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The Balance between Postsynaptic Ca^{2+} -dependent Protein Kinase and Phosphatase Activities Controlling Synaptic Strength

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Abstract

The activities of protein kinases and phosphatases are believed to regulate neuronal activity and synaptic plasticity in brain. Numerous *in vivo* and *in vitro* studies have shown that synaptic strength appears stable under basal conditions and during long-term potentiation (LTP) expression. This may reflect a balance between protein kinase and phosphatase activities. To provide experimental evidence for this hypothesis, and based on our knowledge that Ca^{2+} /CaM activates protein kinases and phosphatases and that postsynaptic Ca^{2+} /CaM signal pathways play important roles in synaptic plasticity, we examined the contribution of postsynaptic Ca^{2+} -dependent protein kinases and calcineurin (CaN) in regulating synaptic strength. We show that inhibiting postsynaptic Ca^{2+} /CaM-dependent protein kinase II (CaM-KII) and Ca^{2+} /phospholipid-dependent protein kinase (PKC) in hippocampal CA1 neurons attenuates significantly the expression of LTP, but not basal synaptic transmission. On the other hand, the inhibition of postsynaptic CaN enhances synaptic transmission at potentiated and naive synapses, and increases significantly the magnitude of synaptic potentiation during the induction phase of LTP. These results indicate that postsynaptic CaM-KII and PKC activities are essential for

maintaining LTP expression, but CaN activity limits synaptic strength at stable levels during both basal and potentiated synaptic transmission; that is, the dynamic balance between protein phosphorylation and dephosphorylation that sets physiological synaptic strength is dominated by CaN activity.

Introduction

Synaptic transmission is one of the basic processes of information exchange among neurons, and activity-dependent enhancement of synaptic efficacy, for example, long-term potentiation (LTP), is suggested to be the cellular basis of learning and memory in the mammalian brain (Bliss and Lynch 1988; Gustafsson and Wigstrom 1988; Bliss and Collingridge 1993; Lisman 1994; Eichenbaum 1995). A detailed understanding of the biochemical processes underlying synaptic transmission and synaptic plasticity is important and awaits experimental elucidation by combining the monitor of synaptic function with molecular manipulation. The process of synaptic transmission is based on the hypothesis that presynaptic terminals release neurotransmitters and postsynaptic receptors respond to them (Kandel 1991; Shepherd 1994). However, it is necessary to know whether intracellular messengers set the strength of "basal" synaptic transmission at certain levels by modulating components responsible for synaptic activity. Furthermore, what mechanisms are responsible for resetting synaptic strength at "saturated" levels during LTP maintenance? Previous results indicated that: (1) Protein phosphorylation and dephosphorylation are ubiquitous reactions control-

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ling biological activities (Krebs 1994; Nestler and Greengard 1984); (2) the activation of many protein kinases and protein phosphorylation are essential to the induction and maintenance of LTP (Akers et al. 1986; Malinow et al. 1988, 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); and (3) protein phosphatase activities are required for long-term depression (LTD) (Mulkey et al. 1993, 1994). The studies herein test the hypothesis that protein phosphatase activity down-regulates synaptic strength by opposing the cascade of kinase activities (second messenger-dependent or constitutively active) that together maintain synaptic transmission at stable levels under basal and potentiated conditions (see Fig. 1A).

It is known that calcium/calmodulin (Ca^{2+} /CaM) and the activities of Ca^{2+} -dependent protein kinases [e.g., Ca^{2+} /CaM-dependent protein kinase II (CaM-KII) and protein kinase C (PKC)] are necessary for LTP induction (Akers et al. 1986; Malinow et al. 1988, Malenka et al. 1989; Feng and Wang 1992; Wang and Feng 1992; Fukunaga et al. 1993). We have further shown that the postsynaptic injection of Ca^{2+} /CaM into hippocampal CA1 neurons induces synaptic potentiation, which uses mechanisms similar to tetanus-induced LTP (Wang and Kelly 1995). These results indicate that postsynaptic Ca^{2+} /CaM signal pathways play critical roles in LTP. Ca^{2+} /CaM activates protein kinases (e.g., CaM-KII) and protein phosphatase [calcineurin (CaN)]; CaN is activated by Ca^{2+} /CaM with a concentration for 50% activation (A_{50}) of 0.1–1 nM, whereas the A_{50} for CaM-KII is 40–100 nM (Cohen 1988; Klee and Cohen 1988; Schulman and Lou 1989; Klee 1991). Because intracellular free Ca^{2+} oscillates in the range of 10–100 nM under resting conditions (Carafoli 1987; Amundson and Clapham 1993; Ghosh and Greenberg 1995), its interaction with B subunit of CaN and CaM makes it likely that CaN is very active under basal conditions. Recent observations have shown that neuronal CaN is active under basal conditions (Steiner et al. 1992; Cameron et al. 1995). Within this context, we hypothesize that postsynaptic CaN activity may contribute significantly to controlling synaptic activity with stable output under basal and potentiated situations. To understand how the balance between protein phosphorylation and dephosphorylation ultimately sets synaptic strength, we examined the role of postsynaptic CaM-KII/PKC and CaN in synaptic transmission by

Figure 1: Tetanus-induced LTP is pathway-specific: experimental protocol. (A) LTP is induced in pathway one (P_1 , $\circ$) by tetanic stimulation (arrow); no change in synaptic strength was observed in nontetanized or naive pathway two (P_2 , $\square$). Synaptic responses in both pathways appear stable. The independence of P_1 and P_2 was determined as described in Materials and Methods. Synaptic responses recorded during the simultaneous stimulation of P_1 plus P_2 under basal conditions are indicated in the bracket (upper left). (B) A diagram (top) showing the protocol used in most experiments (i.e., placement of intra- and extracellular recording electrodes and positions of stimulating electrodes). Alternate stimulation in P_1 and P_2 (Schaffer collateral/commissural fibers) and simultaneous intracellular/field potential recordings in CA1 area of hippocampal slice. The black bar in stratum radiatum between CA1 and CA2 indicates the cut used to “isolate” CA1. Lower panels show waveforms of EPSPs, EPSCs, and f-EPSPs recorded while stimulating P_1 and P_2 individually, or P_1 and P_2 simultaneously.

injecting specific inhibitors into CA1 pyramidal neurons in hippocampal slices. The goal of these manipulations was to determine if activity changes in these signal pathways reset the efficiency of syn-

aptic transmission at basal and potentiated synapses.

