

Postsynaptic Calcineurin Activity Downregulates Synaptic Transmission by Weakening Intracellular Ca^{2+} Signaling Mechanisms in Hippocampal CA1 Neurons

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Protein phosphorylation and dephosphorylation are believed to functionally couple neuronal activity and synaptic plasticity. Our previous results indicated that postsynaptic Ca^{2+} /calmodulin (CaM) signaling pathways play an important role in setting synaptic strength, and calcineurin (CaN) activity limits synaptic responses during basal synaptic transmission and long-term potentiation expression. The inhibition of postsynaptic CaN activity by FK-506 or an autoinhibitory peptide induced synaptic potentiation in hippocampal slices, which occludes tetanus-induced LTP. FK-506-induced synaptic potentiation was expressed in adult but not young rats. To elucidate mechanisms underlying CaN-inhibited synaptic potentiation, we co-injected

certain agents affecting Ca^{2+} signaling pathways with CaN inhibitors into CA1 neurons. Synaptic potentiation induced by FK-506 was significantly attenuated by co-injecting BAPTA, heparin/dantrolene (inhibitors of intracellular Ca^{2+} release), a CaM-binding peptide, or CaM-KII/PKC pseudosubstrate peptides. These results indicate that postsynaptic CaN activity can downregulate evoked synaptic transmission by weakening intracellular Ca^{2+} signals and downstream protein kinase activities.

Key words: synaptic potentiation; calcineurin inhibitor; calcium/calmodulin; IP_3 /ryanodine receptors; positive feedback; postsynaptic manipulation

Synaptic transmission is the basic process of information exchange among neurons in the nervous system, and activity-dependent synaptic plasticity, e.g., long-term potentiation (LTP), is believed to be a cellular model of learning and memory in mammalian brain (Bliss and Lynch, 1988; Gustafsson and Wigstrom, 1988; Bliss and Collingridge, 1993; Lisman, 1994; Eichenbaum, 1995). Much recent experimentation has focused on studying the cellular and molecular mechanisms underlying synaptic transmission and synaptic plasticity. One characteristic of synaptic plasticity is that the strength of synaptic transmission can be enhanced or attenuated by certain electrical or chemical protocols. For example, synaptic strengthening is induced by high-frequency stimulation (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973; Bliss and Collingridge, 1993) or activators of protein kinases (Malenka et al., 1986b; Hu et al., 1987; Wang and Feng, 1992; Wang and Kelly, 1995), and synaptic weakening is induced by low-frequency stimulation (Dunwiddie and Lynch, 1978; Ito, 1982). The molecular mechanisms that reset synaptic strength to new levels after these manipulations may require a shift in balance between protein kinase and phosphatase activities, because many studies have indicated that protein phosphorylation and dephosphorylation regulate the magnitude of many diverse biological activities (Nestler and Greengard, 1984; Krebs, 1994). It is well known that the activities of protein kinases are essential for the induction and maintenance of LTP (Akers et al., 1986; Malinow et al., 1988,

1989; Malenka et al., 1989; O'Dell et al., 1991; Feng and Wang, 1992; Wang and Feng, 1992; Fukunaga et al., 1993; Thomas et al., 1994; Wang and Kelly, 1996a), in which protein phosphorylation is believed to upregulate synaptic strength. The converse of this hypothesis is that protein phosphatases (PrP) may contribute to synaptic weakening, which is supported by results showing that PrP activities are required for long-term depression (LTD) (Mulkey et al., 1993, 1994) and that the inhibition of PrP-1, -2A, or -2B can enhance synaptic transmission (Figurov, 1992; Wyllie and Nicoll, 1994; Wang and Kelly, 1996a).

We have shown that calcium/calmodulin (Ca^{2+} /CaM) signaling pathways are important in regulating the output of synaptic transmission by shifting the balance between postsynaptic Ca^{2+} /CaM-dependent protein kinase II (CaM-KII)/protein kinase C (PKC) and calcineurin (CaN) activities. For example, the injection or perfusion of Ca^{2+} /CaM into postsynaptic neurons induces synaptic potentiation (Wang and Kelly, 1995) (J. Wang and P. Kelly, unpublished observations), the postsynaptic injections of CaN inhibitor (a protocol for inhibiting CaN activity) induces synaptic potentiation (Wang and Kelly, 1996a), which we call CaN-inhibited synaptic potentiation, and the inhibition of postsynaptic CaM-KII and PKC attenuates tetanus-induced LTP (Wang and Kelly, 1996a). Because CaN is preferentially activated by low Ca^{2+} /CaM concentrations ($K_D = \sim 0.1\text{--}1$ nM) compared with CaM-KII ($K_D = \sim 40\text{--}100$ nM) (Cohen, 1988; Klee and Cohen, 1988; Schulman and Lou, 1989; Klee, 1991), and oscillations in intracellular Ca^{2+} range from 10 to 100 nM under resting conditions (Carafoli, 1987; Amundson and Clapham, 1993; Ghosh and Greenberg, 1995), interactions of Ca^{2+} with CaN β -subunit and CaM result in the possibility that CaN is active and limits synaptic strength at a low and stable level under basal conditions. This can explain why postsynaptic injections of Ca^{2+} /CaM primarily activate protein kinases and induce synaptic potentiation without short-term synaptic depression proceeding potentiation (Wang

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and Kelly, 1995) as well as why postsynaptic injections of CaN inhibitors induce synaptic potentiation (Wang and Kelly, 1996a).

What is the mechanism(s) underlying synaptic potentiation induced by inhibiting postsynaptic CaN activity? It may be simply understood as shifting the balance between protein kinase and phosphatase activities toward enhancing the phosphorylation of protein substrates responsible for synaptic transmission without increasing the absolute activity of protein kinases. In other words, after CaN is inhibited, the action of basal kinase activities on synaptic substrates becomes dominant, because Ca²⁺-dependent protein kinases are active under basal conditions in hippocampal slices (Ocorr and Schulman, 1991; Huber, 1995). However, the inhibition of postsynaptic CaM-KII and PKC activities do not significantly alter basal synaptic transmission (Tsien et al., 1990; Feng and Wang, 1992; Wang and Kelly, 1996a), implying that basal CaM-KII and PKC activities are below a threshold for strengthening synaptic transmission. Together with observations that tetanic stimulation produces large increases in protein kinase activities relative to basal conditions (Akers et al., 1986; Fukunaga et al., 1993), it seems a little difficult to understand why CaN-inhibited synaptic potentiation, which presumably requires basal kinase activities, is larger than tetanus LTP (Wang and Kelly, 1996a). We propose that inhibiting postsynaptic CaN enhances positive-feedback cascades that amplify the activities of intracellular signaling pathways and protein kinases more than it shifts the balance between protein kinase and phosphatase activities on synaptic substrates. To better understand these cascades, we examined whether intracellular Ca²⁺ signaling pathways are involved in CaN-inhibited synaptic potentiation. Experiments were conducted by co-injecting agents that block Ca²⁺ signaling cascades with CaN inhibitors into hippocampal CA1 neurons.

MATERIALS AND METHODS

Transverse hippocampal slices were prepared from male Harlan Sprague Dawley rats (6–7 or 2–3 weeks old) with a McIlwain tissue chopper in ice-cold standard medium (gassed with 95% O₂/5% CO₂), as described previously (Wang and Kelly, 1995, 1996a,b). Slices were incubated at 25°C over 1 hr and transferred to a submersion chamber (31°C; 2 ml/min perfusion rate) for electrophysiological experiments. Standard medium contained (in mM): 124 NaCl, 3 KCl, 1.3 NaH₂PO₄, 26 NaHCO₃, 2.4 MgCl₂, 2.4 CaCl₂, 10 dextrose, and 10 HEPES, pH 7.25. To reduce the effects of GABA_A-mediated inhibitory components on excitable synaptic transmission, experiments were conducted in the presence of bicuculline plus picrotoxin (10 μM each). In addition, orthodromic stimulating electrodes were placed as far away from recording sites as possible to avoid evoking monosynaptic GABAergic synaptic activities caused by directly stimulating interneurons. Certain manipulations were used to prevent neuronal hyperexcitability in the presence of GABAergic antagonists as follows. (1) "Isolation" of area CA1 was achieved by cutting presynaptic axons in stratum radiatum but not in oriens/alveus; this optimizes the integrity of CA1 neurons (Wang and Kelly, 1996a) by preventing the stress of neurons from axon injury during preparation of isolated CA1 slices (Kauer et al., 1988; Malenka et al., 1988); and (2) the concentration of Mg²⁺ was 2.4 mM to limit hyperactivity of excitatory synapses. Under these conditions, seizure activity was never observed during basal synaptic transmission and postsynaptic injections of agents; complex waveforms only occurred after tetanic stimulation in some experiments.

