

2004

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Calcium Activation of ERK Mediated by Calmodulin Kinase I*

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Elevated intracellular Ca^{2+} triggers numerous signaling pathways including protein kinases such as the calmodulin-dependent kinases (CaMKs) and the extracellular signal-regulated kinases (ERKs). In the present study we examined Ca^{2+} -dependent "cross-talk" between these two protein kinase families. Using a combination of pharmacological inhibitors and dominant-negative kinases (dnKinase), we identified a requirement for CaMKK acting through CaMKI in the stimulation of ERKs upon depolarization of the neuroblastoma cell line, NG108. Depolarization stimulated prolonged ERK and JNK activation that was blocked by the CaMKK inhibitor, STO-609; this inhibition of ERK activation by STO-609 was rescued by expression of a STO-609-insensitive mutant of CaMKK. However, activation of ERK by epidermal growth factor or carbachol were not suppressed by inhibition of CaMKK, indicating specificity for this "cross-talk." To identify the downstream target of CaMKK that mediated ERK activation upon depolarization, dnKinases were expressed. The dnCaMKI completely suppressed ERK2 activation whereas dnAKT/PKB or nuclear-targeted dnCaMKIV, other substrates for CaMKK, were not inhibitory. ERK activation upon depolarization or transfection with constitutively active (ca) CaMKI was blocked by dnRas. Additionally, depolarization of NG108 cells promoted neurite outgrowth, and this effect was blocked by inhibition of either CaMKK (STO-609) or ERK (UO126). Co-transfection with caCaMKK plus caCaMKI also stimulated neurite outgrowth that was blocked by inhibition of ERK (UO126). These data are the first to suggest that ERK activation and neurite outgrowth in response to depolarization are mediated by CaMKK activation of CaMKI.

One of the most ubiquitous cellular signaling mechanisms is the extracellular signal-regulated kinase (ERK)¹ pathway. ERKs belong to the MAP kinase family of which ERK1/2 is

most closely related to the c-Jun N-terminal kinase (JNK) and the stress-activated kinase, p38 (1). ERK activation plays a role, largely through regulation of gene transcription, in a number of cellular processes including cellular proliferation, DNA synthesis, differentiation, and cellular survival (2–6). In neurons, ERKs also regulate neurite outgrowth, dendritic morphology, and are required for synaptic plasticity in long-term potentiation and in temporal and spatial memory (7–14).

The ERK family is activated by a myriad of extracellular ligands including hormones, neurotransmitters, and growth factors acting through G protein-coupled receptors, tyrosine kinase receptors, and ligand- or voltage-gated ion channels (15, 16). These membrane receptors and channels stimulate the Ras/Rap family of small G proteins that in turn trigger a complex cascade of protein kinases terminating in activation of the MAP kinase family including the ERKs (17–20). The response of the ERK pathway to various stimuli can be cell type-specific and/or -dependent on regulation of different subcellular pools of small G proteins (17, 18, 21). Some of these pathways are mediated in part through elevation of intracellular calcium, which can play an important role in ERK activation, especially in neurons. For example, KCl depolarization of hippocampal neurons activates ERK, and this is required for regulation of gene transcription (5, 22) and for activity-dependent dendritic plasticity and filopodial formation (8).

Several mechanisms for calcium activation of ERK have been reported including pathways through PYK2, EGF receptor transactivation, RasGRF1, and CalDAG-GEFs (23, 24). The family of CaM kinases (Ks), which are activated by stimuli that elevate intracellular calcium, have also been proposed to modulate ERK activation (8, 23). For example, *in vitro* CaMKII can activate SynGAP (25), a Ras inhibitory GTPase expressed in neurons (26), which may inhibit ERK activation, although a regulatory role for CaMKII in the SynGAP pathway has not been demonstrated in cells. In fact, ERK activation by *N*-methyl-D-aspartate receptor stimulation is normal in SynGAP heterozygous knockout mice (27). In thyroid cancer cells, CaMKII may associate with Raf-1 and phosphorylate it downstream of integrin signaling, thereby contributing to ERK activation (28).

In addition to CaMKII, members of the CaMK cascade may also be involved in ERK activation. The CaMK cascade consists of CaMKK and its downstream substrates CaMKI, CaMKIV, and AKT/PKB (29, 30). CaMKI is predominantly cytoplasmic, but its physiological functions are largely undefined (31). CaMKIV is predominantly nuclear, where it phosphorylates transcription factors and regulates gene transcription (32, 33). Glutamate and KCl depolarization persistently activate CaMKI in hippocampal neurons whereas CaMKIV activation is transient (34). AKT/PKB is activated by CaMKK, and this pathway protects NG108 cells from apoptosis (35). Expression of constitutively active (ca) CaMKK or CaMKIV has been proposed to activate ERK and JNK in PC12 cells (36). However,

* This work was supported in part by National Institutes of Health R01 Grants GM 41292 (to T. R. S.) and DK44239 (to T. R. S.) and Training Grant DK007680 (to J. M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: ERK, extracellular signal-regulated kinase; AKT/PKB, protein kinase B; CaM, calmodulin; ca, constitutively active; dn, dominant-negative; CaMK, CaM kinase; JNK, c-Jun N-terminal kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; Ant, antennapedia; PKA, cAMP-dependent protein kinase; CREB, cAMP response element-binding protein; MAP, mitogen-activated protein.

overexpressed constitutively active protein kinases may catalyze non-physiological responses because of their high concentrations, lack of appropriate subcellular localization, and degeneracy of substrate specificity.

It was recently reported that neither CaMKII nor CaMKIV are sufficient to activate ERK-dependent transcription in NG108 cells (37). Interestingly, expression of either nuclear or cytoplasmic CaMKII is unable to activate ERK, and nuclear CaMKII inhibited neurite outgrowth in PC12 cells (38). Thus, the requirement for specific CaMK family members that may mediate calcium effects on ERK remains to be elucidated. Therefore, we have carefully evaluated the roles of CaMKs in ERK activation using more specific molecular and pharmacological inhibitors to determine the physiologic significance of this pathway in neurite outgrowth upon cellular depolarization.

EXPERIMENTAL PROCEDURES

Materials—Epidermal growth factor (EGF), potassium chloride (KCl), phalloidin, and carbachol were purchased from Sigma. Hoechst 33342 solution was purchased from Molecular Probes (Eugene, OR). KN-93, KN-92, H89, ionomycin, and UO126 were purchased from Calbiochem (Riverside, CA). STO-609 was purchased from Tocris Cookson, Inc. (Ellisville, MO). Antibodies specific to phosphorylated and activated ERK (pERK1/2) that bind phosphorylated ERK1 and ERK2 at residues threonine 202 and tyrosine 204 were purchased from Cell Signaling (Beverly, MA). Phosphorylation-specific antibodies to activated AKT (pAKT) that bind phosphorylated AKT at threonine 308 were purchased from Cell Signaling. Antibodies specific to phosphorylated (threonine 286) and activated CaM kinase II (pCaMKII) were purchased from Affinity Bioreagents (Golden, CO). Antibodies specific to phosphorylated and activated JNK (pJNK, threonine 183/tyrosine 185) and p38 (phospho-p38, threonine 180/tyrosine 182) were also purchased from Cell Signaling. Monoclonal antibodies specific to phosphorylated and activated CaMKI (pCaMKI, threonine 178) were generated from immunized mice. Antibodies to ERK2 were purchased from Santa Cruz Biotechnology Inc. Antibodies to CaMKK, CaMKIV, and CaMKII were purchased from Transduction Laboratories (San Jose, CA). Antibodies to CaMKI were generated from immunized rabbits and provided by Dr. Kohji Fukunaga (Sendai, Japan). Agarose-conjugated antibodies to FLAG (M2) and FLAG (M2) antibodies were purchased from Sigma.

