

Region-specific proteolysis differentially regulates type 1 inositol 1,4,5-trisphosphate receptor activity

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Running title: Region specific proteolytic regulation of IP₃R1

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ABSTRACT

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 the current 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 is 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.

INTRODUCTION

Calcium ions (Ca²⁺) are utilized widely as an intracellular second messenger that interact with effectors to induce a diverse array of cellular activities. These events include gene expression,

cell migration, muscle contraction, secretion, autophagy and cell death (1-3). In order to use Ca²⁺ to finely control cellular activities with high specificity and accuracy, cells have evolved a “calcium signaling toolbox” consisting of Ca²⁺ channels, pumps and binding proteins (4). These components function in concert and encode unique information in forms of the amplitude, frequency and subcellular location of Ca²⁺ signals. An important component of the toolbox is the Inositol 1,4,5-trisphosphate receptor (IP₃R). IP₃R are intracellular Ca²⁺ release channels expressed predominately in the membrane of the endoplasmic reticulum (ER) in most of eukaryotic species (5-8). In response to IP₃ binding, IP₃R are activated resulting in Ca²⁺ release from the intracellular store into the cytosol (9).

A series of complex regulatory events allow IP₃R to encode Ca²⁺ signals with distinct temporal and spatial characteristics. First, following binding of IP₃, the interaction of Ca²⁺, nucleotides and binding proteins with IP₃R can regulate channel activity (10-18). Secondly, post-translational modifications including phosphorylation, ubiquitination and oxidation also can influence channel activity and shape the IP₃R Ca²⁺ release profile (19-23). As a further level of complexity, our lab has recently demonstrated that the particular isoform complement of the IP₃R hetero-tetramer can either contribute to, or largely determine the specific

characteristics of IP₃R-mediated Ca²⁺ signals (24,25).

A further potential route of regulation of IP₃R activity is through proteolytic fragmentation (26-28). The functional consequence of IP₃R fragmentation has been a subject of debate for years (29-35). One of the major controversies centered over the question whether IP₃R1 is a preferred substrate of caspase (35). While several reports have demonstrated that caspase and calpain can cleave the IP₃R1 into at least two fragments (26,27), other evidence has suggested that IP₃R1 are not generally cleaved under apoptotic conditions, and is in fact only a late substrate of caspase during intense staurosporine induced apoptosis (35). The latter evidence argued that fragmentation of IP₃R1 does not play a key role in the process of apoptosis.

A further area of contention surrounds the biophysical properties of fragmented IP₃R. Proteolytic fragmentation of IP₃R1 by caspase and calpain results in at least two receptor fragments: The N-terminal fragment consists of the IP₃ binding core and much of the cytosolic peptide chain, while the C-terminal fragment contains the Ca²⁺ permeation pore and the C-terminal cytosolic tail (26,27,33). One proposal suggests that receptor fragmentation physically dissociates the N-terminal region from the ER associated C-terminal channel domain. Such cleavage and dissociation would be predicted to functionally uncouple the regulation of IP₃ binding from channel gating, leading to leaky C-terminal fragments retained in the ER, disruption of Ca²⁺ homeostasis and apoptosis (31,36). However, our lab proposed and demonstrated an alternative scenario by showing that IP₃R1 retains its tetrameric architecture even after proteolytic fragmentation. Moreover, co-expression of complementary IP₃R1 peptides, designed based on caspase and calpain fragmented IP₃R1, can reconstitute tetrameric channel structure that is functionally gated by IP₃ binding (33). These data unambiguously demonstrated that IP₃R1 is still tightly regulated by its endogenous ligand even after proteolytic fragmentation.

In the current 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. The current studies demonstrate that proteolytic fragmentation has profound effects on IP₃-mediated Ca²⁺ signals resulting in altering the signature temporal pattern of Ca²⁺ release through IP₃R1. Further, 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 when compared to IP₃R WT. Our results therefore strongly suggest that proteolytic fragmentation may represent a novel form of regulation of IP₃R activity which expands the repertoire of signaling through IP₃R1 activation.

RESULT

Functional fragmented IP₃R1 are assembled from complementary IP₃R1 fragments.

We have previously reported 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, a cleavage site 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.1 B). Caspase and calpain both cleave the IP₃R1 in the same solvent-exposed region (after tryptic 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) expressing various complementary receptor fragments (Fig. 2A-D) were loaded with Ca²⁺ indicator fura-2 and then were challenged with Gαq/IP₃-linked, Protease Activated Receptor 2 (PAR2) agonist, trypsin. Consistent with our previous work, all types of complementary receptor fragments were capable of inducing an elevation in [Ca²⁺]_i in response to maximal PAR2 activation (Fig. 2 E-I). Further, DT40-3KO cells stably expressing only the channel fragment (IP₃R1 V) showed no Ca²⁺ response when PAR2 was activated (Fig. 2J). Remarkably, functional channels were assembled by co-transfecting the remaining four complementary receptor fragments into these cells (Fig. 2K). To confirm this finding, we performed the co-transfection experiments in HEK cells. We and others have thoroughly characterized and demonstrated that HEK-3KO cells (a HEK cell line *null* for all IP₃R) (41,42) are completely devoid of any functional IP₃R. Consistently, trypsin stimulated PAR2 activation induced Ca²⁺ release in HEK-3KO cells only when complementary five tryptic fragments in a monomer were transiently expressed in to cells (Fig. 2 L). Together, these data confirm that complementary receptor fragments are assembled into functional channels which are gated by IP₃ binding. Next, we investigated whether IP₃R fragmentation results in functionally equivalent IP₃R or alternatively, if the biophysical profile or regulation of the fragmented IP₃R differs from IP₃R formed from conventional peptide monomer chains with linear continuity.

Proteolytic fragmentation alters the temporal pattern of IP₃R1 mediated Ca²⁺ release in a region specific manner.

IP₃R exhibit subtype-specific Ca²⁺ release profiles when continuously exposed to IP₃. This is best exemplified following activation of B cell receptors (BCR) on DT40-3KO cells evoked by crosslinking with α-IgM (43). For example, stimulation of DT40-3KO cells stably expressing IP₃R1 WT with α-IgM characteristically evokes only a few transient increases in [Ca²⁺]_i (Fig. 3A and (41,43,44)), whereas stimulation of cells expressing mouse IP₃R2 WT elicits robust Ca²⁺ oscillations (13,41,43). A higher concentration of α-IgM (2 μg/ml) had no impact on the profile of Ca²⁺ release evoked in cells expressing IP₃R1 WT (Fig. 3G). In addition, this general pattern was observed in

various clones of DT40-3KO cells with different IP₃R1 WT expression levels (data not shown), indicating that the temporal pattern of [Ca²⁺]_i signal likely reflects an intrinsic property of the particular IP₃R. Next, we examined the Ca²⁺ 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 IP₃R1 I-II+III-V (tryp), similar to IP₃R1 WT, a low number of Ca²⁺ transients were evoked upon α-IgM stimulation (Fig. 3B-C). Remarkably, when cleavage sites were introduced further toward the C-terminus of the receptor, for example, IP₃R1 I-III+IV-V (tryp), IP₃R1 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. 3D-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 previously reported 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 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+V (calp). These mutations had no impact on the Ca²⁺ release profile mediated by IP₃R1 I-IV+V (calp) (Fig. 4A-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. 5A-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 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-D). 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. 6E), whereas cells expressing IP₃R1 I-IV+V (calp) display an increase in the population of cells which 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 when compared to IP₃R1 WT in response to photorelease of caged IP₃ (Fig. 6G).

