Nuclear Calpain Regulates Ca\textsuperscript{2+}-dependent Signaling via Proteolysis of Nuclear Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase Type IV in Cultured Neurons* 

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Accumulating evidence indicates that calpains can reside in or translocate to the cell nucleus, but their functions in this compartment remain poorly understood. Dissociated cultures of cerebellar granule cells (GCs) demonstrate improved long-term survival when their growth medium is supplemented with depolarizing agents that stimulate Ca\textsuperscript{2+} influx and activate calmodulin-dependent signaling cascades, notably 20 mM KCl. We previously observed Ca\textsuperscript{2+}-dependent down-regulation of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMK) type IV, which was attenuated by calpain inhibitors, in GCs supplemented with 20 mM KCl (Tremper-Wells, B., Mathur, A., Beaman-Hall, C. M., and Vallano, M. L. (2002) J. Neurochem. 81, 314–324). CaMKIV is highly enriched in the nucleus and thought to be critical for improved survival. Here, we demonstrate by immunolocalization/confocal microscopy and subcellular fractionation that the regulatory and catalytic subunits of m-calpain are enriched in GC nuclei, including GCs grown in medium containing 5 mM KCl. Calpain-mediated proteolysis of CaMKIV is selective, as several other nuclear and non-nuclear calpain substrates were not degraded under chronic depolarizing culture conditions. Depolarization and Ca\textsuperscript{2+}-dependent down-regulation of CaMKIV were associated with significant alterations in other components of the Ca\textsuperscript{2+}-CaMKIV signaling cascade: the ratio of phosphorylated to total cAMP response element-binding protein (a downstream CaMKIV substrate) was reduced by ~10-fold, and the amount of CaMK kinase (an upstream activator of CaMKIV) protein and mRNA was significantly reduced. We hypothesize that calpain-mediated CaMKIV proteolysis is an autoregulatory feedback response to sustained activation of a Ca\textsuperscript{2+}-CaMKIV signaling pathway, resulting from growth of cultures in medium containing 25 mM KCl. This study establishes nuclear m-calpain as a regulator of CaMKIV and associated signaling molecules under conditions of sustained Ca\textsuperscript{2+} influx.

Over 4 decades ago, a neutral Ca\textsuperscript{2+}-activated protease was extracted from the soluble fraction of rat brain (2). Characterized by its calcium dependence and sequence homology to the protease domain of the papain family of cysteine proteases, it was designated calpain. Presently, calpains are recognized as ubiquitous Ca\textsuperscript{2+}-dependent endopeptidases, and more than 1 dozen mammalian gene products have been identified, with expression of some isoforms in all cell types examined (3, 4). Homologs have also been studied in nematodes, insects, yeast, and fungi (3). The calpain family includes ubiquitous and tissue-specific isoforms, the most common being \( \mu \) (calpain I) and \( \mu \) (calpain II), consisting of identical regulatory and distinct catalytic subunits that confer different sensitivities to Ca\textsuperscript{2+}. In vitro, m-calpain binds Ca\textsuperscript{2+} with relatively low affinity (millimolar), and \( \mu \)-calpain binds with higher affinity (micromolar); but their Ca\textsuperscript{2+} requirements in cells and tissues are influenced by several factors that may lower these requirements (5).

Calpains catalyze the proteolysis of numerous and diverse cytosolic, cytoskeletal, and membrane-associated substrates in response to cell injury in vitro and in vivo (6, 7). In the central nervous system, for example, calpain activation is associated with neuronal damage in ischemia or stroke and in Alzheimer’s and Huntington’s diseases and with demyelination in multiple sclerosis (6–8), and protection may be afforded by the judicious use of calpain inhibitors (9, 10). Other studies using cell culture models have provided valuable mechanistic information about activation of calpains, relevant substrates, and their roles in the injury process (11). Consistent with these studies, dysregulation of Ca\textsuperscript{2+} homeostasis, as occurs in many models of neuronal injury, is poorly tolerated by neurons.

Although best known for their roles in neuropathologies, calpains also catalyze a variety of structural and enzymatic responses to physiological alterations in Ca\textsuperscript{2+} (12), including effects on cell proliferation, differentiation, adhesion, and migration; synaptic plasticity; and protein turnover (13, 14). In addition, some calpains are localized in the cell nucleus (15–19), and in vitro, several transcription factors serve as substrates (20–23). Calpains appear to contain nuclear export sequences (1), and although they lack traditional nuclear localization sequences (NLSs),\(^1\) mutation of the two \( \alpha \)-helices in domain I interferes with their nuclear localization (11), suggesting that current definitions of NLSs should be broadened. Taken together, these data indicate that calpains may have important roles in the nuclear compartment, yet we under-

