Calpain-cleaved Type 1 Inositol 1,4,5-Trisphosphate Receptor (InsP$_3$R1) Has InsP$_3$-independent Gating and Disrupts Intracellular Ca$^{2+}$ Homeostasis*§

Received for publication, April 25, 2011, and in revised form, August 2, 2011. Published, JBC Papers in Press, August 22, 2011, DOI 10.1074/jbc.M111.254177

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The type 1 inositol 1,4,5-trisphosphate receptor (InsP$_3$R1) is a ubiquitous intracellular Ca$^{2+}$ release channel that is vital to intracellular Ca$^{2+}$ signaling. InsP$_3$R1 is a proteolytic target of calpain, which cleaves the channel to form a 95-kDa carboxyl-terminal fragment that includes the transmembrane domains, which contain the ion pore. However, the functional consequences of calpain proteolysis on channel behavior and Ca$^{2+}$ homeostasis are unknown. In the present study we have identified a unique calpain cleavage site in InsP$_3$R1 and utilized a recombinant truncated form of the channel (capn-InsP$_3$R1) corresponding to the stable, carboxyl-terminal fragment to examine the functional consequences of channel proteolysis. Single-channel recordings of capn-InsP$_3$R1 revealed InsP$_3$-independent gating and high open probability ($P_o$) under optimal cytoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) conditions. However, some [Ca$^{2+}$]$_i$ regulation of the cleaved channel remained, with a lower $P_o$ in suboptimal and inhibitory [Ca$^{2+}$]$_i$. Expression of capn-InsP$_3$R1 in N2a cells reduced the Ca$^{2+}$ content of ionomycin-releasable intracellular stores and decreased endoplasmic reticulum Ca$^{2+}$ loading compared with control cells expressing full-length InsP$_3$R1. Using a cleavage-specific antibody, we identified calpain-cleaved InsP$_3$R1 in selectively vulnerable cerebellar Purkinje neurons after in vivo cardiac arrest. These findings indicate that calpain proteolysis of InsP$_3$R1 generates a dysregulated channel that disrupts cellular Ca$^{2+}$ homeostasis. Furthermore, our results demonstrate that calpain cleaves InsP$_3$R1 in a clinically relevant injury model, suggesting that Ca$^{2+}$ leak through the proteolyzed channel may act as a feed-forward mechanism to enhance cell death.

Changes in cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) act as a ubiquitous signaling system that is essential to proper neuronal function and survival. Conversely, disrupted [Ca$^{2+}$]$_i$ can serve as a trigger for cell death (1, 2). In particular, compelling evidence suggests that disruption of cellular Ca$^{2+}$ homeostasis, caused in part by dysfunction of Ca$^{2+}$ regulatory proteins, plays a causal role in both acute brain injury and chronic neurodegenerative diseases (3).

The inositol 1,4,5-trisphosphate receptor (InsP$_3$R), a ubiquitous intracellular Ca$^{2+}$ release channel located on the endoplasmic reticulum (ER) membrane, may be an important component of the pathologic cascades leading to disrupted Ca$^{2+}$ homeostasis in many disease states. Cells deficient in InsP$_3$Rs are resistant to apoptosis (4, 5) suggesting that InsP$_3$R-mediated Ca$^{2+}$ signaling plays a mechanistic role in cell death. Altered InsP$_3$R channel function induces aberrant neuronal Ca$^{2+}$ signaling in a variety of neurodegenerative diseases including Alzheimer disease (6, 7), Huntington disease (8), and ischemia (9). Observations in brain ischemia models also suggest altered InsP$_3$R function, specifically, decreased InsP$_3$R binding (10, 11), decreased InsP$_3$-induced Ca$^{2+}$ release (12), and depletion of releasable Ca$^{2+}$ stores (13, 14). Proteolytic cleavage of InsP$_3$R could explain these observations.

The type 1 InsP$_3$R (InsP$_3$R1), the predominant neuronal isoform, is a substrate for both the caspase and calpain families of cysteine proteases (15). These proteases are indirectly (caspase-3 and directly (calpain) activated by Ca$^{2+}$ and are known to play a central role in apoptotic and necrotic cell death pathways (16). Proteolytic activity of these enzymes is limited and site-specific, typically altering rather than eliminating substrate function. In the case of Ca$^{2+}$ regulatory proteins, this may initiate a positive feedback loop that further increases protease activation via increases in [Ca$^{2+}$]$_i$.

Caspase-3 or calpain cleavage of InsP$_3$R1 generates carboxyl-terminal fragments of ~95 kDa (17). Caspase-3 cleaves InsP$_3$R1 at a highly conserved DEVD consensus sequence within the coupling domain (17) (Fig. 1A). Caspase-mediated proteolysis of the channel has been observed in several models of apoptosis (17–19). Previous studies have demonstrated altered ER Ca$^{2+}$ homeostasis in cell lines expressing a recombinant caspase-derived carboxyl-terminal fragment of InsP$_3$R1, suggesting that cleavage generates an unregulated channel that may leak Ca$^{2+}$.

* This work was supported, in whole or in part, by National Institutes of Health Grant Grants NS039481 (to R. W. N.), NS069951 (to R. W. N.), NS071828 (to C. M. K.), MH059937 (to J. K. F.), and GM065830 (D.-O. D. M. and J. K. F.). This work was also supported by American Heart Association Grant Grants NS039481 (to R. W. N.), NS069951 (to R. W. N.), NS071828 (to C. M. K.).

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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§ The abbreviations used are: InsP$_3$R, inositol 1,4,5-trisphosphate (InsP$_3$) receptor; InsP$_3$R1, type 1 InsP$_3$R; ER, endoplasmic reticulum; N2a, Neuro-2A; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N',N' ‑tetraacetic acid; HEDTA, N-(2-hydroxymethyl)ethylendiaminetriacetic acid; SERCA, sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase; EGFP, enhanced green fluorescence protein.
from intracellular stores (20–22). InsP$_3$R1 is also a substrate for calpain, which sequentially cleaves the protein into 200–, 130–, and 95-kDa carboxyl-terminal fragments (23–25). However, the specific calpain cleavage site that generates the stable 95-kDa fragment has not been established, and the functional consequences of proteolysis on channel activity are unknown. We hypothesized that calpain-mediated proteolysis, which removes the amino-terminal ligand binding domain and a large portion of the regulatory domain, generates a channel with InsP$_3$-independent gating that leaks Ca$^{2+}$ from ER stores.

In the present study we have identified the unique calpain cleavage site of InsP$_3$R1 and investigated the electrophysiological and functional properties of the truncated carboxyl-terminal channel. Using a combination of single-channel nuclear patch clamp electrophysiology and single-cell Ca$^{2+}$ imaging, we have determined that the cleaved channel is constitutively active in the absence of InsP$_3$, although its gating retains sensitivity to [Ca$^{2+}$]. Constitutive channel activity accounts for an observed reduction in the Ca$^{2+}$ content of the ER lumen in cells expressing recombinant calpain-cleaved InsP$_3$R1. Importantly, we provide evidence of calpain-mediated proteolysis of InsP$_3$R1 in an in vivo model of ischemic brain injury. These data highlight the important functional consequences of calpain-mediated channel proteolysis, which may critically disrupt intracellular Ca$^{2+}$ homeostasis, particularly under pathologic conditions where other Ca$^{2+}$ regulatory proteins are also compromised.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise noted, all chemical reagents were purchased from Sigma.