One bipolar tungsten stimulating electrode (12 MΩ) was positioned in stratum radiatum of CA1 area for orthodromically stimulating Schaffer collateral/commissural axons. The frequency of test stimuli was 0.05 Hz; high-frequency tetanic stimulation was composed of 10 trains with 5 sec intervals in which each train contained 10 pulses with 200 Hz frequency. Glass microelectrodes (60–85 MΩ) filled with 2 M potassium acetate (KAc) or 2 M KAc plus various agents [FK-506, rapamycin, CaN autoinhibitory peptide (CaN-AIP), Ca²⁺/CaM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid (BAPTA), a mixture of heparin/dantrolene, a CaM-binding peptide (CBP), or CaM-KII/PKC pseudosubstrate inhibitory peptides] were used for current- or voltage-clamp recordings in CA1

pyramidal neurons; pipettes containing 1 M NaCl were routinely used to record field excitatory postsynaptic potentials (f-EPSPs) in stratum radiatum and were positioned near the intracellular recording site to verify the viability of tissue slices and status of synaptic activity.

Experimental results are based only on neurons in which stable recordings were obtained within the initial 2 min after impalement and that exhibited stable membrane potentials between –70 and –73 mV throughout an experiment. During experiments conducted by voltage-clamp recording with 3 KHz sampling rate, membrane potentials were held at –78 mV (i.e., near the GABA_A reversal potential), together with application of GABAergic antagonists, to reduce possible contamination in measuring excitatory postsynaptic currents (EPSCs) from mono- and disynaptic inhibitory activities. Baseline values (the first data point in each figure) of synaptic transmission were averaged from initial slopes of synaptic responses (EPSPs, EPSCs, or f-EPSPs) during the first minute after stable intracellular recordings were established and defined as 100%. Values of CaN-inhibited synaptic potentiation were data points 1–2 min before tetanic stimulation (arrows) compared with baseline. Values of LTP were data points 20 min post-tetanus compared with baseline. Values of synaptic depotentiation were data points 20 min post-tetanus compared with potentiation values. Synaptic responses are represented as mean ± SEM. Series and input resistances were monitored throughout all voltage- and current-clamp experiments by measuring responses to 4 mV and 0.1 nA injections (50 msec). Data were obtained with pClamp 5.5 and analyzed with custom software to compute initial EPSP, EPSC, and f-EPSP slopes. *t* Tests were used for statistical comparisons. Each waveform was averaged from six consecutive responses, and waveforms in each figure were selected from a representative experiment.

CaN-AIP (3 mM stock in distilled water) was diluted to a final concentration of 300 μM in 2 M KAc. Ca²⁺/CaM mixtures were prepared from CaCl₂ and CaM stock solutions and mixed with CaN-AIP; they were diluted to a final concentrations of 20/80 μM and 300 μM in 2 M KAc, respectively. FK-506 and rapamycin were dissolved in 100% ethanol (50 mM stock solutions) and diluted to a final concentration of 50 μM in 2 M KAc. BAPTA was dissolved in 3 M KOH (400 mM in stock, pH 7.2) and diluted to a final concentration of 20 mM in 2 M KAc plus 50 μM FK-506. Heparin and dantrolene were dissolved in 2 M KAc plus 50 μM FK-506 at final concentrations of 300 and 80 μM, respectively. Stock solutions of CBP, or [Ala²⁸⁶]CaM-KII_{281–302}/PKC_{19–31}, were diluted in KAc to final concentrations of 100 μM or 200 μM/100 μM in 2 M KAc, respectively. These solutions filled the entire microelectrode (i.e., back-filling with 2 M KAc was not performed). Bicuculline and BAPTA were obtained from Sigma (St. Louis, MO), and heparin, dantrolene, D(–)-2-amino-5-phosphonopentanoic acid (D-AP5), and picrotoxin were obtained from RBI (Natick, MA). CaM was a gift from Dr. J. Aronowski; CaN-AIP was a gift from Dr. Randall Kincaid (Veritas, Rockville, MD), and FK-506 and rapamycin were gifts from Dr. Stan Stepkowski (University of Texas Medical School at Houston).

RESULTS

Inhibiting postsynaptic CaN induces greater synaptic potentiation than tetanus LTP

Postsynaptic injections of Ca²⁺/CaM into CA1 neurons induce synaptic potentiation by activating CaM-KII and PKC (Wang and Kelly, 1995), and postsynaptic injections of FK-506 induce synaptic potentiation by inhibiting basal CaN activity (Wang and Kelly, 1996a). Thus, we predicted that the co-injection of Ca²⁺/CaM plus CaN inhibitor should induce larger synaptic potentiation than that by Ca²⁺/CaM alone, especially because partial inhibition of postsynaptic CaN activity facilitates the magnitude of tetanus-induced LTP (Wang and Kelly, 1996a). To test this prediction, microelectrodes were used for both intracellular recordings and postsynaptic co-injections of a CaN-AIP (Hashimoto et al., 1990; Perrino, 1995) plus Ca²⁺/CaM into CA1 neurons of hippocampal slices from adult rats. One typical experiment is shown in Figure 1A,a. Postsynaptic co-injection of Ca²⁺/CaM and CaN-AIP induced synaptic potentiation (*solid circles*), whereas synaptic responses in simultaneous field potential recordings were unchanged; tetanic stimulation induced LTP of f-EPSPs (*open circles*). Figure 1A,b shows the average of group results (*n* = 7).

Synaptic potentiation was induced by co-injecting Ca²⁺/CaM plus CaN-AIP (184 ± 10% relative to baseline values 100%); tetanic stimulation given at 60 min produced a small decrease in synaptic strength (165 ± 8% relative to baseline values, i.e., 19% depotentiation). Simultaneous f-EPSPs are shown in Figure 1*A,c*; synaptic transmission was stable for 60 min and then tetanic stimulation induced LTP (172 ± 6%, *n* = 7). Compared with Ca²⁺/CaM-induced synaptic potentiation (183 ± 9%, *n* = 8) (Wang and Kelly, 1995) (see bar graph *inset* in Fig. 1*A,b*), Ca²⁺/CaM plus CaN-AIP-induced synaptic potentiation was not significantly different (*p* = 0.594) 30–35 min after the beginning of injections. This result is different from our prediction. One explanation is that the co-injection of Ca²⁺/CaM plus CaN-AIP may produce an unknown interaction on CaN activity such that synaptic potentiation is primarily attributable to Ca²⁺/CaM-stimulated CaM-KII and PKC activities.

To better understand the effects of CaN-AIP on synaptic activity, we injected CaN-AIP alone into postsynaptic neurons. One typical experiment is shown in Figure 1*B,a*. The postsynaptic injection of CaN-AIP induced a gradual increase in EPSP slopes (*solid circles*); simultaneous f-EPSPs were stable under basal conditions and tetanus LTP (*open circles*). Average results (*n* = 6) of intracellular recordings during postsynaptic injections of CaN-AIP and simultaneous f-EPSPs are shown in Figures 1*B,b* and 1*B,c*, respectively. Synaptic potentiation induced by injecting CaN-AIP was 212 ± 14% relative to baseline. Tetanic stimulation to presynaptic axons making synaptic connections on CaN-AIP-injected neurons after 60 min decreased synaptic potentiation (162 ± 8%, i.e., 50% depotentiation). f-EPSP slopes did not change within the 60 min period of CaN-AIP injections, but increased after tetanic stimulation (177 ± 4%). Interestingly, compared with synaptic potentiation induced by injecting Ca²⁺/CaM plus CaN-AIP, potentiation induced by injecting CaN-AIP alone was significantly greater (*p* = 0.00133). These results urged us to study the mechanisms underlying synaptic potentiation induced by inhibiting postsynaptic CaN activity.