Materials and Methods

Transverse hippocampal slices were prepared from male Harlan Sprague–Dawley rats (6–7 weeks old) with a McIlwain tissue chopper in ice-cold standard medium (gassed with 95% O₂/5% CO₂) as described previously (Wang and Kelly 1995,1996). Slices were incubated at 25°C for at least 1 hr, and transferred to a submersion chamber (31°C, 2 ml/min perfusion rate) for electrophysiological experiments. Standard medium contained (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 routinely in the presence of bicucullin 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 activity caused by directly stimulating interneurons. Certain manipulations were employed to prevent neuronal hyperexcitability in the presence of GABAergic antagonists: (1) Isolation of CA1 area was achieved by cutting presynaptic axons in stratum radiatum but not axons in oriens/alveus of hippocampal slices (see Fig. 1B); this optimizes the integrity of CA1 neurons by preventing stress in CA1 neurons from axon injury during preparation of the isolated CA1 region (Kauer et al. 1988; Malenka et al. 1988); and (2) the concentration of Mg²⁺ used was 2.4 mM to limit synaptic hyperactivity. Under these conditions, seizure activity was never observed during basal synaptic transmission and postsynaptic injections; complex waveforms were observed only after tetanic stimulation in some experiments.

Two bipolar tungsten stimulating electrodes (12 MΩ) were used; one was positioned on each side of the recording site in stratum radiatum of CA1 area for orthodromic stimulation of two independent pathways (P₁ and P₂; see Fig. 1B). The frequency of test stimuli was 0.05 Hz, and stimulation was alternated between these two pathways every 2 min. Independent pathways were verified by the observation that EPSP or EPSC amplitudes measured from simultaneous stimulation of both pathways (data points in brackets in figures) ap-

proximated the algebraic sum of EPSPs or EPSCs when P₁ and P₂ were individually stimulated (Fig. 1B). High-frequency tetanic stimulation consisted of 10 trains with 5-sec intervals, and each train contained 10 pulses at 200 Hz. Recording microelectrodes (60–85 MΩ) filled with 2 M potassium acetate (KAc) or 2 M KAc plus pseudosubstrate inhibitory peptides of CaM-KII and PKC, FK-506 (a potent and specific inhibitor of CaN), or rapamycin (a control agent for FK-506) were used for current- or voltage-clamp recordings. Pipettes containing 1M NaCl were used for extracellular field potential recordings in stratum radiatum, and were positioned near intracellular recording sites to verify the viability of tissue slices and status of synaptic activity (Fig. 1B).

Experimental results are based only on neurons in which stable recordings were obtained within the initial 2-min period after impalement, and which exhibited stable membrane potentials between 70 and 73 mV throughout an experiment. In certain experiments (Fig. 4, below), the possible contamination of EPSC measurements by mono- and di-synaptic inhibitory activities was reduced by voltage-clamp recordings (3-KHz sampling rate) holding membrane potentials at -78 mV (i.e., near the GABA_A reversal potential), together with application of GABAergic antagonists. Baseline values (the first data point in each figure) of synaptic transmission were averaged from the initial slope of three synaptic responses (EPSPs, EPSCs, or f-EPSPs) recorded during the first minute after stable intracellular recordings had been established, and defined as 100%. Synaptic responses are represented as mean ± S.E.M. 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 field EPSP slopes. Student's *t*-tests were used for comparisons within the same treatment at different times, and Mann–Whitney rank-sum tests (Glantz 1993) were used for comparisons among different treatments at comparable experimental times. Each waveform was averaged from six consecutive synaptic responses and waveforms in each figure were selected from a representative experiment.

FK-506 and rapamycin were dissolved in 100% ethanol as 50 mM stocks and then diluted in 2 M KAc to a final concentration of 50 μM. Microelectrode solutions containing [Ala²⁸⁶]CaM-

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Figure 2: Inhibition of postsynaptic CaM-KII and PKC attenuates LTP expression but does not affect basal synaptic transmission. (A) The time course of normalized EPSP slopes recorded in sequential intracellular recordings from two CA1 neurons in response to stimulating independent pathways P_1 (circles) and P_2 (squares). In the first part of each experiment, stable intracellular recordings were monitored for 10 min (KAc filled electrode; open symbols); then tetanic stimulation (arrow) was delivered to P_1 , and nondecremental LTP was monitored for 32 min. The KAc-filled electrode was then withdrawn, and a second electrode containing 200 μM $[\text{Ala}^{286}]\text{CaM-KII}_{281-302}$ and 100 μM PKC_{19-31} in 2M KAc was used to impale a second nearby neuron within 20 min (filled symbols). Manipulations were done very carefully to prevent movement of stimulating electrodes and recording pipette used for field potentials. Synaptic responses in P_1 and P_2 from the second neuron were monitored for an additional 100 min. Stimulation intensity was held constant for P_1 and P_2 . EPSP slopes recorded from the second neuron in the two pathways were normalized to the initial three EPSPs recorded from the first neuron of each experiment for the two pathways (dashed line). Synaptic responses in the potentiated pathway (P_1) decreased to near baseline values, and no significant change in the naive pathway (P_2) was observed. Inset graph shows dynamic changes of synaptic responses in P_1 from all experiments (the different symbols represent individual experiments); inset waveforms show representative waveforms of EPSPs for P_1 and P_2 at indicated times (1–4); calibration is 20 mV/30 msec. (B) Simultaneous field potential recordings show stable synaptic transmission in the LTP pathway (P_1 , $\circ$) and naive pathway (P_2 , $\square$) for the same slices in (A); arrow denotes tetanus to P_1 . Inset shows control and potentiated f-EPSPs in P_1 and P_2 ; calibration is 1 mV/20 msec.