Cell Culture and Treatments—The hybrid neuroblastoma cell line, NG108, was obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) plus 1% HT selection supplement (Invitrogen), 10% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37 °C in 5% CO₂. NG108 cells were serum-starved overnight in DMEM at 37 °C in 5% CO₂ prior to stimulation with the indicated reagents for Western blotting or immunoprecipitation. Cells were pretreated with the inhibitors KN-93 (5 μM), KN-92 (5 μM), UO126 (10 μM), H89 (10 μM), and antennapedia (Ant)-CaM KINtide (5 μM) for 30 min prior to depolarization, unless otherwise indicated. Cells were pretreated with STO-609 (2.6 μM = 1 μg/ml) for 60 min prior to stimulation, unless otherwise indicated. Where indicated, cells were stimulated with ionomycin (500 nM), EGF (100 ng/ml), and carbachol (10 μM) for the indicated times. Cells were stimulated with KCl (60 mM) to elevate intracellular calcium by isotonic depolarization for the indicated times. Sterile-filtered 60 mM isotonic medium consisted of 70 mM NaCl, 60 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 25 mM HEPES-HCl, and 30 mM D-glucose with an osmolality of ~330.

To measure neurite outgrowth, NG108 cells were seeded at low density (~5,000 cells/well) on cover slips in 24-well plates with DMEM plus 1% HT selection supplement, 10% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37 °C in 5% CO₂. After the cells adhered to the plates, the cells were placed in DMEM plus 1% HT selection supplement, 1% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37 °C in 5% CO₂, and then treated with KCl (60 mM) for 5 days to induce differentiation as previously reported (12, 39, 40). NG108 cells were pretreated with STO-609 (1 μg/ml) or UO126 (10 μM) for 1 h and 30 min, respectively, prior to and for the duration of depolarization with KCl. NG108 cells were fixed and analyzed for neurite outgrowth as indicated under "Immunocytochemistry" below.

To measure neurite outgrowth in transfected cells, NG108 cells were seeded on cover slips in 24-well plates with DMEM plus 1% HT selection supplement, 1% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37 °C in 5% CO₂. 40–50% confluent cells were co-trans-

fected with EGFP, along with the control vector pcDNA3, or constitutively active CaMKK and KI-tagged with EGFP using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's guidelines. Each plate received the same amount of DNA. Following transfection, cells were maintained in DMEM plus 1% HT selection supplement, 1% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37 °C in 5% CO₂ for 48 h. Cells were treated with UO126 (10 μM) for the duration of the experiment. NG108 cells were fixed, and GFP-positive cells were analyzed for neurite outgrowth by confocal microscopy.

Western Blotting and Immunoprecipitation—Following NG108 cell stimulations, medium was aspirated, and equivalent amounts of ice-cold lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM MgCl₂) plus freshly added inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 1 mM sodium orthovanadate) were added to each plate of cells. On ice, plates were scraped and cellular proteins placed in ice-cold microcentrifuge tubes. Briefly, cell lysates were spun at 8,000 for 5 min at 4 °C to pellet the cytoskeleton and nuclei. Equivalent amounts of supernatants from each tube were then quantified by the Bradford protein assay on a 96-well plate, and standards and sample protein concentrations were read by a microplate reader (μQuant, BIO-TEK INSTRUMENTS, Inc.). Equivalent amounts of protein were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and examined by Western blotting with the indicated antibodies. Unless otherwise indicated, Western blotting for total ERK2, CaMKII, and CaMKI served as additional loading controls.

Equal amounts of cell lysates per treatment condition were used for immunoprecipitations of FLAG-ERK2. Immunoprecipitations were carried out at 4 °C for 4 h in ice-cold lysis buffer. Precipitated proteins were washed 2× with lysis buffer, resolved by SDS-PAGE, and Western-blotted for phosphorylated FLAG-ERK2 (pFLAG-ERK2). FLAG Western blotting for FLAG-ERK2 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 (New Haven, CT). Band densities were normalized to untreated controls and then to the loading control and presented as fold phosphorylation as indicated.

Statistics—To determine whether significant differences existed among treatments, an analysis of variance 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: a single asterisk indicates *p* ≤ 0.05 and a double asterisk indicates *p* ≤ 0.01.

Immunocytochemistry—NG108 cells were fixed in 4% paraformaldehyde, 4% sucrose, phosphate-buffered saline (PBS), and 50 mM HEPES pH 7.5. at 37 °C for 15 min. Cells were then washed three times for 5 min in PBS and permeabilized with 0.2% Triton X-100 in PBS for 15 min. Cells were then washed three times for 5 min in PBS, stained with phalloidin (1:1,000) and Hoechst 33342 (1:10,000) in PBS for 20 min at room temperature, and washed three times for 5 min in PBS. Coverslips were then mounted on glass slides and analyzed by either fluorescence or confocal microscopy.

