

Recruitment of Calcium-Permeable AMPA Receptors during Synaptic Potentiation Is Regulated by CaM-Kinase I

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Ca²⁺-permeable AMPA receptors (CP-AMPA receptors) at central glutamatergic synapses are of special interest because of their unique biophysical and signaling properties that contribute to synaptic plasticity and their roles in multiple neuropathologies. However, intracellular signaling pathways that recruit synaptic CP-AMPA receptors are unknown, and involvement of CP-AMPA receptors in hippocampal region CA1 synaptic plasticity is controversial. Here, we report that intracellular infusion of active CaM-kinase I (CaMKI) into cultured hippocampal neurons enhances miniature EPSC amplitude because of recruitment of CP-AMPA receptors, likely from an extrasynaptic pool. The ability of CaMKI, which regulates the actin cytoskeleton, to recruit synaptic CP-AMPA receptors was blocked by inhibiting actin polymerization with latrunculin A. CaMK regulation of CP-AMPA receptors was also confirmed in hippocampal slices. CA1 long-term potentiation (LTP) after theta bursts, but not high-frequency tetani, produced a rapid, transient expression of synaptic CP-AMPA receptors that facilitated LTP. This component of TBS LTP was blocked by inhibition of CaM-kinase kinase (CaMKK), the upstream activator of CaMKI. Our calculations show that adding CP-AMPA receptors numbering <5% of existing synaptic AMPA receptors is sufficient to account for the potentiation observed in LTP. Thus, synaptic expression of CP-AMPA receptors is a very efficient mechanism for rapid enhancement of synaptic strength that depends on CaMKK/CaMKI signaling, actin dynamics, and the pattern of synaptic activity used to induce CA1 LTP.

Key words: AMPA receptor; CaM-kinase; LTP; calcium; hippocampus; synaptic plasticity

Introduction

In the mammalian forebrain, AMPA-type glutamate receptors (AMPA receptors), which are tetramers of glutamate receptor 1 (GluR1)–GluR4 subunits, mediate rapid excitatory synaptic transmission. The presence of the edited GluR2 subunit critically determines AMPA receptor properties (for review, see Isaac et al., 2007). AMPA receptors lacking the edited GluR2 subunit are calcium-permeable (CP-AMPA receptors), exhibit rapid kinetics and high conductance, and are inwardly rectifying. Synaptic localization of CP-AMPA receptors can be modulated by neuronal activity, neuronal insults (e.g., ischemia, excitotoxicity), antipsychotics, and drugs of abuse, and are implicated in several neuropathologies (e.g., Alzheimer's) (for review, see Kwak and Weiss, 2006; Isaac et al., 2007; Liu and Zukin, 2007).

The role of CP-AMPA receptors in CA1 long-term potentiation (LTP), a model of associative learning and memory, is currently controversial. LTP can be induced using multiple paradigms including high-frequency tetanic stimulation (HFS), pairing of presynaptic and postsynaptic depolarizations, and theta-burst stimulation (TBS). In the mature hippocampus, CA1 pyramidal cell AMPA receptors are composed predominantly of GluR1 and GluR2 (Monyer et al., 1991; Craig et al., 1993; Geiger et al., 1995;

Wenthold et al., 1996; Sans et al., 2003). Application of CP-AMPA inhibitors has little or no effect on basal CA1 synaptic transmission (Ju et al., 2004; Plant et al., 2006; Sutton et al., 2006; Adesnik and Nicoll, 2007), indicating a lack of synaptic CP-AMPA receptors. One report observed that pairing-induced LTP gave a transient incorporation of synaptic CP-AMPA receptors (Plant et al., 2006), whereas two other reports did not see this subunit recombination using either pairing-induced LTP or HFS-LTP (Adesnik and Nicoll, 2007; Gray et al., 2007). A fourth report indicates that the ability of HFS to induce AMPA receptor subunit recombination may be dependent on the age of the rats (Y. Lu et al., 2007).

Expression of LTP uses multiple signaling cascades, some of which may be age or protocol dependent (Selcher et al., 2003; Yasuda et al., 2003; Schmitt et al., 2005; Li et al., 2006). Thus, identification of pathways that regulate CP-AMPA receptors may explain their variable participation in LTP, will be essential for understanding their physiological roles, and may have implications for neuropathologies. To investigate synaptic recruitment of CP-AMPA receptors, we combined whole-cell recordings from cultured hippocampal neurons with field recordings from acute slices. For LTP, we used a TBS induction protocol because it mimics the endogenous hippocampal firing patterns during active exploration and learning (Feder and Ranck, 1973; Otto et al., 1991), and field recordings of synaptic strength to maintain the physical integrity of the neurons and, therefore, endogenous signaling pathways. We focused on CaM-kinase I (CaMKI) and its upstream activator CaM-kinase kinase (CaMKK), which are critical components of TBS-LTP through cross talk with Ras-extracellular signal-regulated protein kinase (Ras-ERK) signaling (Schmitt et al., 2005). The Ras-ERK pathway contributes to TBS-LTP

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(Selcher et al., 2003) and regulates activity-dependent synaptic trafficking of GluR1 (Zhu et al., 2002) whose C terminus appears to be essential for structural plasticity of synaptic spines (Kopeck et al., 2007).

Materials and Methods

Hippocampal culture and recordings. Rats were housed in the Oregon Health & Science University Animal Care Facility and all procedures were approved by the Institutional Animal Care and Use Committee. Voltage-clamp recordings of spontaneous miniature EPSCs (mEPSCs) were performed at -70 mV on pyramidal cells from 14–21 d *in vitro* (DIV) rat hippocampal cultures at room temperature. For measurements of average channel conductance of synaptic AMPARs, we used nonstationary fluctuation analysis of mEPSCs (see below). To assess the role of CaMKI in synaptic plasticity, constitutively active CaMKI (ca-CaMKI) (Wayman et al., 2004) or heat-inactivated control (inact-CaMKI) was infused through the patch pipette, with or without latrunculin A.

Hippocampal cultures were prepared essentially as described previously (Oh and Derkach, 2005). Cell seeding density was 20,000–25,000 cells/cm². To isolate spontaneous mEPSCs, the extracellular solution contained (in mM) 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 25 HEPES, 33 D-glucose, 0.025 D-APV, 0.003 strychnine, 0.05 picrotoxin or 0.01 gabazine, and 0.0005 TTX. Patch pipettes for whole-cell recordings were filled with (in mM) 100 Cs-methanesulfonate, 25 CsCl, 2 MgCl₂, 10 HEPES, 0.4 EGTA, 4 ATP, 0.4 GTP, 10 phosphocreatine, 0.1 spermine, and 0.05–0.1 Alexa 594, pH 7.3. Glutathione S-transferase-tagged recombinant ca-CaMKI (Wayman et al., 2004) was expressed in *Escherichia coli*, purified on a glutathione-sepharose column, and added to the patch-pipette solution from a concentrated stock solution. Heat-inactivated CaMKI was produced by heating an aliquot of stock ca-CaMKI at 95°C for 15 min; loss of activity was verified by kinase assay (see Fig. 2F). Stock solution (1 mM) of latrunculin A (Sigma) was prepared in ethanol. Currents were recorded at a 2 kHz frequency bandwidth. Individual mEPSCs were detected using the template algorithm (Axiograph 4.0 or pCLAMP9; Molecular Devices), and their representative amplitudes and kinetic properties were determined based on the averaged mEPSC (100–300 currents averaged) or from corresponding cumulative distributions. These measurements of spontaneous activity were made every 5 min. AMPA currents were evoked by 10–15 ms pressure puffs from a patch-pipette filled with 0.3–0.5 mM AMPA, 0.1 mM cyclothiazide, and 0.1 mM Alexa 594. The tip of the patch pipette was positioned 100–150 μ m away from the soma on second-order apical dendrites of the pyramidal neuron for a local activation of dendritic AMPARs. A local suction pipette was also positioned across and in a close proximity to the application pipette to ensure a confined AMPA application. The precision of application was monitored through Alexa 594 fluorescence. Pyramidal neurons were identified by a prominent presence of dendritic spines visualized by fluorescence of intracellular Alexa 594. To further confirm their identity, in several experiments we stained recorded cells with α -GAD65/67 antibody, revealing they are not GAD65/67 positive and, thus, are not interneurons.

