The inositol 1,4,5-trisphosphate receptor (IP₃R) is an intracellular Ca²⁺ release channel expressed predominately on the membranes of the endoplasmic reticulum. IP₃R₁ can be cleaved by caspase or calpain into at least two receptor fragments. However, the functional consequences of receptor fragmentation are poorly understood. Our previous work has demonstrated that IP₃R₁ channels, formed following either enzymatic fragmentation or expression of the corresponding complementary polypeptide chains, retain tetrameric architecture and are still activated by IP₃ binding despite the loss of peptide continuity. In this study, we demonstrate that region-specific receptor fragmentation modifies channel regulation. Specifically, the agonist-evoked temporal Ca²⁺ release profile and protein kinase A modulation of Ca²⁺ release are markedly altered. Moreover, we also demonstrate that activation of fragmented IP₃R₁ can result in a distinct functional outcome. Our work suggests that proteolysis of IP₃R₁ may represent a novel form of modulation of IP₃R₁ channel function and increases the repertoire of Ca²⁺ signals achievable through this channel.

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2 The abbreviations used are: IP₃R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; BCR, B cell receptor; Pₒ, open probability; Z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethyl ketone; ARM, armadillo; TPV, two-promoter vector; ANOVA, analysis of variance.
These data unambiguously demonstrate that IP₃R1 is still tightly regulated by its endogenous ligand even after proteolytic fragmentation.

In this study, we have continued to investigate the consequences of proteolytic fragmentation of IP₃R1. Given that fragmented IP₃R1 are still functional in response to IP₃ binding, we hypothesized that disruption of peptide continuity by proteolytic cleavage may affect the fine regulation of IP₃R1 and, subsequently, alter IP₃R1-mediated Ca²⁺ signals. This study demonstrates that proteolytic fragmentation has profound effects on IP₃-mediated Ca²⁺ signals, resulting in alteration of the signature temporal pattern of Ca²⁺ release through IP₃R1. Furthermore, fragmentation can abolish PKA regulation of the receptor in a cleavage region-specific manner. More importantly, we show that the altered Ca²⁺ signals elicited by fragmented IP₃R1 can specifically activate distinct downstream effectors compared with IP₃R WT. Our results therefore strongly suggest that proteolytic fragmentation may represent a novel form of regulation of IP₃R activity that expands the repertoire of signaling through IP₃R1 activation.

**Results**

**Functional fragmented IP₃R1 are assembled from complementary IP₃R1 fragments**

We have reported previously that heterologous expression of complementary polypeptide fragments, designed based on putative caspase and calpain proteolytic cleavage sites, can be assembled into IP₃R1 tetramers (33). This strategy provides an experimental platform to investigate the functional consequences of IP₃R fragmentation. Our previous work strongly suggested that tetramers assembled from complementary IP₃R1 fragments are functional in terms of IP₃-gated Ca²⁺ release. To further characterize the functional consequence of receptor fragmentation, we designed additional complementary IP₃R1 fragments according to defined IP₃R1 trypsin cleavage sites in the rat IP₃R1 (Fig. 1, A and B). Previous studies have shown that exposure of purified IP₃R1 protein to a low concentration of trypsin in vitro results in five receptor fragments (37–39). These data have been interpreted to indicate that IP₃R1 consists of five compact globular domains connected by four solvent-exposed linker regions. Throughout this work, cleavage sites introduced after the second or third trypsin cleavage sites, respectively, are denoted as IP₃R1 I–II+III–V (tryp) and IP₃R1 I–III+IV–V (tryp) (Fig. 1B). Caspase and calpain both cleave IP₃R1 in the same solvent-exposed region (after trypptic fragment IV) and are denoted as IP₃R1 I–IV+V(casp) and IP₃R1 I–IV+V (calp) (Fig. 1B). To confirm that all fragmented IP₃R1 are functional, DT40-3KO cells (null for all IP₃R) (40) were transfected with linear continuity.

![Region-specific proteolytic regulation of IP₃R1](ASBMBM90185720268fa.png)

**Figure 1. Schematic showing the proteolytic fragmentation sites on IP₃R1. A, 3D structure of IP₃R1 utilizing the cryo-EM structure published in Ref. 50, emphasizing five receptor fragments derived from limited trypsin exposure. Each fragment is color-coded. aa, amino acid. B, ribbons with corresponding colors represent the linear structure of fragmented IP₃R1. Gaps between ribbons indicate the sites of proteolytic cleavages. Numbers above the gap indicate the amino residues at the C-terminal end of cleavage sites. Numbers in the ribbons indicate the relative molecular weight of each receptor fragment. b-TF, β trefoil domain; ARM, armadillo solenoid folds; HD1, helical domain; ILD, intervening lateral domain; TMD, transmembrane domain; C, C-terminal domain.**
Proteolytic fragmentation alters the temporal pattern of IP$_3$R1-mediated Ca$^{2+}$ release in a region-specific manner

