Is Persistent Activity of Calcium/Calmodulin-Dependent Kinase Required for the Maintenance of LTP?

HUAN-XIN CHEN,1 NIKOLAI OTMAKHOV,1 STEFAN STRACK,2 ROGER J. COLBRAN,2 AND JOHN E. LISMAN1

1Volen Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02454; and 2Department of Molecular Physiology and Biophysics and Center for Molecular Neuroscience, Vanderbilt Medical Center, Nashville, Tennessee 37232

Received 29 September 2000; accepted in final form 18 December 2000

Chen, Huan-Xin, Nikolai Otmakhov, Stefan Strack, Roger J. Colbran, and John E. Lisman. Is persistent activity of calcium/calmodulin-dependent kinase required for the maintenance of LTP? J Neurophysiol 85: 1368–1376, 2001. Calcium/calmodulin-dependent protein kinase II (CaMKII) is concentrated in the postsynaptic density (PSD) and plays an important role in the induction of long-term potentiation (LTP). Because this kinase is persistently activated after the induction, its activity could also be important for LTP maintenance. Experimental tests of this hypothesis, however, have given conflicting results. In this paper we further explore the role of postsynaptic CaMKII in induction and maintenance of LTP. Postsynaptic application of a CaMKII inhibitor [autocamtide-3 derived peptide inhibitor (AC3-I), 2 mM] blocked LTP induction but had no detectable affect on N-methyl-D-aspartate (NMDA)-mediated synaptic transmission, indicating that the primary function of CaMKII in LTP is downstream from NMDA channel function. We next explored various methodological factors that could account for conflicting results on the effect of CaMKII inhibitors on LTP maintenance. In contrast to our previous work, we now carried out experiments at higher temperature (33°C), used slices from adult animals, and induced LTP using a tetanic stimulation. However, we still found that LTP maintenance was not affected by postsynaptic application of AC3-I. Furthermore the inhibitor did not block LTP maintenance under conditions designed to enhance the Ca2+ dependent activity of protein phosphatases 1 and 2B (elevated Ca2+, calmodulin, and an inhibitor of protein kinase A). We also tested the possibility that CaMKII inhibitor might not be able to affect CaMKII once it was inserted into the PSD. In whole-brain extracts, AC3-I blocked autophosphorylation of both soluble and particulate PSD CaMKII with similar potencies although the potency of the inhibitor toward other CaMKII substrates varied. Thus we were unable to demonstrate a functional role of persistent Ca2+-independent CaMKII activity in LTP maintenance. Possible explanations of the data are discussed.

INTRODUCTION

Long-term potentiation (LTP) has been widely studied as a cellular model of learning and memory. Induction of LTP is triggered by a transient increase in intracellular Ca2+ concentration, which activates the biochemical cascade leading to enhanced synaptic transmission (reviewed in Bliss and Collingridge 1993; Nicoll and Malenka 1999). There is now substantial evidence for a key role of postsynaptic calcium/calmodulin-dependent protein kinase II (CaMKII) in this induction process. LTP induction produced by a tetanus can be blocked by postsynaptic application of peptide inhibitors of the kinase (Feng 1995; Hvalby et al. 1994; Malinow et al. 1989). Furthermore it was shown that LTP produced by a pairing protocol is also blocked by CaMKII inhibitors (Otmakhov et al. 1997). Since use of this protocol ensures the level of postsynaptic depolarization required for LTP induction, it is clear that CaMKII is involved in the core processes of synaptic plasticity rather than simply in the depolarization processes. More recently, elevation of CaMKII activity by direct introduction of the kinase into a cell or by expression of a constitutively active form was shown to enhance synaptic transmission in a manner that occludes with tetanus-induced LTP (Lledo et al. 1995; Pettit et al. 1994; Shirke and Malinow 1997). This action appears to involve direct phosphorylation of glutamate receptor subunit 1 (GluR1) and the resulting enhancement of AMPA channel conductance (Benke et al. 1998; Derkach et al. 1999) as well as insertion of new GluR1 subunits into the synapse (Hayashi et al. 2000).

