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Calmodulin-Dependent Kinase Kinase/Calmodulin Kinase I Activity Gates Extracellular-Regulated Kinase-Dependent Long-Term Potentiation

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Intracellular Ca^{2+} and protein phosphorylation play pivotal roles in long-term potentiation (LTP), a cellular model of learning and memory. Ca^{2+} regulates multiple intracellular pathways, including the calmodulin-dependent kinases (CaMKs) and the ERKs (extracellular signal-regulated kinases), both of which are required for LTP. However, the mechanism by which Ca^{2+} activates ERK during LTP remains unknown. Here, we describe a requirement for the CaMK-kinase (CaMKK) pathway upstream of ERK in LTP induction. Both the pharmacological inhibitor of CaMKK, STO-609, and dominant-negative CaMKI (dnCaMKI), a downstream target of CaMKK, blocked neuronal NMDA receptor-dependent ERK activation. In contrast, an inhibitor of CaMKII and nuclear-localized dnCaMKIV had no effect on ERK activation. NMDA receptor-dependent LTP induction robustly activated CaMKI, the Ca^{2+}-stimulated Ras activator Ras-GRF1 (Ras-guanyl-nucleotide releasing factor), and ERK. STO-609 blocked the activation of all three enzymes during LTP without affecting basal synaptic transmission, activation of CaMKII, or cAMP-dependent activation of ERK. LTP induction itself was suppressed ~50% by STO-609 in a manner identical to the ERK inhibitor U0126: either inhibitor occluded the effect of the other, suggesting they are part of the same signaling pathway in LTP induction. STO-609 also suppressed regulatory phosphorylation of two downstream ERK targets during LTP, the general translation factors eIF4E (eukaryotic initiation factor 4) and its binding protein 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1). These data indicate an essential role for CaMKK and CaMKI to link NMDA receptor-mediated Ca^{2+} elevation with ERK-dependent LTP.

Key words: calcium; CaM kinase; ERK; LTP; synaptic plasticity; eIF4E

Introduction

Protein phosphorylation is one of the most prevalent mechanisms for modulating neuronal functions, including long-term potentiation (LTP) (Kandel, 2001; Lisman et al., 2002), a cellular model of learning and memory (Malenka, 2003; Lynch, 2004). Two protein kinase families highly expressed in brain that have been implicated in molecular mechanisms regulating LTP are the Ca^{2+}/calmodulin-dependent kinases (CaMKs) and ERK1 and ERK2 (extracellular signal-regulated kinases) (Soderling, 2000; Sweatt, 2004). The CaMK family, which is activated in response to elevations of intracellular Ca^{2+}, includes CaMKII (Soderling et al., 2001; Lisman et al., 2002; Colbran and Brown, 2004) and the CaMK-kinase (CaMKK) cascade consisting of CaMKK and its two major downstream targets CaMKI and CaMKIV (Soderling, 1999; Means, 2000). CaMKII regulates numerous neuronal

functions, including phosphorylation of the AMPA-type glutamate receptor (GluR), resulting in increased conductance during early-phase LTP (E-LTP) (Soderling and Derkach, 2000; Song and Huganir, 2002). CaMKIV is primarily restricted to the nucleus (Jensen et al., 1991; Lemrow et al., 2004), in which it stimulates gene transcription required for late-phase LTP (L-LTP) (Kang et al., 2001) through phosphorylation of transcription factors such as CREB (cAMP response element-binding protein) and CBP (CREB binding protein) (Enslen et al., 1994; Impey et al., 2002). Cytosolic CaMKI modulates cytoskeletal organization (Suizu et al., 2002) and axonal growth cone motility (Wayman et al., 2004), but a potential role for CaMKI in LTP has not been examined previously.

The ERK family of MAP (mitogen-activated protein) kinases are activated by growth factors, neurotransmitters, and hormones, as well as by Ca^{2+}-permeable NMDA receptor (NMDA-R) and voltage-gated channels in neurons (Pearson et al., 2001; Thomas and Huganir, 2004). The NMDA-R gates many forms of synaptic plasticity, including hippocampal CA1 LTP (Malenka and Nicoll, 1999). Activation of ERK during neuronal depolarization or NMDA-R stimulation requires elevations of intracellular Ca^{2+}, and it appears to be mediated through the classical Ras/Raf/MEK (MAP kinase kinase)/ERK cascade (Wu et al., 2001a,b; Agell et al., 2002). Recent studies suggest that Ras-GRF1 (Ras-guanyl-nucleotide releasing factor) may couple the

NMDA-R Ca^{2+} elevation to Ras and ERK activation in neurons (Krapivinsky et al., 2003; Tian et al., 2004).

Induction of LTP activates ERK, and inhibitors of the ERK pathway (e.g., SL327 and U0126) partially suppress LTP induction (Sweatt, 2004; Thomas and Huganir, 2004). A role for ERK in the regulation of gene transcription during L-LTP is well established (Treisman, 1996; Kelleher et al., 2004), and a role of ERK in mRNA translation has been reported recently (Kelleher et al., 2004). Expression of dominant-negative (dn) MEK1 in region CA1 of the hippocampus suppresses hippocampal-dependent memory tasks in mice, ERK activation, and anisomysin-sensitive LTP in hippocampal slices, as well as mRNA translation in cultured hippocampal neurons.

Because of the key role of ERKs in modulating Ca^{2+}-dependent neuronal plasticity, it was important to determine whether the recently identified crosstalk between CaMKK/CaMKI and ERK (Schmitt et al., 2004) regulate LTP. The results of this study provide strong evidence that CaMKK and CaMKI are essential for the full expression of LTP and ERK-dependent translational activation.

Materials and Methods

Antibodies and plasmids. The following reagents were purchased from the indicated sources: U0126 and forskolin from Calbiochem (Riverside, CA); STO-609, NMDA, and APV from Tocris Cookson (Ellisville, MO); and anisomycin from Alexis Biochemicals (San Diego, CA). Phospho-specific antibodies were purchased from the following sources: ERK1/2 (Thr ^{202}, Tyr ^{204}), Ras-GRF1 (Ser ^{916}), eIF4E (eukaryotic initiation factor 4) (Ser ^{209}), 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) (Ser ^{65}), and AKT (Thr ^{308}) from Cell Signaling Technology (Beverly, MA); CaMKII (Thr ^{286}) and GluR1 subunit of the AMPA-R (Ser ^{831}) from Affinity BioReagents (Golden, CO); and CaMKI (Thr ^{177}) from Dr. Naohito Nozaki (Kanagawa Dental College, Yokosuka, Kanagawa, Japan) (Schmitt et al., 2004; Wayman et al., 2004). Other antibodies were purchased from the indicated sources: AKT, Ras-GRF1, eIF4E, and 4E-BP1 from Cell Signaling Technology; ERK2 (D-2) from Santa Cruz Biotechnology (Santa Cruz, CA); CaMKII from Transduction Laboratories (Lexington, KY); CaMKI from Dr. Kohji Fukunaga (Tohoku University, Sendai, Japan); and Flag (M2) from Sigma (St. Louis, MO). The dnCaMKK (K71A, T108A, S458A), dnCaMKI (K49E, T177A, 7IHQS286EDDD, F307A), dnCaMKIV nuclear (T196A, K71E, HMDT305DEDD), and CaMKIIN plasmids have been described previously (Schmitt et al., 2004; Wayman et al., 2004). The Flag-ERK2 plasmid was provided by Dr. Philip Stork (Vollum Institute, Portland, OR). The Ras-GRF1 construct was purchased from Open Biosystems (Huntsville, AL).