$\text{KII}_{281-302}$ (100 μM) and PKC_{19-31} (100 μM) in 2M KAc were similarly prepared. Microelectrodes were filled with a single solution. Bicucullin was purchased from Sigma (St. Louis, MO); picrotoxin was from Research Biochemicals International (Natick, MA); FK-506 and rapamycin were generous gifts from Dr. Stan Stepkowski (University of Texas Medical School at Houston).

Results

The typical behavior of synaptic transmission recorded under basal and potentiated conditions is that synaptic strength is maintained at a stable level (Fig. 1A; see also Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973; Collingridge et al. 1983a,b; Malinow et al. 1988; Malenka et al. 1989; Wang and Feng 1992; Wang and Kelly 1995). We hypothesized that these two stable levels of synaptic strength are mainly controlled by the balance between Ca^{2+} -dependent protein kinases and phosphatase. It was important to establish an experimental model in which basal and potentiated synaptic activities can be recorded in the

same neuron that could also be manipulated biochemically. Such a model allows better statistical comparisons of the effects of certain factors on synaptic function at different synapses under constant experimental conditions. Using these criteria, our experiments were conducted by alternately stimulating two independent Schaffer collateral/commissural (S/C) pathways (P_1 and P_2) while recording synaptic responses from the synapses of one neuron, as well as a population of neurons (Fig. 1B). Tetanic stimulation was given to P_1 to induce LTP, while P_2 was used to monitor basal synaptic transmission (naive pathway) as shown in Figure 1A.

First, we examined the role of protein phosphorylation mediated by postsynaptic CaM-KII and PKC, both Ca^{2+} -dependent protein kinases (Cohen 1988; Huang 1989), in basal and potentiated synaptic transmission. Sharp microelectrodes were used for simultaneous current clamp recordings and postsynaptic injections of pseudosubstrate inhibitory peptides of CaM-KII and PKC. Simultaneous field potential recordings were used to insure that experiments were conducted under

conditions of stable synaptic transmission. Figure 2 shows the experimental protocol and results which involved alternate stimulation in two independent pathways and sequential intracellular recordings from two nearby CA1 neurons. In the first part of every experiment ($n=7$, open symbols), we used a 2M KAc-containing microelectrode to impale a CA1 neuron. After obtaining control values of basal synaptic transmission and being sure that synaptic responses in P_1 and P_2 were independent (see data points in brackets and criteria in Materials and Methods; Bradler and Barriónuevo 1989; Wang and Kelly 1995), tetanic stimulation was delivered to P_1 . Synaptic responses in P_1 displayed LTP in intracellular ($196 \pm 21\%$ relative to base line value 100% ; $P=0.0007$) and field potential recordings ($178 \pm 19\%$; $P=0.0006$) that was monitored for 32 min, whereas synaptic responses in P_2 showed little if any change (Fig. 2A and 2B). Subsequently, the KAc microelectrode was withdrawn from the first neuron and another electrode containing $200 \mu\text{M}$ [Ala²⁸⁶]CaM-KII_{281–302} and $100 \mu\text{M}$ PKC_{19–31} in 2 M KAc was used to impale a second nearby neuron (this manipulation was completed within 20 min). Synaptic responses in P_1 and P_2 were monitored continuously for an additional 120 min and normalized relative to the first data point of basal synaptic responses in P_1 and P_2 recorded from the first neuron.

The effect of pseudosubstrate peptides of CaM-KII and PKC on potentiated and basal synaptic transmission is shown in Figure 2A (filled symbols). The postsynaptic injections of [Ala²⁸⁶]CaM-KII_{281–302} and PKC_{19–31} significantly and selectively attenuated prepotentiated synaptic transmission from $254 \pm 37\%$ to $133 \pm 22\%$ within 90 min ($P=0.0013$; filled circles), but did not significantly affect basal synaptic transmission (116 ± 16 vs. $109 \pm 14\%$; filled squares). The selective attenuation of nondecremental LTP by inhibiting postsynaptic CaM-KII and PKC is consistent with previous results by Feng and Wang (1992). Simultaneous field potential recordings showed that synaptic responses were constant at both potentiated and naive pathways throughout these experiments (Fig. 2B). These results indicate that the activities of postsynaptic CaM-KII/PKC triggered by tetanic stimulation are responsible for maintaining potentiated synaptic transmission, but the level of their activities under basal conditions may not be above a threshold necessary to regulate synaptic transmission, or their function requires certain cofactors that are produced by a tetanus.

Figure 3: Inhibition of postsynaptic CaN activity enhances synaptic transmission in potentiated and naive synaptic pathways. (A) The time course of normalized EPSP slopes of sequential intracellular recordings from two CA1 neurons in response to stimulating two independent pathways P_1 (circles) and P_2 (squares). In the first part of each experiment, a microelectrode containing $50 \mu\text{M}$ rapamycin in 2 M KAc (open symbols) was used to obtain stable intracellular recordings for 10 min; tetani (arrow) were then delivered to P_1 , and enhanced synaptic responses (EPSPs) were monitored for 32 min. The rapamycin electrode was withdrawn, and a second electrode containing $50 \mu\text{M}$ FK-506 in 2M KAc was used to impale a second nearby neuron within 20 min; stable synaptic responses were obtained within a 2-min period (filled symbols). Manipulations were done very carefully to prevent movement of stimulating electrodes and recording pipette for field potentials. Stimulation intensity was held constant for P_1 and P_2 . EPSP slopes recorded from the second neuron were normalized to the initial three EPSPs recorded from the first neuron of each experiment for the two pathways (dashed line). Synaptic responses in P_1 and P_2 were monitored for an additional 70 min and show enhanced EPSP slopes in potentiated (P_1) and naive pathways (P_2). A second tetani (right arrow) was delivered only to P_2 at 100 min and produced no additional potentiation. Inset shows representative waveforms of EPSPs for P_1 and P_2 at indicated times (1–4); calibration is 20 mV/30 msec. (B) Simultaneous field potential recordings show stable synaptic transmission in the LTP pathway (P_1 , $\circ$) and naive pathway (P_2 , $\square$) for the same slices in A; vertical arrows denote tetanus to P_1 and P_2 . Inset shows f-EPSPs for P_1 and P_2 ; calibration is 1 mV/20 msec.

