Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11175–11179, November 1995
Neurobiology

Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism

(glutamate receptor/synaptic plasticity/learning)

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Contributed by Roger A. Nicoll, August 25, 1995

ABSTRACT **Ca^{2+}-sensitive kinases are thought to play a role in long-term potentiation (LTP). To test the involvement of Ca^{2+}/calmodulin-dependent kinase II (CaM-K II), a truncated, constitutively active form of this kinase was directly injected into CA1 hippocampal pyramidal cells. Inclusion of CaM-K II in the recording pipette resulted in a gradual increase in the size of excitatory postsynaptic currents (EPSCs). No change in evoked responses occurred when the pipette contained heat-inactivated kinase. The effects of CaM-K II mimicked several features of LTP in that it caused a decreased incidence of synaptic failures, an increase in the size of spontaneous EPSCs, and an increase in the amplitude of responses to iontophoretically applied α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate. To determine whether the CaM-K II-induced enhancement and LTP share a common mechanism, occlusion experiments were carried out. The enhancing action of CaM-K II was greatly diminished by prior induction of LTP. In addition, following the increase in synaptic strength by CaM-K II, tetanic stimulation failed to evoke LTP. These findings indicate that CaM-K II alone is sufficient to augment synaptic strength and that this enhancement shares the same underlying mechanism as the enhancement observed with LTP.**

Repetitive activation of excitatory glutamatergic synapses results in a long-lasting enhancement in synaptic strength, referred to as long-term potentiation (LTP). The most widespread form of LTP requires the activation of postsynaptic *N*-methyl-D-aspartate (NMDA) receptors and an increase in postsynaptic Ca^{2+}. Considerable evidence suggests that the increase in Ca^{2+} activates Ca^{2+}-dependent kinases (1–4). In particular, Ca^{2+}/calmodulin-dependent kinase II (CaM-K II), which is present at extremely high concentrations in the postsynaptic density (5, 6), is an attractive candidate for mediating the effects of Ca^{2+}. Many lines of indirect evidence support a role for this kinase in mediating the effects of Ca^{2+} on synaptic strength (2, 3). A direct approach to investigating a role for CaM-K II in LTP is to determine its effects on synaptic strength and LTP when the concentration of activated kinase is increased in postsynaptic cells. Recently two groups, one using vaccinia virus in acute hippocampal slices (7) and one using mouse genetics (8), have expressed constitutively active forms of this enzyme. In the vaccinia virus experiments (7), evidence consistent with an enhancement in synaptic transmission was presented, and LTP induction was impaired. In contrast, in the transgenic mouse experiments (8), no change in synaptic transmission or in the ability to generate LTP was found. Aside from the apparent discrepancies in these reports, it is difficult in these experiments to entirely exclude indirect effects associated with the use of the expression systems. In the present experiments we have examined the effects of CaM-K II by directly injecting a constitutively active form of this enzyme into the postsynaptic cell.

METHODS

Experiments were performed at room temperature (21–24°C) on hippocampal slices prepared (9, 10) from 3- to 5-week-old male Hartley guinea pigs or 2- to 3-week-old Sprague–Dawley rats. All bathing solutions contained picrotoxin (100 μM). Standard extracellular, intracellular, and whole-cell patch clamp methods were used (10–12). Field electrodes contained 1 M NaCl and intracellular electrodes contained 2 M potassium acetate with either 1 μM activated CaM-K II or 1 μM heat-inactivated CaM-K II as control. The tips of whole-cell pipettes were filled with a solution containing 123 mM cesium gluconate, 15.5 mM CsCl, 10 mM Hepes, 10 mM CsEGTA, 8 mM NaCl, 1 mM $CaCl_2$, 2 mM MgATP, 0.3 mM Na_3 GTP, 0.2 mM cAMP, 10 mM D-glucose, and 10 μM microcystin-LR (pH 7.3 with CsOH, 280–290 mosM). These patch electrodes were then backfilled with the same solution containing either 200 nM activated CaM-K II or 200 nM heat-inactivated CaM-K II. Cells were maintained at a membrane potential between −70 and −85 mV. Whole-cell experiments were stopped when the series resistance was >30 MΩ or when the series resistance changed >20% during the course of an experiment.