**Antibodies**—The anti-InsP$_3$R1 polyclonal antibody targeted against the 20 carboxyl-terminal residues of rat InsP$_3$R1 was generously provided by Dr. Suresh Joseph (Thomas Jefferson University) (26). The antibody to calpain-cleaved spectrin (Ab38) was generously provided by Dr. Robert Siman (University of Pennsylvania). Anti-calreticulin polyclonal antibody was purchased from Thermo Scientific Pierce Antibodies (PA3–900). Alexa-488 conjugated secondary antibody used for immunofluorescence was purchased from Invitrogen. The antibody against calpain-cleaved InsP$_3$R1 (Ab2054) was generated against the peptide ASAATRKC and is described under “Results.”

**Cerebellar Microsome Isolation**—Male Long-Evans rats were deeply anesthetized with pentobarbital (200 mg/kg) and decapitated. The brain was extracted, and the cerebellum was dissected and homogenized in cold MSHE buffer: 220 mM mannitol, 70 mM sucrose, 2 mM K-HEPES (pH 7.4), 0.5 mM EGTA with 0.1% fatty-acid free bovine serum albumin (BSA), and protease inhibitor mixture. The homogenate was centrifuged at 650 × g for 10 min at 4 °C to remove nuclei (P1). The supernatant from the preceding fraction (S1) was centrifuged again at 8000 × g for 10 min at 4 °C to separate cytoplasm and microsomes from mitochondria and synaptosomes. The remaining supernatant (S2) was centrifuged at 100,000 × g for 1 h in a micro-ultracentrifuge (ThermoFisher Scientific). The pellet from the final spin (P3) containing microsomes was resuspended in either MSHE buffer without BSA for Western blot or digest buffer for subsequent in vitro digest, snap-frozen, and stored at −20 °C.

**In Vitro Caspase-3 and Calpain-1 Digests of Microsomes and InsP$_3$R1 Peptide**—For in vitro caspase-3 digest of cerebellar microsomes, freshly isolated microsomes were resuspended in lysis buffer (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM DTT (Fisher), with 1% Triton X-100) and incubated at 4 °C for 90 min before snap-freezing. For subsequent digest, lysed microsomes were diluted to 1 μg/μl in digest buffer (100 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 20 mM DTT). Samples were preincubated with 1 μM calpastatin peptide (EMD Biosciences) to inhibit endogenous calpain activity and then digested with recombinant active human caspase-3 (0.05 units/μl; Millipore) for 4 h at 37 °C. Loading buffer was added to the digest to stop the reaction.

For in vitro calpain digest of cerebellar microsomes, freshly isolated microsomes were resuspended in a modified digest buffer (25 mM HEPES (pH 7.4), 250 mM sucrose, 1 mM EDTA) and snap-frozen. For subsequent digest, microsomes were diluted to 1 μg/μl in complete digest buffer (25 mM HEPES (pH 7.4), 250 mM sucrose, 1 mM EDTA, 2 mM DTT, and 1 mM CaCl$_2$). Samples were preincubated with 1 mM benzoyl-arginyl-valyl-0-fluoromethyl ketone (Promega) to inhibit endogenous caspase activity and then digested with 0.5 μM calpain-1 (μ-calpain) from human erythrocytes (EMD Biosciences) at 4 °C for various times. Loading buffer was added to the digest to stop the reaction.

For in vitro calpain digest of the GST-InsP$_3$R1 fusion peptides, purified peptide was dialyzed in digest buffer (25 mM HEPES (pH 7.5), 250 mM NaCl, 2 mM DTT, 1 mM CaCl$_2$ with 1.2% CHAPS). Samples were digested with 0.1 μM calpain-1 at 4 °C for various times. Adding loading buffer to the digest terminated the reaction.

**Edman Amino-terminal Sequencing to Determine Cleavage Site**—Amino-terminal automated Edman sequencing was performed on an Applied Biosystems 494 Protein Sequencer using standard programs and reagents (Wistar Institute, Proteomics Core Facility). Samples were excised from stained PVDF membranes, wetted with MeOH, and sonicated in MilliQ water for 5 min. Samples were then removed and placed in MeOH before loading into the upper sample cartridge unit. The cartridge was assembled, and the sample was dried with argon, inserted into the instrument, and processed with a standard pulsed liquid method for 8 cycles.

**Plasmids**—The pIRE2-EGFP (Clontech) expression vector for wild-type (wt) rat InsP$_3$R1 was previously generated (27). To construct the expression vector for calpain-cleaved InsP$_3$R1
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(137 mM NaCl, 2 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 7.3)) to measure Ca$^{2+}$ with or without 100 μg/ml digitonin for 2 min in MgATP-free cytoplasm-like medium (140 mM KCl, 20 mM NaCl, 1 mM EGTA, 0.375 mM CaCl$_2$ (final concentration ≈ 70 nM), 20 mM PIPES (pH 7.3)). Cells were perfused with cytoplasm-like medium for 30 min to wash out digitonin and deplete ER Ca$^{2+}$ stores. ER store loading was activated by the addition of 1.5 mM MgATP to the perfusate to stimulate SERCA-mediated Ca$^{2+}$ uptake. After filling, the passive ER Ca$^{2+}$ leak was evaluated by measuring [Ca$^{2+}$]$_{ER}$ during exposure to 10 μM cyclopiazonic acid (Calbiochem) in the absence of MgATP. Mag-Fura-2 excitation and emission were monitored as described above. Rates for ER loading and release were calculated by fitting individual single-cell responses using single exponential functions and determining the mean of those rates.

Changes in [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_{ER}$ are presented as changes in fluorescence ratio. Dye calibration was achieved by applying experimentally determined constants to the equation [Ca$^{2+}$] = $K_d B(R - R_{\text{min}})/(R_{\text{max}} - R)$. Macrosc for analysis were custom macros written for IGOR Pro (WaveMetrics).

Cardiac Arrest Model—Male Long-Evans rats weighing 300–350 g (Harlan Laboratories, Inc.) were subjected to asphyxial cardiac arrest followed by cardiopulmonary resuscitation and post-cardiac arrest temperature regulation as previously described (32–34). Rats were anesthetized, orotracheally intubated, and mechanically ventilated. Temperature was maintained between 37.0 and 37.5 °C. To initiate cardiac arrest, rats were chemically paralyzed using intravenous vecuronium (2 mg/kg), and asphyxia was induced by discontinuing mechanical ventilation. Cessation of arterial pulse pressure and reduction of mean arterial pressure to ≥20 mm Hg was used to confirm circulatory arrest, which usually occurred within 3–4 min. After the 7-min asphyxia, mechanical ventilation resumed with 100% O$_2$, intravenous epinephrine (0.005 mg/kg) and HCO$_3^-$ (1.0 mEq/kg) were administered, and external chest compressions were performed (350–400 compressions/min). After the return of spontaneous circulation, rats were maintained on mechanical ventilation for 1 h. An intraperitoneal telemetric temperature probe was surgically implanted in the abdomen (Data Systems International). Rats were then extubated and transferred to a computer controlled temperature regulation chamber, which telemetrically monitored intraperitoneal temperature. Post-cardiac arrest body temperature was maintained between 36.5 and 37.5 °C using software-driven relays connected to a heat lamp, water misters, and a cooling fan (32, 35). This study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Immunohistochemical Staining of Cerebellum—At the indicated time points after cardiac arrest (24 or 48 h), rats were

with 10 mM MnCl$_2$ and 10 μM ionomycin). The remaining background fluorescence after Mn$^{2+}$ quench was subtracted during analysis.

