Mechanisms of Potentiation by Calcium-Calmodulin Kinase II of Postsynaptic Sensitivity in Rat Hippocampal CA1 Neurons

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Shirke, Aneil M. and Roberto Malinow. Mechanisms of potentiation by calcium-calmodulin kinase II of postsynaptic sensitivity in rat hippocampal CA1 neurons. J. Neurophysiol. 78: 2682–2692, 1997. Preactivated recombinant α-calcium–calmodulin dependent multifunctional protein kinase II (CaMKII*) was perfused internally into CA1 hippocampal slice neurons to test the effect on synaptic transmission and responses to exogenous application of glutamate analogues. After measurement of baseline transmission, internal perfusion of CaMKII* increased synaptic strength in rat hippocampal neurons and diminished the fraction of synaptic failures. After measurement of baseline responses to applied transmitter, CaMKII* perfusion potentiated responses to kainate but not responses to N-methyl-D-aspartate. Internal perfusion of CaMKII* potentiated the maximal effect of kainate. Potentiation by CaMKII* did not change the time course of responses to kainate, whereas increasing response size by pharmacologically manipulating desensitization or deactivation rate constants significantly altered the time course of responses. Nonstationary fluctuation analysis of responses to kainate showed a decrease in the coefficient of variation after potentiation by CaMKII*. These data support the hypothesis that CaMKII increases postsynaptic responsiveness by increasing the available number of active α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate channels and suggests that a similar process may occur during the expression of long-term potentiation.

INTRODUCTION

Understanding the mechanism of expression of long-term potentiation (LTP) has been a long-standing problem in the field of neurobiology (Malinow 1994; Nicoll and Malenka 1995). The means by which synaptic connections are strengthened on a long-term basis has implications for learning and memory (Bliss and Collingridge 1993), as well as the development of the nervous system (Cline 1991). One proposed mechanism is a change in postsynaptic responsiveness (Bliss and Lomo 1973; Hebb 1949; Lynch and Baudry 1984; Malinow 1994; Nicoll and Malenka 1995; Nicoll et al. 1988).

Considerable evidence supports the hypothesis that at least part of the expression of LTP is mediated by postsynaptic changes. LTP can be expressed primarily by non-N-methyl-D-aspartate (NMDA) receptor mediated transmission. (Aszty et al. 1992; Kauer et al. 1988; Kullmann 1994; Kullmann et al. 1996; Liao et al. 1995; Muller and Luskin 1988; Muller et al. 1988; Perkel and Nicoll 1993; although see Bashir et al. 1991; Clark and Collingridge 1995; O’Connor et al. 1995; Xie et al. 1992). Quantal size, classically thought to be determined postsynaptically (DelCastillo and Katz 1954), increases during LTP (Isaac et al. 1996; Kullmann and Nicoll 1992; Larkman et al. 1992; Liao et al. 1992; Stricker et al. 1996). Other work has shown that synaptic transmission at hyperpolarized potentials undergoes LTP with a decrease in failure rate (Kullmann and Nicoll 1992; Malinow 1991; Malinow and Tsien 1990; Stevens and Wang 1994), whereas transmission at depolarized voltages largely is unaffected (Isaac et al. 1995; Liao et al. 1995; but see Kullmann et al. 1996). One interpretation of these data is that LTP can be expressed predominantly by non-NMDA receptors.

The calcium-calmodulin multifunctional protein kinase II (CaMKII) is a likely postsynaptic effector of this form of plasticity. It is the most common protein in the postsynaptic density (PSD) (Kelly et al. 1984; Kennedy et al. 1983). Protocols that induce LTP increase CaMKII activity (Fukunaga et al. 1993) and result in phosphorylation of several substrates (Fukunaga et al. 1995). Pharmacological blockade of CaMKII prevents LTP (Malenka et al. 1989; Malinow et al. 1989); genetic elimination of αCaMKII diminishes LTP (Silva et al. 1992). Increased postsynaptic activity of CaMKII potentiates synaptic transmission and occludes further LTP (Pettit et al. 1994). In frog optic tectum, increased postsynaptic CaMKII selectively potentiates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–mediated transmission and decreases synaptic failures at hyperpolarized but not depolarized potentials. Quantal size of AMPA responses also is increased (Wu et al. 1996). Autophosphorylation of CaMKII produces a constitutively active enzyme (Hanson and Schulman 1992) that could underlie the persistence of LTP (Lisman 1994). Postsynaptic application of thio-phosphorylated CaMKII (CaMKII*, an in vitro modification of the enzyme that renders it active and resistant to endogenous phosphatases) has been shown to increase somatic sensitivity to kainate in cultured hippocampal cells (Kolaj et al. 1994; McGlade-McCulloch et al. 1993) and to increase the strength of synaptic transmission and mutually occlude LTP in hippocampal slices (Lledo et al. 1995). Together, these studies provide strong evidence that CaMKII is necessary and sufficient to induce LTP.