Primary hippocampal neuronal culture and treatments. Neurons were isolated from the hippocampus of postnatal day 1–2 Sprague Dawley rats. Neurons were grown 5–6 d *in vitro* (DIV) (unless otherwise indicated) and placed in serum-free isotonic media consisting of 130 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES-HCl, and 30 mM D-glucose for 60 min at 37°C with pharmacological inhibitors (5 μM STO-609 for 60 min; 10 μM U0126 for 20 min), as indicated before stimulation. Cells were stimulated with serum- and magnesium-free isotonic media containing NMDA (25 μM) and glycine (1 μM) for the indicated times.

For transfections, 5-d-old hippocampal neurons were cotransfected with Flag-ERK2, along with the control vector pcDNA3, or the indicated plasmids using Lipofectamine 2000 (Invitrogen, San Diego, CA) according to the guidelines of the manufacturer. After transfection, neurons were allowed to recover in complete media for 24 h, placed in serum-free isotonic media for 60 min (see above), treated as indicated, and lysed in ice-cold lysis buffer as detailed below. Lysates were then examined for the presence of Flag-ERK2 or myc-Ras-GRF1 (Open Biosystems) expression by Western blotting, and Flag-ERK2 or myc-Ras-GRF1 were immunoprecipitated and examined by Western blotting for activation.

Western blotting and immunoprecipitation of primary hippocampal neurons. For direct Western blotting after primary hippocampal neuron

stimulations, media was aspirated, and boiling Laemmeli's buffer (supplemented with 200 mM EDTA, 20 mM EGTA, 200 mM NaF, 20 mM β-glycerophosphate, 1 mM PMSF, and 5 μM microcystin) was added to each plate of cells. Plates were scraped, and cellular lysates were placed in microcentrifuge tubes, rapidly vortexed, boiled for 5 min, and centrifuged at 8000 rpm for 3 min to pellet insoluble materials. Equivalent amounts of protein were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes (PVDF), and examined by Western blotting with the indicated antibodies.

For immunoprecipitations after primary hippocampal neuron stimulations, media was aspirated, and ice-cold lysis buffer (10% glycerol, 1% NP-40, 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 2 mM $MgCl_2$) containing freshly added inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 1 mM sodium orthovanadate) was added to each plate of cells on ice. Plates were scraped, and cellular proteins were placed in ice-cold microcentrifuge tubes. Briefly, cell lysates were spun at 8000 rpm for 5 min at 4°C to pellet the cytoskeleton and nuclei. Immunoprecipitations were performed at 4°C for 4 h in ice-cold lysis buffer (Schmitt et al., 2004). Precipitated proteins were washed two times with lysis buffer, placed in Laemmeli's buffer, resolved by SDS-PAGE, and Western blotted for phosphorylated Flag-ERK2 (pFlag-ERK2, pFlag-ERK2, or myc-Ras-GRF1). Western blotting for total Flag-ERK2 with anti-Flag antibody was performed on lysates to serve as a loading and transfection control. For quantitation of Western blots, autoradiographs were scanned and densitized using Kodak ID 3.0.2 system software (Eastman Kodak, New Haven, CT). For each phospho-protein of interest, separate Western blots were run for that phospho-protein (e.g., pCaMKI) and for the total protein (CaMKI). For each Western blot, band densities were first normalized to untreated controls, then phospho-protein was normalized to total protein, and the value was presented as fold increase in phosphorylation relative to the control.

Mouse hippocampal slice production and treatments. Adult male C57BL/6 mice (8–12 weeks old; Charles River Laboratories, Wilmington, MA) were anesthetized with pentobarbital (60 mg/kg, i.p.) and decapitated using procedures reviewed and approved by Institutional Animal Care and Use Committee at the Oregon Health and Sciences University Department of Comparative Medicine. Brains were removed within 1 min of decapitation and immediately submerged in ice-cold, sucrose-modified artificial CSF (ACSF) for hippocampal dissection and slicing (in mM): 110 sucrose, 60 NaCl, 2.5 KCl, 28 $NaHCO_3$, 1.25 NaH_3PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 5 glucose, and 0.6 sodium ascorbate, pH 7.4, at 4°C (presaturated by bubbling with 95% O_2/5% CO_2 at 21–22°C). Hippocampal slices (400 μm, transverse) were prepared using a vibratome and an agar backing (2%), and each slice was transferred to warm ACSF [in mM: 125 NaCl, 2.5 KCl, 22.6 $NaHCO_3$, 1.25 NaH_3PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 11.1 glucose (continuously gas saturated with 95% O_2/5% CO_2, pH 7.4 at 32°C)] for recovery (30 min, 37°C). Slices from the dorsal and ventral thirds of the hippocampus were discarded. After recovery, the chamber was equilibrated at 21–22°C, and slices were held for 2–8 h before recording or 1 h before beginning NMDA and forskolin treatments. For NMDA and forskolin treatments, slices were then placed at 30°C for 1 additional hour, in the presence or absence of pharmacological inhibitors (5 μM ST0–609 for 60 min, 10 μM U0126 for 20 min, and 50 μM APV for 20 min), before chemical stimulations. Hippocampal slices were then stimulated with NMDA plus glycine (25 and 1 μM, respectively) or forskolin (50 μM) for the indicated times in ACSF, in the presence or absence of STO-609 or U0126. After chemical stimulation, and at various time points after the electrical induction of LTP, slices were frozen on a filter paper-covered liquid nitrogen-cooled aluminum block and placed in microcentrifuge tubes in pairs. Slices were kept submerged at all times until frozen. For LTP experiments, area CA1 was isolated by microdissection after freezing. Ice-cold lysis buffer (10% glycerol, 1% NP-40, 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 2 mM $MgCl_2$) plus freshly added inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 5 μM microcystin, 1 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 1 mM sodium orthovanadate) was added to each tube containing frozen slices, which were rapidly homogenized as they thawed in lysis buffer. Laemmeli's buffer (6×) was then added, and tubes were rapidly vortexed and boiled for 5 min. Equivalent volumes of lysate were resolved by SDS-

PAGE, blotted onto PVDF membranes, and examined by Western blotting with the indicated antibodies.

Electrophysiology. For electrophysiological recordings, submerged hippocampal slices were suspended in pairs on a nylon mesh in a small (350–400 μl) recording chamber and perfused with ACSF at a rate of 2.5–3 ml/min. The temperature in the recording chamber was raised to 30–32°C over the course of a few minutes and held for the duration of the experiment. Synaptic responses were evoked by Schaffer collateral stimulation using a bipolar tungsten electrode (tip spacing, 140 μm; Frederick Haer Company, Bowdoinham, ME) and a 100 μs square wave test pulse (typically 30–40 μA) delivered at 60 s intervals. The stimulation intensity was adjusted to produce a basal response of 1.2–1.4 mV [typically 25% of the maximum field EPSP (fEPSP) amplitude]. Recordings were made using ACSF-filled glass micropipettes (2–4 MΩ) placed in the stratum radiatum area of CA1 (see Fig. 2C) and connected via head stages to an A-M Systems (Carlsborg, WA) model 1800 amplifier. Signals were digitized at 100 kHz using the Axon Instruments (Union City, CA) Digidata 1200 series interface running Clampex 8.0, and the initial slope (linear portion of the first millisecond) of the fEPSPs were analyzed with Clampfit 8.0 software. Drug treatments began 30 min (STO-609, U0126, and anisomycin) or 20 min (APV) before and ended (with the exception of anisomycin) 5 min after the induction of LTP using theta bursts. Anisomycin treatments persisted for the duration of the recordings. E-LTP was induced using an acute theta-burst protocol: four pulses per burst (100 Hz), five bursts per train (5 Hz), three trains (beginning 20 s apart). L-LTP was induced using a recurrent theta-burst protocol: four epochs of the acute theta-burst protocol delivered 5 min apart. With the exception of the first data point after LTP induction (posttetanic potentiation), 3 min fEPSP averages are shown. The first data point after LTP induction was taken 20 s after the final train of theta bursts. All treatment groups were balanced such that the average time of the slices in the recording chamber was equal and the average age of animals was equal.