To measure ER luminal Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{ER}$), transfected cells (6 h post-transfection) were loaded with the low affinity Ca$^{2+}$ indicator Mag-Fura-2-AM (Invitrogen; 10 μM) for 30 min at 37 °C in HEPES-HBSS buffer (Hanks’ balanced salt solution supplemented with 10 mM HEPES, 4.2 mM NaHCO$_3$, 1.8 mM CaCl$_2$ and 0.8 mM MgCl$_2$ (pH 7.3)) with 1% BSA. After loading, cells were permeabilized with 10 μg/ml digitonin for 2 min in MgATP-free cytoplasm-like medium (140 mM KCl, 20 mM NaCl, 1 mM EGTA, 0.375 mM CaCl$_2$ (final concentration ≈ 70 nM), 20 mM PIPES (pH 7.3)). Cells were perfused with cytoplasm-like medium for 30 min to wash out digitonin and deplete ER Ca$^{2+}$ stores. ER store loading was activated by the addition of 1.5 mM MgATP to the perfusate to stimulate SERCA-mediated Ca$^{2+}$ uptake. After filling, the passive ER Ca$^{2+}$ leak was evaluated by measuring [Ca$^{2+}$]$_{ER}$ during exposure to 10 μM cyclopiazonic acid (Calbiochem) in the absence of MgATP. Mag-Fura-2 excitation and emission were monitored as described above. Rates for ER loading and release were calculated by fitting individual single-cell responses using single exponential functions and determining the mean of those rates.

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Immunohistochemical Staining of Cerebellum—At the indicated time points after cardiac arrest (24 or 48 h), rats were
anesthetized with pentobarbital (200 mg/kg) and transcardially perfused with cold phosphate-buffered saline (PBS (pH 7.4)) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted, post-fixed in 4% paraformaldehyde overnight, and cryoprotected using serial incubations in 0.1 M phosphate buffer containing 10, 20, and 30% sucrose. Serial sagittal sections through the cerebellum (40 μm) were cut on a freezing sliding microtome and stored in cryoprotectant (0.1 M phosphate buffer with 30% sucrose and 30% glycerol) at −20 °C for future use.

Immunohistochemistry to identify calpain-cleaved InsP3R1 (Ab2054) required heat-induced epitope retrieval. Cerebellar tissue sections were removed from cryoprotectant, rinsed in distilled H2O, and incubated in citrate buffer (10 mM sodium citrate with 0.05% Tween 20 (pH 6.0)) at 95 °C for 20 min. After heat-induced epitope retrieval, tissue was removed from heat and rinsed in PBS (pH 7.2). Standard immunohistochemical staining procedures followed.

Floating cerebellar brain sections were rinsed in PBS (pH 7.2) and blocked using PBS with 4% normal goat serum and 0.5% Triton X-100. Sections were incubated in primary antibody (Ab38 or Ab2054) diluted in block solution overnight at 4 °C. Sections were rinsed with PBS and incubated with Alexa 488 fluorescent secondary antibody (Invitrogen). Finally, sections were rinsed with PBS, counterstained with Hoechst (5 μg/ml in PBS) to label nuclei, mounted on slides, and cover-slipped using Permount (Fisher). Immunofluorescence was studied at 100× using an epifluorescence microscope (Leica DM4500B).

Analyses and Statistics—Data are presented as the mean ± S.E., and statistical significance of differences between the means was assessed using either unpaired t tests or analysis of variance for repeated measures using Barlett’s test for equal variances and a Bonferroni correction. Differences between means were accepted as statistically significant at the 95% level (p < 0.05).

RESULTS

Identifying the Calpain Cleavage Site of InsP3R1—InsP3R1 is a known substrate of calpain that sequentially cleaves the protein into 200-, 130-, and 95-kDa carboxyl-terminal fragments, with the 95-kDa fragment being the stable, predominant cleavage product (Fig. 1B) (23–25). Although the 95-kDa calpain-derived fragment is similar in size to the caspase-derived fragment, the cleavage sites are different. To determine the calpain cleavage site of InsP3R1, we first examined the relative sizes of the caspase- and calpain-cleaved carboxyl-terminal fragments of InsP3R1 using in vitro digests of rat cerebellar tissue, which is enriched in InsP3R1. We performed in vitro digests of cerebellar microsomes with either recombinant caspase-3, or exogenous calpain-1. A Western blot of the digest products using a carboxyl-terminal InsP3R1 antibody demonstrated loss of the full-length protein and generation of stable carboxyl-terminal fragments of ∼95 kDa (Fig. 1C). Shown here for the first time, calpain cleavage of InsP3R1 generates a slightly smaller fragment than the fragment generated by caspase cleavage.

Caspase cleavage of InsP3R1 is known to occur at the carboxy-terminal end of theDEVD consensus sequence (Fig. 1A) (17). Based on the relative sizes of the caspase and calpain InsP3R1 cleavage products, we deduced that the calpain cleavage site responsible for generating the 95-kDa stable fragment was less than 30 residues downstream of the caspase-3 cleavage site. To identify the site we utilized a GST fusion peptide containing residues 1583–1932 of rat InsP3R1, which includes the calpain-3 cleavage site (Fig. 1A). As a control, we used a GST fusion peptide containing an adjacent sequence within InsP3R1 (residues 1932–2257) that does not include the calpain-3 cleavage site (supplemental Fig. 1A). We digested both GST fusion peptides with exogenous calpain-1, separated reaction products using SDS-PAGE, and transferred them to PVDF membranes for protein staining. Protein staining of digest products identified a single proteolytic fragment derived from the 1582–1932-amino acid peptide but not the 1932–2257 amino acid peptide, confirming that the calpain cleavage site is between residues 1582 and 1932 (supplemental Fig. 1B). The addition of the calpain inhibitor calpastatin blocked generation of the proteolytic fragment produced by calpain digest of InsP3R1 peptide 1582–1932 amino acid (Fig. 1D). Eight rounds of Edman degradation and amino-terminal sequencing of the proteolytic fragment from the calpain-digested GST fusion peptide returned four potential sequences, only one of which (1918ASAATRAK) was downstream of the caspase-DEVD site (Fig. 1E). Based on the location of this sequence in InsP3R1, the predominant calpain cleavage product is expected to have a molecular mass of 94.84 kDa. This fragment is 3.02 kDa smaller than the caspase-cleaved fragment, which is consistent with Western blot comparisons of calpain and caspase in vitro digests of microsomal InsP3R1 (Fig. 1C). These data demonstrate that calpain cleaves rat InsP3R1 between residues 1917 and 1918 to generate the 95-kDa carboxyl-terminal fragment. The sequences surrounding the cleavage site are highly conserved in InsP3R1 across species, although it is not present in type 2 or type 3 receptors.