What cellular effects of CaMKII activity could produce such changes? The responsiveness of the postsynaptic membrane would be expected to depend on the proximity of receptors to the release site, the available number of active receptor molecules, and the functional characteristics of those receptors. Receptor characteristics include binding affinity, rate constants among channel states, and single-channel conductance. A modification of any of these factors could affect responsiveness, but each also would have additional...
predictable consequences. Current evidence does not distinguish among these various possibilities. This study examines the mechanism by which CaMKII potentiates responses in the hippocampal slice. Specifically, these experiments test the hypothesis that increased postsynaptic CaMKII activity increases the available number of active postsynaptic receptors. The evidence that increased postsynaptic CaMKII activity underlies LTP argues that a similar mechanism may be responsible for the expression of LTP.

METHOIDS

Hippocampal slices and recording techniques

Hippocampal slices were prepared from rats (Sprague-Dawley, 10- to 16-day old) on a Vibratome (TP1), as described previously (Otmakhov et al. 1993), and were submerged and superfused at 27°C. Whole cell recordings were acquired with a patch electrode (1.5-2.5 MΩ tip resistance) using an Axopatch 1-D (Axon Instruments) and a LM-900 interface (Dagan) operated by a custom program written in a Quickbasic environment (Microsoft) with the Axobasic software library (Axon Instruments). Visualized patch-clamping was performed with a CCD camera (Hamamatsu) attached to a Zeiss Axioskop fixed-stage microscope mounted on a translational stage. Synaptic responses were elicited by monopolar electrical stimulation of stratum radiatum via a glass microelectrode and a Grass SD9 stimulator. Agonist responses were elicited by pressure injection of receptor agonist (kainate or NMDA) using a Picospritzer (General Valve) via a patch pipette (∼7 MΩ) onto the soma of a CA1 neuron once every 10 s, except for saturation experiments when the interval was 20 s. We chose to use kainate to stimulate AMPA/kainate receptors for three reasons: first, kainate has been used previously to assay the effect of active CaMKII (McGlade-McCulloch et al. 1993). Second, because AMPA/kainate receptors do not desensitize completely to kainate, examination of steady state responses is possible (Patneau et al. 1993). Third, because kainate is a partial agonist (Patneau et al. 1993), the maximal response will be smaller and therefore more easily measured under voltage-clamp. Any neurotransmitter analogue may act differently from the endogenous transmitter, however. Agonists were delivered at concentrations (in the puffip pipette) of 0.1 mM for both kainate and NMDA, except where explicitly stated. Injection duration was 4–30 ms. Agonist was applied directly onto the soma as has been described previously (McGlade-McCulloch et al. 1993; Patneau et al. 1993). After establishment of stable baseline responses to either synaptic stimulation or agonist application, a volume of test (or control) solution approximately equal to the amount of solution initially present in the patch pipette (∼4 μl) was perfused into the cell via a small canula placed within the pipette tip.

Recording conditions, including holding current, series resistance, and input resistance, were monitored throughout each experiment. Occasionally conditions changed slightly during perfusion. Only epochs which had passive properties within 20% of baseline values were included for analysis. In puffing experiments, experiments were excluded if response onset time changed by more than 1 ms. Rhodamine-isothiocyanate (RTIC)-dextran (40 kD; Molecular Probes) was added to internal perfusion solution (∼0.1 mg/ml) as a marker for delivery of internal perfusate. This dextran is similar in molecular weight to recombinant CaMKII (33 kDa). The fluorophore was excited by a xenon-mercury arc-lamp filtered to 525–550 nm. Quantitative images of emissions >590 nm were acquired and analyzed using a cooled CCD and PMIS software (Photometrics).

Superfusate contained (in mM) 119 NaCl, 2.5 KCl, 1.3 MgCl2, 2.5 CaCl2, 26.2 NaHCO3, 11 glucose, and 0.1 picrotoxin gassed with 95% O2-5% CO2 (pH 7.4). A cut between the CA3 and CA1 regions prevented epileptiform activity. During synaptic stimulation experiments, the bath also contained 100 μM 2-amino-5-phosphonovalerate. During experiments in which saturating doses of kainate were delivered, bath solution contained 0.3 μM tetrodotoxin. Internal solution contained (in mM) 100 Cs-glucagon, 0.2 ethylene glycol-bis-(β-aminoethoxy ether)-N,N,N′,N′′-tetraacetic acid, 5 MgCl2, 2 ATP (Na2ATP), 0.3 GTP (Na2GTP), and 40 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.2 with CsOH). In some experiments, Cs+ was substituted with K+. Internal perfusion solution contained (in mM) 100 Cs+ (or K+) gluco- nate, 5 MAcetate, 5 reduced glutathione, 0.25 CaCl2, 0.006 cal- modulin, 1 ATP, 0.25 ATPγS, and 0.055 EDTA, plus 0.5 mg/ml bovine serum albumin and 0.005 thio-phosphorylated constitutively active recombinant CaMKII[1-316] (courtesy of T. Sodering, Volum Institute); in control solution, active kinase was replaced with kinase that had been heat inactivated (boiled for 15 min at 100°C) before reacting it with Ca2+. CaM and ATPγS. For experiments with applied agonist, 5 mM 1,2-bis-(2-aminophenoxy)-ethane-N,N′,N″,N‴-tetraacetic acid (Molecular Probes, Eugene, OR) and 10 μM Microcystin-LR (RBI, Natuck, MA), a phosphatase inhibitor, were added to both patch solution and internal perfusion solution. Cyclothiazide (courtesy of Lilly Pharmaceuticals, Indianapolis, IN) was prepared as a 10 mM stock solution in 1% dimethyl sulfoxide and was applied at concentrations of ≤100 μM. All chemicals were from Sigma (St. Louis, MO) except where otherwise noted.