\( ^1 \)The abbreviations used are: NLSs, nuclear localization sequences; CaMK, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase; GCs, cerebellar granule cells; VSCCs, voltage-sensitive calcium channels; DIV, days in vitro; CREB, cAMP response element-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; NF-H, high molecular mass neurofilament; IP3R, type I inositol triphosphate receptor; PBS, phosphate-buffered saline; NFDM, nonfat dry milk; CaMKK, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase kinase; NMDA, N-methyl-D-aspartate.
stand little about relevant substrates and functional consequences of nuclear calpain activity. Previously, we observed Ca2+-dependent proteolysis of Ca2+-/calmodulin-dependent protein kinase (CaMK) type IV in primary cultures of cerebellar granule cells (GCs) when the culture medium was supplemented with 20 mM KCl (1). Investigators who study GCs routinely supplement the growth medium with elevated KCl (25 mM final concentration), which stimulates Ca2+ entry through voltage-sensitive calcium channels (VSCCs), thereby improving long-term survival (24). This phenomenon, referred to as “activity dependence,” is acquired at 2–3 days in vitro (DIV), and subsequent withdrawal of KCl at any time thereafter triggers apoptosis within hours (25, 26). CaMKIV, which is concentrated in GC nuclei and catalyzes the phosphorylation of various transcription factors, i.e. cAMP response element-binding protein (CREB) (27), is thought to be the downstream effector of this depolarization- and Ca2+-dependent survival pathway (24, 27–30). Based on in vitro reconstitution studies using purified calpain, caspase, and CaMKIV, it was determined that CaMKIV is a substrate for both caspase and calpain (31). However, unlike their unsupplemented counterparts and consistent with their improved survival, GCs grown in medium containing elevated KCl have low caspase activity (1). Moreover, pretreatment with cell-permeable calpain inhibitors attenuates depolarization-dependent CaMKIV proteolysis, and several non-nuclear calpain substrates are not down-regulated by chronic exposure to elevated KCl (1). Taken together, these results suggest that a nuclear calpain that selectively cleaves CaMKIV is activated in response to sustained increases in intracellular Ca2+ resulting from growth of cultures in medium containing elevated KCl. Herein, we provide direct evidence for m-calpain localization in the nuclei of GCs and demonstrate that proteolysis of CaMKIV is associated with profound alterations in associated upstream and downstream signaling molecules.

**Experimental Procedures**

**Materials**—Sprague-Dawley neonatal rats were purchased from Taconic Farms (Germanton, NY). Rat brain hippocampal neurons were purchased from QB3 Cell Sciences (Ontario, Canada). Basal Eagle's medium with Earle's salts, Dulbecco's modified Eagle's medium (DMEM), and 1:1 Dulbecco's modified Eagle's medium/Ham's F-12 medium were purchased from Invitrogen. Nifedipine was purchased from Sigma. KN-62 was purchased from EMD Biosciences (La Jolla, CA). MitoTracker Deep Red FM, spectrin, and calpain were purchased from Invitrogen. Alexa Fluor 594-conjugated donkey anti-mouse and Alexa Fluor 647-conjugated goat anti-rabbit secondary antibodies of multiple labels and Alexa Fluor 594-conjugated goat anti-mouse antibody were purchased from Invitrogen. Other fluorescent substrates were provided by Invitrogen. Alexa Fluor 594-conjugated goat anti-rabbit and Alexa Fluor 647-conjugated goat anti-mouse secondary antibodies were purchased from Invitrogen. Texas Red- and Cy2®-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. Alexa Fluor 488- and Alexa Fluor 647-conjugated secondary antibodies were purchased from Invitrogen. Alexa Fluor 594-conjugated goat anti-mouse secondary antibody was purchased from Invitrogen. Alexa Fluor 594-conjugated donkey anti-goat antibody was purchased from Invitrogen. Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody was purchased from Invitrogen.

**Western Blotting**—Cell lysates were washed twice with serum-free DMEM containing 30 mM KCl and incubated in serum-free DMEM containing 5 mM KCl for 16 h, and protein was harvested.

Rat hippocampal cells were thawed and plated according to the manufacturer’s instructions. Briefly, cells were resuspended in Neurobasal medium with 2% B27, 1% antibiotics, and 100 units/ml penicillin/streptomycin. Neurons were seeded at 1 ml/well in 24-well dishes containing coverslips and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air for 4 h. The medium was removed from the cells, and fresh medium was added. Cells were returned to the incubator until 7 DIV.

**Immunocytochemistry**—Cells were grown on glass coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBA (1% bovine serum albumin and 0.1% sodium azide) for 10 min, blocked with 3% NFDM blocking grade in PBA, and incubated with primary antibodies at 1:100-1:1000 dilution in PBA for 1 h at room temperature. After washing, cells were incubated with secondary antibodies (Alexa Fluor 594-conjugated goat anti-mouse, Alexa Fluor 594-conjugated goat anti-rabbit, Texas Red®-conjugated goat anti-rabbit, Texas Red®-conjugated goat anti-mouse, and Texas Red®-conjugated donkey anti-mouse) in PBA for 1 h. Cells were then washed twice with serum-free DMEM containing 30 mM KCl and mounted on slides using Vectashield Antifade and digitally photographed using a Bio-Rad MRC1024ES confocal microscopy system with a Nikon Eclipse E600 microscope (x 40 or oil immersion x 60 objective). Identical parameters (e.g. gain, iris, laser %, etc.) were set when comparing images within each cell preparation.

**Cell Culture**—Primary cultures of GCs were prepared and grown in an atmosphere of 5% CO2 as described (33). Twenty-four hours after plating, serum-containing medium was replaced with a chemically defined medium (1) containing 10 μg/ml cytosine arabinoside to prevent glial overgrowth and, where indicated, supplemented with 20 mM KCl. In a series of experiments involving the activation of cytosolic calpains in response to cell stress, the procedures described previously (34) were used. Briefly, granule cells were cultured in DMEM containing 30 mM KCl (final concentration) or 5 mM KCl, 10% fetal bovine serum, 5 mg/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 24 h, half of the medium was removed and replaced with fresh DMEM containing cytosine arabinoside. At 7 DIV, experimental cultures were washed twice with serum-free DMEM containing 30 mM KCl and incubated in serum-free DMEM containing 5 mM KCl for 16 h, and protein was harvested.

**Confocal Microscopy**—Cells were washed twice with serum-free DMEM containing 30 mM KCl and incubated in serum-free DMEM containing 5 mM KCl for 16 h, and protein was harvested. Rat hippocampal cells were thawed and plated according to the manufacturer’s instructions. Briefly, cells were resuspended in Neurobasal medium with 2% B27, 1% antibiotics, and 100 units/ml penicillin/streptomycin. Neurons were seeded at 1 ml/well in 24-well dishes containing coverslips and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air for 4 h. The medium was removed from the cells, and fresh medium was added. Cells were returned to the incubator until 7 DIV.