Calpain-cleaved InsP3R1 Antibody—To facilitate investigations of calpain-cleaved InsP3R1, we generated an antibody specific to the calpain-derived carboxyl-terminal fragment of the channel using a neoeptope specific approach (36). The peptide ASAATRKAC, corresponding to the new amino terminus of InsP3R1 after calpain cleavage, was synthesized and covalently linked to a keyhole limpet hemocyanin to elicit an immune response (Fig. 2A; Covance ImmunoTechnologies). A New Zealand White rabbit (2054) was immunized by subcutaneous injections of the peptide, and serum ELISA demonstrated a 1:843,000 titer at the time of test-bleed 1.

We tested specificity of Ab2054 for the 95-kDa calpain-derived InsP3R1 fragment by Western blot of rat cerebellar microsomes after in vitro digestion. Using a carboxyl-terminal
InsP$_3$R1 antibody, we detected full-length, caspase-digested and calpain-digested InsP$_3$R1 samples (Fig. 2B). Using Ab2054 on the same blot, we detected no signal in undigested microsomes or microsomes digested with caspase-3, but a single band at $\sim$95 kDa was observed in microsomes digested with calpain-1, indicating the antibody reacts exclusively with the stable 95-kDa calpain-cleaved form of InsP$_3$R1 (Fig. 2C). These data validate Ab2054 as specific antibody for calpain-cleaved InsP$_3$R1.

Expression of Recombinant InsP$_3$R1 Constructs—To examine the consequences of calpain cleavage of InsP$_3$R1 on channel function, we generated a truncated cDNA of the protein (Δ1–1917) corresponding to the stable carboxyl-terminal fragment derived from calpain cleavage (capn-InsP$_3$R1). We used full-length wt rat InsP$_3$R1 as a control (Fig. 3A).

For all functional experiments, we studied wt- and capn-InsP$_3$R1 expressed transiently in N2a mouse neuroblastoma cells. We chose N2a cells because they are neural in tissue origin and have high transfection efficiency. To examine the time course of expression of recombinant rat InsP$_3$R1 in N2a cells, we transiently transfected cells with either wt-InsP$_3$R1 or capn-InsP$_3$R1 and harvested cells 6, 16, 24, or 48 h later. Western blotting of whole-cell lysates using the carboxyl-terminal InsP$_3$R1 antibody demonstrated expression of both wt- and capn-InsP$_3$R1 by 6 h post-transfection. For capn-InsP$_3$R1, expression was maximal at 24 and decreased by 48 h post-transfection, whereas expression of the wt channel continued to increase up to 48 h, suggesting toxicity or cellular regulation of gene expression associated with capn-InsP$_3$R1 (Fig. 3B). In lysates from wt-InsP$_3$R1 cells, we occasionally observed lower molecular weight fragments that are possibly proteolytic degradation products. In lysates from cells expressing capn-InsP$_3$R1, we observed high molecular weight smears that are likely aggregates of capn-InsP$_3$R1. Similar high molecular weight smears have been observed when expressing the caspase-3 cleaved form of InsP$_3$R1 (21). These smears were eliminated by harvesting capn-InsP$_3$R1-transfected cells in lysis buffer containing either 5M urea or 1% SDS (supplemental Fig. 2).

InsP$_3$-independent Gating of Capn-InsP$_3$R1—To determine whether capn-InsP$_3$R1 still functioned as an ion channel, we examined single-channel activity in native ER membranes. We utilized patch clamp electrophysiology of the outer membrane of isolated N2a cell nuclei to study the channel activities of recombinant InsP$_3$R1 constructs (15). To maximize the likelihood of recording recombinant channels, we performed patch clamp experiments at 24 h post-transfection, when expression of capn-InsP$_3$R1 was maximal. We monitored channel activity...
with the pipette solution (cytoplasmic side) containing 2 μM Ca²⁺ and 0.5 mM ATP. In untransfected cells, we observed endogenous InsP₃R with a single-channel conductance of 395 ± 12 pico siemens with saturating 10 μM InsP₃ in the pipette solution (supplemental Fig. 3, A and B). In nuclei from cells transfected with recombinant rat wt-InsP₃R1, we observed channel activities that had a smaller single-channel conductance (230 ± 10 pico siemens; supplemental Fig. 3C). Despite their smaller single-channel conductance, we identified these channels as InsP₃R based on their dependence on InsP₃ for activation (n = 10; Fig. 4A) and their sensitivity to competitive inhibition by heparin (100 μg/ml in pipette solution; n = 5; Fig. 4, A, C, and D). With 10 μM InsP₃ in the pipette solution, the recombinant wt channels had an open probability (pₒ) of 0.69 ± 0.08 (n = 8; Fig. 4, A, C, and D). In cells transfected with capn-InsP₃R1, we observed channel activities with a single-channel conductance (230 ± 10 pico siemens) similar to the recombinant wt-InsP₃R1 (supplemental Fig. 3D). Strikingly, we observed these channels even in the absence of InsP₃ in the pipette solution. Exposed to pipette solution containing no InsP₃, 2 μM Ca²⁺, and 0.5 mM ATP, the spontaneously active capn-InsP₃R1 channels had high pₒ in either the absence (pₒ = 0.82 ± 0.04, n = 9) or presence (pₒ = 0.74 ± 0.09, n = 5) of heparin (Fig. 4, B–D). These results indicate that a truncated InsP₃R1 corresponding to the carboxyl-terminal calpain cleavage fragment forms a functional ion channel with InsP₃-independent gating.

Ca²⁺ Regulation of Capn-InsP₃R1 Gating—Channel activity of InsP₃R1 is both activated and inhibited by Ca²⁺, resulting in a biphasic, bell-shaped pₒ dependence on [Ca²⁺]ᵢ (15). Although InsP₃ regulation of the truncated channel was absent, we asked whether any Ca²⁺ regulation of capn-InsP₃R1 remained. Accordingly, we next examined channel activity at low Ca²⁺ concentration (70 nM), typical of resting cytoplasmic levels. With 10 μM InsP₃ and 70 nM free Ca²⁺ in the pipette solution, pₒ of wt-InsP₃R1 was 0.016 ± 0.004 (n = 8; Fig. 5, A and C), significantly lower than the observed pₒ in 2 μM Ca²⁺ (pₒ ~ 0.7; p < 0.001). With no InsP₃ and 70 nM free Ca²⁺ included in the pipette solution, pₒ of capn-InsP₃R1 was 0.26 ± 0.06 (n = 5; Fig. 5, B and C), also significantly lower than the observed pₒ in 2 μM Ca²⁺ (pₒ ~ 0.8; p < 0.001) but significantly higher than that of wt-InsP₃R1 at low Ca²⁺ (p < 0.001). These

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**FIGURE 2. Antibody targeted against calpain-cleaved InsP₃R1.** A, shown is the calpain cleavage site of rat InsP₃R1 and the corresponding synthetic peptide used to generate polyclonal antibody Ab2054. KLH, keyhole limpet hemocyanin. B, shown is a Western blot of rat cerebellar microsomes after in vitro digestion with caspase-3 or calpain-1 using carboxyl-terminal InsP₃R1 antibody that reacts with unproteolyzed, caspase-cleaved, and calpain-cleaved InsP₃R1. Ab, antibody. C, shown is the Western blot in B, stripped and reprobed using Ab2054 (1:10,000). Ab2054 reacts specifically with the 95-kDa carboxyl-terminal fragment of calpain-cleaved InsP₃R1.