IP$_3$R exhibit subtype-specific Ca$^{2+}$ release profiles when continuously exposed to IP$_3$. This is best exemplified following activation of B cell receptors (BCR) on DT40-3KO cells evoked by cross-linking with α-IgM (43). For example, stimulation of DT40-3KO cells stably expressing IP$_3$R1 WT with α-IgM characteristic evokes only a few transient increases in [Ca$^{2+}$]$_i$ (Fig. 3A and Refs. 41, 43, 44), whereas stimulation of cells expressing mouse IP$_3$R2 WT elicits robust Ca$^{2+}$ oscillations (13, 41, 43). A higher concentration of α-IgM (2 μg/ml) had no impact on the profile of Ca$^{2+}$ release evoked in cells expressing IP$_3$R1 WT (Fig. 3G). In addition, this general pattern was observed in various clones of DT40-3KO cells with different IP$_3$R1 WT expression levels (data not shown), indicating that the temporal pattern of [Ca$^{2+}$]$_i$ signal likely reflects an intrinsic property of the particular IP$_3$R. Next we examined the Ca$^{2+}$ release profile of cells expressing various complementary receptor fragments. When the cleavage site was located closer to the N terminus of the receptor, such as with IP$_3$R1 I–II/III–V (tryp), similar to IP$_3$R1 WT, a low number of Ca$^{2+}$ transients were evoked upon α-IgM stimulation (Fig. 3, B and C). Remarkably, when cleavage sites were introduced further toward the C terminus of the receptor (for example, IP$_3$R1 I–III/IV–V (tryp), IP$_3$R1 I–IV/V (casp), and R1 I–IV/V (calp), a significant increase in the ability of BCR stimulation to
evoke oscillatory activity was observed (Fig. 3, D–G). These data provide evidence that IP₃R fragmentation at sites corresponding to cleavage by calpain and caspase can markedly alter the activity of the receptor and subsequently alter the temporal profile of Ca²⁺ release. We reported previously that a significant increase in the ability of IP₃R1 to support Ca²⁺ oscillations occurred following PKA-mediated phosphorylation of the receptor (44). To investigate whether PKA phosphorylation plays a role in the increased oscillatory activity of particular fragmented IP₃R1, BCR-stimulated [Ca²⁺]ᵢ signals were studied in cells expressing PKA non-phosphorylatable (S1589A, S1755A) and phosphomimetic (S1589E, S1755E) IP₃R1 I–IV (calp). These mutations had no impact on the Ca²⁺ release profile mediated by IP₃R1 I–IV (calp) (Fig. 4, A–D), indicating that PKA phosphorylation does not underlie the gain of oscillatory activity observed in the specific fragmented IP₃R1. In addition, Ca²⁺ oscillations were retained for an extended period of time in the absence of extracellular Ca²⁺ (Fig. 5, A and B), suggesting that Ca²⁺ influx is not necessary to promote Ca²⁺ oscillations from these fragmented IP₃R1.

We next investigated the profile of Ca²⁺ signals mediated by fragmented IP₃R1 more directly by photorelease of a cell-permeable, poorly degradable, caged IP₃ (45, 46). DT40-3KO cells expressing IP₃R1 WT showed a sustained monophasic [Ca²⁺]ᵢ signal in response to photorelease of caged IP₃ (Fig. 6A). In contrast, cells expressing IP₃R2 WT exhibited robust oscillatory Ca²⁺ signals (Fig. 6B). Notably, IP₃R1 I–IV +V (calp) showed both sustained monophasic and oscillatory responses (Fig. 6C). A distribution of the frequency of Ca²⁺ oscillations shows that cells expressing IP₃R1 WT mainly evoked a limited number of Ca²⁺ transients in response to IP₃ exposure (Fig. 6A), whereas cells expressing IP₃R1 I–IV +V (calp) displayed an increase in the population of cells that exhibit robust oscillatory Ca²⁺ release (Fig. 6F). This observation was consistent with the statistics showing that cells expressing IP₃R1 I–IV +V (calp) induced significantly more Ca²⁺ transients
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Figure 4. Altered temporal Ca²⁺ release profile mediated by IP₃R1 I–IV+V (calp) is not due to PKA-mediated receptor phosphorylation. A–C, DT40-3KO cells stably expressing IP₃R1 I–IV+V (calp) (A), IP₃R1 I–IV+V (calp) (S1589A, S1755A) (B), or IP₃R1 I–IV+V (calp) (S1589E, S1755E) (C) were loaded with 2 μM Fura-2/AM, followed by cross-linking the cell surface B cell receptor using α-IgM (500 ng/ml). Two representative Ca²⁺ traces are shown for each set of complementary receptor fragments. D, scatterplot indicating that there is no significant difference among three types of receptor fragments with respect to the number of Ca²⁺ transients in 30 min of recording (one-way ANOVA). Ca²⁺ imaging assays were repeated three times, with more than 40 cells in each experimental run for each complementary receptor fragments. n.s., not significant.