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Thus, the up-regulation of synaptic strength depends on the activation of postsynaptic CaM-KII and PKC. It is interesting that when synaptic responses recorded from the second neuron in P_1 and P_2 are normalized by the first data point in the first neuron, the difference of average values between these two neurons in the potentiated pathway ($254 \pm 37\%$ vs. $196 \pm 21\%$; circles) is larger than that in the naive pathway (squares; Fig. 2A). Although we do not know the exact reason for this, the differences in the number of stimulated synapse between the two neurons, or the diffusion of KAc into the first neuron for 10 min prior to tetanic stimulation for LTP induction are possible. We also noted that the group data for the potentiated pathway (P_1) have large error bars. The primary reason for this variation is caused by one of the experiments shown in the inset in Figure 2A (filled squares). Despite this variability, each experiment showed that tetanus LTP was significantly attenuated by inhibiting postsynaptic CaM-KII and PKC activities (P values ranged from 0.002 to 0.009).

We next examined the role of Ca^{2+} /CaM-dependent protein phosphatase-2B, CaN, in modulating synaptic transmission. If CaN activity is required for synaptic depression, which is suggested by studies on LTD (Mulkey et al. 1994), then inhibiting CaN activity should induce synaptic enhancement and facilitate LTP expression. We selected FK-506, a potent and specific inhibitor of CaN through its interaction with the CaN-anchoring protein FKBP-12 (Wiederrecht et al. 1989; Schreiber and Crabtree 1992; Steiner et al. 1992; MacKintosh and MacKintosh 1994), and used the experimental protocol similar to that in Figure 2, that is, alternate stimulation in two independent pathways and sequential intracellular recordings from two nearby CA1 neurons. In the first part of each experiment ($n = 7$), a microelectrode containing 50 μM rapamycin (which combines with FKBP-12 but does not inhibit CaN activity; Wiederrecht et al. 1989; Schreiber and Crabtree 1992; MacKintosh and MacKintosh 1994) in 2 M KAc was used to impale a CA1 neuron. After obtaining values of basal synaptic transmission and being sure that synaptic responses were recorded from two independent pathways (see symbols exhibiting large synaptic responses before tetanus and criteria in Methods), tetanic stimulation was delivered to P_1 . Nondecremental LTP was expressed in P_1 ($167 \pm 17\%$ in intracellular recordings and $165 \pm 7\%$ in field potential recordings at 40 min;

$P = 0.0009$ and 0.0003 , respectively; open circles in Fig. 3A,B), whereas P_2 showed little if any change. Subsequently, the rapamycin-containing microelectrode was withdrawn and another electrode containing 50 μM FK-506 in 2 M KAc was used to impale a second nearby neuron within 20 min. Synaptic responses in P_1 and P_2 were monitored continuously for an additional 70 min and normalized by the first data points of basal synaptic responses in P_1 and P_2 recorded from the first neuron, respectively.

The effect of inhibiting CaN on potentiated and basal synaptic transmission is shown in Figure 3A. The postsynaptic injection of FK-506 significantly enhanced synaptic transmission in the potentiated pathway (P_1) from $174 \pm 17\%$ to $239 \pm 20\%$ within 20 min after beginning the injection ($P = 0.014$; filled circles); that is, FK-506

Figure 4: Inhibition of postsynaptic CaN activity for 4 min increases the magnitude of tetanus LTP induction. (A) LTP induction under intracellular recordings using microelectrodes containing 50 μM FK-506 in 2 M KAc. After impaling CA1 neurons for 4 min, synaptic potentiation was observed; tetanic stimulation (arrow) was then delivered to presynaptic axons. Potentiated synaptic transmission (monitored as EPSC slopes) showed no decremental component immediately after tetanic stimulation, whereas simultaneous field potential recordings (B) showed a short-term decrease in EPSC slopes (decremental type) during this period. Inset shows representative waveforms of EPSCs (A) and f-EPSPs (B); calibrations are 400 pA/30 msec and 1 mV/20 msec.

facilitated LTP expression. Interestingly, postsynaptic injection of FK-506 also enhanced synaptic strength in the naive pathway (P_2) ($85 \pm 6\%$ vs. $166 \pm 15\%$; $P = 0.0028$; filled squares), and subsequent tetanus given to P_2 did not produce any additional synaptic potentiation. Synaptic responses simultaneously recorded from field potential recordings did not change significantly under the conditions of potentiated or basal synaptic transmission throughout these experiments (Fig. 3B). These results indicate that the function of postsynaptic CaN activity under basal and potentiated conditions appears to down-regulate synaptic strength; that is, CaN activity is a limiting factor in synaptic transmission. It is noteworthy that the time course during development of synaptic potentiation by inhibiting postsynaptic CaN appears different in P_1 versus P_2 (see the second part of experiment in Fig. 3A). A faster raising rate of synaptic potentiation induced in the pretetanzed pathway may be caused by the existence of protein kinase activity which is being maintained at a higher level in the pathway exhibiting LTP expression. We do not know why the additional potentiation induced by FK-506 at potentiated synapses is short-lived, lasting ~ 40 min.