**FIGURE 3. Schematic representation and expression of recombinant capn-InsP₃R1.** A, the domain structure of the full-length (wt) and calpain-cleaved InsP₃R1 recombinant proteins is shown. B, shown is Western blot analysis of whole cell lysates from untransfected N2a cells and N2a cells expressing wt- or capn-InsP₃R1 at 6, 16, 24, and 48 h post-transfection. Carboxyl-terminal InsP₃R1 antibody was used to detect endogenous and recombinant InsP₃R1. Antibody against actin (1:3000) was used as the loading control.
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FIGURE 4. Capn-InsP₃R1 channel activity in the absence of InsP₃. A, shown are representative current traces recorded in the outer membrane of nuclei isolated from N2a cells transfected with wt-InsP₃R1 in the absence (top panel) or presence (middle/bottom panels) of saturating InsP₃ in the pipette solution. The pipette solution contained 2 μM Ca²⁺. The arrows indicate closed channel current level. Channel activity of wt-InsP₃R1 required InsP₃ and was inhibited by heparin; B, shown are representative current recordings in isolated nuclei from N2a cells expressing capn-InsP₃R1 in the absence of InsP₃. Heparin did not inhibit channel activity of capn-InsP₃R1, confirming that InsP₃-independent gating was not caused by local InsP₃ in the patch pipette. C, shown is a summary of the effects of Ca²⁺ concentration on Pₒ of both wt- and capn-InsP₃R1 at 70 nM and 25 μM Ca²⁺. Pₒ of capn-InsP₃R1 was 0.22 ± 0.06 (n = 8; Fig. 5, A and C). In the absence of InsP₃, with heparin (100 μg/ml) and 25 μM free Ca²⁺ in the pipette solution, Pₒ of wt-InsP₃R1 was 0.39 ± 0.08 (n = 8; Fig. 5, B and C). Pₒ of wt- and capn-InsP₃R1 channels in 25 μM Ca²⁺ was significantly lower than the corresponding Pₒ of each channel in 2 μM Ca²⁺ (p < 0.001). Pₒ of capn-InsP₃R1 at 25 μM Ca²⁺ was not, however, significantly different from Pₒ of wt-InsP₃R1 at that Ca²⁺ concentration. These data demonstrate that Ca²⁺ regulation of channel activity is preserved in capn-InsP₃R1.

Decreased Intracellular Ca²⁺ Stores in Cells Expressing Capn-InsP₃R1—To investigate the functional consequences of InsP₃R1 proteolysis on cellular Ca²⁺ homeostasis, we first used single-cell Ca²⁺ imaging to examine [Ca²⁺], and estimate total Ca²⁺ content of intracellular stores. We identified individual transfected cells by EGFP expression driven by the pRES2-EGFP expression vector. To avoid potential complications from compensatory changes in gene expression, we performed Ca²⁺ imaging at an early time point (6 h) after transfection. [Ca²⁺], was monitored with Fura-2 in transiently transfected N2a cells expressing wt-InsP₃R1, capn-InsP₃R1, or a control plasmid (EGFP). We utilized the Ca²⁺ ionophore ionomycin (2 μM) to liberate Ca²⁺ from intracellular stores in the absence of extracellular Ca²⁺ and measured the resulting increase in data suggest that capn-InsP₃R1 may constitute an ER Ca²⁺ leak permeability in resting cells. To determine whether high Ca²⁺ still inhibits capn-InsP₃R1, we examined channel activity at 25 μM Ca²⁺, which corresponds to pathologic neuronal cytoplasmic levels immediately after ischemia (37, 38). With 10 μM InsP₃ and 25 μM free Ca²⁺ in the pipette solution, Pₒ of wt-InsP₃R1 was 0.22 ± 0.06 (n = 8; Fig. 5, A and C). In the absence of InsP₃, with heparin (100 μg/ml) and 25 μM free Ca²⁺ in the pipette solution, Pₒ of wt-InsP₃R1 was 0.39 ± 0.08 (n = 8; Fig. 5, B and C). Pₒ of wt- and capn-InsP₃R1 channels in 25 μM Ca²⁺ was significantly lower than the corresponding Pₒ of each channel in 2 μM Ca²⁺ (p < 0.001). Pₒ of capn-InsP₃R1 at 25 μM Ca²⁺ was not, however, significantly different from Pₒ of wt-InsP₃R1 at that Ca²⁺ concentration. These data demonstrate that Ca²⁺ regulation of channel activity is preserved in capn-InsP₃R1.

FIGURE 5. Ca²⁺ regulation of capn-InsP₃R1. A, representative current traces were recorded in the outer membrane of nuclei isolated from N2a cells transfected with wt-InsP₃R1 with 10 μM InsP₃, and 70 nM (top), 2 μM (middle), or 25 μM (bottom) Ca²⁺ included in the pipette solution. B, representative current traces were recorded in the outer membrane of nuclei isolated from N2a cells transfected with capn-InsP₃R1 in the absence of InsP₃ with 70 nM (top), 2 μM (middle), or 25 μM (bottom) Ca²⁺. C, shown is a summary of the effects of Ca²⁺ concentration on Pₒ of wt- and capn-InsP₃R1 (data at 2 μM Ca²⁺ are the same as shown in Fig. 4). Pₒ of both wt- and capn-InsP₃R1 at 70 nM and 25 μM Ca²⁺ were decreased compared with the Pₒ of either channel at 2 μM Ca²⁺ (unpaired t tests; **, p < 0.001). At 70 nM Ca²⁺, Pₒ of capn-InsP₃R1 was higher than that of wt-InsP₃R1 at 70 nM Ca²⁺ (unpaired t test with unequal variance; **, p < 0.001).
[Ca$^{2+}$], (Fig. 6A). We used ionomycin instead of a sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) inhibitor because it produces a much faster leak and consequent rise in [Ca$^{2+}$]$_i$, providing a better estimate of the Ca$^{2+}$ content of intracellular stores. Expression of capn-InsP3R1 did not effect resting [Ca$^{2+}$]$_i$ in N2a cells (Fig. 6B). However, expression of capn-InsP3R1 significantly decreased the ionomycin-induced peak [Ca$^{2+}$]$_i$ (252 ± 15 nM) compared with control cells (540 ± 30 nM; $p < 0.001$; Fig. 6C). These data indicate that capn-InsP3R1 causes partial depletion of intracellular Ca$^{2+}$ stores, consistent with the hypothesis that calpain-cleaved InsP3R1 is a Ca$^{2+}$ leak channel.

Decreased ER Ca$^{2+}$ Loading in Cells Expressing Capn-InsP3R1—To directly examine the ER Ca$^{2+}$ leak caused by truncated InsP3R1, we monitored [Ca$^{2+}$]$_{ER}$ in N2a cells using Mag-Fura-2 at 6 h post-transfection. We permeabilized the plasma membranes of Mag-Fura-2-loaded EGFP-expressing cells by a brief exposure to digitonin under microscopic observation. After depleting ER stores by washing with ATP-free cytoplasm-like medium for 30 min, we initiated Ca$^{2+}$ filling of ER stores by perfusing permeabilized cells with saturating 1.5 mM MgATP to stimulate the SERCA pump. After a steady-state [Ca$^{2+}$]$_{ER}$ was reached, we observed the ER Ca$^{2+}$ leak by inhibiting the pump with cyclopiazonic acid (10 μM) and removing ATP (Fig. 7A). Expression of capn-InsP3R1 significantly decreased the steady-state loading level of the ER to 26.8 ± 1.1 μM compared with 36.3 ± 0.9 μM in controls and 33.4 ± 1.0 μM in cells expressing wt-InsP3R1 ($p < 0.001$; Fig. 7B).