CaMKII may also have a role in the maintenance of LTP. Since autophosphorylation of Thr286 can promote further autophosphorylation of the kinase, CaMKII activity might be maintained constitutively by a positive feedback process (Lisman 1985, 1994; Miller and Kennedy 1986; Okamoto and Ichikawa 2000; Saitoh and Schwartz 1985; Schworer et al. 1988; Zhabotinsky 2000). Consistent with this hypothesis, biochemical work has shown that LTP induction triggers a long-lasting increase in the autophosphorylated form of CaMKII and in its Ca2+-independent activity (Barria et al. 1997; Fukunaga et al. 1993, 1995; Lee et al. 2000; Ouyang et al. 1997, 1999). Furthermore a mutation of Thr286 prevents LTP induction (Giese et al. 1998).

If persistent CaMKII activity is responsible for LTP maintenance, LTP should be reversed if CaMKII activity is inhibited after induction. Early work tested this prediction in mature rats by infusing CaMKII inhibitors into the postsynaptic neuron through microelectrodes. These studies gave conflicting results (Feng 1995; Malgaroli et al. 1992; Malinow et al. 1989). One difficulty with microelectrode studies is that cells have to be impaled after induction of LTP, and there was, therefore, no direct evidence that LTP had occurred in the neuron that was being recorded from. To address this problem,
we developed a method for controlled inhibitor application using a perfused patch pipette. With this method, CaMKII inhibitor could be applied postsynaptically after verifiable LTP had been induced in a cell. Our results showed that LTP maintenance was not affected by CaMKII inhibitor (Otmakhov et al. 1997), consistent with the results of Malinow et al. (1989), but inconsistent with the work of Feng (1995), showing that LTP can be reversed by this inhibitor.

In this paper, we have attempted to resolve a range of issues relevant to the possible role of CaMKII in LTP induction and maintenance. In the first series of experiments, we explored whether the prevailing notion that CaMKII acts downstream from the NMDA channel might be incorrect. The fact that CaMKII inhibitors block LTP induction has been taken as evidence that CaMKII detects Ca$^{2+}$ entry through the NMDA channel and triggers subsequent processes that strengthen the synapse. However, because the NMDA receptor is phosphorylated by CaMKII (Gardoni et al. 1998; Leonard et al. 1999; Omkumar et al. 1996; Strack and Colbran 1998; Strack et al. 2000), it is possible that CaMKII inhibitors decrease the baseline NMDA conductance and thereby inhibit LTP. We have tested this possibility.

In a second set of experiments, we addressed various technical differences between the experiments of Feng (1995), which did show an effect of CaMKII inhibitor on LTP maintenance, and our previous experiments, which did not (Otmakhov et al. 1997). Feng’s experiments were conducted at higher temperature (32–33°C), used older animals, and induced LTP using a tetanic method. Our work was done at room temperature on younger animals, and LTP was induced by pairing. We have tested whether these factors could account for the discrepancies.

In a third set of experiments, we addressed the hypothesis that reversal of LTP maintenance does not occur because postsynaptic phosphatase activity is too low. The main phosphatase that dephosphorylates PSD-associated CaMKII is protein phosphatase 1 (PP1) (Strack et al. 1997a). PP1 is upregulated by Ca$^{2+}$/calmodulin and downregulated by protein kinase A (PKA) via an enzyme cascade (Shenolikar and Nairn 1991). Specifically, when inhibitor-1 is phosphorylated by PKA, it can effectively inhibit PP1; when it is dephosphorylated by the calcium/calmodulin-dependent protein phosphatase 2B, calcineurin, PP1 becomes active. This makes PP1 dependent on the intracellular free Ca$^{2+}$ concentration and on calmodulin concentration. We therefore attempted to boost postsynaptic PP1 activity by setting Ca$^{2+}$ concentration in pipette solution above the resting level (0.3 μM) and including a PKA inhibitor and calmodulin in this solution.