Brain slice physiology. Young adult rats (4–6 weeks old) were anesthetized with pentobarbital (60 mg/kg, i.p.) and decapitated. CA1 stratum radiatum field EPSPs (fEPSPs) (1.2–1.4 mV basally, \sim 25% maximally) were recorded by Schaffer collateral (SC) stimulation of submerged hippocampal slices (transverse, 400 μ m, 30–32°C). Long-term potentiation was induced using three trains (20 s intertrain interval) of HFS (100 Hz, 1 s) or TBS: four pulses per burst (100 Hz), five bursts per train (5 Hz).

Submerged hippocampal slices were suspended in pairs on a nylon mesh in a small (\sim 350 μ l) recording chamber and perfused with artificial CSF (ACSF) at a rate of 2.5–3 ml/min, allowing rapid drug perfusion of the slices (dead volume of perfusion tubing also \sim 350 μ l) as confirmed in Figure 5A (APV treatment). For rapid application of 1-trimethylammonio-5-(1-adamantane-methylammonio)pentane)dibromide [IEM-1460 (IEM); 30 μ M, prepared from a 100 mM aqueous stock solution; Tocris Bioscience], the flow of solution coming into the chamber was switched from ACSF to ACSF plus IEM immediately after the last

train of HFS or TBS. For 1,8-naphthoyle benzimidazole-3-carboxylic acid (STO-609)-treated slices (Tocris Bioscience; 5 μ M, prepared from a 25 mM stock solution in 0.1N NaOH), treatment began in the recording chamber 30 min before LTP induction and STO washout began immediately after the last train of HFS or TBS. Experiments were otherwise performed as described (Schmitt et al., 2005), except as noted. Test-pulse stimulation was administered once every 20 s. One minute fEPSP averages are shown. NMDAR fEPSPs were pharmacologically isolated at basal test-pulse intensity by blocking AMPA/kainate receptors with 10 μ M CNQX (Tocris Bioscience) in the presence of low Mg²⁺ (0.1 mM). The resulting responses were confirmed to be NMDAR field potentials by blockade with 50 μ M APV (Tocris Bioscience).

In vitro phosphorylation assay. Phosphorylation assays were performed essentially as described using syntide-2 (Tokumitsu et al., 1995; Barria et al., 1997a). The same ca-CaMKI (Wayman et al., 2004) was used as in the infusion experiments described above.

Nonstationary fluctuation analysis. We assumed that the majority of recorded mEPSCs were monoquantal (Bergles et al., 1999). This was also confirmed by the absence of correlation between their amplitude and decay kinetics (data not shown). Because of their monoquantal character, the fluctuations in their waveform should represent the stochastic behavior of synaptic AMPARs, as apposed to fluctuations of evoked EPSCs which are additionally determined by variable quantum number and asynchronization of quantal release. Therefore, we applied nonscaled nonstationary fluctuation analysis (Sigworth, 1980; Derkach et al., 1999; Oh and Derkach, 2005) instead of scaling, which would be preferable for evoked EPSCs (Traynelis et al., 1993; Benke et al., 1998). For each measurement, 100–200 mEPSCs were carefully selected based on the following criteria: stable baseline 10 ms before and 10 ms after mEPSC, fast rise time (<0.5 ms) allowing a precise alignment, and no overlapping mEPSCs or spurious fluctuations during the decay phase (25 ms). mEPSCs were aligned by their point of maximal rise, averaged, and their mean value and variance was calculated (see Fig. 2A,B, insets). In all cells tested, the variance was essentially a linear function of mean current (see Fig. 2A,B), indicating a low open probability of synaptic AMPARs, consistent with their low occupancy by released glutamate (Bergles et al., 1999; Mainen et al., 1999). We therefore fitted the relationship with the following equation: $\text{var} = iI + \text{var}_b$, where i was the mean single-channel AMPA current, I the mean current, and var_b the variance of background noise. Channel conductance was then calculated as $\gamma = i/V$, where V was the holding membrane potential (assuming 0 mV for reversal potential). There was no correlation between conductance and average mEPSC amplitude, mean decay time or background noise. Cells were discarded if access resistance varied during measurements by $>15\%$. We calculated channel conductance under conditions of break in (initial 2–5 min and used as “same cell control”), 15 min of CaMKI infusion ($n = 8$), and an additional 15 min of IEM (30 μ M; $n = 4$) bath application (see Fig. 2C).

Cable filtration can be a factor for underestimation of single-channel conductance (Benke et al., 2001). However, other factors can in contrast lead to overestimation of it during nonstationary fluctuation analysis of evoked EPSCs. In particular, asynchronization in transmitter release in both single synapses and between synapses is an additional contributor to estimated variance (Traynelis et al., 1993) and may result in significantly higher values for γ (V. A. Derkach, unpublished observation). The scaling procedure cannot compensate for that, but the analysis of mEPSCs should produce more correct and lower values. This notion is consistent with observations of others that γ estimates for synaptic AMPARs were several-fold higher if based on EPSCs versus mEPSCs (Mameli et al., 2007). Our estimates of γ for synaptic AMPARs at basal conditions are also in accord with the recent work of Cull-Candy and Gebhardt (2006) (their Fig. 2) on endogenous AMPARs in CA1 neurons. At a low receptor occupancy by agonist, the case for synaptically released glutamate, the main conductance state for AMPARs in CA1 pyramidal neurons was 3.9 pS. Thus, we felt that the low picosiemens values for γ that we and others obtained for endogenous AMPARs under basal conditions were well grounded and were used in our modeling.

Results

CaM-kinase I activity promotes synaptic incorporation of CP-AMPA

The CaMKK/CaMKI cascade is involved in multiple aspects of activity-dependent neuronal development and plasticity (Wayman et al., 2004, 2006; Saneyoshi et al., 2008), including TBS-LTP (Schmitt et al., 2005), in part because of regulation of actin dynamics (Wayman et al., 2004, 2006; Saneyoshi et al., 2008) and cross talk with the Ras/MEK (mitogen activated protein kinase)/ERK pathway (Schmitt et al., 2004). Because actin dynamics and ERK signaling are both thought to regulate activity-dependent trafficking of AMPARs to synapses (Matsuzaki et al., 2004; Korkotian and Segal, 2007), we investigated whether CaMKK/CaMKI might regulate expression of synaptic AMPARs. We focused on CP-AMPA receptors because of their potential role in the initial phase of CA1 LTP (Plant et al., 2006) (but see Adesnik and Nicoll, 2007; Gray et al., 2007), their ability to be uniquely identified, and their potential for subsequent downstream signaling via Ca^{2+} influx.