To evoke synaptic responses, stimuli (100-μs duration at a frequency of 0.05–0.1 Hz) were delivered through fine bipolar stainless steel electrodes placed in stratum radiatum. For two-pathway experiments, two stimulating electrodes were placed on either side of the recording electrodes. To elicit LTP tetani [four trains of 100 pulses (100 Hz) at 20-s intervals] were delivered at test stimulus intensity. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) responses were evoked by iontophoretically applying AMPA (negative 75–150 nA, 1 s) every 120 s with an electrode containing 10 mM AMPA (pH 8) placed close to the cell body layer. For the first 2–3 min after break-in, AMPA pulses were applied more frequently and the iontophoretic current was adjusted to obtain 100- to 200-pA responses. Minimal stimulation recordings at a frequency of 1 Hz were obtained under visual control. Pipette solutions were exchanged using a 2PK+ perfusion kit (Adams & List, Westbury, NY). Fast green (1%) was included in the solution to verify diffusion into the cell, which took 8–10 min. Failure rates were estimated by the method of Liao *et al.* (13), for successive epochs of 180 stimuli. Unless otherwise

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Abbreviations: CaM-K II, Ca^{2+}/calmodulin-dependent protein kinase II; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; fEPSP, field EPSP; LTP, long-term potentiation; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate.

stated, values given in the text are means ± SEM and significance was assessed by either a paired or an unpaired Student's *t* test.

Baseline values of excitatory postsynaptic potentials (EPSPs), excitatory postsynaptic currents (EPSCs), and iontophoretic responses were obtained from averages of responses during the first 2–3 min (time 0 on each graph)—or, for the perfusion experiments, during the 6 min prior to the time of perfusion—and defined as 100% for subsequent analyses. For all experiments neurons injected with activated or heat inactivated CaM-K II were interleaved during daily sessions. The CaM-K II (α subunit) was truncated at residue 316 to give a monomeric (30- to 40-kDa) enzyme, expressed in baculovirus-infected Sf9 insect cells, and purified as described (14). The kinase (2 μM) was converted to its constitutively active form (30–40% of Ca^{2+}-independent activity) by autothiophosphorylation (15). Inactivated kinase was heated for 10 min at 100°C prior to addition to the autothiophosphorylation reaction mixture. All kinase samples were diluted 2-fold (intracellular recording) or 10-fold (whole-cell recording) in pipette solution just before use and were maintained on ice. Aliquots were renewed within 2 hr after their preparation. After some experiments, kinase activity of the internal solution containing the diluted activated CaM-K II was measured and found to retain 80% of its original activity.

RESULTS

We first tested the effects of activated CaM-K II by adding it to the whole-cell pipette solution (200 nM) and measuring the amplitude of EPSCs over time. While a clear growth in the size of the EPSC was seen in some cells, the effect was variable and could not be systematically studied. In an attempt to increase the reliability of this effect, we included the phosphatase inhibitor microcystin-LR (10 μM) in the pipette solution. Under these conditions a very consistent growth in the size of the EPSC was observed (Fig. 1). The size began to increase significantly within 6–8 min after establishment of the whole-cell recording, and the maximum effect occurred at 15–30 min (Fig. 1*B*, ●). The average size of the EPSC reached 164 ± 16% (n = 8) of the initially recorded EPSC. CaM-K II had no effect on the cells' passive membrane properties. To ensure that the enhancement resulted from the CaM-K II and not from nonspecific effects or from the microcystin-LR, interleaved recordings were made with the identical internal recording solution, except that heat-inactivated kinase (200 nM) was used instead of the active kinase. Under these recording conditions, no enhancement was observed (105 ± 6%, n = 15) (Fig. 1*B*, ○).