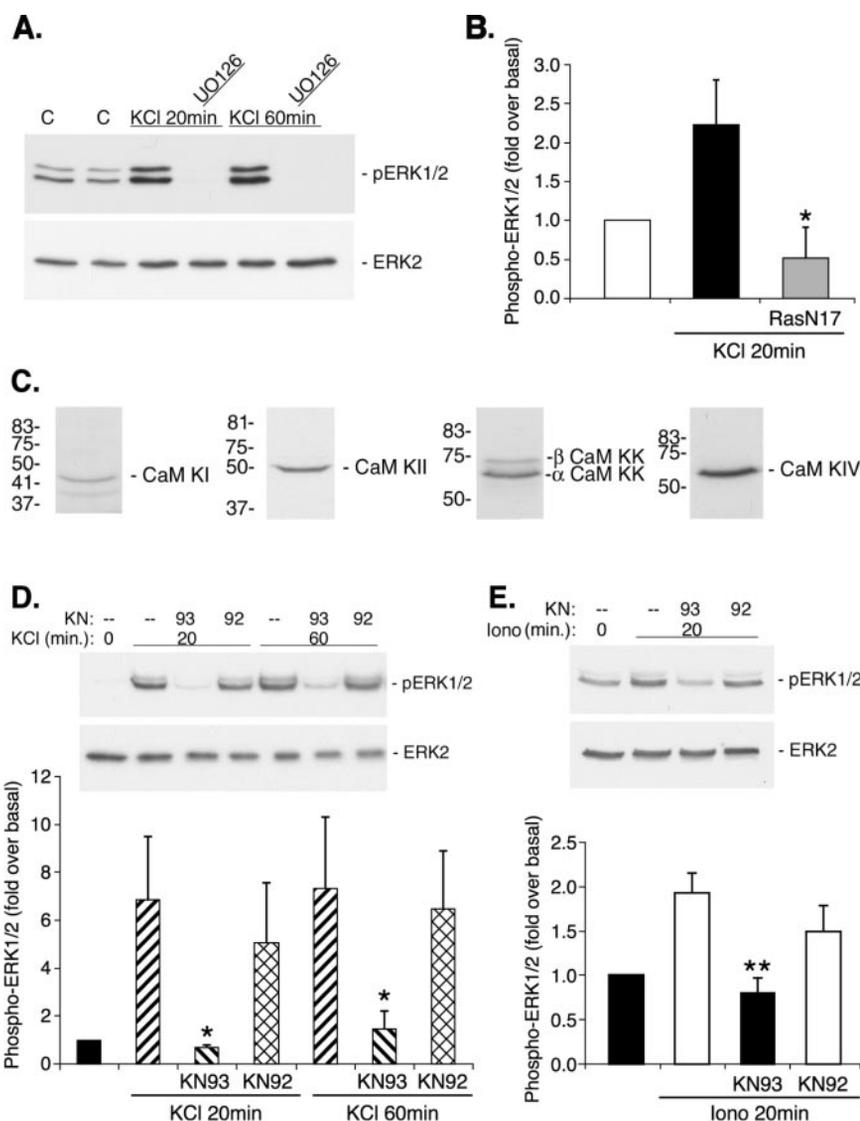
Plasmids and Transfections—The caCaMKK has previously been published (35). The caCaMKI (IHQS286EDDD, F307A), EGFP-tagged caCaMKI, and caCaMKIV (R136A, HMDT305DEDD) with a nuclear localization signal were generated in the Soderling laboratory. EGFP-tagged caCaMKI was constructed using the pEGFP vector (Clontech). The dnCaMKK (K71A, T108A, S458A), dnCaMKI (K49E, T177A, IHQS286EDDD, F307A), and dnCaMKIV nuclear (T196A, K71E, HMDT305DEDD) plasmids were all generated in the Soderling laboratory. The CaMKKαL233F plasmid was provided by Dr. Hiroshi Tokumitsu (Kagawa Medical University, Kagawa, Japan). The FLAG-ERK2 and RasN17 plasmids were provided by Dr. Philip Stork (Vollum Institute, Portland, OR). 70–80% confluent cells were co-transfected with FLAG-ERK2, along with the control vector pcDNA3, or the indicated plasmids using LipofectAMINE 2000 according to the manufacturer's guidelines. Each plate received the same amount of DNA, and following transfection cells were allowed to recover in complete medium for 28 h. Cells were then serum-starved overnight, treated as indicated, and lysed in ice-cold lysis buffer. FLAG-ERK2 was then immunoprecipitated and examined by Western blot for activation (pFLAG-ERK2).

RESULTS

Ca²⁺-dependent ERK Activation in NG108 Neuroblastoma Cells—To investigate the potential role of CaMKs in modulat-

FIG. 1. Activation of ERK by depolarization requires Ras, MEK, and CaM kinases.

A. ERK activation requires MEK. NG108 cells received no pretreatment or were pretreated (20 min) with UO126 (10 μ M) and then stimulated with KCl (60 mM) for the indicated times. Endogenous ERK activation was measured in cell lysates (see "Experimental Procedures") by Western blotting for phosphorylated-ERK1/ERK2, p44, and p42, respectively (pERK1/2). The lower panel is a Western blot of total ERK2. **B.** ERK activation requires Ras. NG108 cells were co-transfected with FLAG-ERK2 along with the control vector pcDNA3 or RasN17. Cells were stimulated with KCl for 20 min, and FLAG-ERK2 phosphorylation was analyzed (see "Experimental Procedures," $n = 4, \pm$ S.E.; *, $p \leq 0.05$). **C.** expression profile of endogenous CaM kinases in NG108 cells. Endogenous CaMKI, CaMKII, CaMKK, and CaMKIV proteins were examined in cell lysates by Western blotting for the indicated proteins. **D.** ERK activation is blocked by the general CaMK inhibitor KN-93 but not the inactive analog KN-92. NG108 cells, with or without KN-93 (5 μ M) or KN-92 (5 μ M) pretreatment for 30 min, were stimulated with KCl for the indicated times. Endogenous ERK activation was measured by Western blotting as in A, and the density of the bands were determined, normalized for total ERK2, and quantified for fold-stimulation relative to the control in the absence of KCl ($n = 4, \pm$ S.E.). **E.** ERK activation by ionomycin is CaM kinase-dependent. NG108 cells received no pretreatment or were pretreated with KN-93 or KN-92 as in D, stimulated with ionomycin (500 nM) for 20 min and endogenous ERK activation was quantified as in D ($n = 3, \pm$ S.D.; **, $p \leq 0.01$).



ing Ca^{2+} -dependent ERK activation, we selected the NG108 neuroblastoma cell line that can be stimulated by depolarization to raise intracellular calcium levels (41, 42). Depolarization of neuronal cells is known to produce activation of CaMKII (43) and CaMKK (35) as well as ERK (5, 44). Several different stimulation paradigms that involve activation of ERK (45, 46) or CaMKs (47) regulate neurite outgrowth of NG108 cells, thereby providing a useful biochemical and physiological indicator of potential "cross-talk" between CaMKs and ERK.

Depolarization of NG108 cells with KCl resulted in robust ERK activation that was evident within 1 min (Fig. 2A) and maintained at both 20 and 60 min (Fig. 1A). In general, we have focused on the sustained activation of ERK (20 and 60 min.) rather than its rapid stimulation (1–5 min) because sustained activation appears to be important for physiological phenomena such as gene transcription and neurite outgrowth (5, 8, 48, 49). As expected, inhibition of MEK, the upstream activator of ERK, by UO126 (50) completely blocked ERK activation (Fig. 1A), as did transfection with the dominant-negative Ras, RasN17 (co-transfected with FLAG-tagged ERK2, Fig. 1B). In some cell types, elevation of intracellular calcium activates adenylyl cyclase to elevate cAMP, and depending on the cell type, cAMP can activate ERK through PKA (20). However, pretreatment of NG108 cells with the PKA inhibitor H89 (10 μ M) did not block ERK activation in NG108 cells but did inhibit ERK activation in hippocampal neurons (data not

shown). Thus, depolarization of NG108 cells activates ERK through a signaling cascade involving Ras and MEK but not PKA.