Analysis

Stimulation artifacts were subtracted digitally from synaptic responses before analysis. Response amplitudes were quantified by calculating the average current during the 10–50 ms around the peak of the response and subtracting the average current during an interval of equal length immediately before the response. Response size after manipulations was measured as a percentage of baseline. The failure rate was estimated as double the percentage of traces in which the measured response was <0 pA after adjustment for stimulation artifact (Liao et al. 1995). Measurements of the rate of decay of responses were produced by least-squares best fit of a single exponential decay to averaged responses using a Levenberg-Marquardt fitting algorithm within the program Origin (Microcal Software). The half-width was measured as the time between half the peak response during the rising phase of the response and half the peak during the decay phase. Nonstationary fluctuation analysis (Sigworth 1980) was performed by calculating the point-by-point mean and standard deviation of a series of traces. The point-by-point coefficient of variant (CV) is calculated as the standard deviation divided by the mean. Theoretically, if N is the number of channels, P is the probability that the channel is open, and i is the single-channel current, then the mean response at any point during the time course of the response is

\[ \mu = N \cdot p \cdot i \]

The coefficient of variation is

\[ CV = \frac{1 - p}{N \cdot p} \]

and

\[ \text{CV'}^2 = \frac{N \cdot p \cdot (1 - p)}{N \cdot p \cdot (1 - i^0)} \]

here the primed terms represent values after potentiation. Note that an increase in i should produce a change in the mean with no change in the CV of responses. Mean and CV were computed over 20–50 consecutive responses during the baseline period, and, after
potentiation had stabilized, over the same number of responses before and after maximum potentiation. Baseline noise was subtracted before computation of CV. If the recording was lost before potentiation had stabilized, the data were excluded for this analysis.

For imaging experiments, the average fluorescence of a rectangle encompassing the soma was calculated, and background fluorescence was subtracted. Fluorescence was measured at several points during an experiment. Values were normalized, using the final value as 100%. Regression analysis was performed to compare normalized fluorescence and normalized potentiation.

\[\text{FIG. 2} \]

Simulations

Simulations were done using a simple five-state model for non-NMDA receptor activation (Ambros-Ingerson and Lynch 1993) based on the acetylcholine receptor. The model has the following states: C = closed, CB = closed and ligand-bound, CD = desensitized, CBD = closed, ligand-bound and desensitized, and O = open. Rate constants are as shown in Fig. 5. Initial estimates for the rate constants were adapted from Ambros-Ingerson et al. (1993). The model was simulated using the program NEURON 3.1 for Windows (M. Hines, http://www.nnc.yale.edu/HTML/YALE/NNC/neuron/nrmsim.html).

RESULTS

Enhancement of synaptic transmission by internally perfused CaMKII*

Standard visualized whole cell patch recordings were obtained from hippocampal CA1 neurons and stable synaptic transmission was elicited for 10–15 min. To test the ability of CaMKII* to potentiate synaptic transmission, the recording pipette was perfused internally with a solution containing either CaMKII* or heat-inactivated CaMKII. Perfusion of active CaMKII* significantly potentiated synaptic transmission [Fig. 1, A–C; 146 ± 17% (mean ± SD) of preperfusion response amplitude; paired t-test: \(P < 0.05\), \(n = 17\)], whereas inactive kinase did not (93 ± 9%; paired \(t\)-test: n.s., \(n = 17\)). Potentiation by active kinase was significantly different from the effect of inactive kinase (independent \(t\)-test: \(P < 0.05\), \(n = 34\)). Some of these experiments were performed with K+ as the major cation instead of Cs+ with no obvious effect on the results.

As LTP produces a decrease in failure rate (Malinow 1991; Malinow and Tsien 1990), we wanted to test the effect of CaMKII* on synaptic failures. Stimulus strength was set so that synaptic failures (trials in which responses were not distinguishable from the background noise; Fig. 2A) were detected. As shown in Fig. 2, perfusion of active CaMKII* decreased the number of synaptic failures (Fig. 2B). In this example, response size increased ~8 min after internal perfusion of CaMKII* (Fig. 2C) with no significant change in series resistance (Fig. 2D). Quantitative failure analysis (see METHODS) showed a decrease in the failure rate, indicated by a decrease in the magnitude of the peak at 0 pA in histograms of response amplitudes (Fig. 2, E and F). The decrease in failure rate was a general finding after perfusion of active CaMKII* (Fig. 2G; before = 55 ± 6.6%, after = 41 ± 8.7%; paired \(t\)-test: \(P < 0.05\), \(n = 11\)). These experiments show that active CaMKII is sufficient to potentiate synaptic transmission and decrease failure rates, as occurs during LTP (Bolshakov and Siegelbaum 1995; Kullmann and Nicoll 1992; Malinow 1991; Malinow and Tsien 1990; Stevens and Wang 1994).

