inhibitor protein 1 (I-1). This renders I-1 incapable of inhibiting protein phosphatase (PP)1, freeing PP1 to dephosphorylate its substrates, which include CaM protein kinase II (CaMKII) and the GluR1 subunit of AMPA receptors. Dephosphorylation of glutamate receptor type 1 (GluR1) leads to LTD. In contrast, high-frequency stimulation (HFS) is thought to lead to LTP as follows. HFS acts through NMDA-R to raise [Ca²⁺] to higher levels (high [Ca²⁺]). This large increase in [Ca²⁺] causes high-level activation of Ca²⁺/CaM. This, in turn, activates not only calcineurin but also CaMKII and adenylyl cyclase (AC). CaMKII phosphorylates itself and GluR1, leading to LTP. On the other hand, activated AC produces cAMP, which activates PKA. Activated PKA counteracts calcineurin activity by phosphorylating I-1, resulting in the inhibition of PP1. We put the question mark on the involvement of I-1 due to the relatively small effect of the I1 knockout mouse in the LTD induction in Shaffer collateral CA 1 synapses (Allen et al. 2000).](http://jn.physiology.org/doi/10.1212/01.JNEUROPHYSiol.0062.04.85)
RESULTS

Effect of PP1/PP2A inhibition on TB depotentiation

To test whether PP1 or PP2A activity is involved in the induction of TB depotentiation, we studied the effect of okadaic acid and calyculin A (PP1/PP2A inhibitors) on TB depotentiation. When HI TB-10 stimulation was applied, depotentiation was induced in control slices pretreated with solvent (0.1% DMSO; Fig. 3, A and B). The magnitude of depotentiation was 70.4 ± 5.92% (P < 0.001) 20 min after the first TB-10 stimulus and 86.6 ± 6.07% (P < 0.001) 20 min after the second TB-10 stimulus (n = 5). In slices pretreated with okadaic acid (1 μM, 2–3 h), the magnitude of depotentiation was 25.5 ± 3.97% (P < 0.001) and 38.1 ± 6.23% (P = 0.001) 20 min after the first and second TB-10 stimulation, respectively (n = 8; Fig. 3, A and B). The magnitude of TB depotentiation was significantly different between control slices and okadaic acid-pretreated slices (P < 0.001 for both the first and the second TB depotentiation; Fig. 3B).

HI TB-5 stimulation induced smaller, but still significant, depotentiation in the control slices (pretreated with solvent 0.075% DMSO) compared with HI TB-10 stimulation (Fig. 4, A and B). The magnitude of depotentiation was 20.5 ± 5.52% (P = 0.008) and 45.9 ± 10.82% (P = 0.002) 20 min after the first and second TB stimulation, respectively (n = 5). In slices pretreated with calyculin A (0.75 μM, 2–3 h), depotentiation by HI TB-5 stimulation was completely blocked (Fig. 4, A and B).

Histological examination of slices incubated with okadaic acid (1 μM) for 3 h showed that the magnitude of depotentiation in the control slices was 70.4 ± 5.92% (P < 0.001), while in slices incubated with okadaic acid for 3 h, the magnitude of depotentiation was 25.5 ± 3.97% (P < 0.001) (Fig. 3B). The magnitude of depotentiation was significantly different between control slices and okadaic acid-pretreated slices (P < 0.001 for both the first and the second TB depotentiation; Fig. 3B).

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Statistical analysis

Statistical comparisons were made using paired or independent Student’s t-test with the level P < 0.05 considered to be significant (SPSS, Chicago, IL). In some cases, two-way ANOVA with repeated measures was used for statistical comparisons. Numerical and graphed data (Origin, OriginLab, Northampton, MA) are presented as a mean ± SE.
Depotentiation 20 min after the first and second TB stimulation was not statistically significant: 4.3 ± 4.66% depotentiation ($P = 0.713$) and 12.0 ± 5.09% depotentiation ($P = 0.200$), respectively ($n = 6$). This result suggests that the activity of PP1/2A is involved in the induction of TB depotentiation.

A previous study reported that in the presence of PP1/PP2A inhibitors, LFS enhanced synaptic transmission through presynaptic mechanisms (Herron and Malenka 1994). To test whether similar presynaptic changes were induced when HI TBS was delivered in the presence of PP1/PP2A inhibitors, we examined the effect of HI TBS in naive (not previously potentiated) synapses. Slices were pretreated with okadaic acid and were also exposed to APV (to block NMDA receptor effects) during the experiment. We found that HI TBS in the presence of PP1/PP2A inhibitors did not enhance synaptic transmission (data not shown). Therefore this result supports the proposal that PP1/PP2A inhibitors block TB depotentiation rather than mask depotentiation via enhancement of synaptic transmission.