If CaN activity is a limiting factor in synaptic transmission, then inhibiting postsynaptic CaN should not only increase the magnitude of LTP expression, but also enhance the level of LTP during the induction phase (i.e., decremental component of LTP). To test this possibility, the experimental protocol shown in Figure 4 was used. In these experiments, synaptic potentiation was observed following postsynaptic injection of FK-506 for only 4 min, presumably because of partial inhibition of CaN activity. Tetanic stimulation administered to presynaptic axons after the 4-min FK-506 injection period induced robust nondecremental synaptic potentiation ($259 \pm 22\%$ and $285 \pm 20\%$ measured at 8 and 20 min after tetanus, $n = 5$; Fig. 4A). Compared with the magnitude of LTP induced during control experiments with postsynaptic injections of rapamycin ($181 \pm 16\%$ and $173 \pm 21\%$ measured at 8 and 20 min after tetanus; see the first part in Fig. 3A), differences in the magnitude of synaptic responses during the induction phase of LTP caused by postsynaptic FK-506 injections were significant ($P < 0.0001$). The facilitation of LTP induction by inhibiting postsynaptic CaN activity (i.e., postsynaptic injection of FK-506 virtually eliminates the decremental component of LTP) indicates that the effect of postsyn-

aptic CaN activity on limiting synaptic transmission may be responsible for the short-term decremental component during LTP induction, and prevents synaptic potentiation from reaching maximum levels by opposing the action of protein kinases.

Discussion

Our results show that the inhibiting postsynaptic CaM-KII and PKC activities by injecting specific pseudosubstrate inhibitory peptides attenuates the expression of LTP (see pretetanus pathway P_1 in Fig. 2A), which is in complete agreement with previous observations (Feng and Wang 1992). This result also supports conclusions from gene knockout experiments which show α CaM-KII or γ PKC genes are necessary for LTP induction (Silva et al. 1992; Abeliovich et al. 1993). Together with other observations showing increased CaM-KII and PKC activities or gene expression during LTP maintenance (Akers et al. 1986; Mackler et al. 1992; Fukunaga et al. 1993; Thomas et al. 1994), or activating postsynaptic PKC- or CaM-KII-induced synaptic potentiation (Hu et al. 1987; Wang and Feng 1992; Pettit et al. 1994; Wang and Kelly 1995), we conclude that the activity of postsynaptic CaM-KII and PKC play a critical role in the induction and maintenance of LTP by up-regulating synaptic strength. Regarding previous studies that showed that postsynaptic injections of PKC and CaM-KII peptide inhibitors did not block LTP expression (Malinow et al. 1989; Tsien et al. 1990), we believe this may be caused by the possibility that effective intracellular concentrations of inhibitors were not achieved because the resistance of microelectrodes was above 100 M Ω ; only the tips of microelectrodes were filled with inhibitors; and the period of observation was shorter. Consistent with previous observations (Feng and Wang 1992), we observed that injecting CaM-KII and PKC inhibitors into postsynaptic neurons did not change basal synaptic responses significantly (see P_2 in Fig. 2A). Together with results showing normal synaptic transmission in slices from γ PKC or α CaM-KII gene knockout mice (Silva et al. 1992; Abeliovich et al. 1993), we propose that basal CaM-KII and PKC activities may not reach a threshold that would significantly increase synaptic strength, or require certain cofactors that are activated by tetanus for their function, because these kinases are active in hippocampal

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slices (Ocorr and Schulman 1991; Huber et al. 1995).

The decrease in potentiated synaptic transmission after inhibiting postsynaptic CaM-KII and PKC activities (Fig. 2A) may result from the dominant effect of protein phosphatases because postsynaptic phosphatase activities are required for the induction of LTD (synaptic weakening) (Mulkey et al. 1993, 1994). If the attenuation of LTP we observed is similar to LTD and requires protein phosphatase activities, it seems reasonable to predict that postsynaptic phosphatase activities should produce a progressive decrease in synaptic strength (below baseline) under basal conditions, and after synaptic potentiation returns to baseline levels during inhibition of CaM-KII and PKC. These predictions were not supported by our results (Fig. 2A). Simple explanations for this difference are that protein phosphatases are inactive under basal conditions, or their activities disappear after inhibiting CaM-KII and PKC. Alternatively, stable basal synaptic transmission may be caused by phosphatase activities that limit synaptic transmission to low levels under both conditions. Our results show that postsynaptic CaN is active in CA1 neurons because its inhibition with FK-506 induces synaptic potentiation at basal and potentiated synapses (Fig. 3A), and increases the magnitude of LTP induction phase (Fig. 4A). Biochemical studies have also shown neuronal CaN activity under basal conditions (Steiner et al. 1992; Liu et al. 1994; Cameron et al. 1995). Thus, substrate dephosphorylation mediated by CaN makes synaptic weakening (i.e., synaptic depression), and postsynaptic CaN is active and functions to limit synaptic strength at a stable and low level under basal conditions.

Our result that potentiated synaptic transmission returns to baseline levels while inhibiting CaM-KII and PKC implies that protein phosphatase activities are high during LTP expression. This implication is supported by our observations that inhibiting postsynaptic CaN activity increases prepotentiated synaptic transmission during LTP maintenance (P_1 in Fig. 3A), and enhances synaptic strength during LTP induction (Fig. 4A). These results further indicate that stable synaptic transmission during LTP expression results from a balance between CaM-KII/PKC and CaN activities, in which CaM-KII and PKC activities up-regulate synaptic strength but CaN activity down-regulates synaptic strength. Thus, the counterbalance between postsynaptic CaM-KII/PKC and CaN activi-

ties produces stable synaptic strength that can be shifted up or down in potentiated pathways by selectively inhibiting either activity, or can be up-regulated in naive pathways by inhibiting CaN. Because the inhibition of postsynaptic CaM-KII and PKC did not affect basal synaptic transmission, other protein kinases may be required to counterbalance CaN activity under basal condition. In this context, we observed that high concentrations of multiple PKA inhibitors in postsynaptic neurons attenuated basal synaptic transmission (Wang and Kelly 1995).