Infusion of activated CaMKI into cultured hippocampal pyramidal cells (14–20 DIV) produced a significant potentiation of AMPAR mEPSC amplitude that plateaued within 15 min (Fig. 1A, B). This potentiation required CaMKI activity, because infusion of inactive CaMKI had no effect (Fig. 1B, C). We did not observe any significant effects of CaMKI or inactive CaMKI infusion on mEPSC frequency (data not shown). To assess the potential involvement of CP-AMPA receptors in mEPSC potentiation, we used IEM-1460, a well characterized rapid and reversible inhibitor, which shows greater selectivity than philanthotoxin-433 for CP-AMPA receptors versus GluR2-containing AMPARs and NMDARs (Magazanik et al., 1997; Buldakova et al., 1999, 2007; Samoilova et al., 1999; Gray et al., 2007). For example, IEM has no effect on basal AMPA EPSCs from CA1 pyramidal neurons, which do not contain CP-AMPA receptors (Monyer et al., 1991; Craig et al., 1993; Geiger et al., 1995; Wenthold et al., 1996; Sans et al., 2003), whereas IEM partially suppresses AMPA EPSCs of CA1 interneurons (Buldakova et al., 2007), which do contain some CP-AMPA receptors (Racca et al., 1996; Laezza and Dingledine, 2004). The best evidence of IEM specificity for synaptic GluR2-lacking AMPARs comes from GluR2 knock-out mice. Basal transmission at CA1 synapses was efficiently inhibited in that mouse, but not in the wild type (Gray et al., 2007). In our experiments, IEM had no effect on mEPSC amplitude when heat-inactivated CaMKI was infused (Fig. 1B), indicating that CP-AMPA receptors do not contribute to basal synaptic transmission in these cultured neurons. In contrast, the CaMKI-mediated potentiation of mEPSC amplitude was completely reversed by IEM (Fig. 1A, B). Because CP-AMPA receptors exhibit more rapid kinetics (Grosskreutz et al., 2003; Oh and Derkach, 2005; Isaac et al., 2007), we also examined the decay kinetics of mEPSCs, which are controlled by deactivation of synaptic AMPARs, as additional confirmation of synaptic CP-AMPA recruitment. Infusion of active CaMKI significantly shortened mEPSC decay time, an effect also reversed by IEM (Fig. 1C). As with mEPSC amplitude, inactive CaMKI with or without IEM had no effect on mEPSC decay kinetics (Fig. 1C). Thus, both the IEM sensitivity of potentiation and the acceleration of mEPSC decay kinetics indicated that CaMKI activity initiated the appearance of CP-AMPA receptors at synapses.

CaMKI enhances synaptic strength by increasing average channel conductance of synaptic AMPARs

GluR2-lacking AMPARs have significantly higher channel conductance than GluR2-containing AMPARs (Swanson et al., 1997;

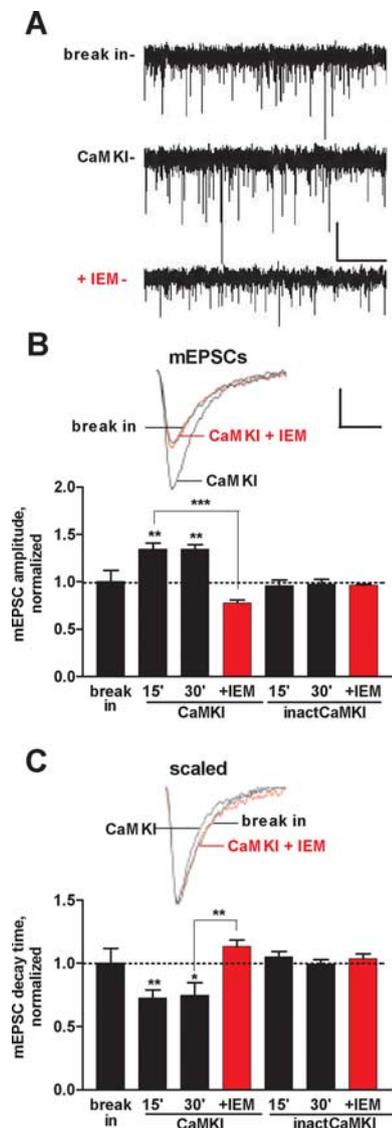


Figure 1. CaMKI activity promotes synaptic CP-AMPA expression. **A**, Representative traces of spontaneous AMPAR mEPSCs from a single experiment during the initial 5 min of infusion of activated CaMKI (500 nM) (break in, top trace). After 15 min of CaMKI infusion, the amplitude of mEPSCs was significantly potentiated (middle trace). This potentiation was reversed by subsequent treatment for 15 min with a selective inhibitor of CP-AMPA receptors, IEM-1460 (30 μ M, bottom trace). Calibration: 10 pA, 2 s. **B, C**, Infusion of activated CaMKI (500 nM) into cultured hippocampal pyramidal cells increased AMPAR mEPSC amplitude (**B**) and decreased decay time (**C**, 90–10% decay time) compared with break in ($n = 7$). In contrast, infusion of inactive CaMKI (heat inactivated; see Fig. 2F) had no effect on the amplitude and decay time of mEPSCs ($n = 4$). Both effects of active CaMKI were reversed by bath application of IEM (30 μ M) beginning 15 min after break in ($n = 4–5$), whereas IEM had no significant effects on mEPSCs recorded from cells infused with inactive CaMKI ($n = 3$). Insets, Representative traces of mEPSCs showing responses during the initial 5 min (break in), after 15 min infusion of active CaMKI, and after an additional 15 min with IEM (red traces). Calibration: 5 pA, 5 ms. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars indicate SEM.

Oh and Derkach, 2005). Furthermore, the channel conductance of GluR1 homomers can be significantly enhanced by CaMKII phosphorylation of Ser831 in the C terminus of the GluR1 subunit in contrast to GluR2–GluR1 heteromers (Derkach et al., 1999; Derkach, 2003). Therefore, one would predict that synaptic incorporation of CP-AMPA receptors by CaMKI should be accompanied by increased averaged channel conductance of synaptic AMPARs.

Under basal conditions, the channel conductance (γ) of syn-

aptic AMPARs, calculated by nonstationary fluctuation analyses (Fig. 2A), was variable from cell to cell but averaged 3.8 ± 0.48 pS ($n = 8$) (Fig. 2C). This value is consistent with the conductance of GluR1/GluR2 (edited) heteromers (3.1 ± 0.3 pS), but not homomeric GluR1 (12.2 ± 1.3 pS) (Derkach et al., 1999; Oh and Derkach, 2005). CaMKI infusion significantly increased channel conductance to 4.9 ± 0.50 pS ($n = 8$; $p = 0.013$), and IEM reversed this increase to 3.5 ± 0.46 pS ($n = 4$; $p = 0.031$) (Fig. 2B,C). To further clarify these analyses, effects of CaMKI infusion and IEM treatment were normalized for each cell to the initial conductance after break in. These normalized changes showed a significant increase in conductance by CaMKI by $\sim 30\%$ that was fully reversed by IEM (Fig. 2D). Importantly, changes in mEPSC amplitude strongly correlated with changes in channel conductance (Fig. 2E). This suggests that the major mechanism for potentiation of synaptic strength by CaMKI was regulation of average channel conductance, likely through synaptic incorporation of IEM-sensitive CP-AMPA.