CaN-inhibited synaptic potentiation is developmentally regulated

Recent reports have shown that the manipulation of inhibiting postsynaptic CaN activity produces different actions on synaptic plasticity. FK-506 or cypermethrin prevents LTD induction in hippocampal slices from young rats (2–3 weeks old) (Mulkey et al., 1994) or LTP induction in hippocampal slices from young guinea pigs (2 weeks old) (Wang and Stelzer, 1994). In these studies, postsynaptic injections of CaN inhibitors did not affect basal synaptic transmission. These results together with our own findings (Fig. 1) (Wang and Kelly, 1996a) suggest that CaN activity regulates basal synaptic transmission only when hippocampal CaN has reached more mature levels at approximately postnatal day 24 (P24) (Tallant and Cheung, 1983; Polli et al., 1991; Wang and Kelly, 1996a). To examine this hypothesis, we injected FK-506 into CA1 neurons in hippocampal slices from either adult or young rats. FK-506 inhibits CaN activity by binding to FK-506-binding proteins (e.g., FKBP-12) and is a much more potent (IC₅₀ = ~30 nM) (Wiederrecht et al., 1989; Schreiber and Crabtree, 1992; Steiner et al., 1992; Dawson et al., 1994; MacKintosh and MacKintosh, 1994) than CaN-AIP (IC₅₀ = ~10 μM) (Hashimoto et al., 1990). Under voltage-clamp recordings with pipettes containing 50 μM FK-506, synaptic responses (EPSC slope) gradually increased over 32 min (220 ± 16%; *n* = 6) (Fig. 2*A, solid circles*) in adult CA1 neurons; subsequent tetanic stimulation produced an attenuation of synaptic potentiation (189 ±

17%, i.e., 31% depotentiation; *p* = 0.021). Simultaneous extracellular f-EPSPs were stable during the initial 32 min and expressed LTP after tetanic stimulation (155 ± 5%; *n* = 6) (Fig. 2*B*). In control experiments using 50 μM rapamycin (in 0.1% ethanol/2 M KAc), which combines with the CaN anchoring protein FKBP-12 but does not inhibit CaN activity (Wiederrecht et al., 1989; Schreiber and Crabtree, 1992; MacKintosh and MacKintosh, 1994), synaptic responses were unchanged for 60 min (Fig. 2*A, gray circles*) and were potentiated by tetanic stimulation (184 ± 10%; *n* = 5, 3 from 22- to 28-d-old rats and 2 from adult rats). These controls indicate that postsynaptic injections of 50 μM rapamycin in 0.1% ethanol into CA1 neurons from young and adult slices did not affect basal synaptic transmission or tetanus LTP.

Next we examined the effect of postsynaptic injections of FK-506 into CA1 neurons on synaptic transmission in hippocampal slices from young rats (2–3 weeks old). As shown in Figure 2*C*, EPSC slopes did not significantly change during voltage-clamp recordings with pipettes containing 50 μM FK-506, and tetanic stimulation at 32 min induced no synaptic potentiation (*n* = 6). Simultaneous f-EPSPs showed tetanus-induced LTP (138 ± 7% relative to baseline, *n* = 6) (Fig. 2*D*). The block of tetanus LTP by FK-506 in young slices was not attributable to the ethanol solvent (see end of preceding paragraph). These results support the notion that the inhibition of postsynaptic CaN activity enhances basal synaptic transmission only in adult animals; i.e., synaptic potentiation induced by inhibiting postsynaptic CaN activity is age-dependent. Our results also support a previous study in which postsynaptic injections of cypermethrin (a CaN inhibitor with IC₅₀ = ~40 pM) prevented LTP induction in hippocampal slices from young guinea pigs (Wang and Stelzer, 1994), although another study showed that bath application of FK-506 did not affect LTP induction in young rats (Mulkey et al., 1994). It is noteworthy that the magnitude of tetanus LTP (f-EPSP slopes) in slices from adult (155–184%) was significantly greater than that from young rats (138%; *p* < 0.01). This indicates that mechanisms underlying LTP magnitude are still developing during this early stage.

Synaptic potentiation induced by inhibiting postsynaptic CaN requires Ca²⁺

One explanation for CaN-inhibited synaptic potentiation is that the basal activity of protein kinases that strengthen synaptic transmission becomes dominant during the inhibition of CaN. We have shown that inhibiting postsynaptic CaM-KII and PKC activities under basal conditions does not alter synaptic strength (Wang and Kelly, 1996a), which suggests that basal CaM-KII and PKC activities are not high enough to modulate basal synaptic transmission, or their function is masked by high CaN activity. Because CaM-KII and PKC activities increase after LTP induction (Akers et al., 1986; Fukunaga et al., 1993) and are required for tetanus-induced LTP (Malinow et al., 1988, 1989; Malenka et al., 1989; Feng and Wang, 1992; Wang and Feng, 1992; Wang and Kelly, 1996a), it would seem logical that tetanus-induced LTP should be greater than synaptic potentiation induced by inhibiting postsynaptic CaN. This prediction was not supported by our results, which showed that CaN-inhibited synaptic potentiation is greater in magnitude than tetanus LTP (Wang and Kelly, 1996a) (see also Figs. 1, 2*A*).

Alternatively, inhibiting postsynaptic CaN activity may trigger an amplification cascade (positive feedback) that produces greater CaN-inhibited synaptic potentiation compared with tetanus LTP. Because synaptic potentiation induced by inhibiting postsynaptic CaN activity appears in a few minutes (Figs. 1, 2), it seems likely

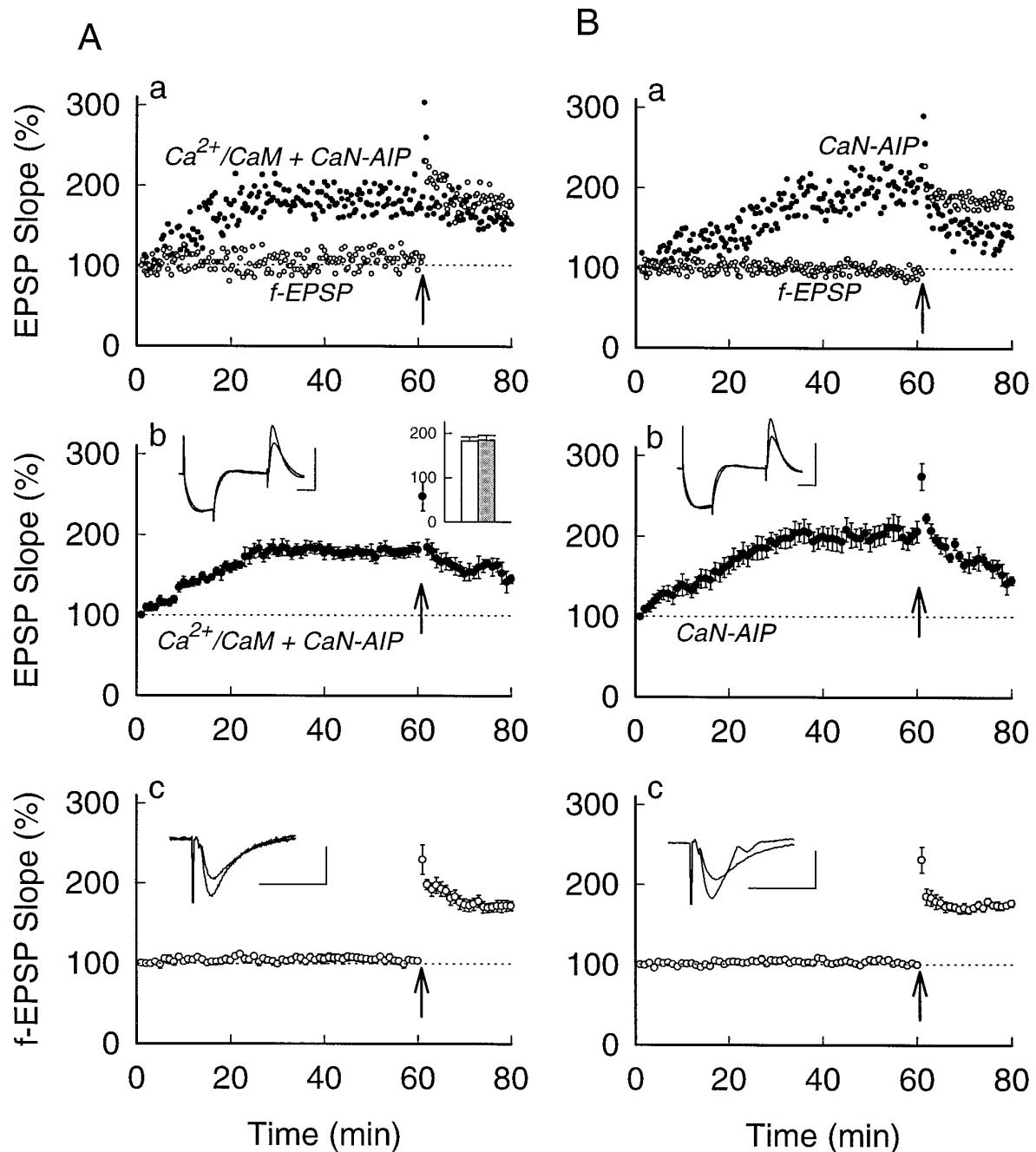


Figure 1. Postsynaptic injections of Ca²⁺/CaM and/or CaN-AIP induce synaptic potentiation. *A, a*, Representative experiment showing potentiated EPSPs induced by postsynaptic injection of Ca²⁺/CaM plus CaN-AIP (solid symbols) and stable synaptic transmission followed by tetanus-induced LTP during simultaneous f-EPSPs (open symbols). *A, b*, Average synaptic potentiation induced by injecting Ca²⁺/CaM plus CaN-AIP ($n = 7$). Inset waveforms show potentiated EPSPs and unchanged input resistance. Calibration: 15 mV/30 msec. Inset bar graph compares the magnitude between Ca²⁺/CaM-induced synaptic potentiation (white bar; value from Wang and Kelly, 1995) and Ca²⁺/CaM plus CaN-AIP-induced synaptic potentiation (gray bar). *A, c*, Normalized f-EPSP slopes recorded during injection of Ca²⁺/CaM plus CaN-AIP ($n = 7$). Waveforms show tetanus-induced LTP. Calibration: 1 mV/20 msec. *B, a*, Representative experiment showing synaptic potentiation induced by postsynaptic injection of CaN-AIP (solid symbols) and simultaneous f-EPSPs with stable baseline followed by tetanus-induced LTP (open symbols). *B, b*, Average synaptic potentiation induced by injection of CaN-AIP ($n = 6$). Waveforms show potentiated EPSPs and unchanged input resistance. Calibration: 15 mV/30 msec. *B, c*, Normalized f-EPSP slopes recorded during CaN-AIP injections ($n = 6$). Waveforms show tetanus-induced LTP. Calibration: 1 mV/20 msec. Vertical arrows at 60 min denote tetanic stimulation.