Proteolytic fragmentation alters the single channel open probability of IP₃R1

One caveat of 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 (P_o) of IP₃R1 to ~20% (Fig. 7A, G). Addition of active caspase-3 (3 ng/ml) in the patch pipette in the presence of IP₃ (1 μM) significantly augmented channel P_o to ~70% (Fig. 7B, 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 due to nonspecific digestion followed by destruction of the receptor (Fig. 7C, D, G). Addition of the caspase inhibitor z-VAD (20 μM) completely blocked the effect of active caspase on channel P_o (Fig. 7E, 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. 7F, G) (30), no increase in P_o was observed in the presence of active caspase-3. These data strongly suggest that specific receptor fragmentation by caspase-3 at Asp1891 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.

While 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 P_o 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 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 to DMSO treated controls in response to all PAR2 agonist concentrations (Fig. 8A-B and F). The increase in phosphorylation and regulation of Ca²⁺ signals was completely abolished in cells expressing non-phosphorylatable IP₃R1 mutants at both PKA phosphorylation sites (Fig. 8C and F). Notably, when a disruption of peptide continuity was introduced at the third trypsin cleavage site, IP₃R1 I-III+IV-V (tryp), PKA phospho-regulation was maintained (Fig. 8D and F). In contrast, although forskolin pretreatment increased the level of phosphorylated IP₃R1 I-IV+V (calp), no significant difference in terms of the amplitude of Ca²⁺ response was observed. (Fig. 8E and G). These data demonstrate that PKA regulation of IP₃R 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 IP₃R1 activity may be altered depending on the specific site of fragmentation.

Fragmented IP₃R1 can activate distinct downstream effectors.

Based on the observation that specific fragmented IP₃R1 can induce different Ca²⁺ signals, we next investigated if fragmented IP₃R1 can specifically activate distinct downstream effectors. Oscillatory Ca²⁺ signals, but not single Ca²⁺ transients, have been shown to specifically activate the transcription factor NFAT. Ca²⁺ 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 IP₃R1, but not IP₃R1 WT, might provide the necessary Ca²⁺ signal to activate NFAT translocation. GFP tagged NFAT (NFAT-GFP) was transfected into cells expressing either IP₃R1 WT, IP₃R1 I-IV+V (casp) or IP₃R1 I-IV+V (calp). In the quiescent state, NFAT-GFP was mainly located in the cytosol (Fig. 9B, E, H). Upon BCR stimulation, NFAT-GFP efficiently translocated from the cytosol into the nucleus in cells expressing either IP₃R1 I-IV+V (casp) or IP₃R1 I-IV+V (calp) (Fig. 9A-F, J and K). Translocation of NFAT-GFP was not observed in cells expressing IP₃R1 WT (Fig. 9G-K). These data strikingly illustrate that the distinct patterns of Ca²⁺ signal evoked through particular fragmented IP₃R have the ability to activate distinct downstream events when compared to 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 which correspond to any pair of fragments representing the 5 globular domains generated *in vitro* by limited tryptic exposure similarly result in functional channels (37). Indeed, co-expression of individual cDNA encoding the 5 domains separately, remarkably, leads to the assembly of a functional channel, gated by IP₃. While 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, namely augmented Ca²⁺ release following PKA phosphorylation, is lost when fragments are expressed which mimic calpain cleavage to generate fragmented IP₃R1 I-IV + V. However, regulation by PKA is clearly evident if 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 Ser1589 and Ser1755 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 if 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 integrated 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 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, while signals through the IP₃R1 WT could not. Taken together these data provide support 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 (CTD) 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 helical domain (HD), armadillo solenoid folds (ARM) and intervening later domain (ILD), 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. This concept was again strongly supported by the recent structure which 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 fragmentation site for IP₃R1 I-III+IV-V are located in ARM regions. Given the critical role of coupling domain in terms of receptor regulation, it is conceivable that disruption of peptide continuity in the ARM regions (IP₃R1 I-III+IV-V and IP₃R1 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 IP₃R1-induced Ca²⁺ signals.

What is the mechanism underlying the ability of particular fragmented IP₃R1 to support robust Ca²⁺ oscillations? A canonical model for class I Ca²⁺ oscillations provides a possible explanation for this

remarkable alteration (51). This model suggests that cells can control the Ca²⁺ oscillation period by modulating the rate of Ca²⁺ activation of IP₃R in response to the change in [Ca²⁺]_i. Of note, although there was no EF-hand Ca²⁺ binding motif found in IP₃R1, the ARM3 region in the coupling domain contains a putative Ca²⁺ binding region with a highly conservative Glu 2100 critical for Ca²⁺ binding and regulation of IP₃R1 (52,53). Based on the model and the location of Ca²⁺ sensor in the ARM regions, we speculate that fragmentation of IP₃R1 at particular ARM regions impact the Ca²⁺ modulation of channel gating, and consequently alters the Ca²⁺ activation rate for IP₃R1. This hypothesis is supported by mathematical simulations showing that by solely changing the rate of IP₃R1 activation by Ca²⁺, the normal monotonic Ca²⁺ release pattern through wild type IP₃R1 Ca²⁺ can be converted into robust oscillatory Ca²⁺ signals observed with IP₃R1 I-III+IV-V and IP₃R1 I-IV+V (51).

While IP₃R1 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 labs report that even when caspase and calpain are massively activated during apoptosis, only a small fraction of IP₃R1 is actually fragmented. Therefore, we envision that when caspase or calpain are mildly activated in non-apoptotic conditions, a small proportion of a particular fragmented IP₃R1 is generated in cells that might then locally activate specific downstream effectors and fulfill unique roles. When might lower levels of protease activity occur? Recent reports have suggested that caspase and calpain activity is also essential for processes other than initiating cell death. These include protein maturation, cell proliferation and differentiation, and myoblast fusion (54-61). More importantly, there is burgeoning evidence suggesting that, beyond negating protein activity, caspase can activate its substrates by proteolysis (54,56,60,62). Consistent with this idea, this study provides evidence that enzymatic cleavage *per se* might uniquely regulate IP₃R1 activity and thus might potentially contribute to the regulation of different cellular activities rather than cell death.

In summary, our work provides the first evidence showing that proteolytic fragmentation may serve

as a novel regulatory event for the IP₃R1 by altering its Ca²⁺ release profile. Future work is necessary to specifically investigate the pathophysiological conditions where IP₃R1 is cleaved and the corresponding significance of receptor fragmentation for those processes. We envision that spatially confined protease activity may cleave the IP₃R1 to obtain a transient alteration of receptor mediated Ca²⁺ signals, which can rapidly switch on alternative signaling pathways for unique cellular activities. In addition, there is evidence showing that both IP₃R2 and IP₃R3 can also be fragmented by proteases (63). We are currently investigating the functional consequence of proteolytic fragmentation for IP₃R2 and IP₃R3. Our study will answer the important question whether proteolytic fragmentation may be a general regulatory event for all isoforms of IP₃R.

MATERIALS AND METHODS

Reagents

All restriction enzymes and T4-DNA ligase were from New England Lab. RPMI-1640 medium, penicillin/streptomycin, G418 sulfate, β -mercaptoethanol and chicken serum were purchased from Invitrogen. Fetal bovine serum was from Gemini Bio-products. iso-Ins(1,4,5)P₃/PM (caged), Z-VAD-FMK and active caspase-3 were from Enzo Life Science. Fura-2 was from TEFLABS. All reagents for SDS-PAGE were from Bio-rad. N-terminal antibody for IP₃R1 and phospho- IP₃R1 were from Cell Signaling Technology. DyLightTM 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 IP₃R1 was generated by Pocono Rabbit Farms and Laboratories.