In a final set of experiments, we investigated whether CaMKII inhibitors can affect CaMKII activity in the PSD. The PSD is an array of scaffolding proteins and attached enzymes (Kennedy 1998), which could make some PSD proteins inaccessible from cytosol. Thus CaMKII inhibitor might effectively inhibit the activity of soluble CaMKII but might have restricted access to the PSD such that PSD-associated CaMKII is not effectively inhibited. If LTP maintenance requires activity of PSD-associated CaMKII, this could explain why we have been unable to reverse maintenance with CaMKII inhibitor. To test this possibility, biochemical experiments were conducted in which we examined the effect of inhibitors on PSD CaMKII.

The work presented in this paper poses a puzzle: biochemical experiments show that CaMKII is persistently activated after LTP induction. However, all the experiments that we have reported previously and that we have extended in this paper seem to indicate that constitutive CaMKII activity does not play a role in LTP maintenance. Possible resolutions to this puzzle are presented in the Discussion.

**METHODS**

**Electrophysiology**

Transverse hippocampal slices were prepared from male Long-Evans rats (6–8 wk old) as described previously (Chen et al. 1999). The CA3 region of each slice was removed from the slice by a surgical cut. Slices were incubated on cell culture inserts (Falcon, 8 μm pore diameter) covered by a thin layer of artificial cerebrospinal fluid (ACSF containing 2 mM Ca$^{2+}$ and 6 mM Mg$^{2+}$) and surrounded by a humidified 95% O$_2$–5% CO$_2$ atmosphere at room temperature (~22°C). For recording, a single slice, after 2- to 6-h incubation, was transferred to a submerged recording chamber with continuous flow (1.5–2 ml/min) of ACSF. The ACSF contained (in mM) 124 NaCl, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2.5 KCl, 2 CaCl$_2$, 2 MgSO$_4$, 10 d-glucose, and 0.05 picrotoxin, gassed with 95% O$_2$–5% CO$_2$ giving pH 7.4. All experiments were carried out at 32–33°C.

Whole-cell current-clamp recordings were performed from CA1 pyramidal cells located 50–90 μm beneath the slice surface under visual control using infrared dark-field illumination and a CCD TV camera. The patch electrodes were made from borosilicate glass and filled with (in mM) 125 K-glucocinate, 10 HEPES, 8 NaCl, 0.2 EGTA, 2 MgATP, 0.3 Na$_3$GTP, and 10 phosphocreatine (pH 7.3 with KOH, osmolality 290–296 mOsm). The electrodes had resistance 3–5 MΩ when filled with internal solution. Whole-cell recordings were made in current-clamp mode using an Axopatch-1D (Axon Instruments, Foster City, CA). Only cells with membrane potential more negative than ~65 mV were used. To evoke synaptic responses, two glass electrodes filled with ACSF (300 KΩ) were placed in the dendrite region 70 and 150 μm away from the cell body layer to stimulate two separate groups of Schaffer collaterals. Stimuli (100 μs) were delivered alternatively to each input pathway through current output isolation units. The interval between stimuli in each pathway was 6 s with 3-s interval between pathways. Stimulation intensity was adjusted to produce an excitatory postsynaptic potential (EPSP) with amplitude of ~30–50% of threshold for an action potential. Whole-cell voltage-clamp recording was used in experiments in which the isolated synaptic NMDA component was studied. In these experiments, K-glucocinate was replaced by Cs-glucocinate in the internal solution, 1.2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo-[f]quinoxaline-7-sulfonamide disodium (NBQX) (10 μM) was used to block AMPA component and membrane potential was held at ~45 mV. In all experiments, series and input resistances during the recording were monitored every 3 s by applying depolarizing voltage pulses. The series resistance ranged from 6 to 12 MΩ. The input resistance ranged from 70 to 150 MΩ.