CaMKII Does Not Mediate ERK Activation—NG108 cells contain endogenous CaMKI, CaMKII, and CaMKIV as well as CaMKK (Fig. 1C), the upstream activating kinase for CaMKI and CaMKIV. Therefore, NG108 cells are a useful tool for examining which CaMKs may mediate ERK activation by depolarization. The potential role of CaMKs in depolarization-dependent ERK activation was initially investigated using a general pharmacological CaMK inhibitor, KN-93. KN-93 (and KN-62) are known to inhibit binding of Ca^{2+} /CaM to CaMKs, thereby blocking their activation (51). Depolarization of NG108 cells resulted in an ~4–5-fold increase in ERK activation at 20 and 60 min that was significantly blocked by KN-93 but not by its inactive analog KN-92 (Fig. 1D). The ineffectiveness of the inactive analog KN-92 suggests that KN-93 inhibition may be specific for the CaMK family. To assure that KN-93 was not acting indirectly (e.g. to inhibit voltage-dependent Ca^{2+} channels), we used ionomycin to elevate Ca^{2+} and activate ERK, and this was also significantly blocked by KN-93 but not KN-92 (Fig. 1E). Taken together, these data demonstrate that ERK activation requires CaMKs in response to elevation of intracellular calcium.

Previous studies have confirmed that depolarization of NG108 cells results in activation of the two major classes of

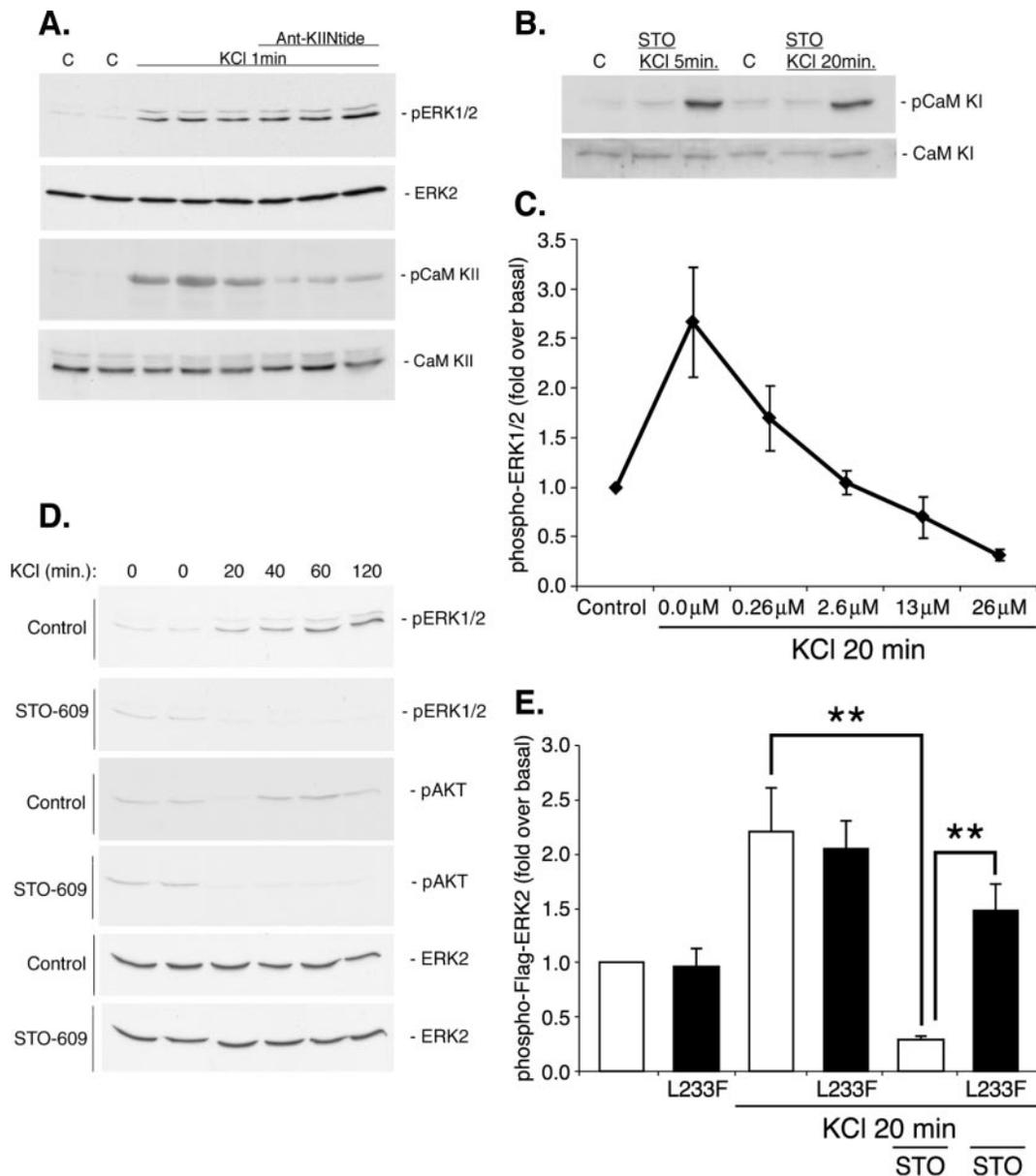


FIG. 2. ERK activation requires CaMKK. *A*, ERK activation by depolarization is CaMKII-independent. NG108 cells received no pretreatment or were incubated for 30 min with the cell-permeable CaMKII inhibitor Ant-KIINtide (5 μ M), stimulated with KCl for 1 min, and endogenous ERK activation (pERK1/2, *panel 1*) or CaMKII (pCaMKII, *panel 3*) were measured. The *second* and *fourth* panels are loading controls showing total ERK2 and CaMKII, respectively. *B* and *C*, ERK activation requires CaMKK. NG108 cells, without or with pretreatment for 60 min with STO-609 (2.6 μ M or as indicated), were depolarized for the indicated times. Endogenous CaMKI activation by CaMKK (*B*) was measured by Western blotting for phosphorylated CaMKI (pCaMKI). *C* illustrates the dose-response of inhibition by STO-609 for endogenous ERK activation upon depolarization as measured in Fig. 1 ($n = 3$, \pm S.D.; 26 μ M, $n = 6$, \pm S.E.). *D*, time course of ERK activation. NG108 cells, without or with pretreatment with STO-609 (2.6 μ M, 60 min), were depolarized for the indicated times, and activation of endogenous ERK and AKT (another CaMKK substrate) were measured (pERK1/2 and pAKT, respectively). The two *bottom* panels are Western blots of ERK2, demonstrating equal loading of proteins. *E*, CaMKK_{L233F} reverses STO-609 inhibition of ERK activation. NG108 cells were co-transfected with FLAG-ERK2 along with the control vector pcDNA3 or the CaMKK_{L233F} (STO-609 insensitive mutant) plasmid and pretreated with STO-609 as indicated. Cells were then stimulated with KCl for 20 min, and FLAG-ERK2 phosphorylation was analyzed ($n = 7$, \pm S.E.).

general CaMKs, CaMKII (52) and CaMKK (35). CaMKII is the best known multifunctional CaMK that mediates many cellular responses from Ca^{2+} /CaM (43, 53). Therefore, we wanted to specifically block CaMKII to assess its role in mediating Ca^{2+} -dependent activation of ERK. CaMKII is rapidly activated within 1 min upon depolarization of NG108 cells (Fig. 2A, *lower panel*) and returns to basal levels by 5–10 min (data not shown). CaMKIIN is an endogenous brain protein that specifically inhibits CaMKII with an IC_{50} of 0.1 μ M whereas at 10 μ M it exhibits little or no inhibition of CaMKI, CaMKIV, CaMKK, PKA, or protein kinase C (54). CaM KIINtide, a 27-residue peptide derived from CaMKIIN, retains the potency and spec-

ificity of CaMKIIN, and we have also determined that *in vitro* it does not directly inhibit ERK (data not shown). CaM KIINtide can be made cell-permeable by attachment of an Ant sequence (55). Incubation of NG108 cells with 5 μ M Ant-CaM KIINtide effectively blocks activation of CaMKII upon depolarization, but has little effect on ERK activation at 1 min (Fig. 2A) or 20 min (data not shown). Ant-CaM KIINtide also had no effect on activation of CaMKI by CaMKK (data not shown). Taken together, these results suggest that ERK activation by calcium is CaMKII-independent in NG108 cells.