To gain insight into the mechanism(s) involved in the CaM-K II-induced enhancement, we carried out experiments using minimal stimulation so that we could record failures of evoked responses (16–18). In these experiments, the pipette was internally perfused under visual control to deliver the CaM-K II in a temporally controlled manner (see *Methods*). In the example shown in Fig. 2*A* the stimulus intensity was initially adjusted so that it produced mostly failures. After a stable baseline was established, perfusion of the pipette with CaM-K II was initiated, and about 10 min later the failure rate began to decrease, accompanied by an increase in the mean size of the response (Fig. 2*A2*). A summary of five experiments with the active kinase (Fig. 2*B1*, ●) demonstrates that the failure rate on average decreased by 27 ± 10% ($P < 0.02$), whereas the heat-inactivated kinase had no effect on either the failure rate (Fig. 2*B1*, ○) or the mean size of the EPSC (Fig.

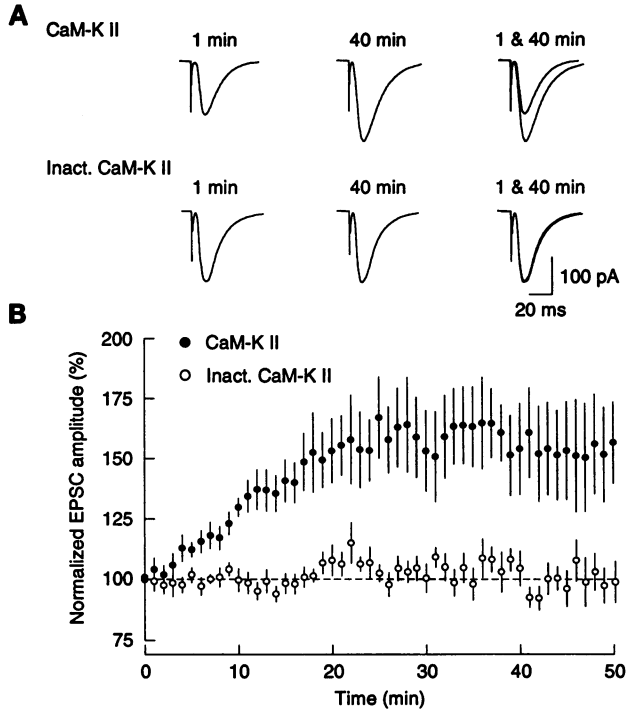

FIG. 1. Activated form of CaM-K II potentiates evoked EPSCs recorded in hippocampal CA1 neurons. (*A*) Average of six consecutive EPSCs obtained during whole-cell recordings at the indicated times following break-in. Recordings were made with pipette solutions containing activated (upper traces) or heat-inactivated (lower traces) CaM-K II. EPSCs are superimposed to the right. (*B*) Summary data illustrating the time course of EPSC amplitudes (see *Methods* for normalization procedure) in the presence of activated (●) or heat-inactivated (○) CaM-K II.