To compare the kinetics of ER Ca$^{2+}$ loading and passive leak in cells expressing various InsP3R1 constructs, we determined the average rate of ER loading and release by fitting each respective portion of single-cell responses with a single exponential equation. Expression of capn-InsP3R1 reduced the rate of ER Ca$^{2+}$ loading compared with cells expressing wt-InsP3R1, although the rate was similar to that observed in EGFP-expressing cells (Fig. 7C), suggesting that SERCA activity was sufficient to overcome the Ca$^{2+}$ leak through the truncated channel. Expression of capn-InsP3R1 and, to a lesser extent, wt-InsP3R1 significantly increased the ER Ca$^{2+}$ leak rate compared with control ($p < 0.001$; Fig. 7D). Taken together, these data demonstrate that calpain-cleaved InsP3R1 alters ER Ca$^{2+}$ homeostasis.

Calpain-mediated Proteolysis of InsP3R1 after in Vivo Ischemia—To determine whether calpain proteolysis of InsP3R1 occurs in vivo in a clinically relevant injury model, we examined
rat cerebellum after 7 min of asphyxial cardiac arrest. We chose this model because cerebellar Purkinje neurons express high levels of InsP$_3$R1 and are selectively vulnerable to transient global ischemia caused by cardiac arrest (39). We first performed Western blot analysis of rat cerebellar microsomes from naïve animals or from animals resuscitated from cardiac arrest. Using the carboxyl-terminal InsP$_3$R1 antibody, which reacts with both unproteolyzed and cleaved InsP$_3$R1, we observed evidence of InsP$_3$R1 proteolysis and the appearance of a faint $\sim$95-kDa fragment at both 1 and 24 h after return of spontaneous circulation (Fig. 8A). Using Ab2054, which specifically recognizes the stable 95-kDa carboxyl-terminal fragment generated by calpain proteolysis, we identified calpain-cleaved InsP$_3$R1 in subpopulations of Purkinje neurons in a distribution similar to that of calpain-cleaved spectrin (Fig. 8C). The intensity of Ab2054 staining was comparable at 24 and 48 h after cardiac arrest, suggesting that the calpain-derived InsP$_3$R1 fragment is more stable than the spectrin fragment.

Finally, to evaluate the relationship between calpain cleavage of InsP$_3$R1 and cell death, we used Fluoro-Jade B to identify degenerating neurons in the cerebellum (Fig. 8C). At both time points examined after cardiac arrest, we observed selective Fluoro-Jade labeling of subpopulations of Purkinje neurons. The intensity of Fluoro-Jade staining was greater at 48 h compared with 24 h post-cardiac arrest and was accompanied by a shrunken somatic morphology consistent with neurodegeneration. The morphological changes and increased Fluoro-Jade intensity in Purkinje cells at 48 h post-injury suggests that both
enhanced calpain activity and cleavage of \( \text{InsP}_3 \text{R1} \) precede neurodegeneration. We did not observe any Ab38, Ab2054, or Fluoro-Jade labeling in naïve tissue. Together, these data demonstrate that calpain cleaves \( \text{InsP}_3 \text{R1} \) in vivo in a clinically relevant model of brain ischemia and that calpain-mediated proteolysis of \( \text{InsP}_3 \text{R1} \) is an early molecular event in the injury cascade.

**DISCUSSION**

The present study is the first functional investigation of calpain-cleaved \( \text{InsP}_3 \text{R1} \). Based on our results from single-channel electrophysiology and \( \text{Ca}^{2+} \) imaging experiments, we conclude that capn-\( \text{InsP}_3 \text{R1} \) forms a dysregulated channel with \( \text{InsP}_3 \)-independent gating that functions as a leak channel in the ER. Moreover, evidence of calpain-mediated \( \text{InsP}_3 \text{R1} \) proteolysis in the brain after cardiac arrest demonstrates that \( \text{InsP}_3 \text{R1} \) proteolysis is a clinically relevant cellular pathway that is active in a neurodegenerative disease.

**Calpain Proteolyzes \( \text{InsP}_3 \text{R1} \) at a Unique Cleavage Site**—Previous reports (23–25) and data presented here (Fig. 1) establish that \( \text{InsP}_3 \text{R1} \) is a proteolytic target of calpain that cleaves the channel at multiple sites to form 200-, 130-, and 95-kDa carboxy-terminal fragments. Calpains, unlike caspases, do not use consensus sequences for target recognition and proteolysis.
Our data show that calpain cleaves InsP$_3$R1 between residues 1917 and 1918 to generate the stable 95-kDa channel fragment (Fig. 1, D and E). The InsP$_3$R$_1$ calpain cleavage site and surrounding residues are highly conserved in mouse and human homologs and are unique to the type 1 isoform of the channel. Our cleavage specific antibody (Ab2054; Fig. 2) targeting the new amino terminus of InsP$_3$R$_1$ after calpain proteolysis provides a useful tool for studying the truncated form of the channel in multiple species, including human.

To begin characterizing the functional consequences of calpain proteolysis of InsP$_3$R$_1$, we utilized a recombinant channel construct corresponding to the 95-kDa carboxyl-terminal fragment of InsP$_3$R$_1$ produced by calpain cleavage (Fig. 3). We expected the recombinant truncated InsP$_3$R$_1$ to tetramerize in the ER, as previous studies demonstrated that the transmembrane domains and carboxyl-terminal tail of the channel are sufficient for ER localization and oligomerization (40, 41). We had attempted to study capn-InsP$_3$R$_1$ on a null-background using the InsP$_3$R-deficient DT40 cell line. The extremely low transfection efficiency of these cells requires working in stable lines. However, we were unable to generate stable InsP$_3$R$_1$-deficient DT40 lines expressing capn-InsP$_3$R$_1$ despite our laboratory and others being able to generate stable lines expressing the similarly sized caspase-3 cleaved form of the channel (data not shown) (20, 22). The apparent toxicity associated with expression of capn-InsP$_3$R$_1$ in the DT40 cell system suggests that the truncated channel may cause cell death or attenuate proliferation. Instead of using stable lines, we studied capn-InsP$_3$R$_1$ in transiently transfected N2a cells at early time points after transfection to minimize the impact of potentially confounding compensatory changes as seen in stable lines overexpressing wt-InsP$_3$R$_1$ (42–44). Furthermore, studying capn-InsP$_3$R$_1$ in transiently transfected cells expressed under the CMV promoter strongly increases the probability that the recombinant truncated subunits form homotetramers. Thus, heteroligomerization of truncated subunits with endogenous, full-length InsP$_3$R$_1$ was unlikely in the studies presented here. However, understanding the possible existence and implications of heteroligomerization of truncated and full-length InsP$_3$R requires additional studies.