Proteolytic fragmentation alters the single-channel open probability of IP₃R1

One caveat of the single-cell imaging assay above is that fragmented IP₃R1 is not generated from the proteolytic cleavage of full-length IP₃R1 WT but from the assembly of complementary receptor fragments. Therefore, we next performed patch-clamp recording in the “on nucleus” configuration to fragment IP₃R1 WT in situ and directly investigate the biophysical consequences of IP₃R1 fragmentation at the single-channel level (18). A submaximal concentration of IP₃ (1 μM) resulted in an increase in the steady-state open probability (Pₒ) of IP₃R1 to ~20% (Fig. 7, A and G). Addition of active caspase-3 (3 ng/ml) in the patch pipette in the presence of IP₃ (1 μM) significantly augmented the channel Pₒ to ~70% (Fig. 7, B and G). Higher concentrations of active caspase-3 (10 ng/ml or 30 ng/ml) either diminished the conductance of the channel or completely inactivated the receptor, likely because of nonspecific digestion followed by destruction of the receptor (Fig. 7, C, D, and G). Addition of the caspase inhibitor Z-VAD (20 μM) completely blocked the effect of active caspase on channel Pₒ (Fig. 7, E and G). In addition, in cells expressing constructs where the caspase cleavage site (DEVD at amino acids 1888–1891) was mutated to be non-cleavable (IEVA) (Fig. 7, F and G) (30), no increase in Pₒ was observed in the presence of active caspase-3. These data strongly suggest that specific receptor fragmentation by caspase-3 at Asp-1891 enhances IP₃R1 channel activity (Fig. 7G), and this likely reflects the single-channel correlate of the increase in oscillatory activity observed in intact cells expressing fragmented IP₃R1 following stimulation with IP₃.

PKA regulation is abolished in calpain-fragmented IP₃R1

Although fragmented IP₃R is still gated by IP₃ binding, how important individual modes of regulation of IP₃R1 are impacted by receptor fragmentation remains unclear. Phosphorylation of IP₃R1 at Ser-1589 and Ser-1755 by PKA significantly increases channel Pₒ at the single-channel level (21, 22). This biophysical alteration is manifested as an increase in Ca²⁺ release at the single-cell level determined in Ca²⁺ imaging assays. Interestingly, the calpain fragmentation site is located between the PKA phosphorylation sites and the receptor channel domain. As a result, proteolytic receptor fragmentation by calpain separates PKA phosphorylation sites and the Ca²⁺ permeation pore into two different peptide fragments. Given the location of the calpain fragmentation site in IP₃R1, we next investigated the effects of fragmentation on PKA regulation of IP₃R1. Activation of PKA resulted in IP₃R1 phosphorylation and, subsequently, a significant increase in Ca²⁺ release in cells expressing IP₃R1 compared with DMSO-treated con-
controls in response to all PAR2 agonist concentrations (Fig. 8, A, B, and F). The increase in phosphorylation and regulation of Ca\(^{2+}\) signals was completely abolished in cells expressing non-phosphorylatable IP\(_3\)R1 mutants at both PKA phosphorylation sites (Fig. 8, C and F). Notably, when a disruption of peptide continuity was introduced at the third trypsin cleavage site, IP\(_3\)R1 I–III/H11001 IV–V (tryp), PKA phosphoregulation was maintained (Fig. 8, D and F). In contrast, although forskolin pretreatment increased the level of phosphorylated IP\(_3\)R1 I–IV/H11001 V (calp), no significant difference in terms of the amplitude of Ca\(^{2+}\) response was observed. (Fig. 8, E and G). These data demonstrate that PKA regulation of IP\(_3\)R activity requires the phosphorylated residues to be proximal or, possibly, in the same fragment as the channel domain and further suggest that other particular modes of regulation of IP\(_3\)R1 activity may be altered depending on the specific site of fragmentation.