Statistics. To determine whether significant differences existed among treatments, an ANOVA was performed on the data with significance set at 0.05. To compare whether significant differences existed between two treatments, a Student's *t* test was performed on the data with significance set at 0.05. Significance levels (*p* value) are indicated in the figures: $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Results

NMDA activation of ERK requires CaMKK and CaMKI

To investigate the potential role of CaMKs in NMDA-dependent ERK activation in hippocampal neurons, we used the membrane-permeable CaMKK inhibitor STO-609 (Tokumitsu et al., 2002) or expression of dnCaMKs or the CaMKII inhibitor protein CaMKIIN (Chang et al., 1998). STO-609 appears to be highly specific for CaMKK: (1) it has an *in vitro* IC$_{50}$ of 0.13–0.38 μM for CaMKK and 32 μM for CaMKII with little or no inhibition of CaMKI, CaMKIV, PKA (protein kinase A), PKC, ERK, or myosin light chain kinase (Tokumitsu et al., 2002); and (2) in cultured hippocampal neurons (Wayman et al., 2004) and NG108 cells (Schmitt et al., 2004) subjected to depolarization, STO-609 inhibits CaMKK activation, as assessed by its ability to block phosphorylation of the activation loop Thr^{177} in CaMKI, with no effect on CaMKII activation (i.e., autophosphorylation of Thr^{286}). Furthermore, we used dnCaMKs as an independent confirmation of the involvement of this pathway. CaMKIIN is an endogenous CaMKII inhibitor protein (IC$_{50}$ of 50–100 nM) that exhibits no *in vitro* inhibition of CaMKI, CaMKIV, CaMKK, PKA, PKC (Chang et al., 1998), or ERK (S. Nygaard and T. R. Soderling, unpublished result). When transfected into cultured hippocampal neurons, CaMKIIN potently blocks CaMKII activation (Wayman et al., 2004) but not ERK activation (Schmitt et al., 2004) during depolarization.

Stimulation of cultured hippocampal neurons with NMDA activated endogenous ERK within 5 min, an effect that was com-

pletely blocked by either the MEK inhibitor U0126 or by STO-609 (Fig. 1A), indicating involvement of the CaMKK cascade. To confirm the specificity of STO-609, we used a mutant (L233F) of CaMKK (Tokumitsu et al., 2002) that is insensitive to inhibition by this compound. Transfection of neurons with CaMKK$_{L233F}$ completely rescued inhibition by STO-609 (Fig. 1A), establishing that STO-609 was exerting its effect through CaMKK. To further characterize which member of the CaMKK cascade mediated ERK activation, hippocampal neurons were cotransfected with Flag-ERK2 plus various dominant-negative CaMK constructs. ERK activation was completely inhibited by dnCaMKK, as well as by dnCaMKI (Fig. 1B). Endogenous CaMKIV is predominantly nuclear restricted (Lemrow et al., 2004), and nuclear-localized (nuc) dnCaMKIVnuc did not block ERK activation (Fig. 1B), although we have shown previously that it suppressed NMDA-stimulated CREB-dependent transcription in neurons (Wayman et al., 2004). To examine the role of CaMKII, we expressed the specific CaMKII inhibitor protein CaMKIIN (Chang et al., 1998) that completely blocked neuronal CaMKII activation in response to depolarization (Wayman et al., 2004). CaMKIIN had no effect on NMDA-dependent ERK activation (Fig. 1B). These results demonstrate in cultured hippocampal neurons that NMDA-dependent activation of ERK was mediated by the CaMKK and CaMKI pathway but not by CaMKII or nuclear CaMKIV.

Next, we examined the role of CaMKK in ERK activation using acute mouse hippocampal slices. The rapid activation of ERK in response to NMDA treatment was blocked by the NMDA receptor antagonist APV, the MEK inhibitor U0126, and the CaMKK inhibitor STO-609 (Fig. 1C,D). CaMKK and CaMKI were also rapidly activated by NMDA as assessed by phosphorylation of the activation loop Thr^{177} in CaMKI (a target of CaMKK), and, as expected, these were also inhibited by STO-609 but not U0126 (Fig. 1E). In agreement with previous studies (Fukunaga et al., 1992), CaMKII was also activated (i.e., autophosphorylation of Thr^{286}) by NMDA stimulation, and one of its substrates, Ser^{831}, in the GluR1 subunit of the AMPA-R was phosphorylated (Barria et al., 1997), but neither of these reactions was inhibited by STO-609 or U0126 (Fig. 1F). This result confirms our previous observation that STO-609 does not block CaMKII activation in hippocampal neurons (Wayman et al., 2004) or NG108 cells (Schmitt et al., 2004). The specificity of STO-609 for CaMKK was further demonstrated by the fact that activation of ERK in response to forskolin treatment, which was NMDA-receptor independent (Fig. 1C), was not blocked by STO-609 but was suppressed by U0126 (Fig. 1G).

We have shown previously a requirement for the small G-protein Ras in the CaMKK/CaMKI activation of ERK during depolarization (Schmitt et al., 2004). Ras is required for NMDA receptor-dependent activation of ERK in neurons (Zhu et al., 2002), and the dnRas RasN17 blocked ERK activation in response to NMDA treatment of cultured hippocampal neurons in our experiments (data not shown). Furthermore, recent studies demonstrate the involvement of the Ca^{2+}-dependent Ras activator Ras-GRF1 in ERK activation (Krapivinsky et al., 2003; Tian et al., 2004). Phosphorylation of Ras-GRF1 at Ser^{916} appears to play a pivotal role in its physiological functions and serves as a readout for activation (Mattingly, 1999; Yang et al., 2003). Endogenous Ras-GRF1 was rapidly phosphorylated on Ser^{916} in response to NMDA treatment, an effect that was blocked by STO-609 but not U0126 (Fig. 1H). To confirm the roles for CaMKK and CaMKI in Ras-GRF1 phosphorylation, cultured hippocampal neurons were cotransfected with myc-Ras-GRF1 plus dnCaMKI or CaMKIIN. Ras-GRF1 phosphorylation was completely suppressed by

dnCaMKI but not by the CaMKII inhibitor (Fig. 1*I*). Together, the data of Figure 1 support the specificity of STO-609 for CaMKK and suggest that the requirement for CaMKK/CaMKI in NMDA-dependent ERK activation in hippocampal neurons is upstream of Ras-GRF1.

Induction of LTP requires the CaMKK pathway

The CaMKK cascade can mediate NMDA receptor-dependent ERK activation (Fig. 1) and ERK plays an important role in LTP induction (Sweatt, 2004; Thomas and Huganir, 2004), so we investigated the role of CaMKK in LTP induction. Mouse hippocampal slices treated with STO-609 exhibited a normal input–output relationship over a wide range of stimulus intensities (Fig. 2*A*), as well as normal paired-pulse facilitation (Fig. 2*B*), indicating that acute blockade of the CaMKK pathway does not effect basal synaptic transmission. Additionally, we integrated the area above the curve of the fEPSPs during stimulation with theta-burst trains (E-LTP protocol, see Materials and Methods) and found no significant difference among the dendritic field responses of STO-609- or U0126-treated and untreated slices during E-LTP induction (Fig. 2*C*). This finding held true for the first (naive) theta bust, each individual train, the plasticity within each train (area of burst 5/burst 1), as well as the sum of all trains. These results indicate that STO-609 does not impair normal synaptic transmission nor the dendritic field response to high-frequency theta-burst trains, important prerequisites for our study of the role of CaMKK in LTP.