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**Western Blotting**—Cell lysates were harvested in 1 mM Na2VO4, 0.3 mM phenylmethylsulfonyl fluoride, 2% SDS, 62.5 mM Tris, and 10% glycerol and sonicated. Samples were either homogenized by 10 strokes in a Dounce homogenizer with 1% saponin or sonicated at the lowest setting for 20 s (Vibracell, Sonics & Materials, Inc., Newton, CT) and centrifuged at 10,000 × g for 10 min. The pellet (crude nuclear fraction) was resuspended in 0.32 M sucrose buffer. The supernatant was removed and resuspended at a second set point of 10,000 × g for 10 min. The supernatant (crude cytoplasmic fraction) was collected. Nuclear and cytoplasmic fractions were equilibrated for protein based on the Micro BCA™ assay.

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anti-phosphorylated CREB (diluted 1:500 in 3% NFDM and TTBS), and anti-cleaved caspase-3 (diluted 1:1000 in 5% NFDM and TTBS). Blots were incubated for 1–2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted in 5% NFDM as follows: mouse anti-CaMKIV, anti-NF-H, and anti-spectrin (diluted 1:750); anti-calpain (Santa Cruz Biotechnology, Inc.); goat anti-protein phosphatase 2A and anti-CaMKIIβ (diluted 1:4000); anti-p35, anti-c-Jun, and anti-calpain (Triple Point Biologies Inc.); rabbit anti-cleaved caspase-3 and anti-CREB (diluted 1:1000); and rabbit anti-IP3R (diluted 1:500). SuperSignal® West Pico or West Dura and x-ray films were used to visualize immunoreactivity. To ensure linearity of the assays, multiple protein concentrations were routinely analyzed.

Reverse Transcription-PCR—Whole cell RNA was isolated from cultures using a QiAGEN RNeasy® mini kit. The concentration and purity of RNA were assessed with a spectrophotometer using nucleotide absorbance at 260 nm and a ratio of 260/280 nm. Harvested RNA was used in a reverse transcription reaction to produce cDNA for use as template in PCR. RNA (0.1–0.2 μg/lane), virus reverse transcriptase, reverse transcription buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 10 mM dithiothreitol), the four dNTPs (0.5 mM each), 20 units of RNasin® ribonuclease inhibitor, of RNA were assessed with a spectrophotometer using nucleotide absorbance at 260 nm and a ratio of 260/280 nm. Harvested RNA was used in a reverse transcription reaction to produce cDNA for use as template in PCR. RNA (0.1–0.2 μg/lane), virus reverse transcriptase, reverse transcription buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 10 mM dithiothreitol), the four dNTPs (0.5 mM each), 20 units of RNasin® ribonuclease inhibitor, and 5 μM hexamer random primers was added to each RNA sample (final volume of 10 μl). The reverse transcription reaction was initiated by incubating the total mixture at 37 °C for 60 min to promote cDNA synthesis and terminated by heating to 95 °C for 5 min and then placing the tubes on ice and diluting to 0.1–5.0 ng/μl using sterile water. Following reverse transcription, PCR was performed in a final volume of 100 μl containing Taq buffer (10 mM Tris-HCl (pH 8.8) and 50 mM KCl), 2 mM MgCl2, 0.17 mg/ml bovine serum albumin, 2.5 units of Taq polymerase (AmpliTaq Gold® (CaMKIV) or Taq (actin and CaMK kinase (CaMKV)), the four dNTPs (0.05 mM each), and oligonucleotide primers (25 pmol each) as follows: CaMKIV, 5′-TGGCAAGGTTAGAGGGACTCG-3′ (upstream antisense); actin, 5′-TCATGAAGTGTGACGTTGAG-3′ (downstream antisense); actin, 5′-TCAATGAAGTTAGAGGGACTCG-3′ (upstream antisense); actin, 5′-TCATGAAGTGTGACGTTGAG-3′ (downstream antisense); actin, 5′-TCAATGAAGTTAGAGGGACTCG-3′ (upstream antisense); actin, 5′-TCAATGAAGTGTGACGTTGAG-3′ (downstream antisense); actin, 5′-TCAATGAAGTTAGAGGGAC-3′ (upstream antisense); actin, 5′-TCAATGAAGTGTGACGTTGAG-3′ (downstream antisense); actin, 5′-TCAATGAAGTGTGACGTTGAG-3′ (downstream antisense); actin, 5′-TCAATGAAGTGTGACGTTGAG-3′ (downstream antisense). Prior to PCR, samples were incubated at 95 °C for 7 min (CaMKIV), 5 min (actin), or 2 min (CaMKK). The CaMKIV PCR protocol consisted of 36 cycles in a PerkinElmer Life Sciences Model 480 Tempcycler as follows: denaturing for 30 s at 95 °C, annealing for 30 s at 56 °C, and extension for 45 s at 72 °C, ending with a final extension for 5 min at 72 °C. The actin PCR protocol consisted of 26 cycles of denaturing for 60 s at 94 °C, annealing for 60 s at 60 °C, and extension for 2 min at 72 °C, ending with a final extension for 10 min at 72 °C. The amplified products were resolved on an 8% polyacrylamide or a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination.
ependent process and requires prior activation of a CaMK, perhaps CaMKIV itself.