**Figure 8. Photorelease of caged IP\(_3\) induces distinct Ca\(^{2+}\) signals mediated by complementary receptor fragments.** A and B, DT40-3KO cells stably expressing IP\(_3\)R1 WT, IP\(_3\)R2 WT, and IP\(_3\)R1 I–IV+V (calp) were loaded with 1 \(\mu\)M Fluo-8/AM and 2 \(\mu\)M caged 6-O-[(4,5-dimethoxy-2-nitrophenyl)methyl]-2,3-O-(1-methylethylidene)- \(\alpha\)-myo-inositol 1,4,5-tris[bis(1-oxoproxy)methyl]phosphate (ci-IP3) for 30 min. A UV flash (200 ms) was introduced at the indicated time to photolyse caged IP\(_3\), and Ca\(^{2+}\) signals were recorded for 15 min. In response to ci-IP\(_3\), cells stably expressing IP\(_3\)R1 WT mainly evoked a sustained single Ca\(^{2+}\) release event (A), whereas cells stably expressing IP\(_3\)R2 WT evoked robust Ca\(^{2+}\) oscillations (B). C and D, both types of Ca\(^{2+}\) signals in A and B were observed in cells stably expressing IP\(_3\)R1 I–IV+V (calp). E and F, distribution plots indicate that the majority of cells expressing IP\(_3\)R1 WT induced either a sustained single Ca\(^{2+}\) release or few Ca\(^{2+}\) transients (E) whereas cells expressing IP\(_3\)R1 I–IV+V (calp) gave rise to an increased level of Ca\(^{2+}\) transients during the 15-min recording. (F). G, box plot with whiskers showing the 10–90 percentile suggests a significant increase in the ability of IP\(_3\)R1 I–IV+V (calp) to induce Ca\(^{2+}\) oscillations compared with IP\(_3\)R1 WT (Student’s t test). Experiments were repeated four times for each IP\(_3\)R or complementary pairs of receptor fragments.

**Fragmented IP\(_3\)R1 can activate distinct downstream effectors**

Based on the observation that specific fragmented IP\(_3\)R1 can induce different Ca\(^{2+}\) signals, we next investigated whether fragmented IP\(_3\)R1 can specifically activate distinct downstream effectors. Oscillatory Ca\(^{2+}\) signals, but not single Ca\(^{2+}\) transients, have been shown to specifically activate the transcription factor nuclear factor of activated T cells (NFAT). Ca\(^{2+}\) oscillations are thought to deliver signals with the appropriate spatial and temporal properties necessary to activate the phosphatase calcineurin, which dephosphorylates NFAT and facilitates its translocation to the nucleus (45, 47, 48). We hypothesized that caspase- or calpain-fragmented IP\(_3\)R1, but not IP\(_3\)R1 WT, might provide the necessary Ca\(^{2+}\) signal to activate NFAT translocation. GFP-tagged NFAT (NFAT-GFP) was transfected into cells expressing either IP\(_3\)R1 WT, IP\(_3\)R1 I–IV+V (casp), or IP\(_3\)R1 I–IV+V (calp). In the quiescent state, NFAT-
GFP was mainly located in the cytosol (Fig. 9, B, E, and H). Upon BCR stimulation, NFAT-GFP efficiently translocated from the cytosol into the nucleus in cells expressing either IP3R1 I–IV/H11001V (casp) or IP3R1 I–IV/H11001V (calp) (Fig. 9, A–F, J, and K). Translocation of NFAT-GFP was not observed in cells expressing IP3R1 WT (Fig. 9, G–K). These data strikingly illustrate that the distinct patterns of Ca2+/H11001 signal evoked through particular fragmented IP3R have the ability to activate distinct downstream events compared with the intact IP3R1.

Discussion

The data presented here expand on our earlier findings exploring the functional consequences of fragmentation of IP3R1 by intracellular proteases. We now demonstrate that expression of complementary polypeptides that correspond to any pair of fragments representing the five globular domains generated in vitro by limited trypsin exposure similarly result in functional channels (37). Indeed, co-expression of individual cDNA encoding the five domains separately, remarkably, leads to the assembly of a functional channel gated by IP3. Although these data firmly establish that peptide continuity is not required for channel opening per se, the major finding of this study is that fragmentation of IP3R1 markedly alters allosteric regulation of the channel by key modulators to alter Ca2+ release activity. Specifically, as an example, we show that a prominent mode of regulation of IP3R1, augmented Ca2+ release following PKA phosphorylation, is lost when fragments are expressed that mimic calpain cleavage to generate fragmented IP3R1 I–IV + V. However, regulation by PKA is clearly evident when peptide continuity is lost more proximal to the N terminus to yield IP3R1 I–III + IV–V. Notably, in this case, the key phosphorylation sites at Ser-1589 and Ser-1755 are present in the same fragment as the channel pore in fragment V, suggesting that peptide continuity is required to communicate the conformation change imparted by phosphorylation to modulation of gating of the pore. Further, these data are consistent with the observation that PKA phosphorylation does not alter IP3 binding to the binding core in the N terminus, which is present in the complementary fragment (49). These data clearly demonstrate that alterations in allosteric modulation of IP3R activity are dependent on the site of cleavage.