To determine whether CaMKK and its downstream targets are involved in the induction of early-phase LTP (E-LTP), we treated acute hippocampal slices with 5 μM STO-609. Analysis of the initial slope of fEPSPs taken from area CA1 (Fig. 2*C*) before and after theta-burst stimulation (see Materials and Methods) revealed a significant ($\sim$50%) STO-609 sensitivity of E-LTP out to 60 min (Fig. 2*D*). Our acute theta-burst protocol produced activation of CaMKI and ERK within 5 min, effects that were completely blocked by the NMDA receptor antagonist APV, as was the induction of E-LTP (Fig. 2*E*).

The role of CaMKK in L-LTP lasting 3 h was examined using a recurrent theta-burst protocol spaced over 15 min (see Materials and Methods). Again, STO-609 treatment gave $\sim$50% suppression over the first hour and completely obviated LTP at 3 h (Fig. 2*F*), demonstrating that the requirement for CaMKK in the first hour of LTP was not overcome by a more robust induction protocol. Although CaMKIV is also activated by CaMKK, it does not
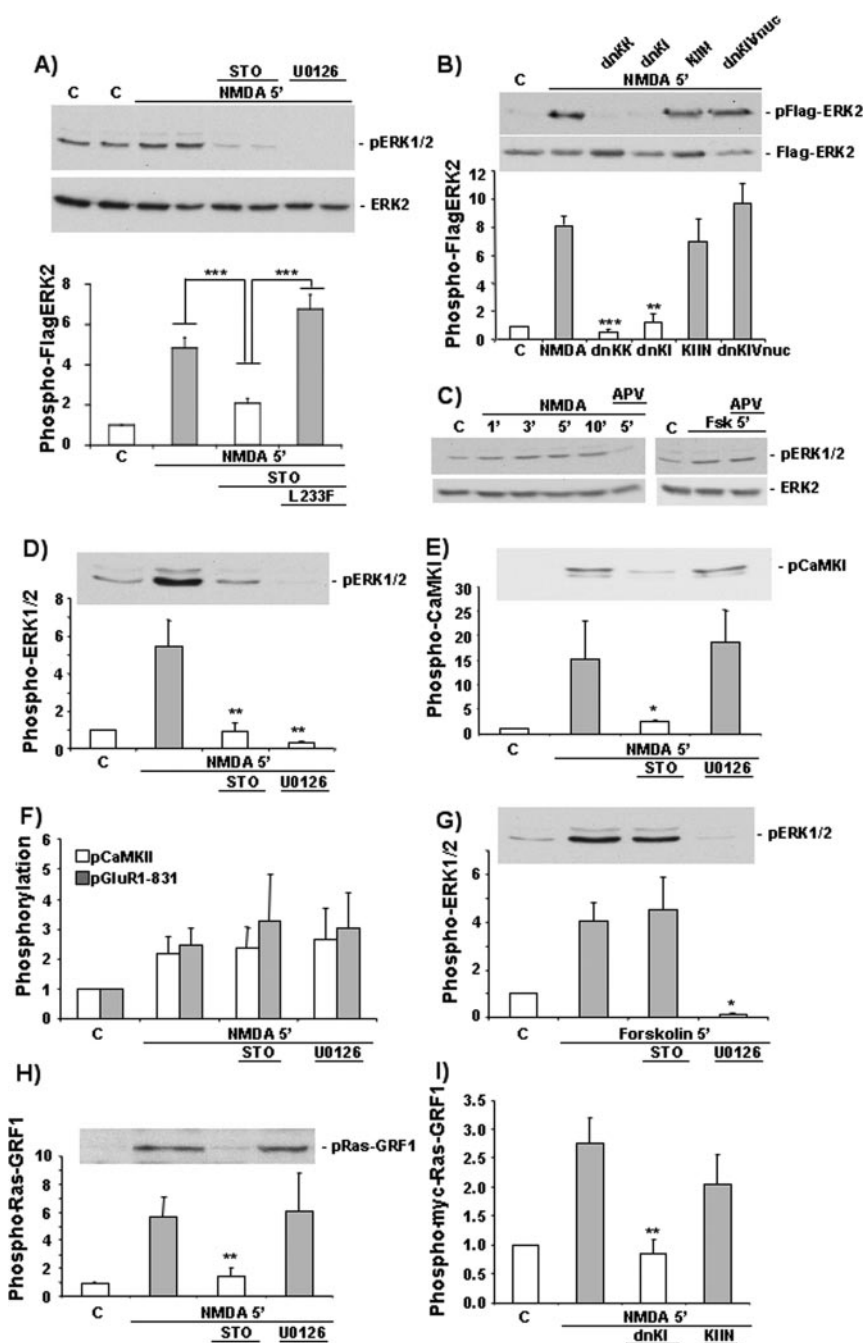

Figure 1. NMDA activation of ERK and Ras-GRF1 in hippocampus requires CaMKK and CaMKI. Primary cultures of rat hippocampal neurons (*A*, *B*, *I*, 6 DIV) or acute mouse hippocampal slices (*C–H*, 8 –12 weeks old) were preincubated with the indicated pharmacological reagents [STO-609 (STO), 5 μM, 60 min; U0126, 10 μM, 20 min; APV, 50 μM, 20 min] before stimulation by NMDA/glycine (25 and 1 μM, respectively) or forskolin (Fsk; 50 μM) for 5 min. *A*, *B*, *I*, Cultured rat hippocampal neurons (5 DIV) were cotransfected with Flag-ERK2 and either CaMKK$_{L233F}$ (L233F, a STO-609-insensitive mutant; *A*, bottom graph) or myc-Ras-GRF1 plus control vector pcDNA3 or the indicated dnCaMKs or CaMKIIN as indicated. Cells were stimulated with NMDA, and Flag-ERK2 or myc-Ras-GRF1 phosphorylation was determined (see Materials and Methods). In all experiments, the activation states of the indicated proteins were determined by Western blots using phospho-specific antibodies (see Materials and Methods). The ratio of the phospho-protein to total amount of that same protein was set equal to 1 for the control, and relative values for treatments are shown as fold stimulation over basal. Means ± SD; $n = 6$ (*A*), $n = 3$ (*B*), $n = 5$ (*D–H*), or $n = 6$ (*I*). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. C, Control.