The Regulatory and Catalytic Subunits of m-calpain Are Enriched in the Nuclei of Granule Neurons—In Fig. 2A, the KCl-mediated down-regulation of immunoreactive CaMKIV is contrasted with its lack of effect on CaMKIIδ, assessed using double immunostaining and confocal laser scanning microscopy. Accordingly, GCs were grown for 5 DIV in medium containing 5 or 25 mM KCl and then fixed and incubated with antibodies recognizing CaMKIV (red), which is predominantly nuclear, and CaMKIIδ (green), which is predominantly cytosolic. In vitro, both are excellent calpain substrates (7). Consistent with Western immunoblot data (Fig. 1), CaMKIV was down-regulated in neurons grown in 25 mM KCl compared with 5 mM KCl. In contrast, the staining intensity of CaMKIIδ was not substantially different in GCs grown in 25 or 5 mM KCl. Fig. 2A also highlights the prominent nucleus and scant cytoplasm that characterize granule neurons of the cerebellar cortex and that are consistent with their proportionately smaller size (~8–10 μm) compared with most other neuronal types.

The majority of published reports on calpains demonstrate activity toward substrates in non-nuclear compartments (7, 36), but there is also compelling evidence supporting nuclear localization of calpains, at least in some cell types (8, 11, 19, 37–39). If calpain selectively degrades CaMKIV in neuronal nuclei, then it should be present in the nuclear compartment. To examine this, antibodies recognizing the regulatory subunit that is common to m-calpain and μ-calpain (green) were used in a series of double immunostaining studies together with antibodies recognizing CaMKIV (red). Fig. 2B is a composite fluo-
rescent photomicrograph showing that the regulatory subunit was concentrated in the nuclei of GCs grown in medium containing 5 or 25 mM KCl. In contrast, CaMKIV was enriched in the nuclear compartment in GCs grown in 5 mM KCl, but was substantially down-regulated in those grown in 25 mM KCl. Note that the anti-calpain antibody used for immunolocalization was shown by Western immunoblotting to recognize a major immunoreactive protein with an apparent molecular mass of ~28 kDa, as predicted for the regulatory subunit of calpain (40), with minor differences in staining intensities between GCs grown in 5 or 25 mM KCl (Fig. 2C, left panel). The specificity of the anti-CaMKIV antibody, which recognizes an immunoreactive protein with an apparent molecular mass of ~65–67 kDa, as predicted, is also shown (Fig. 2C, middle panel).

If calpain-mediated CaMKIV proteolysis occurs in the nuclear compartment, then the catalyt subunit of m-calpain and/or μ-calpain, like their common regulatory subunit, should be detectable in GC nuclei. To examine this, cultures were grown for 5 DIV in medium containing 5 or 25 mM KCl and then analyzed for μ-calpain or m-calpain localization using a battery of selective antibodies. Fig. 2D illustrates that m-calpain was enriched in the nuclei of GCs grown in 5 mM KCl, where it was colocalized with CaMKIV. Comparable results were obtained using antibodies recognizing epitopes in the C or N terminus of the catalytic subunit of m-calpain and using GCs grown in medium containing 25 mM KCl (data not shown). There was also evidence of μ-calpain immunoreactivity in nuclear and cytosolic compartments in GCs grown in 5 or 25 mM KCl, but the staining intensity was weak with both antibodies that were tested (data not shown). This difference was not unexpected since m-calpain is the more abundant transcript in neurons (41).

Activation of caspases (in particular, caspase-3) has a decisive role in GC apoptosis, which is triggered upon removal from the culture medium of elevated extracellular potassium (42–46). Also, caspase-mediated apoptosis occurs spontaneously beginning at 3–4 DIV in a subpopulation of GCs grown in standard medium containing 5 mM KCl (25). Consistent with this, caspase activity in general (1) and caspase-3 activity in particular (Fig. 2C, right panel) were significantly elevated in whole cell homogenates derived from GCs grown for 3–4 DIV in medium containing 5 mM versus 25 mM KCl. It is therefore unlikely that activation of caspase-3, which can also catalyze the proteolysis of CaMKIV in vitro (31), accounts for the observed down-regulation of CaMKIV in GCs grown under survival-promoting conditions, i.e. 25 mM KCl. To further examine this, the activation state of caspase in GCs grown for 5 DIV in medium containing 5 or 25 mM KCl was evaluated using an antibody that exclusively recognizes the cleaved (i.e. activated) form of caspase-3 (Fig. 2E, left panel, red). Cultures were also incubated with antibody against the catalytic subunit of m-calpain (Fig. 2E, middle panel, green). As shown, the majority of neurons were calpain-positive and caspase-negative. Interestingly, in the small number of dead or dying GCs that were caspase-positive, calpain immunoreactivity was dramatically reduced or absent. Qualitatively similar results were obtained in cultures grown in 5 mM KCl (data not shown), except that a greater proportion of neurons were caspase-positive, consistent with the fact that a subpopulation of these neurons spontaneously undergoes apoptosis. These data rule out a role for caspase-3 in KCl-mediated down-regulation of CaMKIV in GCs. Notably, there is ample support for cross-talk between caspase- and calpain-mediated signaling pathways in various cell types as well as evidence of calpain-mediated cleavage of caspases (47–50). The data in Fig. 2E suggest that the converse may also occur, that calpain may be a substrate for caspase in GCs undergoing apoptosis.

In healthy hippocampal pyramidal neurons, calpain is enriched in the cytosolic compartment (51, 52). To further verify the specificity of the anti-calpain antibodies used herein, rat hippocampal neurons were grown for 7 DIV and processed for immunocytochemistry using antibodies recognizing the catalytic subunit of m-calpain (Fig. 2F, middle panel, green). Neuronal nuclei were counterstained with an antibody against CREB (Fig. 2F, left panel, red) and analyzed by confocal laser microscopy. Fig. 2F shows that m-calpain expression in hippocampal neurons was predominantly cytoplasmic or membrane-associated, whereas CREB expression was predominantly nuclear. Similar results were obtained using antibodies against the common regulatory subunit of m-calpain and μ-calpain or the catalytic subunit of μ-calpain (data not shown).