*CaMKK Is Required for ERK Activation by Depolarization—*CaMKK is the upstream activator of the two other multifunc-

tional CaMKs, CaMKI and CaMKIV (29, 30). Inhibition of CaMKK was achieved using a recently described inhibitor STO-609 (56). *In vitro* this compound has an IC_{50} of 0.26 μM (0.1 $\mu g/ml$) for CaMKK and 26 μM for CaMKII with little or no inhibition of CaMKI, CaMKIV, PKA, protein kinase C, or ERK. At a concentration of 2.6 μM it inhibits CaMKK but not CaMKII in cultured hippocampal neurons (57). CaMKK activates CaMKI, through phosphorylation of Thr-177 (58), and depolarization of NG108 cells for 5 and 20 min resulted in CaMKI phosphorylation at this site that was completely blocked by 2.6 μM STO-609 (Fig. 2B). These data confirm the efficacy of STO-609 to block CaMKK activity.

We next wanted to determine if inhibition of the CaMK cascade would effect ERK activation. Fig. 2C shows a STO-609 dose-response curve, and ERK activation was completely blocked by 2.6 μM , the same concentration that inhibited CaMKK activation of CaMKI. Interestingly, the inhibitory concentration of STO-609 on ERK activation by depolarization was nearly 0.26 μM , which is similar to the IC_{50} for CaMKK (56). Since STO-609 up to 26 μM has no effect on ERK *in vitro*, it is likely that STO-609 was blocking ERK activation by inhibiting CaMKK. The effect of 2.6 μM STO-609 was further characterized in Fig. 2D, which shows a time course of ERK activation out to 2 h (*top panel*); STO-609 completely blocked ERK activation at all time points examined (*second panel*). We have previously shown, both *in vitro* and in NG108 cells, that the protein kinase AKT/PKB is also a direct substrate of CaMKK (35). CaMKK activation of AKT/PKB upon depolarization was maximal at about 60 min, whereas activation of CaMKIV occurred within 1–5 min. Fig. 2D (*third panel*) confirms the slow activation of AKT upon depolarization, and this response was also suppressed by STO-609 (*fourth panel*) to below basal (*i.e.* no depolarization) levels.

With pharmacological compounds a concern is that the molecule modulates not only the protein of interest, but it may also have other effects. To confirm the specificity of STO-609 toward CaMKK in our system, we used a point mutant of CaMKK that is insensitive to STO-609, CaMKK_{L233F} (59). NG108 cells were transfected with FLAG-tagged ERK2 without or with co-transfection with CaMKK_{L233F}. In the absence of STO-609, CaMKK_{L233F} had no effect on ERK with or without depolarization (Fig. 2E). However, STO-609 strongly suppressed activation of ERK upon depolarization, and this inhibition was rescued by expression of CaMKK_{L233F} (Fig. 2E). Taken together, these data strongly suggest that activation of ERK upon depolarization proceeds primarily through CaMKK in NG108 cells.

To examine whether STO-609 blocks ERK activation in response to other stimulation paradigms, we treated NG108 cells with EGF or the muscarinic receptor agonist, carbachol. Depending on the cell type, both of these agonists can activate multiple signaling pathways including elevation of intracellular Ca^{2+} from external or internal stores. As can be seen in Fig. 3A, depolarization, EGF, and carbachol all activated ERK in NG108 cells, but only ERK activation through depolarization was blocked by STO-609. As a control for calcium influx and STO-609 specificity, we also examined the ability of KCl, EGF, and carbachol to activate CaMKII. Activation of CaMKII was maximal at 1 min and rapidly declined to near basal levels within 5–10 min. As illustrated in Fig. 3B, all treatments activated CaMKII, and this activation was insensitive to STO-609. These data confirm the specificity of STO-609 for inhibiting CaMKK but not CaMKII, and for blocking ERK activation in response to depolarization but not EGF or carbachol stimulation in NG108 cells. ERK activation by EGF and carbachol are presumably mediated by signaling pathways not involving CaMKK and perhaps even independently of Ca^{2+} . In addition,