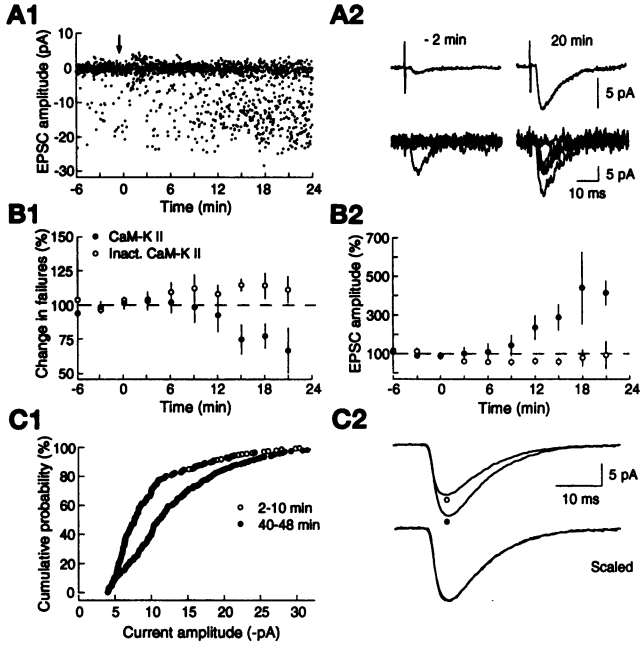

FIG. 2. CaM-K II-induced potentiation is associated with both a change in the number of synaptic failures and the size of spontaneous EPSCs. (*A1*) An individual example of the decrease in failure rate following exchange of the pipette solution with one containing activated CaM-K II. Time 0 is defined as when the solution reaches the tip of the electrode. (*A2*) Average of 100 (upper) or composite of 6 consecutive (lower) traces at times indicated. (*B*) Summary of failures (*B1*) and average amplitude (*B2*) for experiments using activated (●) or heat-inactivated (○) CaM-K II. (*C1*) Cumulative amplitude distributions comparing events collected 2–10 min (○) and 40–48 min (●) after break-in with a CaM-K II-containing pipette. (*C2*) Averages of events shown in *C1* are superimposed (upper traces) and scaled (lower traces).

2*B2*) (n = 5). Consistent with the decrease in failure rate, we also found that, associated with the increase in the evoked response (Fig. 1), the frequency of spontaneous EPSCs increased 32 ± 6% (n = 4) [compared with 6 ± 4% (n = 3)] for the heat-inactivated CaM-K II ($P < 0.02$) (data not shown).

The results presented thus far can be explained by an increase in the probability of transmitter release (18) and/or an all-or-none upregulation of clusters of AMPA receptors (13, 19). Indeed, we also found that CaM-K II increased the size of the spontaneous EPSCs (Fig. 2*C*) [active, 36 ± 3%, n = 4; inactive, 6 ± 4%, n = 3 ($P < 0.002$)]. This observation suggested that CaM-K II might be enhancing the EPSCs, at least in part, by increasing the sensitivity of AMPA receptors to synaptically released glutamate. This possibility was tested directly by monitoring the responses of CA1 cells to inotophoretically applied AMPA. The response to AMPA slowly increased when the recording pipette contained CaM-K II (151 ± 10% measured at 40–50 min, n = 8) (Fig. 3 *A1* and *B*, ●), but not when it contained the inactive form (101 ± 14%, n = 8) (Fig. 3 *A2* and *B*, ○) ($P < 0.02$). These results indicate that CaM-K II applied directly into the cell can increase the sensitivity of AMPA receptors, an effect that most likely contributes to the enhancement of the EPSC.

Are the effects of CaM-K II related to LTP? To address this question, we designed occlusion experiments in which the effects of CaM-K II on control synapses were compared with its effects on synapses expressing LTP. In these experiments, two independent inputs onto the same population of pyramidal cells were monitored with field electrode recordings (Fig. 4*A*). A saturating level of LTP was induced in one of the pathways (S1), while the other pathway (S2) served as a control (Fig. 4*A*). Once LTP was observed to be stable (typically within 60 min), a whole-cell recording was made from a cell within the population sampled by the field electrode (Fig. 4*B*). The effect of CaM-K II on the LTP-expressing pathway was then compared with the effect on the control pathway (Fig. 4 *B1* and *B2*), while we continued to monitor fEPSPs from both pathways (Fig. 4*B3*). CaM-K II had its normal enhancing effect on the control pathway but was without effect on the pathway expressing LTP (Fig. 4 *B1* and *B2*). A summary graph of all the experiments (Fig. 5) demonstrates that the enhancing action of CaM-K II was markedly attenuated on synapses expressing LTP (an increase of 17 ± 9%, n = 6 vs. 67 ± 16%, n = 6, measured at 40–55 min from tetanized and untetanized pathways, respectively, $P < 0.0001$) (Fig. 5*B1*). The average amplitude of the CaM-K II-induced potentiation was less than that for saturated LTP (compare Fig. 5 *A1* and *B1*). This most likely was due to the variable loading of the cells with the kinase, as suggested by the fact that the potentiation observed in some of the loaded cells was equivalent to that observed with saturated LTP (data not shown). To verify that this selective effect was, in fact, due to CaM-K II, a series of control experiments with heat-inactivated enzyme were interleaved with the above experiments (Fig. 5*B2*) (n = 6) and no change in the EPSCs in either pathway occurred. The simultaneously recorded extracellular responses to both the control and LTP-expressing pathways remained constant in all of these experiments (Fig. 5*A2*).