LTP was induced by tetanic stimulation including five trains, each containing 20 pulses at 100 Hz. The interval between trains was 10 s. The specific inhibitor of CaMKII used was an autacotamide-3 derived inhibitory peptide (Ac-KKALHRQEAVDAL-NH$_2$), which resists proteolysis (AC3-I) (Otmakhov et al. 1997). Peptide inhibitors of this class are 100-fold more specific for CaMKII than for PKC (Braun and Schulman 1995). A peptide with the reversed amino acid sequence relatively to AC3-I served as a control peptide (gift of Dr. Leslie Griffith). For inhibition of PKA, a specific PKA peptide inhibitor PKI(6-22) amide was used. Aliquots of the peptide stock solutions were thawed only once immediately before an experiment. The pH of internal perfusion solution was adjusted to 7.2–7.4. Internal perfusion was performed as described elsewhere (Otmakhov et al. 1997).
Data were acquired using a 486 PC computer, Labmaster DMA ADC, and program written in Axobasic. The amplitude of a synaptic response was calculated as the difference between the average of data points in a window before the stimulus and in a window around the peak of the synaptic response. The average of responses during a 5-min period before LTP induction was taken as the baseline, and all values were normalized to this baseline. Values were expressed as means ± SE. Two-tailed paired t-test was used for calculation of the statistical significance of differences. Drugs used included D-2-amino-5-phosphonovaleric acid (D-AP5, RBI), Picrotoxin (Sigma), NBQX (RBI), AC3-I, and PKI(6-22) (QCB, Hopkinton, MA), Calmodulin (Sigma).

Biochemistry

Rat whole forebrain extracts and PSDs were prepared (Strack et al. 1997), and baculovirus-expressed murine CaMKIIz was purified as described (McNeill and Colbran 1995). Purified CaMKII and extracts were diluted to similar kinase activities (∼100 pmol · ml⁻¹ · min⁻¹) and assayed for autocamtide-2 phosphorylation (10 μM) in the presence of calcium/calmodulin (0.5 mM/1 μM) and varying concentrations of AC3-I or inactive control peptide essentially as described (Colbran 1993). Less than 1% kinase activity was detected in the absence of calcium/calmodulin. Controls omitting peptide substrate showed that phosphorylation of endogenous proteins did not contribute significantly to total 32P-incorporation at these dilutions.

Endogenous substrate assays were carried out following a two-phase protocol. To autophosphorylate CaMKII, whole rat forebrain extracts (0.25 mg/ml) were incubated for 15 s on ice in the presence of 0.5 mM CaCl₂/3 μM calmodulin or 1 mM EGTA (as a control) with 5 μM nonradioactive ATP, 2 mM Mg acetate, 20 mM HEPES, pH 7.5. 0.5% (vol/vol) Triton X-100, 1 mM dithiotreitol, 20 μg/ml leupeptin, 1 mM benzamidine, 1 μM microcystin-LR. Calcium/calmodulin-dependent activities of 20–30% (using the autocamtide-2 assay as described in the preceding text) were typically achieved during this incubation. After 15 s, [γ-32P]ATP (5 μM, ∼20 cpm/fmol) was added without or with 1.5 mM NBQX to radiolabel proteins by calcium/calmodulin-dependent or -independent phosphorylation, respectively, and incubation was continued for 45 s on ice before reactions were stopped by addition of 20 mM EDTA. Varying concentrations of AC3-I and other kinase inhibitors were also present during the second phase. Extracts were microcentrifuged (30 min, 4°C) to separate soluble from particulate proteins, and analyzed by SDS-PAGE and autoradiography. 32P incorporation into individual bands was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

CaMKII inhibitor does not affect the NMDA receptor-dependent synaptic conductance

The induction of LTP depends on the activation of the NMDA receptor (Bliss and Collingridge 1993). It has been reported that CaMKIIz directly binds to NMDA receptor and phosphorlates it, suggesting that NMDA activity can be regulated by CaMKII (Gardoni et al. 1998; Leonard et al. 1999; Omkumar et al. 1996; Strack and Colbran 1998; Strack et al. 2000). If activation of NMDA receptor is required for basal CaMKII activity, blocking CaMKII activity may reduce the basal NMDA conductance and therefore block LTP induction. To test this possibility, we have examined the effect of the inhibitory peptide on the NMDA component of the synaptic response isolated by blocking the AMPA component with NBQX (20 μM). The recordings were done under voltage-clamp mode at holding potential −45 mV. As shown in Fig. 1, perfusion of 2 mM AC3-I had no effect on the basal NMDA excitatory postsynaptic current (EPSC) at 30 min after the perfusion of AC3-I were 98 ± 5% of the baseline (n = 6; P > 0.05).