Collectively, these studies validate the fidelity of the antibodies used to demonstrate calpain localization in the nuclei of GCs.

The Regulatory and Catalytic Subunits of m-calpain Are Enriched in Nuclear Fractions Prepared from GC Cultures—To independently examine the localization of m-calpain in GCs, subcellular fractionation studies were performed. The scant cytosolic compartment relative to the prominent nuclear compartment in GCs made it particularly difficult to separate these fractions while conserving the integrity of the nuclei and the nuclear proteins therein. Nevertheless, two methods yielded nuclear enriched fractions. Cultures were grown for 5 DIV in medium containing 5 mM KCl, and whole cell homogenates were collected in 0.32 M sucrose buffer and subjected to Dounce homogenization (designated as Method 1) or sonication (designated as Method 2), followed by low speed centrifugation (see “Experimental Procedures”). Samples corresponding to the cytosol/supernatant or nuclei/pellet were then compared for the expression of immunoreactive “marker” proteins by Western immunoblotting (Fig. 3). The nuclear fractions were defined by the relative enrichment of the c-Jun transcription factor coupled with the de-enrichment of CaMKIIß. Conversely, the cytosolic fractions were defined by the relative enrichment of CaMKIIß coupled with the de-enrichment of c-Jun. Phase-contrast microscope examination of samples confirmed the presence of numerous nuclei in the nuclear enriched fractions and their absence in the cytosolic fractions (data not shown). Fig. 3A shows that antibodies recognizing both the regulatory and catalytic subunits of m-calpain were enriched in the nuclear fractions compared with the cytosolic fractions. Furthermore, when cultures grown for 5 DIV in medium containing 5 or 25 mM KCl (overnight or chronic exposure) were compared for immunoreactive CaMKIV, it was often possible to detect a proteolytic fragment of 33–40 kDa in the nuclear fractions (Fig. 3B). Note that a fragment with this apparent molecular mass is obtained after cleavage of CaMKIV with caspase or calpain in vitro (31). Together, these data provide independent evidence that m-calpain is localized in GC nuclei and that proteolysis of CaMKIV occurs in this compartment.

A Non-nuclear Calpain Is Activated in GC Cultures Exposed to a Stressful Stimulus—Nath et al. (34) reported previously that caspase, m-calpain, and μ-calpain catalyze the proteolysis of non-nuclear substrates in GCs subjected to trophic factor withdrawal, a stressful stimulus. No reference to a nuclear isoform of calpain was made in their report. Furthermore, they distinguished between activation of calpain and caspase under these conditions by the appearance of distinct α-spectrin breakdown products based on Western immunoblot analysis. Specifically, both enzymes catalyze the formation of a 150-kDa fragment, whereas each additionally produces a distinct fragment: a 145-kDa calpain cleavage product and a 120-kDa caspase
Fractions, obtained by low speed centrifugation, were subsequently processed for Western immunoblot analysis (30 μg/lane) using antibodies recognizing the regulatory (reg-calp) and catalytic (m-calp) subunits of m-calpain (Santa Cruz Biotechnology, Inc.), c-Jun (a predominantly nuclear protein), and CaMKIIβ (a predominantly cytosolic protein). A, granule neurons were grown for 5 DIV in medium containing 5 mM KCl and harvested in 0.32 M sucrose buffer with 1% saponin and then subjected to Dounce homogenization (Method 1 (Mtd.1)) or without saponin and then sonicated for 15 s at the lowest setting (Method 2 (Mtd.2)). Nuclear enriched (nuc) and cytosolic (cyto) fractions, obtained by low speed centrifugation, were subsequently processed for Western immunoblot analysis (30 μg/lane) using antibodies recognizing the regulatory (reg-calp) and catalytic (m-calp) subunits of m-calpain (Santa Cruz Biotechnology, Inc.), c-Jun (a predominantly nuclear protein), and CaMKIIβ (a predominantly cytosolic protein). B, granule neurons were grown for 5 DIV in medium containing 5 or 25 mM KCl (overnight or chronic) and harvested in 0.32 M sucrose buffer without saponin and then subjected to sonication and low speed centrifugation (i.e. Method 2). The nuclear enriched fraction was processed for Western immunoblot analysis (30 μg/lane) using antibodies recognizing intact (CaMKIV) and proteolyzed (fragment) CaMKIV.

Cleavage product (34, 53). In the following series of experiments, the protocol described by Nath et al. (34) was used to verify the presence of a non-nuclear calpain in the preparations used herein. This is an important control because, in our cultures, calpain immunoreactivity was detected primarily in the nucleus, and it was often necessary to “overexpose” the autoradiograms to reveal calpain in the cytosolic fractions (data not shown). GCs were grown for 7 DIV in medium containing 5 or 30 mM KCl, and then elevated KCl was withdrawn from a subset of the cultures grown in 30 mM KCl to induce apoptosis. After 24 h, whole cell homogenates were prepared, equalized for protein content, and compared by Western immunoblotting for intact and specific fragments of α-spectrin. Immunoreactive CaMKIV in the same samples is shown for comparison (Fig. 4, middle panel). Withdrawal of 30 mM KCl triggered apoptosis (assessed by phase-contrast microscopy and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) (data not shown) as well as the appearance of 150-, 145-, and 120-kDa α-spectrin breakdown products compared with cultures grown continuously in 30 or 5 mM KCl (Fig. 4, upper panel). This result supports the coactivation of calpain and caspase in response to KCl withdrawal and is in good agreement with the findings of Nath et al. (34). Furthermore, a second calpain substrate and allosteric activator of Cdk5 (54, 55), p35, was also analyzed under these treatment conditions (Fig. 4, lower panel). Consistent with the activation of a non-nuclear calpain in response to KCl withdrawal, p35 was cleaved to its truncated form, p25. Note that a modest increase in p35 and a profound decrease in CaMKIV were observed in cultures that were chronically exposed to 30 mM KCl, a growth condition that promotes long-term survival. Taken together, these data verify the presence in GCs of a non-nuclear calpain activity that is selectively activated in response to trophic factor withdrawal. They also provide additional evidence that, unlike CaMKIV, non-nuclear calpain substrates (spectrin and p35) are not down-regulated in cultures chronically exposed to elevated KCl.