that the primary mechanisms underlying this potentiation rely on intracellular second messenger pathways rather than on the activation of gene expression. To examine the possibility that increases in intracellular Ca²⁺ serve as this amplification cascade, we co-injected BAPTA with FK-506 into postsynaptic CA1 neurons. Figure 3*A* shows the time course of synaptic responses

(EPSC slopes) observed while injecting 50 μ M FK-506 plus 20 mM BAPTA, or 50 μ M FK-506 alone. Compared with FK-506-induced synaptic potentiation at 60 min ($207 \pm 11\%$; $n = 6$), the co-injection of BAPTA significantly attenuated FK-506-induced potentiation ($117 \pm 5\%$; $n = 6$, $p = 0.00079$) and prevented subsequent tetanus-induced LTP ($119 \pm 8\%$ relative to baseline at 20

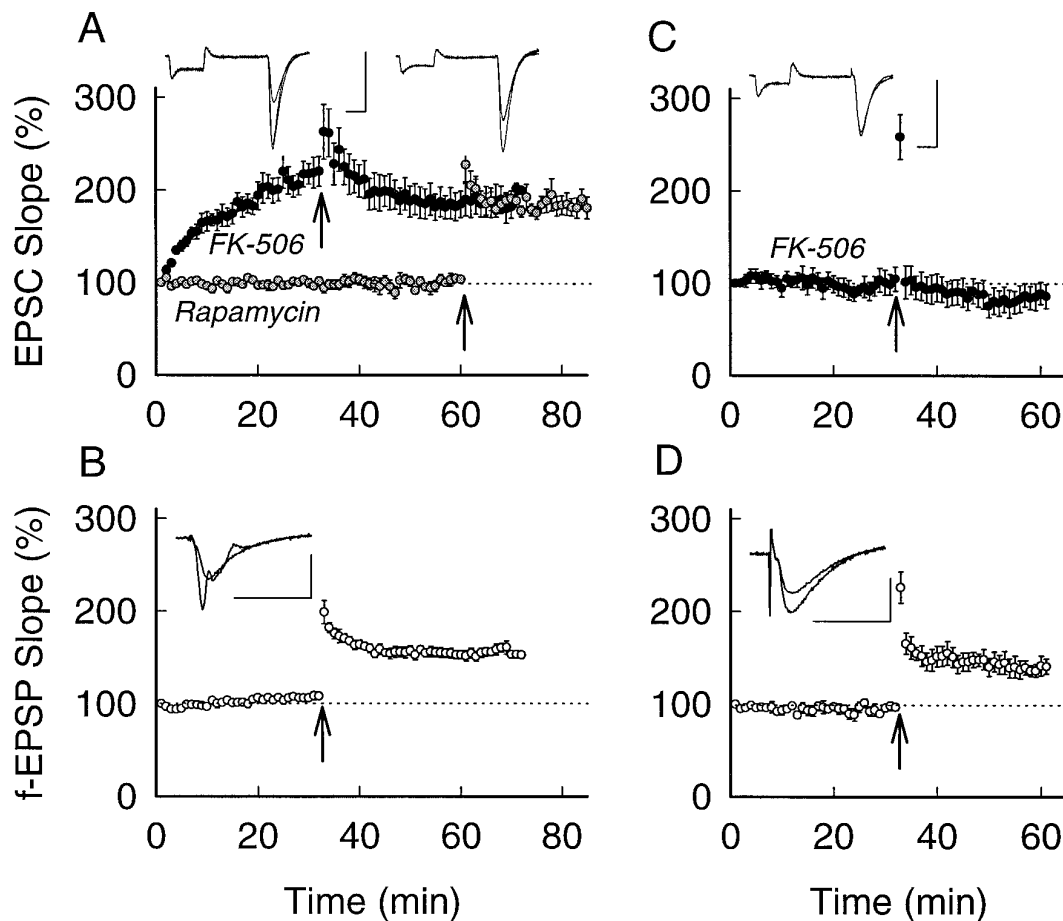


Figure 2. Synaptic potentiation induced by postsynaptic injections of FK-506 is age-dependent. *A*, Increases in EPSC slopes induced by postsynaptic injections of FK-506 and partial depotentiation after tetanic stimulation (solid symbols; $n = 6$); gray symbols show stable synaptic transmission and subsequent tetanus-induced LTP during postsynaptic injections of rapamycin ($n = 5$). Waveforms show FK-506-induced synaptic potentiation (left) and tetanus LTP in rapamycin controls (right). Calibration: 250 pA/30 msec. *B*, Stable f-EPSP slopes recorded during FK-506 injections and subsequent tetanus-induced LTP. Waveforms show tetanus LTP. Calibration: 1 mV/20 msec. Results in *A* and *B* were obtained using slices from adult rats. *C*, Postsynaptic injections of FK-506 into CA1 neurons in young slices (14–21 d) did not significantly change synaptic transmission and prevented tetanus LTP ($n = 6$). Inset shows representative waveforms. Calibration: 200 pA/30 msec. *D*, f-EPSPs during FK-506 injections in young slices show stable synaptic transmission and tetanus LTP. Waveforms show tetanus LTP. Calibration: 1 mV/20 msec. Vertical arrows denote tetanic stimulation.

min post-tetanus). Simultaneous f-EPSPs showed stable synaptic transmission and tetanus-induced LTP ($167 \pm 6\%$). This result indicates that postsynaptic Ca^{2+} is involved in CaN-inhibited synaptic potentiation. Because the dissociation constant of BAPTA (K_d) for Ca^{2+} is 107 nM (Tsien, 1980), and postsynaptic injection of BAPTA does not affect basal synaptic transmission (Wang and Kelly, 1996b), BAPTA may not efficiently chelate intracellular Ca^{2+} under resting conditions (10–100 nM). Thus, the block of CaN-inhibited synaptic potentiation by BAPTA indicates that an increase in intracellular Ca^{2+} may be associated.

Ca^{2+} release by IP_3 R and RyR is required for FK-506-induced synaptic potentiation

What are potential sources of intracellular Ca^{2+} required for FK-506-induced synaptic potentiation? The increase of cytoplasmic Ca^{2+} may come from influx via NMDA receptor channels or voltage-gated calcium channels and/or release from intracellular stores via inositol-1,4,5-triphosphate (IP_3) and ryanodine receptor channels (Ghosh and Greenberg, 1995). Our results suggest that voltage-gated calcium channels may not play an important role in FK-506-induced potentiation, because they should not be significantly activated during our experiments under the condi-

tions of voltage-clamp recordings and low frequency (0.05 Hz) of test stimuli without depolarizing pulses (Figs. 2–4). Reports have shown that phosphatase inhibitors enhanced currents through NMDA receptor channels (Lieberman and Mody, 1994; Wang and Salter, 1994; Wang et al., 1994), and CaN activated by Ca^{2+} influx shortens opening time (Lieberman and Mody, 1994). It seems reasonable that the inhibition of postsynaptic CaN activity could enhance NMDA receptor currents and produce increases in intracellular Ca^{2+} . To test this possibility, we conducted experiments injecting FK-506 into postsynaptic neurons in the presence of 40 μ M D-AP5 (an NMDA receptor antagonist). Hippocampal slices were perfused with D-AP5-containing standard solution starting 30 min before recordings. Figure 4*A* shows the effect of D-AP5 on postsynaptic injections of FK-506 (50 μ M in pipettes). Robust synaptic potentiation was routinely induced by FK-506 ($211 \pm 12\%$ at 40 min; $n = 5$) (Fig. 4*A*). It is interesting that FK-506-induced synaptic potentiation in the presence of D-AP5 appeared not to be attenuated by tetanic stimulation ($237 \pm 20\%$ at 72 min; $p = 0.0383$). This is different from our results in the absence of D-AP5 in that tetanic stimulation produced synaptic depotentiation (19–50%, Figs. 1, 2). Simultaneous f-EPSPs