Attempts to reverse LTP maintenance

Recordings were made in hippocampal slices from 6- to 7-wk-old rat at 32°C in current-clamp mode, and LTP was induced by a tetanus to match conditions used by Feng (1995). To determine the effect of CaMKIIz inhibitory peptide, AC3-I, on the maintenance of LTP, we used a two-pathway protocol. After 5 min of baseline recording, LTP was induced in one pathway and then AC3-I (2 mM) was perfused into the patch pipette. Fifteen minutes later, LTP was induced in the second pathway (Fig. 2A). This protocol allowed us to measure in the same experiment the inhibitor effect on both the maintenance of LTP in the first pathway and on LTP induction in the second pathway. We found that in the first pathway, the level of LTP at 60 min after perfusion of AC3-I was not significantly different (201 ± 14%, Fig. 2A, n = 6) from the level of LTP in experiments in which control peptide (2 mM) was perfused (249 ± 54%, Fig. 2B, n = 6, P > 0.05). However, because the initial levels of LTP in the test and control experiments were slightly different, it was desirable to check for an effect of the inhibitor using a procedure that normalized these initial levels.

![Figure 1](http://jn.physiology.org/)

**Fig. 1.** Perfusion of calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitory peptide does not affect the N-methyl-D-aspartate (NMDA) response. A: a representative experiment. The NMDA excitatory postsynaptic current (EPSC) was isolated in voltage-clamp mode by using the AMPA antagonist, NBQX (20 μM), and holding the membrane potential at −45 mV. (a) the perfusion of CaMKII inhibitor; (c) bath application of NMDA antagonist, APV (50 μM). Inset: examples of NMDA responses (average of 20 consecutive responses) taken at time marked by letters. Calibration, 100 pA, 200 ms. B: summary data (n = 6).
Characterization of CaMKII inhibition by AC3-I

LTP is associated with a rapid and maintained increase in calcium/calmodulin-independent activity of CaMKII as a consequence of autophosphorylation of Thr286/287 (Barria et al. 1997; Fukunaga et al. 1993, 1995; Lee et al. 2000; Ouyang et al. 1997, 1999). This autophosphorylation also promotes trans-
location of CaMKII to postsynaptic densities where it enhances the phosphorylation of several postsynaptic substrates including the GluR1 subunit of the AMPA-type glutamate receptor (Shen and Meyer 1999; Shen et al. 2000; Strack et al. 1997b).

The failure of AC3-I to block maintenance of LTP could conceivably be attributed to an inability of this compound to inhibit the PSD-associated form of the kinase. To address this issue, we first compared the inhibitory potency of AC3-I toward purified recombinant CaMKII and endogenous CaMKII in whole-brain lysates and PSDs, using as a substrate the specific CaMKII substrate autocamtide-2 (AC2). AC3-I inhibited AC2 phosphorylation in the presence of calcium/calmodulin with similar IC50s (20–80 µM) for different sources of kinase (Fig. 4A). Thus CaMKII in PSDs, crude brain lysates, and purified soluble CaMKII are similarly accessible to inhibition by AC3-I.

We next investigated the effect of AC3-I on phosphorylation of endogenous CaMKII substrates. Whole forebrain extracts were briefly incubated with calcium/calmodulin and nonradioactive ATP under conditions that lead to selective autophosphorylation of CaMKII (see METHODS). To label proteins by calcium/calmodulin-dependent and -independent phosphorylation, incubation was continued by adding [γ-32P]ATP in the absence or presence of calcium/calmodulin (Fig. 4A). Thus CaMKII in PSDs, crude brain lysates, and purified soluble CaMKII are similarly accessible to inhibition by AC3-I.