A further demonstration that the activity of IP3R1 is dramatically altered by cleavage is the observation that the temporal profile of Ca2+ release following agonist stimulation is temporally transformed when the channel is assembled from particular complementary polypeptide chains. We and others have consistently reported that sustained stimulation of cells expressing individual IP3R subtypes in isolation supports specific patterns of Ca2+ release that can be considered a “signature” for that subtype (13, 41, 43, 44). As the signal is largely independent of extracellular Ca2+, these signatures are the result of the inte-
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Figure 8. The increase in Ca²⁺ release in IP₃R1 by PKA phosphorylation is regulated by receptor fragmentation in a cleavage region-specific manner. A, cells were loaded with 1 μM Fluo-2/AM for 1 h, followed by a FlexStation assay to monitor the change in [Ca²⁺],. Preincubation of cells stably expressing IP₃R1 WT with forskolin significantly increased the amplitude of Ca²⁺ release in response to PAR2 activation. B and D, this increase was observed at all agonist concentrations tested for IP₃R1 WT and at high agonist concentrations tested for IP₃R1 I–III + IV–V (tryp). C and E, forskolin had no effect on the amplitude of Ca²⁺ release in cells expressing IP₃R1 (S1589A, S1755A) (C) or IP₃R1 I–IV+V (calp) (E). F and G, pretreatment of forskolin results in IP₃R1 phosphorylation at residue Ser-1755 in both full-length and fragmented receptors. Statistics were performed using one-way ANOVA followed by Dunnett post-test. Experiments were repeated three times for each set of IP₃R1 or complementary receptor fragments. WB, Western blot.

grated regulatory input received by the particular IP₃R expressed. Notably, in this study, we show that expression of pairs of complementary fragments, in particular those corresponding to products derived from caspase or calpain proteolytic fragmentation, results in a transformation of the Ca²⁺ signal from a largely transient increase into an oscillatory profile consisting of numerous organized transients present throughout stimulation. In addition, single-channel recordings indicate that in situ cleavage of IP₃R1 by caspase significantly increased the channel open probability. This provides a potential underlying mechanism for altering IP₃R1 activity through proteolytic fragmentation. Consistent with the widely held view that the spatial and temporal properties of Ca²⁺ signals are important for the activation of downstream effectors, these oscillatory Ca²⁺ signals were capable of driving the nuclear localization of NFAT-GFP whereas signals through the IP₃R1 WT could not. Taken together, these data provide support for the hypothesis that cleavage by proteases can potentially have significant consequences for IP₃R1 activity and might be considered a novel mode of regulation influencing multiple modulatory inputs.

The cryo-EM structure of IP₃R1 was recently solved to near atomic resolution and provides structural details consistent with our findings (50). The suppressor domain (TFβ1) and the IP₃ binding core (TFβ2 and the N-terminal region of ARM1) at the N terminus of the receptor physically interact with the C-terminal domain of the adjacent subunit. This interaction
provides a physical basis for a direct communication between IP₃ binding at the N terminus and the distant channel opening at the C terminus without requiring signaling propagation along the whole peptide sequence. These data are consistent with our conclusion that peptide continuity in the receptor coupling domain, which comprises the helical domain, armadillo solenoid folds (ARM), and the intervening later domain, is not required for channel opening following IP₃ binding. However, the coupling domain is crucial for integrating intracellular input and, accordingly, imposing regulations on the channel.

Figure 9. Ca²⁺ signals induced by IP₃R1 I–IV + V can activate distinct downstream effectors. B, E, and H, DT40-3KO cells stably expressing IP₃R1 I–IV + V (casp), IP₃R1 I–IV + V (calp), and IP₃R1 WT were transiently transfected with NFAT-GFP, followed by 12-h recovery in a 39 °C, 5% CO₂ incubator. Cells were then mounted in a perfusion chamber, the location of NFAT-GFP was monitored following excitation with 488 nm, and emitted fluorescence recorded above 510 nm. Prior to stimulation, NFAT-GFP was mainly located in the cytosol (B, E, and H). A, D, and G, 3D heat plots, with the x and y axes indicating spatial coordinates and the z axis indicating the amplitude of the GFP signal. A, C, D, F, G, I, and J, addition of α-IgM (500 ng/ml) activated and translocated NFAT-GFP from the cytosol into the nucleus in cells expressing IP₃R1 I–IV + V (casp) (A, C, and J) and IP₃R1 I–IV + V (calp) (D, F, and J). This translocation was not observed in cells expressing IP₃R1 WT (G, I, and J). C, F, and I, nucleus NFAT-GFP signals are shown in red and cytosolic NFAT-GFP signals in black and were used to calculate the nucleus/cytosol ratio (N/C, blue). J, the percentage of cells where translocation could be observed in individual trials in which more than 20 cells were imaged per trial. K, the magnitude of the N/C ratio in individual cells from each trial that showed translocation. These data demonstrate a significant increase in nucleus/cytosol ratio for fragmented IP₃R1 compared with IP₃R1 WT in response to α-IgM stimulation. Experiments were repeated four times for IP₃R1 and three times for each type of complementary receptor fragments. *, P < 0.01.
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This concept was again strongly supported by the recent structure that shows that flexible structures of the ARM domains were amenable to generating interfaces for recognition and binding of various regulators. Noticeably, both caspase and calpain cleavage sites as well as the fragmentation site for IP$_3$R1 I–III+IV–V are located in ARM regions. Given the critical role of the coupling domain in terms of receptor regulation, it is conceivable that disruption of peptide continuity in the ARM regions (IP$_3$R1 I–III+IV–V and IP$_3$R1 I–IV+V) are likely to either interfere with the intrinsic regulation of ARM on the channel domain or affect other mediators that regulate channel activity through binding to or modifying ARM domains and thus alter IP$_3$R1-induced Ca$^{2+}$ signals.