Enhancement of responsiveness to glutamate analogues by CaMKII*

Changes in synaptic failure rates traditionally have been interpreted to indicate presynaptic modifications. However, more recently, postsynaptic mechanisms have been postulated to explain this finding (Isaac et al. 1995; Liao et al. 1995). We wanted to examine more closely possible postsynaptic modulations produced by CaMKII*. First, we studied the specificity of CaMKII* potentiation for NMDA or non-NMDA receptor responses. Responses were monitored under voltage-clamp while glutamate analogues were pressure-applied to the soma. Figure 3 shows an experiment in which kainate and NMDA were puffed alternately. After achieving stable baseline responses to the two agonists of \(\geq 50\) sweeps, CaMKII* was perfused into the patch pipette. About 10 min subsequent to perfusion, the response to kai-
nate began to grow (Fig. 3, A–C). Overall, active CaMKII* significantly potentiated responses to kainate (Fig. 3H; 265 ± 54%; paired t-test: \( P < 0.05, n = 8 \)), whereas inactive kinase had no significant effect (104 ± 21%; paired t-test: n.s., \( n = 5 \)). The effect of active kinase was significantly different from inactive kinase (independent t-test: \( P < 0.05, n = 13 \)). In the experiment shown in Fig. 3, a fluorescent marker (40 kDa RITC-dextran) was included in the internal perfusate. The time course of the increase in fluorescence intensity of marker in the cell body (Fig. 3G) closely matched potentiation of responses to kainate (Fig. 3C). This was a general finding (Fig. 3I; multiple regression analysis: \( P < 0.05, R = 0.95, n = 17 \)).

In contrast to the effect seen on kainate responses, active kinase produced a rapid, long-lasting decrease of NMDA responses (Fig. 3, D–F). This depression occurred before the appearance of fluorescence (Fig. 3G). Perfusion of active kinase depressed NMDA responses (64 ± 8%; paired t-test: \( P < 0.05, n = 4 \)). Perfusion of inactive kinase also depressed NMDA responses (75 ± 4%; paired t-test: \( P < 0.05, n = 5 \)). The depression lasted for the duration of the experiment; response size at the end of the experiment was the same as that immediately after perfusion (active: 102 ± 13% of immediate postperfusion level, paired t-test: n.s., \( n = 4 \); inactive: 108 ± 6% of immediate postperfusion level, paired t-test: n.s., \( n = 5 \)). There was no difference between the effect of active and inactive kinase, (independent t-test: n.s., \( n = 9 \)) implying that this effect was not produced by CaMKII. This effect may be due to calmodulin, which is included in both test and control internal perfusate and has been shown to depress NMDA responses (Ehlers et al. 1996). No further characterization of this effect was attempted.

**Increase of the maximal effect of kainate**

To investigate the mechanism of potentiation of kainate responses, several hypothetical mechanisms were tested. CaMKII* might increase responsiveness to kainate via a simple increase in receptor affinity for agonist; however, this hypothesis also implies that responses to a saturating dose of kainate, a measure of maximum efficacy, should not be potentiated by CaMKII* (Fig. 4A, Table 1). In contrast, an increase in the number of AMPA/kainate receptors would cause an increase of responses to all concentrations of kainate as well as of the maximal effect of kainate (Fig. 4B).

Calculations from previous estimates of the AMPA/kainate receptor dose-response curve predict that 10 mM kainate will produce ~99% of a saturated response (Jonas and Sakmann 1992; Patneau and Mayer 1991). Puffed application of 10 mM kainate produced a much larger response than the 100 μM concentration used in other experiments in this study (Fig. 4C). As the duration of pressure application was increased, peak response size increased but eventually reached a plateau (Fig. 4C).

Note that when application time was increased further, it only increased the late portion of the response, which was probably due to agonist diffusing to sites distant from the application site. To ensure that the response to application of 10 mM kainate for 20 ms was the maximal effect of kainate, it was compared with the response to 50 mM kainate applied in a similar fashion. Two application pipettes, one containing 10 mM kainate, the other containing 50 mM kainate, were positioned at equal distance from the cell membrane of a patched cell. Responses were evoked from each on alternate pathways. The time course of the increase in fluorescence intensity of marker in the cell body (Fig. 3G) closely matched potentiation of responses to kainate (Fig. 3C). This was a general finding (Fig. 3I; multiple regression analysis: \( P < 0.05, R = 0.95, n = 17 \)).
E–G: 114 ± 3%, paired t-test: $P < 0.05$, $n = 4$). The increase of the maximal effect of kainate is evidence against a simple change in affinity.

Although CaMKII* increased the maximal effect of kainate, the magnitude of potentiation was considerably smaller than potentiation of responses to 100 μM kainate (see preceding text). We noticed that positioning a puffer pipette filled with 10 mM kainate close to the soma of a target cell, in the absence of puffing, produced a large standing current on the order of 1 nA, reversibly decreasing input resistance from 198.5 ± 26.9 to 95.1 ± 10.9 MΩ (paired t-test: $P < 0.05$, $n = 4$). On puffing agonist, the input resistance dropped as low as 30 MΩ. Exchanging K⁺ for Cs⁺ in internal solutions did not affect this response. This low resistance, presumably due to activation of AMPA/kainate conductances, will compromise the voltage clamp. Note that potentiation of responses to 10 mM kainate is evidence that the voltage-clamp was not completely compromised before application of CaMKII*. Thus although potentiation could be detected, interpretation of responses must take this into account.