On the other hand, we found that LTP in the presence of calyculin A was significantly decreased compared with control ($66.8 ± 6.02%$ vs. $100.5 ± 11.76%$, $P = 0.0248$). Considering that LTP in slices pretreated with okadaic acid is comparable to the control, this decrease in LTP in slices pretreated with calyculin A may not be attributed to the inhibition of PP1/2A. In fact, inhibition of PP1/2A is expected to positively modulate LTP induction (Blitzer et al. 1998), even though disinhibition of PP1/2A through I-1 knockout did not affect the LTP induction in Shaffer collateral CA 1 synapses (Allen et al. 2000). Further studies may be required to understand this effect of calyculin A.

**Effect of calcineurin inhibition on TB depotentiation**

Since synaptic activation of PP1 may be mediated indirectly through the activation of calcineurin (the Ca$^{2+}$/CaM-dependent protein phosphatase that dephosphorylates and inactivates I-1) (Oliver and Shenolikar 1998), we tested whether calcineurin was involved in TB depotentiation using FK506, a specific calcineurin inhibitor. Prior studies have reported complex effects of FK506 on LTP—including developmental differences. In hippocampal slices from adult animals, FK506 caused LTP induction, whereas in hippocampal slices from young animals, FK506 prevented LTP induction (Wang and Kelly 1997; Wang and Stelzer 1994). However, in other studies using hippocampal slices from young animals, LTP induction was not prevented in FK506-treated slices (Mulkey et al. 1994). In our experiments using young animals, pretreatment of rat hippocampal slices with FK506 did not interfere with LTP induction, in agreement with Mulkey et al. (1994) (Fig. 5, A and B).

In some cases, fEPSPs were followed by population spikes after LTP induction. Even though we selectively analyzed the fEPSP by measuring the slope in the rising phase of the responses, one may argue that the drug effect could be affected by the development of population spikes. Therefore in a separate set of experiments, we analyzed the effect of FK 506 on depotentiation at different stimulus intensities. The fEPSP slopes were plotted as a function of the fiber volley amplitudes (Fig. 5C). When the magnitudes of depotentiation were compared among different stimulus intensities, there was no significant difference [$F(5,18) = 0.483$, $P = 0.784$], while there is significant difference in the depotentiation magnitude between control and FK506-treated slices [$F(1,18) = 67.237$, $P < 0.001$].

To ensure specific effects of FK506 on calcineurin rather than on a calcineurin-independent pathway, we studied the effect of rapamycin on HI TB-10 depotentiation. Rapamycin is known to bind to the FK506 binding protein and act through FK506-calcineurin-independent mechanisms. In this study, we found that HI TB-10 stimulation induced significant depotentiation ($52.6 ± 13.9\%$, $P = 0.02$, $n = 4$) in slices pretreated with rapamycin (1 $\mu$M). The magnitude of depotentiation in slices pretreated with rapamycin was not significantly different from the magnitude of depotentiation in control slices pretreated with solvent (DMSO 0.1%), which was $46.0 ± 9.4\%$ ($P = 0.012$, $n = 4$; $P = 0.708$ between control and rapamycin). This suggests that calcineurin activity is also required for the induction of TB depotentiation.
Effect of PKA activation on TB depotentiation

Since PKA can also result in PP1 inactivation by phosphorylating endogenous I-1 (Blitzer et al. 1998; Oliver and Shenolikar 1998), we tested the effect of PKA activation on TB depotentiation. We used 8-Br-cAMP (300 μM) and phosphodiesterase inhibitor IBMX (50 μM) based on the report that 8-Br-cAMP blocked LTD when applied in conjunction with IBMX (Mulkey et al. 1994). In addition, DPCPX (1 μM), an A1 adenosine receptor antagonist, was included in both the control and test solution to avoid any confounding effect of IBMX on adenosine receptors (Mulkey et al. 1994). Under this condition, as noted by Mulkey et al. (1994), LTD induced by LFS was blocked (data not shown).

HI TB-10 stimulation induced significantly smaller depotentiation in the presence of 8-Br-cAMP and IBMX (P = 0.003). The magnitude of depotentiation in the presence of 8-Br-cAMP and IBMX was 27.1 ± 2.60% (P = 0.016, n = 4), whereas the magnitude of depotentiation in the control solution (0.25% DMSO and DPCPX, 1 μM) was 52.2 ± 5.58% (P = 0.0073, n = 4; Fig. 6, A and B). DPCPX alone prevented the posttetanic depression, but did not affect TB depotentiation. This result suggests that TB depotentiation was partially inhibited by PKA activation.