What is the mechanism underlying the ability of particular fragmented IP$_3$R1 to support robust Ca$^{2+}$ oscillations? A canonical model for class I Ca$^{2+}$ oscillations provides a possible explanation for this remarkable alteration (51). This model suggests that cells can control the Ca$^{2+}$ oscillation period by modulating the rate of Ca$^{2+}$ activation of IP$_3$R in response to the change in [Ca$^{2+}$]. Of note, although there was no EF-hand Ca$^{2+}$ binding motif found in IP$_3$R1, the ARM3 region in the coupling domain contains a putative Ca$^{2+}$ binding region with a highly conservative Glu-2100 critical for Ca$^{2+}$ binding and regulation of IP$_3$R1 (52, 53). Based on the model and the location of the Ca$^{2+}$ sensor in the ARM regions, we speculate that fragmentation of IP$_3$R1 at particular ARM regions impacts the Ca$^{2+}$ modulation of channel gating and, consequently, alters the Ca$^{2+}$ activation rate for IP$_3$R1. This hypothesis is supported by mathematical simulations showing that, by solely changing the rate of IP$_3$R1 activation by Ca$^{2+}$, the normal monotonic Ca$^{2+}$ release pattern through wild-type IP$_3$R1 Ca$^{2+}$ can be converted into robust oscillatory Ca$^{2+}$ signals observed with IP$_3$R1 I–III+IV–V and IP$_3$R1 I–IV+V (51).

Although IP$_3$R1 has been reported to be a substrate of caspase and calpain for more than a decade, any cellular role of cleavage has yet to be firmly established. We and other laboratories report that, even when caspase and calpain are massively activated during apoptosis, only a small fraction of IP$_3$R1 is actually fragmented. Therefore, we envision that, beyond negating protein activity, caspase can act upon the Ca$^{2+}$ signaling, that caspase and calpain activity is also essential for processes that modulate the Ca$^{2+}$ signaling. New reports have suggested that functional consequence of proteolytic fragmentation for IP$_3$R2 and IP$_3$R3. Our study will answer the important question of whether proteolytic fragmentation may be a general regulatory event for all isoforms of IP$_3$R.

Materials and methods

Reagents

All restriction enzymes and T4-DNA ligase were from New England Biolabs. RPMI 1640 medium, penicillin/streptomycin, G418 sulfate, β-mercaptoethanol, and chicken serum were purchased from Invitrogen. Fetal bovine serum was from Gemini Bioproducts. Iso-Ins(1,4,5)P$_3$/PM (caged), Z-VAD-fmk, and active caspase-3 were from Enzo Life Science. Fura-2 was from Tefabs. All reagents for SDS-PAGE were from Bio-Rad. The N-terminal antibody for IP$_3$R1 and phospho-IP$_3$R1 were from Cell Signaling Technology. DyLight™ 800CW secondary antibody was from Thermo Scientific. Forskolin was from Sigma-Aldrich. Mouse α-chicken IgM was from Southern Biotech. The antibody against the C-terminal 19 amino acids of IP$_3$R1 was generated by Pocono Rabbit Farms and Laboratories.