Data are representative of 3 similar experiments.
Two major $^{32}$P-labeled bands present in both soluble and particulate fraction correspond to the autophosphorylated $\alpha$ and $\beta$ isoforms of CaMKII. Additional CaMKII substrates included a doublet of 76K and 78K molecular weight in the soluble extract (S76/78) and 180K in the particulate fraction (P180). Based on molecular weight, fractionation profile, and previous characterization as CaMKII substrates, these bands were tentatively identified as synapsin 1a/b (S76/78) (Huttner et al. 1981) and a combination of the NR2B subunit of the NMDA receptor (Omkumar et al. 1996) and the PSD protein, densin-180 (Apperson et al. 1996). Calcium-independent autophosphorylation of CaMKII$\beta$ in both fractions was potently inhibited by AC3-I (IC$_{50}$ 0.6 ± 0.3 and 1.1 ± 0.4 $\mu$M, in soluble and particulate fractions, respectively, $n = 3$; Fig. 4C) but unaffected by the PKA inhibitor PKI-tide and the PKC inhibitor PKC$_{19-32}$ (10 $\mu$M, not shown). Similar results were obtained for AC3-I inhibition of $^{32}$P incorporation into CaMKII$\alpha$ (IC$_{50}$ 1 $\mu$M) and the soluble substrate S76/78 (IC$_{50}$ 0.3 ± 0.1 $\mu$M, $n = 3$ not shown). The phosphorylation of a particulate CaMKII substrate, P180, was much more difficult to inhibit with AC3-I (IC$_{50}$ 8 ± 1.2 $\mu$M, $n = 3$) than S76/78. While the potency of inhibition of phosphorylation of different substrates by AC3-I varied, calcium-dependent and -independent activities toward a given substrate were inhibited similarly by AC3-I (Fig. 4C).

DISCUSSION

This work is a continuation of the investigation into the role of CaMKII in the maintenance of LTP. Our previous results indicated that perfusion of CaMKII inhibitor after LTP induction did not block LTP maintenance (Otmakhov et al. 1997), contrary to the results of Feng (1995), who found that the maintenance of LTP could be blocked by a similar CaMKII peptide inhibitor. Here we have investigated whether age, temperature, or method of LTP induction might account for this discrepancy. In particular, we now have used mature animals, induced LTP using a tetanus, and performed our experiments at 32–33°C to make our conditions more similar to those used by Feng. We also followed the recording for $\geq$1 h from the start of the perfusion because Feng reported the effect occurred on this slow time scale. Our method of drug delivery (diffusion from a patch pipette) is more efficient than the infusion from the microelectrode used by Feng, and our inhibitor concentration (2 mM) is higher than used in his work (100 $\mu$M). Nevertheless, contrary to Feng’s finding, we did not see a block of LTP maintenance within the 1-h period after inhibitor application. The fact that the same application of the inhibitor completely blocked LTP induction indicates that the inhibitor was effective inside of these cells.

We have also addressed several other possible reasons for the discrepancy with Feng. One specific concern is that reversals of maintenance might depend on phosphatase activity and that phosphatas might “wash out” of the cell during whole-cell recording procedure that we have used. It seems doubtful, however, that phosphatases have completely washed out or been irreversibly inactivated since cells still expressed normal long-term depression (LTD). This was also true in the presence of AC3-I (data not shown). Previous work has shown that LTD depends on phosphatase activity (Mulkey et al. 1993. 1994).

Another possibility is that the intracellular Ca$^{2+}$ concentration may have been higher in Feng’s experiments than in our experiments. This could potentially affect the dominant PSD phosphatase, PP1, which is turned on by a cascade activated by the Ca$^{2+}$/calmodulin-dependent phosphatase 2B (calcineurin) and turned off by PKA (Shenolikar and Nairn 1991). The difference in Ca$^{2+}$ concentration may be because in Feng’s experiments, intracellular Ca$^{2+}$ concentration was artificially elevated because the relatively low-resistance microelectrode (50 MΩ) that impaled the cell might not provide tight seal with the membrane and thus make the membrane leaky to Ca$^{2+}$. Alternatively, the Ca$^{2+}$ concentration in our experiments may be lowered below normal as a consequence of the low Ca$^{2+}$ levels in our internal solution (due to use of a small concentration of Ca$^{2+}$ chelator in the internal solution). To address these possibilities, we set the Ca$^{2+}$ level in the internal solution slightly above (0.3 $\mu$M) resting concentration. We were still unable to reverse LTP with CaMKII inhibitor. This was also true when additional steps to activate PP1 were taken (addition of calmodulin and PKA inhibitor).