Synaptic AMPARs can be potentiated by multiple mechanisms: modulation of receptor number through trafficking, alteration of subunit composition, and phosphorylation of the GluR1 C terminus (Derkach et al., 2007). For example, phosphorylation of Ser831 by CaMKII (Barria et al., 1997a) can increase single channel conductance of homomeric GluR1 AMPARs (Derkach et al., 1999; Oh and Derkach, 2005). Because there can be overlap in substrate phosphorylation by the CaMK family members, we examined whether CaMKI might also phosphorylate the C terminus of GluR1, which might account for the observed enhancement of channel conductance. Although CaMKI and CaMKII both phosphorylated the synthetic peptide syntide (Syn), only CaMKII phosphorylated the GluR1 C terminus of GluR1 (Fig. 2F). Lack of *in vitro* phosphorylation of Ser831 by CaMKI is not surprising because the sequence surrounding Ser831 (PQQQS) does not conform to a consensus CaMK phosphorylation motif which contains an Arg in the P-3 position (RXXS/T). This result is also consistent with the finding that enhanced Ser831 phosphorylation during TBS-LTP is not suppressed by the CaMK inhibitor STO-609 (Schmitt et al., 2005). Thus, based on four independent criteria (IEM-sensitivity, faster decay kinetics, increased channel conductance, and lack of phosphorylation of Ser831 by CaMKI) we conclude that CaMKI potentiates mEPSC amplitude through recruitment of CP-AMPA at synapses.

Recruitment of synaptic CP-AMPA by CaMKI requires actin polymerization

We investigated the potential regulatory role of actin polymerization for several reasons. First, actin polymerization is essential for activity-dependent AMPAR trafficking, including Ca^{2+} -

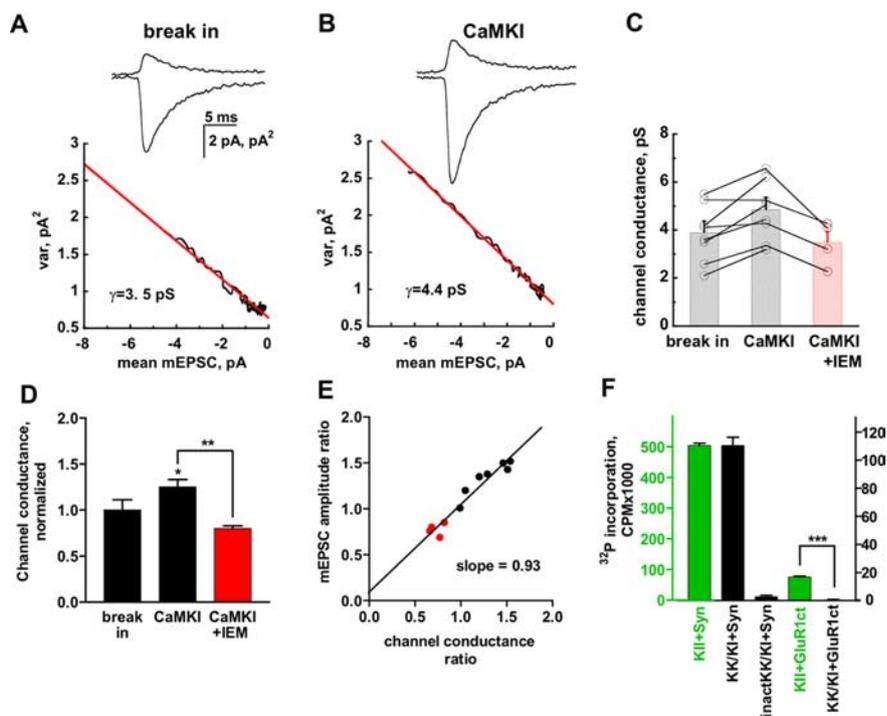


Figure 2. CaMKI increases channel conductance of synaptic AMPARs. **A, B**, Representative nonstationary fluctuation analyses for mEPSCs recorded in the same cell after break in (**A**) and after 15 min of caCaMKI infusion (**B**). Insets, Bottom traces are averages of 158 and 199 mEPSCs for **A** and **B**, respectively; top traces are the corresponding variances. Calibrations are the same for **A** and **B**. Graphs are variance–mEPSC relationships for traces shown in insets. Red lines are fittings by the equation described in Materials and Methods. Estimated channel conductances are indicated as γ . **C**, Changes in single-channel conductance in individual cells (open circles) after break in, infusion of CaMKI alone for 15 min ($n = 8$), or followed by another 15 min of CaMKI in the presence of $30 \mu\text{M}$ IEM-1460 ($n = 4$) (see also Fig. 1). Error bars show mean values of γ and SEMs. **D**, Relative changes in channel conductance for cells in **C** with break in values normalized to 1. **E**, Changes in mEPSC amplitude as a function of channel conductance. For each cell, ratios of mean mEPSCs for CaMKI infusion and break in ($n = 8$; black circles), and IEM application with CaMKI infusion ($n = 4$; red circles), were plotted against corresponding ratios of channel conductances. Points were fitted by a linear function: (mEPSC amplitude ratio) = $0.12 + 0.93 \times$ (channel conductance ratio). **F**, CaMKI does not phosphorylate the C terminus of GluR1. CaMKII and CaMKI (activated by CaMKK) were assayed for ^{32}P incorporation into the C terminus of GluR1 (Barria et al., 1997) and the synthetic peptide substrate syntide-2. CPM, Counts per minute. Note the different scales for CaMKI and CaMKII and that heat-inactivated CaMKI (used in Fig. 1) was catalytically inactive. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

dependent GluR1 insertion (Korkotian and Segal, 2007), into spines and synapses (Matsuzaki et al., 2004; Korkotian and Segal, 2007). Second, actin polymerization significantly increases in CA1 hippocampal spines during TBS-LTP (Lin et al., 2005) and is involved in LTP consolidation (Messaoudi et al., 2007; Rex et al., 2007). Last, CaMKI can modulate spine actin dynamics directly through activation of Rac (Saneyoshi et al., 2008) or indirectly through activation of Ras-ERK signaling (Schmitt et al., 2004), which in turn regulates actin polymerization (Futter et al., 2005).