participate in E-LTP induced with either high-frequency stimulation (HFS) or recurrent theta bursts in mouse hippocampal region CA1 but is thought to contribute to CREB/CBP-dependent transcription during L-LTP (Ho et al., 2000; Kang et al., 2001). Based on these observations, we conclude that CaMKI mediated the down-
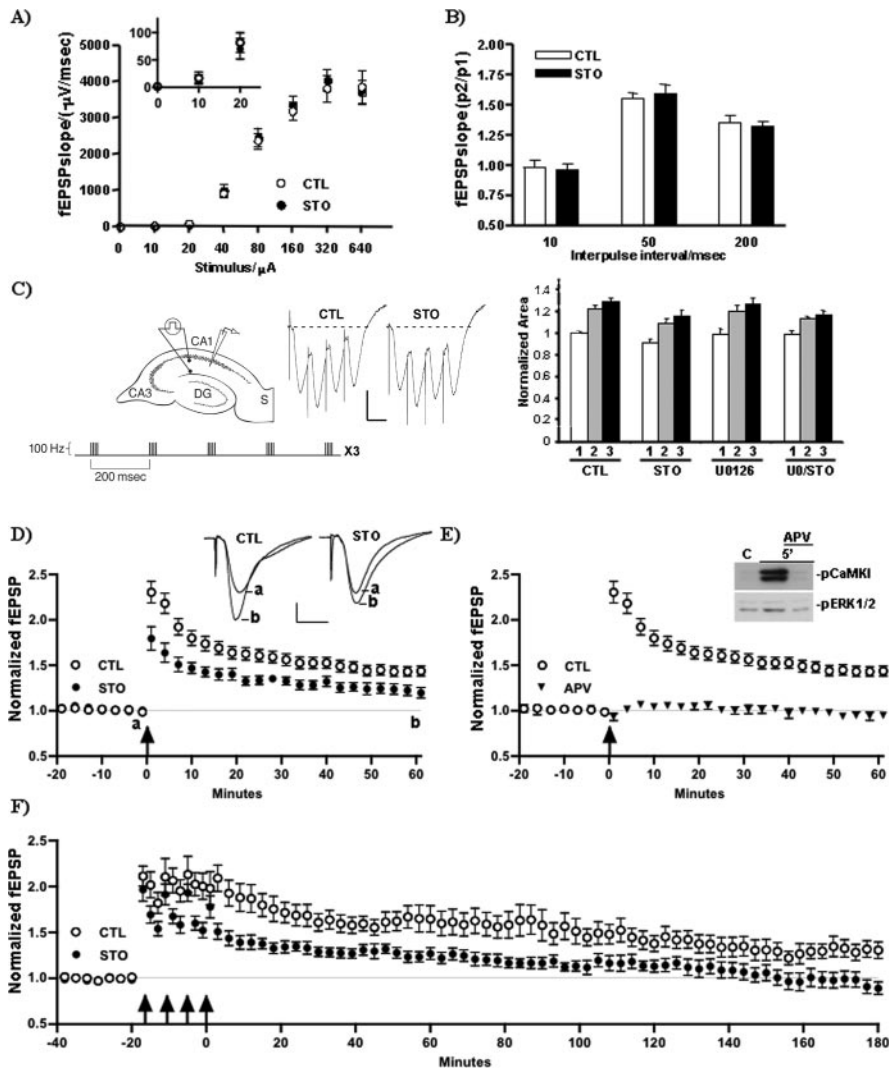
Figure 2. Inhibition of CaMKK markedly attenuates NMDA receptor-dependent LTP. *A*, STO-609 does not affect basal synaptic transmission. Input–output relationship for Schaffer collateral stimulation and fEPSP initial slopes recorded from area CA1 of mouse hippocampal slices preincubated without or with 5 μM STO-609 (STO) for 30 min. Inset, 0–20 μA; $n = 8$. *B*, Paired-pulse facilitation is normal during STO-609 treatment. Paired-pulse facilitation at 10, 50, and 200 ms interpulse intervals. Values presented are ratios of fEPSP initial slopes (pulse 2/pulse 1). $n = 8$. *C*, Left, Schematic drawing of electrode placements during recording and E-LTP induction paradigm (see Materials and Methods). DG, Dentate gyrus; S, stratum radiatum. Middle, Representative first burst responses from mock-treated (control) and treated (STO) slices. Calibration: 0.5 mV, 10 ms. Right, Integrated dendritic theta-burst responses of control slices and treated slices: $n = 24$, control; $n = 11$, STO-609; $n = 10$, U0126; $n = 10$, U0126 plus STO-609 U0/STO. The theta-burst responses were calculated by integrating the fEPSP response during stimulation of naive slices with our E-LTP-generating protocol. *D*, *E*, Inhibition of CaMKK partially inhibits NMDA receptor-dependent E-LTP. After 20 min of stable baseline recording (1 test pulse per minute min), slices were treated for 30 min without or with 5 μM STO-609 for 30 min (*D*) or with 50 μM APV for 20 min (*E*) before and 5 min after theta-burst stimulation [4 pulses per burst (100 Hz), 5 bursts per train (5 Hz), 3 trains (20 s apart)]. STO-609 had no significant effect on baseline fEPSP amplitude or initial slope kinetics. Control slices (LTP, 144 $\pm$ 5.4%; $n = 24$) exhibited approximately twofold greater LTP than STO-treated slices (LTP, 120 $\pm$ 5.9%; $n = 11$) at 60 min, whereas APV treatment ($n = 8$) completely blocked LTP. *D*, Inset, Average of 10 responses, 1–10 min before (*a*) and 50–60 min after (*b*) LTP induction. *E*, Inset, Western blots from microdissected region CA1 showing pCaMKI and pERK 5 min after mock stimulation (control) or theta-burst stimulation without or with APV treatment. *F*, Inhibition of CaMKK blocks L-LTP. Slices were treated without or with STO-609 as in *D* before stimulation with recurrent theta-burst patterned activity [4 pulses per burst (100 Hz), 5 bursts per train (5 Hz), 6 trains (20 s apart), 4 epochs (5 min apart)] to generate late-phase LTP. Control, $n = 6$; STO-609, $n = 6$. CTL, Control.

stream effects of CaMKK during E-LTP, whereas CaMKIV may contribute significantly to L-LTP.

CaMKK mediates ERK activation in E-LTP
Induction of E-LTP in hippocampal slices activates ERK, and this activation is required for full expression of LTP (Selcher et al.,

2003). In light of our findings that CaMKI functions upstream of ERK activation, we examined whether the effects of STO-609 on LTP occurred upstream of ERK. Induction of LTP with our acute theta-burst protocol (see Materials and Methods) (Fig. 2*D*,*E*) resulted in threefold to fourfold activation of CaMKI and ERK within 5 min that was sustained for at least 60 min (Fig. 3*A*,*B*). Both CaMKI and ERK activation were suppressed by STO-609 at all time points examined. Another cytoplasmic target for CaMKK is the protein kinase AKT, which is slowly phosphorylated during depolarization of NG108 cells (Yano et al., 1998; Schmitt et al., 2004). A dnAKT (Yano et al., 1998) did not block ERK activation by NMDA treatment of cultured hippocampal neurons (data not shown) or during depolarization of NG108 cells (Schmitt et al., 2004). Induction of LTP in hippocampal slices produced a slow phosphorylation of AKT that was blocked by STO-609 (Fig. 3*B*). ERK was maximally activated within 5 min after LTP induction, whereas robust AKT activation was not observed until after 15 min (Fig. 3*B*). Therefore, we propose that CaMKK was not acting through AKT to activate ERK or to mediate LTP induction. However, endogenous Ras-GRF1 was significantly phosphorylated within 5 min, and this was obviated by STO-609 treatment (Fig. 3*C*). LTP induction also resulted in rapid activation of CaMKII and phosphorylation of its substrate Ser[831] in the GluR1 subunit of the AMPA-R (Barria et al., 1997), but these effects were not suppressed by STO-609 (Fig. 3*D*). This confirms that STO-609 does not suppress other Ca^{2+}-dependent pathways, such as CaMKII. These data support our conclusion that ERK activation during LTP is mediated by CaMKK acting through CaMKI.

CaMKK and ERK mediate E-LTP by the same pathway
As reported previously (Selcher et al., 2003), the MEK inhibitor U0126 produced a partial suppression of E-LTP elicited with theta bursts (Fig. 4*A*). The same drug application and acute theta-burst protocol was used here as in Figure 2, *D* and *E*. The inhibition of E-LTP by U0126 (Fig. 4*A*) was strikingly similar to that produced by STO-609 (Fig. 2*D*), consistent with the data in Figure 3 demonstrating that ERK activation during LTP is mediated by CaMKK. If this model is correct and the role of CaMKK in LTP induction is upstream of ERK, then the effect of U0126 should be occluded by STO-609 and visa versa. As shown in Figure 4*B*, combined bath treatment with U0126 plus STO-609 was no more effective at blocking E-LTP than treatment

with STO-609 alone. In these experiments, U0126 completely blocked ERK activation at both 5 and 60 min (Fig. 4C). The specificity of U0126 was confirmed in that it did not inhibit activation of CaMKI, AKT, CaMKII, or Ras-GRF1 (Fig. 4D). These data demonstrate that the role of CaMKK in E-LTP is mediated by an ERK-dependent mechanism.