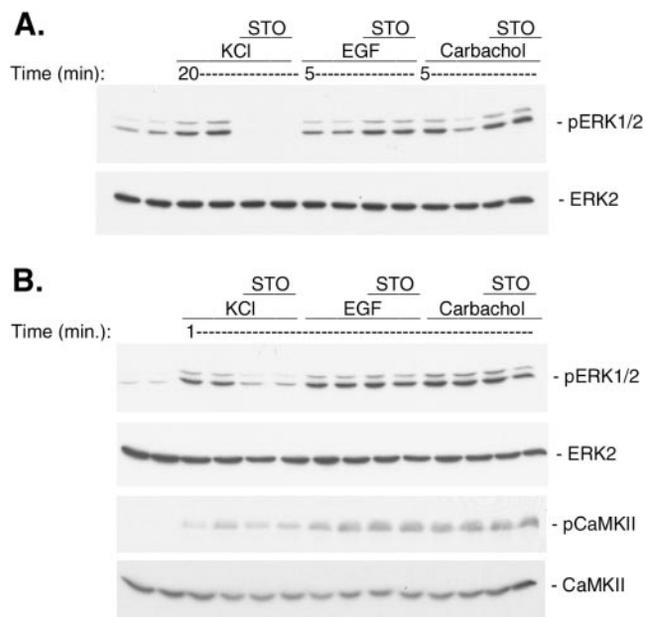


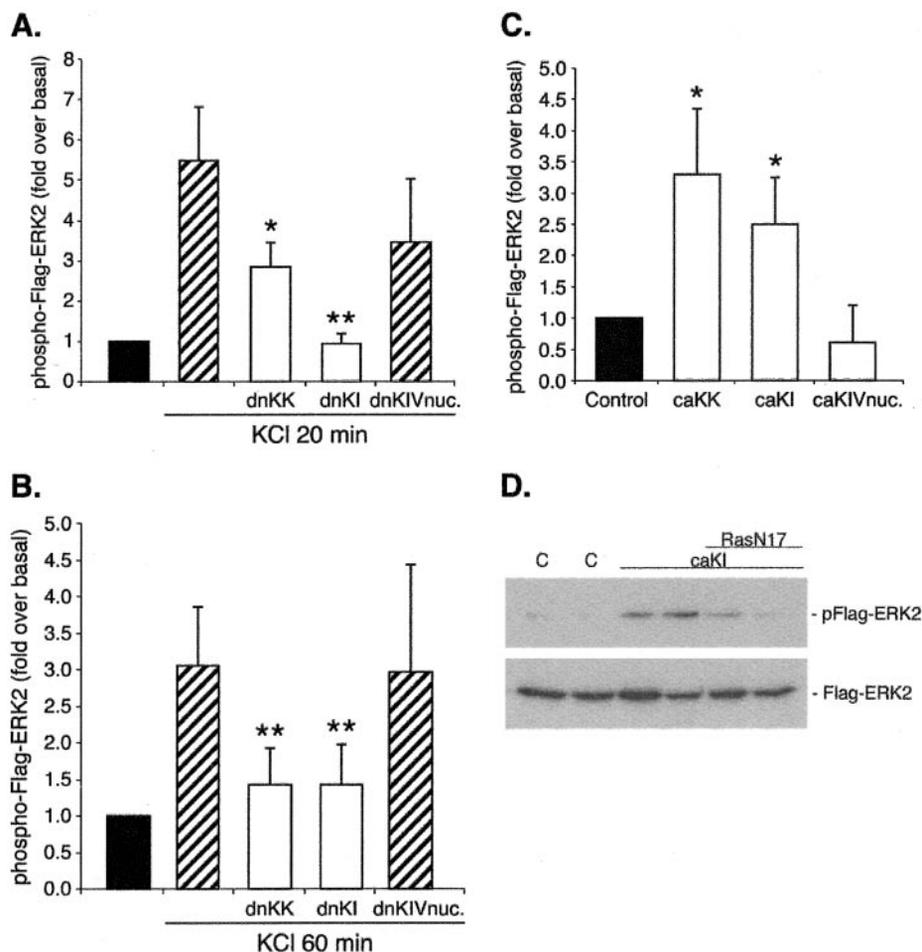
FIG. 3. Depolarization, but not EGF or carbachol, activation of ERK depends on CaMKK. A, STO-609 specifically blocks KCl activation of ERK. NG108 cells, with or without STO-609 pretreatment (2.6 μM , 60 min), were stimulated with KCl, EGF (100 ng/ml), or carbachol (10 μM) for the indicated times, and endogenous ERK activation was measured (pERK1/2). B, STO-609 does not block CaMKII activation. NG108 cells were treated identically to A for 1 min and analyzed for pERK1/2 and pCaMKII as well as total ERK2 and CaMKII.

since CaMKII activation occurred in the presence of STO-609, this indicates that Ca^{2+} influx in response to depolarization is not blocked by STO-609.

Involvement of CaMKI, but Not CaMKIV or AKT/PKB, in ERK Activation—The above data strongly implicate a major role for CaMKK in ERK activation upon depolarization of NG108 cells. The two major known targets of CaMKK are CaMKI and CaMKIV. AKT/PKB is a relatively weak substrate for CaMKK and does not exhibit significant CaMKK-mediated activation upon depolarization in NG108 cells until ~30–40 min. (Fig. 2C). Since CaMKK-mediated activation of ERK occurs by 5 min or less (Figs. 2A and 3B), AKT/PKB is an unlikely intermediate candidate to activate ERK. Furthermore, dnAKT/PKB had no inhibitory effect on ERK activation (data not shown).

We have previously shown that expression of constitutively active CaMKIV in PC12 cells results in activation of ERK (36), but more recent studies have shown that some expressed CaMKIV constructs, especially dominant-negatives (60), are not properly localized to the nucleus, like the endogenous CaMKIV, unless a nuclear localization signal is provided (57). Therefore, to determine which CaMK may be responsible for CaMKK-mediated activation of ERK, NG108 cells were co-transfected with FLAG-ERK2 along with dnCaMKI and dnCaMKIV constructs, and ERK activation was examined. Depolarization resulted in 3–5-fold activation of transfected FLAG-ERK2 at both 20 and 60 min (Fig. 4, A and B), and this was largely to completely inhibited by dnCaMKI or dnCaMKK. The dnCaMKIV with a nuclear localization signal did not significantly suppress ERK activation at 20 or 60 min, although it did block *N*-methyl-D-aspartate-stimulated CREB-mediated transcription in hippocampal neurons (57). These results confirm an obligatory role for CaMKK in depolarization-mediated activation of ERK in these cells and strongly implicate CaMKK acting through CaMKI but not CaMKIV or AKT/PKB. Moreover, caKK and caKI, but not nuclear localized caCaMKIV, significantly increased ERK activation (Fig. 4C). The ability of

FIG. 4. CaMKI mediates activation of ERK by CaMKK. *A* and *B*, dnCaMKK and dnCaMKI block KCl activation of ERK. NG108 cells were co-transfected with FLAG-ERK2 along with the control vector pcDNA3 or vectors encoding dnCaMKK, dnCaMKI, or dnCaMKIV containing a nuclear-localization signal (dnKIVnuc). Cells were stimulated with KCl for 20 (*A*) or 60 min (*B*), and FLAG-ERK2 phosphorylation was analyzed ($n = 6$, \pm S.E.). *C*, caCaMKK and caCaMKI are sufficient to activate ERK. NG108 cells were co-transfected with FLAG-ERK2 along with the control vector pcDNA3 or vectors expressing caCaMKK, caCaMKI, or caCaMKIVnuc, and FLAG-ERK2 phosphorylation was analyzed ($n = 6$, \pm S.E.). *D*, CaMKI activation of ERK is Ras-dependent. NG108 cells were co-transfected with FLAG-ERK2 along with the control vector pcDNA3 or caCaMKI with or without RasN17 as indicated, and FLAG-ERK2 phosphorylation (pFLAG-ERK2) was analyzed.



caCaMKI to activate ERK was blocked by RasN17, suggesting that CaMKI activates ERK through Ras in NG108 cells (Fig. 4D), similar to ERK activation upon depolarization (Fig. 1B). Taken together, these data demonstrate that CaMKK and CaMKI are both necessary and sufficient for ERK activation in NG108 cells.

To investigate whether CaMKK may also be important for calcium signaling to other MAP kinase family members, we analyzed JNK and p38 activation in NG108 cells treated with STO-609. Depolarization robustly activated both endogenous ERK and JNK at 20 and 60 min (Fig. 5, *A* and *B*). Similar to ERK, JNK activation by depolarization was completely blocked by STO-609 at both time points. Although depolarization may have slightly activated p38 at 20 min, an effect that was reduced by STO-609, at 60 min there was no apparent activation (Fig. 5, *A* and *B*). Thus, it appears that activation of both ERK and JNK by depolarization is mediated by CaMKK. The activation of p38 was too weak to assess the role of CaMKK in its activation.