To assess the role of actin, we infused latrunculin A, which specifically binds actin monomers and potently inhibits actin polymerization, with or without coinfusion of active CaMKI. Infusion of latrunculin A alone gave a small ($\sim 15\%$) but significant suppression of mEPSC amplitude (Fig. 3A), consistent with a role for the actin cytoskeleton in constitutive recycling of a pool of synaptic AMPARs (Park et al., 2004; Schulz et al., 2004; J. Lu et al., 2007). There was no effect of latrunculin on mEPSC kinetics (Fig. 3B), indicating no involvement of CP-AMPA as expected, because they are absent in synapses under basal conditions (Fig. 1). However, coinfusion of latrunculin A with CaMKI completely blocked the effects of CaMKI alone (dashed lines) on AMPAR mEPSC amplitude and decay kinetics (Fig. 3C,D). Because both the changes in amplitude and decay kinetics were strongly

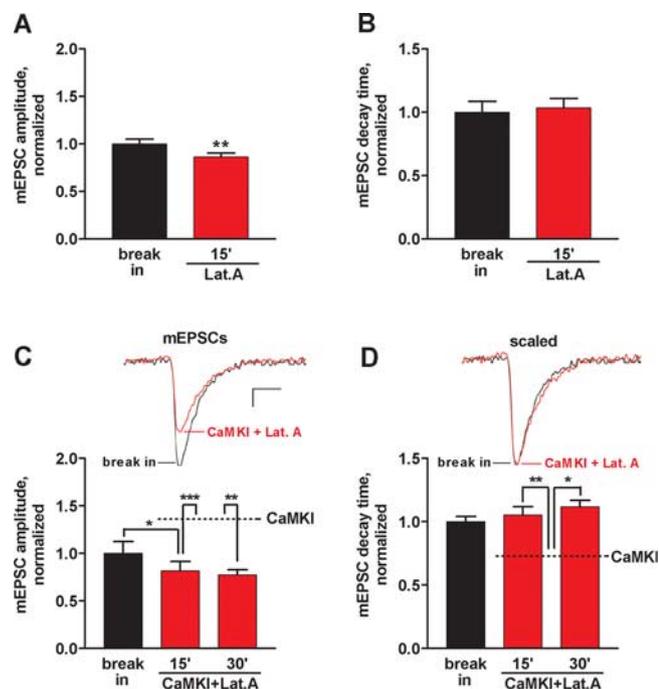


Figure 3. Actin polymerization is required for CaMKI-mediated synaptic expression of CP-AMPA receptors. **A, B**, Under basal conditions, intracellular infusion of latrunculin A ($2 \mu\text{M}$, $n = 7$) to inhibit actin polymerization resulted in a small inhibition of mEPSC amplitude (**A**) but not decay kinetics (**B**). **C, D**, Coinfusion of latrunculin A (Lat.A) with activated CaMKI blocked both the increases in mEPSC amplitude (**C**, $n = 7$ for 15 min and $n = 3$ for 30 min) and acceleration in kinetics (**D**) caused by infused CaMKI alone (effects of CaMKI alone are indicated by dashed lines and taken from Fig. 1). Insets, Averaged mEPSCs recorded during first 2 min (break in) and after 15 min of coinfusion (CaMKI + latrunculin). Calibration: 1 pA, 5 ms. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars indicate SEM.

inhibited, this indicates an essential role of actin polymerization in CaMKI-mediated recruitment of synaptic CP-AMPA receptors.

Dendritic CP-AMPA receptors are excluded from the synapse basally

Our experiments in Figure 1 demonstrate a lack of synaptic CP-AMPA receptors under basal conditions, but they are recruited to the synapse within 15 min of active CaMKI infusion. Recruitment of AMPARs to synapses can occur from intracellular pools or via membrane diffusion from extrasynaptic sites (Cognet et al., 2006). Therefore, we wanted to know whether these CP-AMPA receptors preexist extrasynaptically under basal conditions or whether they are assembled de novo by CaMKI stimulation and trafficked into the synapse. When the lysate from mature basal hippocampal neurons is immunodepleted of GluR2, there is still significant GluR1 remaining in the supernatant (Oh and Derkach, 2005), indicating a significant fraction of preexisting GluR2-lacking, GluR1-containing AMPARs (i.e., CP-AMPA receptors). To test whether these nonsynaptic (Fig. 1) CP-AMPA receptors might reside on the surface of dendrites, AMPA was ionophoretically applied. Consistent with this possibility, local dendritic application of AMPA under basal conditions revealed significant current rectification (Fig. 4A, B), a hallmark of CP-AMPA receptors (Oh and Derkach, 2005; Isaac et al., 2007). Furthermore, IEM suppressed this rectifying component of dendritic AMPA-currents, but did not block synaptic mEPSCs in the same cells (Fig. 4C, D). Unfortunately, we were unable to measure rectification of AMPA mEPSCs because increased basal noise at positive potentials and concerns over the long duration of depolarization (several minutes) required for averaging outward mEPSCs. Prolonged membrane

depolarization can result in secretion of neurotrophic factors, such as BDNF, from hippocampal pyramidal neurons and activation of Ras (Iida et al., 2001; Kolarow et al., 2007). In turn, BDNF can induce trafficking to and insertion of GluR1 homomers at synapses of hippocampal neurons, and phosphorylation of Ser831 (Caldeira et al., 2007; Nakata and Nakamura, 2007). Thus, such manipulations could lead to potentially altered trafficking of synaptic AMPARs and aberrant rectification. Nevertheless, our observations indicate a significant reservoir of dendritic, extrasynaptic CP-AMPA receptors in hippocampal pyramidal neurons.

Hippocampal CA1 LTP induced by TBS, but not HFS, transiently recruits synaptic CP-AMPA receptors via CaM-kinase kinase

Having established a biochemical role for CaMKI in the recruitment of CP-AMPA receptors to synapses in cultured hippocampal neurons, we next asked whether this also occurs under more physiological circumstances such as hippocampal slice LTP. We have shown previously that inhibition of CaMKK, the upstream activator of CaMKI, partially suppresses TBS-LTP (Schmitt et al., 2005). Under our experimental conditions, $30 \mu\text{M}$ IEM was selective for CP-AMPA receptors: it did not inhibit NMDARs (Fig. 5A) and gave no inhibition of basal synaptic transmission (Fig. 5B). This latter result is consistent with the absence of CP-AMPA receptors at naive Schaffer collateral–CA1 synapses in wild-type rodents whereas IEM gives almost complete inhibition (90%) of basal transmission in the GluR2 knock-out mouse (Gray et al., 2007), consistent with the specificity of IEM for GluR2-lacking AMPARs. Importantly, IEM treatment immediately after TBS stimulation suppressed LTP by $\sim 40\%$ (Fig. 5C), indicating that rapid synaptic incorporation of CP-AMPA receptors had occurred. Intriguingly, the reduction in TBS-LTP by IEM is similar in magnitude and onset to what we observed previously after inhibition of CaMKK by STO-609 (Schmitt et al., 2005). STO-609 potently inhibits CaMKK *in vitro* with little effect on numerous other protein kinases (Tokumitsu et al., 2002). Furthermore, STO-609 has no effect on basal synaptic transmission, paired-pulse facilitation, or CaMKII activation during LTP, yet it prevents activation of CaMKI by CaMKK during TBS-LTP (Schmitt et al., 2005). As expected, STO-609 pretreatment suppressed TBS-LTP identical to IEM-treatment (Fig. 5C). Importantly, in STO-treated slices IEM had no additional effect on LTP, indicating that synaptic incorporation of CP-AMPA receptors requires CaMKK signaling.

It was reported previously that incorporation of CP-AMPA receptors after pairing-induced LTP is transient and lasts < 30 min (Plant et al., 2006). Likewise, IEM had no effect when applied 20 min after TBS-LTP (Fig. 5D), indicative of a very transient expression of CP-AMPA receptors in the early phase of TBS-LTP. In contrast, HFS-LTP has been reported to occur independently of CP-AMPA receptors (Adesnik and Nicoll, 2007; Gray et al., 2007), although this finding appears to be age-dependent (Y. Lu et al., 2007), so we also investigated this protocol. Indeed, we also did not observe any effect of IEM on HFS-LTP (Fig. 5E), indicating that CP-AMPA receptors were not recruited to synapses. Our TBS- and HFS-LTP experiments were similar except for the pattern of activity: both used three one second long trains of either TBS (4 pulses/burst at 100 Hz, 5 bursts at 5 Hz) or HFS (100 Hz, 1 s) spaced 20 s apart. If CaMKK and CaMKI regulate LTP specifically through the recruitment of CP-AMPA receptors, one would predict that CaMKK inhibition by STO-609 would have no effect on HFS-LTP, which did not use CP-AMPA receptors, and this is exactly what we observed (Fig. 5E). Thus, the pattern of activity used to generate LTP appears to

be critical for the recruitment of synaptic CP-AMPA receptors via CaMKK/CaMKI.