Role of CaMKK in translational activation during LTP

A recent study (Kelleher et al., 2004) shows that protein synthesis-dependent L-LTP induced by four trains of HFS is partially suppressed in mice expressing a dominant-negative MEK1 (the upstream activator of ERK) in region CA1. In fact, the degree and kinetics of L-LTP suppression in the dnMEK1 mice was equivalent to suppression of L-LTP in wild-type mice by the mRNA translation inhibitor anisomycin. These authors did not observe any decrement in E-LTP induced by two trains of HFS in the dnMEK1 mice, but the decrement of L-LTP (induced by four trains of HFS) developed within minutes in both dnMEK1 and anisomycin-treated mice. This is consistent with our results because E-LTP induced by two trains of HFS in mouse is not sensitive to the MEK inhibitors U0126 or SL327 (Selcher et al., 2003). Therefore, we tested whether E-LTP induced with our acute theta-burst protocol was dependent on protein synthesis using anisomycin. Not only did anisomycin produce a partial suppression of this E-LTP, but also both the magnitude and kinetics of E-LTP blockade with anisomycin (Fig. 5A) were nearly identical to those of U0126 (Fig. 5B) and STO-609 (Fig. 5C), indicating that the CaMKK/ERK pathway in E-LTP may exert its effects primarily through a translation-dependent mechanism. Furthermore, anisomycin treatment did not significantly affect the dendritic field responses (area under the curve) during theta-bursts (data not shown), as was the case with STO-609 and U0126 (Fig. 2C). Our observation that E-LTP generated by theta-burst stimulation are equally sensitive to STO-609 (Fig. 2D) and anisomycin (Fig. 5A) suggest that protein synthesis during theta-burst E-LTP is regulated by CaMKK and is required for concurrent CaMKK-dependent LTP expression. Indeed, treatment of slices with STO-609 was able to occlude further inhibition of E-LTP by U0126 (Fig. 5D).

Regulation of general mRNA translation by ERK during LTP may be related to phosphorylation of several translation fac-
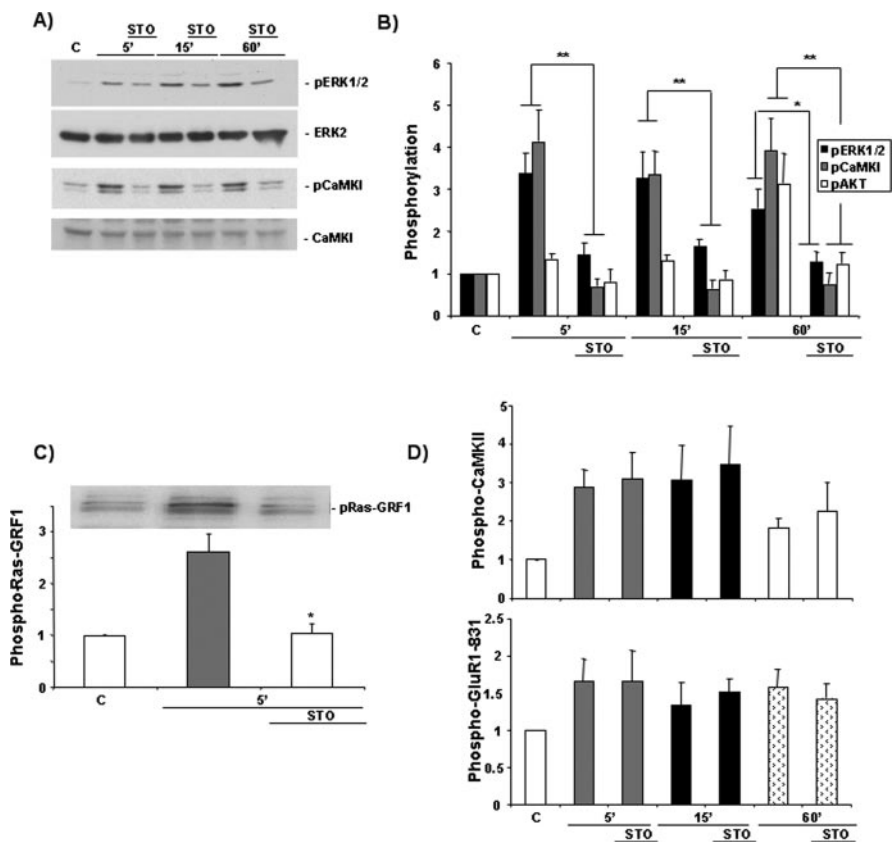

Figure 3. LTP-activation of CaMKI, Ras-GRF1, and ERK requires CaMKK. Mouse hippocampal slices were preincubated without or with STO-609 (STO; 5 μM, 30 min) and subjected to theta-burst stimulation as in Figure 2D. The activation states, assessed by phospho-specific antibodies (see Fig. 1), of ERK1/2, CaMKI, and AKT (A, B); Ras-GRF1 (C); and CaMKII and the GluR1 subunit of the AMPA-type glutamate receptor (a CaMKII substrate) (D) were determined at the indicated times. Mean $\pm$ SE; $n = 6$. *$p \leq 0.05$; **$p \leq 0.01$. C, Control.
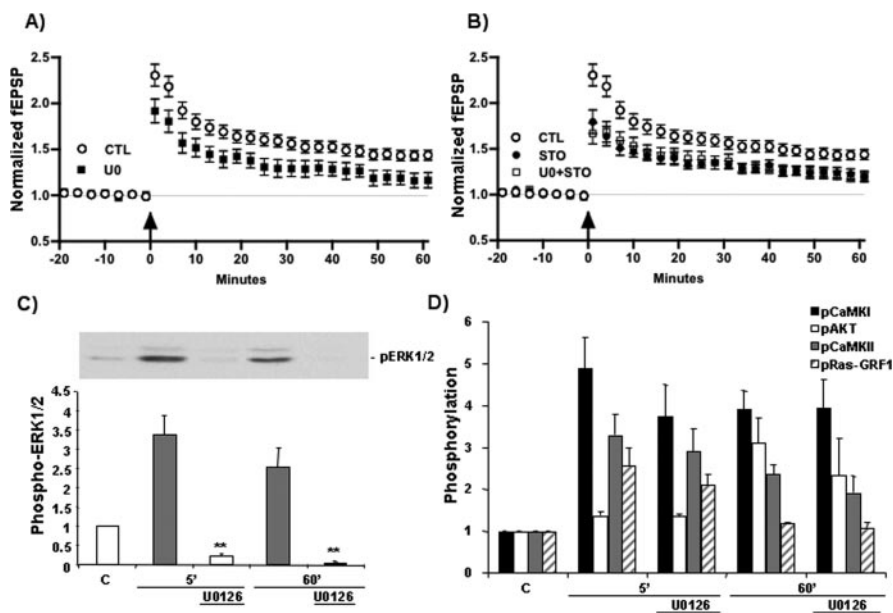