In a final set of experiments we performed the reverse occlusion experiment, in which we first potentiated the synapses with CaM-K II and then asked whether this potentiation affected the ability to induce LTP. Whole-cell recording techniques could not be used for this experiment, because the ability to induce LTP washes out over the 30–40 min required for the CaM-K II effect to stabilize. We therefore loaded sharp microelectrodes with the enzyme (1 μM). Because this method of loading neurons with CaM-K II was less successful than that used with the whole-cell approach, we examined only the ability to generate LTP in those cells in which CaM-K II caused an enhancement. Fig. 6*A1* shows a summary graph of these cells (n = 5). We also simultaneously monitored field potentials, so that the magnitude of LTP in the field could be compared with that generated in the cell. While tetanization caused substantial LTP in the field (Fig. 6*A2*), it produced no LTP in the cells loaded with CaM-K II (Fig. 6*A1*) (208 ± 40% vs. 79 ± 33% of baseline, $P < 0.0001$, for field potential and intracellular recordings, respectively). In a series of control experiments with the heat-inactivated enzyme, the magnitude of the LTP in the cells (Fig. 6*B1*) was similar to that recorded in the field (Fig. 6*B2*) (n = 5). These findings provide further evidence that LTP and CaM-K II enhance EPSCs by the same underlying mechanism(s).

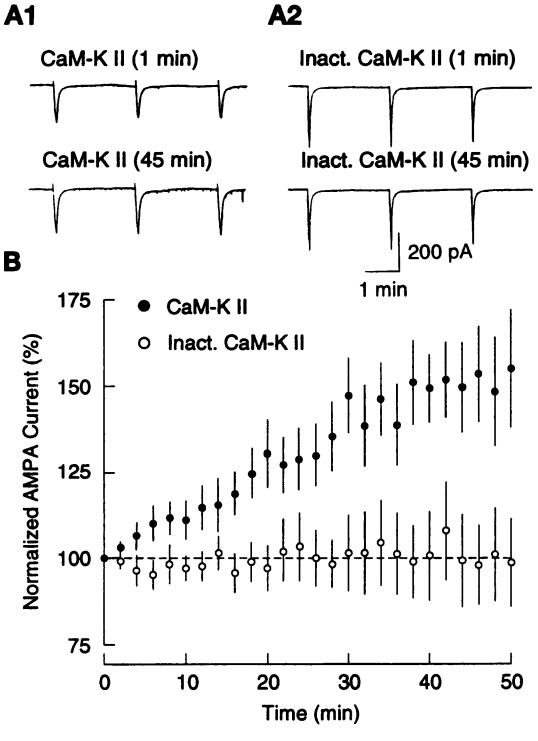

FIG. 3. Postsynaptic sensitivity to AMPA is increased by CaM-K II. (*A*) Chart records of membrane current from voltage-clamped CA1 pyramidal cells held at −75 mV show responses to brief iontophoretic pulses of AMPA. The presence of activated (*A1*), but not heat-inactivated (*A2*), CaM-K II potentiated AMPA responses 45 min after recordings were initiated. (*B*) Plots of summarized data illustrating the time courses of the mean amplitude of AMPA-induced current expressed as percentage of the mean control amplitude, in the presence of either activated (●) or heat-inactivated (○) CaM-K II.