Quantification of synaptic potentiation by subunit recomposition

How efficient is subunit recomposition for synaptic potentiation? We estimated the relative number of CP-AMPA receptors, which exhibit high conductance compared with (edited)GluR2-containing AMPARs residing at the synapse basally, needed to account for both potentiation and rectification of AMPARs at SC–CA1 synapses (see Appendix). CP-AMPA receptors are likely to be predominantly GluR1 homomers because expression levels of GluR3 and GluR4 in the mature hippocampus are very low compared with GluR1 and GluR2 (Monyer et al., 1991; Craig et al., 1993; Geiger et al., 1995; Wenthold et al., 1996; Sans et al., 2003). We calculated the fraction of CP-AMPA receptors added to the existing synaptic population of Ca^{2+} -impermeable AMPARs to account for changes in both rectification properties of synaptic AMPARs and synaptic strength. Based on rectification measurements (Appendix, Equation 5), the fraction is ≤ 0.015 . Thus, the addition of only 2% of GluR1 homomers can produce the rectification seen at CA1 synapses during pairing-induced early LTP (E-LTP) (Plant et al., 2006).

The fraction of newly inserted GluR1 homomers can also be calculated using an alternative approach based on the increase in synaptic strength at negative membrane potentials (Appendix, Equation 8). For pairing-induced LTP (Plant et al., 2006), this ratio is $\leq 4.3\%$. Furthermore, our observations independently suggest that for both TBS-LTP and potentiation by CaMKI infusion, the ratio is 1.6–2.1%, considering ~ 30 –40% increases in synaptic strength attributable to CP-AMPA receptors (Figs. 1, 5). Importantly, these calculations provide relative changes in receptor number and are therefore independent of their initial number at synapses. This is noteworthy because excitatory synapses are heterogeneous with respect to AMPAR number (Nusser et al., 1998; Matsuzaki et al., 2004). Thus, both approaches substantiate each other and indicate that a small fraction ($< 5\%$) of newly recruited GluR1 homomers can produce the observed changes in both rectification and synaptic strength for the three different stimulation paradigms (pairing-induced LTP, TBS-LTP, and active CaMKI infusion) (Fig. 6). Such an efficient mechanism for enhancing postsynaptic strength via trafficking of GluR1 homomers may be critical for rapid postsynaptic potentiation before subsequent slower structural remodeling and enlargement of synaptic spines (Matsuzaki et al., 2004; Nagerl et al., 2004; Kopec et al., 2007; Korkotian and Segal, 2007).

Discussion

Our principal finding is that the CaMKK/CaMKI signaling pathway can recruit CP-AMPA receptors to synapses (Figs. 1, 2, 5). Based on the unique properties of CP-AMPA receptors (Isaac et al., 2007), we used several criteria to detect their presence in synapses. Thus, inhibition by IEM1460 of potentiated mEPSCs, acceleration in the decay kinetics, changes in channel conductance of synaptic AM-

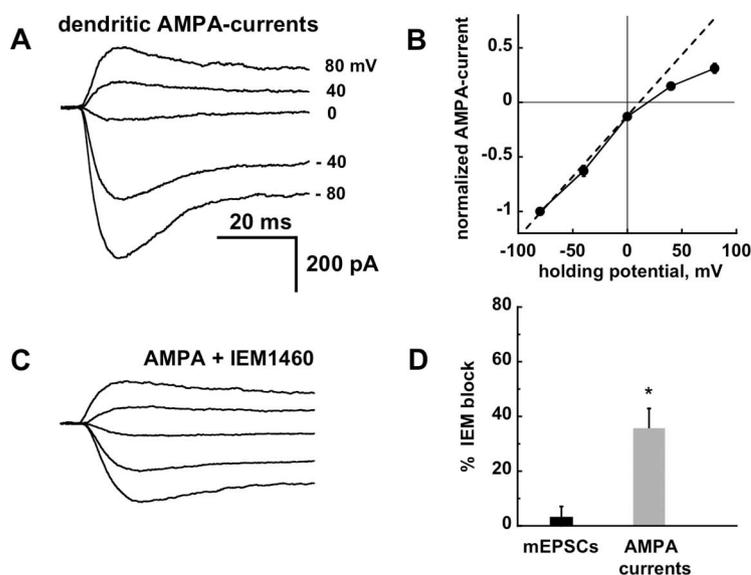


Figure 4. Extrasyntaptic CP-AMPA receptors are expressed on dendrites under basal conditions. The experiments in this figure were performed without infusion of kinase. **A**, AMPA (0.5 mM) was briefly (10 ms) applied to a dendrite 100 micron from the soma, and AMPA currents were measured at different holding potentials (from -80 to 80 mV, step 40 mV). **B**, Current–voltage (I – V) relationships for dendritic AMPA currents ($n = 4$, SEs are less than the size of symbols). The dashed line indicates a linear I – V extrapolated from points at negative potentials. A significant rectification of the I – V curve at positive potentials indicates that CP-AMPA receptors are present on the dendritic surface. **C**, In the presence of IEM-1460 ($100 \mu\text{M}$), dendritic AMPA currents were significantly inhibited (the same cell as in **A**), independently confirming the presence of CP-AMPA receptors. **D**, In contrast, mEPSCs ($n = 3$) were not affected by IEM-1460 (see also Fig. 1 *B*) when measured in the same cells as AMPA currents, indicating that CP-AMPA receptors are not in synapses. * $p < 0.05$. Error bars indicate SEM.

PARs, and lack of GluR1 phosphorylation by CaMKI all point to the recruitment of synaptic CP-AMPA receptors mediated by CaMKK/CaMKI. Although CaMKII can phosphorylate Ser831 of GluR1 in GluR2-containing AMPARs that exist at synapses under basal conditions, this does not result in increased channel conductance as occurs with homomeric GluR1 (Derkach et al., 1999; Derkach, 2003). Thus, it requires CaMKI-mediated recruitment of synaptic GluR2-lacking AMPARs, which by themselves have higher conductance, for the CaMKII phosphorylation to further increase channel conductance. Thus, these two CaM-kinases act in concert to achieve synaptic potentiation through recruitment and phosphorylation of CP-AMPA receptors (Fig. 6).

It is likely that CaMKK/CaMKI-regulated actin dynamics (Saneyoshi et al., 2008) are necessary for Ca^{2+} -dependent trafficking of GluR1 homomers (Korkotian and Segal, 2007) from a dendritic, extrasynaptic pool to synapses (Figs. 3, 4). This would be consistent with the observation that actin polymerization significantly increases in CA1 hippocampal spines during TBS-LTP (Lin et al., 2005). Thus, TBS-LTP, which is likely more physiological than HFS- or pairing-induced LTP, promoted rapid but transient synaptic incorporation of CP-AMPA receptors that required CaMKK signaling (Fig. 5). Importantly, our LTP experiments used native AMPARs and endogenous signaling pathways in intact neurons. Although studies of transfected GluR1 in neurons can allow unique analyses of receptor trafficking, it is likely this manipulation promotes nonphysiological levels of GluR1 that could artificially promote formation of GluR1 homomers.