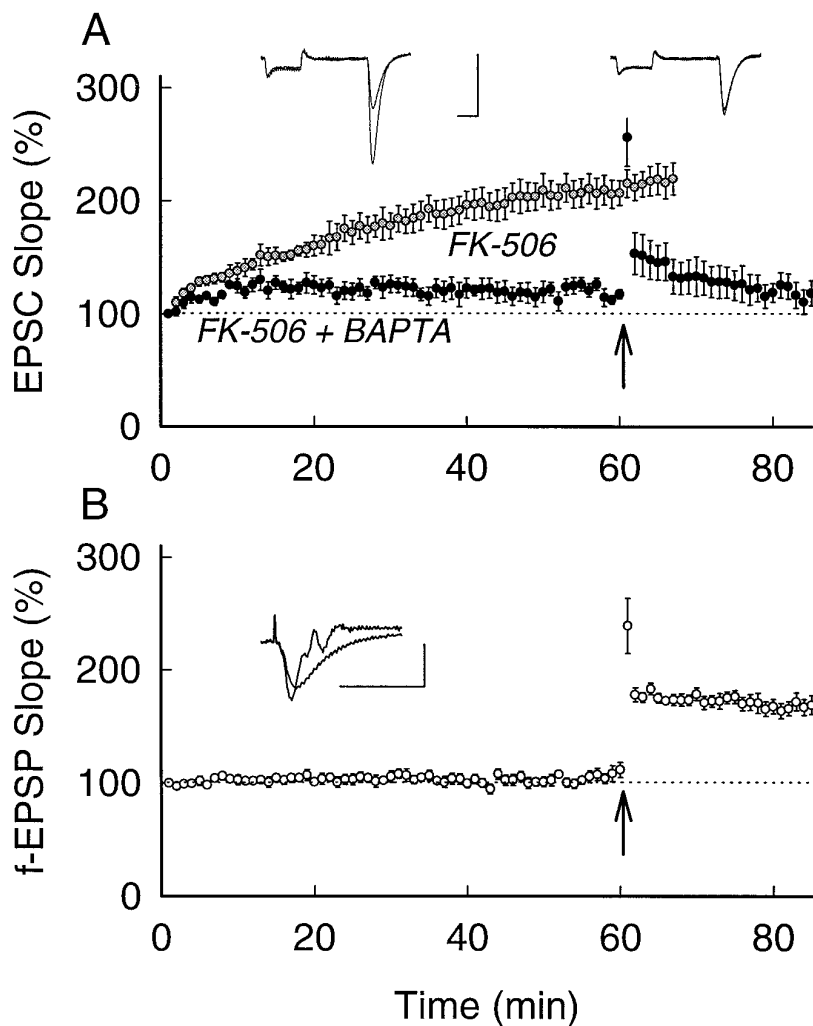


Figure 3. Postsynaptic co-injection of BAPTA attenuates FK-506-induced synaptic potentiation. *A*, Gray symbols show FK-506-induced synaptic potentiation without tetanic stimulation (this data also present in Figs. 4C, 5A, 5C) ($n = 6$); solid symbols show results from co-injections of BAPTA with FK-506, which attenuates FK-506-induced potentiation and blocks tetanus LTP. Waveforms show FK-506-induced synaptic potentiation (left) and its attenuation by BAPTA (right). Calibration: 250 pA/30 msec. *B*, f-EPSP slopes recorded during co-injection of BAPTA with FK-506 are stable and exhibit tetanus LTP. Waveforms show LTP. Calibration: 1 mV/20 msec. Vertical arrows denote tetanic stimulation.

showed stable synaptic transmission, and tetanic stimulation failed to induce LTP ($127 \pm 7\%$ relative to $111 \pm 9\%$ before tetanus; $p = 0.11$) (Fig. 4B) in the presence of D-AP5. These results indicate that the postsynaptic Ca²⁺ requirement for FK-506-induced synaptic potentiation does not depend on NMDA receptor activity; however, the depotentiation of FK-506-induced synaptic potentiation after tetanic stimulation seems to require NMDA receptor activity.

Next we examined the possibility that Ca²⁺ release from intracellular stores via IP₃ and ryanodine receptor channels contributes to FK-506-induced synaptic potentiation. Heparin and dantrolene, which block activities of IP₃ and ryanodine receptors, respectively (Ohta, 1990; Smith and Gallacher, 1994), were co-injected with FK-506 into CA1 neurons. Figure 4C shows the time course of synaptic responses (EPSC slopes) while injecting 50 μ M FK-506 plus 300 μ M heparin/80 μ M dantrolene, or FK-506 alone. Compared with FK-506-induced synaptic potentiation at 60 min ($207 \pm 11\%$; $n = 6$), postsynaptic co-injections of heparin/dantrolene significantly attenuated FK-506-induced synaptic potentiation ($124 \pm 6\%$; $n = 8$, $p = 0.0009$) and prevented additional potentiation by subsequent tetanic stimulation ($119 \pm 8\%$). Simultaneous f-EPSPs showed stable synaptic transmission and tetanus-induced LTP ($164 \pm 9\%$) (Fig. 4D). Postsynaptic injections of heparin plus dantrolene did not affect basal synaptic transmission but did prevent the induction of tetanus LTP ($n = 5$;

data not shown). These results indicate that postsynaptic Ca²⁺ required for FK-506-induced synaptic potentiation is primarily released from intracellular stores via IP₃ and ryanodine receptor channels.

Ca²⁺/CaM signaling pathways and CaM-KII/PKC activities are required for FK-506-induced synaptic potentiation

Because CaM is an important and ubiquitous target of free calcium, i.e., Ca²⁺/CaM formation during Ca²⁺ increases, the role of Ca²⁺/CaM signal pathways in FK-506-induced synaptic potentiation was examined. We first used a high-affinity CBP (Hanley et al., 1988; Kelly et al., 1989) to block intracellular cascades initially triggered by Ca²⁺/CaM. Microelectrodes containing 100 μ M CBP plus 50 μ M FK-506 in 2 M KAc were used for intracellular recordings and postsynaptic co-injections into CA1 neurons. Figure 5A shows the time course of synaptic responses (EPSC slopes) while injecting FK-506 plus CBP, or FK-506 alone. Compared with FK-506-induced synaptic potentiation after 60 min of injection ($207 \pm 11\%$; $n = 6$), postsynaptic co-injections with CBP significantly eliminated FK-506-induced synaptic potentiation ($105 \pm 14\%$; $n = 7$, $p = 0.00004$) and prevented subsequent tetanus-induced LTP ($97 \pm 6\%$). Simultaneous f-EPSPs showed stable synaptic transmission and tetanus-induced LTP ($165 \pm 5\%$) (Fig. 5B). These results indicate that biologically