Calpain-mediated Proteolysis of CaMKIV Is Associated with Significant Alterations in Upstream and Downstream Components of the CaMKIV Signaling Cascade—Activation of CaMKIV in response to increased intracellular Ca2+ is dependent on phosphorylation of its activation loop by an upstream CaMK, CaMKK, which increases its catalytic activity by ~10–30-fold (56). In turn, CaMKIV regulates nuclear transcription of numerous genes via phosphorylation of transcription factors such as CREB (at Ser133), its best characterized substrate, and also activator protein-1, serum response factor, and activating transcription factor-1 (27, 57). As a first step in exploring the functional consequences of calpain-mediated CaMKIV proteolysis, the effects of elevated KCl on immunoreactive CaMKK and CREB (phosphorylated and total) compared with GCs grown in 5 mM KCl were evaluated. Whole cell homogenates were prepared from cultures grown for 5 or 9 DIV; equalized for protein content; and compared by Western immunoblotting for intact and specific fragments of p35 that were obtained by low speed centrifugation, were subsequently processed for Western immunoblot analysis (30 μg/lane) using antibodies recognizing intact (CaMKIV) and proteolyzed (fragment) CaMKIV.

**Fig. 3.** m-calpain is enriched in the nuclear fractions of granule neurons. A, granule neurons were grown for 5 DIV in medium containing 5 mM KCl and harvested in 0.32 M sucrose buffer with 1% saponin and then subjected to Dounce homogenization (Method 1 (Mtd.1)) or without saponin and then sonicated for 15 s at the lowest setting (Method 2 (Mtd.2)). Nuclear enriched (nuc) and cytosolic (cyto) fractions, obtained by low speed centrifugation, were subsequently processed for Western immunoblot analysis (30 μg/lane) using antibodies recognizing the regulatory (reg-calp) and catalytic (m-calp) subunits of m-calpain (Santa Cruz Biotechnology, Inc.), c-Jun (a predominantly nuclear protein), and CaMKIIβ (a predominantly cytosolic protein). B, granule neurons were grown for 5 DIV in medium containing 5 or 25 mM KCl (overnight or chronic) and harvested in 0.32 M sucrose buffer without saponin and then subjected to sonication and low speed centrifugation (i.e. Method 2). The nuclear enriched fraction was processed for Western immunoblot analysis (30 μg/lane) using antibodies recognizing intact (CaMKIV) and proteolyzed (fragment) CaMKIV.

**Fig. 4.** Non-nuclear calpain is activated in granule neurons after KCl withdrawal. Shown are Western immunoblots comparing intact spectrin and its breakdown products (upper panel), CaMKIV (middle panel), and p35 (lower panel) in granule neurons grown in 5 mM KCl, 30 mM KCl, or 30 mM KCl followed by KCl withdrawal at 7 DIV (30–5 mM) as indicated. Whole cell homogenates were harvested at 8 DIV and processed for immunoblot analysis (30 μg/lane).
phorylation of its substrate CREB and, perhaps as a consequence, ultimately leads to a compensatory increase in the synthesis of total CREB protein.

Chronic exposure of GCs to elevated KCl also has a profound effect on immunoreactive CaMKK, an upstream regulator of CaMKIV. Note that there are two distinct CaMKK isoforms designated α (~64 kDa) and β (~70 and 73 kDa, representing alternative splice variants) (58, 59). The olfactory bulb predominately expresses CaMKKα, whereas the cerebellum and cerebral cortex express both isoforms, with proportionately more CaMKKβ in adult cerebral cortex (59). These region-specific differences were verified in the Western immunoblots shown in Fig. 5C. As exemplified in Fig. 5A (second row), both isoforms of CaMKK were significantly down-regulated by at least 80% (n = 5) in GCs grown in 25 mM KCl versus 5 mM KCl. Interestingly, although GCs are reported to express proportionately more CaMKKβ, there appears to be a maturation-dependent switch from the predominant expression of CaMKKα to CaMKKβ between 5 and 9 DIV (Fig. 5A). At least two possibilities could account for the observed down-regulation of CaMKK: (i) that calpain-mediated proteolysis of CaMKIV interferes with CaMKIV-mediated transcription, including a decrease in the synthesis of mRNA encoding CaMKK, and (ii) that CaMKK, a substrate for calpain, is directly down-regulated/proteolyzed by calpain. The latter possibility seems unlikely since CaMKK is localized in the cytosolic compartment (confirmed herein using immunocytochemistry; data not shown), which should not contain activated calpain under survival-promoting culture conditions. Nevertheless, to distinguish between these possibilities, the expression of CaMKKβ mRNA was evaluated by reverse transcription-PCR in GCs grown in 5 mM KCl or after overnight exposure of cultures to 25 mM KCl (i.e. supplemented with 20 mM KCl). For comparison, expression of mRNAs encoding CaMKIV and actin was also assessed in the same samples. As exemplified in Fig. 6A, comparable amounts of a 193-bp amplicon, the size predicted for CaMKK, were observed in cultures grown in 5 mM KCl or supplemented with 20 mM KCl. This result is consistent with the theory that depolarization-dependent down-regulation of CaMKIV is due to calpain-mediated proteolysis and not reduced CaMKIV synthesis. In contrast, a decrease in the 928-bp amplicon, the size predicted for CaMKKβ, was seen in cultures receiving KCl supplementation compared with 5 mM KCl (observed in three different cell preparations). There were no apparent intersample differences when amplicons corresponding to actin mRNA were compared. Fig. 6B verifies that the amount of immunoreactive CaMKK/mg of homogenate protein was substantially reduced in GCs following overnight incubation in medium containing 25 mM KCl compared with 5 mM KCl. Shown for comparison in the same samples are relative amounts of immunoreactive CaMKIV, calpain (regulatory subunit), and NF-H. As expected, there was a reduction in CaMKIV, an increase in neurofilament protein, and no apparent change in calpain in 25 mM KCl-containing cultures compared with those grown in 5 mM KCl. Taken together, these data suggest that the observed depolarization-mediated reduction in immunoreactive CaMKK is due to a decrease in mRNA and not calpain-mediated proteolysis of cytosolic CaMKK.