Constructs

The method for generation of IP₃R1 I-IV+V (casp) and IP₃R1 I-IV+V (calp) was first described elsewhere (33). In brief, to create IP₃R1 I-IV+V (casp), cDNA encoding rat IP₃R1 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 IP₃R1 at the DEVD1891 consensus site, IP₃R1 cDNA was modified by PCR (forward, 5'-GAAAGATGATGAAGTGGACTAGAATTCGC GGCCGCGCTAGCATGCGGGATGCCCCATC CCGAA3'). 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, the NheI-NotI IP₃R1 I-IV (casp) was inserted into the TPV digested with NheI and PspOMI (NotI and PspOMI have compatible cohesive ends). Second, the NotI-NotI fragment coding IP₃R1 V (casp) was inserted into the TPV that was digested with NotI. TPV encoding IP₃R1 I-II+III-V (tryp), IP₃R1 I-III+IV-V (tryp) and IP₃R1 I-V+V (calp) were constructed in identical manner using corresponding primers (forward, 5'-GGCAGCAACGTGATGAGATAGGCGGCCCGC GCTAGCATGTCTATCCATGGAGTTGG-3') for IP₃R1 I-II+III-V (tryp), (forward 5'-CTGGCGG TTATCAGCCCGCTAGGCGGCCGCGCTAGC ATGAACGCTGCTCGTAGAG-3') for IP₃R1 I-III+IV-V (tryp), and (Forward 5'-CCGGGATCAG CTCTTGGAATAGAATTCGCGGCCGCGCTAG CATGGCATCTGCTGCCACCAGAAAAGCC-3') for IP₃R1 I-V+V (calp). To generate caspase non-cleavable rIP₃R1, primer (5'-GGGAAACAAAA GAAAGATATCGAAGTGGCCAGGGATGCCC C-3') was used to mutate the sequence encoding amino acid DEVD to be IEVA. Two-step PCR was performed sequentially using primers (5'-TCAGGAAGAAGAGAGGCTCTTACCAGCTT TGGCA-3') and (5'-GCTGCTCGTAGAGACGC TGTCCTGGCAGCTTCC-3') to generate rIP₃R1 (S1589A, S1755A), and (5'-TCAGGAAGAAGAG AGGAGCTTACCAGCTTTGGCA-3') and (5'-GCTGCTCGTAGAGACGAGGTCCTGGCAGC TTCC-3') to generate rIP₃R1 (S1589E, S1755E).

Western blot

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, 1mM EDTA, 1 mM NaF, 20 mM

Na₄P₂O₇, 2mM Na₃VO₄, 1% Triton X-100(v/v), and 10% glycerol with 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_c protein assay kit (Bio-Rad). Protein were resolved on 5-7.5% SDS-PAGE gels, transferred to nitrocellulose, and processed for immunoblotting with the indicated primary antibodies and corresponding secondary antibodies. Proteins were detected using an Odyssey infrared imaging system (LI-COR Biosciences).

Fluorescence imaging assay

DT40 cells expressing defined IP₃R constructs were loaded with 2 μM Fura-2 (AM) on a glass cover-slip 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 CaCl₂, 1mM Na₂HPO₄, 0.56 mM MgCl₂, 10 mM HEPES, 5.5 mM glucose, pH 7.4) and stimulated with the indicated agonist. Ca²⁺ imaging was performed using an inverted epifluorescence Nikon microscope with a X40 oil immersion objective (NA=1.3). Cells were alternately excited at 340 and 380 nm, and emission was monitored at 505 nm. Images were captured every second with an exposure of 10 ms and 4 by 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 IP₃R 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 39°C with 5% CO₂. 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% CO₂. HEK-3KO cells were transfected using Lipofectamine 2000 (Invitrogen) following the protocol of the manufacturer.

FlexStation assay

DT40-3KO cells (5X10⁵ per 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 one hour. Cells were then spun down, washed with imaging buffer containing 1% BSA and seeded onto 96 well plate that was pre-coated with 0.1% Poly-L-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 same volume of DMSO for 3 min followed by stimulation with different concentrations of PAR2 agonist. For each types of IP₃R or receptor fragments, a control response was defined as the amplitude of Ca²⁺ response stimulated by 500 μM PAR2 agonist following DMSO pre-incubation. The amplitude of Ca²⁺ response 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 was transiently transfected with NFAT-GFP. 12 hr after recovery, cells were transferred onto cover-slips mounted in a Warner chamber. Cells were perfused with HEPES imaging buffer (137 mM NaCl, 4.7 mM KCl, 1.26 mM CaCl₂, 1 mM Na₂HPO₄, 0.56 mM MgCl₂, 10 mM HEPES, 5.5 mM glucose, pH7.4) and stimulated with the mouse α-chicken IgM. GFP imaging was recorded using an inverted epifluorescence Nikon microscope with a X40 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 by 2 binning using a digital camera driven by TILL Photonics software.

Preparation of DT40 cell nuclei.

Isolated DT40 nuclei were prepared by homogenization as previously described (64). The homogenization buffer (HB) contained 250 mM sucrose, 150 mM KCl, 10 mM Tris, pH 7.5. Cells were washed and resuspended in HB prior to nuclear isolation using a RZR 2021 homogenizer (Heidolph Instruments, Germany) with 25 strokes at 1200 rpm. A 3 μl aliquot of nuclear suspension was placed in 3 ml of bath solution (BS) which contained 140 mM KCl, 10 mM Hepes, 500 μM

BAPTA and 246 nM free Ca²⁺, pH 7.1. Nuclei were allowed to adhere to a plastic culture dish for 10min prior to patching.

On-nuclei patch clamp experiments.

Single InsP₃R channel currents using Potassium ions as the charge carrier (*i_k*) were measured in the on nucleus patch clamp configuration using pCLAMP 9 and an Axopatch 200B amplifier (Molecular Devices, Sunnydale, CA, USA) as previously described (64). Pipette solution contained 140 mM KCl, 10 mM Hepes, 1 μM InsP₃, 5 mM ATP, 200 nM free Ca²⁺, pH 7.1. Free [Ca²⁺] 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 were considered for data analyses. Pipette resistances were typically 20 MΩ and seal resistances were >5 GΩ.

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. Probability of opening (P_o), unitary current (*i_k*), and open and closed times were calculated using Clampfit 9 and Origin 6 software (Origin Lab, Northampton, MA, USA). 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 every 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 0.1ms were ignored.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

This work was performed in the Department of Pharmacology and Physiology at the University of Rochester. LW designed and stably expressed some of the constructs, collected and analyzed the data, drafted the manuscript and prepared the figures. LEW collected and analyzed data obtained through single channel electrophysiology (Fig. 7). KJA designed and expressed some of the constructs, and performed experiments in figure 1J. DIY was responsible for conception and design of all experiments as well as data analysis, generation of figures and editing manuscript. All authors approved the final version

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Figure 1. Schematic diagram showing the proteolytic fragmentation sites on IP₃R1. 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 (A). Ribbons with corresponding colors (B) 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 fragments (B).

Figure 2. **IP₃R1 reconstituted from complementary receptor fragments are functional.** Expression of each types of IP₃R1 receptor fragments in DT40-3KO cells were confirmed by western blot using IP₃R1 antibodies probing the N-terminus (A and C) or the C-terminus of the receptor (B and D). Each lane was loaded with 18 µg protein. Arrows indicate the fragments of interest. DT40-3KO cells stably expressing IP₃R1 WT (E), IP₃R1 I-II+III-V (tryp) (F), IP₃R1 I-III+IV-V (tryp) (G), IP₃R1 I-IV+V (calp) (H) or IP₃R1 I-IV+V (casp) (I) were loaded with 2 µM fura-2 (AM) followed by stimulation with PAR2 agonist trypsin (500 nM). Averaged traces of Ca²⁺ release was measured as a change in the 340/380 fluorescence ratio. DT40-3KO cells stably expressing IP₃R1 V (tryp) didn't respond to trypsin (500 nM) stimulation (J). Transient co-expression of IP₃R1 I (tryp), IP₃R1 II (tryp), IP₃R1 III (tryp) and IP₃R1 IV (tryp) in to DT40-3KO cells stably expressing IP₃R1 V (tryp) constituted functional Ca²⁺ release channels (K). (L) is three representative HEK-3KO cells demonstrating that functional IP₃R1 can be assembled from 20 IP₃R1 polypeptide fragments corresponding to those produced by trypsinization of each monomer into 5 fragments. Ca²⁺ imaging assays were repeated five times for each set of complementary receptor fragments.