Our approach to studying the functional consequence of calpain proteolysis of InsP$_3$R$_1$ using capn-InsP$_3$R$_1$ does have limitations. Foremost, it is unknown if the amino terminus of InsP$_3$R$_1$ completely dissociates from the channel domain after proteolysis. Limited digestion of cerebellar microsomes with trypsin results in channel fragments that remain associated via noncovalent or indirect interactions (45, 46). The trypsinized channel also remains functional, demonstrated by InsP$_3$-induced Ca$^{2+}$ release from microsomes (46). Thus, expression of capn-InsP$_3$R$_1$ may not accurately reflect the conformation that exists after proteolysis in vivo. Caspase-3 proteolysis of InsP$_3$R$_1$, however, results in loss of InsP$_3$-mediated Ca$^{2+}$ release from microsomes in a manner that corresponds to the percentage of digestion (17). The same is likely true for calpain-cleaved InsP$_3$R$_1$. By studying capn-InsP$_3$R$_1$, we are likely examining a model of an InsP$_3$R$_1$ channel in which all four subunits have been completely proteolysed by calpain. Understanding the behavior of InsP$_3$R$_1$ channels with both intact and calpain-cleaved subunits warrants future investigation.

**Calpain-cleaved InsP$_3$R$_1$ Has InsP$_3$-independent, Ca$^{2+}$-dependent Channel Gating**—To explore how calpain cleavage affects InsP$_3$R$_1$ channel function, we examined the single-channel properties of recombinant capn-InsP$_3$R$_1$ using nuclear patch clamp electrophysiology. Recordings from N2a nuclei expressing recombinant InsP$_3$R$_1$ revealed channels with smaller single-channel conductance than that of endogenous InsP$_3$R$_1$. This difference is potentially mediated by interactions with endogenous proteins, which may modulate recombinant rat InsP$_3$R$_1$ activity differently than endogenous mouse InsP$_3$R. The low conductance of recombinant InsP$_3$R$_1$ was cell-type specific, as we did not observe such reduction in conductance when the channels were expressed in HEK293 cells. Nevertheless, it provided a means to distinguish recombinant and endogenous InsP$_3$R channels in N2a cells. In examining capn-InsP$_3$R$_1$ specifically, we observed activity of single channels in patches in the absence of InsP$_3$, indicating that calpain cleavage does not eliminate channel function but instead leads to constitutive InsP$_3$-independent gating (Fig. 4B). Previous studies of the caspase-3 cleaved form of InsP$_3$R$_1$ similarly suggested that the proteolyzed channel retained activity (21, 22).

In addition to InsP$_3$, Ca$^{2+}$ is the most important ligand for InsP$_3$R$_1$ (15). Even in saturating concentrations of InsP$_3$, [Ca$^{2+}$]$_i$ greater than 100 nm is required for the InsP$_3$R$_1$ channel to be appreciably activated (15). Unlike the InsP$_3$ binding site in InsP$_3$R$_1$, which is known and missing in the carboxyl-terminal fragment generated by calpain proteolysis, the location of functionally important Ca$^{2+}$ binding sites within the primary sequence of InsP$_3$R$_1$ is largely unknown (15). Therefore, although we expected that capn-InsP$_3$R$_1$ activity would be InsP$_3$-independent if it formed a functional channel, we made no a priori assumptions about Ca$^{2+}$ regulation of capn-InsP$_3$R$_1$ activity. To experimentally determine if the Ca$^{2+}$ requirement for channel activation was retained in capn-InsP$_3$R$_1$, we also studied recombinant channel gating at non-optimal Ca$^{2+}$ concentrations (70 nm and 25 μm). As expected, the $P_o$ of wt-InsP$_3$R$_1$ was significantly decreased at 70 nm and 25 μM Ca$^{2+}$ compared with channel $P_o$ at optimal 2 μM Ca$^{2+}$ (Fig. 5). Notably, the constitutively active capn-InsP$_3$R$_1$ channel demonstrated a similar behavior (Fig. 5). This result suggests that the carboxyl-terminal part of InsP$_3$R$_1$ beyond residue 1917 contains functionally relevant Ca$^{2+}$ binding sites involved in channel activation and inhibition. The $P_o$ of capn-InsP$_3$R$_1$ at 70 nm Ca$^{2+}$ was, however, significantly greater than that of wt-InsP$_3$R$_1$ at this Ca$^{2+}$ concentration. This difference may reflect loss of the high affinity, InsP$_3$-regulated Ca$^{2+}$ binding site in the truncated channel (15). However, because of the complexity of Ca$^{2+}$ regulation of InsP$_3$R channel activity, involving multiple functional Ca$^{2+}$ binding sites (47), future studies are required to determine what elements of Ca$^{2+}$ regulation are modified in the truncated channel. These investigations may provide critical clues needed to identify additional Ca$^{2+}$ binding sites within the primary InsP$_3$R sequence.

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3 C. M. Kopil, H. Vais, K.-H. Cheung, A. P. Siebert, D.-O. D. Mak, J. K. Foskett, and R. W. Neumar, unpublished observation.
Expression of capn-InsP$_3$R1 Decreases Ca$^{2+}$ Content of Intracellular Ca$^{2+}$ Stores—How does InsP$_3$-independent activity of capn-InsP$_3$R1 affect cellular Ca$^{2+}$ regulation? Using changes in [Ca$^{2+}$], to indirectly measure the Ca$^{2+}$ content of intracellular stores, we observed that expression of capn-InsP$_3$R1 significantly reduced ionomycin-releasable Ca$^{2+}$ compared with wt-InsP$_3$R1 and EGFP controls, although it did not completely deplete intracellular stores (Fig. 6, A and C). The kinetics of the ionomycin response in capn-InsP$_3$R1-expressing cells was also slower than in EGFP wt-InsP$_3$R1 controls, which suggests a smaller Ca$^{2+}$ driving force consistent with a smaller intracellular Ca$^{2+}$ store. On the other hand, expression of capn-InsP$_3$R1 was not associated with an increased basal [Ca$^{2+}$]$_i$, suggesting that Ca$^{2+}$ transport mechanisms were able to compensate for enhanced ER Ca$^{2+}$ leak through the cleaved channel, at least at the early times studied after transfection (Fig. 6B). In ER Ca$^{2+}$ imaging experiments, expression of capn-InsP$_3$R1 significantly reduced the steady-state Ca$^{2+}$ loading capacity of the ER compared with wt-InsP$_3$R1 and EGFP controls (Fig. 7, A and B). The steady-state [Ca$^{2+}$]$_{ER}$ represents the equilibrium between the passive Ca$^{2+}$ leak and Ca$^{2+}$ uptake by SERCA. Under steady-state filling conditions, the enhanced Ca$^{2+}$ leak induced by expression of capn-InsP$_3$R1 exceeds the SERCA-mediated Ca$^{2+}$ uptake rate, resulting in lower [Ca$^{2+}$]$_{ER}$. The small effect of capn-InsP$_3$R1 expression on the ER-filling rate may reflect the negligible driving force for Ca$^{2+}$ leak under these conditions (Fig. 7C). Of note, the ER leak rate in capn-InsP$_3$R1 was considerably higher than that observed in control or wt-InsP$_3$R1-expressing cells (Fig. 7D). Taken together, our results suggest that capn-InsP$_3$R1 acts as a Ca$^{2+}$ leak channel that perturbs normal ER Ca$^{2+}$ homeostasis.