Figure 4. CaMKK and ERK mediate E-LTP via a common mechanism. A, B, Mouse hippocampal slices were treated without or with the MEK inhibitor U0126 (UO; 10 μM) and/or the CaMKK inhibitor STO-609 (STO; 5 μM) by bath application as in Figure 2D for 30 min before and 5 min after E-LTP induction. Control (CTL), $n = 24$; U0126, $n = 10$; STO-609, $n = 11$; U0126 plus STO-609, $n = 10$. C, D, At various times after E-LTP induction (as in Fig. 2D), the activation status of ERK (C) and CaMKI, AKT, CaMKII, and Ras-GRF1 (D) was determined at the indicated times. Mean $\pm$ SE; $n = 5$. **$p \leq 0.01$.
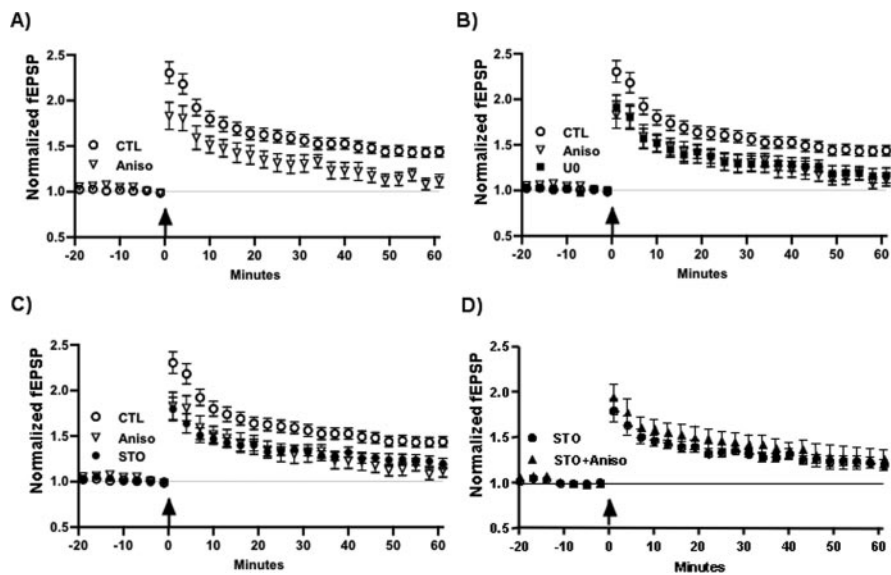
Figure 5. Inhibitors of CaMKK and MEK suppress LTP by a translation-dependent pathway. *A*, Mouse hippocampal slices were treated by bath application with the translation inhibitor anisomycin (Aniso; 40 μM) 30 min before and for the duration of recording after E-LTP (as in Fig. 2*D*). *n* = 8. Note that the magnitude and kinetics of anisomycin suppression of LTP are identical to those of U0126 (U0; *B*) and STO-609 (STO; *C*) and are occluded by STO-609 (*D*). CTL, Control.
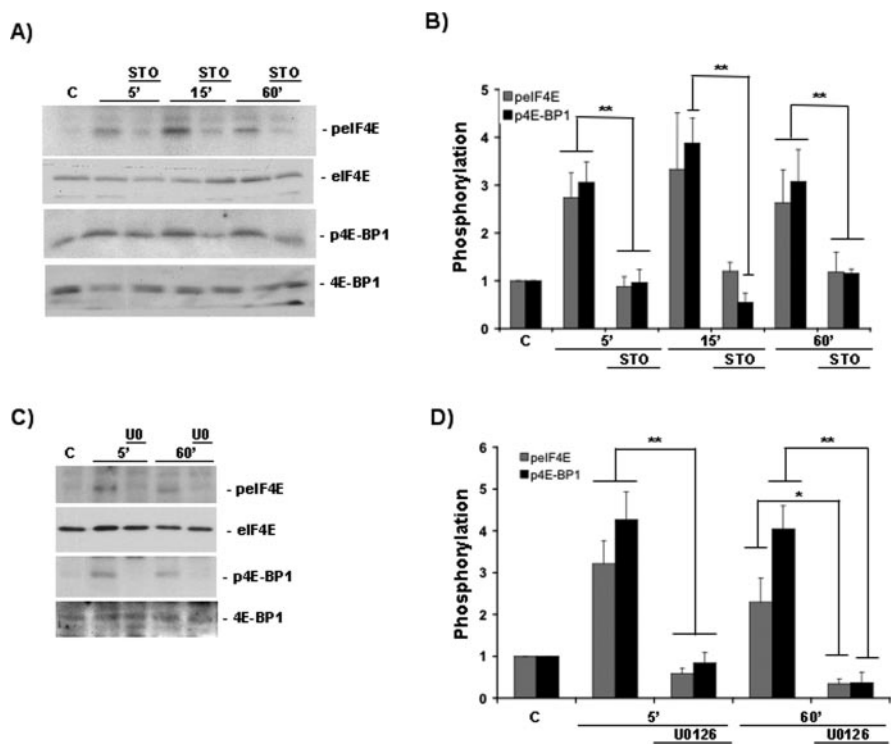

Figure 6. Translation factor activation in E-LTP requires CaMKK and ERK. Mouse hippocampal slices were preincubated with the indicated pharmacological reagents (see Fig. 1) and then subjected to E-LTP induction (as in Fig. 2*D*). The activation state of translation factors eIF4E and its inhibitory binding protein 4E-BP1 were determined using phospho-specific antibodies against their respective activation sites. Mean ± SE; *n* = 6 (*B*) or *n* = 5 (*D*). STO, STO-609; U0, U0126; C, Control. *p ≤ 0.05; **p ≤ 0.01.

tors, including eIF4E and its inhibitory binding protein 4E-BP1 (Kelleher et al., 2004). Induction of LTP with our acute theta-burst protocol produced a rapid (5 min) and sustained (60 min) phosphorylation of eIF4E and 4E-BP1 (Fig. 6*A–D*), both of which required CaMKK activity because STO-609, when present during LTP induction, was inhibitory at all time points examined. U0126 completely blocked phosphorylation of eIF4E and

4E-BP1 (Fig. 6*D*), highlighting the importance of eIF4E and 4E-BP1 in translation-dependent LTP. We conclude that regulatory eIF4E and 4E-BP1 phosphorylations are mediated by the CaMKK/ERK pathway in NMDA receptor-dependent theta-burst LTP.

Discussion

Ca^{2+} signaling and protein phosphorylation play essential roles in regulating the multiple mechanisms that produce hippocampal CA1 LTP, and recent studies have focused on the roles of CaMKII (Soderling et al., 2001; Lisman et al., 2002), CaMKIV (Kang et al., 2001; Kasahara et al., 2001), and ERK (Morozov et al., 2003; Selcher et al., 2003; Kelleher et al., 2004). Although ERK activation during LTP induction is Ca^{2+} dependent, the mechanisms responsible for this are unclear (Thomas and Huganir, 2004). Using cultured hippocampal neurons and acute slices, we reveal a robust Ca^{2+}-mediated activation of CaMKI by CaMKK that is required for both the activation of ERK and the full expression of NMDA-receptor dependent LTP. Additionally, our study suggests that CaMKK mediates mRNA translation, as measured by regulatory phosphorylation of several translation initiation factors, via ERK during LTP. Together, our data demonstrate that CaMKK and CaMKI are required for ERK activation, regulatory phosphorylation of two limiting general translation factors, and E-LTP at the Schaeffer–collateral CA1 synapse.

A role for CaMKI in E-LTP

Previous studies have examined the role of the CaMKK cascade in LTP, focusing on the role of CaMKIV in CREB/CBP-mediated gene transcription. LTP induction does result in activation of CaMKIV (Kasahara et al., 2001), but, because CaMKIV is predominantly nuclear, it is unlikely to play a major role in cytoplasmic ERK activation. Indeed, nuclear-localized dominant-negative CaMKIV has no effect on ERK activation in NG108 cells (Schmitt et al., 2004) or cultured hippocampal neurons (Fig. 1*B*). Furthermore, Ca^{2+}-dependent ERK activation in dnCaMKIV-expressing (Kang et al., 2001) and CaMKIV knock-out mice (Ho et al., 2000) was normal. Moreover, expression of dnCaMKIV in region CA1 of hippocampus does not inhibit E-LTP induced with theta burst (Kang et al., 2001), nor does knocking out the CaMKIV gene inhibit E-LTP induced by HFS (Ho et al., 2000). These findings support the conclusion that CaMKI, but not CaMKIV or AKT, mediates the CaMKK-dependent portion of LTP induction and E-LTP.

