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Calcium Activation of ERK Mediated by Calmodulin Kinase I*

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Elevated intracellular Ca\textsuperscript{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\textsuperscript{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)\textsuperscript{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/Raf 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 (CaMs), which are activated by stimuli that elevate intracellular calcium, have also been proposed to mediate 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 acting through G protein-coupled receptors 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,
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 clear 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 carbocannel were purchased from Sigma. Hoechst 33342 solution was purchased from Molecular Probes (Eugene, OR). KN-93, KN-92, H89, ionomycin, and U0126 were purchased from Calbiochem (Riverside, CA). STO-699 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 and activated AKT (pAKT) at threonine 308 were purchased from Cell Signaling. Antibodies specific to phosphorylated (threonine 286) and activated CaM kinase II (pCaMKII) were purchased from Santa Cruz Biotechnology Inc. Antibodies to CaMKK, CaMKIV, and CaMKII were purchased from Transduction Laboratories (San Jose, CA). Antibodies to CaMKII 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—

To investigate the potential role of CaMKs in modulating ERK activation, we used the CaMKK and KI-tagged with EGFP using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s guide lines. 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 1-glutamate at 37 °C in 5% CO2 for 48 h. Cells were treated with U0126 (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. Western blotting was performed from Western blots, and gels were stained with Ponceau S to determine even loading. Immunoblots were visualized using a Chemidoc gel documentation system (BIO-RAD). Immunoreactive protein bands were quantified using Quantity One software. Antibodies were used at the following dilutions: anti-FLAG (1:3,000), anti-pERK1/2 (1:10,000), anti-pAKT (1:2,000), anti-pCaMKII (1:2,000), anti-phospho-jun N-terminal (pJNK) (1:1,000), and anti-pCaM kinase II (1:1,000). Immunoblotting of whole-cell lysates was performed using antibodies specific to CaMKK (1:1,000), CaMKIV (1:1,000), CaMKII (1:1,000), CaMKI (1:1,000), and CaMK in (1:1,000). Immunoprecipitations were performed with 20 μg ml of IgG (1:1,000) antibody supernatants, which were incubated with 1:10 dilution of xylol. Western blotting was performed using antibodies specific to pCaM kinase II (1:1,000), pCaMKII (1:1,000), pCaMKIV (1:1,000), and pCaMKIII (1:1,000). Immunoprecipitations were performed with 20 μg ml of IgG (1:1,000) antibody supernatants, which were incubated with 1:10 dilution of xylol.

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 10 min at room temperature. Cells were then washed three times for 5 min in PBS. Coverslips were then mounted on glass slides and analyzed by either fluorescence or confocal microscopy.

Plasmas and Transfections—

The CaMKK has previously been published (35). The CaMKK (H/Q228EEDDD, F307A), EGFP-tagged CaMKK, and CaMKIV (R136A, HMID356D) with a nuclear localization signal were generated in the Soderling laboratory. EGFP-tagged CaMKK was constructed using the pEGFP vector (Clontech). The dnCaMKK (K71A, T108A, S458A), EGFP-tagged CaMKK, and CaMKIV (K136A, HMID356D) with a nuclear localization signal were generated in the Soderling laboratory. The CaMKK was transfected using LipofectAMINE 2000 (Invitrogen) 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 24 h. Cells were then serum-starved overnight, treated as indicated, and lysed in ice-cold lysis buffer. The 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-
ing Ca²⁺-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) and 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 U0126 (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 adenyl 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 μM) 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.

CaMKI 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-94) and KN-92 (and KN-94) are known to inhibit binding of Ca²⁺/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 Ca²⁺ channels), we used ionomycin to elevate Ca²⁺ 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
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). CaMKII is an endogenous brain protein that specifically inhibits CaMKII with an IC_{50} of 0.1 μM whereas at 10 μM it exhibits little or no inhibition of CaMKI, CaMKIV, CaMKK, PKA, or protein kinase C (54). CaMKII, a 27-residue peptide derived from CaMKII, retains the potency and specificity of CaMKII, and we have also determined that in vitro it does not directly inhibit ERK (data not shown). CaMKII can be made cell-permeable by attachment of an Ant sequence (55). Incubation of NG108 cells with 5 μM Ant-CaMKII 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-CaMKII 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-

![Image](image-url)
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 IC50 of 0.26 μM (0.1 μg/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 CaMKK 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 IC50 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<sub>L233F</sub> (59). NG108 cells were transfected with FLAG-tagged ERK2 without or with co-transfection with CaMKK<sub>L233F</sub>. In the absence of STO-609, CaMKK<sub>L233F</sub> 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<sub>L233F</sub> (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<sup>2+</sup> 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<sup>2+</sup>. In addition, since CaMKII activation occurred in the presence of STO-609, this indicates that Ca<sup>2+</sup> 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 caII, but not nuclear localized caCaMKIV, significantly increased ERK activation (Fig. 4C). The ability of
CaMKI 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, lower left and B). Depolarization is known to stimulate neurite extension in NG108 cells (12, 40), and in our experiments ~85% of cells displayed neurites under these conditions (Fig. 6, A, upper right and B). Importantly, the stimulation of neurite production upon depolarization was completely 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 CaMKKI. 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
activated the downstream substrates of CaMK (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 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 Ca²⁺-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
CaMK inhibitor KN-93 (8). We speculate that the relevant formation in response to repetitive pulses of KCl requires a PC12 cells, KCl stimulation of neurite formation has been parallel pathways. A requirement for ERK activation in neurons of CaMKK (STO-609) or MEK (U0126). Importantly, neuritogenesis in NG108 cells in an ERK-dependent manner (45, 46). We show established model for examining neurite outgrowth by various broad and includes neurite outgrowth. NG108 cells are an during overlapping time points (30–60 min) in NG108 cells. AKT activation is not inhibiting ERK activation in this system (17, 20). Stork, P. J., and Schmitt, J. M. (2002) Trends Cell Biol. 12, 258, –266.

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). Fibronec- tin, constitutively active Ras, and angiogenins 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 neurogenesis is blocked by inhibitors of CaMKK (STO-609) or MEK (U0126). Importantly, neurite formation in response to co-transfection with caMKK plus CaMKK is blocked by U0126. This indicates that CaMKK/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 βCaMKII (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.

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