Our results show that inhibiting postsynaptic CaN activity increases synaptic strength under basal and potentiated conditions. Comparing synaptic potentiation in naive (P_2) and potentiated pathways (P_1 ; see Fig. 3A), in which values are normalized to the first data point for the respective pathways in the second neuron, potentiation in P_2 is 96%, whereas prepotentiated P_1 increases just 36%. Furthermore, comparing the magnitude of tetanus-induced LTP in control experiments ($167 \pm 17\%$; see rapamycin control, Fig. 3A), potentiation induced by FK-506 ($196 \pm 15\%$) is significantly larger ($P = 0.028$). If we accept that LTP expression (persistent up-regulation of synaptic strength) depends mainly on protein kinase activities, how do we explain that synaptic potentiation induced by simply inhibiting CaN is larger than potentiation induced by tetanic stimulation (presumably activating CaM-KII, PKC and other kinases), or the additional potentiation induced by inhibiting CaN after tetanus LTP? Our interpretation of these results is that (1) CaN activity under basal conditions plays a dominant role in limiting synaptic strength compared with potentiation induced primarily by kinase activities; (2) CaN activity limits synaptic strength during LTP expression more strongly than during FK-506-induced synaptic potentiation; and (3) the inhibition of postsynaptic CaN triggers certain mechanisms that contribute to potentiating synaptic transmission. Biochemical results have shown that neuronal inositol-1,4,5-triphosphate (IP_3) and ryanodine receptor-mediated mobilization of intracellular Ca^{2+} is stimulated in a Ca^{2+} -dependent manner in a bell-shaped range of Ca^{2+} concentrations, and these receptors are phosphorylated by PKC and CaM-KII and dephosphorylated by CaN (Cameron et al. 1995; Furuichi and Mikoshiba 1995; Snyder and Sabatini 1995). The phosphorylation of IP_3 receptors appears to increase their sensitivity to IP_3 (Cameron et al. 1995). Thus, inhibiting

postsynaptic CaN activity may result in a progressive Ca^{2+} release from these intracellular stores, and this Ca^{2+} would then activate Ca^{2+} -dependent protein kinases and increase the phosphorylation of substrate proteins to facilitate synaptic transmission. This scheme is supported by our recent observations in which inhibiting cascades of postsynaptic Ca^{2+} signal pathways with 1,2-bis(2-aminophenoxy)-ethane- N,N,N',N' -tetraacetic acid (BAPTA), heparin/dantrolene (inhibitors of IP_3 and ryanodine receptors), a CaM-binding peptide (CBP), or CaM-KII/PKC pseudosubstrate peptides significantly attenuated FK-506-induced potentiation (in prep.).

Our conclusion is that postsynaptic CaN activity limits synaptic transmission at a low and stable level. Does this compromise the findings that CaN activity is required for LTD induction (Mulkey et al. 1994)? One simple interpretation is that low-frequency stimulation required to induce LTD may activate CaN to near maximum levels and depress synaptic transmission. In addition, because LTD is usually induced in young animals (Mulkey et al. 1993, 1994; Bashir and Collingridge 1994; Thiels 1994) where presynaptic release probability appears maximal (Bolshakov and Siegelbaum 1995), we predict that LTD may be induced primarily at the most active synapses in young animals. In the other words, the potentiated synaptic responses in adult animals, which can be depotentiated by low-frequency stimulation (O'Dell and Kandel 1994), may be similar to basal synaptic transmission in young animals, which is depressed by low-frequency stimulation. This scenario is supported by our recent observations that postsynaptic injections of a CaN inhibitor in young rats did not induce synaptic potentiation, presumably because of low CaN levels during this developmental stage (Tallant and Cheung 1983; Polli et al. 1991); in addition, the magnitude of tetanus-induced LTP in young rats is significantly lower than in adult rats (in prep.).

We conclude that postsynaptic CaM-KII and PKC activities are essential for maintaining LTP expression by up-regulating synaptic strength, but the down-regulating action of CaN activity limits synaptic strength at stable levels during both basal and potentiated synaptic transmission, i.e., the dynamic balance among their activities that sets physiological synaptic strength is dominated by CaN activity in adult brain (see Fig. 5). The role of CaN activity in limiting synaptic transmission under basal conditions gives synaptic transmission

Figure 5: Hypothetical model showing the regulation of synaptic strength by the balance between postsynaptic phosphorylation and dephosphorylation of AMPA receptors. Receptor phosphorylation is directly and/or indirectly controlled by CaM-KII and PKC and phosphatase activities (CaN). The level of free Ca^{2+} /CaM in postsynaptic neurons (from injections or intracellular mobilization) is a key element in this control. Ca^{2+} /CaM predominantly activates CaN at lower concentrations (i.e., 0.1–1 nM Ca^{2+} /CaM; thick arrow) and also activates CaM-KII pathways at concentrations of ~ 100 nM (thin arrow). This model incorporates the conclusions from our results (see text and Wang and Kelly 1995).

the potential to become enhanced by certain input signals to increase efficiency of information storage. These conclusions represent an important extension to the hypothesis of bidirectional control of synaptic efficacy during LTP and LTD (Lisman 1994; Schulman 1995). It is important to note, however, that other protein kinases and phosphatases also should be considered in regulating synaptic output by such mechanisms, as many protein kinases are required for LTP production (Akers et al. 1986; Malinwo et al. 1988, 1989; Malenka et al. 1989; O'Dell et al. 1991; Feng and Wang 1992; Wang and Feng 1992; Frey et al. 1993; Fuku-naga et al. 1993; Zhuo et al. 1994) and phosphatase type-1 and type-2A can facilitate synaptic transmission (Figurov 1992; Wyllie and Nicoll 1994).