The results from nuclear patch clamp electrophysiology and Ca$^{2+}$ imaging experiments together elucidate the physiologic relevance of calpain-mediated InsP$_3$R1 proteolysis. Evidence that InsP$_3$-independent gating of capn-InsP$_3$R1 remains Ca$^{2+}$-dependent may explain why expression of capn-InsP$_3$R1 induces only a moderate, albeit significant decrease in intracellular and ER Ca$^{2+}$ stores, at least at the early time point examined after transfection (6 h). The electrophysiology data suggest that resting [Ca$^{2+}$]$_i$ is insufficient to fully activate the truncated channel, thus reducing the Ca$^{2+}$ leak. It is interesting to speculate that this result may also account for conflicting findings regarding the effects of caspase-3 cleavage of InsP$_3$R1 on Ca$^{2+}$ homeostasis. Previous studies have reported that expression of caspase-cleaved InsP$_3$R1, which is only 26 residues longer than the calpain cleaved form, either depletes (21) or does not deplete (22) Ca$^{2+}$ stores in the resting state. Although it was agreed that caspase-3-cleaved InsP$_3$R1 represents a leak channel, the size and impact of that leak remains disputed. It is possible that different [Ca$^{2+}$]$_i$ in the two studies resulted in channels with different $P_a$, which would likely lead to distinct effects on cellular Ca$^{2+}$ homeostasis. Electrophysiological recordings of caspase-cleaved InsP$_3$R1, similar to those performed here for capn-InsP$_3$R1, may clarify the functional consequences of caspase-mediated channel proteolysis.

InsP$_3$R1 Is Cleaved by Calpain after Ischemic Brain Injury—Under pathologic conditions, particularly those associated with elevated [Ca$^{2+}$]$_i$, and impaired ATP-dependent Ca$^{2+}$ removal mechanisms, as in ischemia, calpain cleavage of InsP$_3$R1 may be an important mechanistic component of cell death. Brain ischemia and reperfusion dramatically disrupt neuronal Ca$^{2+}$ homeostasis, and there is compelling evidence for a causal role of both Ca$^{2+}$ overload and consequent pathologic calpain activation in ischemic neurodegeneration (3, 39). As both a Ca$^{2+}$ regulatory protein and calpain substrate, InsP$_3$R1 is at a critical intersection between protease activation and disruption of cellular Ca$^{2+}$ homeostasis during the molecular injury cascade.

Here, we demonstrate that InsP$_3$R1 proteolysis occurs in vivo in an animal model of ischemic brain injury (Fig. 8). In this cardiac arrest model, calpain cleavage of InsP$_3$R1 occurred in cerebellar Purkinje neurons, which are selectively vulnerable to post-ischemic neurodegeneration (39). Moreover, identification of calpain-cleaved InsP$_3$R1 in cerebellar microsomes as early as 1 h after cardiac arrest and reperfusion demonstrates that channel proteolysis is an early event in the cell death cascade rather than merely reflecting broad cellular degradation. The persistence and stability of calpain cleaved InsP$_3$R1 at the latest time point examined after cardiac arrest (48 h) also suggests that the leaky, proteolyzed channel may act as a feed-forward mechanism for increased Ca$^{2+}$ overload and calpain activation that eventually leads to neuronal death. In addition to InsP$_3$R1, a number of Ca$^{2+}$ regulatory proteins are also calpain substrates, including the N-methyl-D-aspartate receptor, plasma membrane Ca$^{2+}$ ATPase, Na$^+$/Ca$^{2+}$ exchanger, L-type Ca$^{2+}$ channel, SERCA, and ryanodine receptor (3). Thus, calpain cleavage of InsP$_3$R1 may be an important component of a broader pathway of calpain-mediated disruption of neuronal Ca$^{2+}$ homeostasis in neurodegenerative diseases associated with Ca$^{2+}$ dysregulation.

Conclusions—In summary, our results indicate that calpain proteolysis of InsP$_3$R1 creates an ER Ca$^{2+}$ release channel that is InsP$_3$-independent and constitutively active. Expression of the truncated channel reduces the content of intracellular Ca$^{2+}$ stores, which may have detrimental effects on Ca$^{2+}$ signaling and buffering under pathologic conditions. Evidence of calpain cleavage of InsP$_3$R1 in neurons after cardiac arrest provides a potential mechanism to account for decreased InsP$_3$ binding (10, 11), depletion of ER Ca$^{2+}$ stores (13), and disruption of Ca$^{2+}$ homeostasis reported in previous studies of in vivo ischemia and reperfusion. Together, these results provide important insights into a molecular pathway that may act as a feed-forward mechanism to enhance cell death. Furthermore, the results presented here identify a novel target for therapeutic intervention after brain ischemia or in other neurodegenerative disorders associated with Ca$^{2+}$ dysregulation and protease activation.

Acknowledgments—We thank Dr. Suresh Joseph (Thomas Jefferson University) and Dr. Robert Simon (University of Pennsylvania) for supplying antibodies. We thank Jun Yang for the GST-InsP$_3$R1 peptides in pGEX expression vectors and for excellent technical expertise in cloning. We also thank Nicole Gorman and the Wistar Institute for help with amino-terminal sequencing.
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REFERENCES

1. Merril, C. R., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell Biol. 1, 11–21
2. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) Nat. Rev. Mol. Cell Biol. 4, 552–565
3. Bevers, M. B., and Neumar, R. W. (2000) J. Cereb. Blood Flow Metab. 20, 32533–32538
4. Leissring, M. A., Paul, B. A., and Neumar, R. W. (1999) J. Biol. Chem. 274, 32535–32538
5. Leissring, M. A., and Neumar, R. W. (1999) J. Neurosci. 22, 9416–9427
6. Sugawara, H., Kurosaki, M., Takata, M., and Kuroski, T. (1997) EMBO J. 16, 583–590
7. Nagasawa, H., and Kogure, K. (1991) J. Biol. Chem. 266, 16652–16655
8. Joseph, S. K., and Samanta, S. (1993) J. Biol. Chem. 268, 6477–6486
9. Fis, D., and Bourguignon, L. Y. (2000) J. Biol. Chem. 275, 379–388
10. Wang, K. K. (2000) J. Biol. Chem. 275, 3934–3944
11. Foskett, J. K., White, C., and Neumar, R. W. (1995) J. Cereb. Blood Flow Metab. 15, 1032–1039
12. Galvan, D. L., Borrego-Diaz, E., Perez, P. J., and Mignery, G. A. (1999) J. Biol. Chem. 274, 39427–39436
13. Davis, R. J., Challiss, J., and Nahorski, S. R. (1999) J. Biol. Chem. 274, 39437–39446
14. Sayers, L. G., Miyawaki, A., Takeshita, H., Yamamoto, A., and Foskett, J. K. (2005) Biochem. J. 389, 273–280
15. Davis, R. J., Challiss, J., and Nahorski, S. R. (1999) Biochem. J. 341, 813–820
16. Fischer, G. A., Clementi, E., Raichman, M., Südhof, T., Ulrich, A., and Meldolesi, J. (1994) J. Biol. Chem. 269, 19216–19224
17. Mackrill, J. J., Wilcox, R. A., Miyawaki, A., and Foskett, J. K. (1996) Biochem. J. 318, 871–878
18. Joseph, S. K., Pierson, S., and Samanta, S. (1993) J. Biol. Chem. 268, 859–865
19. Yoshikawa, F., Iwasaki, H., and Furuuchi, T., and Mikoshiba, K. (1997) J. Biol. Chem. 272, 5190–5193
20. Foskett, J. K., and Mak, D. O. (2010) in Current Topics in Membranes (Serysheva, I. I., ed) Vol. 66, pp. 235–272, Academic Press, Burlington