Figure 3. **Fragmentation pattern determines the temporal Ca²⁺ release profile of the complementary receptor fragments.** DT40-3KO cells stably expressing IP₃R1 WT (A), IP₃R1 I-II+III-V (tryp) (B), IP₃R1 I-III+IV-V (tryp) (D), IP₃R1 I-IV+V (calp) (E) or IP₃R1 I-IV+V (casp) (F) were loaded with 2 µM fura-2 (AM) followed by crosslinking the B cell receptor using α-IgM (500 ng/ml). Two representative Ca²⁺ traces are shown for each pair of complementary receptor fragments. Number of Ca²⁺ transients in 15 min of experiments were calculated. Scatter plots indicate that a cleavage site in solvent exposed region II has no effect on the temporal Ca²⁺ release profile (C) (*student's t-test*) while fragmentation sites more toward to the C-terminus significantly increase the number of Ca²⁺ transients mediated by complementary receptor fragments (*one-way ANOVA* followed by *Dunnnett post-test*) (G). * denotes statistical significance determined by *Dunnnett post-test*. Ca²⁺ imaging assays were repeated seven times with more than 40 cells in each experimental run for each set of complementary receptor fragments.

Figure 4. **Altered temporal Ca²⁺ release profile mediated by IP₃R1 I-IV + V (calp) is not due to PKA mediated receptor phosphorylation.** DT40-3KO cell 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 crosslinking the B cell receptor using α-IgM (500 ng/ml). Two representative Ca²⁺ traces are shown for each set of complementary receptor fragments. Scatter plot indicates that there is no significant difference among three types of receptor fragments with respect to number of Ca²⁺ transients in 30 min of recording (*one-way ANOVA*) (D). Ca²⁺ imaging assays were repeated three times with more than 40 cells in each experimental run for each complementary receptor fragments.

Figure 5. Ca²⁺ influx is not necessary for complementary receptor fragments to induce robust Ca²⁺ oscillation. DT40-3KO cells stably expressing IP₃R1 I-IV+ V (calp) were loaded with 2 μ M fura-2 (AM) followed by crosslinking the cell surface B cell receptor using α -IgM (500 ng/ml). Perfusion buffers with or without extracellular Ca²⁺ were alternated every 10 min during recording as indicated (A). Column statistics suggests that there is no significance between conditions with or without extracellular Ca²⁺ with respect to number of Ca²⁺ transients (*Student's t-test*) (B). Experiments were repeated five times with more than 40 cells in each experimental run.

Figure 6. Photorelease of caged IP₃ induces distinct Ca²⁺ signals mediated by complementary receptor fragments. DT40-3KO cells stably expressing IP₃R1 WT, IP₃R2 WT and IP₃R1 I-IV+V (calp) were loaded with 1 μ M fluo-8 (AM) and 2 μ M caged ci-IP₃ for 30 min. A UV flash (200 msec) was introduced at the indicated time to photolyse caged IP₃ and Ca²⁺ signals were recorded for 15 min. In response to ci-IP₃, cells stably expressing IP₃R1 WT mainly evoked a sustained single Ca²⁺ release event (A) while cells stably expressing IP₃R2 WT evoked robust Ca²⁺ oscillations (B). Both types of Ca²⁺ signals in (A) and (B) were observed in cells stably expressing IP₃R1 I-IV+V (calp) (C and D). Distribution plots indicate that the majority of cells expressing IP₃R1 WT induced either a sustained single Ca²⁺ release or few Ca²⁺ transients (E) whereas cells expressing IP₃R1 I-IV+V (calp) gave rise to an increased level of Ca²⁺ transients during the 15 min recording. (F). Box plot with whiskers showing 10-90 percentile suggests a significant increase in the ability of IP₃R1 I-IV+V (calp) to induce Ca²⁺ oscillations when compared to IP₃R1 WT (*Student's t-test*, $p < 0.0001$) (G). Experiments were repeated four times for each IP₃R or complementary pairs of receptor fragments.

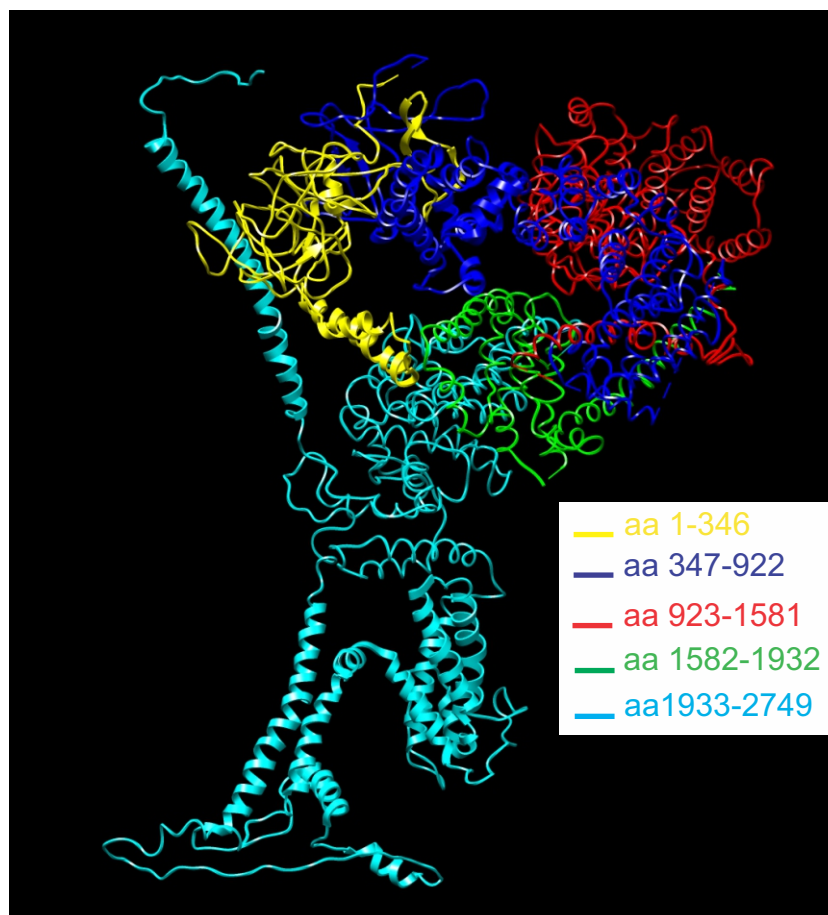
Figure 7. Fragmentation of IP₃R1 by caspase-3 increases channel open probability. Patch clamp recording in the “on nucleus” configuration demonstrated that low concentration of active caspase-3 (3 ng/ml) significantly increased IP₃R1 single channel open probability in the presence of 1 μ M IP₃ (A and B) while high concentrations of active caspase-3 (10 and 30 ng/ml) abolished channel activity (C and D). The increase in channel open probability mediated by low concentrations of active caspase (3 ng/ml) can be blocked by addition of caspase-3 inhibitor, ZVAD-FMK (E) or mutating the putative caspase cleavage motif DEVD to IEVA to make the IP₃R1 non-cleavable (F). (G) show the pooled data (*heteroscedastic t-test*). Each condition was repeated six times.