We considered whether these large conductances could compromise the somatic voltage clamp to a degree that blunts the amount of potentiation seen of responses to 10 mM kainate compared with responses to lower concentrations (100 μM kainate produced responses that were ~2% of the maximal effect of kainate). To address this issue, we used the simulation program NEURON to model the voltage-clamp configuration (Fig. 5A) and the reaction scheme for AMPA/kainate channel activation (Fig. 5B). If the fraction of receptors in the open state is large, the membrane resistance will decrease markedly. Because the driving force for currents is determined by the ratio of membrane resistance to total circuit resistance, the magnitude of large conductances will be proportionately underestimated. Thus potentiation of large conductances also could be underestimated.

Simulations of various modifications that doubled the magnitude of observed synaptic responses to 100 μM kainate
produced a considerably smaller potentiation of currents observed in response to 10 mM kainate (Table 1). This supports the view that the smaller CaMKII*-induced potentiation seen with saturating applications of 10 mM kainate could be explained by the compromise in voltage clamp. Because of this potential problem with saturating applications of kainate, all other experiments were done using applications of kainate that were well below saturation level.

**Fig. 4.** Perfusion of active CaMKII* increases the maximal effect of kainate. A: hypothetically, an increase of the binding affinity of the receptor would result in a leftward shift of the dose-response curve and could produce potentiation of subsaturated responses. However, such a change would not change the maximal effect of the agonist. B: an increase of receptor number could produce potentiation of responses to all concentrations of agonist and would increase the maximal effect of the agonist. C: responses to 10 mM kainate as the puff duration is increased are larger up to the maximal effect. D: responses to saturating applications of kainate during the same experiment (subsaturating doses of kainate). E: Potentiation of subsaturated responses. However, such a change would not change the maximal effect of the agonist. F: responses to application of 10 mM kainate are mechanistic changes in receptor function as well as artifactual. 

**Table 1.** Theoretical estimates of the effect of voltage-clamp compromise on measured potentiation

<table>
<thead>
<tr>
<th>Simulated Modification Mechanism</th>
<th>Potentiation, %</th>
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<tbody>
<tr>
<td>Increased binding affinity</td>
<td>196</td>
</tr>
<tr>
<td>Partial block of desensitization</td>
<td>196</td>
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<tr>
<td>Increased open probability</td>
<td>195</td>
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<tr>
<td>Increased maximum conductance</td>
<td>196</td>
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Theoretical estimates of the effect of voltage-clamp compromise on measured potentiation demonstrate that voltage-clamp effects can diminish observed potentiation. The modeling program NEURON was used to simulate voltage-clamp configuration, synaptic receptor function, and the effect of several different possible changes in receptor function as well as the effect of voltage-clamp compromise on the magnitude of observed potentiation. The cell was modeled as a simple cylinder of 10 μm in length and diameter; the model synapse and the voltage clamp were positioned at the middle of the cell. Parameters for passive cell membrane properties were as follows: series resistance, $R_s = 15 \, \text{MΩ}$; membrane conductance $= 0.001 \, \text{mho/cm}^2$; membrane capacitance $= 1 \, \mu\text{F/μm}^2$; input resistance, $R_i = 320 \, \text{MΩ}$. Initial parameters for receptor kinetic model (see Fig. 5 for a diagram of the model) were $f_1 = 2$, $b_1 = 1$, $f_3 = 10$, $b_3 = 0.1$, $f_4 = 0.001$, $b_4 = 0.002$, $r_d = 2$, $r_o = 0.016$, $r_{0.45} = 0.45$, $r_c = 1$, maximum conductance $= 1 \, \mu\text{S}$. Potentiation was simulated by changing model parameters to double synaptic current magnitude at the membrane. Values in table indicate expected observed measures of currents obtained in simulated voltage clamp. To simulate an increase in binding affinity, the binding rate, $f_1$, was doubled from 2 to 4. A partial block of desensitization was simulated by decreasing desensitization rate, $r_d$, from 2 to 4. An increase in open probability was produced by doubling the opening rate, $r_o$, from 0.45 to 0.9. To simulate either a doubling of single-channel conductance or of total channel number, maximum conductance was doubled. In simulations, 100 μM agonist produced 0.1% receptor binding and 10 mM agonist produced 99% receptor binding. Potentiation is indicated as a percentage of baseline response.

**Analysis of time course of kainate responses before and after CaMKII* perfusion**

For a ligand-gated channel, the time course of the whole-cell response depends on the time course of ligand concentration and on single-channel open time distribution (Colquhoun and Hawkes 1995; Jonas and Spruston 1994). Therefore, time course analysis may provide a method of detecting mechanistic changes in receptor function as well as artifactual parameters for receptor kinetic model (see Fig. 5 for a diagram of the model).
CaMKII\(^*\) potentiation of responsiveness to non-NMDA agonists does not change the time course of responses. A: time course of responses changes when puff duration is varied between 6, 9, 12, and 20 ms. Raw averages are shown top; normalized responses (bottom) show the gross changes in time course associated with changing the time course of agonist concentration. B: moving the puffer pipette from 6 \(\mu\)M (thick line) to 3 \(\mu\)M (thin line) produces an increase in the magnitude of the response (top) as well as an alteration of the time course (bottom, normalized). Note the change in onset time, time to peak, and decay time course. C: CaMKII\(^*\) potentiates responses (top) without changing the time course of responses (bottom, normalized). Heavy line, after potentiation, is nearly superimposable on the scaled fine line, before potentiation. D: cyclothiazide increases response magnitude (top) and slows decay time course (bottom, normalized). E and F: quantitative analysis of time course of responses augmented by cyclothiazide (■) and potentiated by CaMKII\(^*\) (○). Time course measures (E: decay tau; F: half-width) normalized to baseline values are plotted against normalized amplitude increase.