CaMKK/CaMKI Regulate Neurite Outgrowth through ERK— To examine the physiologic significance of ERK activation by CaMKK in response to depolarization, we analyzed neurite outgrowth stimulated by depolarization in NG108 cells. A neurite was defined as a thin protrusion from the cell soma extending at least one cell diameter in length. Only 5% of NG108 cells generated neurites while maintained in low serum for 5 days (Fig. 6, *A*, upper left and *B*). Depolarization is known to stimulate neurite extension in NG108 cells (12, 40), and in our experiments ~60% of cells displayed neurites under these conditions (Fig. 6, *A*, upper right and *B*). Importantly, the stimulation of neurite production upon depolarization was com-

pletely blocked by inhibition of either CaMKK (STO-609, Fig. 6, *A*, lower left and *B*) or ERK (UO126, Fig. 6, *A*, lower right and *B*). STO-609 and UO126 treatments did not affect basal levels of neurite outgrowth of cells maintained in low serum (Fig. 6B). As a control for cell viability, NG108 cells were stained with Hoechst to examine nuclear condensation. Treatment of cells with KCl, STO-609, or UO126 in low serum did not affect cell viability (data not shown).

To determine whether ERK is downstream of CaMKK in mediating neurite outgrowth, cells were transfected with caCaMKK and EGFP-caCaMKI in the presence or absence of UO126. About 15% of NG108 cells transfected with EGFP vector alone generated neurites (Fig. 7, *A*, left and *B*). Cells expressing caCaMKK/KI generated neurites in ~85% of the cells (Fig. 7, *A*, middle and *B*), an effect that was completely blocked by inhibiting ERK (UO126, Fig. 7, *A*, right and *B*). These results suggest that calcium-evoked neurite outgrowth is mediated by CaMKK and CaMKI acting through MEK and ERK.

DISCUSSION

A number of pathways exist for how ERK may be regulated by calcium signaling including the modulation of small G proteins, phosphatases, and protein kinases (23). The present study demonstrates a new pathway, the CaMK cascade of CaMKK/CaMKI, in ERK and JNK activation upon depolarization of NG108 cells. This conclusion is based on the use of STO-609, a pharmacological inhibitor of CaMKK, as well as dominant-negative kinases. Importantly, the inhibition of ERK activation by STO-609 was rescued by transfection with a STO-609-insensitive mutant of CaMKK. Although depolarization

FIG. 5. Activation of JNK upon depolarization also requires CaMKK. *A* and *B*, KCl activation of JNK is blocked by STO-609. NG108 cells, without or with STO-609 pretreatment (2.6 μ M, 60 min), were stimulated with KCl for 20 min (*A*) or 60 min (*B*), and activation of endogenous ERK (*pERK1/2*), JNK (*pJNK*), and p38 (*phospho-p38*) was determined.

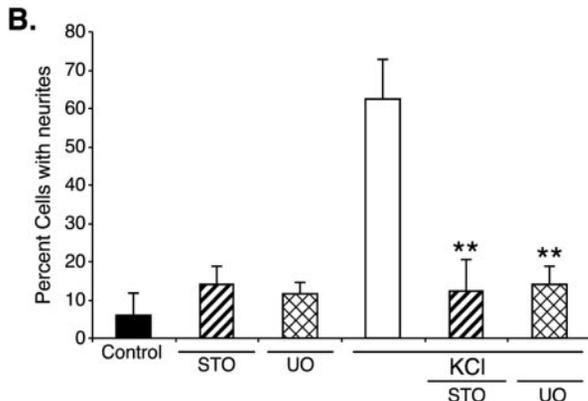
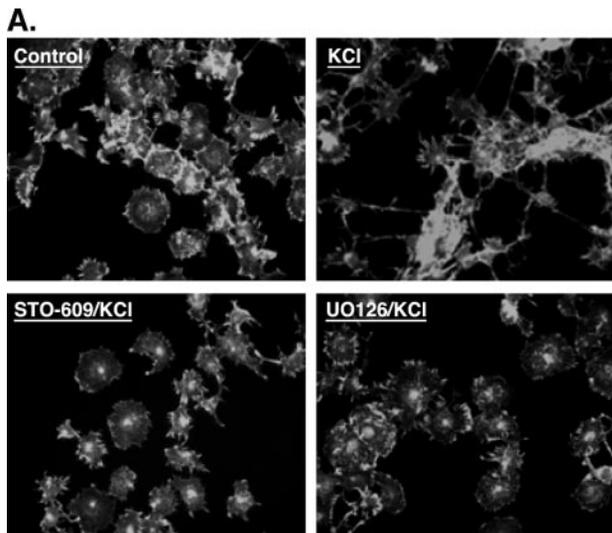
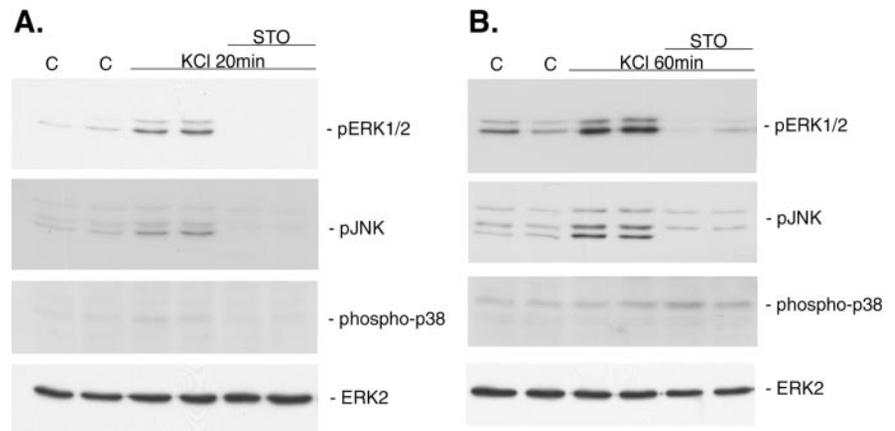


FIG. 6. Calcium-stimulated neurite outgrowth in NG108 cells requires CaMKK and ERK. *A*, neurite outgrowth in NG108 cells is CaMKK and ERK-dependent. NG108 cells were plated and grown in 1% serum without or with 60 mM KCl, to induce neurite formation (see "Experimental Procedures"), in the absence of presence of STO-609 (2.6 μ M) or UO126 (10 μ M) for 5 days. Fixed cells were stained with fluorescein isothiocyanate-phalloidin to visualize the actin cytoskeleton, and representative cells are shown in *A*. Note the presence of neurites induced by KCl (*upper right panel* compared with *upper left panel*) that are blocked by inhibition of CaMKK (*lower left panel*) or ERK (*lower right panel*). *B*, data from three separate experiments are quantified in terms of the percentage of cells with neurites more than 1 cell body in length ($n = 3$, >300 cells, \pm S.D.).

activated the downstream substrates of CaMKK (CaMKI, CaMKIV, and AKT/PKB), only the dominant-negative construct for CaMKI blocked ERK activation, indicating a central function for CaMKI. It is known that CaMKIV is involved in