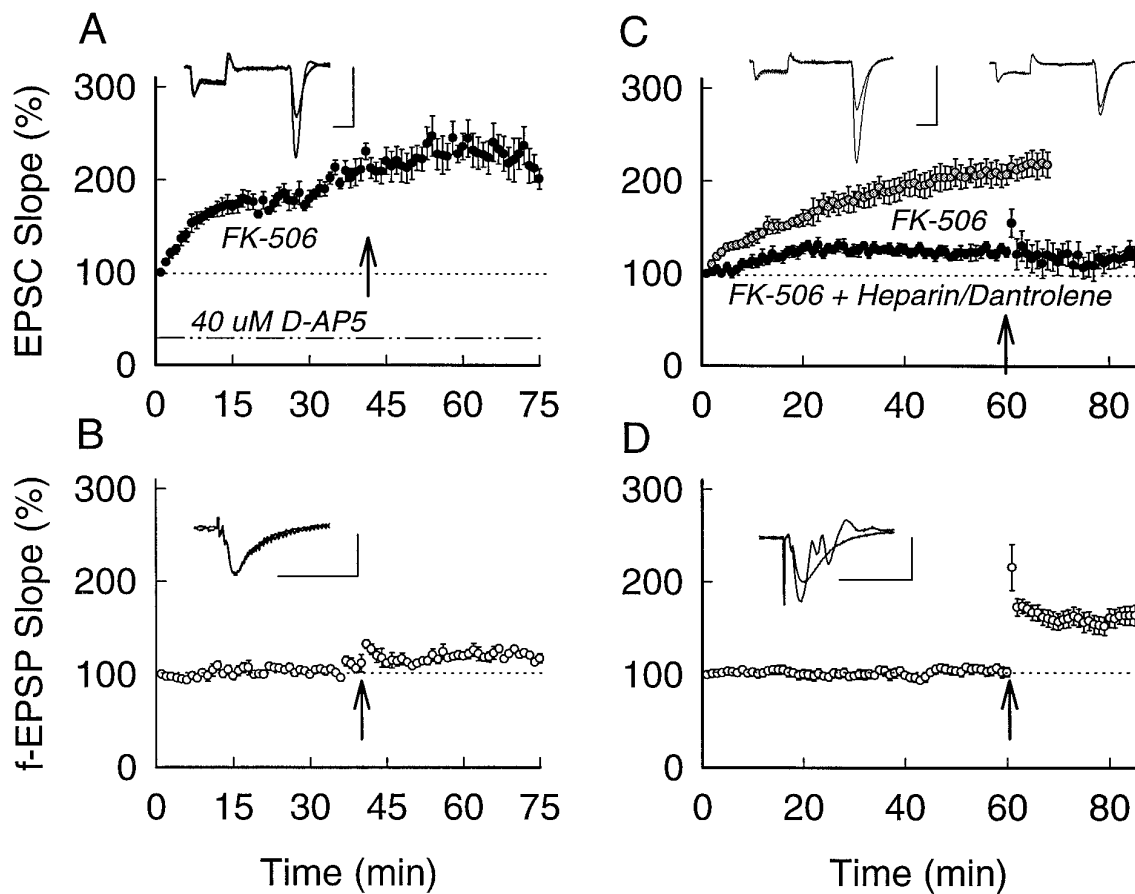


Figure 4. FK-506-induced synaptic potentiation does not require NMDA receptor activation and is attenuated by co-injection of heparin/dantrolene. *A*, Postsynaptic injections of FK-506 in the presence of $40 \mu M$ D-AP5; D-AP5 and tetanic stimulation at 40 min did not affect FK-506-induced synaptic potentiation ($n = 5$). Waveforms show potentiated EPSCs. Calibration: $250 \text{ pA}/30 \text{ msec}$. *B*, Stable f-EPSP slopes during FK-506 injections, and tetanic stimulation at 40 min produced no LTP in the presence of D-AP5. Inset shows representative waveforms. Calibration: $1 \text{ mV}/20 \text{ msec}$. *C*, Co-injections of heparin/dantrolene with FK-506 attenuate FK-506-induced potentiation and block tetanus-induced LTP (solid symbols, $n = 8$); gray symbols show FK-506-induced synaptic potentiation ($n = 6$). Waveforms show FK-506 potentiation (left) and its attenuation by heparin/dantrolene (right). Calibration: $250 \text{ pA}/30 \text{ msec}$. *D*, f-EPSP slopes recorded during co-injections of heparin/dantrolene with FK-506 are stable and exhibit tetanus-induced LTP. Inset shows representative waveforms. Calibration: $1 \text{ mV}/20 \text{ msec}$. Vertical arrows denote tetanic stimulation.

active Ca^{2+} /CaM complexes serve as functional elements in FK-506-induced synaptic potentiation.

We have shown that Ca^{2+} /CaM-induced synaptic potentiation requires CaM-KII and PKC activities (Wang and Kelly, 1995). In addition, neuronal IP_3 and ryanodine receptors, which mediate the mobilization of intracellular Ca^{2+} stores, are phosphorylated by PKC and CaM-KII, and the distinct PKC phosphorylation site on IP_3 receptors is dephosphorylated by CaN (Cameron et al., 1995; Furuichi and Mikoshiba, 1995; Hain et al., 1995; Snyder and Sabatini, 1995). The phosphorylation of IP_3 receptors by either of these kinases appears to increase their sensitivity to IP_3 (Cameron et al., 1995). We speculated that inhibiting postsynaptic CaN activity could result in increased release of Ca^{2+} from intracellular stores, and subsequent increases in Ca^{2+} /CaM levels and CaM-KII and PKC activities, which then enhance protein phosphorylation and induce synaptic potentiation. To test this possibility, we co-injected autoinhibitory peptides of CaM-KII and PKC, [Ala²⁸⁶]CaM-KII_{281–302} ($100 \mu M$) and PKC_{19–31} ($100 \mu M$), together with $50 \mu M$ FK-506 into postsynaptic neurons. Figure 5C shows the time course of synaptic responses (EPSC slopes) while injecting FK-506 plus [Ala²⁸⁶]CaM-KII_{281–302} and PKC_{19–31}, or FK-506 alone. Compared with FK-506-induced synaptic potenti-

ation at 60 min ($207 \pm 11\%$; $n = 6$), CaM-KII and PKC inhibitors significantly attenuated FK-506-induced synaptic potentiation ($123 \pm 11\%$; $n = 7$, $p = 0.0004$) and prevented subsequent tetanus-induced LTP ($109 \pm 11\%$). Simultaneous f-EPSPs showed stable synaptic transmission and tetanus-induced LTP ($173 \pm 13\%$) (Fig. 5D). These results indicate that postsynaptic CaM-KII and PKC activities are required for synaptic potentiation induced by inhibiting postsynaptic CaN activity.

DISCUSSION

Our results clearly show that the inhibition of postsynaptic CaN activity by injecting CaN-AIP or FK-506 induces synaptic potentiation at CA1 synapses. This potentiation appears to require Ca^{2+} release from intracellular stores, the participation of Ca^{2+} /CaM, and the activities of CaM-KII and PKC in postsynaptic neurons. Together with biochemical studies showing that IP_3 and ryanodine receptors are phosphorylated by PKC and CaM-KII and dephosphorylated by CaN (Cameron et al., 1995; Furuichi and Mikoshiba, 1995; Hain et al., 1995; Snyder and Sabatini, 1995) and the phosphorylation of IP_3 receptors appears to increase their sensitivity to IP_3 (Cameron et al., 1995), we outline a scheme responsible for CaN-inhibited synaptic potentiation (Fig. 6). The