In contrast to these possible artifactual effects, internal perfusion of CaMKII\(^*\) potentiated responses to kainate but did not change their time course (Fig. 6C). To show that the time course of responses to applied agonist is sensitive to changes in single-channel open time, the kinetics of AMPA/kainate channels were altered pharmacologically. Cyclothiazide is known to block desensitization and deactivation of non-NMDA receptors (Partin et al. 1993; Patneau et al. 1993). Blockade of desensitization and/or deactivation has been shown to augment response size and to change the time course of responses to exogenously applied transmitter in isolated preparations (Tang et al. 1991). Application of cyclothiazide increased the magnitude of responses to puffed kainate and slowed their time course (Fig. 6D).

To quantify the relationship between potentiation and time course changes, the decay rate (\(\tau\)) and half-width of responses were measured and compared with the magnitude of potentiation (Fig. 6, E and F). CaMKII\(^*\) potentiation did not correlate with changes in \(\tau\) (regression analysis: n.s., \(R = -0.01, n = 6\)) while cyclothiazide-mediated augmentation was correlated with larger \(\tau\) values (regression analysis: \(P < 0.05, R = 0.97, n = 4\)). CaMKII\(^*\) potentiation also did not correlate with changes in half-width (regression analysis: n.s., \(R = 0.02, n = 6\)). Cyclothiazide potentiation correlated with a measurable change in half-width (regression analysis: \(P < 0.05, R = 0.97, n = 4\)).

**Nonstationary fluctuation analysis of kainate responses before and after CaMKII\(^*\) perfusion**

Nonstationary fluctuation analysis (Sigworth 1980) was used to test the hypothesis that CaMKII\(^*\) potentiates kainate responsiveness by changing single-channel current. Because the number of receptors exposed to agonist is not likely to be constant during an individual response in this experimental paradigm, plots of variance versus mean derived from entire traces were not parabolic. However, over the time course of a response, the number of active channels at any point during the time course of a response should be constant from trial to trial. We therefore analyzed the point-by-point CV (standard deviation divided by mean) around the peak of the response. To determine if this analysis method is sensitive to a modification of single-channel current, the holding potential was changed. This is expected to alter the driving force and the single-channel current. Changing the driving force from -35 to -70 mV increased average response size, but had no effect on the CV (Fig. 7A), as expected (Hille 1992). If CaMKII\(^*\) potentiates kainate responsiveness by increasing the single-channel current, no change in the nonstationary CV is predicted with potentiation. However, CaMKII\(^*\)-mediated potentiation of responses to kainate decreased the CV (Fig. 7B), consistent with an increase in the number of AMPA receptors. The ratio of CV\(^-2\) before and after potentiation will be constant and equal to one for changes in single-channel current. Potentiation of kainate responses by CaMKII\(^*\) produced CV\(^-2\) ratio values greater than one, and the ratio correlated with the amount of potentiation (Fig. 7C; regression analysis: \(P < 0.05, R = 0.89, n = 8\)). Mean potentiation for experiments used for CV analysis was 1.72 ± 0.19. The mean change in CV\(^-2\) was 2.15 ± 0.58. The change in CV\(^-2\) was significantly different from 1 and is therefore inconsistent with a hypothesis involving a change in single-channel conductance.

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**Fig. 6.** CaMKII\(^*\) potentiation of responsiveness to non-NMDA agonists does not change the time course of responses. A: time course of responses changes when puff duration is varied between 6, 9, 12, and 20 ms. Raw averages are shown top; normalized responses (bottom) show the gross changes in time course associated with changing the time course of agonist concentration. B: moving the puffer pipette from 6 \(\mu\)M (thick line) to 3 \(\mu\)M (thin line) produces an increase in the magnitude of the response (top) as well as an alteration of the time course (bottom, normalized). Note the change in onset time, time to peak, and decay time course. C: CaMKII\(^*\) potentiates responses (top) without changing the time course of responses (bottom, normalized). Heavy line, after potentiation, is nearly superimposable on the scaled fine line, before potentiation. D: cyclothiazide increases response magnitude (top) and slows decay time course (bottom, normalized). E and F: quantitative analysis of time course of responses augmented by cyclothiazide (■) and potentiated by CaMKII\(^*\) (○). Time course measures (E: decay tau; F: half-width) normalized to baseline values are plotted against normalized amplitude increase.
MECHANISMS OF POSTSYNAPTIC CaMKII-DIRECTED POTENTIATION

Ci®cally potentiated kainate but not NMDA responsiveness. The maximal effect of kainate also was potentiated. Potentiation was not associated with a change in the time course of responses. Further, CaMKII* potentiation was accompanied by a decrease in the point-by-point CV.