Still another possibility to be considered is that the activity of PSD-associated CaMKII is less effectively inhibited by AC3-I than that of soluble CaMKII. We initially demonstrated that the abilities of soluble and PSD-associated CaMKIIs to phosphatase an exogenous synthetic peptide substrate (10 $\mu$M autocamtide-2) were similarly inhibited by AC3-I (IC$_{50}$ 20–80 $\mu$M; Fig. 4A). Furthermore AC3-I also similarly inhibited autophosphorylation of soluble and particulate CaMKII in whole-brain extracts (IC$_{50}$ 0.6–1.1 $\mu$M). These results indicate that the inhibitor does not have difficulty entering the PSD and interacting with CaMKII. This conclusion is consistent with the report that phosphorylation of GluR1 by PSD-associated CaMKII can be potently inhibited by a related CaMKII inhibitor peptide (Hayashi et al. 1997). However, further analysis of CaMKII-mediated phosphorylation in whole-brain extracts revealed significant differences in the susceptibility of various substrate phosphorylations toward inhibition by AC3-I: while phosphorylation of the soluble P76/78 proteins was potentely inhibited (IC$_{50}$ 0.3 $\mu$M), phosphorylation of particulate/PSD-associated proteins P180 was much less potently inhibited (IC$_{50}$ 8 $\mu$M). Therefore there is a possibility that LTP induction in our experiments is blocked due to inhibition of phosphorylation of a substrate with high sensitivity to AC3-I, but LTP maintenance might not be blocked because the persistent phosphorylation of a substrate with low sensitivity to AC3-I, like P180, was not inhibited. Indeed, we found that just a twofold reduction in concentration of AC3-I already caused a significant decrease in its effect on LTP induction (Fig. 2), suggesting that the concentration of the inhibitor in dendrites may be too low to block reactions that are less sensitive to the inhibitor. The preceding interpretation, however, requires that intramolecular phosphorylation of CaMKII should not be blocked by this concentration of CaMKII inhibitor, or, if it is blocked, that the kinase remains phosphorylated on T286, perhaps, due to low phosphatase activity (see following text).

One remaining technical difference between our work and that of Feng (1995) is that he used a microelectrode for intracellular application of inhibitor whereas we used the whole-cell patch-clamp method. However, our results are in agreement with Malinow et al. (1989) using microelectrodes to deliver CaMKII inhibitory peptide; they also did not find that LTP maintenance could be reversed.
Possible roles of CaMKII in LTP

Although the role of CaMKII in LTP induction is clear, the crucial substrates are not known with certainty. One of CaMKII substrates in the PSD is the NMDA channel (Gardoni et al. 1998; Leonard et al. 1999; Omkumar et al. 1996; Strack and Colbran 1998; Strack et al. 2000), the function of which may be upregulated as a result of this phosphorylation (Kitamura et al. 1993; Kolaj et al. 1994). It is therefore possible that if the basal phosphorylation of the NMDA channel enhances its conductance that the CaMKII inhibitor blocks LTP induction simply because the basal NMDA conductance is reduced. We have tested this possibility by determining whether CaMKII inhibitor reduces the baseline NMDA conductance and found that it does not. A more complex possibility that we have not tested is that the NMDA conductance becomes enhanced by a CaMKII-dependent process during the LTP induction protocol. If this occurs, this enhancement might be necessary for LTP induction. In general, little is known about the time-dependent changes in the NMDA conductance during LTP induction, but it is suspected that it is upregulated during induction through a PKC- and Src-dependent process and that blocking this up-regulation blocks LTP induction (W. Y. Lu et al. 1999; Y. M. Lu et al. 1998; Yu and Salter 1999). However, it is also possible that the NMDA conductance could be upregulated during LTP induction by a CaMKII-dependent phosphorylation of the NMDA channel or indirectly through the cascade (Weng et al. 1999) initiated by the CaMKII-dependent phosphorylation of SynGap (Chen et al. 1998).