FIG. 5. Effect of FK506 on TB-10 depotentiation. Depotentiation was induced with HI TB-10 stimulation. A: representative traces before LTP induction, 20 min after LTP induction, and 20 min after depotentiation induction in slices incubated in solvent (0.66% ethanol) (A), and in slices incubated with FK506 (50 μM) for 1–3 h (B). B: summary of experiments is shown in control slices (○, n = 6) and in FK506-treated slices (●, n = 9). Responses were recorded once per 30 s and each point represents the mean ± SE of the respective time points. Upward arrows mark LI TB-10 stimulation and downward arrow marks HI TB-10 stimulation. TB depotentiation was prevented in slices incubated with FK506 (−7.3 ± 8.60% depotentiation, P = 0.677), while significant depotentiation was observed in control slices (49.7 ± 9.04%, P = 0.007). Scale bars = 0.5 mV, 10 ms. C: effect of FK506 on depotentiation was examined at the different stimulus intensities. The fEPSP slopes were plotted as a function of the fiber volley amplitudes. ○, ○, and ⌜ represent fEPSP slopes before LTP induction, 20 min after LTP induction, and 20 min after depotentiation induction, respectively. There was no significant difference in the magnitude of depotentiation among different stimulus intensities [F(5,18) = 0.483, P = 0.784], while there is a significant difference in the depotentiation magnitude between control and FK506-treated slices [F(1,18) = 67.237, P < 0.001].

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FIG. 6. Effect of 8-Br-cAMP on TB-10 depotentiation. Depotentiation was induced with HI TB-10 stimulation. A: representative traces before LTP induction, 20 min after LTP induction, and 20 min after depotentiation induction in slices with bath application of solvent (0.25% DMSO) and the adenosine receptor antagonist (1 μM DPCPX; n = 4) (A), and in slices with bath-application of 8-Br-cAMP (300 μM) (B). 8-Br-cAMP was applied along with IBMX (50 μM) and DPCPX (1 μM) 35 min before depotentiation induction and throughout the experiments. B: summary of experiments is shown in control slices (○, n = 4), and in 8-Br-cAMP-treated slices (●, n = 4). Responses were recorded once per 30 s and each point represents the mean ± SE of the respective time points. Upward arrows mark LI TB-10 stimulation and downward arrows marks HI TB-10 stimulation. The magnitude of depotentiation was 52.2 ± 5.58% (P = 0.013) in control slices and 27.1 ± 2.60% (P = 0.016) in 8-Br-cAMP-perfused slices. TB-10 depotentiation was significantly decreased (P = 0.003) by application of 8-Br-cAMP with IBMX (P = 0.02). Scale bars = 0.5 mV, 10 ms.
In this study we showed that inhibitors of PP1/2A and calcineurin block the induction of TB depotentiation, as does PKA activation using 8-Br-cAMP and IBMX. These results are consistent with the well-accepted role of protein phosphatases in the down-regulation of synaptic strength (Mulkey et al. 1993, 1994) and the involvement of PKA in negatively modulating protein phosphatase activity (Blitzer et al. 1998; Mulkey et al. 1994; Oliver and Shenolikar 1998).

What determines whether phosphatases or kinases predominate to regulate synaptic strength? It has been proposed that weak stimulation (like LFS) induces LTD by producing a modest rise in $[Ca^{2+}]_i$, which in turn, activates a protein phosphatase pathway. This level of $[Ca^{2+}]_i$ may be below the threshold for activating protein kinases such as CaMKII. Stronger stimulation (like HFS) induces LTP by producing a higher level of $[Ca^{2+}]_i$, and thus activates protein kinases, including CaMKII (Cormier et al. 2001; Hansel et al. 1997; Lisman 1989; Yang et al. 1999).

Given these relationships, how does strong stimulation (HI TBS) activate the protein phosphatases pathway? One possibility is that HI TBS deactivates or desensitizes NMDA receptors during stimulation, leading to only a small elevation of $[Ca^{2+}]_i$. In a preliminary study we observed even higher elevation of $[Ca^{2+}]_i$ in the hippocampal proximal dendrites during HI TBS than during LTP-inducing stimulation (Kang et al. 1998). However, we did not visualize spines in that study, so it is conceivable that the $[Ca^{2+}]_i$ increase in spines (which is primarily NMDA receptor-dependent) might be smaller during HI TBS than during LTP-inducing stimulation. In other words, HI TBS would increase $[Ca^{2+}]_i$ in spines to a low level, similar to LFS. This would activate a common pathway for the down-regulation of synaptic strength, namely, the activation of protein phosphatases. This possibility is currently under investigation.