These results confirm findings that increased postsynaptic CaMKII activity is sufficient to potentiate synaptic transmission in rodent hippocampus (Lledo et al. 1995; Pettit et al. 1994; Wang and Kelly 1995) and frog optic tectum (Wu et al. 1996). In contrast to those studies, the patch perfusion technique used in the present study allowed direct comparison of responses before and after a delimited increase of CaMKII activity. Interestingly, CaMKII*-mediated potentiation occurred long after the typical "washout" period for LTP (Malinow and Tsien 1990), implying that the processes underlying washout are before CaMKII activation. The delay between internal perfusion and potentiation varied from experiment to experiment; part of this delay may be attributable to diffusion of CaMKII* (33 kDa) to the synapses being stimulated.

Somatic receptors have been used previously as models for synaptic receptors (Jonas and Sakmann 1992; Tang et al. 1991; Wyllie et al. 1993); in this study, we assume that effects seen at somatic receptors will have analogous effects at synaptic receptors. We also assume that the observed changes of responses to kainate will be accompanied by similar changes of responses to endogenous transmitter. Two advantages of measuring responses to somatic application of agonist are that we have complete control over the distance between the application pipette and the response site and that diffusion of intrapipette perfusate to the cytoplasm underlying the response site is more rapid. Note that our application method is quite sensitive to distance. In fact, we were unable to evoke detectable responses with 100 μM kainate application unless the application pipette was within 10 μm of the cell membrane. Diffusion of kainate to greater distances (e.g., the dendrites) is thus unlikely to contribute significantly to the measured response. The greater magnitude of potentiation of somatic responses compared with synaptic responses might be due to the greater concentration of perfused CaMKII* in the soma compared with dendritic arbors and spines. Another possibility is that somatic AMPA/kainate receptors may be naive to CaMKII because CaMKII is found in the highest concentration at the PSD (Kelly et al. 1984; Kennedy et al. 1983) and are therefore more sensitive to its effect.

Previous studies have demonstrated that protocols necessary for potentiation of non-NMDA responses may be distinct from those necessary for potentiation of NMDA responses (Aniksztejn and Ben-Ari 1995; Kullmann et al. 1996). The finding that CaMKII* specifically potentiated responses to exogenous application of a non-NMDA agonist is consistent with the idea that plasticity mechanisms are specific to receptor subtype. This result is also consistent with the finding that enhancement of kinase activity in CA1 cells by inhibiting phosphatases also selectively potentiated non-NMDA receptor-mediated responses (Figurov et al. 1993). Although we found that active CaMKII* had no specific effect on extra-synaptic NMDA responses, Kolaj et al. (1994) found that NMDA responses in dorsal horn neurons potentiated when activated CaMKII was included in

DISCUSSION

To summarize, perfusion of CaMKII* potentiated synaptic transmission and decreased failure rates. CaMKII* specifically potentiated kainate but not NMDA responsiveness. The maximal effect of kainate also was potentiated. Potentiation was not associated with a change in the time course of responses. Further, CaMKII* potentiation was accompanied by a decrease in the point-by-point CV.

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FIG. 7. CaMKII* potentiation of responsiveness to non-NMDA agonists is accompanied by a decrease in the nonstationary coefficient of variation (CV). A: 50 consecutive responses to kainate at −35 mV (fine line) and −70 mV (heavy line) are shown bottom; the CV calculated over the entire time course of the responses is shown top. Driving force changes the size of kainate responses, but the point-by-point CV at each potential is similar. B: CaMKII* potentiates responses to exogenous kainate and decreases point-by-point CV. Mean response before (fine line) and after (heavy line) potentiation are shown bottom. Point-by-point CV is shown top. Note that CV decreased over the length of the response. C: ratio of CV² after to CV² before potentiation increases with the amount of potentiation. Individual points show the ratio value and amount of potentiation for experiment used for CV analysis. Circle with error bars is the average potentiation and specific to receptor subtype. This result is also consistent with the finding that enhancement of kinase activity in CA1 cells by inhibiting phosphatases also selectively potentiated non-NMDA receptor-mediated responses (Figurov et al. 1993). Although we found that active CaMKII* had no specific effect on extra-synaptic NMDA responses, Kolaj et al. (1994) found that NMDA responses in dorsal horn neurons potentiated when activated CaMKII was included in
patch solution. Different NMDA receptor subunit compositions in dorsal horn neurons and hippocampal CA1 neurons may explain this difference. In addition, Kolaj et al. did not use internal perfusion; as a result, they could not have easily observed the rapid decrease of NMDA responsiveness immediately after application of kinase solution. The rapid, kinase-independent decrease seen of NMDA responses preceded appearance of RITC-dextran (40 kDa). This effect may have been mediated by calmodulin (10 kDa), a constituent of both test and control perfusion solutions, which can down-regulate directly NMDA responses (Ehlers et al. 1996). Elucidation of this effect will require further studies. If the factor mediating this nonspecific effect can be found and excluded, the effect of CaMKII* on synaptic NMDA responses could be measured directly.