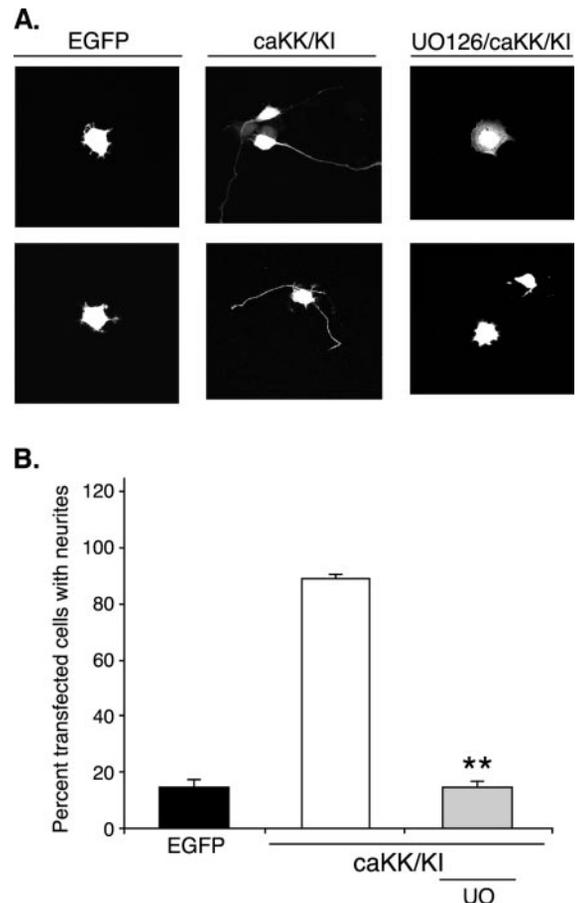


FIG. 7. Stimulation of neurite outgrowth by caCaMKK plus caCaMKI is mediated by ERK. *A*, NG108 cells, cultured in the presence of 1% serum, were transfected with EGFP (*left panels*) or EGFP-tagged constructs of caCaMKK plus caCaMKI without (*middle panels*) or with (*right panels*) treatment of UO126 (10 μ M) for 2 days. Cells were examined by confocal microscopy for EGFP expression, and representative cells are shown. *B*, results from six experiments were analyzed in terms of numbers of EGFP-positive cells exhibiting neurites more than one cell body in length ($n = 6$, >300 cells, \pm S.E.).

transcriptional regulation, and AKT/PKB activation by CaMKK suppresses apoptosis. The specific inhibitor of CaMKII, CaM KIIntide, did not suppress ERK activation, eliminating a role for this regulatory enzyme. These results provide a cellular function for CaMKI in modulating Ca^{2+} -dependent responses, such as neurite outgrowth, mediated by the ERK/JNK pathways in NG108 cells.

ERK activation connects extracellular stimulation to cell growth, proliferation, survival, and differentiation by the activation of small G proteins (15). Since ERK activation in NG108

cells, in response to either depolarization or transfection with caCaMKI, was blocked by dominant-negative RasN17, this indicates that Ras is downstream from CaMKI. ERK activation was not dependent on PKA, so it is likely that the Rap1/B-Raf pathway is not involved in activating ERK in this system (17, 49, 61). Active Ras triggers the MAP kinase kinase kinase, Raf-1, at the plasma membrane, which in turn phosphorylates and activates the MAP kinase kinase, MEK (16). MEK is a dual specificity serine/threonine kinase that phosphorylates and activates ERK1/2, and the MEK inhibitor UO126 also suppressed Ca²⁺-dependent ERK activation in our experiments. Thus, we conclude that Ras and MEK are downstream of CaMKI in the ERK activation pathway.

Depolarization of NG108 cells produced a prolonged activation of ERK that persists for at least one hour. In striatal neurons the duration of ERK activation may be regulated by its dephosphorylation catalyzed by the tyrosine phosphatase STEP (62). Calcium regulation of STEP appears to function primarily in the prolonged activation of ERK by *N*-methyl-D-aspartate and glutamate signaling but not depolarization signaling pathways. STEP regulation of ERK by KCl depolarization has not been reported in NG108 cells, perhaps accounting in part for the sustained ERK activation we observed. Prolonged ERK activation in response to depolarization of hippocampal neurons is critical to regulation of gene transcription through phosphorylation of transcription factors such as CREB (5, 44, 63). Rapid phosphorylation of CREB after depolarization may be due to activation of nuclear CaMKIV (64), an excellent CREB kinase (65). Slow, sustained CREB phosphorylation for up to an hour requires a Ras/MEK pathway that also involves a CaM kinase (*i.e.* is blocked by KN-93) (44). Based on our results, it is likely that CaMKI mediates this sustained activation of ERK.

Our data demonstrate that both AKT and ERK are activated during overlapping time points (30–60 min) in NG108 cells. AKT has been proposed to inhibit the Raf-1/MEK/ERK pathway in differentiated myotubes (66). However, recent studies in neurons would suggest that AKT does not inhibit Raf-1-mediated signaling and axon outgrowth (14). Our data would suggest that AKT activation is not inhibiting ERK activation in NG108 cells. Indeed, AKT activation by CaMKK may function in an independent pathway that mediates neuronal survival (35), consistent with the data presented here that prolonged depolarization (*i.e.* 5 days for neurite outgrowth) does not stimulate apoptosis in NG108 cells.

The role of ERK activation in neuronal physiology is very broad and includes neurite outgrowth. NG108 cells are an established model for examining neurite outgrowth by various stimuli (11, 38, 39). Fibronectin, constitutively active Ras, and angiotensin signals have all been shown to stimulate neurites in NG108 cells in an ERK-dependent manner (45, 46). We show that prolonged treatment of NG108 cells with KCl stimulates neurite outgrowth, and this neuritogenesis is blocked by inhibitors of CaMKK (STO-609) or MEK (UO126). Importantly, neurite formation in response to co-transfection with caCaMKK plus caCaMKI is blocked by UO126. This indicates that CaMKK/KI and MEK are part of the same pathway rather than parallel pathways. A requirement for ERK activation in neurite outgrowth has also been reported in PC12 cells (67, 68). In PC12 cells, KCl stimulation of neurite formation has been shown to depend on PKA, Ras, and Rap1, and it is blocked by ERK inhibitors (49, 69–71). In hippocampal neurons, filopodial formation in response to repetitive pulses of KCl requires a prolonged activation of ERK that is blocked by the general CaMK inhibitor KN-93 (8). We speculate that the relevant CaMK in this system may be CaMKI. Prolonged incubation of

neurons in KCl also stimulates neurite extension, perhaps through CaMKIV (72). In the absence of depolarization, immature neurons also express neurites, one of which becomes the axon, and the remainder develop into dendrites. Recent detailed studies in hippocampal neurons have shown that dendritic arborization depends on β CaMKII (50) whereas CaMKI mediates axonal outgrowth and growth cone motility (57). Thus, it appears that various members of the CaMK family regulate different aspects of neurite development.

Although CaMKI is a rather ubiquitously expressed kinase and some *in vitro* substrates (*e.g.* synapsin 1) are known (73–75), few *in vivo* substrates have been identified (76, 77) and therefore physiological roles for CaMKI have been largely unexplored. Our results provide a cellular role for CaMKI in regulating the Ca²⁺-dependent ERK/JNK pathway.

Acknowledgments—We thank Dr. Philip J. S. Stork for critically reading and reviewing the manuscript. We also wish to thank the members of the Soderling laboratory for scientific and technical assistance.

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