Figure 8. Increase of Ca²⁺ release in IP₃R1 by PKA phosphorylation is regulated by receptor fragmentation in a cleavage region specific manner. Cells were loaded with 1 μ M of fluo-2 (AM) for 1hr followed by performing a Flexstation assay to monitor the change of [Ca²⁺]_i. Pre-incubation of cells stably expressing IP₃R1 WT with forskolin significantly increased the amplitude of Ca²⁺ release in response to PAR2 activation (A). This increase was observed at all agonist concentrations tested for IP₃R1 WT (B) and at high agonist concentrations tested for IP₃R1 I-III+IV-V (tryp) (D). 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) show that pretreatment of forskolin results in IP₃R1 phosphorylation at residue ser1755 in both full length and fragmented receptors. Statistics was performed using *one-way ANOVA* followed by *Dunnnett post-test*. Experiments were repeated three times for each set of IP₃R1 or complementary receptor fragments.

Figure 9. Ca²⁺ signals induced by IP₃R1 I-IV+V can activate distinct downstream effectors. 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 hr recovery in a 39°C, 5% CO₂ incubator. Cells were then mounted in a perfusion chamber and 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) are 3D heat plots with x and y axes indicating spatial coordinates, and z axis indicating the amplitude of GFP signal. Addition of α -IgM (500 ng/ml) activated and translocated NFAT-GFP from 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). In figure (C), (F) and (I), nucleus NFAT-GFP signals were in red and cytosolic NFAT-GFP signals were in black, which were used to calculate N/C ratio (blue). (J) shows the percentage of cells that translocation could be observed in individual trials where greater than 20 cells were imaged in each trial. (K) shows the magnitude of the N/C ratio in individual cells from each trial which showed translocation. These data demonstrate a significant increase in N/C ratio for fragmented IP₃R1 when compared to 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.