Constructs

The method for generation of IP$_3$R1 I–IV+V (casp) and IP$_3$R1 I–IV+V (calp) was first described elsewhere (33). In brief, to create IP$_3$R1 I–IV+V (casp), cDNA encoding rat IP$_3$R1 flanked by the NheI and NotI sites at the 5’ and 3’ ends in pcDNA3.1 was used as the template. All PCR modifications described here were conducted using Pfu Ultra II Hotstart 2X Master Mix (Agilent), and only forward primers are shown here. To generate the construct coding for N-terminal and C-terminal fragments predicted to result from caspase cleavage of IP$_3$R1 at the DEVDD1891 consensus site, IP$_3$R1 cDNA was modified by PCR (forward, 5’-GAAAGATGATGAAGTGGA-CATAAATTCGGGCGCGTACTGATCGGGATGCC- CATCCGAA-3’). This modification introduced a stop codon after residue Asp-1891 and also a Kozak sequence and an initiation methionine in-frame with the sequence coding for the membrane fragment, designed to ensure efficient expression. To obtain a two-promoter vector (TPV) encoding both N- and C-terminal fragments, two-step ligation was performed (33). First, NheI-NotI IP$_3$R1 I–IV (casp) was inserted into the TPV digested with NheI and NotI at the 5’ and 3’ ends in pcDNA3.1 and then cloned into the TPV digested with NotI. The TPV encoding IP$_3$R1 I–II+III–V (tryp), IP$_3$R1 I–III+IV–V (tryp), and IP$_3$R1 I–IV+V (calp) were constructed in an identical manner using corresponding primers: forward 5’-GGCAACAGCAGGACGTGATGATAGTAGCGGCGCGTGAGCATCTCATTCATGGGATTTG-3’ for IP$_3$R1 I–II+III–V (tryp), forward 5’-CTGGCGGTATATCAGCCCGCTAGG-
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CGGCCCGTACGATCGAAGCTGCGTAGAG-3' for IP$_3$R1–I–III+IV–V (tryp), and forward 5'-CCGGGATCCTGTTGGAATAGAATTCGCGGCCGCGCTAGCATGGCAT-G for IP$_3$R1 I–V+V (calp).

To generate caspase non-cleavable rIP3R1, forward primer 5'GGGAAAAAAGGAAAAGATCGAATGGCAGGATGCCCGC-3' was used to mutate the sequence encoding amino acid DEVD to be IEVA. Two-step PCR was performed sequentially using forward primers 5'-TCAGGAAAGAAGAGGAGCTTTACCAGCTTGGA-3' and 5'-GCTGCTGCTGAGGCTCCAGCTCC-3' to generate rIP$_3$R1 (S5189A, S1755A) and forward primers 5'-TCAGGAAAGAAGAGGAGCTTTACCAGCTTGGA-3' and 5'-GCTGCTGCTGAGGCTCCAGCTCC-3' to generate rIP$_3$R1 (S1589E, S1755E).

Western blot analysis

Cells were harvested by centrifugation, washed once with ice-cold PBS, and solubilized in cell lysis buffer containing 10 mM Tris-HCl, 10 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM NaF, 20 mM Na$_3$PO$_4$, 2 mM Na$_3$VO$_4$, 1% Triton X-100 (v/v), and 10% glycerol with a mixture of protease inhibitors. After 30 min on ice, cell lysates were precleared by centrifugation at 16,000 × g for 10 min at 4 °C. Cleared lysates were transferred into fresh tubes, and protein concentrations were measured using a D$_2$-protein assay kit (Bio-Rad). Protein were resolved on 5–7.5% SDS-PAGE gels, transferred to nitrocellulose, and pro-cessed for immunoblotting with the indicated primary antibod-ies and corresponding secondary antibodies. Proteins were detected using an Odyssey infrared imaging system (LI-COR Biosciences).

Fluorescence imaging assay

DT40 cells expressing defined IP$_3$R constructs were loaded with 2 μM Fura-2/AM on a glass coverslip mounted onto a Warner chamber at room temperature for 20–30 min. Loaded cells were perfused with HEPES imaging buffer (137 mM NaCl, 4.7 mM KCl, 1.26 mM CaCl$_2$, 1 mM Na$_3$PO$_4$, 0.56 mM MgCl$_2$, 10 mM HEPES, and 5.5 mM glucose (pH 7.4)) and stimulated with the indicated agonist. Ca$^{2+}$ imaging was performed using an inverted epifluorescence Nikon microscope with a ×40 oil immersion objective. Images were captured every second with an exposure of 10 ms and 4 × 4 binning using a digital camera (Cooke Sensicam QE) driven by TILL Photonics software.

Cell culture and plasmid transfection

DT40-3KO cells, a chicken B lymphocyte line with targeted deletion of the three endogenous IP$_3$R isoforms, were grown in RPMI 1640 medium supplemented with 1% chicken serum, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO$_2$. DT40-3KO cell transfection and generation of stable cell lines was performed as described previously using the Amaxa nucleofector (Lonza Laboratories). HEK-3KO cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO$_2$. HEK-3KO cells were transfected using Lipofectamine 2000 (Invitrogen) following the protocol of the manufacturer.