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References

- Abeliovich, A., C. Chen, Y. Goda, A.J. Silva, C.F. Stevens, and S. Tonegawa. 1993. Modified hippocampal long-term potentiation in PKC γ -mutant mice. *Cell* **75**: 1253–1262.
- Akers, R., D. Lovinger, P. Colley, D. Linden, and A. Routtenberg. 1986. Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science* **231**: 587–589.
- Amundson, J. and D. Clapham. 1993. Calcium waves. *Curr. Opin. Neurobiol.* **3**: 375–382.
- Bashir, Z.I. and G.L. Collingridge. 1994. An investigation of depotentiation of long-term potentiation in the CA1 region of the hippocampus. *Exp. Brain Res.* **100**: 437–443.
- Bliss, T.V.P. and G.L. Collingridge. 1993. A synaptic model of memory: LTP in the hippocampus. *Nature* **361**: 31–39.
- Bliss, T.V.P. and A.R. Gardner-Medwin. 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* **232**: 357–374.
- Bliss, T.V.P. and T. Lomo. 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* **232**: 331–356.
- Bliss, T.V.P. and M.A. Lynch. 1988. Long-term potentiation of synaptic transmission in the hippocampus, properties and mechanisms. In *Long-term potentiation: From biophysics to behavior* (ed. P.W. Landfield and S.A. Deadwyler, pp. 3–72. Alan R. Liss, New York, NY.
- Bolshakov, V. and S. Siegelbaum. 1995. Regulation of hippocampal transmitter release during development and long-term potentiation. *Science* **269**: 1730–1734.
- Bradler, J.E. and G. Barrionuevo. 1989. Long-term potentiation in hippocampal CA3 neurons: Tetanized input regulates heterosynaptic efficacy. *Synapse* **4**: 132–142.
- Cameron, A.M., J.P. Steiner, A.J. Roskams, S.M. Ali, G.V. Ronnett, and S.H. Snyder. 1995. Calcineurin associated with the inositol 1,4,5-triphosphate receptor-FKBP12 complex modulates Ca²⁺ flux. *Cell* **83**: 463–472.
- Carafoli, E. 1987. Intracellular calcium homeostasis. *Ann. Rev. Biochem.* **56**: 395–433.
- Cohen, P. 1988. The calmodulin-dependent multiprotein kinase. In *Calmodulin* (ed. P. Klee and C.B. Cohen, pp. 145–193. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
- Collingridge, G.L., S.J. Kehl, and H. McLennan. 1983a. The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones in vitro. *J. Physiol.* **334**: 19–31.
- . 1983b. Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J. Physiol.* **334**: 33–46.
- Eichenbaum, H. 1995. The LTP-memory connection. *Nature* **378**: 131.
- Feng, T.P. and J.-H. Wang. 1992. PKC(19–31) and CaMKII(273–302) given together intracellularly to the postsynaptic neuron synergistically block LTP in hippocampal CA1 region. *Soc. Neurosci. Abstr.* **18**: 760.
- Figurov, A., H. Boddeke, and D. Muller. 1992. Enhancement of AMPA-mediated synaptic transmission by protein phosphatase inhibitor Calyculin A in rat hippocampal slices. *Eur. J. Neurosci.* **5**: 1035–1041.
- Frey, U., Y.-Y. Huang, and E. Kandel. 1993. Effects of cAMP stimulate a late stage of LTP in hippocampal CA1 neurons. *Science* **260**: 1661.
- Fukunaga, K., L. Stoppini, E. Miyamoto, and D. Muller. 1993. Long-term potentiation is associated with an increased activity of Ca²⁺/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **268**: 7863–7870.
- Furuichi, T. and K. Mikoshiba. 1995. Inositol 1,4,5-triphosphate receptor-mediated Ca²⁺ signaling in the brain. *J. Neurochem.* **64**: 953–960.
- Ghosh, A. and M.E. Greenberg. 1995. Calcium signaling in neurons: Molecular mechanisms and cellular consequences. *Science* **268**: 239–247.
- Glantz, S.A. 1993. *Primer of biostatistics*, 3rd edition. McGraw Hill, New York, NY.
- Gustafsson, B. and H. Wigstrom. 1988. Physiological mechanisms underlying long-term potentiation. *Trends Neurosci.* **11**: 156–162.
- Hu, G.Y., O. Hvalby, S.I. Walaas, K.A. Albert, P. Skjeflo, P. Andersen, and P. Greengard. 1987. PKC injected into hippocampal pyramidal cells elicits features of long term potentiation. *Nature* **328**: 426–429.
- Huang, K.P. 1989. The mechanism of protein kinase C activation. *Trends Neurosci.* **12**: 425–432.
- Huber, K.M., M.D. Mauk, C. Thompson, and P.T. Kelly. 1995. A critical period of protein kinase activity after tetanic stimulation is required for the induction of long-term potentiation. *Learn. & Mem.* **2**: 81–100.
- Kandel, E.R. 1991. Synaptic transmission. In *Principles of neural science* (ed. E.R. Kandel, J.H. Schwartz, and T.M. Jessell), pp. 123–193. Elsevier, New York, NY.
- Kauer, J.A., R.C. Malenka, and R.A. Nicoll. 1988. A

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persistent postsynaptic modification mediates long-term potentiation in the hippocampus. *Neuron* **1**: 911–917.

Klee, C.B. 1991. Concerted regulation of protein phosphorylation and dephosphorylation by calmodulin. *Neurochem. Res.* **16**: 1059–1065.

Klee, C.B. and P. Cohen. 1988. The calmodulin-regulated protein phosphatase. In *Calmodulin* (ed. P. Klee and C.B. Cohen), pp. 225–248. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Krebs, E.G. 1994. The growth of research on protein phosphorylation. *Trends Biochem. Sci.* **19**: 439.

Lisman, J. 1994. The CaM kinase II hypothesis for the storage of synaptic memory. *Trends Neurosci.* **17**: 406–412.

Liu, J.-P., A.T.R. Sim, and P.J. Robinson. 1994. Calcineurin inhibition of dynamin I GTPase activity coupled to nerve terminal depolarization. *Science* **265**: 970–973.