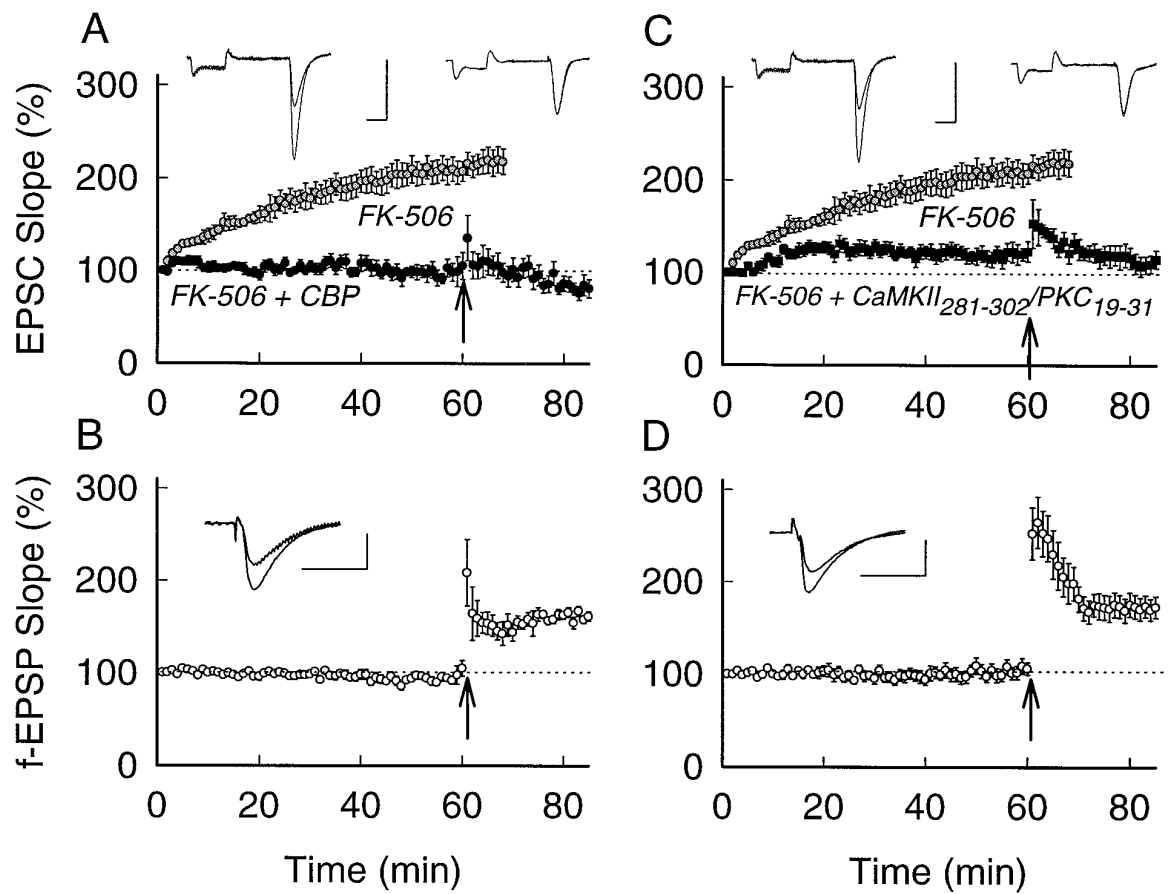


Figure 5. CaM antagonist (*CBP*) or autoinhibitory peptides of CaM-KII/PKC attenuate FK-506-induced synaptic potentiation. *A*, Co-injections of *CBP* with FK-506 block FK-506-induced potentiation and tetanus LTP (solid symbols, $n = 7$); gray symbols show FK-506-induced synaptic potentiation ($n = 6$). Waveforms show FK-506 potentiation (left) and its inhibition by *CBP* (right). Calibration: 250 pA/30 msec. *B*, f-EPSP slopes recorded during co-injections of *CBP* with FK-506 are stable and exhibit tetanus-induced LTP. Inset shows representative waveforms. Calibration: 1 mV/20 msec. *C*, Co-injections of [Ala^{286}]CaM-KII_{281–302} and PKC_{19–31} with FK-506 attenuate FK-506-induced potentiation and block tetanus LTP (solid symbols, $n = 7$); gray symbols show FK-506-induced synaptic potentiation ($n = 6$). Waveforms show FK-506 potentiation (left) and its attenuation by [Ala^{286}]CaM-KII_{281–302} and PKC_{19–31} (right). Calibration: 250 pA/30 msec. *D*, f-EPSP slopes recorded during co-injections of [Ala^{286}]CaM-KII_{281–302} and PKC_{19–31} with FK-506 are stable and exhibit tetanus-induced LTP. Inset shows representative waveforms. Calibration: 1 mV/20 msec. Vertical arrows denote tetanic stimulation.

inhibition of postsynaptic CaN increases the ratio of active CaM-KII and PKC to CaN and facilitates the action of CaM-KII and PKC in phosphorylating protein substrates that contribute to synaptic potentiation (e.g., glutamate receptors) and Ca^{2+} increases from intracellular stores by IP_3 and ryanodine receptor channels. Increased intracellular Ca^{2+} promotes the formation of Ca^{2+} /CaM complexes and further activation of CaM-KII and PKC. This network constitutes an amplification cascade (positive feedback) that is triggered by inhibiting CaN activity and may lead to unlimited potentiation of synaptic transmission. However, IP_3 and ryanodine receptor-mediated mobilization of intracellular Ca^{2+} is stimulated in a Ca^{2+} -dependent manner with a bell-shaped range of Ca^{2+} concentrations; i.e., high Ca^{2+} inhibits Ca^{2+} release (Bootman and Berridge, 1995; Ehrlich, 1995; Furuichi and Mikoshiba, 1995; Simpson et al., 1995). This may explain why synaptic potentiation appeared to be saturated in our experiments (Figs. 1–5).

One could argue that CaN-inhibited synaptic potentiation only results from basal CaM-KII/PKC activities (Ocorr and Schulman, 1991; Huber, 1995) instead of from an absolute increase in their activities by positive feedback. Because inhibiting postsynaptic CaM-KII/PKC activities does not significantly alter basal synaptic

transmission (Tsien et al., 1990; Feng and Wang, 1992; Wang and Kelly, 1996), basal CaM-KII/PKC activities are not high enough to strengthen synaptic transmission. In addition, tetanus LTP is associated with increases of protein kinase activities (Akers et al., 1986; Fukunaga et al., 1993), and CaN-inhibited synaptic potentiation, which requires CaM-KII/PKC activities, is larger than tetanus LTP (Wang and Kelly, 1996). We suggest that basal CaM-KII and PKC activities may not be the major contributor to the large synaptic potentiation induced by inhibiting CaN activity. Instead, our results suggest that inhibiting CaN not only shifts the equilibrium between protein kinase and phosphatase activities to favor the phosphorylation of substrates responsible for synaptic transmission but, more importantly, triggers positive-feedback cascades that produce long-term increases in synaptic strength.

Postsynaptic application of CaN inhibitors prevented the induction of LTD (Mulkey et al., 1993, 1994), LTP, and synaptic disinhibition (Wang and Stelzer, 1994, 1996) in young animals without changing basal synaptic transmission. Why do postsynaptic injections of CaN inhibitors not induce synaptic potentiation in young animals? Examining the effect(s) of inhibiting CaN on excitatory synaptic transmission in CA1 neurons of hippocampal slices from young rats (14–21 d old), we observed that postsyn-

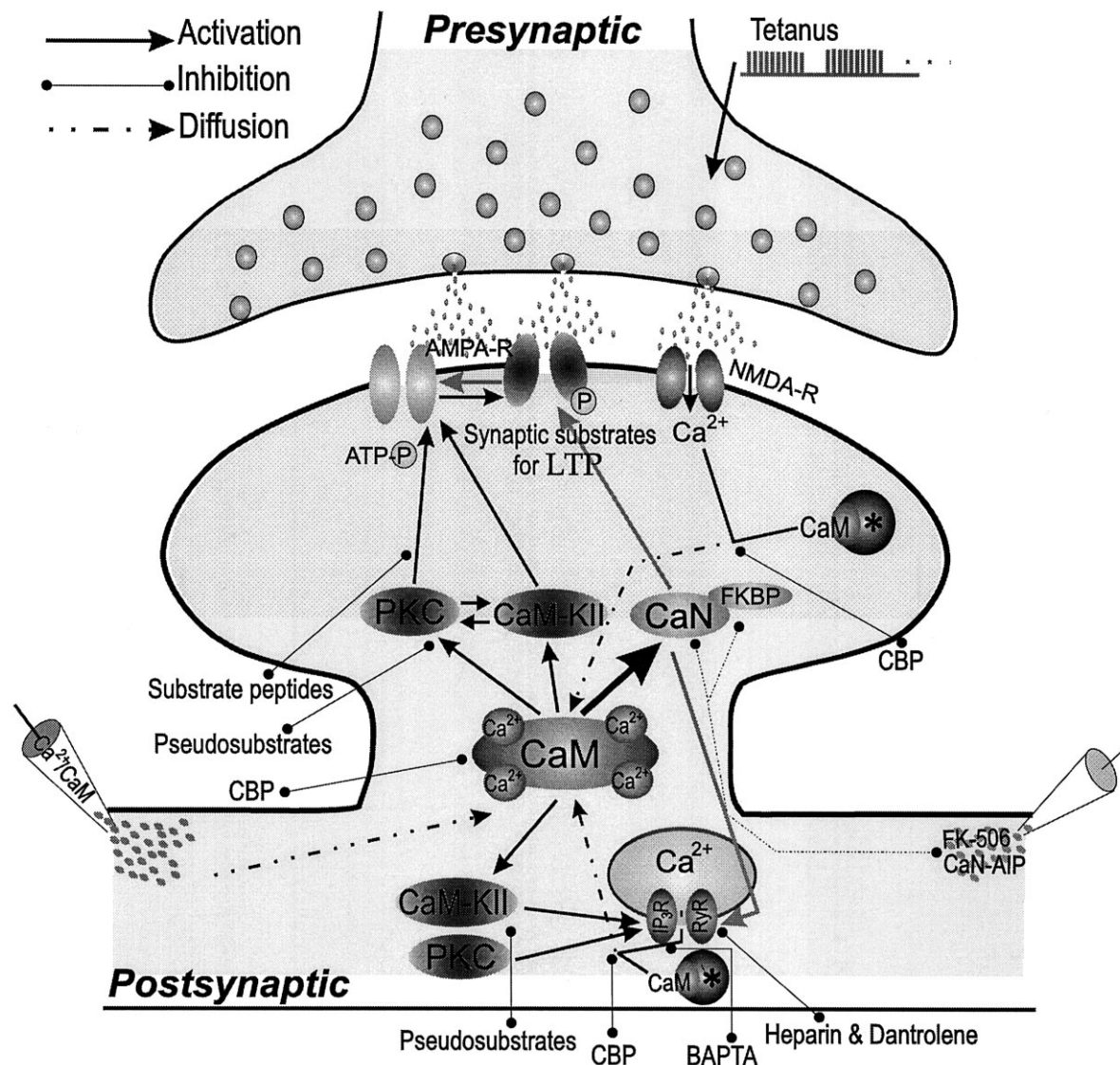


Figure 6. Postsynaptic Ca²⁺/CaM pathways play key roles in regulating synaptic strength. Ca²⁺/CaM is involved in three forms of synaptic potentiation, including tetanus LTP, Ca²⁺/CaM-induced potentiation, and CaN-inhibited potentiation. (1) NMDA receptor (*NMDA-R*) activation by tetanus permits Ca²⁺ influx into postsynaptic neuron and increases Ca²⁺/CaM complexes. (2) Postsynaptic injection of Ca²⁺/CaM (*bottom left*) elevates Ca²⁺/CaM concentrations. (3) Postsynaptic injection of CaN inhibitors (*CaN-AIP* or *FK-506*; *bottom right*) reduce basal CaN activity, facilitates the action of CaM-KII and PKC activities on IP₃ and ryanodine receptors, produces more Ca²⁺ release, and elevates Ca²⁺/CaM concentrations. The Ca²⁺ and Ca²⁺/CaM concentrations under basal conditions make CaN highly active (*thick arrow*), whereas Ca²⁺/CaM increased by LTP-inducing protocols primarily stimulates CaM-KII and/or PKC. Synaptic substrates phosphorylated by CaM-KII and PKC contribute to synaptic potentiation; the phosphorylation of IP₃ and ryanodine receptors by CaM-KII and PKC further facilitate Ca²⁺ release from intracellular stores and activation of Ca²⁺/CaM pathways. The decreased dephosphorylation of synaptic substrates during CaN inhibition shifts the equilibrium to phosphorylation for synaptic potentiation. *Asterisks*, Endogenous CBP; *AMPA-R*, AMPA receptors; *light gray arrows*, dephosphorylation. This scheme is supported by our results using postsynaptic co-injections of inhibitors that block these cascades (see text) (Wang and Kelly, 1995, 1996a).