FlexStation assay

DT40-3KO cells (5 × 10$^5$ cells/well) expressing the receptor of interest were washed with PBS once, followed by incubation in imaging buffer containing 1% BSA and 5 μM Fluo-2/AM for 1 h. Cells were then spun down, washed with imaging buffer containing 1% BSA, and seeded onto a 96-well plate that was precoated with 0.1% poly-1-lysine. The plate was spun at 500 × g for 2 min and rested at room temperature for 15 min. During FlexStation recording, cells were treated with 20 μM forskolin (final concentration) or the same volume of DMSO for 3 min, followed by stimulation with different concentrations of DMSO for 3 min, following by stimulation with different concentrations of PAR2 agonist for 3 min. For each type of IP$_3$R or receptor fragments, a control response was defined as the amplitude of Ca$^{2+}$ response stim-u-lated by 500 μM PAR2 agonist following DMSO preincuba-tion. The amplitudes of Ca$^{2+}$ responses for all other conditions were calculated and displayed as the percentage of the control response.

NFAT-GFP translocation assay

DT40-3KO cells expressing the desired receptor were transiently transfected with NFAT-GFP. 12 h after recovery, cells were transfected onto coverslips mounted in a Warner chamber. Cells were perfused with HEPES imaging buffer (137 mM NaCl, 4.7 mM KCl, 1.26 mM CaCl$_2$, 1 mM Na$_3$PO$_4$, 0.56 mM MgCl$_2$, 10 mM HEPES, and 5.5 mM glucose (pH 7.4)) and stimulated with mouse α-chicken IgM. GFP imaging was recorded using an inverted epifluorescence Nikon microscope with a ×40 oil immersion objective. Cells were excited at 488 nm, and emission was monitored at 509 nm. Images were captured every second with an exposure of 10 ms and 2 × 2 binning using a digital camera driven by TILL Photonics software.

Preparation of DT40 cell nuclei

Isolated DT40 nuclei were prepared by homogenization as described previously (64). The homogenization buffer contained 250 mM sucrose, 150 mM KCl, and 10 mM Tris (pH 7.5). Cells were washed and resuspended in homogenization buffer prior to nuclear isolation using a RZR 2021 homogenizer (Hei-dolph Instruments) with 25 strokes at 1200 rpm. A 3-μl aliquot of nuclear suspension was placed in 3 ml of bath solution that contained 140 mM KCl, 10 mM HEPES, 500 μM BAPTA and 246 nM free Ca$^{2+}$, pH 7.1. Nuclei were allowed to adhere to a plastic culture dish for 10 min prior to patching.

On-nuclei patch clamp experiments

Single InsP$_3$R channel currents using Potassium ions as the charge carrier (i$_V$) were measured in the on nucleus patch clamp configuration using pCLAMP 9 and an Axopatch 200B amplif-ier (Molecular Devices, Sunnydale, CA, USA) as previously described (64). Pipette solution contained 140 mM KCl, 10 mM HEPES, 1 μM InsP$_3$, 5 mM ATP, and 200 nM free Ca$^{2+}$ (pH 7.1). Free [Ca$^{2+}$] was calculated using Max Chelator freeware and verified fluorometrically. Active caspase-3 and/or Z-VAD-fmk were included in the pipette solution for the corresponding experiments. Traces were consecutive 3-s sweeps recorded.
at $-100$ mV, sampled at 20 kHz, and filtered at 5 kHz. A minimum of 15 s of recordings was considered for data analyses. Pipette resistances were typically 20 mehmogs, and seal resistances were $>5$ gigaohms.

**Data analysis**

Single-channel openings were detected by half-threshold crossing criteria using the event detection protocol in Clampfit 9. We assumed that the number of channels in any particular nuclear patch is represented by the maximum number of discrete stacked events observed during the experiment. Even at low $P_o$, stacking events were evident (data not shown). Only patches with one apparent channel were considered for analyses. $P_o$ unitary current ($i_u$) and open and closed times were calculated using Clampfit 9 and Origin 6 software (Origin Lab, Northampton, MA). All-points current amplitude histograms were generated from the current records and fitted with a normal Gaussian probability distribution function. The coefficient of determination (R2) for each fit was >0.95. The $P_o$ was calculated using the multimodal distribution for the open and closed current levels. Channel dwell time constants for the open and closed states were determined from exponential fits of all-points histograms of open and closed times. The threshold for an open event was set at 50% of the maximum open current, and events shorter than 0.1 ms were ignored.

**Author contributions**—This work was performed in the Department of Pharmacology and Physiology at the University of Rochester. L. W. designed and stably expressed some of the constructs, collected and analyzed the data, drafted the manuscript, and prepared the figures. L. E. W. collected and analyzed data obtained through single-channel electrophysiology (Fig. 7). K. J. A. designed and expressed some of the constructs and performed the experiments shown in Fig. 1J. D. i. Y. was responsible for the conception and design of all experiments as well as data analysis, generation of figures, and editing of the manuscript. All authors approved the final version.

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