On the other hand, if HI TBS increases $[Ca^{2+}]_i$, in dendritic spines to levels higher than LTP-inducing stimulation (as observed in the proximal dendrites), then we must conclude that protein phosphatase pathways can be predominantly activated not only by small elevations of $[Ca^{2+}]_i$, but also by large elevation of $[Ca^{2+}]_i$, whereas intermediate levels of $[Ca^{2+}]_i$ represent the optimal conditions for activating protein kinases.

How might high levels of $[Ca^{2+}]_i$ activate the protein phosphatases? We speculate that the following mechanism is involved. Moderate elevations of $[Ca^{2+}]_i$, as seen during LTP induction activate protein kinases such as CaMKII and also suppress phosphatase activity by the concomitant activation of PKA (Blitzer et al. 1995, 1998; Makinson et al. 1999). PKA, which negatively regulates PP1 activity by phosphorylating the endogenous PP1 inhibitor, I-1, responds to the activation by Ca$^{2+}$/CaM-dependent adenylyl cyclases (AC) present in CA1 neurons (Ahlijanian and Cooper 1988; Piascik et al. 1980; Potter et al. 1980). These forms of AC show a bell-shaped activity curve relative to increasing calcium concentrations (Ahlijanian and Cooper 1988; Piascik et al. 1980; Potter et al. 1980). Therefore AC activity may be decreased by large elevations of $[Ca^{2+}]_i$, induced by HI TB stimuli. In addition, PKA activity can be decreased by Ca-dependent activation of phosphodiesterase (PDE). The resulting low PKA activity is accompanied by a disinhibition of PP1, which dephosphorylates CaMKII (Shenolikar and Narin 1991; Shields et al. 1985), NMDAR (Westphal et al. 1999), and glutamate receptor type 1 (GluR1) subunit of AMPA receptors (Lee et al. 2000) to promote depotentiation.

If HI TB stimulation favors phosphatases, why then does HI TB stimulation not produce LTD in naive synapses, as seen with LFS (Barr et al. 1995)? This suggests that LTD and depotentiation are mechanistically distinct. Growing experimental evidence supports this viewpoint. For example, Lee et al. (2000) reported that identical stimulation conditions recruit different signal-transduction pathways depending on prior synaptic history. In naive synapses, LTD induction results from the preferential dephosphorylation of GluR1 at PKA sites. In contrast, depotentiation in previously potentiated synapses results from the dephosphorylation of GluR1 at CaMKII sites (Lee et al. 2000). There is also evidence from gene disruption studies that the specific calcineurin isoforms (i.e., calcineurin Aα) mediate depotentiation, but not LTD, even though both LFS-induced LTD and depotentiation can be blocked by pharmacological inhibition of calcineurin (Huang et al. 1999; O’Dell and Kandel 1994; Zhuo et al., 1999). In a similar manner, HI-TB stimulation may selectively depotentiate signals mediated by CaMKII (but not PKA), or may utilize specific phosphatase isoforms to transduce signals at postsynaptic sites. Clearly, other mechanisms could be postulated, but further work analyzing second messengers and postsynaptic signaling pathways will be required to resolve this issue.

What is the functional significance of depotentiation? LTP is studied as the molecular mechanism that underlie learning and memory, and the disruption of LTP such as depotentiation may subserve the mechanism underlying memory loss, i.e., amnesia. In support of this point of view, the protein phosphatase pathways that mediate depotentiation, was shown to mediate amnesic effects (Genoux et al. 2002). Depotentiation produced by HI TB stimuli may have functional consequences that differ from depotentiation induced by other means. For example, in LFS-induced depotentiation, the stimuli are relatively low in intensity and resemble some naturally occurring neuronal firing patterns. As such, LFS-induced depotentiation may be relevant to normal physiological processes such as desaturation of potentiated pathway or natural decay of memory. In contrast, HI TB stimuli are, by definition, of pathologically high intensity, and the stimuli are given as theta bursts—a specific pattern that enhances neuronal responsiveness. These two factors work together; the net result of which is that HI-TB stimuli activate many afferent fibers and evoke neuronal responses that include robust population spikes. These burst-like responses resemble activity recorded during seizures. In fact, in vitro studies showed that seizure activity or seizure-like extracellular conditions induced depotentiation (Harrison and Alger 1993; Hesse and Teyler 1976; Moore et al. 1993).

In humans, seizure activity is commonly associated with memory impairment or amnesia (Halgren and Wilson 1985; Squire 1986; Thompson 1991). Patients with epilepsy have episodes of memory loss such as difficulty remembering past events (retrograde amnesia) and retaining newly learned information (anterograde amnesia). The degree of memory loss in epilepsy patients has generally been correlated with the frequency and/or severity of seizure. The consequences of seizures on memory are also well documented in patients who undergo electroconvulsive therapy.