Our observations also demonstrate that recruitment of native CP-AMPA receptors depends on the pattern of synaptic activity used to induce LTP because TBS, but not HFS (Adesnik and Nicoll, 2007; Gray et al., 2007), resulted in transient expression of CP-AMPA receptors (Fig. 5). It is well known that various LTP induction protocols can trigger different signaling pathways including

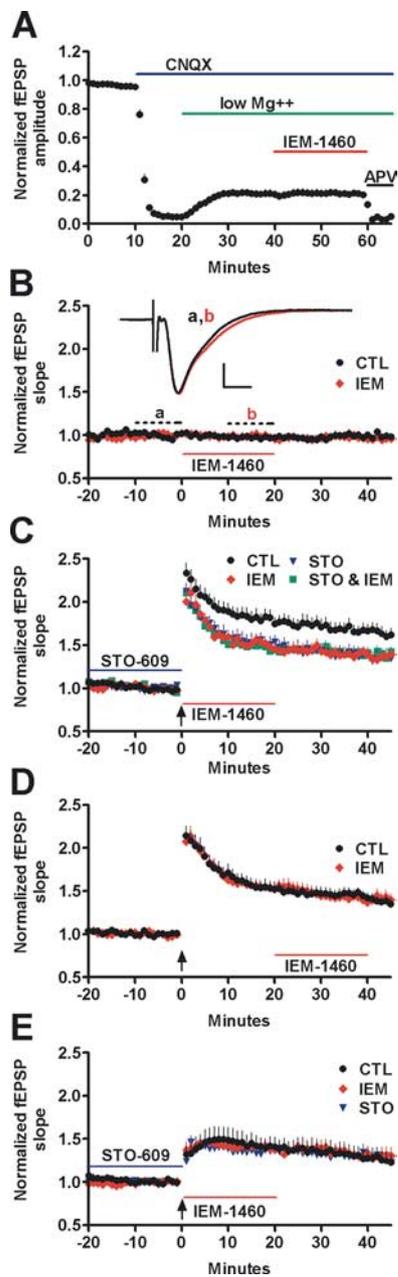


Figure 5. CaMKK regulates transient synaptic CP-AMPA expression during TBS-LTP. Field recordings (fEPSPs) from area CA1 (stratum radiatum) elicited by SC-stimulation (see Materials and Methods) in acute hippocampal slices (4- to 6-week-old rat). **A** confirms that IEM ($30 \mu\text{M}$) does not inhibit NMDARs (pharmacologically isolated with $10 \mu\text{M}$ CNQX in 0.1 mM Mg^{2+}), and **B** demonstrates that IEM ($30 \mu\text{M}$) has no effect on basal synaptic transmission in naive slices ($n = 6$). Inset, Representative fEPSP averages before (*a*, black trace) and during (*b*, red trace). Calibration: 0.5 mV , 5 ms . **C**, Application of IEM immediately after TBS (Materials and Methods) suppressed LTP $\sim 40\%$ compared with control (CTL). This IEM-sensitive component of LTP was absent in slices pretreated with the CaMKK inhibitor STO-609 ($5 \mu\text{M}$), demonstrating rapid recruitment of synaptic CP-AMPA receptors downstream of CaMKK ($n = 9-10$). **D**, Application of IEM 20 min after TBS did not alter LTP expression, indicating that synaptic incorporation of CP-AMPA receptors is transient ($n = 9$). **E**, In contrast to TBS-LTP, HFS-LTP (see Materials and Methods) was not affected by STO-609 pretreatment or treatment with IEM immediately after HFS ($n = 10$).

CaMKI, CaMKII, PKA (protein kinase A), PKC, and ERK (Selcher et al., 2003; Yasuda et al., 2003; Schmitt et al., 2005). Even the same protocol can use different pathways depending on the developmental age (Yasuda et al., 2003; Y. Lu et al., 2007). It appears that multiple signaling pathways may mediate TBS-LTP because inhibition of CaMKK only gives a 40% suppression.

Why do synapses use CP-AMPA receptors to initiate plasticity? Insertion of highly conductive synaptic CP-AMPA receptors from a dendritic extrasynaptic pool, would allow postsynaptic potentiation via AMPAR trafficking to occur rapidly. This higher conductance of CP-AMPA receptors compared with GluR2(edited)-containing AMPARs, and their ability to be further potentiated by phosphorylation of GluR1 at Ser831 (Oh and Derkach, 2005), make them ideally suited to initiate LTP where both rapid trafficking and increased conductance of postsynaptic AMPARs are established mechanisms (Benke et al., 1998; Luthi et al., 2004). Based on these considerations, we calculated that $<5\%$ of existing synaptic GluR1/GluR2 receptors would have to be augmented by homomeric GluR1 (Fig. 6) to account for the degree of potentiation observed in three different forms of synaptic potentiation by CP-AMPA receptors: pairing-induced LTP (Plant et al., 2006), TBS-LTP (Fig. 5), and CaMKI infusion (Fig. 1). This mechanism for initial LTP expression appears to be the inverse of what happens during LTD at young (<3 weeks) cerebellar stellate synapses and mossy fiber-CA3 pyramidal cell synapses. These synapses contain CP-AMPA receptors that are replaced by GluR2-containing AMPARs (Ho et al., 2007) from an extrasynaptic pool (Liu and Cull-Candy, 2000, 2005). This subunit recombination appears to involve interaction of the GluR2 subunit with the proteins PICK1 (PKC-interacting protein 1) (Ho et al., 2007) and GRIP (glutamate receptor-interacting protein) (Liu and Cull-Candy, 2005).

Is rapid regulation of synaptic strength the only function of CP-AMPA receptors, and why does their expression in CA1-LTP last $<20-25$ min (Fig. 5D) (Plant et al., 2006)? Because these receptors have high Ca^{2+} and Zn^{2+} permeability at resting membrane potentials, their prolonged synaptic activation could lead to cell death (Calderone et al., 2004). Indeed, several neuropathologies associated with cell death are attributed to the expression of CP-AMPA receptors (for review, see Kwak and Weiss, 2006; Liu and Zukin, 2007). It is also possible that either the transient Ca^{2+} influx through CP-AMPA receptors or the synaptic insertion of the GluR1 C terminus (Kopeck et al., 2007) regulate unique signaling events important for subsequent stable LTP expression and structural plasticity of spines. One attractive candidate that we are investigating is modulation of structural plasticity resulting from actin and CaMKs, which mediate changes in spine morphology (Matsuzaki et al., 2004; Nagerl et al., 2004; Saneyoshi et al., 2008).

Appendix

Quantifying the fraction of CP-AMPA receptors for synaptic potentiation

We estimated how many CP-AMPA receptors would need to be inserted into a CA1 synapse to account for the observed synaptic potentiation. Our calculations are based on the conclusion that these CP-AMPA receptors are predominantly GluR1 homomers because expression levels of GluR3 and GluR4 in the mature hippocampus are very low compared with GluR1 and GluR2 (Monyer et al., 1991; Craig et al., 1993; Geiger et al., 1995; Wenthold et al., 1996; Sans et al., 2003).