DISCUSSION

The hypothesis that CaM-K II mediates the effects of the NMDA receptor-dependent increase in postsynaptic Ca^{2+} that triggers LTP is attractive and is based on a variety of experimental approaches. (*i*) Biochemical studies have shown that the kinase can act as a molecular switch, conferring properties that are advantageous for long-lasting storage of changes initiated by brief Ca^{2+} signals (2, 20, 21). (*ii*) Manipulations that interfere with CaM-K II function interfere with LTP. These experiments include the use of inhibitors (12, 22–24) and knockout of the α subunit of CaM-K II in mice (25). (*iii*) LTP is associated with an increase in the activity of the Ca^{2+}-independent form of CaM-K II (26). (*iv*) One of the targets for CaM-K II is the AMPA receptor itself. Thus, activated CaM-K II can phosphorylate AMPA receptors in the postsynaptic density and can enhance responses to AMPA receptor agonists in cultured hippocampal neurons (15) and acutely isolated dorsal root ganglion neurons (27). Responses

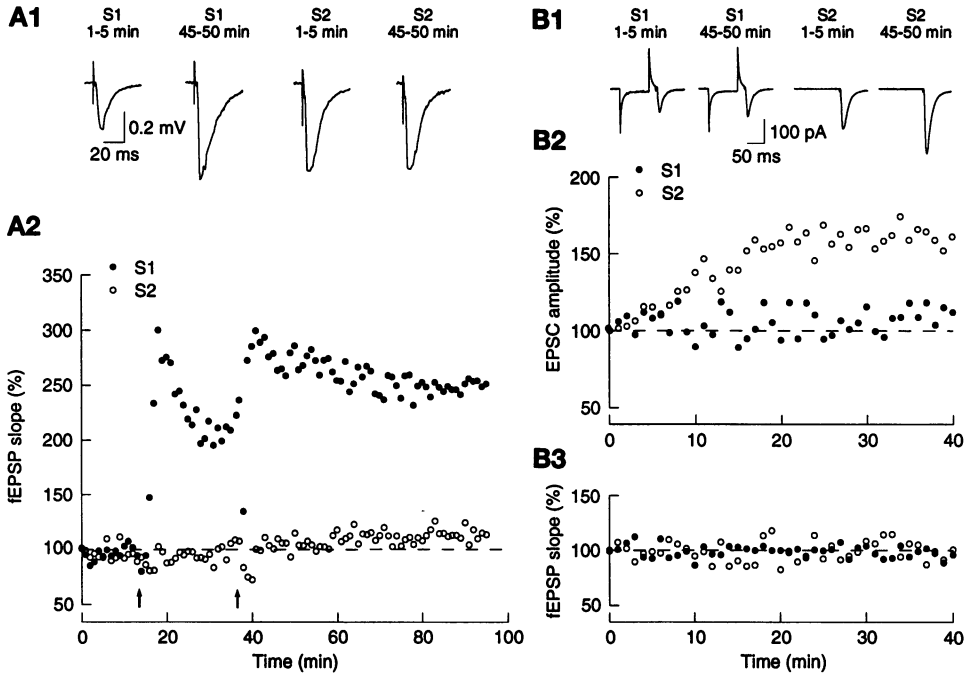

FIG. 4. CaM-K II-induced potentiation of evoked EPSCs is occluded at synapses expressing saturated LTP. Two independent inputs (S1 and S2) onto the same population of CA1 neurons were alternatively stimulated. LTP was induced by tetanic stimulation of S1; S2 served as the control input. (*A1*) Field potential recordings (average of 15 consecutive traces) from a representative experiment taken at the indicated times. (*A2*) Normalized field EPSP (fEPSP) slopes evoked in the tetanized pathway (S1; ●) and in the untetanized control pathway (S2; ○). Two tetanizations were given in this experiment (arrows). (*B*) Simultaneous recordings of whole-cell EPSCs obtained with a pipette containing CaM-K II (*B1* and *B2*) and field potentials (*B3*) 80 min after having induced LTP (shown in *A2*). EPSCs and fEPSPs were normalized to the initial responses at the beginning of the whole-cell experiment (dotted baseline). *B1* shows representative EPSCs recorded at the indicated times from the two independent pathways. A voltage step was given 85 ms before S1 to monitor series and input resistance.

evoked by activating GluR1 receptors expressed in oocytes are also enhanced by CaM-K II (28).