**DISCUSSION**

Calpains are generally considered to be cytoplasmic enzymes that degrade cytosolic, membrane, and cytoskeletal substrates in response to cell injury or stress. As such, most published reports describe their involvement in pathologies, including ischemia, cataract formation, muscular dystrophies, Alzheimer’s and Huntington’s diseases, and myocardial infarcts (Refs. 6–8; reviewed in Ref. 36). Additionally, some investigations have focused on the physiological functions of calpains, which include regulation of adhesion, migration, differentia-

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2 K. K. W. Wang, personal communication.
tion, and progression through the cell cycle (Ref. 60; reviewed in Refs. 4 and 14). A limited but growing body of evidence indicates that calpains are present in the nucleus of many cell types, which, as presented herein, GCs. In some cases, nuclear localization of calpains is developmentally regulated or sensitive to changes in the environment. For example, both m-calpain and μ-calpain are predominantly nuclear in myoblasts, but become cytosolic as they differentiate into myotubes (38, 62); and translocation of cytosolic calpains to the nucleus has been observed in Schwann cells treated with growth factors (63), in lens epithelial cell lines treated with ionomycin (64), and in hippocampal neurons exposed to ischemia (65). In A431 cells, nuclear translocation of calpain is ATP-dependent (37). These and similar observations (39, 66) support the notion that calpains can be shuttled between nuclear and cytosolic compartments and have distinct functions in proliferating and differentiated cells.

Structural analyses of calpains support immunolocalization studies, and various isoforms have been evaluated for NLSs and nuclear export sequences. For example, the predicted gene product of calpain-10 contains an NLS in domain III (77), comprised of a stretch of positively charged residues (67). Likewise, calpain-3, present in ~45% of human muscle cells (68), contains an NLS (69). Although lacking conventional NLSs, two α-helices in domain I of the ubiquitous calpains are positively charged and necessary for nuclear localization (11). Consistent with this, positively charged helical structures are nuclear targeting structures in other proteins (70, 71). Nuclear export sequences are characterized by multiple leucine residues with the spacing LX_{2,3}LX_{2,3}LX_{4} (72, 73), and CRM-1 substrates containing such sequences include the human immunodeficiency virus type 1 Rev protein (LPLLPERTLTL), protein kinase inhibitor-α (LALKLAGLDI), and p53 (FRELH9262NEALEL) (73). Notably, m-calpain (LDDLWLSLF) and μ-calpain (FDLFWKQLQ) contain similar sequences in their C-terminal catalytic domains. We previously demonstrated that inhibition of CRM-1, a receptor for proteins containing nuclear export sequences (74), with leptomycin B exacerbates CaMKIV proteolysis in GCs grown in medium containing 25 mM KCl or N-methyl-d-aspartate (NMDA) and triggers CaMKIV proteolysis in GCs grown with either of two trophic agents that enhance long-term survival, elevated KCl (25 mM final concentration) or NMDA. We showed that CaMKIV down-regulation is dependent on Ca^{2+} entry through VSCCs (inhibited by nifedipine or nimodipine in 25 mM KCl-containing cultures) or NMDA receptor channels (inhibited by MK-801 or DL-2-amino-5-phosphonopentanoic acid in NMDA-containing cultures) and an active CaMK-dependent signaling cascade (inhibited by KN-62 or KN-93 in 25 mM KCl-containing cultures). It was often possible to distinguish an ~33–40 kDa proteolytic fragment of CaMKIV (e.g. Fig. 3B), predicted only to result from the activity of calpain or caspase-3 (31). However, the cleaved/active form of immunoreactive caspase-3 was detectable only in a small subset of dying granule neurons (Fig. 2E), consistent with its role as a terminal executioner of apoptosis (42–46). It was therefore ruled out as producing depolarization-dependent CaMKIV proteolysis in healthy viable GCs. Moreover, two-cell-permeable calpain inhibitors attenuated the depolarization-dependent loss of CaMKIV after overnight exposure of cultures to elevated KCl (1). Notably, these inhibitors are selective for calpains and do not target the proteasome, another major proteolytic pathway in cells. In fact, proteasome inhibitors themselves are quite toxic and rapidly trigger activation of caspase-3 and apoptosis in GCs (78–80) and in other cultured neurons (81–84).