Figure 1

A



B

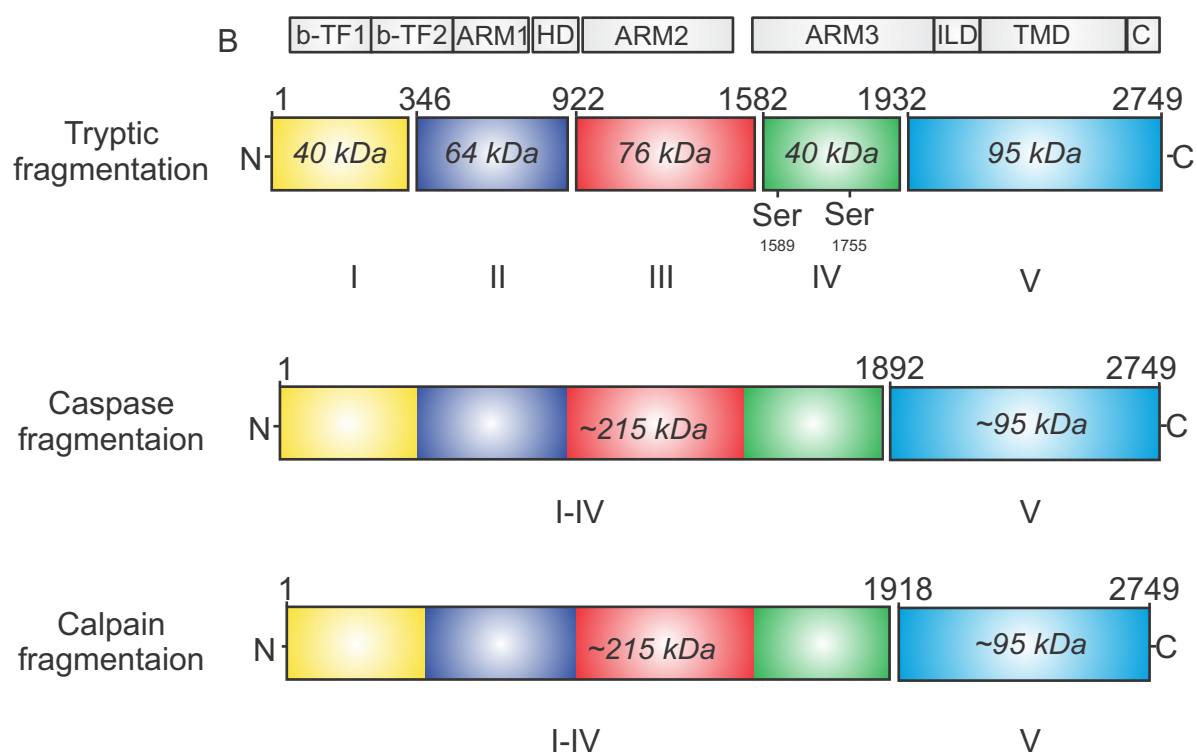


Figure 2

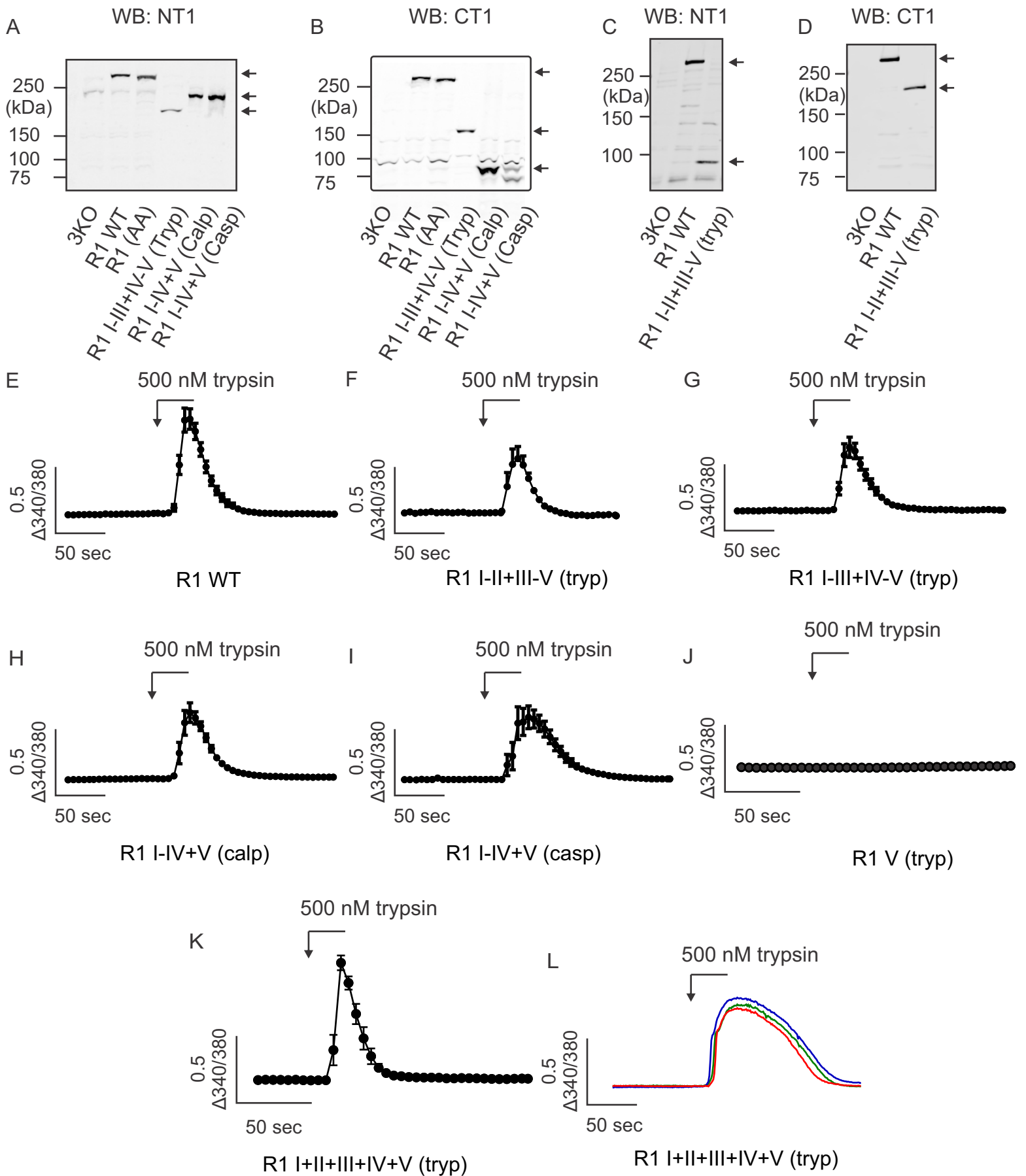


Figure 3