The present results demonstrate that in hippocampal pyramidal cells *in situ*, CaM-K II enhances EPSCs and that this enhancement is due, at least in part, to an enhancement of AMPA receptor sensitivity, thus extending previous results in cultured neurons (15) and spinal cord neurons (27). CaM-K II also caused an increase in the frequency of spontaneous EPSCs and a reduction in the number of failures of evoked responses. These results can be explained by a presynaptic enhancement in transmitter release (18) and/or an all-or-none upregulation of clusters of AMPA receptors (13, 19). The finding

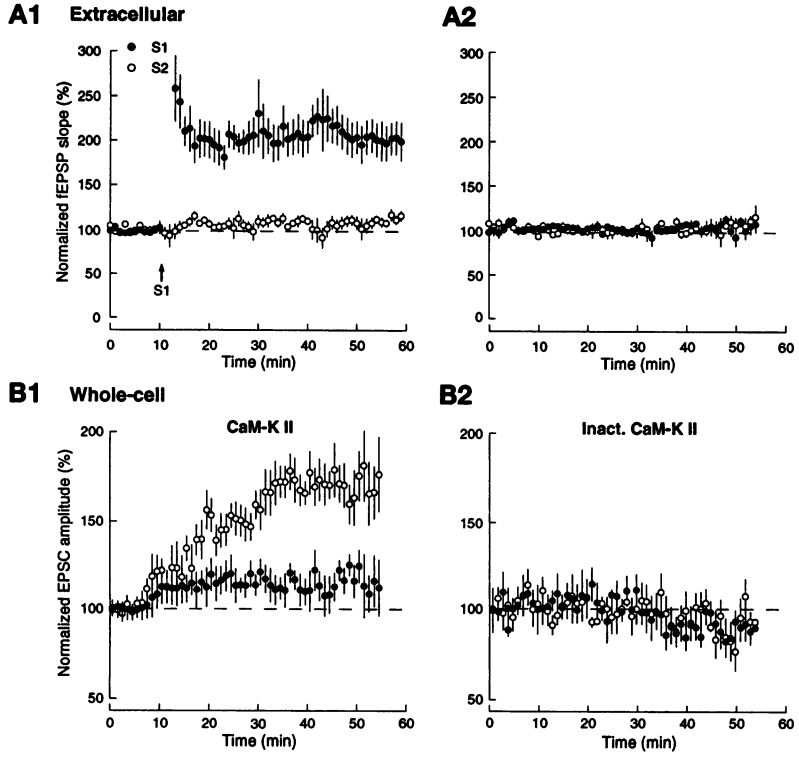

FIG. 5. Summary data showing occlusion of the CaM-K II-induced potentiation by LTP. The time course of changes in extracellular fEPSPs (*A*) and evoked whole-cell EPSCs (*B*) is shown. *A1* illustrates that LTP was induced in S1, but not S2, prior to whole-cell recordings; and *A2* shows the stability of fEPSPs during the whole-cell recordings (normalized to the initial recordings at the beginning of whole-cell experiments). Arrow indicates the time of the first tetanus given to S1. (*B*) Graph illustrating potentiation of EPSC amplitudes by CaM-K II only in the untetanized control pathway (S2; ○) (*B1*). EPSCs did not change when the pipette solution contained heat-inactivated CaM-K II (*B2*). Because there was no difference in the magnitude of LTP induced in the two sets of experiments (active kinase, 117 ± 11%, $n = 6$; inactive kinase, 102 ± 10% increase, $n = 6$), the data from these experiments were combined in *A*.