MacKintosh, C. and R.W. MacKintosh. 1994. Inhibitors of protein kinases and phosphatases. *Trends Biochem. Sci.* **12**: 444–448.

Mackler, S.A., B.P. Brooks, and J.H. Eberwine. 1992. Stimulus-induced coordinate changes in mRNA abundance in single postsynaptic hippocampal CA1 neurons. *Neuron* **9**: 539–548.

Malenka, R.C., J.A. Kauer, R.S. Zucker, and R.A. Nicoll. 1988. Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science* **242**: 81–84.

Malenka, R.C., J.A. Kauer, D.J. Perkel, M.D. Mauk, P.T. Kelly, R.A. Nicoll, and M.N. Waxham. 1989. An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature* **340**: 554–557.

Malinow, R., D.V. Madison, and R.W. Tsien. 1988. Persistent protein kinase activity underlying long-term potentiation. *Nature* **335**: 820–824.

Malinow, R., H. Schulman, and R.W. Tsien. 1989. Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* **245**: 862–866.

Mulkey, R.M., C.E. Herron, and R.C. Malenka. 1993. An essential role for protein phosphatases in hippocampal long-term depression. *Science* **261**: 1051–1055.

Mulkey, R.M., S. Endo, S. Shenolikar, and R.C. Malenka. 1994. Involvement of a calmodulin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* **369**: 486–488.

Nestler, E.J. and P. Greengard. 1984. Introduction. In *Protein phosphorylation in the nervous system* (ed. E.J. Nestler and P. Greengard), pp. 1–16. John Wiley & Sons, New York, NY.

Ocorr, K.A. and H. Schulman. 1991. Activation of

multifunctional Ca^{2+} /calmodulin-dependent kinase in intact hippocampal slices. *Neuron* **6**: 907–914.

O'Dell, T.J. and E.R. Kandel. 1994. Low-frequency stimulation erases LTP through an NMDA receptor-mediated activation of protein phosphatases. *Learn. & Mem.* **1**: 129–139.

O'Dell, T.J., E.R. Kandel, and S.G. Grant. 1991. Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature* **353**: 558–560.

Pettit, D.L., R. Periman, and R. Malinow. 1994. Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neuron. *Science* **266**: 1881–1885.

Polli, J.W., M.L. Billingsley, and R.L. Kincaid. 1991. Expression of the calmodulin-dependent protein phosphatase, calcineurin, in rat brain: Developmental patterns and the role of nigrostriatal innervation. *Dev. Brain Res.* **63**: 105–119.

Schreiber, S.L. and G.R. Crabtree. 1992. The mechanism of action of cyclosporin A and FK506. *Immunol. Today* **13**: 136–142.

Schulman, H. 1995. Protein phosphorylation in neuronal plasticity and gene expression. *Curr. Opin. Neurobiol.* **5**: 375–381.

Schulman, H. and L.L. Lou. 1989. Multifunctional Ca^{2+} /calmodulin-dependent protein kinase: Domain structure and regulation. *Trends Biochem. Sci.* **14**: 62–66.

Shepherd, G.M. 1994. The synapse. In *Neurobiology* (ed. G.M. Shepherd), pp. 102–107. Oxford University Press, New York, NY.

Silva, A., C. Stevens, S. Tonegawa, and Y. Wang. 1992. Deficient hippocampal LTP in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257**: 201–206.

Snyder, S.H. and D.M. Sabatini. 1995. Immunophilins and the nervous system. *Nature Med.* **1**: 32–37.

Steiner, J.P., T.M. Dawson, M. Fotuhi, C.E. Glatt, A.M. Snowman, N. Cohen, and S.H. Snyder. 1992. High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358**: 584–587.

Tallant, E.A. and W.Y. Cheung. 1983. Calmodulin-dependent protein phosphatase: A developmental study. *Biochemistry* **22**: 3630–3635.

Thiels, E., G. Barrionuevo, and T.W. Beger. 1994. Excitatory stimulation during postsynaptic inhibition induces long-term depression in hippocampus in vivo. *J. Neurophysiol.* **72**: 3009–3016.

Thomas, K.L., S. Laroche, M.L. Errington, T.V.P. Bliss, and S.P. Hunt. 1994. Spatial and temporal changes in signal transduction pathways during LTP. *Neuron* **13**: 737–745.

POSTSYNAPTIC CALCINEURIN LIMITS SYNAPTIC STRENGTH

Tsien, R.W., H. Schulman, and R. Malinow. 1990. Peptide inhibitors of PKC and CaMKII block induction but not expression of long-term potentiation. *Adv. Second Messenger Phosphoprotein Res.* **24**: 101–107.

Wang, J.H. and D.P. Feng. 1992. Postsynaptic protein kinase C essential to induction and maintenance of long-term potentiation in the hippocampal CA1 region. *Proc. Natl. Acad. Sci.* **89**: 2576–2580.

Wang, J.H. and P.T. Kelly. 1995. Postsynaptic injection of Ca^{2+} /CaM induces synaptic potentiation requiring CaM-KII and PKC activity. *Neuron* **15**: 443–452.

———. 1996. Regulation of synaptic facilitation by postsynaptic Ca^{2+} /CaM pathways in hippocampal CA1 neurons. *J. Neurophysiol.* **76**: 276–286.

Wiederrecht, G., E. Lam, H. Shirley, M. Martin, and N. Sigal. 1989. The mechanism of action of FK-506 and cyclosporin A. *Ann. NY Acad. Sci.* **104**: 9–19.

Wyllie, D.J.A. and R.A. Nicoll. 1994. A role for protein kinases and phosphatases in the Ca^{2+} -induced enhancement of hippocampal AMPA receptor-mediated synaptic responses. *Neuron* **13**: 635–643.

Zhuo, M., Y. Hu, C. Schultz, E. Kandel, and R. Hawkins. 1994. Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation. *Nature* **368**: 635–638.

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References

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