There appear to be several CaMKII substrates crucial for LTP induction that are downstream from Ca$^{2+}$ entry through the NMDA channel. First, during induction of LTP, CaMKII phosphorylates the GluR1 subunit of AMPA channel (on S831) (Barria et al. 1997; Lee et al. 2000; Mammen et al. 1997), and this causes the AMPA channel conductance to increase (Benke et al. 1998; Derkach et al. 1999). Second, new AMPA channels appears to be inserted into the synapse during LTP induction and this insertion is CaMKII dependent (Hayashi et al. 2000; Maletic-Savatic et al. 1998). This last process, however, is not due to CaMKII-dependent phosphorylation of S831, indicating that other targets of CaMKII must be involved (Hayashi et al. 2000). Third, it appears that CaMKII is responsible for the addition of new synapses after LTP induction (Tonii et al. 1999). This idea is consistent with the data suggesting the role of CaMKII in restructuring of dendritic branches, synapse shape, and synapse number (Koh et al. 1999; Rongo and Kaplan 1999; Wu and Cline 1998).

Despite our evidence against a role for CaMKII activity in the maintenance of LTP, there remain some reasons for suspecting that CaMKII does play a role: biochemical experiments show a persistent activation of CaMKII and a persistent phosphorylation of GluR1 (Barria et al. 1997; Fukunaga et al. 1993, 1995; Lee et al. 2000; Ouyang et al. 1997, 1999). Why then, do our data not provide any support for the functional role of this persistent activity? Several hypotheses need to be considered. One possibility is that the relevant phosphatase activity may still be too low and that higher Ca$^{2+}$ concentrations than we have tested in our experiments (0.3 μM) are required to activate the phosphatase. Indeed, it has been suggested that calcium elevation as much as ~0.8 μM (Yang et al. 1999) are needed to induce LTD, which is known to involve phosphatase activation. These considerations emphasize that the mechanisms maintaining low phosphatase activity could be as important for LTP maintenance as mechanisms maintaining the kinase activity. There are in fact indications that the basal phosphatase activity decreases after induction of LTP and therefore can contribute to LTP maintenance (Blitzer et al. 1998; Fukunaga et al. 2000; Klann and Thiels 1999). Another possibility is that there is a redundant kinase (PKC) that phosphorylates the LTP target even after CaMKII is inhibited (Feng 1995; Wang and Feng 1992; Wang and Kelly 1996, but see Malgaroli et al. 1992). Finally, it cannot be excluded that constitutive CaMKII activation and GluR1 phosphorylation do not occur at synapses and are thus not directly relevant to synaptic function. More work is needed to distinguish among these possibilities.

We thank Dr. Simon Levy for measuring Ca$^{2+}$ concentration in the internal solution and Dr. Leslie Griffith for providing AC3-control peptide. Present address of S. Strack: Dept. of Pharmacology, University of Iowa, 2-432 BSB, Iowa City, IA 52242.

REFERENCES


Blitzer RD, Connor JH, Brown GP, Wong T, Shenolikar S, Iyengar R, and Landau EM. Gating of CaM-KII by CAMP-regulated protein phospha-


Derkach V, Barria A, and Soderling TR. Ca$^{2+}$/calmodulin kinase II en-
hances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isox-


Rongo C and Kaplan JM. CaMKII regulates the density of central gluta

Saitoh T and Schwartz JH. Phosphorylation-dependent subcellular transloca
tion of a Ca2+/calmodulin-dependent protein kinase produces an autonomou


Shen K and Meyer T. Dynamic control of CaMKII translocation and localiza


Strack S, Barbana MA, Waizenke BE, and Colbran RJ. Differential inactiva
tion of postsynaptic density-associated and soluble Ca2+/calmodulin-depen

Strack S, Choi S, Lovinger DM, and Colbran RJ. Translocation of auto