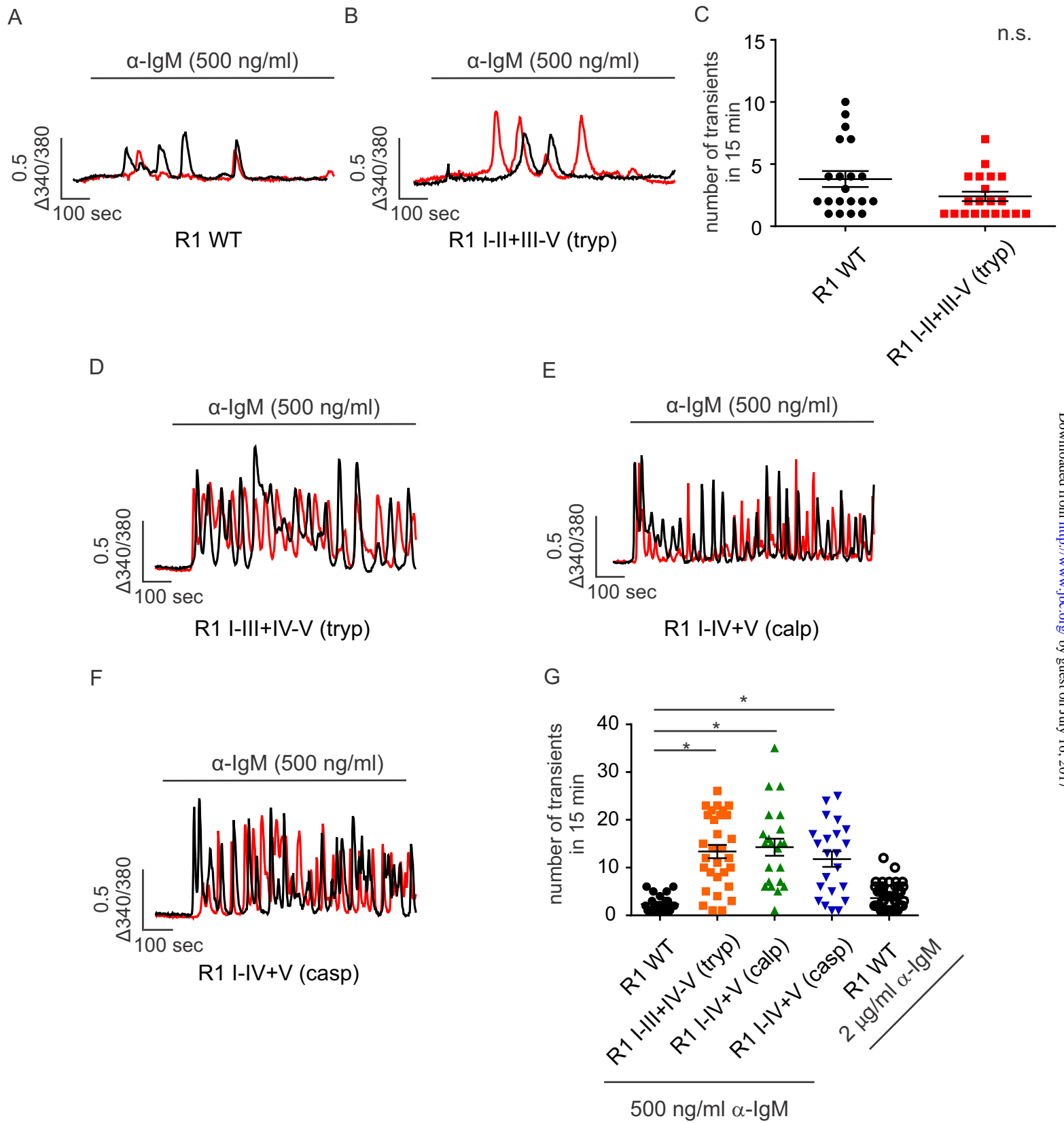


Figure 4

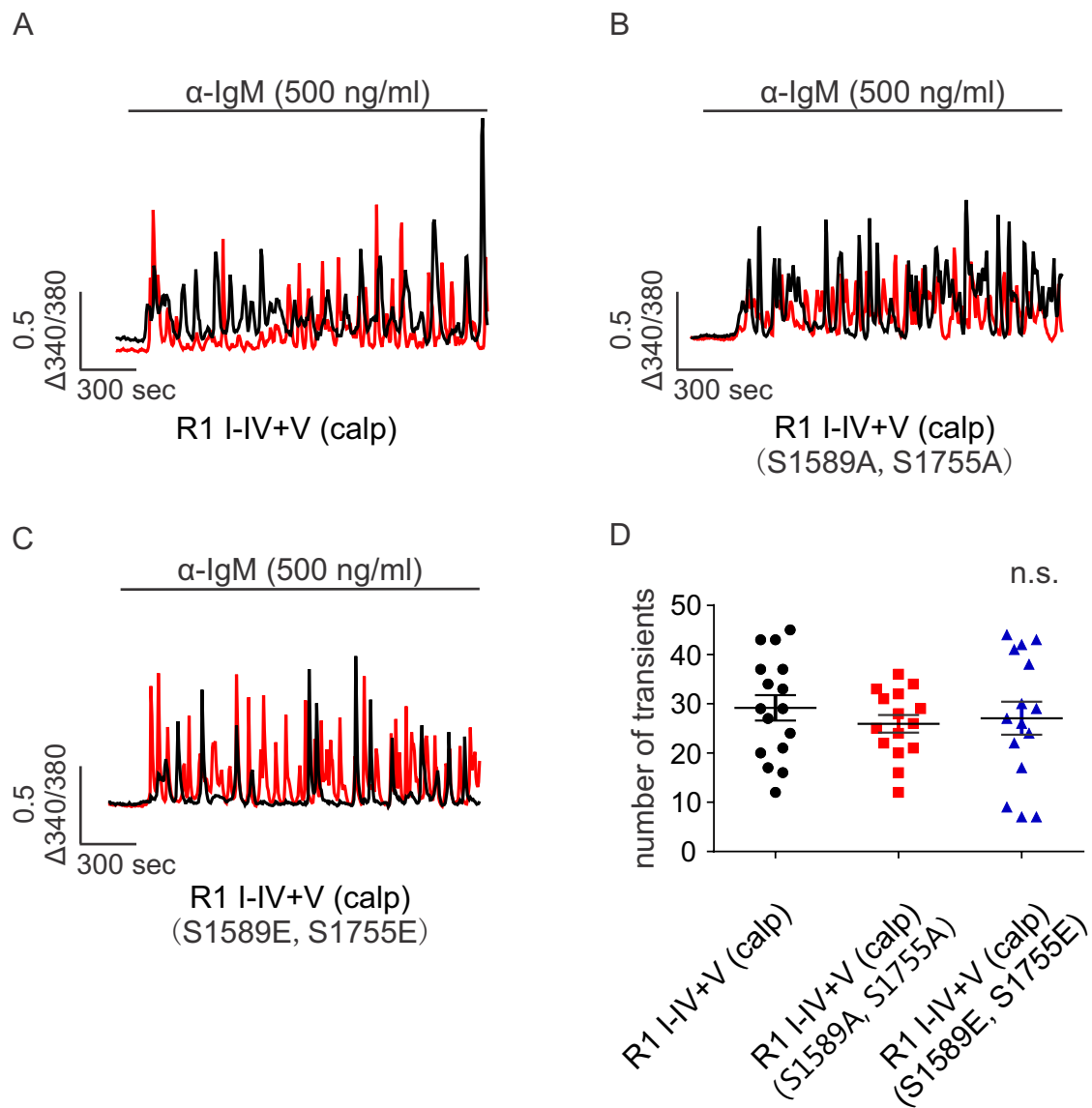
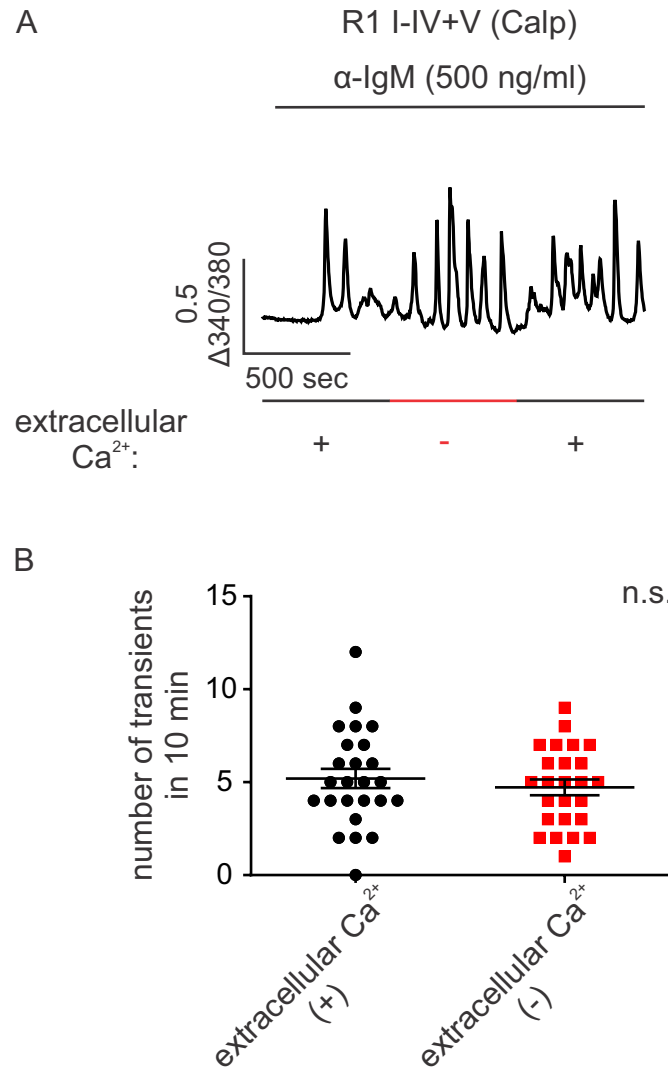