Subunit recombination is accompanied by increased rectification of AMPAR-mediated synaptic currents (Plant et al., 2006), and one can calculate the rectification index, RI, as follows:

$$\text{RI} = \text{EPSC}_{\text{LTP}}(-60\text{mV}) / \text{EPSC}_{\text{LTP}}(40\text{mV}), \quad (1)$$

where $\text{EPSC}_{\text{LTP}}(-60 \text{ mV})$ and $\text{EPSC}_{\text{LTP}}(40 \text{ mV})$ are amplitudes of EPSCs at -60 and 40 mV of membrane potential, respectively, during E-LTP. One can write for the enhanced EPSC at -60 mV as follows:

$$EPSC_{LTP}(-60mV) = (\gamma_{CI}P_{o,CI}N_{CI} + k\gamma_{CP}P_{o,CP}N_{CP})E(-60), \quad (2)$$

where γ_{CI} and γ_{CP} are channel conductances, $P_{o,CI}$ and $P_{o,CP}$ are open probabilities and N_{CI} and N_{CP} are the number of GluR2-containing heteromers (designated CI for calcium impermeable) and GluR1 homomers (designated CP for calcium permeable) in a synapse, respectively. $E(-60)$ is the electrochemical potential for GluR2-containing AMPARs, and k is a coefficient accounting for the difference in electrochemical potentials between GluR2-containing heteromers and GluR1 homomers. However, in contrast to -60 mV, the contribution of GluR1 homomers to postsynaptic current at 40 mV is negligible because it is reduced by a factor of 45 (Derkach et al., 1999; Oh and Derkach, 2005) as these channels are blocked at this membrane potential by intracellular polyamines (Donevan and Rogawski, 1995; Bowie et al., 1998), producing rectification of the postsynaptic current during E-LTP (Plant et al., 2006). This is translated into

$$EPSC_{LTP}(40mV) = \gamma_{CI}P_{o,CI}N_{CI}E(40), \quad (3)$$

where $E(40)$ is an electrochemical potential for GluR1 homomers. Substituting Equations 2 and 3 into 1 yields the following:

$$RI = [1 + (k\gamma_{CP}P_{o,CP}N_{CP})/(\gamma_{CI}P_{o,CI}N_{CI})]E(-60)/E(40). \quad (4)$$

In this equation, the ratio N_{CP}/N_{CI} represents the fraction of new GluR1 homomers incorporated into a potentiated synapse. Solving for this ratio gives the following:

$$N_{CP}/N_{CI} = [RI E(40)/E(-60) - 1] \gamma_{CI}P_{o,CI}/(k\gamma_{CP}P_{o,CP}). \quad (5)$$

There are, however, two different types of CA1 synapses: those that showed an increase in single-channel conductance after LTP induction and those that did not (Benke et al., 1998; Lüthi et al., 2004). Importantly, these two types of synapses were also different in their basal channel conductances. Those synapses showing an increase in γ had baseline γ values of 4.8 pS versus 7.2 pS in those synapses without changes in γ (Benke et al., 1998). We therefore used the value 4.8 pS for nonpotentiated synapses in our modeling (see also comments of low γ estimates in Materials and Methods). AMPARs are phosphorylated at Ser831 in GluR1 during LTP (Barria et al., 1997b; Lee et al., 2000), and channel conductance of phosphorylated GluR1 has been measured [taken as 18.9 pS from the studies by Derkach et al. (1999) and Oh and Derkach (2005)]. GluR2 subunit also controls open probability of AMPARs, and we found that GluR1/GluR2 heteromers have at least a fourfold lower open probability compared with GluR1 homomers (Oh and Derkach, 2005), so we can write $P_{o,CI}/P_{o,CP}$ as ≤ 0.25 . The value of k was given as 1.15, assuming that currents through GluR2-containing AMPARs and GluR1 homomers reverse at 0 and 9 mV, respectively. Substitution of all of these values and RI [taken as 1.85 from the study by Plant et al. (2006)]

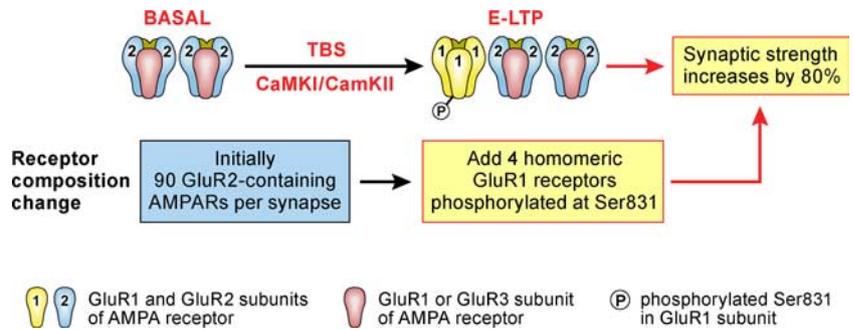


Figure 6. Model for potentiation of synaptic strength through CaM-kinase-mediated recruitment and regulation of GluR1 homomeric CP-AMPA receptors. Calculations (see Appendix) show that for a typical hippocampal SC-CA1 pyramidal cell synapse consisting of ~ 90 GluR2-containing AMPARs, CaMKI-mediated recruitment of four homomeric GluR1 AMPARs and their phosphorylation on Ser831 by CaMKII is sufficient to increase synaptic strength by 80%. If these CP-AMPA receptors are recruited from an existing extrasynaptic pool, as indicated by our results, this process provides a very rapid and efficient mechanism for the initial synaptic potentiation (E-LTP) after induction of LTP by appropriate stimuli. Because the presence of the dominant GluR2 subunit precludes functional regulation of GluR1-containing heteromeric AMPARs because of S831 phosphorylation (Oh and Derkach, 2005), this model may also explain how CaMKI and CaMKII cooperate to maximally increase single channel conductance of AMPARs during LTP (Benke et al., 1998).

in Equation 5 yields $N_{CP}/N_{CI} \leq 0.015$. Thus, insertion of $\sim 2\%$ of GluR1 homomers is sufficient to produce the rectification seen at CA1 synapses during E-LTP.

The number of newly inserted GluR1 homomers can be also calculated in an alternative way based on the increase in synaptic strength at negative membrane potentials. Thus,

$$EPSC_{LTP}(-60mV)/EPSC_{base}(-60mV) = 1 + \Delta EPSC, \quad (6)$$

where $EPSC_{basal}(-60$ mV) is a baseline EPSC before LTP induction and can be expressed as follows:

$$EPSC_{basal}(-60mV) = \gamma_{CI}P_{o,CI}N_{CI}E(-60), \quad (7)$$

where $\Delta EPSC$ is an increase in synaptic strength during E-LTP. After substituting Equations 2 and 7 into 6 and solving for N_{CP}/N_{CI} , one can write

$$N_{CP}/N_{CI} = \Delta EPSC(\gamma_{CI}P_{o,CI}/k\gamma_{CP}P_{o,CP}). \quad (8)$$

At 80% of potentiation during pairing-induced E-LTP (Plant et al., 2006), this ratio is 4.3%. Furthermore, for both TBS-LTP and potentiation by CaMKI infusion, the ratio is 1.6–2.1%, assuming ~ 30 – 40% increases in synaptic strength associated with CP-AMPA receptors (Figs. 1, 5). It is noteworthy that Equations 5 and 8 are general expressions estimating relative changes in the number of receptors and, thus, are independent of the initial number of AMPARs at a synapse.

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