aptic injections of FK-506 did not induce synaptic potentiation and prevented tetanus LTP (Fig. 2*B*). The inability of FK-506 to induce potentiation at this age may be attributable to CaN and/or FKBP being functionally immature, because CaN expression (activity and protein mass) dramatically increases during P10–21 and reaches plateau levels by P24 Polli et al., 1991; Tallant and Cheung, 1983), and the developmental expression of FKBP-12 parallels CaN (P. Kelly and J.-H. Wang, unpublished observations). Therefore, we postulate that CaN activity limits synaptic transmission at basal levels only after CaN and FKBP expression is mature and they are functionally co-localized. Why does inhib-

iting CaN activity in young animals produce multiple results, i.e., prevent induction of LTD, LTP, and synaptic disinhibition (LTD of inhibitory synapses by tetanus)? We propose that although the developing pattern of Ca²⁺-dependent protein kinases in brain is similar to that for CaN, their expression is not synchronous (Turner et al., 1984; Kelly and Vernon, 1985; Kelly et al., 1987; Hashimoto, 1988; Yoshida et al., 1988; Hirata et al., 1991) such that shifting the equilibrium between kinases and CaN in postnatal versus adult brain produces different effects on synaptic plasticity. CaN activity may play a dual role in synaptic plasticity during postnatal development and is required for LTD and LTP.

In terms of physiological significance, the role of CaN activity in excitatory synaptic potentiation and inhibitory synaptic depression may facilitate the excitatory output of neurons.

Our results indicate that CaN-inhibited synaptic potentiation requires Ca^{2+} release from intracellular stores by IP_3 and ryanodine receptor channels, but not influx via NMDA receptor channels. Because tetanus LTP at CA1 synapses requires NMDA receptor activation (Collingridge et al., 1983a,b), one could argue that CaN-inhibited synaptic potentiation is mechanistically different from tetanus LTP. Because CaN-inhibited synaptic potentiation occludes LTP (Figs. 1, 2A), we suggest that they share common mechanisms. This suggestion is supported in that postsynaptic injections of heparin and dantrolene attenuate CaN-inhibited synaptic potentiation (Fig. 4C) and prevent LTP induction (data not shown), and bath application of dantrolene blocked tetanus LTP (Obenaus et al., 1989). At mechanistic levels, how do we explain the similarities and differences between CaN-inhibited synaptic potentiation and tetanus LTP? Although positive results from occlusion experiments suggest that two forms of synaptic potentiation share common mechanisms (Malenka et al., 1986; Gustafsson et al., 1988; Muller et al., 1988; Wang and Feng, 1992; Wang and Kelly, 1995), this does not mean that they are identical at every step in sequential and/or parallel pathways. The best understanding of occlusion results is that the mechanisms underlying two forms of synaptic potentiation are “common” only in the final key expression element(s). CaN-inhibited synaptic potentiation starts at Ca^{2+} and Ca^{2+}/CaM and employs downstream targets (e.g., CaM-KII, PKC and IP_3 receptors), which are essential for tetanus LTP. Thus, CaN-inhibited synaptic potentiation and tetanus LTP share common signaling pathways beginning with increased intracellular Ca^{2+} and Ca^{2+}/CaM to their final expression element(s) through multiple postsynaptic protein kinase cascades.

Tetanic stimulation given 32–60 min after CaN-inhibited synaptic potentiation does not induce additional synaptic potentiation (Figs. 1, 2A). However, tetanus after 4 min of injecting CaN inhibitor increases the magnitude of tetanus LTP (Wang and Kelly, 1996a). We postulate that the postsynaptic injection of FK-506 for 4 min results in partial inhibition of CaN activity and small synaptic potentiation, which is not sufficient to initiate secondary mechanisms such as Ca^{2+} release from intracellular stores. Under this condition, tetanic stimulation activates protein kinases and induces larger synaptic potentiation than tetanus LTP. The injection of FK-506 over 30 min produces substantial inhibition of CaN and secondary activation of protein kinases to induce large synaptic potentiation. Under this situation, additional kinase activation by tetanus does not enhance synaptic potentiation but causes depotentiation. Thus, the different ratios of postsynaptic protein kinase and phosphatase activities may determine the efficiency of an individual synapse as well as the percentage of sampled synapses that actually express plasticity (potentiation or depotentiation).

Our model may also explain why CaN-inhibited synaptic potentiation is greater than tetanus LTP (Wang and Kelly, 1996a), although one could argue that tetanus LTP induced by our protocols does not reach saturation (i.e., the percentage of sampled synapses that are potentiated by tetanus is lower than that by inhibiting CaN activity). Based on the understandings that protein kinase activities play a critical role in synaptic potentiation and that the percentage of potentiated synapses is determined by the phosphorylation of synaptic substrates, the higher ratio of CaM-KII and PKC to CaN activities produced by inhibiting CaN

relative to tetanus increases the proportion of potentiated synapses for a larger synaptic potentiation. However, if the percentage of potentiated synapses in tetanus LTP or CaN-inhibited synaptic potentiation is constant, then higher kinase activities during CaN inhibition enhance synaptic strength to a greater degree at each synapse.

Tetanic stimulation produced partial depotentiation of CaN-inhibited synaptic potentiation (Figs. 1, 2A); this depotentiation did not occur in D-AP5 (Fig. 4A). This indicates that NMDA receptor activation by tetanus during CaN-inhibited synaptic potentiation is responsible for the synaptic depotentiation. Because NMDA receptor activation is essential for induction of tetanus LTP under basal conditions (Collingridge et al., 1983), why does tetanus induce synaptic depotentiation during CaN-inhibited synaptic potentiation? Studies showed that phosphatase inhibitors enhance current through a single NMDA receptor channel (Lieberman and Mody, 1994; Wang and Salter, 1994; Wang et al., 1994) and CaN activated by Ca^{2+} influx shortens opening time of NMDA channels (Lieberman and Mody, 1994). Although our results show that CaN-inhibited synaptic potentiation does not depend on NMDA receptor channels for Ca^{2+} , inhibiting postsynaptic CaN activity may facilitate NMDA receptor activity. Thus, tetanic stimulation during CaN-inhibited synaptic potentiation may evoke excessive NMDA receptor activity and massive Ca^{2+} influx. Such a great increase in intracellular Ca^{2+} may produce Ca^{2+} -dependent inhibition of Ca^{2+} release by IP_3 and ryanodine receptor channels (Bootman and Berridge, 1995; Ehrlich, 1995; Furuichi and Mikoshiba, 1995; Simpson et al., 1995) and/or neurotoxicity attributable to the superactivities of protein kinases (Meldrum and Garthwaite, 1990), which contribute to the depotentiation of CaN-inhibited synaptic potentiation.

CaN also dephosphorylates type II regulatory subunits of cAMP-dependent protein kinase, inhibitor-1 (I-1) of PrP-1, microtubule-associated proteins, and neuromodulin (Klee, 1991), which may indirectly affect synaptic plasticity. Thus, the equilibrium between CaN and CaM-KII/PKC activities may constitute a pivotal balance in regulating synaptic transmission. Phosphorylated I-1 actively inhibits PrP-1, whereas the dephosphorylation of I-1 by CaN increases PrP-1 activity, which dephosphorylates substrate proteins at synapses (Shields et al., 1985). Thus, inhibiting CaN will also decrease PrP-1 activity, and the attenuation of two phosphatase pathways during CaN-inhibited synaptic potentiation may synergistically facilitate the phosphorylation of substrate proteins that contribute to very large synaptic potentiation.

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