To address mechanisms of potentiation, we attempted to obtain single-channel AMPA/kainate responses of outside-out patches while perfusing CaMKII*. However the signal-to-noise resolution was not sufficient, likely due to the relatively small conductance of AMPA/kainate receptors (Edmonds et al. 1995) and the increase in noise produced by the perfusion canula within the patch pipette. As an alternative, the time course and trial-to-trial variability of whole cell currents were studied as a method of examining single-channel properties (Hessler et al. 1993; Li et al. 1993; Lu et al. 1993; Numann et al. 1991; Tang et al. 1991). The finding that CaMKII*-mediated potentiation does not change kainate response time course parallels the finding that the time course of non-NMDA synaptic responses does not change during LTP (O’Connor et al. 1995). Analysis of trial-to-trial variability showed that the CV increased with potentiation; by itself, this finding is consistent with a change in channel number or open probability.

Our results argue against several hypothetical mechanisms whereby CaMKII increases postsynaptic responsiveness indirectly. Selective potentiation of extrasynaptic non-NMDA responsiveness by CaMKII* is inconsistent with hypotheses that CaMKII alters nonspecifically the membrane or signal conduction from the membrane to the soma. In addition, these data are inconsistent with hypotheses that require specialized synaptic elements after CaMKII activation to express potentiation. The observation that potentiation of responses is concurrent with appearance of marker dye suggests that the effect of CaMKII* on non-NMDA responses is unlikely to be mediated by a series of messenger cascades or attributable to activation of transcription or translation. CaMKII has been demonstrated to phosphorylate directly GluR1, a component of hippocampal non-NMDA receptors (Hayashi et al. 1997; McGlade-McCulloch et al. 1994; Tan et al. 1994), in the PSD as well as in vitro, supporting the hypothesis that CaMKII acts directly on AMPA/kainate receptors to potentiate responsiveness during LTP.

The results presented here also constrain hypotheses for specific potentiation of AMPA/kainate responsiveness. A simple hypothesis that the affinity of non-NMDA receptors is inconsistent with two observations. Tighter binding of ligand by receptors would be expected to have no effect on a saturating dose of agonist. Although potentiation of saturated responses is smaller than potentiation of responses to 10 μM kainate, the observation of significant potentiation of the maximal effect of kainate demonstrates that the voltage clamp was sufficiently intact and that a simple change in receptor affinity is unlikely to underlie potentiation by CaMKII*. Furthermore, the time course of responses is not changed. Increased receptor affinity for ligand also would be expected to produce longer lasting responses, which was not seen. Consistent with our analysis of CaMKII*-induced potentiation, previous studies have shown no change in binding affinity of AMPA receptors after LTP (Maren et al. 1993). A second hypothesis is that other rate constants of the channel change with CaMKII activity. One specific change might be a decrease in the desensitization rate. Experiments with cyclothiazide demonstrate that decreasing desensitization and/or deactivation results in a slowing of time course of the kainate-evoked responses. CaMKII activation, in contrast, potentiates responses without changing time course, implying that CaMKII does not potentiate transmission by reducing desensitization as cyclothiazide does. It is possible that a kinetic change distinct from that mediating the cyclothiazide effect results in increased open probability; kinetic changes that increase response size without affecting the macroscopic response time course are not ruled out by our results. Finally, the hypothesis that the single-channel conductance of receptors increases with CaMKII activity would predict an increase in the mean response with no change in the nonstationary CV; our finding that CV decreases is evidence against this case.

A mechanism that would explain the findings of this study, as well as many in the literature, is a specific increase in the number of active non-NMDA receptors after CaMKII activation. Increasing the number of active receptors at synapses lacking active receptors would be manifested as the observed decrease in synaptic failure rate. More active receptors would increase the maximal effect of kainate. This mechanism of potentiation also would predict a direct “scaling-up” of responses, maintaining time course. In addition, it would decrease the nonstationary CV. Consistent with this view, LTP has been demonstrated to be coincident with an increase of the number of binding sites for AMPA without a change in binding affinity (Maren et al. 1993).

The exact mechanism by which such an increase in the number of active channels might occur is not elucidated by the experiments in this study, except that the necessary molecular machinery subsequent to CaMKII activation is not specific to synapses. The properties of several ligand-gated channels are modified by direct phosphorylation (Mei and Si 1995; Raymond et al. 1993; Swope et al. 1992). Phosphorylation studies show that GluR1 is phosphorylated by CaMKII (Hayashi et al. 1997; McGlade-McCulloch et al. 1993; Tan et al. 1994). Activation of previously silent channels may occur by changing open probability from 0 to a nonzero value or increasing conductance from 0 to a nonzero value. In addition, phosphorylation has been shown to induce acetylcholine receptor assembly (Green et al. 1991) as well as to cause active translocation of glucose transporters to the cell surface (Haruta et al. 1995; Pessin and Bell 1992). The phosphorylation state of synapsin I influences targeting of presynaptic vesicles and is regulated by CaMKII (Llinas et al. 1991). The phosphorylation state of the NR1 subunit of the NMDA receptor also has been shown to regulate its localization (Ehlers et al. 1995). CaMKII phosphorylation
also may regulate targeting of AMPA/kainate receptors to the cell surface (Malinow 1995). Recent work in our laboratory demonstrates calcium-evoked exocytosis of dendritic vesicles that is CaMKII dependent (M. Maletic-Savatic, T. Koothan, and R. Malinow, unpublished observations). The complete resolution of this problem is of great importance to an understanding of synaptic physiology.

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