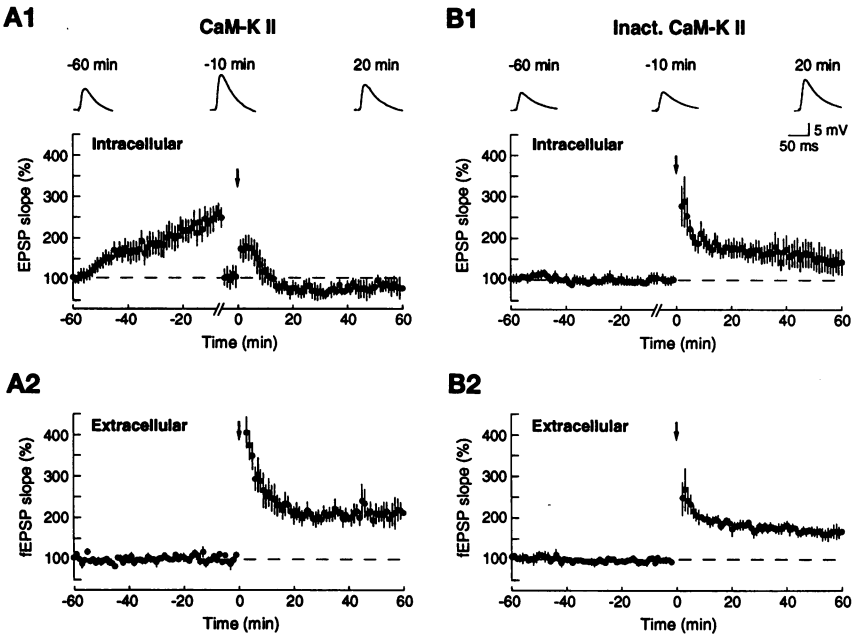

FIG. 6. Summary data from occlusion experiments examining LTP at synapses already potentiated by activated CaM-K II. (*A*) Time course of EPSP changes recorded with intracellular electrodes containing CaM-K II (*A1*) with simultaneous recordings of field potentials (*A2*). (*B*) Similar experiments using heat-inactivated CaM-K II as control. Arrow indicates the tetanus given at time 0. Slopes of EPSPs in LTP were then renormalized 5 min before the tetanus (dotted baseline). *Insets* show averaged EPSPs taken at indicated time.

that the enhancement of responses to exogenously applied AMPA was of similar magnitude to that observed with the evoked EPSC favors the latter hypothesis. Most importantly, occlusion experiments establish that the synaptic enhancement evoked by CaM-K II shares the same underlying mechanisms as LTP. Two recent reports examined the effects of overexpression of constitutively active CaM-K II on LTP. One study found that overexpression with vaccinia virus in the hippocampal slice impaired the ability to induce LTP (7), whereas the other study, using transgenic mice, found no impairment in LTP (8), even though significant effects on long-term depression were observed. The results of our acute experiments are more consistent with the former study.

Inhibitor studies have led to the proposal that protein kinase C activity (23, 29–31) and protein-tyrosine kinase activity (32) are also required for LTP. Since our results indicate that CaM-K II alone mimics LTP, either CaM-K II can, in some unknown manner, activate these enzymes, or certain levels of basal protein kinase and tyrosine kinase activity are required for CaM-K II to cause a synaptic enhancement.

In summary, the present results indicate that the enhancement of synaptic strength caused by postsynaptic injection of activated CaM-K II shares the same underlying mechanism(s) as LTP. It appears that elucidating the substrates targeted by CaM-K II should shed considerable light on the molecular mechanisms underlying LTP. A likely target is the AMPA receptor itself, which is colocalized in the postsynaptic density with CaM-K II. Thus, defining the mechanism by which CaM-K II increases AMPA receptor function should be particularly informative.

We thank Drs. D. Copenhagen and D. Dixon for comments on the manuscript. P.-M.L. was supported by the Centre National de la Recherche Scientifique and by a North Atlantic Treaty Organization fellowship. G.O.H. was supported by a National Science Foundation predoctoral fellowship. T.R.S. was supported by National Institutes of Health Grant NS27037. R.C.M. and R.A.N. were supported by grants from the National Institutes of Health. R.A.N. is a member of the Keck Center for Integrative Neuroscience and the Silvio Conte Center for Neuroscience Research. R.C.M. is a member of the Center for Neurobiology and Psychiatry.

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