Although LTP induced with theta bursts was sensitive to U0126 or STO-609, it was not completely inhibited during the first hour (~50% inhibition) (Fig. 2E,F). This observation is consistent with the well established role in LTP of CaMKII to phosphorylate the AMPA receptor subunit GluR1, thereby enhancing its conductance (Derkach et al., 1999). This mechanism is thought to mediate ~50% of the change in synaptic efficacy that occurs during E-LTP (Benke et al., 1998; Poncer et al., 2002). We found that neither the activation of CaMKII nor the phosphorylation of GluR1 at Ser[831] were affected by STO-609 during LTP, demonstrating both the specificity of STO-609 and that CaMKK and ERK effects were independent of CaMKII actions. We interpret these findings as evidence that the CaMKK/ERK and CaMKII pathways mediate E-LTP by distinct mechanisms. However, STO-609 did completely suppress L-LTP after ~2 h, consistent with a dual role for CaMKK in regulation of CaMKI (E-LTP) and CaMKIV (L-LTP). This is consistent with the report that a mouse lacking the β isoform of CaMKK, which is thought to be predominantly nuclear and to regulate CaMKIV, showed no deficit in E-LTP but lacked L-LTP (Peters et al., 2003)

CaMKI has been shown previously to promote hippocampal axon outgrowth and growth cone dynamics (Wayman et al., 2004), structural components of plasticity. In this study, we find that CaMKI is robustly activated by CaMKK after induction of E-LTP with theta bursts. This stimulation of the CaMKK/CaMKI pathway was required for ERK activation, providing a compelling mechanism for Ca^{2+}-mediated ERK activation during LTP. Accordingly, the effects of pharmacological inhibition of CaMKK and ERK (with STO-609 and U0126, respectively) on E-LTP are mutually occlusive, indicating that these kinases share the same pathway. We chose theta burst rather than HFS to induce E-LTP because it has been shown in mice that U0126 does not block E-LTP resulting from HFS (two trains of 100 Hz) but is partially inhibited using theta-burst induction (Selcher et al., 2003). However, we should point out that we used lower stimulation intensity, as well as older animals, than used by Selcher et al. to demonstrate a role for ERK in temporal integration during LTP induction. These differences may help explain why inhibition of the ERK pathway did not significantly reduce the integrated field responses during E-LTP induction in our study. It is interesting that induction of LTP by both of our theta-burst protocols was partially suppressed by STO-609 (Fig. 2F) and induction of L-LTP by HFS (4 trains of 100 Hz, 5 min apart) was partially suppressed by anisomysin (Kelleher et al., 2004), even during the E-LTP phase (first 60 min). These results suggest that the CaMKK, ERK, and protein synthesis-dependent processes can affect LTP within minutes depending on the induction protocol.

In addition to CaMKII-mediated phosphorylation of AMPA-Rs to enhance conductivity (Derkach et al., 1999), recruitment of additional AMPA-Rs to synapses is thought to be a second major postsynaptic mechanism of E-LTP expression at the CA1 synapse (Malinow and Malenka, 2002). One possible explanation for the rapid deficit of LTP after STO-609, U0126, or anisomycin treatment could be that synaptic delivery of AMPA receptors may be coupled to translation. Indeed, it has been shown that the Ras/ERK pathway is required for this rapid trafficking of AMPA-Rs (Zhu et al., 2002). AMPA-R trafficking is also dependent on CaMK activity, with current evidence supporting a role for CaMKII (Poncer et al., 2002). However, because recent work indicates that CaMKII probably has either an inhibitory effect on ERK (Oh et al., 2004) or no effect (Krapivinsky et al., 2004), we propose that the requirement for a CaMK in AMPA-R trafficking may be attributable to CaMKK/CaMKI activation of ERK.

ERK regulation in synaptic plasticity
A role for ERK1/2 in NMDA receptor-dependent hippocampal LTP is well established (Sweatt, 2004; Thomas and Huganir, 2004), and a number of mechanisms for how Ca^{2+} may activate ERK have been proposed, including roles for Pyk2 and Src, CalDAG-GEFs (calcium- and diacylglycerol-regulated guanine nucleotide exchange factors), EGF (epidermal growth factor) receptor transactivation, and Ras-GRF1/2. The data presented here demonstrate that NMDA receptor-dependent ERK activation during E-LTP requires CaMKK acting through CaMKI. Other components of NMDA receptor-dependent ERK activation include Ras (Thomas and Huganir, 2004) and its activator Ras-GRF1 that directly interacts with the NMDA-receptor and is required for ERK activation (Krapivinsky et al., 2003; Tian et al., 2004). Ras-GRF is regulated through phosphorylation of Ser[916] by several kinases, including PKA and CaMKII, and this phosphorylation is required for full activation of Ras and for neurite outgrowth (Mattingly, 1999; Baouz et al., 2001; Yang et al., 2003). We find that Ser[916] is rapidly phosphorylated in response to NMDA treatment and theta-burst stimulation in a CaMKK-dependent manner. It should be noted that Ras-GRF1 contains multiple phosphorylation sites (Baouz et al., 2001), and activation appears complex and may be regulated by the binding of cofactor(s) such as CaM and/or by its subcellular localization (Buchsbaum et al., 1996; Arozarena et al., 2004). In this context, Ras-GRF1 is a very poor *in vitro* substrate for activated CaMKI relative to PKA (T. Saneyoshi, unpublished observation). The precise mechanism for CaMKK-mediated activation of the Ras pathway remains an area of investigative interest.

Regulation of translational activation by CaMKK and ERK
ERK activation has been implicated in regulating multiple aspects of LTP, including gene transcription (Thomas and Huganir, 2004), AMPA-R trafficking (Zhu et al., 2002), dendritic excitability (Morozov et al., 2003), and translation factor phosphorylation (Kelleher et al., 2004). The data presented here are consistent with a role of ERK in regulation of mRNA translation via phosphorylation of both eIF4E and 4E-BP1 (Fig. 6). Phosphorylation of 4E-BP1 frees up additional eIF4E, a limiting translation factor, and phosphorylation of eIF4E enhances its functionality (Raught et al., 2000). Moreover, the partial inhibition of E-LTP by the protein synthesis inhibitor anisomycin was identical to inhibition by U0126 and STO-609 and occluded by STO-609, implying that CaMKK and ERK regulate E-LTP by a translation-dependent mechanism.

There are several reports documenting inhibition of NMDA receptor-dependent LTP by anisomycin and other translation inhibitors during the early phase in hippocampus and amygdala (Huang et al., 2000; Kelleher et al., 2004). The partial inhibition of E-LTP by anisomycin in our acute theta-burst protocol was very rapid (Fig. 5), but it should be noted that translation of a typical 50 kDa protein, at a rate of seven amino acids per second, would only require ~1 min. Indeed, a significant increase in dendritic CaMKII, attributable to localized synthesis, was observed within 5 min of LTP induction (Ouyang et al., 1999). Additionally, anisomycin was present for 30 min before LTP induction, and, although it had little effect on basal synaptic transmission, it could suppress basal synthesis of some component necessary for the rapid insertion or modulation of AMPA-Rs during induction (Malinow and Malenka, 2002). Alternatively, it is possible that

translation and postsynaptic exocytosis are coupled through the secretory pathway and that inhibiting one process may affect the other. Furthermore, it is now recognized that protein synthesis from mRNAs localized within dendrites and even dendritic spines is essential for synaptic plasticity, so the possibility that protein synthesis may have acute regulatory effects has to be considered. CaMKK, CaMKI (Wayman et al., 2004), ERK (Wu et al., 2001b), and eIF4E (Asaki et al., 2003; Smart et al., 2003) are all localized in dendrites, as is the mRNA for the GluR1 subunit of the AMPA-R (Job and Eberwine, 2001). Thus, it will be important to determine whether the CaMKK/CaMKI pathway regulates a pool of ERK that in turn stimulates AMPA-R synthesis and/or trafficking to synapses.

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