Figure 5



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Figure 7

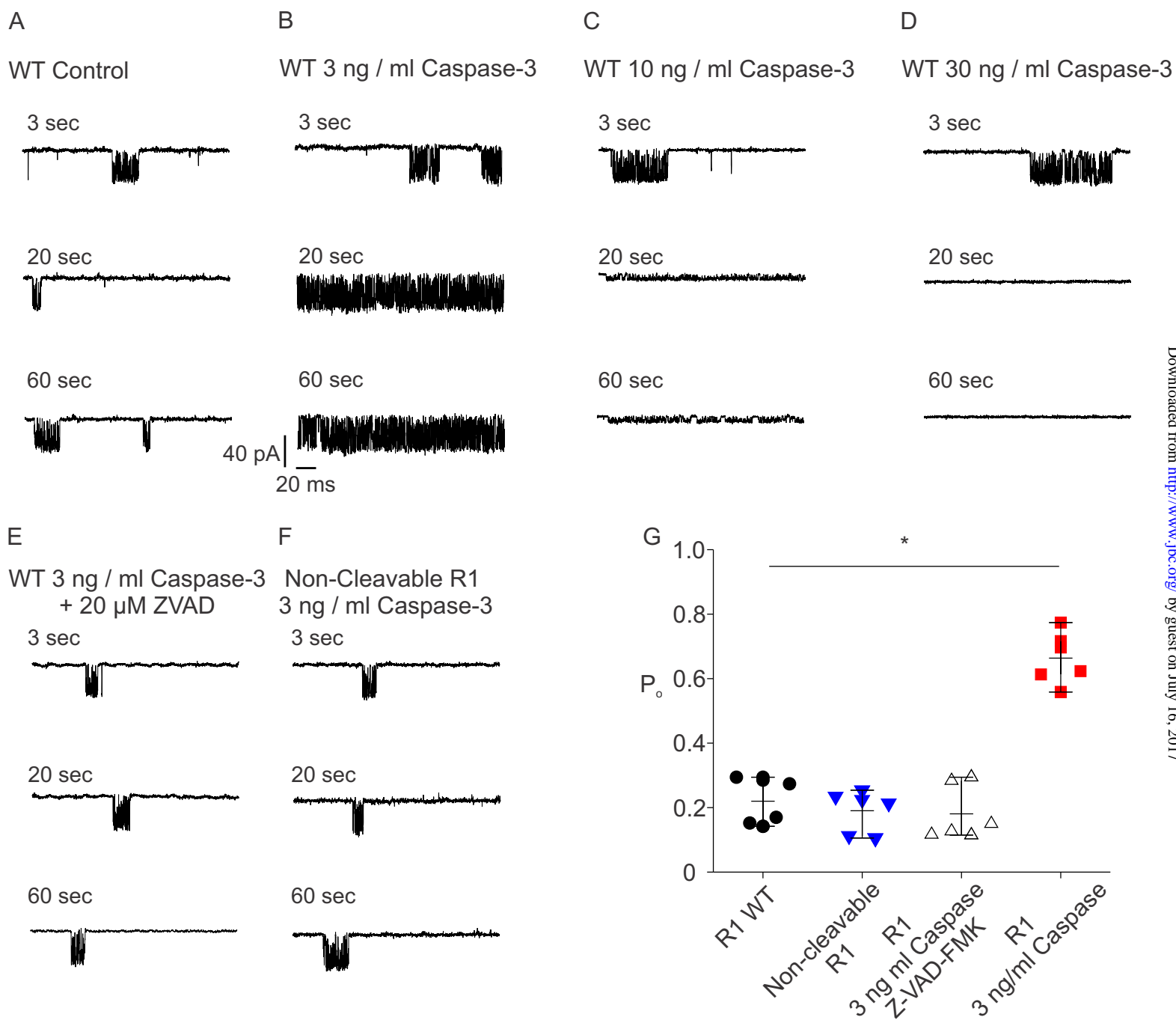


Figure 8

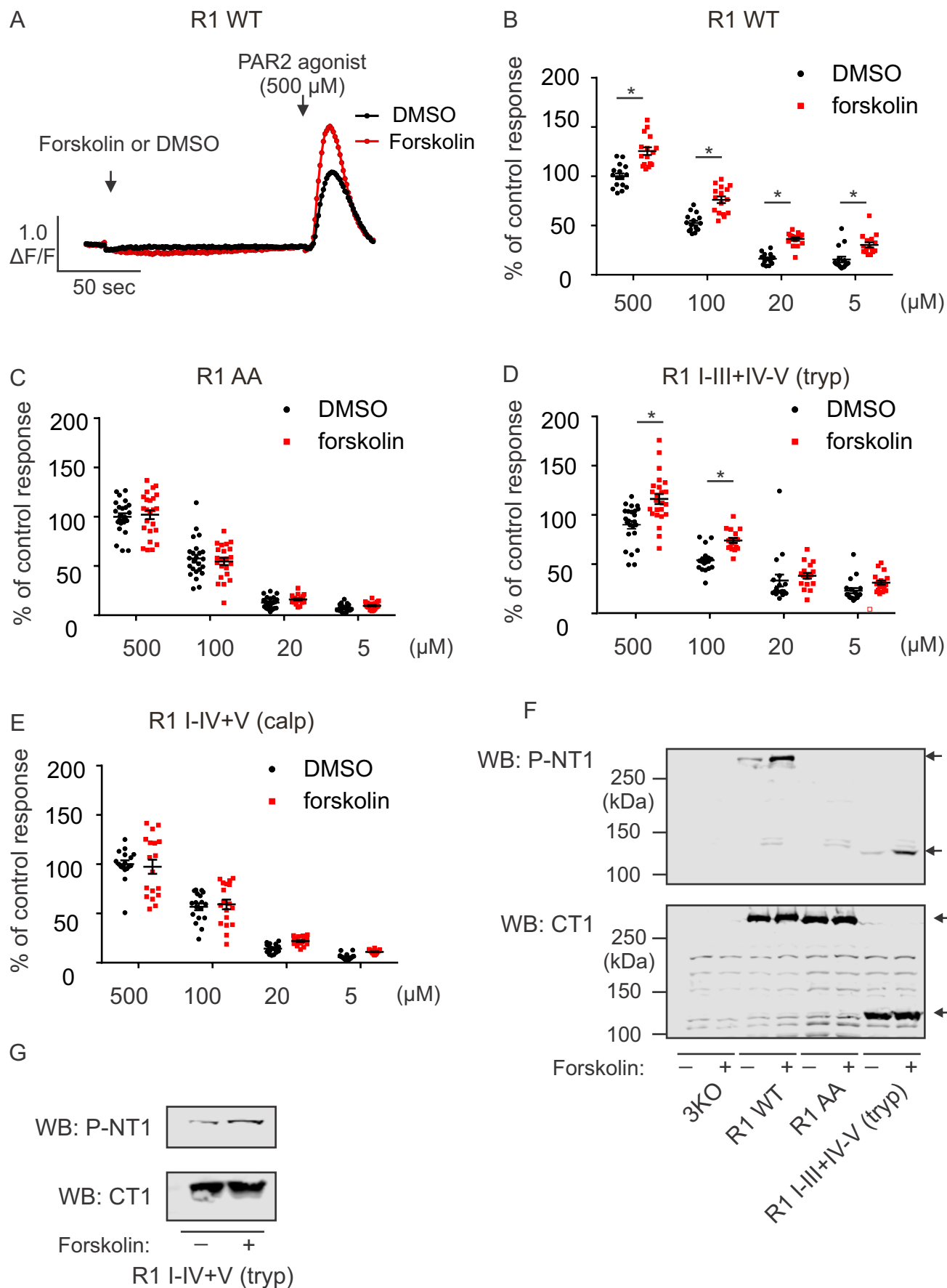
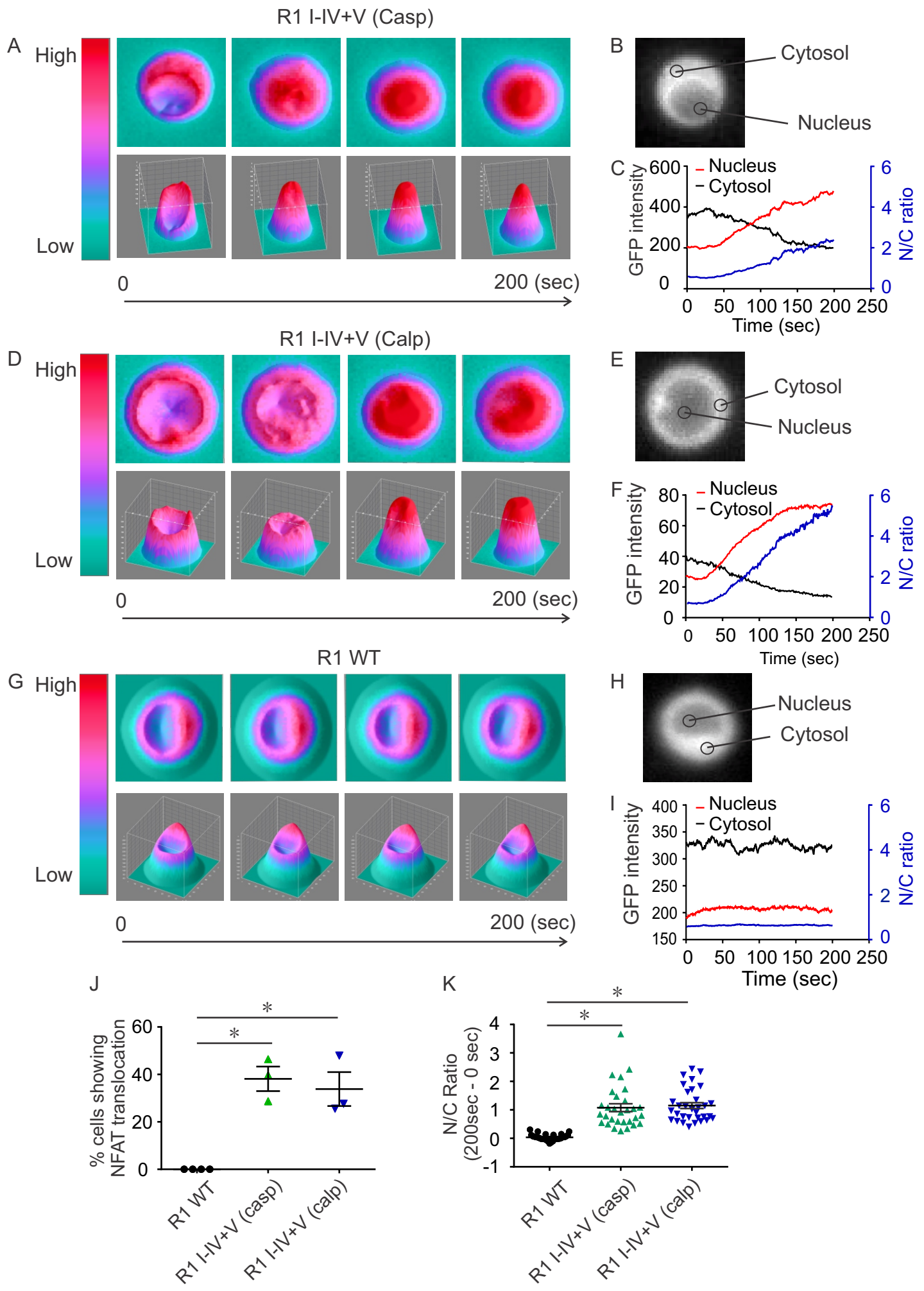


Figure 9



Region-specific proteolysis differentially regulates type 1 inositol 1,4,5-trisphosphate receptor activity

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