We utilized several distinct and selective antibodies against the regulatory and catalytic subunits of m-calpain and μ-calpain in conjunction with laser confocal microscopy to directly demonstrate the enrichment of m-calpain in GC nuclei (Fig. 2). Subcellular fractionation analysis of nuclear enriched fractions also supported the nuclear localization in GCs of both regulatory and catalytic subunits of m-calpain (Fig. 3). In the same cultures, we verified previous reports in GCs (53, 54) that non-nuclear calpains catalyze the proteolysis of spectrin and p35 in response to trophic factor withdrawal (Fig. 4). Moreover, these substrates remained intact under trophic conditions of culture (i.e. chronic growth in medium containing 30 mM KCl), whereas nuclear CaMKIV was degraded. Similarly, several other non-nuclear as well as two nuclear substrates remained unchanged or increased in amount in GCs grown in medium containing 25 mM KCl compared with those grown in 5 mM KCl. These results indicate that calpain activation is restricted to the nuclear compartment under survival-promoting conditions of growth and that proteolysis of nuclear CaMKIV is a selective process. The observation that cotreatment of GCs with 20 mM KCl plus a potent and selective CaMKII/VII inhibitor (85), KN-62, attenuated KCl-mediated CaMKIV down-regulation (Fig. 1B) (1) suggests that calpain may specifically recognize the activated (i.e. phosphorylated or calmodulin-bound) form of CaMKIV. Possibly, activation of CaMKIV leads to structural modifications that render it susceptible to calpain-mediated proteolysis. Indeed, phosphorylation and/or calmodulin binding can target other substrates for calpain-mediated proteolysis (4, 36). It has been established that CaMKK phosphorylation of CaMKIV at Thr^{186} converts it to a more active form, increasing its affinity for calmodulin (27, 86). CaMKIV also undergoes autophosphorylation at several sites in its N-terminal serine-rich region (residues 8–20) with unknown consequences that

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remain to be explored (87, 88). Possibly then, CaMKIV in GCs grown with elevated KCl (or NMDA) becomes a target for calpain-mediated proteolysis as a result of its phosphorylation state or prolonged association with calmodulin. By down-regulating CaMKIV under conditions of sustained Ca\(^{2+}\) activation, nuclear calpain could modulate CaMKIV-dependent gene transcription in GCs, thereby preventing an excessive transcriptional response. This possibility is supported by the dramatic ∼10-fold reduction in the ratio of phosphorylated to total CREB in GCs grown in 25 mM KCl compared with those grown in 5 mM KCl (Fig. 6). Also, using microarray analysis of GCs grown in medium containing 5 mM versus 25 mM KCl, we observed depolarization-dependent induction and repression of dozens of gene products (89). An intact Ca\(^{2+}\)-CaMKIV-CREB signaling system is operative in neurons in vitro (90, 91) and in vivo (92, 93). Furthermore, the profound depolarization-dependent down-regulation of CaMKK mRNA and protein hints at the possibility that CaMKIV-mediated regulation of gene transcription may include the CaMKK gene. Additional studies should be revealing since, at present, little is known about the transcriptional regulation of CaMKK.

How can the depolarization-dependent down-regulation of CaMKIV be reconciled with its putative role in supporting survival? As depicted schematically in Fig. 7, enhanced long-term survival of GCs is observed in response to medium supplementation with elevated KCl and activation of L-type VSCCs (26). Medium supplementation with NMDA (or glutamate) also supports survival by enhancing Ca\(^{2+}\) influx through NMDA receptor channels (94). Addition of cell-permeable agents that inhibit calmodulin or CaMKII/IV interferes with survival under these growth conditions (24). Moreover, transfection with a dominant-negative construct of CaMKIV, but not CaMKII, interferes with depolarization-dependent survival (29, 30). As previously discussed, phosphorylation of CaMKIV by CaMKK increases its activity by 10–30-fold (56), and recent work indicates that catalytically active CaMKIV is the form that gains access to the cell nucleus (95). CaMKIV-mediated phosphorylation of CREB and other transcription factors regulates expression of prosurvival/anti-apoptosis genes (29, 57, 96). A mechanism known to regulate the activity of CaMKIV under transient conditions of Ca\(^{2+}\)-influx is dephosphorylation by protein phosphatase 2A, which is complexed with the inactive form of CaMKIV in the nucleus (97, 98). Our data support an additional level of regulation under conditions of sustained Ca\(^{2+}\) activity, viz. calpain-mediated proteolysis of CaMKIV. We hypothesize that calpain-mediated proteolysis of nuclear CaMKIV is an autoregulatory feedback response to the sustained activation of a Ca\(^{2+}\)-CaMKIV signaling pathway by 25 mM KCl or NMDA. Although this seems paradoxical in light of evidence supporting a critical role for CaMKIV in survival, there may remain a sufficient amount of intact CaMKIV to support the survival-promoting effects of elevated KCl and NMDA. This suggestion is based on evidence that addition of the CaMKII/IV inhibitor KN-62 triggers apoptosis and also inhibits acute KCl-mediated phosphorylation of CREB in GCs grown in medium containing 25 mM KCl (1). Further studies are needed to establish whether the target of KN-62 under these conditions is CaMKIV.

In summary, the ubiquitous expression of calpains in distinct subcellular compartments at different maturational stages and the diversity of substrates indicate that they are multifunctional effectors of myriad intracellular processes. Accumulating
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evidence indicates that some calpains are localized or translocated to the cell nucleus, where they influence the availability of transcription factors and other nuclear proteins. This study establishes the importance of calpains in regulating a Ca2+/calmodulin-dependent signaling cascade in granule neuronal nuclei. It also highlights the value of cerebellar granule neurons as a model to characterize these unique functions.

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Nuclear Calpain Regulates Ca\(^{2+}\)-dependent Signaling via Proteolysis of Nuclear Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase Type IV in Cultured Neurons
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